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CONTENTS

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DNA Repair Mechanisms as Drug Targets in Prokaryotes

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Abstract

Nowadays, a great amount of pathogenic bacteria has been identified such as *Mycobacterium* sp. and *Helicobacter pylori* and have become a serious health problem around the world. These bacteria have developed several DNA repair mechanisms as a strategy to neutralize the effect of the exposure to endogenous and exogenous agents that will lead to two different kinds of DNA damage: single strand breaks (SSBs) and double strand breaks (DSBs). For SSBs repair, bacteria use the base excision repair (BER) and nucleotide excision repair (NER) mechanisms, which fix the damaged strand replacing the damaged base or nucleotide. DSBs repair in bacteria is performed by homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). HRR uses the homologous sequence to fix the two damaged strand, while NHEJ repair does not require the use of its homologous sequence. The use of unspecific antibiotics to treat bacterial infections has caused a great deal of multiple resistant strains making less effective the current therapies with antibiotics. In this review, we emphasized the mechanisms mentioned above to identify molecular targets that can be used to develop novel and more efficient drugs in future.

Key words: DNA damage, antibiotic resistance, SSB, DSB, antimicrobial drugs, drug-resistant mutants, BER, NER, HRR, NHEJ.

1. Introduction

During the last decades, our knowledge of DNA structure and function has increased dramatically. For example, a recent publication of Wolfe-Simon *et al.* (1) shows a bacterium strain "GFAJ-1" of the *Halomonadaceae*, can use arsenic, a substance that is highly toxic to almost all life on this planet, instead of phosphorus to sustain its growth and incorporate it to its DNA. It is predicted that these bacteria may have formed more than 760,000 years ago. Until now, O_2, C, H, N, P and S have been the basic ingredients of the chemistry of life. However, the present evidence shows this bacterium uses arsenic to maintain its life machinery (1). This information has allowed us to gain a better understanding of life in general, as well as of human diseases (2,3,4). As a matter of fact, with the rise of molecular biotechnology and genetic engineering, scientists have started to develop more effective tools against diseases, targeting key components of molecular mechanisms and even targeting the origin of any living process: the genes. Nevertheless, our knowledge about DNA still remains limited, as well as the options that we need to explore on the path to developing drugs and vaccines against infectious diseases (5).

One aspect that could help us in our fight against infectious diseases is to understand DNA

repair mechanisms in pathogens and evaluate, if DNA repair mechanisms can be targets of antimicrobial drugs. So far, the DNA repair mechanisms that have been described in prokaryotes, can be classified in three main groups (6).

The first group is the direct repair, where DNA damaged part is restored to its original form *in situ*. An example of this kind of repair is photoreactivation. The DNA damage repair through photoreactivation consists of an enzymatic-dimer complex that is activated by its close-proximity exposure to near UV and visible light (6).

The second group of mechanisms share as a main characteristic that they remove the damaged section of the DNA and replace it with normal nucleotides, using the complementary strand as template to restore the sequence (7,8,9). This group of mechanism could repair damages such as mismatches, inter and intra-strand cross-links, and insertion and deletion loops originated from photoproducts of UV radiation and chemical reactions that would lead to oxidation, deamination of bases, and alkylation. It is also the first type of mechanism discovered that is totally independent of UV radiation induction. Examples of this type of mechanism include the base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR).

The third group repairs the DNA damage through recombination processes. This kind of system is called post-replication repair. These mechanisms are the homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). These pathways could repair double strand breaks caused by reactive oxygen species, nuclease action or collapsed replication forks (10).

It is widely known that classical drugs and vaccines are becoming less effective due to the increase of resistant bacteria strains worldwide. The selective pressure put on these organisms and their high rates of mutation are the main causes of the increase of resistance. When a pathogen undergoes genotoxic stress, it activates specialized DNA copying enzymes that copy in an error-prone way. This process originates mutations that are in most cases lethal, but some mutations in genes linked to drug action may be beneficial. For this reason, the idea of targeting components of the DNA repair mechanisms of these pathogens is attractive. Nevertheless, we have a long way to go in our understanding of DNA repair in pathogens and the main reason for this is the complexity of DNA repair pathways. These mechanisms fight against different kinds of damage such as single strand breaks, double strand breaks, and base modifications that lead to mutations, loss of information, transitions and transversions. In addition, a huge battery of enzymes on these mechanisms is related, with very specific functions and signaling pathways that are not well-understood so far. Consequently, the inherent complexity of DNA repair mechanisms leaves us an abundance of unanswered questions, as well as the need to increase our understanding of some unknown and known processes. It is crucial that we achieve a good comprehension of key processes like damage detection, activation of checkpoints pathways, cell cycle arrest, DNA repair mechanism initiation and the mechanism pathway and signaling routes. This understanding will allow us to choose the right molecules involved in these mechanisms as drug targets against pathogens. Targeting these mechanisms could compromise the survival of pathogens to the oxidative damage caused by the immune response, and consequently decrease the proliferation of drug-resistant mutants.

The objectives of this review are: i) present the mechanisms of DNA damage repair in

prokaryotes and ii) propose potential targets within these mechanisms for the developing of novel drugs.

2. Genetic Elements that Lead to Variability and Mutations

2.1. In Prokaryotes, Plasmid DNA is Abundant: Plasmids are considered transferable genetic elements capable of replicating themselves within a host. They are double stranded and in most cases circular and are found in *Archea* and *Bacteria* domains as well as in some eukaryotes like yeasts. Their size varies from 1 to over 1000 kilobases. Each cell may harbor from one to thousands of copies of the same plasmid within it. Plasmids are a mechanism for horizontal gene transfer within a population of microbes that normally confer a selective advantage in certain environmental conditions. The process of transference of plasmids is known as conjugation. In this context, there are two main types of plasmids: conjugative plasmids and non-conjugative plasmids. Conjugative plasmids are those that contain transfer genes that perform the complex process of conjugation. Non-conjugative plasmids cannot initiate conjugation, and they can only be transferred with the assistance of conjugative plasmids. Plasmid of different types can coexist in a single cell, offering more selective pressure advantages to that cell (11). According to their function, plasmids could be classified in 5 main categories: 1) resistance plasmids, which contain genes of resistance against antibiotics or toxic substances; 2) fertility plasmids, which contain the trans-genes that allow conjugation; 3) col-plasmids, which encode for the production of bacteoricines, which are substances that kill other bacteria of the same genus; 4) virulence plasmids, which turn bacteria into pathogens; and 5) degradative plasmids, which enable the digestion of unusual substances like hydrocarbons or other contaminants (11).

2.2. Transposable Genetic Elements in Bacteria

2.2.1. Insertion Sequences (IS): Insertion sequences are small elements of approximately 1000 base pairs or less, with ends of 15 to 25 base pairs inversely repeated (IR). They have just one gene that encodes for the transposase enzyme. In many bacteria genomes exists a great amount of insertion sequences. In addition, conjugative plasmids are rich in insertion sequences. The frequency of insertion of an insertion sequence in a determined gene is about 10^{-5} to 10^{-7} (12).

2.2.2. Transposons: Transposons are sequences of 3000 to 20000 base pairs, that have at least one gene that encodes for transposase and one gene with a certain function unrelated to transposition. These other genes can encode for antibiotic resistance or heavy metals resistance (12). Two main kinds of transposons exist: The first kind is 1) composite transposons. These have two identical or almost identical insertion sequences in both ends. The transposition of these elements depends on one or both copies of the insertion sequences. The second kind is non-composite transposons, which lack the insertion sequences, and their ends are just two short sequences inversely repeated. In their central part they have the gene that encodes the transposase, and sometimes a gene for transposition regulation. They also contain genes for antibiotic resistance (12).

2.2.3. Integrons: Integrons are transposable elements with inversely repeated ends and antibiotics or heavy metals resistance genes that also have a gene that encodes for integrase. Their transposition mechanism is similar to the insertion sequences or transposons mechanisms, but in this case the integrase have allowed them to perform site specific recombination. For this reason, they have acquired genes from other

genetic elements. Integrons are usually part of greater transposons (12).

3. Molecular Basis of DNA Damage and Mutation

In nature, DNA molecules have to deal with many chemical and physical agents that are likely to cause severe damage on them and originate mutations. The two main types of DNA damage are: 1) damage that is originated within the cell because of the cell's natural physiology, known as spontaneous or endogenous damage; and 2) damage that is originated from the environment, known as environmental damage. In the spontaneous damage category, it could be mentioned the mismatches that arise during DNA replication, the damage resulting from spontaneous modifications of DNA (incorporation of uracil, deamination of bases, depurination and depyrimidination), and oxidative damage caused by oxygen reactive species (ROS). In the environmental damage category, they are the base damage and strand breaks caused by ionizing radiation, photoproducts originated by UV radiation and the alkylation of bases caused by alkylating agents. In addition, genetic transposable elements could cause mutations in bacterial genomes (13).

In the following sections the molecular mechanisms of these different kinds of damage were discussed.

3.1. Mismatches: Mismatches are mainly originated when DNA polymerase commits an error that is not corrected by the 3' exonuclease activity. The incorrect pairing occurs because of rare and less stable forms of nitrogenous bases appearing during replication: the tautomers (mentioned above). The proton's change of position alters the bonding properties of the base. Table-1 shows the bonding properties of tautomeric forms of nitrogenous bases.

Table 1. Bonding properties of tautomeric forms of nitrogenous bases.

Tautomeric Form	Behaves as	Bond Formed
A (Imine)	G	A-C
G (Enol)	A	C-A
C (Imine)	T	T-G
T (Enol)	C	G-T

Transitions can also be caused by deamination of cytosines. Deamination turns cytosine into uracil, which can then base-pair with adenine. Therefore, in the next round of replication, the complementary strand containing the adenine will serve as a template to pair a thymine, originating a transition G:C to A:T. The Deamination can occur spontaneously, but it appears less frequently in double stranded DNA than in single stranded DNA. Normally, uracil is not added to DNA due to the process of dUTPase being encoded by the *dut* gene, but strains lacking this gene have higher chances to incorporate uracils originated from the deamination of cytosines (13).

3.2. Depurination and Depyrimidination: Depurination and depyrimidination are alterations of the DNA structure, in which a purine or a pyrimidine is removed respectively by hydrolysis of the glycosidic bond from the deoxyribose sugar. After this phenomenon, the absence of information from the complementary strand will lead to mutation because the BER (base excision repair) will add an incorrect base. This would originate transition or transversions mutations (change of a purine-pyrimidine pair for a pyrimidine-purine pair). The main cause of depurination and depyrimidination is the presence of endogenous metabolites going through chemical reactions. Depyrimidination occurs less frequently than depurination. This happens because purine is a susceptible group, and the anomeric carbon is especially reactive towards nucleophilic substitution, making the

carbon-purine bond longer and weaker, and therefore susceptible to hydrolysis (13).

3.3. Oxidative Damage: Oxidative damaged is caused when reactive oxygen species (ROS) attack the DNA (14). ROS are generated in cells as byproducts of respiration and by ionizing radiation. The two main radicals involved in DNA damage are the peroxide radicals (H_2O_2) and the hydroxyl radicals (OH^-). A large variety of chemical derivates of the nitrogenous bases are produced when DNA is attacked by ROS. For example, OH^- radicals attack C5=C6 double bonds. If this occurs in thymine, it leads to formation of thymine glycol that could block DNA replication (15). Another common example of ROS's effect is the conversion of guanine into 7, 8-dihydro-8-oxoguanine which is mutagenic because it mispairs with adenine. Figure 1 shows examples of DNA damage induced by reactive oxygen species.

3.4. Environmental Damage: Several environmental factors could damage DNA. The first one is ionizing radiation. Eighty percent of ionizing radiation in cells takes electrons from water forming the H_2O^+ radical. If oxygen is present, hydroxyl and peroxide radicals are also formed. Radicals formed by ionizing radiation cause the same base damage that is caused by radicals formed from the metabolism of the cell. Furthermore, ionizing radiation can cause damage to the sugar residues. Such damage leads to single or double strand breaks, which can result in cell death or mutagenesis if they are not repaired. A break in one strand is repaired easily using the opposite strand as a template. Breaks in both strands are repaired as single breaks if they are well spaced. But if breaks in both strands are directly opposite or separated by few base pairs, it leads to a double strand break that separates chromatin (16).

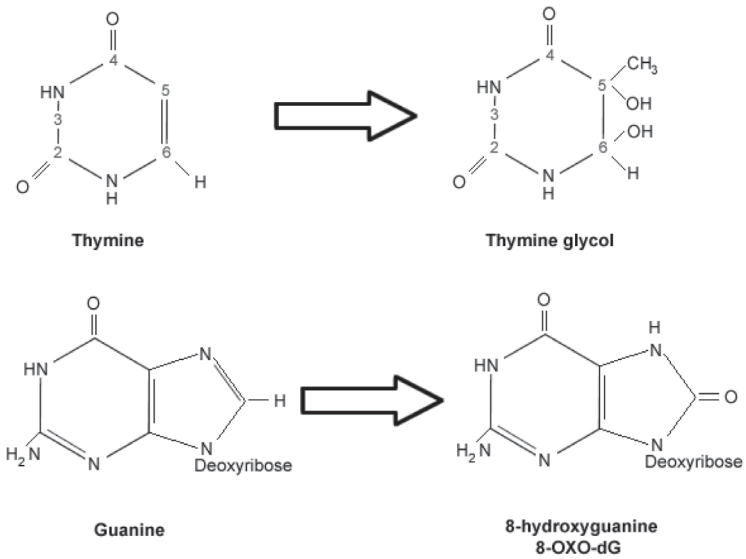

Fig. 1. Examples of DNA damaged bases induced by reactive oxygen species. Upper panel shows the conversion of thymine into thymine glycol, this form could block DNA replication. Lower panel shows the conversion of guanine into 8-hydroxyguanine which is mutagenic because it mispairs with adenine.

The second main environmental factor that damages DNA is UV radiation. Photoproducts are most efficiently induced with 254nm UV light. UV radiation produces cyclobutane pyrimidine dimers. These dimmers are form when adjacent pyrimidines are covalently linked through carbons 5 and 6. This damage interferes with DNA transcription. The most common pyrimidine dimer is the thymine dimer (17).

The last environmental factor that leads to DNA damage is alkylation. An alkylating agent is an electrophilic compound with high affinity for nucleophilic centers in organic macromolecules. We can distinguish between mono-functional alkylating agents, which have one reactive group and can react with one nucleophilic center in DNA, and bi-functional alkylating agents, which have two reactive groups and can react with two sites in DNA. Alkylation of DNA can result in mutations in several ways. The addition of alkyls groups will distort the DNA double helix. As well, alkylation can lead to mismatches that result in transitions.

3.5. Mutations Caused by Genetic Transposable Elements: The insertion of a transposon or an insertion sequence originates the inactivation of the gene because of the reading frame shift. After the insertion, deletions of the transposable element and adjacent genetic sequences can occur. On the other hand, two insertion sequences of the same type, located at a certain distance can go through recombination of the inversely repeated ends or translocation of the genetic material between them (12).

4. Single strand break DNA repair mechanisms

Single strand breaks is the most common type of DNA damage that is found in cells. SSB have an arising frequency in tens of thousands per cell per day. SSBs consist in one or more discontinuities in a single strand of the DNA double helix. The most important consequence of SSB in chromosome is the collapse of the DNA replication fork in the S phase during the cell cycle leading in some cases to the arise of double strand breaks (18).

4.1. Base Excision Repair in prokaryotes: Base excision repair is a cellular mechanism that fixes damaged DNA throughout the entire progression of the cell cycle. This mechanism exists because there is a high spontaneous-mutation rate present in organisms during DNA replication. There also exist mutagens in the environment which can further increase this inherent mutation rate that all organisms posses. BER is important for removing damaged bases that could cause mutations by incorrect pairing or lead to breaks in DNA during replication. It is more specifically directed to repairing single-strand breaks of DNA. This means that the damaged base is located in only one of the helixes; it is eliminated and then correctly synthesized using the complementary base on the other helix. The other mechanisms that specialize in single strand repair are the nucleotide excision repair system and mismatch repair system. These damages affect the fidelity of DNA replication. Depending on the nucleotide to be removed, the pathway has different approaches and variations but the specificity is conferred by the DNA-N-Glycosylase to be utilized (19).

4.1.1. BER mechanism in E. coli: There are several enzymes that are documented for *E. coli* to continue with its repair mechanism and correct DNA replication. In *E. coli*, 2, 6-dihydroxy-5N-formamidopyrimidine-DNA glycosylase (also known as Fapy or Fpg DNA glycosylase) removes the mutagenic adduct from DNA. Fpg is a DNA glycosylase that removes Fapy and 8-oxo-G from DNA (20,21,22). In *E. coli*, several DNA repair enzymes, known as the GO system,

prevent mutagenesis caused by 8-oxo-G. This system consists of: MutM [2, 6-dihydroxy-5N-formamidopyrimidine (Fapy)-DNA glycosylase, Fpg], MutT (8-oxo-dGTPase), and MutY (adenine-DNA glycosylase) (23,24). Predominantly, ROS has proven to cause DNA damage and affect damage repair. *E. coli* has two families of AP endonucleases: the family of endonuclease III and the family of endonuclease IV. Endonuclease III is coded by the *xthA* gene and endonuclease IV is coded by the *nfo* gene (25,26,27). These two endonucleases have great relationship with the Fpg protein which removes damaged purines from DNA (28). If either of these proteins is eliminated or bypassed, it could potentially hinder the organisms' ability to manage the damages in the DNA. For this reason, these proteins are excellent candidates for continued study.

Several mutagens affect DNA repair efficiency. For example, hydrogen peroxide (H_2O_2) causes sensitivity in the gene (*zthA*) that code for endonuclease III (29). This generates many AP sites that, if not eliminated by the DNA-glycosylases, will result in the decrease in the survival rate of *E coli* (30). Endonuclease III (which makes up for 90% of nucleolytic activity) has the function of AP-lyase and it cleaves 3´ to the AP site leaving a 5´ phosphate and a 3´ ring opened sugar. H_2O_2 generates free radicals that cause DNA strands to break, leaving 3' phosphate groups. Once these products are formed, they block the action of polymerases and are susceptible only to endonuclease III (Fig 2). *E. coli* mutant strains for the *zthA* gene (31), have demonstrated that, with these conditions, the survival rate for cultures subjected to treatments with H_2O_2 decreased. Comparatively, *nfo* mutants have suggested that endonuclease IV also bears some responsibility in the repair of lesions caused by this agent, but the bacteria depends more on the function performed by endonuclease III (32). It has been described recently that endonuclease IV plays a role in an alternative pathway to classic BER, called nucleotide incision repair (NIR), in which it cleaves DNA generating terminus which constitutes the DNA polymerase target. Therefore, the advantage of this pathway is avoiding the genotoxic intermediates generated in BER mechanism (32).

4.2. Nucleotide Excision Repair in prokaryotes: The first evidence of the existence of the NER repair system is shown in the 1960s, when researchers observed the excision and damage repair induced by UV in bacteria. This damage is mainly cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (6-4PPs). If these damages persists in the bacteria genome, eventually can cause the impediment of DNA replication and transcription that can cause cell death. For this reason, cells posses several mechanisms that contribute to survival after UV irradiation. These mechanisms include UV-absorbing pigmentation, repair or removal of the UV photoproducts, cell-cycle checkpoints and some grade of damage tolerance that permit the replication of the cells even when damage is still unrepaired (33).

In 1965, Howard-Flanders *et al* (34) showed that on *E. coli* mutant strains and they found evidence suggesting that, the repair of thymine dimers and other damages caused by UV radiation in the DNA helix is controlled by three genetic loci: *uvrA, uvrB, uvrC* (34). In the first instance, the expression of *uvrA* and *uvrB* were only related with the SOS response to damage caused by mutagenic agents to DNA. However, more recent observations determined that the inducibility of UvrC expression is also regulated by the SOS response at the cellular level (35). All three *uvrA, uvrB* and *uvrC* are regulated in the same way and they are jointly involved in the synthesis of the UvrABC

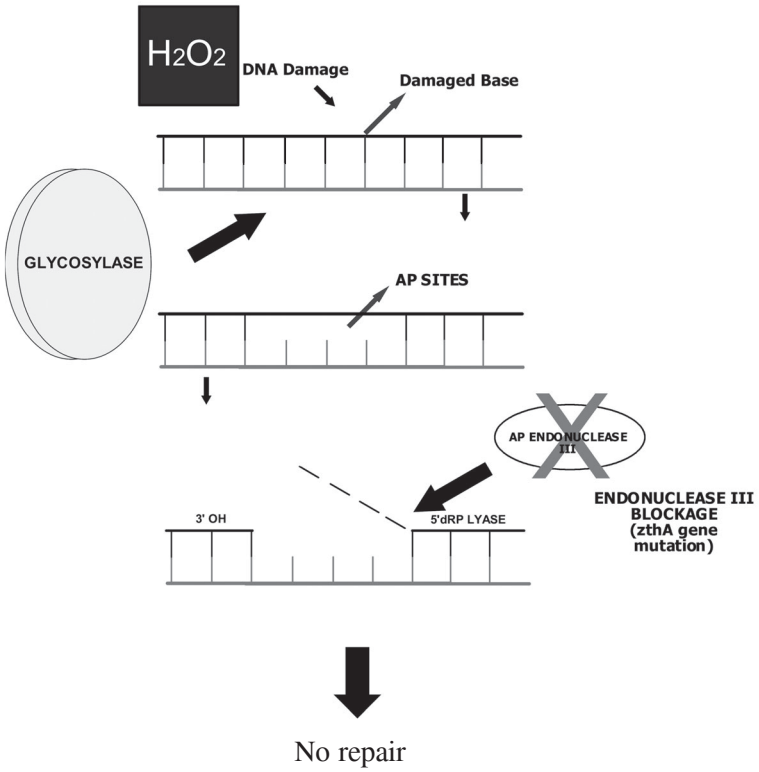

Fig. 2. Proposed target to inhibit the base excision repair mechanism. DNA is damaged by several agents, among them, hydrogen peroxide. This produces damaged bases that are excised by a DNA glycosylase which generate an apurinic/apyrimidinic site (AP site). The only way that *E. coli* can repair a damaged caused by H_2O_2 is through endonuclease III and endonuclease IV. If the synthesis of either of these enzymes is inhibited, the bacteria is incapable of remedying the damage caused by this agent.

endonuclease (35,36). The UvrABC proteins recognize and cleave damaged DNA in a multistep adenosine triphosphate (ATP)-dependent reaction. Bacteria undergoing repair have such a late requirement for the *uvrC* gene product, unless UvrC plays a semi detached, independent role in the detection and processing of DNA damage (37). It has been showed that the UvrABC catalyzed *in vitro* repair reaction can be separated into two consecutive steps: an ATP-dependent UvrAB-catalyzed binding to the damaged template, followed by an ATP-independent UvrC-catalyzed endonucleolytic step (37). Sharma and Moses (37) concluded from repair experiments in permeable cells, that the UvrC protein might be required late in the incision step. *In vitro* studies on DNA incised by *Micrococcus luteus* pyrimidine dimer-N-glycosylase, revealed that extracts from UvrC + cells catalyzed repair replication by DNA polymerase I (38).

The studies mention above has shown that the expression of *uvrA* and *uvrB* genes responsible for the NER is regulated by the SOS system, which acts in the presence of agents that cause extensive damage to double-stranded DNA (34,35). In the first instance, the expression of

uvrA and *uvrB* were only related with the SOS response to damage caused by mutagenic agents to DNA. However, more recent observations determined that the inducibility of *uvrC* expression is also regulated by the SOS response at the cellular level (35,37).

4.2.1. NER repair pathway

4.2.1.1. NER repair in prokaryotes: The a function in DNA replication. The UvrABC complex recognizes DNA damage and repairs it in a series of ATP-dependent reactions. Roughly, the NER system consists of three main steps: recognition of damaged DNA, excision and repair, and finally ligation of the repaired segment (40). Although NER is conserved in prokaryotes, archae bacteria and eukaryotes, the simplicity of the proteins make a difference in this system in relation to humans and prokaryotes. In prokaryotes, the NER acts in a much more simple way. It only requires the presence of three enzymes: UvrA, UvrB and UvrC, which make up the UvrABC complex. (40)

The first step is the recognition of the DNA lesion. It is the most important, as it has proven to be the key step for success of the entire repair system. Unlike other repair systems, NER is the only one in which recognition is given to the open threads. Initially the UvrA dimer recognizes sites of DNA damage and causes a twist in the double strand of DNA that allows the binding of UvrB to form a complex called UvrA2B. Given the characteristics previously studied in the recognition of lesions in DNA by this complex, it is thought that recognition is not only chemically, but transcends to recognize the impact that these injuries have in the flexibility of DNA (changes in DNA topology) (Fig. 3).

One of the features that make the NER one of the most important repair systems and one of the most used of all is because of its great ability to recognize many different types of lesions in double-stranded DNA. It has a wide range of injury recognition. The mechanism used is uncertain but there are many speculations. It has been supposed that the UvrA does not recognize the injury itself, but rather the distortion of the double helix of DNA induced by the lesion's presence. This helix distortion may include the disruption of base pairing as well as the bending of DNA strands (41). UvrA protein has two DNA binding sites, one located at the N-terminal and the second located at the C-terminal. It has been shown that this protein binds to damaged DNA both in the presence or absence of nucleotide cofactors, thus forming a double mark on the string that marks the site of injury that needs to be repaired. The size of the footprint is about 33 bp (42). For a proper recognition process, the functionality of the ATPase domains of the system is vital, so the UvrA protein can successfully recognize the lesion in the DNA.

After the lesion sites are detected, both enzymes work together in an ATP-dependent reaction, resulting in a stable complex between UvrB and damaged DNA. The UvrA dimer dissociates the complex and returns to be used for further recognition of DNA lesions (39). In this way the complex is formed between the injured DNA pre-incision and UvrB protein.

After this occurs, the UvrC endonuclease recognizes the pre-incision complex formed by the damaged DNA and the UvrB protein, and together they cut the piece of DNA chain damaged. The process is this; make two incisions, one in the eighth phosphodiester bond in the direction 5' downstream to the injury, and the second incision at the fourth phosphodiester bond 3' upstream the injury. The first incision is made in the last 3' through the UvrB while the second takes place at the 5´ and goes through the UvrC. The resulting fragment is finally removed. (43) While UvrC protein dissociates

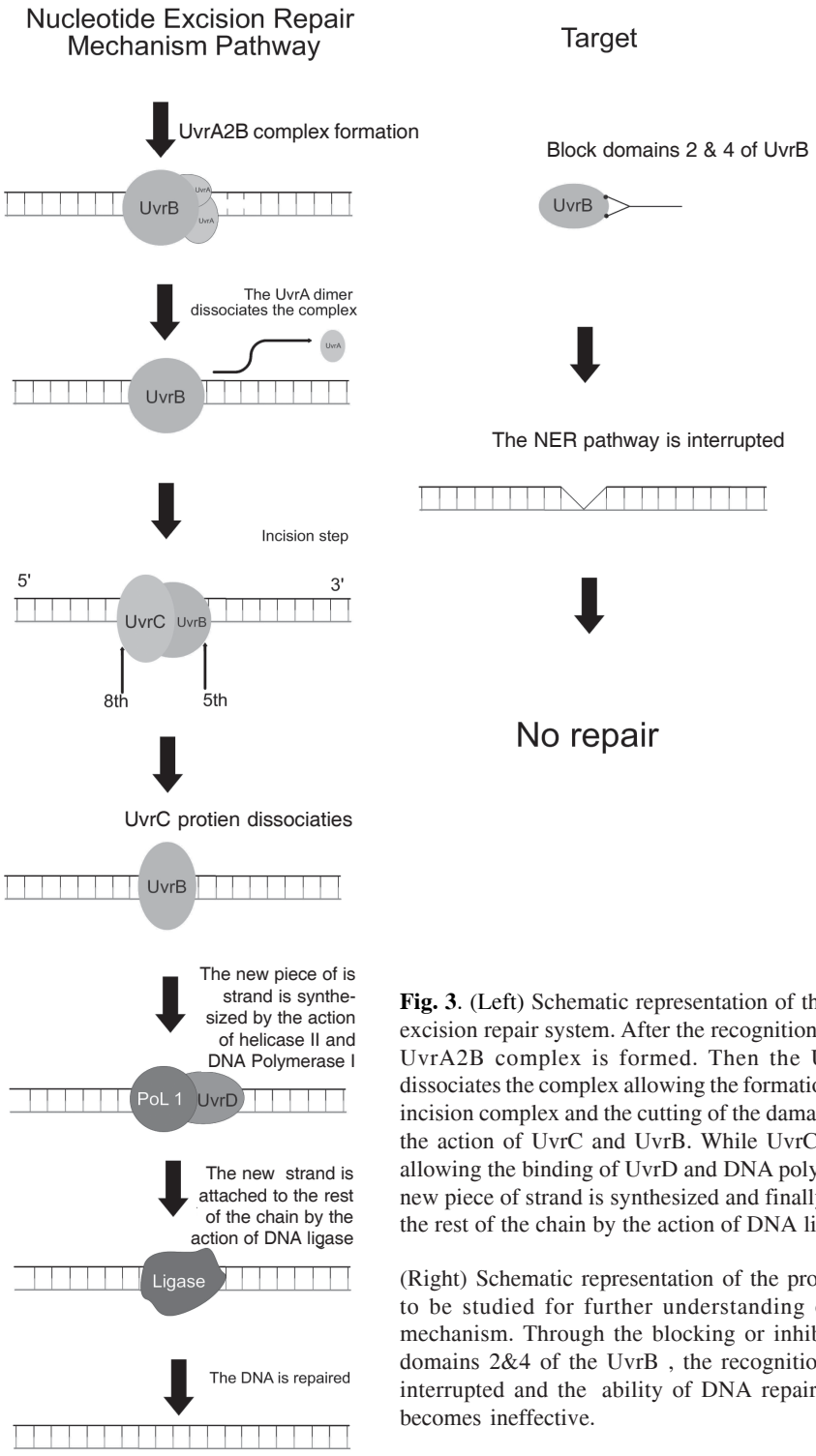

Fig. 3. (Left) Schematic representation of the nucleotide excision repair system. After the recognition process, the UvrA2B complex is formed. Then the UvrA dimer dissociates the complex allowing the formation of the pre-incision complex and the cutting of the damaged DNA by the action of UvrC and UvrB. While UvrC dissociates allowing the binding of UvrD and DNA polymerase. The new piece of strand is synthesized and finally attached to the rest of the chain by the action of DNA ligase.

(Right) Schematic representation of the proposed target to be studied for further understanding of the NER mechanism. Through the blocking or inhibition of the domains 2&4 of the UvrB , the recognition process is interrupted and the ability of DNA repair using NER becomes ineffective.

allowing the binding of UvrD (a helicase II), the UvrB is displaced by DNA polymerase. The new piece of strand is synthesized by the action of UvrD and DNA polymerase I restructuring the new strand in the presence of dNTPs. The new strand is attached to the rest of the chain by the action of DNA ligase.

***4.2.2. NER** in Mycobacterium* sp.: Among some of the pathogens that use this pathway to repair their DNA is the *Mycobacterium* genus. The studies previously done in *Mycobacterium tuberculosis* and *M. smegmantis* lead to the conclusion that the NER repair system is very important in the *Mycobacterium* genus to repair their DNA resulting from external damage (44).

4.2.2.1. Mycobacterium* sp. *Case: *Mycobacteria* are an important group of pathogens that affect humans. It resides in host macrophages, and due to the hostile conditions in which lives it can be considered one of the most successful pathogens. It is prone to survive in a hard environment of macrophages, supports low temperatures, low pH, and environmental stress caused by external factors like UV exposure, among others (44). Additionally, in recent years the coinfection rate has grown between *M. tuberculosis* and HIV, as well as the emergence of strains resistant to the drugs being used (45).

It is well known that DNA is a biological target for ROS and NIS, in addition to the many toxic radicals that are mutagenic. The DNA of the pathogen within the host is exposed to all kind of damages and injuries, which jeopardizes the integrity of its genome. It can be seen clearly how the DNA repair systems are critical for virulence and survival of intracellular pathogens so they can maintain the integrity of its genome and to remain within host cells (45). Previous studies have established the role of DNA repair systems in the success that has this pathogen to persist in host macrophages (45). By means of gene knock-out it has been determined that the strains that are deficient in NER are the most sensitive to situations that cause DNA damage. For this reason it is suggested that the NER repair system is extremely important in the genus *Mycobacterium* (46). Through studies conducted in *Mycobacteria* has indicated that UvrB is the central part of the repair system. In studies conducted by Darwin *et al* (47) demonstrate that UvrB-deficient strains of *M. tuberculosis* showed a marked non-survival pattern in mice (47).

4.2.3. UvrB as drug target: Studies by Deisenhofer *et al* (48) are the first to elucidate the structure of one of the components of the NER. They elucidated the structure of the enzyme UvrB from *Thermus thermophilus*. The enzyme UvrB consists of three domains: H1, H2, and P1. The H1 and H2 domains are very similar and they share the same aminoacid sequence. Both are connected by a linker or connector. They have a large central beta sheet flanked by many alpha helices (48).

Helicase activity has been held by the UvrB because the H1 and H2 UvrB domains are similar to those that have helicase activity. Therefore it should have the ability to "sense and scan" double-stranded DNA for lesions. But we know the limited ability of the ATPase activity in UvrB, merely moving about 22 base pairs depending on the DNA melting temperature. For this reason some authors describe this activity not as a helicase but as "destabilizing thread" (48).

The UvrB protein repair system plays an important role in the process of recognition of the injury (49). Their interaction with UvrA and DNA strands causes a series of conformational changes in the structure of the double chain waste leaving the arms uncovered in A1 and A2 of the UvrB. This process is decisive in the recruitment of UvrC to the DNA injured (50). We can notice how UvrB actively participates in the three main

steps that make up the NER, and thus can be regarded as a central molecule in this repair system. For this reason we can consider the UvrB to be a good target to inhibit the NER repair system and thus be used as a DNA based antimicrobial target. As previously mentioned in the text, the enzyme UvrB is actively involved throughout the process of repair system for excision of nucleotides. It interacts with all other key parts in the system. Ranging from the recognition phase, pre-incision complex, cleavage and sealing piece has been cut by the DNA polymerase and ligase.

Due to its vital importance in the system, reversing the activity of the NER should be viewed as a realistic and possible objective (Fig. 3). It can be used as a therapy against pathogens that use this pathway to maintain the integrity of its genome inside the host. Because of the importance of UvrB and the role it plays in NER, using it to inhibit the repair system in pathogenic prokaryotes might be a good objective that should be considered.

5. Double strand breaks DNA repair mechanisms

The DBS can cause cell death and cellular transformation, and deleterious mutations in bacteria (51,52). In addition, antibiotics can generate DSB, hence activating the DNA repair mechanisms in bacteria, a situation that is not desirable in pathogens. The treatment of bacterial infections diseases has become a difficult task since the arising of bacteria antibiotic resistance. In the case of pathogenic bacteria, not only the antibiotics apply selective pressure, but also the immune response plays an important role (53).

The two main mechanisms that repair the DSB are the HRR and the NHEJ. The HRR is considered a more accurate repair mechanism, because the homologous sequence is used to direct a faithful repair of the DSB. The HRR is triggered during the sister chromatids phase of the cell division. In contrast, the NHEJ repair acts in the G1 phase and does not require a DNA template for the DNA repair (54). These two pathways, HRR and NHEJ are described as "error-free" and "error-prone" respectively (55).

5.1. Homologous Recombination Repair in prokaryotes: The comparative and evolutionary studies of bacterial homologous recombination systems, realized by Rocha *et al*, have shown that almost all bacteria groups possessed homologous recombination machinery (56). Studies insight that the homologous recombination repair mechanism is highly conserved among prokaryotes, archae and eukaryotes. In the repair mechanism, a variety of enzymes work together to perform the DNA reparation. Of these enzymes, the most conserved is the RecA, which is a recombinase. The other enzymes that participate in the DNA repair are the RecBCD holoenzyme, and the enzymes that resolve the Holliday junction. The main functions of RecA are: strand exchange, and promoting the annealing of the 3´ single strand DNA from the broken chromosome with its homologous (57,58).

5.1.1. Homologous Recombination repair mechanism: In the mid 1940´s, the homologous recombination mechanism is first described in *E. coli*. And so far three main pathways have been described: the RecBCD, RecFOR and the AddAB. The RecBCD (primary pathway), which promotes the repair of DSB, the RecFOR (secondary pathway), which is involved in the ssDNA gaps repair and which is supposed to work when the primary pathway is inactive, and last but not least the AddAB pathway. Since the re-combinational repair mechanism is highly conserved among bacteria, the two pathways are present in very different species like *E. coli* and *Bacillus subtilis* (56,58,59). Both pathways

provide the ssDNA with RecA and permit the invasion of the homologous molecule. Although these pathways have the same function, they repair different types of DNA damage (56).

5.1.1.1. RecA, the HRR central protein: RecA is an important protein that participates in the DNA SOS induction mechanism, DNA repair and DNA recombination. Unlike the RecBCD complex, the RecA enzyme is not part of an operon (59). The RecA protein (and RecA homologs) is highly conserved in prokaryotes and in other organisms, and this protein catalyzed the key step of strand invasion and strand exchange in HRR (56). An important step in the RecA function in the cell is the ATP hydrolysis in a DNA dependant mode (60). To induce the conformational rearrangements for the strand exchange reaction, the RecA helps pairs the ssDNA with its homologous duplex DNA and hydrolyzes ATP, and this reaction produces two new DNA molecules (53). The RecA is a 38kDa enzyme and functions as part of a helical nucleoprotein filament; in addition, the main function of this protein is the search of homologous sequence both catalytically and stoichiometrically (59). This repressor binds to a promoter that activates the transcription of nearly 40 genes that are involved in repairing DNA damage (53,61). In *E. coli,* RecA expression is up-regulated by events that challenge the integrity of the bacteria genome. When the HRR is activated, this pathway can follow two different ways: de-repression of the SOS regulon or re-combinational DNA strand exchange (53). If errors are generated during the DNA replication, RecA will inhibit cell division by SOS induction. Hence, damaged DNA activates RecA and its activated form catalyzes self-cleavage of LexA. The SOS response increases the ability of the cell to repair DNA damage and delays cell division (59).

5.1.1.2. RecBCD pathway: The central step in this pathway is the synapsis between the homologous DNA molecules. The RecA enzyme catalyzes the DNA strand exchange, and forms a filament on the ssDNA, which is the active species in the exploration of homology. Besides, it is an important component in future invasions of the homologous duplex DNA. Therefore, a DNA lesion that requires recombination repair must first be processed in ssDNA by the action of helicase and nuclease. This complex has many biochemical activities such as DNA binding, DNA helicase, RecA binding, DNA dependent ATPase, ssDNA endonuclease, Chi regulated nuclease, dsDNA exonuclease, and helicase activity (62).

RecBCD is a heterodimer and consists of three different polypeptides. The RecB is a 134kDa protein, which is a DNA dependent ATPase, a weak helicase that operates in the 3'-5' direction. In addition, RecB plays an essential role in the RecA loading mechanism onto Chi containing ssDNA. The Chi sequence (5'-GCTGGTGG-3') is a regulatory sequence and a critical cis-acting DNA element. RecC can stimulate the ATPase and helicase activities of the RecB protein. Further, RecD has two important activities, which are the ssDNA dependent ATPase activity and the 3'-5' DNA helicase activity (51,62). The RecBCD substrate is a free blunt or almost blunt duplex DNA end. The RecBCD enzyme possesses a helicase and nuclease function. This enzyme complex initiates the DSB repair by converting a blunt dsDNA end into a duplex molecule leaving a 3‘ terminated ssDNA tail. RecBCD directs the RecA protein onto this ssDNA (56,62).

The RecBCD holoenzyme binds to the damaged dsDNA. This enzyme unwinds and degrades DNA from one end until it finds the Chi sequence site in the correct orientation (63). When the RecBCD enzyme recognizes the Chi

site, its function is modified, stops degrading the 3'ending strand and begins to produce a 3'single strand extension. These extensions are needed for the RecA ssDNA strand invasion, and this step is the central event in HRR (64). Then the RecA protein binds to the Chi terminated 3' ssDNA tail (56,63). When this loading mechanism is not present, the resulting ssDNA product is rapidly and tightly bound by ssDNA binding protein, which binds ssDNA nonspecifically. When the RecA nucleoprotein filament is formed, the next step is the most crucial phase of HRR, which is the search of the homologous sequence donor, where the RecA enzyme performs a scanning process of the whole genome looking for the sequence homology (64). This eventually results in loading of replication DNA helicase (62). The strand exchange reaction is propagated uniquely 3'- 5' relative to the ssDNA substrates (Fig. 4) (65).

Another protein that participates in the HRR, is the single strand DNA binding protein (SSB). This protein can inhibit or enhance the RecA filament formation (65). The filament assembly on ssDNA occurs with distinct nucleation and extension steps with extension proceeding 5'- 3'. During the DNA strand exchange the SSB binds to the displaced strand of the duplex substrate. The effect of SSB depends on when it's added (65).

So far, two independent enzymatic systems for DNA junction in *E. coli*, have been described: the RuvABC resolvasome and the RecG helicase. In addition, it is found that the DNA interaction mechanisms of these systems in some measure complement each other, even though they are quite different, since mutants with single mutations in one or the other system show only a fair defect in recombination repair. This suggests that there exists more than one way of DNA junction resolution *in vivo* (59).

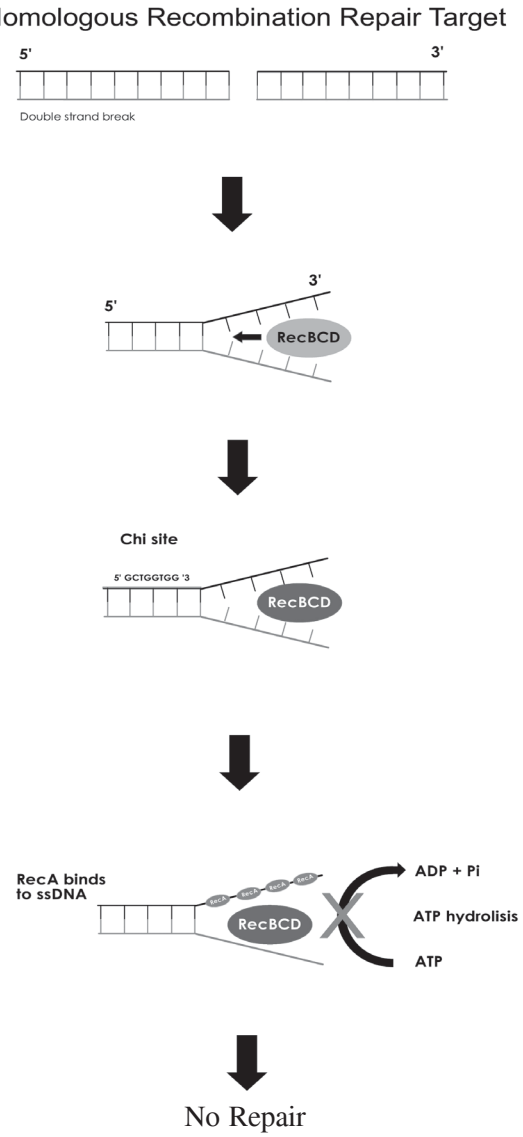

Fig. 4. The RecBCD holoenzyme binds to the damaged dsDNA. Then this enzyme began to unwind and separates both strands until it finds the Chi site in the correct orientation, converting a blunt dsDNA end into a 3, terminated ssDNA tail. When the RecBCD enzyme recognizes the Chi site, its function is modified, stops degrading the 3, ending strand and begins to produce a 3, single strand extension. Then the RecA protein binds to the Chi terminated 3, ssDNA tail. RecA is activated in the presence of ssDNA and ATP. A RecA ATPase inhibitor could be a adjuvant for the inhibition of the homologous recombination pathway.

The biochemical activities of RuvABC proteins and the ways they interact with DNA junctions, both structurally and functionally are now well characterized. In the HRR, the RuvC and RuvAB proteins catalyze the resolution of the Holliday junction and branch migration (56).

5.1.1.3. RecFOR pathway: It has been found that in the RecFOR pathway, the RecF, RecO and RecR, interact with each other, but do not form an enzyme complex as the RecBCD holoenzyme. The RecJ enzyme also interacts in this pathway, and it is important in the RecBCD pathway. This enzyme is an exonuclease that helps extend the ssDNA region when is needed. It degrades the 5' strand from a duplex DNA end during unwinding by the helicase RecQ, generating a 3' overhang. In *E. coli*, RecJ is an exonuclease specific for 5'ssDNA. RecFOR, helps RecA bind to the ssDNA (66,56). In addition, as in the RecBCD pathway the RecA protein catalyzes the strand exchange (56). In the RecF pathway, RecQ and RecJ, work in conjunction for the DNA repair (62).

The RecO protein has diverse functions, like stimulation onto the RecA's conjunction of filaments, and onto SsbA coated single stranded DNA. This protein also modulates RecA mediated DNA strand exchange, and promotes the annealing of complementary DNA strands. In addition, RecO possesses an important role in the RecA plasmid transformation (67).

The single strand binding protein, SsbA is essential for cell proliferation, because it can inhibit the spontaneous annealing of complementary DNA strands (67). SsbA facilitates the RecO mediated strand annealing by means of the accumulation of non productive ternary complexes. How RecO mediates the DNA strand annealing occurs, could be as follows: The ssDNA-SsbA-RecO ternary complex, it is formed when the SsbA binds to the ssDNA and recruits the RecO. After the formation of the ternary complex, RecO interacts with SsbA-ssDNA and with itself leading to the formation of bridge structures, and the RecO protein decreases the half life of the SsbA-ssDNA complex. When RecO binds to the naked ssDNA, it distorts the ssDNA structure and prevents the SsbA binding or relieves it from ssDNA (67).

Studies performed by Manfredi *et al* (67), suggest that RecO has three main activities coordinated by SsbA: 1) can recruit RecA onto SsbA coated ssDNA; 2) can modulate the extent of RecA mediated DNA strand exchange; and 3) bridges SsbA coated ssDNA molecules, when the complementary promotes annealing (67). The strand annealing mediated by RecO is critical for the RecA filament extension and strand exchange during recombination mediated by RecA (67).

5.1.1.4. AddAB pathway: The AddAB pathway which is found in *Bacillus subtilis*. The AddAB is a nuclease/helicase protein complex that generates a ssDNA region at the DSB, and acts upstream the RecA. Downstream RecA, the RecG and RuvABC complex are involved in the formation of the Holliday junction and the crossover resolution (57). The AddA subunit of the AddAB protein complex presents homology regions with the RecB, which contains an N-terminal helicase domain and a C-terminal nuclease domain. The AddB subunit also contains a conserved nuclease domain at its C-termini. The helicase and nuclease domains of the AddAB, coordinate the bindings of dsDNA ends, and before the Chi sequences are encountered by the RecBCD, the AddAB catalyzes the unwinding and degradation of both DNA strands. The 3' strand cleavage activity ends when the AddAB recognizes the Chi sequence and the degradation of the opposite strand in the 5'- 3'. direction is not affected (58). Studies realized so far have elucidated different

HRR pathways, indicating a great diversity in proteins involves in the repair mechanism of DSB (58).

5.1.2. HRR in Helicobacter pylori and Mycobacterium tuberculosis: Here we briefly describe the HRR characteristics of these bacteria that make them so resistant to stressful environments.

***5.1.2.1. Helicobacter pylori* case** : The DNA recombination and repair mechanism of the *H. pylori* has been extensively studied, since this bacterium has successfully colonized the human stomach. Hence, it is a very useful microorganism for the understanding of bacteria pathogenicity. The results of an investigation led by Dorer *et al* (61), suggest that *H. pylori* requires the RecA and AddAB proteins for efficient stomach colonization. AddA is required for DSB repair by homologous recombination and the RecA expression is frequently induced by DNA damage, thus increasing induction of SOS (61). Since *H. pylori* is exposed to DNA damage in the stomach, this bacteria requires RecA and AddAB for DNA repair and other recombination events, and in this way it can accomplish the stomach colonization (61).

Genome sequence studies revealed that *H. pylori* lacks LexA, low fidelity polymerases, and a cell cycle repressor, suggesting that *H. pylori* is deficient in the SOS response. An investigation made by Dorer *et al* (61), indicates that individual induction of competence is a key component of the *H. pylori* reaction to DNA damage and implies the existence of a close connection between DNA damage and genetic variability during stomach colonization. Further, Dorer *et al* (61) have demonstrated that genes involved in DNA repair are only one of the many types of genes that are regulated by DNA damage. In response to DNA damage, a variety of genes with different functions are regulated, like the genes required for energy metabolism, membrane protein and fatty acids biosynthesis. But how these genes help the bacteria survival in the case of DNA damage is not well understood (61).

It could be possible that RecA may be necessary for a transcriptional response to DNA damage in *H. pylori*, even though this bacterium seems to lack lexA gene, by means of sensing and transmission of the damage signal. Also Dorer *et al* (61), suggest that under stressful conditions the *H. pylori* strain tested maintains a low mutation rate, which supports the hypothesis proposed by Schwarz and Salama, that *H. pylori* variation is driven by recombination among diverse strains. Further, Dorer *et al* (61) revealed that in *H. pylori* exists a relation between the natural competence and the response to DNA damage (61). An important finding was that in a heterogeneous population of *H. pylori,* a genetic exchange can be induced by signals produced by extreme environments that occasioned DNA damage. The selection of a fitter variation through the re-assortment of pre-existing alleles and the exchange of antibiotic resistance can be increased by the up-regulation of natural competence (61).

In *H. pylori*, has been found two groups of genes that could resolve the branch migration of the Holliday junction, the *ruvABC* and the *recG*. The RecG and RuvB enzymes are helicases, which are a common recombination intermediates and can branch migrate the Holliday junction. The RuvC is an exonuclease, of the RuvABC pathway, that nicks DNA, thus generating the Holliday junction resolution into dsDNA. Studies realized by Kang *et al* (51), try to elucidate which pathway is more prominent in *H. pylori*. These studies revealed that RecG competes with RuvABC for the DNA substrate. Nevertheless, RecG initiates an incomplete pathway in *H. pylori*. Consequently, the Holliday junction cannot be solved in the RecG pathways

causing a failure to repair the replication fork, naturally producing a dead end (51). It has been found that, in *H. pylori* the major recombination repair pathway is the RuvABC, and it is critical for DNA damage repair. Even though the RecG has a role in branch migration, this enzyme interferes with the recombination repair (51).

***5.1.2.2. Mycobacterium tuberculosis* case:** It has been found that during the different stages of the *Mycobacterium* infection, genes from the NER, BER, NHEJ and HRR were expressed; allowing the *Mycobacterium* genome to maintain a great stability. For example, gene expression experiments performed with microarrays suggest that genes involved in the HRR pathway were expressed during the infection active phase (68).

The ability to repair damaged DNA in *M. tuberculosis* is very important in these bacteria because it helps protects them from the immune cells attack. The RuvC enzyme is a specific endonuclease that has a function in the final step of the Holliday junction resolution. The *ruvC* gene is induced following DNA damage (69). It has also been proposed elsewhere that *M. tuberculosis* has at least two mechanisms that control gene expression in response to DNA damage. The first mechanism is mediated by the LexA protein that binds to the SOS box, which is up-stream the regulated gene with liberation of this repression, and requires RecA. The second mechanism is independent of RecA (69).

The *ruvC* gene is part of a group of 28 genes that were identified by genomic analyses and it's thought that it can potentially regulate by both LexA/RecA and alternative mechanisms. The control of the DNA cleavage activity of RuvC is important to facilitate DNA repair (69).

It has also been found in *E. coli* that RuvC is expressed in a very low level and is not induced by DNA damage like in *Mycobacterium*, however its activity is stimulated by interaction with the RuvAB branch migration complex. It was also found that in *E. coli* the RuvC enzyme is controlled post-translationally, and not at the transcriptional level. In the other hand, *ruvC* is transcriptional, regulated with *ruvAB* in *M. tuberculosis* (69).

5.1.3. RecA as a drug target: One of the main problems for the treatment of diseases caused by bacterial pathogen is their ability to become resistant to antibiotics whereby causing an important health problem. As mentioned above, the RecA enzyme plays a very important role in the DNA repair and stalled replication fork, and participates in processes that promote mutations induced by stress and horizontal gene transfer (70).

An important characteristic of the RecA protein is their activation in the presence of ssDNA and ATP. This protein is loaded to the ssDNA with ATP, and forms a helical homopolymeric filament (the RecA-ssDNA filament), which has enzymatic and signaling properties. This ATP molecule is later hydrolyzed (70). This enzyme has been proposed as a bacterial drug target by Sexton *et al* (53), as an adjuvant with the potential to inhibit the mechanism by which bacteria obtain the very harmful drug resistance. The DNA damage repair and stalled replication is the central activity of the RecA protein, but also participates in the adaptive mutagenesis and horizontal gene transfer (53). It has been found that the SOS and recombination processes mediated by RecA protein can be activated in response to antibiotic treatment. Hence, instead of treating diseases we are probably helping the prokaryotic pathogen develop a greater variability, originating a drug resistance strain (53).

Sexton *et al* (53) found that RecA inhibition on bacterial drug resistance is through a high throughput screening that potentially identifies

a RecA inhibitor. The investigators found 40 possible inhibitors of the RecA ATPase activity. Further structured activity relation analysis would be needed for the development of a successful RecA inhibitor (53).

5.2. Non-Homologous end Joining (NHEJ) in prokaryotes: For a long time, NHEJ had not been considered as a mechanism of DNA repair in bacteria. However, NHEJ has recently been identified in bacteria (71). Homologous elements of eukaryotes have been identified in prokaryotes. Some authors accepted that the NHEJ repair pathway is not present in prokarytic and archaeal organisms. However, as we will discuss, this theory has now been refuted by a number of recent studies (72). Current studies have in sighted the role that NEHJ plays in protecting against bacterial DBS of the chromosome via homologous to proteins found in eukaryotes (73,74).

The first evidence for the existence of this pathway in prokarya came from *In silico* studies that identified many bacterial genomes that possess genes encoding putative Ku orthologs. Although the eukaryotic Ku is a heterodimer, the bacterial Ku is usually encoded by a single gene, and biochemical studies have confirmed that the Ku is indeed a homodimer that binds to the termini of DSBs (74). Even if, an amount of bacteria encoded a potential heterodimeric *ku* operonic system suggesting that a gene duplication event occurred early on in the evolution of the NHEJ apparatus. The *ku* genes are often genetically linked in operons with another gene that encodes a putative ATP-dependent DNA ligase (75). Apparently, bacteria possess a few NHEJ-specific genes, including a gene encoding a homodimeric Ku. Generally, the prokaryotic *ku* genes are located in operons containing a conserved ATP-dependent DNA ligase, LigD (ligase D) but the mechanism is not clear.

5.2.1. NHEJ repair mechanisms: A Ku homodimer binds to the ends of the DNA break and recruits LigD. The polymerase domain of LigD binds to a 5´-phosphate (P) and, together with Ku, promotes end-synapsis. The nuclease and polymerase activities of LigD, if necessary, can require other factors to process the break termini to re-establish complementary ends. Finally, ligation of the nick by LigD repairs the break (Fig. 5) (76). Still the biochemical pathways are not clear.

5.2.2. NHEJ in Bacillus subtilis and Mycobacterium sp.: Genes have been identified with homology to Ku70 and Ku80 in some genomes of bacteria, which demonstrates that prokaryotes might have a NHEJ pathway that is homologous to that of eukaryotic cells. Notably, the *ku*-like gene exists in some bacterial species in an operon that includes a gene predicted to encode an ATP-dependent DNA ligase. The operons frequently co-regulate functionally relating protein. These ligases interact with the Ku-like proteins (74). The widespread of the repair mechanism of eukaryotic and prokaryotic NHEJ is dependent on the DNA end-binding protein Ku and a dedicated ATP-dependent DNA ligase (Lig4 in eukarya, LigD in bacteria). Only some sets of bacteria have genes encoding Ku and LigD whereas Ku and Lig4 are present in almost all eukaryal species, among which are the human pathogens *Bacillus* sp., *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (77).

5.2.2.1. Bacillus subtilis case: DSB are the most critical damage in DNA, caused by ionizing radiation and desiccation in vegetative cells, and are also induced in spores of bacteria. It is important to mention that it requires the participation of two homologous chromosomes for homologous recombination pathway. The spores of *B. subtilis* contain only one chromosome (as toroidal) therefore DSB repair

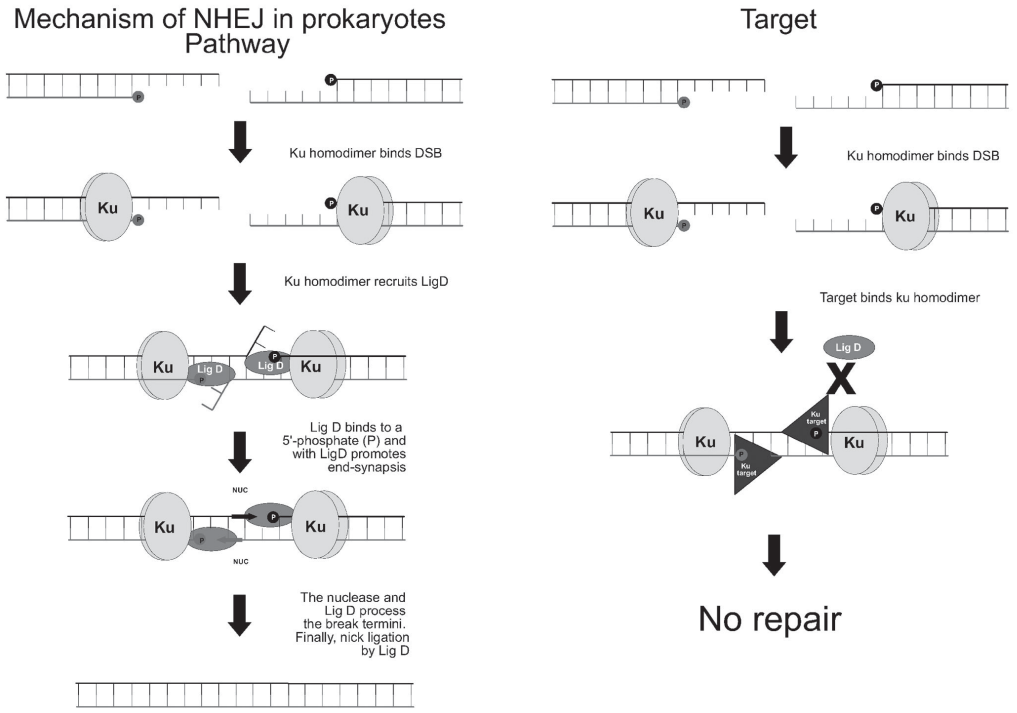

Fig. 5. Signaling pathways in NHEJ repair mechanism prokaryotes with Ku protein and LigD. Possible mechanisms that could be a drug target in the Ku protein. A. Pathway: Ku homodimer binds DBS, then LigD binds to a phosphate 5´ and promotes end-synapsis, which with the nuclease process break termini and finally nick ligation. B. Target: Ku homodimer binds DBS, then Ku target bind to Ku and disrupts repair.

by homologous recombination cannot function during the germination of spores (78).

A different repair pathway to HR for DSB induced in spore DNA, nonhomologous-end joining (NHEJ) has recently been identified in *B. subtilis,* a Ku homolog (encoded by the *ykoV* gene). The bacterial Ku recruits a DNA ligase (encoded by *ykoU*) to DNA ends and in this manner stimulates DNA ligation (78). *Bacillus subtilis* has a heterodimer Ku 70/80 YkoU and a protein with few homologies to ADN ligase IV YkoV.

5.2.2.2. Mycobacterium* sp. *Case: Nonhomologous end-joining (NHEJ) pathway with protein Ku and DNA ligase (LigD) is used by *Mycobacteria* to repair DNA double-strand breaks (DSBs). The mechanisms of mycobacterial NHEJ depend on the structures of the DSBs and end-processing and end-sealing components (77). The Ku like genes are often genetically linked in operons with another gene that encodes an ATP-dependent DNA ligase. It has been also established in *Mycobacterium tuberculosis* that Ku (Mt-Ku) and ligase (Mt-lig) proteins, together reconstitute a mechanism with capacity for ligation (79). Ku in *Mycobacterium tuberculosis* is a homodimer, which binds only to linear DNA ends.

5.2.2.3. E. coli: Other bacteria, where Ku-like and Ligase-D-like proteins have not been found,

are only generally accepted to be end-joining and as the recombination-mediated mechanisms to repair DNA breaks and integrate exogenous sequences. In fact, this bacterium is used as a negative control for experiments in other bacterial NHEJ (80). However, as a strategy for the horizontal transfer of genes in the genome of this bacterium, *E. coli* can integrate unrelated sequences by, non-homologous end-joining. Hence, alternative end-joining (A-EJ) contributes to bacterial genome evolution and adaptation to environmental challenges, but the most interesting fact is that characteristics of A-EJ also come into view in A-NHEJ (80).

5.2.3. Ku protein as a drug target: The key DNA end-binding component of NHEJ is Ku protein. Ku, is a heterodimer of two subunits [Ku70 (69 kD) and Ku80 (83kD)] (*3–5*) present in eukaryotic cells that form a structure through which a diversity of DNA end, has NHEJ has not been reported in prokaryotes. Nevertheless, genes with significant homology to Ku70 and Ku80 have been identified in some bacterial genomes (74).

Several studies have demonstrated that the Ku protein is the homologous most common in bacteria for the NHEJ repair of mechanisms. Here are some examples: the *Bacillus subtilis* gene *ykoV* is adjacent to the *ykoU* gene, which encodes a two domain protein: a catalytic subunit of the eukaryotic-archeal DNA primase (EP) and their juxtaposed with a gene for a eukaryotic-archeal ATP-dependent DNA ligase (ADDL) domains; the YkoV protein is conserved in bacteria that encode an EP. The combination of the *ykoV* and the genes coding for EP or ADDL is maintained in some bacteria. This suggests that these genes belong to the same operon. However, the possible operon position gene arrangement is variable. Therefore, it seems most likely that YkoV form a functional complex with EP, ADDL (75).

These studies insight that prokaryotes might have a NHEJ apparatus that is fundamentally homologous to that of eukaryotic cells. Considerably, the *ku*-like gene exists in some bacterial species in an operon that includes a gene predicted to encode an ATP–dependent DNA ligase. The operons co-regulate the participation of proteins in the same metabolic pathway; this creates the potential for putative ligases to interact with the Ku-like proteins**.** Part of the structure of the Ku protein homologue present in prokaryotes could be targeted for use as drugs and treatments against these pathogens. This protein is highly conserved in bacteria (Fig. 5).

The Ku heterodimer's subunits Ku70 and Ku80 form a dyad-symmetrical molecule with a preformed ring that encircles duplex DNA. Ku does not have interactions with DNA bases and sometimes with the sugar-phosphate (81). This suggests that these structurally support broken DNA ends and that the DNA helix in phase across the junction during end processing and ligation. Moreover it does not interfere with heterodimer present in eukaryotes because homolog proteins are present in prokaryotic homodimers.

Although not previously reported, this mechanism in prokaryotes, could shed us light on repair the damage of double-stranded DNA. One of the most important mechanisms is the NHEJ, which is mainly involved in two components: to find the Ku protein damage (homologous to eukaryotic) and the ligD ligand. NHEJ have been found as highly conserved in bacteria of the genus *Mycobacterium*, *Bacillis subtilis* to be working in operons. More studies are needed to understand this phase.

6. Conclusion

Microorganisms have developed a wide range of DNA repair mechanisms that make them

able to survive the hostile conditions in which they are found when colonizing a host (immune response, production of reactive oxygen species, etc.). These repair mechanisms could be classified mainly by the type of DNA lesion that they fix. For example, for single strand breaks we have the BER and NER pathways whereas, for double strand breaks we have the HRR and NHEJ.

Even though a great deal of studies have been made to gain a better understanding of the repair mechanism in prokaryotes, the new drug discovery studies should focus on inhibition of these mechanisms to avoid the generation of more genetic variation on pathogens. This genetic variation can be translated in the development of multi drug resistant strains, hence generating a serious public health problem.

A critical aspect that should be considered by further studies is what direct consequences will arise from the inhibition of a determined DNA repair mechanism due to its intrinsic characteristics. For example, as we have seen before, BER and NER mechanisms act on single strand breaks to maintain the fidelity of the DNA sequence.

But if we inhibit these mechanisms we could induce errors in DNA replication that in most cases could cause the death of the pathogen but in some cases could lead also to favorable mutations. On the other hand, if we inhibit NHEJ and HRR mechanisms we could surely induce the death of the pathogen without cause any genetic variation because we are leaving a lethal double strand break that could not be repaired.

Another point to consider is to perform more studies related to DNA damage checkpoints. These pathways are the first activated in response to DNA damage producing a cell cycle arrest to allow DNA repair. Recently it has been demonstrated that proteins involved in DNA repair have a role in DNA damage checkpoints (82).

An important aspect is way to develop drugs directed specifically to pathogens that do not affect the microbiota of the gastrointestinal track and oral cavity. The current treatments with nonspecific antibiotics do not discriminate between non-pathogenic and pathogenic organisms within the host (83). The chosen targets should be molecules highly conserved within the groups of pathogen bacteria but with low homology with the molecules of the host. These novel drugs may act as adjuvants of current antibiotics to help delay the development of resistance.

Further studies are highly essential in understanding NHEJ and HRR pathways to develop an effective antimicrobial strategy against pathogens which is of importance for public health.

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References

1. Wolfe-Simon, F. A. (2010). Bacterium that Can Grow by Using Arsenic Instead of Phosphorus. Science, 2 December 2010 (10.1126/Science. 1197258).
2. Witkin, E.M. (1976). Ultraviolet mutagenesis and inducible DNA repair in *E. coli*. Bacteriological Reviews, 40: 867-907.
3. Hart, R. W. (1976) In Aging, Carcinogenesis and Radiation Biology (Edited by K.C. Smith) pp. 537-556, Plenum Press, New York.

4. Trosko, J.E. and Chang, C.C. (1978). The role of mutagenesis in carcinogenesis. Photochemistry and Photobiology, 28:157-168.

5. 2010. Targeting TB in South Africa. Twas Newsletter. Vol 22, No.1.

6. Smith K.C. (1978). Multiple Pathways of DNA Repair in Bacteria and their Roles in Mutagenesis. Photochemistry and Photobiology, 28:121-129.

7. Krokan, H.E. (1997). DNA glycosylase in the base excision repair of DNA. Biochemistry Journal, 325: 1-16.

8. Prakash, S. and Prakash, L. (2000). Nucleotide excision repair in yeast. Mutation Research, 451: 13-24.

9. Marti, T.M., Kunz, C. and Fleck, O. (2002). DNA mismatch repair and mutation avoidance pathways. Journal of Cell Physiology, 191: 28-41.

10. Fleck, O. and Nielsen, O. (2004). DNA repair. Journal of Cell Science, 117: 515-517.

11. Lipps, G. (editor). (2008). Plasmids: Current Research and Future Trends. Caister Academic Press.

12. Wilson III, D.M. (2003). Repair Mechanisms for oxidative DNA damage. Frontiers in Bioscience, 8:963-981.

13. Cadet, J., Delatour, T., Douki,T., Gasparutto, D., Pouget, J., Ravanat, J. and Sauvaigo, S. (1999). Hydroxyl radicals and DNA base damage. Mutation Research, 424 (1–2): 9–21.

14. Cathcart, R., Schwiers, E., Saul, R. and Ames, B. (1984). Thymine glycol and thymidine glycol in human and rat urine: a possible assay for oxidative DNA damage. Proceedings of the National Academy of Science of the United States of America, 81 (18): 5633–7.

15. Valerie, K. and Povirk, L. (2003). Regulation and mechanisms of mammalian double-strand break repair. Oncogene, 22 (37): 5792–812.

16. Douki, T., Reynaud-Angelin, A., Cadet, J. and Sage, E. (2003). Bipyrimidine photoproducts rather than oxidative lesions are the main type of DNA damage involved in the genotoxic effect of solar UVA radiation. Biochemistry, 42 (30): 9221–6.

17. Kim, J.M. (1998). Transposable elements and genome organization: a comprehensive survey of retrotransposons revealed by the complete *Saccharomyces cerevisiae* genome sequence. Genome Research. 8(5):464-478.

18. Caldectt, K.W. (2008). Single strand breaks repair and genetic disease. Nature Review Genetics, 9:619-631.

19. Seeberg, E., Eide, L. and Bjørås, M. (1995). The Base Excision Repair Pathway. Trends in Biochemical Sciences, 20: 391–397

20. Shinmura, K. and Yokota, J. (2001). The OGG1 Gene Encodes a Repair Enzyme for Oxidatively Damaged DNA and is involved in Human Carcinogenesis. Antioxidants & Redox Signaling; 3:597–609.

21. Kohno, T., Shinmura, K., Tosaka, M., Tani, M., Kim, S.R., Sugimura, H., Nohmi, T., Ksai, H. and Yokota, J. (1998). Genetic Polymorphisms and Alternative Splicing of the hOGG1 Gene, that is involved in the Repair of 8-hydroxyguanine in Damaged DNA. Oncogene; 16:3219–25.

22. Tchou, J., Kasai, H., Shibutani, S., Chung, M.H., Laval, J., Grollman, A.P. and Nishimura, S. (1991). 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its Substrate Specificity. Proceedings of the National Academy of Sciences of the United States of America. 88 (11):4690-4.

23. Michaels, M.L. and Miller, J.H. (1992). The GO System Protects Organisms from the Mutagenic Effect of the Spontaneous Lesion 8-hydroxyguanine (7, 8-dihydro-8-oxoguanine). The Journal Bacteriology; 174:6321-5.

24. Bhagwat, M. and Gerlt, J. (1996). A. 3'- and 5'-strand Cleavage Reactions Catalyzed by the Fpg Protein from *E. coli* Occur via Successive Beta- and Delta-Elimination Mechanisms, Respectively. *Biochemistry*, **35: 659–**665.

25. Barzilay, G. and Hickson, I. D. (1995). Structure and Function of Apurinic/ Apyrimidinic Endonucleases. BioEssays, 17:713-719.

26. Popoff, S. C.; Spira, A. I.; Johnson, A. W. and Demple, B. (1990). Yeast Structural Gene (APN1) for the Major Apurinic Endonuclease: Homology to *Escherichia coli* Endonuclease IV. Proceedings of the National Academy of Sciences of the United States of America, 87:4193-4197.

27. Bennett, R. A. O. (1999). The *Saccharomyces cerevisiae* ETH1 Gene, an Inducible Homolog of Exonuclease III that Provides Resistance to DNA-damaging Agents and Limits Spontaneous Mutagenesis. Molecular and Cellular Biology, 19:1800-1809.

28. Boiteux, S., O'Connor, T. R., Gouyette, F. A. and Laval, J. (1990). Homogeneous *Escherichia coli* FPG Protein. The Journal of Biological Chemistry, 265:3916-3922.

29. Todar, K. (2010). Pathogenic *E. coli*. Online Textbook of Bacteriology. University of Wisconsin–Madison Department of Bacteriology.

30. Demple, B., Halbrook, J. and Linn, S. (1983). *Escherichia coli* xth Mutants are Hypersensitive to Hydrogen Peroxide. The Journal Bacteriology, 153:1079-1082.

31. Demple, B. and Linn, S. (1982). 5, 6-saturated Thymine Lesions in DNA: Production by Ultraviolet Light or Hydrogen Peroxide. Nucleic Acids Research, 10:3781-3789.

32. Ischenko, A. A. and Saparbaev, M. K. (2002). Alternative Nucleotide Incision Repair Pathway for Oxidative DNA Damage. Nature 415 (6868): 183–187.

33. Crowley, D.J., Boubriak, I., Berquist, B.R., Clark, M., Sullivan, R. and DasSarma, M.(2006). The uvrA, uvrB and uvrC genes are light induced DNA photoproducts in *Halobacterium* sp. Saline Systems, 13:2-11

34. Howard-Flanders, P., Boyce, R. and Lee T. (1966). Three loci in *Escherichia coli K-12* that control the excision of pyrimidine dimers and certain other mutagen products from DNA. Genetics, 53: 1119-113.

35. Van Sluis, C.A., Moolenaar, G.F. and Backendorf, C. (1983). Regulation of the uvrC gene of *Escherichia coli K12*: localization and characterization of a damage-inducible promoter. EMBO Journal. pp.2313-2318

36. Seeberg, E., Steinum, A.L. and Linn, S. (1983). Journal of Cellular Biochemistry. Suppl.7B,175.

37. Sharma, S. and Moses, R. (1979). uvrC Gene Function in Excision Repair in Toluene-Treated *Escherichia coli* . The Journal of Bacteriology, 137(1): 397-408

38. Sharma, R.C., Barfknecht, T.R., Smith, K. (1982). Postreplication repair in uvrA and uvrB strains of *Escherichia coli K-12* is inhibited by rich growth medium. Photochemistry and Photobiology, 36(3): 307–311.

39. Truglio, J.J., Croteau, D.,Van Houten, B., and Kisker, C. (2006). Prokaryotic nucleotide excision repair: the UvrABC system. Chemical Reviews, 106: 233-252

40. Janion, C. (2001). Some aspects of the SOS response system- a critical survey. Acta Biochimica Polonica, 48(3):599-610.

41. Sancar, A. (1994). Mechanism of DNA excision repair. Science, 266(5193):1954-6.

42. Sancar, A., Tang, M.S. (1993). Nucleotide Excision Repair. Photochemistry and Photobiology, 57(5):905-921.

43. Sancar, A. and Rupp, W.D. (1983). A novel repair enzyme: UvrABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region. Cell, 33(1):249-260

44. Manganelli, R., Proveddi, R., Rodriguez, S., Beaucher, J., Gaudreau, L. and Smith, I. (2004). Factors and Global Gene Regulation in *Mycobacterium tuberculosis*. Journal of Bacteriology, 186(4):895-902.

45. Warner, D.F. and Mizrahi, V. (2006). Tuberculosis chemotherapy: the influence of bacillary stress and damage response pathways on drug efficacy. Clinical Microbiology Reviews, 19(3):558-70.

46. Kurthkoti, K., Kumar, P., Jain, R., and Varshney, U. (2008). Important role in nucleotide excision repair pathway in *Mycobacterium smegmatis* in conferring protection against commonly encountered DNA-damaging agents. Microbiology,154: 2776–2785.

47. Darwin, K. H., Ehrt, S., Gutierrez-Ramos, J. C., Weich, N. and Nathan,C. F. (2003). The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. Science, 302: 1963–1966.

48. Machius, M., Henry, L., Palnitkar, M., and Deisenhofer, J. (1999). Crystal structure of the DNA nucleotide excision repair enzyme UvrB from *Thermus thermophilus*. Biochemestry, 96(21):11717-11722

49. Skorvaga, M., Della Vecchia, M.J., Croteau, D.L., Theis, K., Truglio, J.J., Mandavilli, B.S., Kisker, C. and Van Houten, B. (2004). Identification of residues within UvrB that are important for efficient DNA binding and damage processing. The Journal of Biological Chemistry, 279(49):51574-80.

50. Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S. and Hanaoka, F. (2001). A multistep damage recognition mechanism for global genomic nucleotide excision repair. Genes and Development,15(5):507-21

51. Kang, J. and Blaser, M.J. (2008). Repair and Antirepair DNA heliase in *Helicobacter pylori*. Journal Bacteriology, 190(12):4218-24.

52. Haber, J.E. (2000). Partners and pathways repairing a double-strand break. Trends in Genetics, 16(6):259-64.

53. Sexton, J.Z., Wigle, T.J., He, Q., Hughes, M.A., Smith, G.R., Singleton, S.F., Williams, A.L., Yeh, L.A. (2010). Novel inhibitors of *E. coli* RecA ATPase activity. Current Chemical Genomics, 4:34-42.

54. Helleday, T., Lo, J., van Gent, D.C. and Engelward, B.P. (2007). DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair (Amst). 6(7):923-35.

55. Shrivastav, M., De Haro, L.P. and Nickoloff, J.A. (2008). Regulation of DNA double-strand break repair pathway choice. Cell Research, 18(1):134-47.

56. Rocha, E.P., Cornet, E. And Michel, B. (2005). Comparative and evolutionary analysis of the bacterial homologous recombination systems. PLoS Genetics, 1(2):e15.

57. Mascarenhas, J., Sanchez, H., Tadesse, S., Kidane, D., Krisnamurthy, M., Alonso, J.C. and Graumann, P.L. (2006). *Bacillus subtilis* SbcC protein plays an important role in DNA interstrand cross-link repair. BMC Molecular Biology, 7:20.

58. Niu, H., Raynard, S. and Sung, P. (2009). Multiplicity of DNA end resection machineries in chromosome break repair . Genes Development, 23(13):1481-6.

59. Kuzminov, A. (1999). Recombinational repair of DNA damage in *E. coli* and bacteriophage *lamda.* Microbiology and Molecular Biology Review, 63(4):751-813.

60. Gruenig, M.C., Renzette, N., Long, E., Chitteni-Pattu, S., Inman, R.B., Cox, M.M. andSandler, S.J. (2008). RecA mediated SOS induction requires an extended filament conformation but no ATP hydrolysis. Molecular Microbiology, 69(5):1165-79.

61. Dorer, M.S., Fero, J. and Salama, N.R. (2010). DNA damage triggers genetic exchange in *Helicobacter pylori.* PLoS Pathogens, 6(7):e1001026.

62. Dillingham, M.S. and Kowalczykowski, S.C. (2008). RecBCD enzyme and the repair of double strand breaks. Microbiology and Molecular Biology Review, 72(4):642-71.

63. Cox, M. M. (2001). Historical Overview: Searching for The Replication Help in All of The Rec Places. Proceedings of the National Academy of Sciences of the United States of America, 98(15):8173-80.

64. Saladin, A., Amourda, C., Poulain, P., Férey, N., Baaden, M., Zacharias, M., Delalande, O. and Prévost, C. (2010). Modeling the early stage of DNA sequence recognition within RecA nucleoprotein filaments. Nucleic Acids Research, 38(19):6313-23

65. Haruta, N., Yu, X., Yang, S., Egelman, E.H. and Cox, M.M. (2003). A DNA pairing enhanced conformation of bacterial RecA proteins. Journal Biological Chemistry, 278(52):52710-23

66. Kickstein, E., Harms, K. and Wackernagel, W. (2007). Deletions of RecBCD or recD influence genetic transformation differently and are lethal together with RecJ deletion in *Acinetobacter bayli*. Microbiology, 153(Pt 7):2259-70.

67. Manfredi, C., Suzuki, Y., Yadav, T., Takeyasu, K. and Alonso, J.C. (2010). RecO mediated DNA homology search and annealing is falitated by SsbA. Nucleic Acids Research, 38(20):6920-9.

68. Gorna, A.E., Bowater, R.P. and Dziadek, J. (2010). DNA repair systems and the pathogenesis of *Mycobacterium tuberculosis*: varying activities at different stages of infection. Clinical Science, 119(5):187-202.

69. Dawson, L. F., Dillury, J. and Davis, E.O. (2010). RecA independent DNA damage induction of *Mycobacterium tuberculosis ruvC* gene despite an appropriately located SOS box. Journal Bacteriology, 192(2):599-603

70. Wigle, T.J., Sexton, J.Z., Gromova, A.V., Hadimani, M.B., Hughes, M.A., Smith, G.R., Yeh, L.A., Singleton, S.F. (2009). Inhibitors of RecA Activity Discovered by High-Throughput Screening: Cell-Permeable Small Molecules Attenuate the SOS Response in *Escherichia coli.* Journal Biomolecular Screening, 14(9):1092-101

71. Pitcher, R.S., Brissett, N.C. and Doherty, A.J. (2007). Nonhomologous end-joining in bacteria: a microbial perspective. Annual Review of Microbiology, 61:259-82.

72. Doherty, A.J., Jackson, S.P. and Weller, G.R. (2001). Identification of bacterial homologues of the Ku DNA repair proteins. FEBS Letters, 6;500(3):186-8.

73. Shuman, S. and Glickman, M.S. (2007). Bacterial DNA repair by non-homologous end joining. Nature Reviews Microbiology, 5(11):852-61.

74. Weller, G.R., Kysela, B., Roy, R., Tonkin, L.M., Scanlan, E., Della, M., Devine, S.K., Day, J.P., Wilkinson, A., d'Adda di Fagagna, F., Devine, K.M., Bowater, R.P., Jeggo, P.A., Jackson, S.P. and Doherty, A.J. (2002). Identification of a DNA nonhomologous end-joining complex in bacteria. Science, 6; 297(5587):1686-9.

75. Aravind, L. and Koonin, E.V. (2001). Prokaryotic homologs of the eukaryotic DNA-end-binding protein Ku, novel domains in the Ku protein and prediction of a prokaryotic double-strand break repair system. Genome Research, 11(8):1365-74.

76. Brissett, N.C. and Doherty, A.J. (2009). Repairing DNA double-strand breaks by the prokaryotic non-homologous end-joining pathway. Biochemical Society Transactions, 37(Pt 3):539-45.

77. Aniukwu, J., Glickman, M.S. and Shuman, S. (2008). The pathways and outcomes of mycobacterial NHEJ depend on the structure of the broken DNA ends. Genes & Development, 15;22(4):512-27.

78. Moeller, R., Stackebrandt, E., Reitz, G., Berger, T., Rettberg, P., Doherty, A.J., Horneck, G. and Nicholson, W.L. (2007). Role of DNA repair by nonhomologous-end joining in *Bacillus subtilis* spore resistance to extreme dryness, mono- and polychromatic UV, and ionizing radiation. The Journal of Bacteriology, 189(8):3306-11.

79. Gong, C., Bongiorno, P., Martins, A., Stephanou, N.C., Zhu, H., Shuman, S. and Glickman, M.S. (2005). Mechanism of nonhomologous end-joining in *mycobacteria*: a low-fidelity repair system driven by Ku, ligase D and ligase C. Nature Structural & Molecular Biology, 12(4):304-12.

80. Chayot, R., Montagne, B., Mazel, D. and Ricchetti, M. (2010). An end-joining repair

mechanism in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America, 2;107(5):2141-6.

81. Walker, J.R., Corpina, R.A. and Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature, 9;412(6847):607-14.

82. Kondo, S. and Perrimon, N. (2011). A Genome-wide RNA Screen Identifies Core Components of the G2-M DNA damage Checkpoint. Science Signaling, 4, rs1.

83. Reid, G., Younes, J., Van dor Mei, H., Gloor, G., Knight, R. and Busscher, H. (2011). Microbiota restoration: natural and supplemented recovery of human microbial communities. Nature Reviews Microbiology, 9:27-28.

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Headway in Reproductive Biotechniques for Genetic Improvement of Buffaloes

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Abstract

Buffaloes are one of the major contributors in refining the rural economy, particularly in South and South-East Asia countries. There are about 177.5 million buffaloes in the world and more than 96 % of which are found in Asia. Though genetic improvement of this animal resource is imperative for milk and meat, it is limited by inherent biological attributes such as, long generation interval, long inter-calving period, longer age at sexual maturity, delay in puberty, distinct seasonal reproductive pattern, silent estrous, repeat breeding and eventually low reproductive rates. However, advancement in reproductive bio-techniques offers greater prospects to achieve the desired genetic improvement in buffaloes. Artificial insemination (AI) using frozen semen is well established and manipulation of ovarian function for estrus synchronization is used in large farms commonly. Super ovulation to produce embryos *in vivo* yielded average transferable embryos, 1.8-2.1 per collection. With advancement, embryo production using ovum pick-up (OPU) in combination with *in vitro* maturation (IVM) and fertilization (IVF) has been tried to a limited extent, but it is impending to be an alternative to earlier one. Recently advanced "Hand guided Cloning Technique", a landmark technique developed at National Dairy Research Institute (NDRI), Karnal could go a long way for faster multiplication of superior milch buffaloes in India.

Keywords: Buffalo; Reproductive biotechniques; Super ovulation; Genetic improvement.

Introduction

India is primarily an agrarian country with livestock playing a pivotal role in uplifting the socioeconomic conditions of rural masse. Presently, there are about 177.5 million water buffaloes in the world and 96% of them are found in Asia alone. India ranks first in respect of buffalo *(Bubalus bubalis)* population in the world and has more than 50% of the world's buffalo population (~98 million) which contributes 56% of total milk production, 104.8 million tonnes (1). Buffaloes are multi-purpose animals and preferred over other animals due to their ability fulfill the protein requirements of human population through milk and meat, and also for their great share in providing the traction power for various agricultural purposes.

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Improving the genetic potential of buffaloes for milk and/or meat has been a major concern for decades in many buffalo-producing countries, and has become a recent development focus in several more. But there are many biological conditions inherent in the buffalos such as high age at first calving, longer dry period, poor reproductive performance resulting in high service period and longer calving interval, embryonic mortality, inadequate health care, genetic slippage of superior germplasm from the breeding tracts and poor response to artificial breeding are impeding the efforts put forth in genetic improvement of buffaloes (2). However, the fast pace of developments transpiring in reproductive biotechniques can be exploited for improvement in buffaloes so as to vanquish the above cited limitations. Emerging biotechniques can be effectively employed to enhance the production performance and reproductive efficiency of buffaloes by reducing the generation interval, which in turn accelerate the genetic improvement in desired direction.

Associated to the enhancement in reproductive efficiency, techniques used to achieve genetic improvement make possible to obtain herds with better productive characteristics, such as growth rate, carcass quality, milk yield, feed conversion and precocity. Thus, multiplication of superior animals by using reproductive biotechniques can provide greater economic return. Moreover, increased reproductive rates associated to genetic improvement must be the main objective to improve buffalo productivity and farm income. However, most of them are not as efficient as in cattle. Genetic improvement is possible when potential animals are selected based on their pedigree, karyotyping (free from chromosomal aberration), tests for various diseases like; tuberculosis, brucellosis, John's disease and any other genetic disorders. The chromosomal aberrations are one of the reasons for reduced fertility (3). In this review, hence, the focus will be given on research advances transpired in main reproductive biotechniques applied for buffalo reproduction such as AI, estrus synchronization, super ovulation, *in vivo* and *in vitro* production (IVP) of embryos, embryo transfer, cloning, semen and embryo sexing. Along with the advancement made, the prospective areas of research one should be aimed to make these advanced reproductive biotechniques, of buffalo in particular and livestock's as a whole, as more efficient and economical for their wider application will be deliberated.

Artificial Insemination (AI) and Associated Techniques : The quality of semen before and after freezing should be evaluated by various parameters before a bull is introduced for AI programme (4, 5, 6). AI in either natural or synchronized estrus is the earliest reproductive biotechnique in farm animals including buffalo. For many reasons, AI can be used in buffalo, to inseminate magnitudes of female using proven bull, to use a male genetic material separated by distance or time, to overcome difficulties of physical breeding, to control the offspring's paternity, to synchronize births, to avoid injuries of natural mating, and to avert the need to rear a male (such as for small numbers of females or in species whose fertile males may be difficult to manage).

Yet, the technique of AI in overall genetic improvement programs of water buffalo is not exploited widely. The reported low efficiency of AI in water buffaloes is mainly the result of human factors such as inability to detect estrus properly, improper handling and usage of semen in the field by technicians, and most common of all, poor management and nutrition of inseminated animals. Therefore, the development of a sustainable and effective delivery system of

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AI service is the only panacea to the wide- scale use of this technique, particularly in relatively dispersed buffalo populations in most of the small farms in Asia. Many researchers have underpinned various techniques to produce quality semen and there by the better pregnancy rates following AI in water buffaloes. Some of the alternate techniques of AI to accomplish higher pregnancy rate are deliberated below.

Fixed-time artificial insemination (FTAI) : The AI in bovine has been widely pursued and successfully used in breeding programs around the world. However, FTAI has been used to limited extent in buffaloes due to certain difficulties in the estrus detection and imperfect timing of AI. The failure to detect estrus is one of the main factors that impair reproductive development in artificially inseminated herds. Therefore, the use of protocols that do not require the estrus detection contributes to wax the use of AI in buffalo herds. Hence, in this review we will consider two hormonal treatments that endorse FTAI.

Synchronization of ovulation using GnRH (Gonadotropin Releasing Hormone) and prostaglandins (PG) for FTAI : In cattle, "Ovsynch" method has demonstrated efficient synchronization of ovulation and production of satisfactory pregnancy rates in the inseminated herd, without the need of estrus detection. This protocol has also been tested in buffaloes, in sequential follicular dynamics and field studies buffaloes received a treatment based on GnRH and PG according to the protocol scheduled. In these experiments it was verified that buffaloes respond to hormonal treatment, in which a new follicular wave emerged due to the ovulation of dominant follicle present at the time of the first GnRH. On day 7, buffaloes respond to PGF2α (luteolysis), and on day 9 around 80% have a synchronized ovulation within 12 hours. Additionally, a pregnancy rate (PR) of about 50% can be obtained during the breeding season, in cycling buffaloes.

Synchronization of ovulation using progesterone and/or progestin plus estradiol : The Ovsynch protocol has been demonstrated to be efficient during the breeding season in cycling buffaloes. However, the same protocol has resulted in the low pregnancy rates of 6.9 to 28.2% during off- season breeding in anestrous buffaloes. Several studies were conducted in order to establish an appropriate protocol for the off- season breeding (7, 8). The association of progesterone/progestin to estradiol in the beginning of the protocol (day 0) has been demonstrated to be effective in inducing a new follicular wave, due to the suppression of both follicle stimulated hormone (FSH) and luteinizing hormone (LH), promoting atresia of all follicles present in the ovary. Treatment of postpartum anestrous cows with progesterone results in greater follicular fluid and circulating concentrations of estradiol, increased pulsatile release of LH and increased numbers of receptors for LH in granulosa and theca cells in pre-ovulatory follicles, compared with untreated animals (9). Further, a short period of elevated progesterone concentrations during anestrus period is important for the expression of estrus as well as subsequently normal luteal function. On day 9, the progesterone device was removed and a $PGF_2\alpha$ and an eCG (equine chorionic gonadotropin) dose was administered to decrease the progesterone levels in circulation and to improve the follicular growth, which is compromised in non cycling buffaloes during the off- season breeding. On day 11 the GnRH promotes a synchronized ovulation. In previous studies, the eCG treatment improved ovulation and pregnancy rate in anestrous females (10). These protocols resulted in approximately 50% of pregnancy at first insemination, which compel

their use in anestrous buffaloes around the year, without detecting the estrus. This strategy of breeding the buffaloes during the off season is economically viable to suffice the market demands of milk and its products.

In vivo* Production of Buffalo Embryos *: The second advancement made in the field of reproductive biotechnology is the *in-vivo* and *in-vitro* production of embryos. In both systems, still immense scope is there to improve efficiency so that the cost of production of a calf will be reduced. Methods for in vivo embryo production are single ovulation and embryo transfer (SOET) and multiple ovulation and embryo transfer (MOET). In SOET embryos are produced *in-vivo* without any superovulatory treatments using exogenous hormones. Therefore, no disturbance in the physiological milieu of the donor animals is expected. Singla and Madan (1990) produced the embryos through SOET with 60% efficiency (11). Although, the efficiency of the flushing was limited by the animal's estrous cycle, harvested embryos were of high quality. Depending on the efficiency of collection and success of transfers, a donor can potentially produce 3-6 calves per year. Currently, MOET is commonly used method over SOET and hence we are describing this method and known factors affecting.

MOET in combination with AI was the first advanced concept in the late 1970s, to achieve more rapid gains in cattle genetics than are provided by conventional progeny testing (12). Thereafter, superovulation through the exogenous hormones administration was used in buffaloes to produce the genetically superior embryos at higher rates. However, the process relied mostly on protocols that were originally used in cattle (11). Superovulation followed by insemination of the donor, embryo collection and their transfer have allowed researchers and dairy producers to obtain multiple offspring from only a few genetically superior donors.

Buffalo donor presented low embryo recovery rate compared to cattle. Embryo transfer, as well, has many problems such as recipient estrus detection, presence of a corpus luteum at the time of embryo transfer and synchronization donor-receptor. Poor response to superovulation and low embryo recovery is attributed to the low primordial follicle pool in buffaloes as compared to cattle and high rate of follicular atresia. As a consequence, 10% buffalo failed to respond (0-2 corpus luteum; CL) and nearly half responded poorly (0-5 CL) to superovulation treatment (13). These problems make buffalo embryo recovery rate lower than cattle. Hence, considerable attention has been focused on developing the most appropriate treatment for induction of multiple ovulations in buffaloes. After the first report of buffalo calf birth through MOET technique in the world, many works were embarked with an aim to increase embryo production. Trials have included the studies on effects of different gonadotropin hormones, forms of medicine administration and pretreatments on superovulation in buffalo. Results from such trials were not very consistent, while ovulatory responses were found to be lower than those achieved in cattle. Pregnant mare serum gonadotropin (PMSG) and FSH were usually adopted for buffalo superovulation. There have been many researches on the effects of PMSG and FSH on superovulation, and almost all the experiments demonstrated that FSH is superior over PMSG and the transferable embryos per superovulation were 2.09 and 0.56, respectively. On the other hand, the development of a new purified FSH preparation has further improved the consistency of ovulatory response in buffaloes (14). This comes in the form of 600 mg NIH-FSH-P1 {Equivalent to National Institutes of Health (U.S.A.) Reference Standard NIH-FSH-P1} administered in 10 divided and decreasing doses at an interval of 12 hours. This is followed by PGF2α administration after

Vol. 5 (3) 1233-1250 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

72hours of initiation of superovulatory treatment. This superovulatory regimen resulted in an average of 4.2 total embryos and 2.1 viable embryos per flushing (14).

Some studies on the application of synchronization have been done in recent years, and many schemes have been developed such as administrations of progesterone, PGF2α+ PGF2α, GnRH + prostaglandin and progesterone + eCG. Jiang *et al.* (5) adopted controlled internal releasing device (CIDR) PGc to synchronize native buffalo cows, and resulted in a synchronous estrus rate of 85.13%, while when the single drug of PGc and CIDR was applied, the synchronization rates were 64.18% and 73.01%, respectively (15). Liang *et al.* (16) reported GnRH+PGF2α+GnRH is the most effective combination for buffalo estrus synchronization and it resulted a synchronous rate of 88.46% (16). Qin *et al.* (17) reported that the synchronous estrus rate reached 91.7% when GnRH+PG+GnRH was adopted for buffalo estrus synchronization (17). The synchronous effect is better during breeding season, and combination of progesterone during anestrus season can promote synchronous rate. The seasons seem to affect the response to superovulatory treatment, with summer having a depressive effect. On the whole, the synchronization effect in our nation remains to be improved.

In recent years, the average yield has been greatly enhanced from less than 1 viable embryo to 2.5-3.0 but most were still in the range of 1.0-2.0. But, in isolated cases over 4.0 (32) and 5.9 transferable embryos have been reported.

***In vitro* Production (IVP) of Buffalo Embryos:** IVP can mass-produce embryos of higher genetic merit and is an efficient tool. This new approach ensures the supply of ample embryos for genetic improvement of buffalo. Several factors influence the success of *in vitro* embryo production, which either increase or decrease the yield and gestational viability of embryos. Differences between donors and recipients with respect to breed, age and other physiological factors should be taken into account during IVP. Use of animals that are well adapted to the given environment will certainly assist to overcome the effect of environmental factors such as heat on oocyte quality and embryo yield. *In vitro* culture environment is another factor that immensely influences embryo production. There are different culture systems and culture media available for production of *in vitro* fertilized bovine embryos, and embryo yield generally varies among them. The efficiency of culture systems may also vary with labs/place, making data comparisons difficult. Therefore, it may be advisable to test what culture system or culture medium will provide better results for a given lab. For IVP of embryos, oocytes were amassed mainly from the ovaries collected at abattoirs and by OPU procedure. Collection of ovaries from abattoirs is very easy, but their genealogical records are not lucid. In contrary, the transvaginal ultrasound guided OPU allows repeated collection of oocytes from live donors of high genetic value.

***In vitro* Production of Buffalo Embryos from Ovaries of Slaughtered Animals** *:* The yield of immature oocyte and quality vary considerably depending, more or less, on species and health, size of the ovary, number of follicles accessible and retrieval methods. For example, the mean number of oocytes collected per ovary from buffalo and subsequently used for embryo production in vitro has been reported to vary between 0.43 and 0.70 in India (18), compared to 2.4 and 3.3 in Italy (19). The common method of IVP of embryos involves in vitro maturation (IVM) of the immature oocytes, *in vitro*

Vol. 5 (3) 1233-1250 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

fertilization (IVF) of the mature oocytes and *in vitro* culture (IVC) of the presumptive embryos until a portion forms morulae and blastocyst which can be transferred non-surgically to recipients (ET) or cryopreserved for future use.

***In vitro* maturation :** The ability of aspirated immature buffalo oocytes to mature *in vitro* is influenced to a large extent by maturation media and supplements. Many studies have divulged that the benefits from the supplements are media-dependent. Buffalo oocytes are mostly cultured in groups (5–20 oocytes) for 24 h in 50–100 ul droplets of tissue culture medium such as TCM-199 (20) or Ham's F-10 medium (7) or minimum essential medium and Waymouth medium (21) or Modified synthetic oviductal fluid (mSOF) media (Our unpublished data) under paraffin oil in a CO_2 incubator at 38.5°C (5% CO_2 and 90–95% relative humidity). Maturation medium may be supplemented with serum from different sources (22, 23, 24), human chorionic gonadotrophin (25), FSH (26), PMSG (27), LH and estradiol 17b, either alone or in combination (28, 29), buffalo follicular fluid (30) and feeder cells (granulosa cells, oviductal epithelial cells; 23). Various growth factors such as epidermal growth factor (EGF; 31), EGF plus fibroblast growth factor (32), insulin-like growth factor-I (IGF-I; 33), insulin-like growth factor-II (IGF-II; 34) and cysteamine (35) in oocyte culture media have been shown to increase cumulus expansion, nuclear maturation and post-fertilization events. The maturation rate in vitro of buffalo oocytes are assessed by methods such as staining the oocytes (metaphase II stage; 36) and degree of expansion of cumulus cell mass (30). Oocytes with full or moderate cumulus cell expansion and those with slight or no cumulus cell expansion but having the first polar body extruded in the perivitelline space may be considered as matured (28). Buffalo oocytes complete both meiotic divisions earlier than cattle oocytes in an in vitro system (37).

It remains a challenge to develop techniques, which would permit a swift assessment of the maturity stage of oocyte without under-estimating the actual maturation rate. More research is needed to describe the molecular and cellular events during oocyte maturation; the role of regulatory factors, gene expression and translation on completion of buffalo oocyte maturation in vitro.

***In vitro* Fertilization (IVF) :** IVF is carried out in Tyrode's modified medium (MTALP) or Brackett or Oliphant (BO) or mSOF medium by *in vitro* matured buffalo oocytes co-incubated with frozen-thawed *in vitro* capacitated spermatozoa. Although fresh semen gives better fertilization rates than frozen-thawed semen, the practicality of using fresh buffalo semen in IVF is negligible due to drastic changes in buffalo semen quality with season. BO medium supported higher fertilization and cleavage rates than MTALP medium (29, 38) with rates of 29.8% - 78.2% and 27.6% - 68.5%, respectively. In mSOF based IVF medium, it was reported that the fertilization and cleavage rate as 40-70% (Our unpublished data). Swim up or Percoll gradient technique are used for *in vitro* capacitation of buffalo spermatozoa from frozen-thawed semen in media containing sperm motility enhancers (22, 39, 40, 41). A marked difference in the sperm concentrations and sperm–oocyte co-incubation period has been reported in buffalo IVF with varying fertilization and cleavage rates (10, 22, 25, 38, 39, 42). The hindrance to production of IVF embryos is the occurrence of polyspermy among IVF buffalo oocytes at higher rate. For graded increase of sperm concentration from 1, 5 and 10 x 10^6 there was a corresponding increase in the occurrence of polyspermy from 24.0%, 43.2% and 64.0%, respectively (29). Perhaps,

Vol. 5 (3) 1233-1250 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

this high incidence of polyspermy will be modulated by improving the efficiency at the level of oocyte maturation. One critical aspect of a buffalo oocyte IVM/IVF system, that demands meticulous work, is improving the culture system for the development and formation of viable and transferable blastocysts. The methodology and molecular mechanism of capacitation remains poorly defined in buffalo. Recent advances in experimental techniques such as monoclonal antibodies and other specific probes of cell function together with methods adopted to study sperm function directly (43) may provide valuable information for further investigation of buffalo sperm capacitation at the molecular level.

In vitro **culture of embryos** : Culture of buffalo embryos are carried out in ligated oviduct of rabbits or complex medium (TCM-199) supplemented with serum and somatic cells (cumulus cells (38) or oviductal epithelial cells (44) or simple defined media (45) and semi-defined media (46)). In some studies simple defined media have been used for culture of buffalo embryos (19, 45) but most investigators have used complex media containing serum and somatic cells (22, 34, 39, 47). The most common defined medium for *in vitro* culture is synthetic oviductal fluid (SOF) or a modified form of SOF (mSOF). Our unpublished data showed that blastocyst yield up to 35 % of the cleaved embryos in mSOF media. Kumar et al. (2007) compared different media and reported that mSOFaa is the most effective medium for supporting the development of buffalo zygote to the morula and blastocyst stages (48). Supplementation of the embryo culture media with IGF-1 (49), insulin (47), cysteamine (35) and a combination of BSA, EGF and insulin transferrin selenium (ITS; 46) has been found to increase the blastocyst production and decrease the number of degenerated/arrested at the 2–16-cell stage embryos. In most laboratories, blastocyst production *in vitro* is 15–30% of inseminated oocytes.

Culturing embryos in somatic cell monolayer facilitated their development through the 8 to 16 cell block. Although the pattern of embryonic development up to the blastocyst stage is similar in cattle and buffalo, buffalo embryos had a faster rate of development to the blastocyst stage than cattle embryos. Buffalo embryos that complete first cleavage before 30h post insemination, are more likely to develop into blastocysts and the quality and viability of the these blastocysts are superior in terms of total cell number (TCN) than those complete first cleavage after 30h post insemination . The TCN, and trophoectoderm (TE) and inner cell mass (ICM) cell numbers are higher in blastocysts developing on or before Day 7 post fertilization than in those which develop after Day 7 (49). New concepts of *in vitro* cattle and human embryo culture that can be applied in buffalo IVP are the use of sequential media systems. In this system, the media components and physical components could be alerted during the culture. A further development of sequential media is the use of perfusion culture as the vehicle to introduce changes in media composition.

As sub-optimal culture conditions may lead to the production of embryos with developmental abnormalities and reduced viability, renewed research into the interaction of factors contributing to the development of a viable embryo and signal transduction mechanisms influencing embryo development may assist in the understanding of the relationship between the embryo and culture environment.

In vitro **Production of Buffalo Embryos aspirated from Live Animals** *:* Following successful trials of ultrasound guided oocyte aspiration from live cattle, commonly known as

Ovum Pick-Up, efforts were made to reproduce the same technique in buffalo. The transvaginal ultrasound-guided OPU technique is a noninvasive and repeatable procedure for recovering large numbers of meiotically competent oocytes from antral follicles of genetically proven live animals without sacrificing the animal for future production. Along with IVP, OPU can produce an average of 2.0±0.6 transferable embryos from per buffalo per month (50). Therefore, OPU augment the reproductive efficiency of females and expedite breeding progress when compared to abattoir-derived ovaries which have very little impact on genetic improvement although these provide a cheap and abundant source of oocytes for research and propagation of desired breed of animal. The association of OPU and IVP can be promising and feasible technique in cattle. However, in buffaloes, even after pursuing several studies (19, 50, 51), blastocyst rates are still low which ranges from 9.5% to 30.0% (50, 52). So far, the low rate of oocyte recovery (27.3% to 31.3%) and poor quality of oocyte and embryo seem to be interconnected to this inefficiency (51).

Various workers have compared the *in vitro* development ability of *in vitro* fertilized oocytes derived from abattoir ovaries and OPU. Zhang *et al.* (53) found out that the cleavage rates in the two groups were similar (57.73 versus 54.81 %), while the blastocyst rate of *in vitro* fertilized oocytes derived from abattoir ovaries was significantly higher than that of OPU (28.78 vs 21.34)(53). Neglia *et al.* (54) accounted that retrieval of good quality oocytes was lower in OPU than aspirating oocytes from slaughter house-derived ovaries but of a higher blastocyst yield (29.7% versus 19.9%) (54). Also, embryos arrested at tight morula stage (11.1% versus 22.3%) were of a lower proportion in live animals than abattoir ovaries. Pang *et al.,* (55) studied the role of seasonal effect playing on the efficiency of OPU and found that the average number of oocytes recovered per session and cleavage rate of oocytes after IVF in various season was not different with each other, while the blastocyst rate of IVF embryos in autumn-winter (27.09%) was significantly higher than those in spring (20.95%) and summer (20.45%) (55). In buffaloes, Sa Filho *et al.* (52) tested recombinant bovine somatotropin (rbST) on OPU-IVP aiming to improve recovery and quality of oocytes and they found that higher number of follicles aspirated (12.2 ± 0.1 vs 8.7 ± 0.04) and oocytes retrieved (5.2 ± 0.5 vs. and 4.1 ± 0.5) in rbST treated group than control group (52). However, blastocyst rate were similar in both the groups (19.7% for bST group and 26.0% for control group). Regarding pregnancy rates of IVP embryos, higher rate was observed for fresh embryos (14.3%) than vitrified embryos (8.0%), when embryo transfer was done at fixed time, in previously synchronized donors (GnRH/ PGF2á/GnRH). Further studies are still needed to develop strategies that improve embryo production in buffaloes, in an attempt to facilitate the commercial use of this technology.

Transgenesis, Nuclear Transfer and Cloning *:* Genetic modifications in buffalo using transgenesis, nuclear transfer and cloning have a huge potential for animal agriculture as well as biomedical science. Transgenic animals are generated by injecting desirable DNA into the pronuclei of *in vitro*-matured and fertilized zygotes, which are subsequently cultured *in vitro* to the blastocyst stage before transferring them to the recipient. Nuclear transfer involves the introduction of the nucleus from a totipotent donor cell into matured enucleated oocytes. The resulting embryo is transferred to a surrogate mother for development to term. Nuclear transfer

using embryonic, fetal and/or somatic cells as karyoplasts resulted in the production of cloned animals.

Transgenesis will accelerate the speed of genetic improvement and establish mammary gland bioreactors in buffalo, a potential way to improve the reproductivity and milk yield of buffalo. However, there are few reports on buffalo transgenesis. Verma *et al.* (56) reported transgenic embryos expressing green fluorescent protein (56). Huang *et al*. (57) produced transgenic buffalo embryos by chimera and nuclear transfer (NT) using buffalo embryonic germ (EG)-like cells expressing enhanced green fluorescent protein (EGFP) (57). Scientists of Animal Science and Technology of Guangxi University, China produced the world's first transgenic cloned buffalo male twin calves on 19th December, 2010 in Guangxi by lentivirus-mediated gene transfer. Somatic cell nuclear transfer (SCNT) is an emerging technology with many applications in animal breeding, from multiplying superior genotypes to making genetically engineered animals and genotyping to select the best genomes for breeding. The researches in buffalo SCNT has been started and got some successful outcome. Shi *et al.* (58) transferred 42 SCNT embryos into 21 recipients and got the first live cloned buffalo in the world (58). Yang *et al.* (59) reported the research on swamp and river buffalo inter subspecies SCNT, the SCNT embryos were cryopreserved and then transferred into 15 recipients, 5 recipients were confirmed to be pregnant, and one recipient delivered a cloned river buffalo (59).

Conventional Cloning Technique that was employed for the production of cloned sheep "Dolly" demands sophisticated and expensive equipments like micromanipulators etc. Hence, the scientists of Animal Biotechnology Centre (ABTC) at NDRI, Karnal have developed the landmark technique called "Hand guided Cloning Technique" which is an advanced modification of "Conventional Cloning Technique" (60). This new cloning technique is a simple and less demanding in terms of equipment, time and skill. Using this technique, scientists have produced the world's first cloned buffalo calf (60, 61), which died later, and the first live cloned buffalo is named as GARIMA. In this technique, immature oocytes isolated from ovaries were matured *in vitro*. Then they were denuded and treated with an enzyme to digest the zona pellucida. The oocytes were then treated with chemicals to push their genetic material to one side of the oocyte. This protruded side was then cut off with the help of "hand held fine blade" for removing the original genetic material of the oocyte. The enucleated oocytes were then electrofused with single cell taken from colony of embryonic stem cells or somatic cells. The resulting embryos were cultured and grown in the laboratory for seven days to develop them to the stage of blastocyst. The blastocysts were transferred to recipient buffaloes. One of the invaluable advantages of this technique is the production of sex predetermined calf, which will revolutionize the animal husbandry in near future.

Till date, four buffalo calves have produced by hand made guided technique. The first one in the world has born on 6th February, 2009, followed by second (GARIMA-I) on June 6th, 2009, third (GARIMA-II) on August 22, 2010 (61) and fourth (SHRESTH) on August 26, 2010 (61). Earlier three are female and the fourth one is male. This cloned buffalo calf is different from the earlier clone calves as, here the foster mother was provided opportunity for normal delivery with slight external assistance, the cloned calf was from ear somatic cell of 2 week old buffalo calf, and the embryo which led to successful pregnancy and normal delivery had remained frozen at -196^{0}C for one week in liquid nitrogen

and brought back to active life upon thawing at room temperature. The earlier three calves were born through caesarean operation and produced by using somatic, foetus and embryonic stem cell, respectively (60, 61, 62). The scientists are thinking to make the cryopreservation of embryos will need to be made as part of technique, so that the embryos could be transported and used at several places. The scientists are of the opinion that the embryonic stem cells have better cloning ability as compared to somatic cells, as such the epigenetic reprogramming of these cells is much more efficient than the somatic cells, which are already differentiated and lineage committed. Although India has a largest world's buffalo population and they are contributing about 55% of total milk production in country, but the percentage of elite animals is very low and there is an urgent need to enhance the population of these elite buffaloes. This technology could go a long way in helping scientist to multiply the superior milch buffaloes at a faster rate in India to face the challenges of increasing demands of milk in view of the ever growing human population. Furthermore, this technology will decrease gap between supply and demand of breeding bulls in the shortest possible time.

Embryo and gamete cryopreservation : Cryopreservation is also an important tool for the management of supernumerary embryos or embryos destined to be transferred under more appropriate conditions. This is particularly relevant in the buffalo, in which synchronization of recipients may be less efficient than in cattle, meaning that fewer surrogate females may be available for the transfer of embryos (63). Furthermore, *in vitro* culture and cryopreservation of cells for nuclear transfer apparently increase the chance of application of cloning technique to basic research and commercial purpose. The combination of SCNT technique with cryopreservation of cloned embryos is very important to enlarge the well-bred buffalos. However, intrinsic biological problems such as poor freezability of semen, high chilling sensitivity and high lipid content in buffalo oocytes and/or embryos have impeded the progress in cryopreservation. Few studies have been performed on embryo cryopreservation in buffalo. Cryopreservation techniques for preserving buffalo oocytes (64), spermatozoa (65), and *in vivo* embryos (66) have vastly improved in recent times. Recent reports have highlighted improvements in the cryopreservation of both OPU- and abattoir-derived oocytes, with morulae and blastocysts obtained following IVF of oocytes cryopreserved using slow-freezing or vitrification, although the embryo production efficiency was lower than that for fresh oocytes (67). Both conventional freezing and vitrification (68) are used for buffalo embryo cryopreservation and pregnancies from conventionally frozen (53) and vitrified (54) embryos, as well as live calves from conventionally frozen (53) and vitrified–warmed embryos (69), have been reported. Yang *et al.* (59) reported that 33.3% SCNT embryos survived programmed cryopreservation after thaw, and one cloned buffalo calf was obtained after transfer of thawed embryos (59). NDRI has produced the world's first male cloned buffalo calf (SHRESTH) from cryopreserved embryo at -196°C for one week in liquid nitrogen on August 26, 2010 by landmark technique "Hand guided Cloning Technique". Although the above researches have testified the feasibility of embryo cryopreservation in buffalo, this technique is not yet used as commercially. More works should be done in the future to promote the cryopreservation efficiency of buffalo gametes and embryos.

Sperm and embryo sexing : Pre-selection of sex of offspring at the time of conception is the most

Vol. 5 (3) 1233-1250 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

sought-after reproduction techniques of all time. Application of sexed semen allows the production of replacement dairy heifers from genetically superior dams. Because dairy farmers have less use for male calves, the use of sexed semen to produce only females would make buffalo production more attractive. Alternatively, because male calves exhibit higher growth rates, sexed semen can also be used for the preferential production of males for meat or draft animals. Sperm- sexing methods are based on the differential amount of DNA being present in the X- and Y-chromosome bearing sperm. Sort analysis of DNA content by flow cytometry, fluorescence in situ hybridization (FISH) and use of polymerase chain reaction (PCR) on individual sperm are effective for assessing samples to find the proportion of X and Y sperm. At the present time the flow cytometric sorting method and modified high-speed sorting provide the only fully validated means of sperm sexing in cattle. Though much of the work has been done in cattle, the information on sperm sexing in buffalo is very scanty.

Di *et al.* (70) cross-hybridized river buffalo spermatozoa using double color FISH with bovine X and Y-chromosome painting probes, prepared by degenerate oligonucleotide primed PCR (DOP-PCR) of laser-micro-dissected-catapulted chromosomes, to investigate the possibility of using bovine probes for sexing sperm (70). Presicce *et al.* (71) were the first to report flow cytometric sorting and freezing of buffalo spermatozoa, as well as calves born after insemination with sexed spermatozoa in Mediterranean Italian buffaloes (71). In other studies, Murrah and Nili-Ravi buffaloes were selected for flow cytometric sperm separation, with sorting accuracies for X- and Y-bearing spermatozoa of 94% and 89%, respectively, being reported (72). Transfer of the presumed X-embryos derived from IVF using sexed spermatozoa resulted in the birth of female twins, indicating the feasibility of sperm sorting by flow cytometry, the *in vitro* production of sex-preselected embryos and the birth of subsequent offspring, as well as the potential use of the technology in buffaloes (72). Liang *et al.* (16) reported that OPU and abattoir-derived oocytes fertilized *in vitro* with sexed spermatozoa had similar developmental competence with regard to cleavage (57.6% *v.* 50.4%, respectively) and blastocyst development (16.0% *v.* 23.9%, respectively) rates (73). Lu *et al.* (72) evaluate the efficiency of AI by using the sexed sperm to produce sex-preselected calves in buffalo species (74).

Pre-implantation embryos can be sexed or screened for genetic abnormalities using techniques such as polymerase chain reaction (PCR), fluorescence *in situ* hybridisation (FISH) and karyotyping. Pre-implantation genetic diagnosis (PGD) allows the continued use of valuable germplasm, even from carrier animals, by screening embryos before transfer and excluding affected embryos. The sexing of buffalo embryos has been reported by various workers using the polymerase chain reaction to amplify buffalo Y-chromosome-specific primers (75, 76). Fu *et al.* (77) reported embryo sexing by multiplex-nested PCR and this method is extremely reliable and useful in sexing embryos before transfer (77). For example, loop-mediated isothermal amplification (LAMP), a novel DNA amplification method that amplifies a target sequence specifically under isothermal conditions, has been developed for the rapid sexing of buffalo embryos through the identification of Y-chromosome-specific sequences (78). Although embryo sexing is routinely used in the multiple ovulation embryo transfer scheme in cattle, the development of accurate embryo splitting and sexing procedures

in buffalo requires optimization.

Results of above studies indicate the feasibility of the application of the sperm and embryo sexing technology to accelerate the genetic improvement in buffaloes. These some studies provide proof of concept for further research and wider field application in buffalo.

Conclusion

Water buffalo will remain as a crucial component of the Asian economy. As a provider of milk and source of meat, the research should be focused on genetic improvement of this important animal resource. However, the genetic improvement was impeded by the inherent biological parameters like long generation interval, which can be overpowered by using recently developed and refurbished reproductive biotechniques. Among them, AI will remain a major technology for buffalo genetic improvement programmes. Hence, there is a scope to refine AI technique with respect to its efficiency and other avoidable human related factors such as appropriate timing of AI, suitable semen handling and hygiene. Less number of ovarian follicles sets another limit to the efficient use of superovulatory regimens for buffalo production programmes. There is an urgent need, therefore, to develop more efficient systems in order to harness the available potential oocytes and maximize the utility of the superior members of the population. As an alternative, embryos can be produced *in vitro* through the aspiration of *in vivo* oocytes from superior donors and fertilizing them *in vitro*. *In vitro* oocyte fertilization and their culture appear to be the feeble point and hence efficiency of IVP of embryos has to be improved considerably to transform this technology as a practical tool in buffalo improvement. There are also various ways to maximize the efficient use of already available embryos, such as splitting the embryos prior to transfer. Once these technologies are made competent, then the current buffalo genetic resources can be ameliorated at the faster rate and the animal husbandry will be made an economical endeavor. But there is a long way before it is realized.

References

1. Department of Animal Husbandry, Dairying & Fisheries, Ministry of Agriculture, Government of India, New Delhi , Annual Report, 2007–2008.
2. Campanile, G., Neglia, G., Gasparrini, B., Galiero, G., Prandi, A., Di Palo, R., D'Occhio, M.J. and Zicarelli, L. (2005). Embryonic mortality in buffaloes synchronized and mated by AI during the seasonal decline in reproductive function. Theriogenology, 63: 2334-2340.
3. Patel, R. K., Singh, K.M., Soni, K. J. and Chauhan, J. B. (2006). Novel Cytogenetic finding: Unusual translocations X; X in Mehsana buffalo (*Bubalus bubalis*). Cytogenetic and Genome Res. 115 (2): 186-188.
4. Singh, M., Shukla, M. K. and Patel, R. K. (2006). Morphological defects of spermatozoa in frozen semen of *Bos taurus* (HF), *Bos indicus* (Sahiwal and Gir) and their crossbreds. Ind. J Comparative Animal Physiology. 24 (1): 91-94.
5. Singh, M., Shukla, M. K. and Patel, R. K. (2006). Sperm livability in frozen semen of *Bos taurus* (HF), *Bos indicus* (Sahiwal and Gir) and their crossbreds. Ind. J Comparative Animal Physiology. 24 (1): 63-65.
6. Shukla, M. K. (2011). Applied Veterinary Andrology and Frozen Semen Technology(ISBN: 978-93-80235-64-6).

7. Baruselli, P.S., Carvalho, N.A.T., Henriquez, C.E.P., Amaral, R., Nichi, M. and Reichert, R.H. (2003). Use of progesterone associated to "ovsynch" protocol for timed artificial insemination in buffalo (bubalus bubalis). Proc. 2nd Congresso Nazionale Sull'allevamento Del Buffalo, roma, 1: 265-268.

8. Carvalho, N.A.T., Nagasaku, E.M., Vannucci, F.S., Toledo, L.M. and Baruselli, P.S. (2007). Ovulation and conception rates according intravaginal progesterone device and hCG or GnRH to induce ovulation in buffalo during the off breeding season. Ital. J .Anim. Sci, 6 (2): 646-648.

9. Rhodes, F. M., McDougall, S., Burke, C. R., Verkerk, G. A. and Macimillan, K. L. (2003). Invited Review: Treatment of cows with an extended postpartum anestrous interval. J. Dairy Sci, 86: 1876-1894.

10. Baruselli, P. S., Carvalho, N.A.T., Gimenes, L.U. and Crepaldi, G.A. (2007). Fixed-time artificial insemination in buffalo. Italian J. Anim. Sci, 6: 107-118.

11. Singla, S.K. and M.L. Madan. (1990). Single ovulation and embryo transfer (SOET) in non-superovulated buffaloes. Theriogenology, 33: 329.

12. Gordon, I. (2003). Embryos and oocytes in research and commerce. In 'Laboratory Production of Cattle Embryos'. (Ed. G. J. Persley.) pp. 322–381. (CAB International:Wallingford, UK.)

13. Misra, A.K. (1996). Studies on improving certain aspects of embryo biotechnology in the buffalo. Ph. D. Thesis, CSA University of Agriculture and Technology, Kanpur, India.

14. Misra, A.K., Kasiraj, R., Mutha Rao, M., Ranga Reddy, N.S. and Joshi, B.V. (1994). Embryo transfer in buffalo in India: Progress in the last five years. Proc. IVth World Buffalo Congress, 3: 501-504.

15. Jiang R.M., Wei, Y.M. and Ling, Z.J. (2003). The Estrous and Conception Rates in Swamp Buffaloes after Synchronization by PGc CIDR alone or Cimbined with other Hormones. Chinese J. Anim. Sci, 39 (6): 17-18.

16. Liang, X., Qin, G. and Chen, M. (2007). Study on synchronization in buffalo. Animal Husbandry & Veterinary Medicine, Longjiang Animal Husbandry & Veterinary Medicine, 2: 34.

17. Qin, G., Chen M. and Liang, X. (2008). Experiments on synchronization induced by GnRH combining with PGF2á in buffalo. Hei Longjiang Animal Husbandry & Veterinary Medicine, 2: 34.

18. Das, G. K., Jain, G. C., Solanki, V. S., and Tripathi, V. N. (1996). Efficacy of various collection methods for oocyte retrieval in buffalo. Theriogenology, 46: 1403–1411.

19. Boni, R., Rovello, S., Gasparrini, B., Langella, M. and Zicarelli, L. (1999). *In vitro* production of buffalo embryos in chemically defined medium. Buffalo J, 1: 115 120.

20. Madan ML, Singla SK, Chauhan MS, Manik RS, (1994a). *In vitro* production and transfer of embryos in buffaloes. Theriogenology, 41, 139–143.

21. Ravindranatha, B.M., Nandi, S., Gupta, P.S.P. and Sarma, P.V. (2001). Comparison of three different media on maturation of buffalo oocytes in vitro. Ind. J. Anim. Sci, 71: 841–843.

22. Nandi, S., Chauhan, M.S. and Palta, P.

(1998). Influence of cumulus cells and sperm concentration on cleavage rate and subsequent embryonic development of buffalo (Bubalus bubalis) oocytes matured and fertilized *in vitro*. Theriogenology, 50: 1251–1262.

23. Nandi, S., Ravindranatha, B.M., Gupta, P.S.P. and Sarma, P.V. (2001b). Effect of somatic cells monolayer on maturation of buffalo oocytes *in vitro*. Ind. J. Anim. Sci, 71: 936–937.

24. Samad, H.A., Khan, I.Q., Rehmanb, N.U. and Ahmed, N. (1998). The recovery, *in vitro* maturation and fertilization of Nilli-Ravi buffalo follicular oocytes. Asian Aust. J. Anim. Sci, 11: 491 497.

25. Chuangsoongneon, U. and Kamonpatana, M. (1991). Oocyte maturation, *in vitro* fertilization and culture system for developing preimplantation Swamp buffalo embryos using frozenthawed semen. Buffalo J. l7: 189–198.

26. Pandey, A., Gupta, S.C. and Gupta, N. (2010). Effect of FSH and LH hormones on oocyte maturation of buffalo and gene expression analysis of their receptors and Cx43 in maturing oocytes. Zygote, 18: 231-234.

27. Gupta, P.S.P., Nandi, S., Ravindranatha, B.M. and Sarma, P.V. (2001). Effect of commercially available PMSG on maturation, fertilization and embryo development of buffalo oocytes *in vitro*. Reprod. Fertil. Dev, 13: 355–360.

28. Nandi, S., Ravindranatha, B.M., Gupta, P.S.P. and Sarma, P.V. (2002). Timing of sequential changes in cumulus cells and first polar body extrusion during *in vitro* maturation of buffalo oocytes. Theriogenology, 57 (3): 1151-1159.

29. Ocampo, L., Ocampo, M.B., Aquino, F.P., de Vera, R. and Cruz, L. (1996). Blastocyst formation of swamp buffalo embryos in co-culture system. Proc. 2nd ABA Congress, Manila, Phil., 412-416.

30. Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K. and Madan, M.L. (1997a). Replacement of serum and hormone additives with follicular fluid in the IVM medium: effects of maturation, fertilization and subsequent development of buffalo oocytes *in vitro*. Theriogenology, 48: 461–469.

31. Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S. and Madan, M.L. (1999). Effect of EGF on cumulus expansion, meiotic maturation and development of buffalo oocytes *in vitro*. Vet. Rec, 144: 266–267.

32. Gupta, P.S.P., Nandi, S., Ravindranatha, B.M. and Sarma, P.V. (2002). *In vitro* maturation of buffalo oocytes with epidermal growth factor and fibroblast growth factor. Ind. J. Anim. Sci, 72: 20–23.

33. Pawshe, C.H., Appa Rao, K.P.C.A. and Totey, S.M. (1998). Effect of insulin-like growth factor I and its interaction with gonadotrophins on *in vitro* maturation and embryonic development, cell proliferation and biosynthetic activity of cumulusoocyte complexes and granulosa cells in buffalo. Mol. Reprod. Dev, 49: 277–289.

34. Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S. and Tomer, O.S. (1998c). IGF-II stimulation of *in vitro* maturation, in vitro fertilization and subsequent development of buffalo (Bubalus bubalis) oocytes *in vitro*. Vet. Rec, 142: 727–728.

35. Anand, T., Kumar, D., Chauhan, M.S.,

Manik, R.S. and Palta, P. (2008). Cysteamine supplementation of *in vitro* maturation medium, *in vitro* culture medium or both media promotes in vitro development of buffalo (Bubalus bubalis) embryos. Reprod. Fertil. Dev, 20(2):253-257.

36. Yadav, B.R., Katiyar, P.K., Chauhan, M.S. and Madan, M.L. (1997). Chromosome configuration during *in vitro* maturation of goat, sheep and buffalo oocytes. Theriogenology, 47: 943– 951.

37. Neglia, G., Marino, M., Di Palo, R., Wilding, M., Caracciolo di Brienza, V., Dale, B., Gasparrini, B. and Zicarelli, L. (2001). A comparison of *in vitro* maturation in buffalo (Bubalus bubalis) bovine oocytes using confocal microscopy. Theriogenology, 55: 488.

38. Madan, M.L., Chauhan, M.S., Singla, S.K. and Manik, R.S. (1994b). Pregnancies established from water buffaloes (Bubalus bubalis) blastocysts derived from *in vitro* matured, in vitro fertilized oocytes and co-cultured with cumulus and oviductal cells. Theriogenology, 42: 591-600.

39. Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S. and Madan, M.L. (1998d). Influence of theophylline on cleavage rate and embryonic development following *in vitro* fertilization of buffalo oocytes. Ind. J. Anim. Sci, 68: 920–922.

40. Jainudeen, M.R., Takahashi, Y., Nihayah, M. and Kanagawa, H. (1993). *In vitro* maturation and fertilization of swamp buffalo (Bubalus bubalis) oocytes. Anim. Reprod. Sci, 31: 205–212.

41. Ramesha, K.P., Balakrisnan, M, Murthy, L.K. and Balakrisnan, C.R. (2000). Additive effect of pentoxyfylline and heparin on buffalo sperm motility and fertilization of oocytes matured in culture. Buffalo J, 16: 63–71.

42. Suzuki, T., Singla, S.K. Sujata, J. and Madan, M,L. (1992). *In vitro* fertilization of water buffalo follicular oocytes and their ability to cleave in vitro. Theriogenology, 38:1187–1194.

43. Kaul, G., Sarma, G.S., Singh, B. and Gandhi, K.K. (2001). Capacitation and acrosomal reaction in buffalo bull spermatozoa assessed by chlortetracycline and Pisum sativum agglutinin fluorescence assay. Theriogenology, 55: 1457–1468.

44. Yadav, P.S., Khanna, S., Hooda, O.K. and Sethi, R.K. (2000). Effect of oviductal cell co-culture on cleavage and development of buffalo IVF embryos. Asian Aust. J. Anim. Sci, 13: 894–896.

45. Abdoon ASS, Kandil OM, Otoi T, Suzuki T, 2001) Influence of oocyte quality, culture media and gonadotrophins on cleavage rate and development of *in vitro* fertilized buffalo embryos. Anim. Reprod. Sci, 65: 215–223.

46. Raghu, H.M., Nandi, S. and Reddy, S.M. (2002). Development of buffalo oocyte to the blastocyst stage *in vitro* in serumfree, semi-defined media: effect of insulin-transferrin-selenium and epidermal growth factor. Vet. Rec, 151 (9): 260-265.

47. Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K. and Tomer, O.S. (1998b). Effect of culture conditions on the hatching ability of *in vitro* produced buffalo (Bubalus bubalis) oocytes in vitro. Vet Rec 142, 169–171.

48. Kumar, D., Palta, P., Manik, S.R. and

Chauhan, M.S. (2007). Effect of culture media ans serum supplementation on the development of *in vitro* fertilized buffalo embryos. Ind. J. anim. Sci, 77 (8):679-701.

49. Narula, A., Taneja, M. and Totey, S.M. (1996). Morphological development, cell member and allocation of cells to trophoectoderm and inner cell mass of *in vitro* fertilized and parthenogenetically developed buffalo embryos: the effect of IGF-I. Mol. Reprod. Dev, 44: 343–351.

50. Gasparrini, B. (2002). *In-vitro* embryo production in buffalo species: state of the art. *Theriogenology*, 57: 237-256.

51. Campanile, G., Baruselli, P.S., Carvalho, N.A.T, Di Palo, R., Cavalcanti, R.B. and Vecchio, D. (2003). Effect of feed restriction on ovarian activity and recovered oocyte in murrah buffalo heifers. Bubalus Bubalis, 275-278.

52. Sa Filhoa, M.F., Carvalhoa, N.A.T., Gimenesa, L.U., Torres-Juniora, J.R., Nassera, L.F.T., Tonhatib, H., Garciab, J.M., B. Gasparrinic, B., L. Zicarellic, L. and P.S. Barusellia, P.S. (2009). Effect of recombinant bovine somatotropin (bST) on follicular population and on in vitro buffalo embryo production. Anim. Reprod. Sci, 113 (1): 51-59.

53. Zhang, X., Liang, X. and Chen, M. (2007). *In vitro* fertilization and subsequent embryo development using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration in water buffalo. Animal Husbandry & Veterinary Medicine, 11: 15-17.

54. Neglia, G., Gasparrini, B., Caracciolo di Brienza, V., Di Palo, R., Campanile, G., Presicce, G.A. and Zicarelli, L., (2003). Bovine and buffalo *in vitro* embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology, 59: 1123–1130.

55. Pang, C.Y., Yang, C.Y., Li, R.C., Li, J. H., Huang, F.X., Zhang, X.F., Qin, G.S., Qin, M.C., Yang, B.Z. and Liang, X.W. (2009). The Efficiency of Ovum Pick Up and the Embryo Development of *in vitro* Fertilization in Buffaloes (Bubalus bubalis) in Different Seasons. Pakistan J. Zool. Suppl. Ser., 9:323-327.

56. Verma, V., Gautam, S. K., Palta, P., Manik, R. S., Singla, S. K. and Chauhan, M. S. (2008). Development of a pronuclear DNA microinjection technique for production of green fluorescent protein-expressing bubaline (Bubalus bubalis) embryos. Theriogenology, 69: 655–665.

57. Huang, B., Cui1, K., Li, T., Wang, X., Lu, F., Liu, Q., da Silva, F. M. and Shi, D. (2010). Generation of Buffalo (Bubalus bubalis) Transgenic Chimeric and Nuclear Transfer Embryos Using Embryonic Germ-Like Cells Expressing Enhanced Green Fluorescent Protein. Reprod. Dom. Anim, 45: 103–108 (2010).

58. Shi, D., Lu, F.,Wei,Y., Cui, K.,Yang, S.,Wei, J. and Liu, Q. (2007). Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. Biol. Reprod, 77: 285–291.

59. Yang, C., Li, R. and Pang, C. (2008). New advances in buffalo SCNT. Southwest China J. Agri. Sci, 21: 61-64.

60. Shah, R.A., George, A., Singh, M.K., Kumar, D., Anand, T., Chauhan.M.S., Manik, R.S., Palta, P., Singla, S.K. (2009). Pregnancies established from handmade cloned blastocysts reconstructed using skin

fibroblasts in buffalo (Bubalus bubalis). Theriogenology. 1(8):1215-1219.

61. A quarterly newsletter of dairy science and technology. (2010). NDRI, News, 15(2): 7-8.

62. George, A., Sharma, R., Singh, K. P., Panda, S. K., Singla, S.K., Palta, P., Manik, R. S. and Chauhan, M. S. (2011). Production of Cloned and Transgenic Embryos Using Buffalo (Bubalus bubalis) Embryonic Stem Cell-Like Cells Isolated from *in Vitro* Fertilized and Cloned Blastocysts. Cellular Reprogramming. 13 (3): 1-11.

63. Presicce, G. A. (2007). Reproduction in water buffalo. Reprod. Domest. Anim, 42 (2), 24–32.

64. Dhali, A., Manik, R.S., Das, S.K., Singla, S.K. and Palta, P. (2000). Vitrification of buffalo (Bubalus bubalis) oocytes. Theriogenology, 53: 1295–1303.

65. Fabbrocini, A., Sorbo, C.B., Fasano, C. and Sansone G. (2000). Effect of differential addition of glycerol and pyruvate to extender on cryopreservation of Mediterranean buffalo (Bubalus bubalis) spermatozoa. Theriogenology, 54: 193–207.

66. Apimeteetumrong, M., Songsasen, N., Yiengvisavakul, V., Sukawongs, Y. and Techakumphu, M. (1998). Cryopreservation of embryos collected from elite swamp buffalo cows (Bubalus bubalis).Thai J. Vet. Med, 28: 39–48.

67. Gautam, S. K., Verma, V., Palta, P., Chauhan, M. S. and Manik, R. S. (2008*b*). Effect of type of cryoprotectant on morphology and developmental competence of *in vitro* matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or vitrification. Reprod. Fertil. Dev, 20: 490–496.

68. Gasparrini, B., Neglia, G., Di Palo, R., Campanile, G. and Zicarelli, L. (2000). Effect of cysteamine during *in vitro* maturation on buffalo embryo development. Theriogenology, 54: 1537–1542.

69. Hufana-Duran, D., Pedro, P.B., Venturina, H.V., Hufana, R.D., Salazar, A. L., Duran, P. G. and Cruz, L. C. (2004). Post-warming hatching and birth of live calves following transfer of *in vitro*-derived vitrified water buffalo (*Bubalus bubalis*) embryos. Theriogenology, 61: 1429–1439.

70. Di Berardino, D., Vozdova, M., Kubickova, S., Cernohorska, H., Coppola, G., Coppola, G., Enne, G. and Rubes, J. (2004). Sexing river buffalo (Bubalus bubalis L.), sheep (Ovis aries L.), goat (Capra hircus L.), and cattle spermatozoa by double color FISH using bovine (Bos taurus L.) X- and Y-painting probes. Mol. Reprod. Dev, 67(1):108-15.

71. Presicce, G. A., Rath, D., Klinc, P., Senatore, E. M. and Pascale, M. (2005*b*). Buffalo calves born following AI with sexed semen. Reprod. Domest. Anim, 40: 349.

72. Lu, Y. Q., Liang, X. W., Zhang, M., Wang, W. L., Kitiyanant, Y., Lu, S. S., Meng, B. and Lu, K. H. (2007). Birth of twins after *in vitro* fertilization with flow-cytometric sorted buffalo (*Bubalus bubalis*) sperm. Anim. Reprod. Sci, 100: 192–196.

73. Liang, X. W., Lu, Y. Q., Chen, M. T., Zhang, X. F., Lu, S. S., Zhang, M., Pang, C. Y., Huang, F. X. and Lu, K. H. (2008). *In vitro* embryo production in buffalo (*Bubalus*

bubalis) using sexed sperm and oocytes from ovum pick up. Theriogenology, 69: 822–826.

74. Lu, Y., Zhang, M., Lu, S., Xu, D., Huang, W., Meng, B., Xu, H. and Lu, K. (2010). Sex-preselected buffalo (Bubalus bubalis) calves derived from artificial insemination with sexed sperm. Anim. Reprod. Sci, 119(3-4):169-71.

75. Appa Rao, K.B.C. and Totey, S.M. (1999). Cloning and sequencing of buffalo male-specific repetitive DNA: sexing of *in vitro* developed buffalo embryos using multiple and nested polymerase chain reaction. Theriogenology, 51: 785–797.

76. Sood, S.K., Chauhan, M.S. and Tomer, O.S. (2001). A direct duplex PCR assay sex determination of Murrah buffalo (Bubalus bubalis) embryos. Buffalo J, 17: 113–124.

77. Fu, Q., Zhang, M., Qin,W. S., Lu,Y. Q., Zheng, H.Y., Meng, B., Lu, S. S. and Lu, K. H. (2007). Cloning swamp buffalo SRY gene for embryo sexing with multiplex-nested PCR. Theriogenology, 68: 1211–1218.

78. Hirayama, H., Kageyama, S., Takahashi, Y., Moriyasu, S., Sawai, K., Onoe, S., Watanabe, K., Kojiya, S., Notomi, T. and Minamihashi, A. (2006). Rapid sexing of water buffalo (*Bubalus bubalis*) embryos using loop-mediated isothermal amplification. Theriogenology, 66: 1249–1256.

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Does Nature has the Cure for Hypertension?: Endothelin Receptors as Drug Targets

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Abstract

Nature remains the major source of drugs with complex structures and novel biological activities. The search for bioactive molecules from nature remains the most important strategy to find new medicinal agents. Recent findings indicate that hypertension is a major health burden in economically developing countries. Thus a source of less expensive treatments needs to be identified. Current hypertension pharmacotherapy consists in administration of drugs capable of interacting with one or several molecular mediators. Within the group of drugs acting as inhibitors of receptors involved in blood pressure regulation, endothelin receptor antagonists have been the most prominent class containing drugs which were first found in nature. Thus the present review focuses on endothelin receptor as drug targets.

Key words: endothelin receptors, hypertension, marine natural products, plants, traditional medicine.

Introduction

Nature remains unrivaled in its ability to produce organic molecules with structural complexity and biological potency, which is why it has been a major source of drugs for centuries. Interest in natural product research within the new era of drug discovery includes issues like unmet medical needs, diversity of both chemical structures and biological activities (1). During the early decades of the last century, natural substances played a leading role as therapeutic agents. However, this role has declined in modern medicine; nowadays the search for bioactive molecules from nature (plants, animals, microflora) remains the most important strategy to find new medicinal agents (2). Hypertension is a public-health concern worldwide, because of its high frequency and its role as leading factor for mortality (3, 4). Approximately 8 million deaths each year worldwide are blood-pressure related (5). In the year 2000, nearly one billion of the world´s population had hypertension and the projections for 2025 estimate an increase to 1.56 billion people (6). Interestingly, recent findings indicate that hypertension is a major health burden in economically developing countries (7), where health-care resources are especially inadequate (6). For these countries, investment in primary prevention strategies such as weight loss, reduced intake of dietary sodium, moderate alcohol consumption, potassium supplementation, modification of eating habits, and increased physical activity would yield the greatest benefit (6) and should be the first line of prevention of chronic diseases such as

hypertension. A second alternative could be used taking in consideration the advice of the World Health Organization, which has been promoting a rational use of traditional medicine as a source of less expensive medical care (8).

Current pharmacotherapy of hypertension consists mainly on the use of two strategies. The first strategy is the application of a single drug capable of interacting with molecular mediators involved in the rennin-angiotensin system (9) and the endothelin system (10); with adrenergic (11) and urotensin II receptors (12); and with the atrial natriuretic peptide (13). The second strategy consists in the use of a "multi-target approach" (14) focused on the simultaneous treatment of more than one target involved in blood pressure regulation and the development of the disease. This second approach has been successfully tested combining drugs capable of inhibiting the activity of ACE and NEP; ECE and NEP; AT_1 and ET_A receptors (14).

From the group of drugs acting as inhibitors of receptors involved in blood pressure regulation, endothelin receptor antagonists have been the most prominent group containing drugs which were first found in nature (e.g. microorganisms, plants); thus the present review focuses on endothelin receptor as drug targets.

Pathophysiology of hypertension: the endothelin system

Endothelins are a family of vasoconstrictive peptides synthesized from larger precursors and expressed in different tissues (15). They consist of three isoforms, namely, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), being ET-1 the most widely distributed, best studied, and most powerful (16). ET-1 is synthesized in endothelial cells by means of a specific endothelin-converting enzyme (ECE) (15, 17). ET-1 appears to be the predominant member of the family with direct vascular effects, inotropic and mitogenic properties; it influences homeostasis of salts and water, alters central and peripheral sympathetic activity and stimulates the renin-angiotensin-aldosterone system (10).

Generation of endothelins : The major site of generation of ET-1 is in endothelial cells and to a lesser extent in smooth muscle cells, the heart, kidneys, posterior pituitary and central nervous systems (10). The initial product of the human endothelin-1 gene is preproendothelin-1 (212 amino acid peptide), which is then transformed into proendothelin-1 by removal of a short secretory sequence. Proendothelin-1 is further cleaved by a furin-like enzyme generating big endothelin-1 (38 amino acid peptide) (18). The formation of mature ET-1 requires cleavage of big endothelin-1 by one of several unique ECE. Several forms of ECE have been identified to have distinct roles and tissue distribution (10). ECE-1 and ECE-2 are relatively selective for big endothelin-1, having much less activity in cleaving big endothelin-2 and big endothelin 3. A third type of ECE was purified from bovine iris and named ECE-3, which selectively cleaved big endothelin-3 (15).

Endothelin receptors : Endogenous ET-1 contributes to maintenance of basal vascular tone and blood pressure through activation of two G-protein coupled receptors (GPCR), namely, endothelin ET_A (ET_A) and endothelin ET_B (ET_B) (19), the only two receptor subtypes identified in mammalian species, as mediating the different actions of endothelins (20). As a member of the GPCR superfamily, the ET_A receptor has seven hydrophobic membrane-spanning domains and a relatively long extracellular N terminal (10). A model for the ET-1/ET_A receptor interaction showed that ET-1 docks principally at the extracellular ends of the transmembrane domain 1 (TM1), TM2 and TM7 of the receptor as well as the extracellular loop 1 (EXL1) and, to a lesser

extent, EXL2 and part of the N terminus. Residues 140-156 were clearly involved in the binding interaction (21).

The binding of ET-1 to the ET_A receptor activates phospholipase C (PLC), which hydrolyses phosphatidylinositol-4,5-biphosphate (PIP_2) into two products, inositol-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to an initial increase of calcium concentration from the intracellular calcium stores. Afterwards a maintained increase in intracellular calcium levels is brought about by the opening of specific calcium channels which can be directly activated by the binding of endothelin to its receptor or by the IP_3 metabolite, IP_4. The increase in intracellular calcium promotes contraction which could also be partially affected by the action of DAG which is an activator of protein kinase C (PKC) (17). The activation of the ET_A receptor also induces cell proliferation in different tissues. In contrast, the activation of endothelial ET_B receptor stimulates the release of nitric oxide (NO) and prostacyclin, prevents apoptosis, and inhibits ECE-1 expression in endothelial cells (22).

Pathophysiology of endothelins: Resistance vessels and veins are particularly sensitive to the effects of ET-1. On smooth muscle cells, the vasoconstrictor effect caused by ET-1 is mediated by activation of both ET_A and ET_B receptors present on these cells (10, 23). The opposite effect is mediated by stimulation of the ET_B receptor on the endothelial cells by means of NO and prostacyclin (24). Due to this situation the net effect of ET-1 depends not only on the balance between ET_A and ET_B, but also on vessel type-size and the receptors localization (10, 23). This is the reason why available literature suggests that dual ET_A/ET_B receptor antagonism is more effective than selective ET_A receptor antagonism in order to fully prevent the deleterious actions of ET-1 in cardiovascular disease (23). It has been demonstrated that ET-1 is involved as an etiologic or aggravating factor in a number of cardiovascular diseases, including essential hypertension, pulmonary hypertension, acute renal failure, cerebral vasospasm after subarachnoid hemorrhage, vascular remodeling, cardiac hypertrophy, and congestive heart failure (25). During the development of cardiovascular disease the expression and biological activities of ET-1 and its receptors are altered. For instance, patients with pulmonary hypertension, a devastating disease with a median life expectancy of less than 2.8 years postdiagnosis, have increased plasma levels of ET-1 due to either an increase in its synthesis or a reduction in its clearance (26). In patients with congestive heart failure, ET_A receptors are upregulated, while ET_B receptors are downregulated (27, 28); and in patients with systemic hypertension ET-1 plasma levels are normal, with a local increase of ET-1 levels in the vascular wall (28, 29).

Endothelin receptor antagonists: Structural differences

The development of endothelin receptor antagonists is a rapid evolving area (30). Many pharmaceutical companies have discovered a large number of endothelin receptor antagonists by random screening of their compound libraries. This section intends to review those of most common use in clinical and preclinical investigations (Table 1).

Peptides and peptidomimetics : The first endothelin receptor antagonists developed were of a peptide nature. It was discovered that the ET_A receptor recognizes the tertiary structure of the N-terminal and C-terminal portion of the endothelin molecules. A major advance was made with the discovery of two cyclic pentapeptides BE-18257A and BE-18257B isolated from the bacteria *Streptomyces misakiensis* (Table 2) (31, 32). Chemical modifications on the structure of compound BE-

18257B originated the first selective endothelin ET_A receptor antagonist BQ-123, a cyclic pentapeptide possessing three diaminopropionic acids (Table 1) (33). Another endothelin ET_A receptor antagonist of a peptide nature is the compound BQ-610, which is used in cerebral vasoconstriction produced by subarachnoid haemorrhage (34). Three examples more are the cyclic hexapeptide TAK-044, a non-selective endothelin ET_A/ET_B receptor, and the linear peptides PD-145065 and FR-139317 (Table 1) (35).

Myriceric acid derivatives : Non-peptide endothelin antagonists have also been developed. The first was myriceric acid A (compound 50-235) (Table 2), an oleanane triterpene selective for the endothelin ET_A receptor, which was isolated from the bayberry *Myrica cerifera* (36). Its analog, S-0139, faced problems of oral bioavailability and it was developed in parenteral formulations as neuroprotector in cerebral ischemia (Table 1) (35, 37).

Carboxylic acid derivatives : In the search of highly potent endothelin receptor antagonists, pharmaceutical companies incorporated carboxylic acid moieties. Within this group, Abbott synthesized atrasentan, a pyrrolidine-3-carboxylic acid derivative selective for the endothelin ET_A receptor; SmithKline Beecham synthesized enrasentan and SB-209670, two indane-2-carboxylic acids derivatives (38), and compounds J-104132 and PD-156707, which were further derived (Table 1) (35).

Phenyl acetic acid derivatives: The most prominent compounds of this group are the highly potent non-selective endothelin ET_A/ET_B receptor antagonists L-754142 developed by Merck and PABSA developed by Shionogi (Table 1) (35).

Heteroaryl sulfonamide derivatives : This group contains the well-known oral and parenteral endothelin ET_A selective antagonist TBC-11251 (sitaxsetan) and the orally active endothelin ET_A selective antagonists BMS-181874 and BMS-193884. Also from this group is ZD-1611, a selective endothelin ET_A antagonist which has been discontinued from further development (Table 1) (35).

3,3-Diphenylpropionic acid derivatives : Darusentan is the best-known compound of this group, which shows endothelin ET_A selective antagonism and is used in clinical studies for congestive heart failure and hypertension (35).

Tetra-substituted pyrimidines : From this group, the most important non-peptide endothelin antagonist is the compound Ro-47-0203 (bosentan) (39), a more potent analog of Ro-46-2005 (40, 41) which was discovered by high-throughput screening. Both compounds Ro-46-2005 and Ro-47-0203 are non-selective endothelin ET_A/ET_B receptor antagonists (39, 42). Other examples are tezosentan (Ro-61-0612) a non-selective ET_A/ET_B receptor antagonist for parenteral use, TA-0201 an orally active ET_A selective antagonist and the non-selective ET_A/ET_B receptor antagonist YM-598 for oral and parenteral administration (Table 1) (35).

Non-sulfonamide derivative : Ambrisentan is the first non-sulphonamide ET_A receptor antagonist with proven efficacy for the treatment ofpulmonary arterial hypertension. Its chemical structure (propanoic-acid class), may confer benefits over other endothelin antagonists in terms of a lower risk of drug interactions (Table 1) (43).

Structural requirements for endothelin ET_A receptor antagonism

Rational design of selective receptor antagonists requires a good understanding of the binding properties and binding sites of the endogenous agonist. Early attempts to determine specific conformational features necessary for receptor binding and vasoconstricting activity of

Table 1. Applications, structural requirements and origin of most common endothelin ET_A receptor antagonists.

Name	Structure	Base	Origin	Selectivity	Structural requirements for the activity	Application
Peptides and peptidomimetics						
BQ-123		Cyclic pentapeptide	Semi-synthetic	ET_A	β, γ-backbone conformation and common features with C-terminus of ET-1 (58)	Arrhythmias, cerebral ischemia, diabetic complications, hypertension (33, 35)
BQ-610		Linear peptide	Synthetic	ET_A	common features with C-terminus of ET-1 (58)	Subarachnoid haemorrhage (34, 44)
TAK-044		Cyclic hexapeptide	Synthetic	ET_A/ET_B	common features with C-terminus of ET-1 (58)	Renal failure, acute myocardial infarction (35, 45)

PD-145065		Linear hexapeptide	Synthetic	ET_A/ET_B	common features with C-terminus of ET-1 (58)	Hypertension (35, 46, 47)
FR-139317		Linear tripeptide	Natural (*Streptomyces sp.*)	ET_A	common features with C-terminus of ET-1 (58)	Hypertension (35, 48)
Non-peptide, triterpene derivative						
S-0139 (SB-737004)		Non-peptide, triterpene	Semi-synthetic	ET_A	Carbonyl group at C-3; carboxylic acid group at C-17, trans-caffeoyloxy group at C-27, dimethyl group at C-20 (64)	Cerebral ischemia (37), hemorrhagic and ischemic stroke (35, 49)
Carboxylic acid derivatives						

Atrasentan (A-127722)		Carboxylic acid	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Prostate cancer, heart failure, diabetic nephropathy (38, 50)
Enrasentan		Carboxylic acid	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Pulmonary hypertension, congestive heart failure (38)
SB-209670		Carboxylic acid	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Acute renal failure (38)

J-104132		Carboxylic acid	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Congestive heart failure, hypertension (35, 45)
PD-156707		Carboxylic acid	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Hypertension (35, 45)
Phenyl acetic acid						
L-754142		Phenyl acetic acid	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Renal protection in pre-clinical model (35, 51)
PABSA		Phenyl acetic acid	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Hypertension, renal failure (35)

Heteroaryl sulfonamide						
BMS-182874		Heteroaryl sulfonamide	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Coronary disorders, hypertension (35)
BMS-193884		Heteroaryl sulfonamide	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Congestive heart failure (35, 45)
TBC-11251 (sitaxsetan)		Heteroaryl sulfonamide	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Congestive heart failure (45), pulmonary hypertension (35)
ZD-1611		Heteroaryl sulfonamide	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Discontinued, pulmonary hypertension (35)

3,3-Diphenylpropionic acid derivatives						
LU-135252 (darusentam)		3,3-diphenylpropionic acid	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Congestive heart failure (45), resistant hypertension (35, 52)
Non-peptide, substituted pyrimidines						
Ro-47-0203 (bosentan)		Non-peptide, tetra-substituted pyrimidines	synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Essential and pulmonary hypertension (35, 39, 53, 54, 55)
Ro-46-2005		Non-peptide, tri-substituted pyrimidines	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Hypertension (40, 41)

Ro-61-0612 (tezosentan)		Non-peptide, tetra-substituted pyrimidines	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Acute heart failure (35, 45)
TA-0201		Non-peptide, tri-substituted pyrimidines	Synthetic	ET_A	Acidic group between two aromatic rings (35, 64)	Heart failure (35)
YM-598		Non-peptide, tetra-substituted pyrimidines	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Hypertension (35)
Macitentan (Actelion-1, ACT-064992)		Non-peptide, tri-substituted pyrimidines	Synthetic	ET_A/ET_B	Acidic group between two aromatic rings (35, 64)	Idiopathic pulmonary fibrosis, pulmonary arterial hypertension (56, 57)
Non-sulphonamide, propanoic acid						
Ambrisentan		Propanoic acid	Synthetic	ET_A	Possible same mechanism as diphenylpropionic acid derivatives	Pulmonary hypertension (43)

ET-1 utilized the help of circular dichroism (CD) studies, nuclear magnetic resonance (NMR) and molecular dynamics simulation (59). ET-1 was identified to be 30-35% helical between residues Lys9 and Cys15, being the disulfide the responsible for this helicity (60). Additionally, the cyclic structure of the peptides would seem to be essential for binding and functional activity, where the outer disulfide bond Cys1-Cys 15 would appear to be indispensable (61). L-Stereochemistry of the C-terminal of ET-1 is also crucial for binding and functionality, thus antagonists have been designed to have D-stereochemistry at position 16 (59).

Findings from structure-activity relationship studies of cyclic pentapeptide antagonists showed that these antagonists required a type II β,γ backbone conformation with DDLDL chirality to show affinity for the ET_A receptor (62). However, the amino acid residue at position 3 can be deleted to generate linear peptides while retaining the β-turn structure (63). Moreover, addition of functional groups on the side chain of the amino acid at position 3 residue can generate an antagonist with a more preferable pharmacokinetics. The D-Val^4 position is critical for activity, and a lipohilic D-amino acid with a β-position branched alkyl side chain such as D-Val or D-Cpg or a lipophilic D-heteroarylglycine such as D-Thg is preferable (62).

Plenty of studies have shown that diverse structural classes of molecules can function as endothelin receptor antagonists (35). These studies showed common features for the different chemical groups. For instance, most of non-peptidic endothelin receptor antagonists like carboxylic acids, tetra-substituted pyrimidines and sulfonamides have at least two aromatic groups placed around one acidic functional group (35, 64). Chemical improvements have been made on carboxylic acid derivatives by replacement of the indan ring for a pyrrolidine ring, like compound SB-209670. Moreover, the type of N substituent has been shown crucial for endothelin receptor antagonism, like featured by atrasentan (A-127722) (64).

For the case of triterpene derivatives like myriceric acid A four functional groups have been identified as essential for their activity and affinity to the ET_A receptor; namely, the carbonyl group at C-3, carboxylic acid group at C-17, dimethyl group at C-20 and *trans*-caffeoyloxy group at C-27. Moreover, the study revealed that when the hydroxyl groups on the *trans*-caffeoyl moiety where sulfated, the binding affinity increased up to 20-fold (65).

Diverse origins for endothelin receptor antagonists

The need to find selective, potent and safe endothelin ET_A receptor antagonists has moved forward the development of drug discovery programs within natural product research. Since random screening of natural products followed by rational chemical design have played a major role in the discovery of successful antihypertensive drugs acting on the rennin-angiotensin system (angiotensin coverting enzyme inhibitors and angiotensin II AT_1 blockers) (66) and the endothelin system (35), it is important to keep screening natural resources for new molecules with new properties.

Microorganisms : The first cyclic pentapeptide ET_A receptor antagonists were discovered within a screening program for endothelin-binding inhibitors from the culture broths of microorganisms. Compounds BE-18257A and BE-18257B were isolated from the bacteria *Streptomyces misakiensis* obtained from soil samples (Table 2) (67). These novel cyclic pentapeptides gave origin to the most commonly used selective ET_A receptor antagonist, the

analogue BQ-123 (Table 1) (68). The observed potency and selectivity of these cyclic pentapeptides has promoted the development of several tripeptide analogues having N-terminal amide, urethane and urea moieties or derivatives of the tryptophan indole ring, such as BQ-788 and BQ-017 (selective ET_B receptor antagonists), and BQ-928 a non-selective ET_A/ET_B antagonist (69). More recently, within a screening program for compounds which inhibit binding of endothelin to its receptor, three novel pentapeptolides named aselacin A, aselacin B and aselacin C were isolated from the fungus *Acremonium spp*. grown in stationary culture. Aselacin A (Table 2) was found to be a selective inhibitor of the ET_A receptor. These compounds have a ring formed by cyclo[Gly-D-Ser-D-Trp-β-Ala-L-Thr] and an additional exocyclic D-Gln to which is attached to a functionalized long chain fatty acid (70, 71).

Plants : Myriceric acid A was isolated from the twigs of the southern bayberry (*Myrica cerifera*) during a screening program of approximately 400 different plants (36, 72, 73).

Since random screening of natural products has led to the discovery of several bioactive compounds (41), diverse screening programs have been designed to search for potent endothelin antagonists. Example of this is a study carried out using crude drugs from Chinese medicines, in which pheophorbide a was found (Table 2) (74). In the same line, 149 extracts from nineteen plants described by traditional Panamanian medicine has also led to identification of potential sources of new active molecules. The results suggest further investigation of the chemical constituents in the ethanolic extracts of the leaves of *Cecropia cf.obtusifolia* Bertol., the leaves of *Hedyosmum bonplandianum* H.B.K., the roots of *Bocconia frutescens* L., the stem of *Cecropia cf.obtusifolia* Bertol. and the branches of *Psychotria elata* (Sw.) Hammel (75).

Additionally, a random screening of triterpenoid saponins and the corresponding aglycons was carried out on the human ET_A receptor. The results showed that selectivity for the ET_A receptor was exhibited by asiatic acid and its saponins; and to a lesser extent by betulinic acid, β-amyrin and friedelin (Table 2) (76).

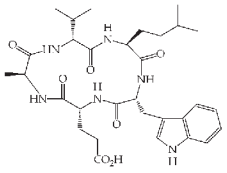
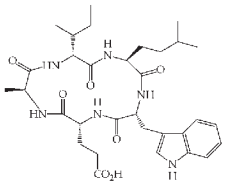
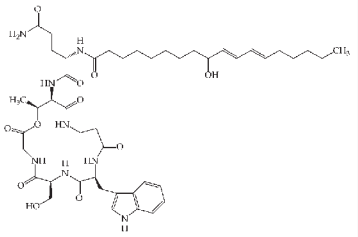
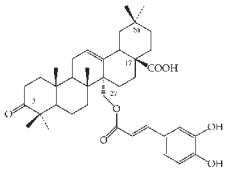
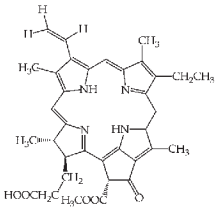
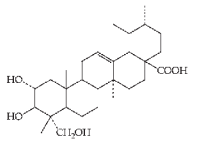
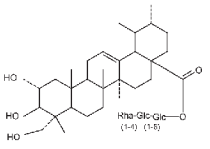
Animals : Attempts at finding more selective and potent ET_A receptor antagonists have led researchers to study natural sources of diverse habitats. Sponges of the genus *Iantbella* have been reported to contain bromotyrosine derivatives, called bastadins, showing variable numbers of tyrosine units as well as different substitution patterns on the tyrosine units. These bastadins possesses a different ring pattern with an alternative oxidative cyclization of the general bastadin backbone. The first isolated sulfated compound of the bastadin series was 34-sulfobastadin 13, which showed some inhibition of endothelin binding to its receptor (Table 2) (77). In the same line, further screening of marine natural products showed that plakortide N isolated from the sponge *Plakortis halichondrioides* caused a moderate inhibition of [^{3}H] BQ-123 binding to the ET_A receptor (Table 2) (78).

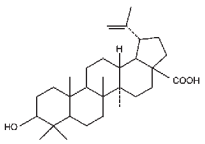
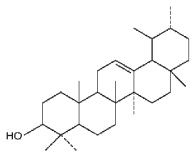
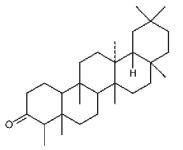
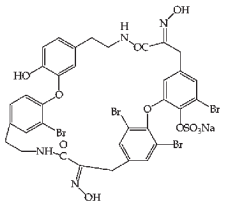
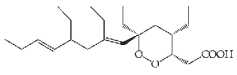
Other mechanisms by which natural products inhibit the endothelin system

Polyphenols from red wine (Cabernet Sauvignon grapes) decreased ET-1 synthesis in cultured bovine aortic endothelial cells by suppressing transcription of the ET-1 gene. Red-grape juice also inhibits ET-1 synthesis, but it is markedly less potent than red wine. On the other hand, the white and rosé wines had no effect (79).

Freeze-dried garlic powder caplets were tested on rat´s isolated pulmonary arteries and

Table 2. Natural occurring compounds found to inhibit binding of specific ligands to the ET_A receptor.

Name	Structure	Base	Origin	Inhibition of binding to ET_A receptor (IC_{50} values or % of binding inhibition)	Reference
BE-18257A		Cyclic pentapeptide	Bacteria (*Streptomyces misakiensis*)	3.00 µM (IC_{50})	(31)
BE-18257B		Cyclic pentapeptide	Bacteria (*Streptomyces misakiensis*)	1.40 µM (IC_{50})	(31, 32)
Aselacin A		cyclopentadepsipeptide	Natural (*Acremonium spp.*)	24.20 µM (IC_{50})	(70, 71)
Myriceric acid A (50-235)		Non-peptide, triterpene	Natural (*Myrica cerifera*)	0.06 µM (IC_{50})	(65, 72, 73)
Pheophorbide a			Plant (*Artemisia capillaris* Thunb.)	0.08 µM (IC_{50})	(74)
Asiatic acid		Triterpene	Plant	74% (10µg/mL)	(76)
Asiaticoside		Triterpenoidal saponin	Plant	44% (10µg/mL)	(76)

Betulinic acid		Triterpene	Plant	45 % (10µg/mL)	(76)
β-amyrin		Triterpene	Plant	49% (10µg/mL)	(76)
friedelin		Triterpene	Plant (*Garcinia livingstonei*)	36% (10µg/mL)	(76)
34-sulfatobastadin 13		bromotyrosine	Marine sponge (*Ianthella sp.*)	39.00 µM (IC_{50})	(77)
Plakortide N		Polyketide endoperoxide	Marine sponge (*Plakortis halichondrioides*)	44% (100 µg/mL)	(83)

showed both an endothelium (NO)-dependent and –independent relaxation with inhibition of ET-1 contraction (80). These preliminary findings cannot differentiate whether this inhibition was at the receptor level or by direct relaxation on vascular smooth muscle cells. Thus further studies are required to determine the molecular interactions responsible for this effect.

Compound CPU 86017 (*p*-chloro benzyl tetra hydroberberine), obtained as a derivative of berberine, is capable of indirectly inhibiting the endothelin system by blocking voltage-dependent calcium channels coupled to ET_A receptors by G proteins. Additionally, CPU 86017 suppresses the the endothelin-reactive oxygen species pathway responsible for the increase in mRNA of prepro-ET-1, eNOS, and iNOS due to increments of ROS (81).

Conclusions

Pharmaceutical companies have invested huge resources screening thousands of compounds from their chemical libraries in search for novel and more active molecules. Nevertheless, plenty of evidence supports the fact that nature remains the first class source of diverse, complex and active new chemical structures (2). The case of endothelin receptor antagonists is a good example of the uniqueness of nature´s ability to create chemical diversity, and how the researchers´ work has been focused to only perform small modifications of these natural occurring substances in order to improve their activities.

Remarkable chemical diversity has been found when analyzing endothelin receptor antagonists from different sources. For instance,

microorganisms and marine sponges showed to produce related peptide-like structures, capable of mimicking the binding of endogenous peptides without displaying their functionality. Interestingly, it has been proven that many bioactive compounds isolated from marine sponges are produced by its associated microorganisms (82), suggesting that more research should be done in isolating the chemical entities amongst the microbiota of marine sponges.

Additionally, selectivity for one receptor was found in plant-derived compounds, suggesting that they are a good source of potential lead compounds and that compounds identified during screening programs could end up as novel, more active and safer structures and should be then promoted for further evaluation.

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References

1. Sandsborg, R. (1999). Natural products in drug discovery and development. In: Bioassay Methods in Natural Product Research and Drug Development. Bohlin, L., Bruhn, J.G., editors. Kluwer Academic Publishers, pp 143-149.
2. Dev S. (2010). Impact of natural products in modern drug development. Indian J Exp Biol, 48: 191-198.
3. He, J. and Whelton, P.K. (1997). Epidemiology and prevention of hypertension. Med Clin North Am., 81: 1077–1097.
4. Ezzati, M., Lopez, A.D., Rodgers, A., Vander Hoorn, S. and Murray, C.J. (2002). Selected major risk factors and global and regional burden of disease. Lancet, 360: 1347–1360.
5. Sood, N., Reinhart, K.M. and Baker, W.L. (2010). Combination therapy for the management of hypertension: A review of the evidence. Am J. Health. Syst. Pharm., 67: 885-894.
6. Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K. and He, J. (2005). Global burden of hypertension: analysis of worldwide data. Lancet, 365: 217–223.
7. Murray, C.J. and Lopez, A.D. (1997). Mortality by cause for eight regions of the world: Global Burden of Disease Study. Lancet, 349: 1269–1276.
8. Schuster, B.G. (2001). A New Integrated Program for Natural Product Development and the Value of an Ethnomedical Approach. Journal of Alternative & Complementary Medicine, 7: 61-72.
9. Dinh, D.T., Frauman, A.G., Johnston, C.I. and Fabiani, M.E. (2001). Angiotensin receptors: distribution, signaling and function. Clinical Science, 100: 481-492.
10. Haynes, W.G., Webb, D.J. (1998). Endothelin as a regulator of cardiovascular function in health and disease. Journal of Hypertension, 16: 1081-1098.
11. Langer, S.Z., Cavero, I. and Massingham, R. (1980). Recent developments in noradrenergic neurotransmission and its relevance to the mechanism of action of

certain antihypertensive agents. Hypertension, 2: 372-382.

12. Douglas, S.A., Dhanak, D. and Johns, D.G. (2004). From 'gills to pills': urotensin-II as a regulator of mammalian cardiorenal function. Trends Pharmacol Sci, 25: 76-85.

13. Johnston, C.I., Hodsman, P.G., Kohzuki, M., Casley, D.J., Fabris, B. and Phillips, P.A. (1989). Interaction between atrial natriuretic peptide and the renin angiotensin aldosterone system. Endogenous antagonists. Am J Med, 87: 24S-28S.

14. Balsamo, A., Calderone, V. and Rapposelli, S. (2008). New emerging prospects in the pharmacotherapy of hypertension. Cardiovascular & Hematological Agents in Medicinal Chemistry, 6: 1-19.

15. Masaki, T. (2000). The endothelin family: an overview. Journal of Cardiovascular Pharmacology, 35: S3-S5.

16. Hunley, T.E. and Kon, V. (2001). Update on endothelins – biology and clinical implications. Pediatr Nephrol, 16: 752–762.

17. Ortega, A. and De Artiñano, A. (1997). Highlights on endothelins: a review. Pharmacological Research, 36: 339-351.

18. Yanagisawa, M., Kurihawa, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K.and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature, 332: 411-415.

19. Takigawa, M., Sakurai, T., Kasuya, Y., Abe, Y., Masaki, T. and Goto, K. (1995). Molecular identification of guanine-nucleotide-binding regulatory proteins which couple to endothelin receptors. Eur J Biochem., 228: 102-108.

20. Goldie, R.G. (1999). Endothelins in health and disease: an overview. Clinical and Experimental Pharmacology and Physiology, 26: 145-148.

21. Orry, A.J. and Wallace, B.A. (2000). Modeling and docking the endothelin G-protein-coupled receptor. Biophysical Journal, 79: 3083-3094.

22. Lüscher, T.F. and Barton, M. (2000). Endothelins and endothelin receptor antagonists. Therapeutic considerations for a novel class of cardiovascular drug. Circulation, 102: 2434-2440.

23. Böhm, F. and Pernow, J. (2007). The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. Cardiovasc Res., 76: 8-18

24. DeNucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C. and Warner, T.D. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium derived relaxing factor. Proc Natl Acad Sci USA, 85: 9797-9800.

25. Goto, K. (1999). History of endothelin. In: Pulmonary actions of the endothelins. Goldie, R.G., Hay, D.W., editors. Kirkhäuser Verlag Basel, Switzerland, pp 1-20

26. Pullamsetti, S.S. and Schermuly, R.T. (2009). Endothelin receptor antagonists in preclinical models of pulmonary hypertension. European Journal of Clinical Investigation, 39: 3-13.

27. Zolk, O., Quattek, J., Sitzler, G., Schrader, T., Nickening, G., Schnabel, P., Shimada, K., Takahashi, M. and Bohm, M. (1999). Expression of endothelin-1, endothelin-

converting enzyme, and endothelin receptors in chronic heart failure. Circulation, 99: 2118-2123

28. Kawanabe, Y.and Nauli, S. (2011). Endothelin. Cellular and Molecular Life Sciences, 68: 195-203.

29. Schiffrin, E.L. (2001). Role of endothelin-1 in hypertension and vascular disease. Am J Hypertens, 14: 83S-89S

30. Opgenorth, T.J. (1995). Endothelin receptor antagonism. Adv Pharmacol, 33: 1-65.

31. Nakajima, S., Niiyama, K., Ihara, M., Kojiri, K. and Suda, H. (1991). Endothelin-binding inhibitors, BE-18257A and BE-18257B: II. Structure determination. J Antibiot., 44: 1348-1356.

32. Ihara, M., Fukuroda, T., Saeki, T., Nishikibe, M., Kojiri, K., Suda, H. and Yano, M. (1991). An endothelin receptor (ET_A) antagonist isolated from *Streptomyces misakiensis*. Biochem Biophys Res Commun., 178: 132-137.

33. Ishikawa, K., Fukami, T., Nagase, T., Fujita, K., Hayama, T., Niiyama, K., Mase, T., Ihara, M. and Yano, M. (1992). Cyclic pentapeptide endothelin antagonists with high ET_A selectivity. Potency and solubility-enhancing modifications. J Med Chem., 35: 2139-2142.

34. Zuccarello, M., Lewis, A.I. and Rapoport, R.M. (1994). Endothelin ET_A and ET_B receptors in subarachnoid haemorrhage-induced cerebral vasospasm. Eur J Pharmacol., 259: R1-R2.

35. Boss, C., Bolli, M. and Weller, T. (2002). Endothelin receptor antagonists: structures, synthesis, selectivity and therapeutic applications. Current Medicinal Chemistry, 9: 349-383.

36. Mihara, S. and Fujimoto, M. (1993). The endothelin ET_A receptor-specific effect of 50-235, a nonpeptide endothelin antagonist. Eur J Pharmacol, 246: 33-38.

37. Zhang, R.L., Zhang, C., Zhang, L., Roberts, C., Lu, M., Kapke, A., Cui, Y., Ninomiya, M., Nagafuji, T., Albala, B., Zhang, Z.G. and Chopp, M. (2008). Synergistic effect of an endothelin type A receptor antagonist, S-0139, with rtPA on the neuroprotection after embolic stroke. Stroke, 39: 2830-2836.

38. Elliot, J.D., Lago, M.A., Cousins, R.D., Gao, A., Leber, J.D., Erhard, K.F., Nambi, P., Elshousbaby, N.A., Kumar, C., Lee, J.A., Bean, J.W., De Brosse, C.W., Eggleston, D.S., Brooks, D.P., Beuerstein, G., Ruffolo, R.R., Weinstock, J., Gleason, J.G., Peishoff, C.E. and Ohlstein, E.H. (1994). 1,3-Diarylindan-2-carboxylic acids, potent and selective nonpeptide endothelin receptor antagonists. J Med Chem., 37: 1553-1557.

39. Clozel, M., Breu, V., Gray, G., Kalina, B., Loffler, B.M., Burri, K., Cassal, J.M., Hirth, G., Muller, M., Neidhart, W. and Ramuz, H. (1994). Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. J Pharmacol Exp. Ther., 270: 228-235.

40. Clozel, M., Breu, V., Burri, K., Cassal, J.M., Fischli, W., Gray, G.A., Hirth, G., Loffler, B.M., Muller, M., Neidhart, W. and Ramuz, H. (1993). Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. Nature, 365: 759-761.

41. Clozel, M., Breu, V., Gray, G.A. and Loffler, B.M. (1993). *In vivo* pharmacology of Ro 46-2005, the first synthetic nonpeptide

endothelin receptor antagonist: Implications for endothelin physiology. J Cardiovasc Pharmacol., 22: S377-S379.

42. Breu, V., Loffler, B.M. and Clozel, M. (1993). In vitro characterization of Ro 46-2005, a novel synthetic non-peptide endothelin antagonist of ET_A and ET_B receptors. FEBS Lett., 334: 210-214.

43. Chong, C.C. (2009). Ambrisentan: A new, nonsulphonamide, selective endothelin receptor antagonist for the treatment of pulmonary arterial hypertension. Current Treatment of Pulmonary Vascular Diseases, 1: 31-33.

44. Cao, S., Wang, L.C., Kwansa, H., Roman, R.J., Harder, D.R. and Koehler, R.C. (2009). Endothelin rather than 20-HETE contributes to loss of pial arteriolar dilation during focal cerebral ischemia with and without polymeric hemoglobin transfusion. Am J Physiol Regul Integr Comp Physiol., 296: R1412-1418.

45. Clozel, M. (2000). Endothelin receptor antagonists: current status and perspectives. J Cardiovasc Pharmacol., 35: S65-68.

46. Wellings, R.P., Warner, T.D., Thiemermann, C., Cristol, J.P., Corder, R. and Vane, J.R. (1993). Vasoconstriction in the rat kidney induced by endothelin-1 is blocked by PD 145065. J Cardiovasc Pharmacol., 22: S103-106.

47. Dobrowolski, L., Endlich, K., Sadowski, J. and Steinhausen, M. (1997). Cardiovascular and renal effects of endothelin receptor blockade with PD 145065 and interaction with urodilatin. Acta Physiol Scand., 159: 7-13.

48. Cardell, L.O., Uddman, R. and Edvinsson, L. (1993). A novel ET_A-receptor antagonist, FR 139317, inhibits endothelin-induced contractions of guinea-pig pulmonary arteries, but not trachea. Br J Pharmacol., 108: 448-452.

49. Wu-Wong, J.R. (2002). S-0139 (Shionogi). Curr Opin Investig Drugs, 3: 1051-1056.

50. Opgenorth, T.J., Adler, A.L., Calzadilla, S.V., Chiou, W.J., Dayton, B.D., Dixon, D.B., Gehrke, L.J., Hernandez, L., Magnuson, S.R., Marsh, K.C., Novosad, E.I., Von Geldern, T.W., Wessale, J.L., Winn, M. and Wu-Wong, J.R. (1996). Pharmacological characterization of A-127722: an orally active and highly potent ET_A-selective receptor antagonist. J Pharmacol Exp Ther., 276: 473-481.

51. Krause, S.M., Walsh, T.F., Greenlee, W.J., Ranaei, R., Williams, D.L. and Kivlighn, S.D. (1997). Renal protection by a dual ET_A/ET_B endothelin antagonist, L-754,142, after aortic cross-clamping in the dog. J Am Soc Nephrol., 8: 1061-1071.

52. Enseleit, F., Luscher, T.F. and Ruschitzka, F. (2008). Darusentan: a new perspective for treatment of resistant hypertension? Expert Opin Investig Drugs, 17: 1255-1263.

53. Krum, H., Viskoper, R.J., Lacourciere, Y., Budde, M.and Charlon, V. (1998). The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. Bosentan Hypertension Investigators. N Engl J Med., 338: 784-790.

54. Dwyer, N. and Kilpatrick, D. (2011). Bosentan for the treatment of adult pulmonary hypertension. Future Cardiol., 7: 19-37.

55. Dingemanse, J. and van Giersbergen, P.L.M. (2004). Clinical pharmacology of

Bosentan, a dual endothelin receptor antagonist. Clin Pharmacokinet, 43: 1089-1115.

56. Iglarz, M., Binkert, C., Morrison, K., Fischli, W., Gatfield, J., Treiber, A., Weller, T., Bolli, M.H., Boss, C., Buchmann, S., Capeleto, B., Hess, P., Qiu, C. and Clozel, M. (2008). Pharmacology of macitentan, an orally active tissue-targeting dual endothelin receptor antagonist. J Pharmacol Exp Ther., 327: 736-745.

57. Raja, S.G. (2010). Macitentan, a tissue-targeting endothelin receptor antagonist for the potential oral treatment of pulmonary arterial hypertension and idiopathic pulmonary fibrosis. Curr Opin Investig Drugs, 11: 1066-1073.

58. Miller, R.C., Pelton, J.T.and Huggins, J.P. (1993). Endothelins – from receptors to medicine. TiPS, 14: 54-60.

59. Doherty, A.M. (1992). Endothelin: a new challenge. Journal of Medicinal Chemistry, 35: 1493-1508.

60. Reily, M.D.and Dunbar, J.B. (1991). The conformation of endothelin-1 in aqueous solution. NMR-derived constrains combined with distance geometry and molecular mechanisms calculations. Biochem Biophys Res Commun., 178: 570-577.

61. Takasaki, C., Aimoto, S., Tkayanagi, R., Ohashi, M. and Nawata, H. (1990). Structure-receptor binding relationships of sarafotoxin and endothelin in porcine cardiovascular tissues. Biochem Int., 21: 1059-1064.

62. Fukami, T., Nagase, T., Fujita, K., Hayama, T., Niiyama, K., Mase, T., Nakajima, S., Fukuroda, T., Saeki, T., Nishikibe, M., Ihara, M., Yano, M. and Ishikawa, K. (1995). Structure-activity relationships of cyclic pentapeptide endothelin A receptor antagonists. J Med Chem., 38: 4309-4324.

63. Ishikawa, K., Fukami, T., Nagase, T., Mase, T., Hayama, T., Niiyama, K., Fujita, K., Urakawa, Y., Kumagai, U., Fukuroda, T., Ihara, M. and Yano, M. (1992). Endothelin antagonistic peptide derivatives with high selectivity for ET_A receptors. In Proceedings of the 22nd European Peptide Symposium; ESCOM Science Publishers B.V.: Leiden, The Nederlands, 1992, pp 685-686.

64. Winn, M., von Geldern, T.W., Opgenorth, T.J., Jae, H., Tasker, A.S., Boyd, S.A., Kester, J.A., Mantei, R.A., Bal, R., Sorensen, B.K., Wu-Wong, J.R., Chiou, W.J., Dixon, D.B., Novosad, E.I., Hernandez, L. and Marsh, K.C. (1996). 2,4-Diarylpyrrolidine-3-carboxylic acids-potent ET_A selective endothelin receptor antagonists. 1. Discovery of A-127722. J Med Chem., 39: 1039-1048.

65. Sakurawi, K., Yasuda, F., Tozyo, T., Nakamura, M., Sato, T., Kikuchi, J., Terui, Y., Ikenishi, Y., Iwata, T., Takahashi, K., Konoike, T., Mihara, S. and Fujimoto, M. (1996). Endothelin receptor antagonist triterpenoid, myriceric acid A, isolated from *Myrica cerifera*, and structure activity relationships of its derivatives. Chem Pharm Bull (Tokyo), 44: 343-351.

66. Timmermans, P.B. (1999). Angiotensin II receptor antagonists: an emerging new class of cardiovascular therapeutics. Hypertens Res., 22: 147-153.

67. Kojiri, K., Ihara, M., Nakajima, S., Kawamura, K., Funaishi, K., Yano, M. and Suda, H. (1991). Endothelin-binding inhibitors, BE-18257A and BE-18257B. I.

Taxonomy, fermentation, isolation and characterization. J Antibiot (Tokyo), 44:1342-1347.

68. Ihara, M., Ishikawa, K., Fukuroda, T., Saeki, T., Funabashi, K., Fukami, T., Suda, H. and Yano, M. (1992). *In vitro* biological profile of a highly potent novel endothelin (ET) antagonist BQ-123 selective for the ET_A receptor. J Cardiovasc Pharmacol., 20: S11-14.

69. Fukami, T., Yamakawa, T., Niiyama, K., Kojima, H., Amano, Y., Kanda, F., Ozaki, S., Fukuroda, T., Ihara, M., Yano, M. and Ishikawa, K. (1996). Synthesis and structure-activity relationships of 2-substituted D-tryptophan-containing peptidic endothelin receptor antagonists: importance of the C-2 substituent of the D-tryptophan residue for endothelin A and B receptor subtype selectivity. J Med Chem., 39: 2313-2330.

70. Hochlowski, J.E., Hill, P., Whittern, D.N., Scherr, M.H., Rasmussen, R.R., Dorwin, S.A. and McAlpine, J.B. (1994). Aselacins, novel compounds that inhibit binding of endothelin to its receptor. II. Isolation and elucidation of structures. J Antibiot (Tokyo), 47: 528-535.

71. Jackson, M., Burres, N.S., Karwowski, J.P., Alder, L.A., Humphrey, P.E., Kohl, W.L. and McAlpine, J.B. (1994). Aselacins, novel compounds that inhibit binding of endothelin to its receptor. I. The producing organism, fermentation and biological activity. J Antibiot (Tokyo), 47: 523-527

72. Fujimoto, M., Mihara, S., Nakajima, S., Ueda, M., Nakamura, M. and Sakurai, K. (1992). A novel non-peptide endothelin antagonist isolated from bayberry, *Myrica cerifera*. FEBS Lett., 305: 41-44.

73. Maguire, J., Bacon, C., Fujimoto, M. and Davenport, A. (1994). Myricerone caffeoyl ester (50-235) is a non-peptide antagonist selective for human ET_A receptors. J Hypertens., 12: 675-680.

74. Ohshima, T., Hirata, M., Oda, T., Sasaki, A. and Shiratsuchi, M. (1994). Pheophorbide a, a potent endothelin receptor antagonist for both ET_A and ET_B subtypes. Chem Pharm Bull., 42: 2174-2176.

75. Caballero-George, C., Vanderheyden, P.M.L., Solis, P.N., Pieters, L., Shahat, A.A., Gupta, M.P., Vauquelin, G. and Vlietinck, A. (2001). Biological screening of selected medicinal Panamanian plants by radioligand-binding techniques. Phytomedicine, 8: 59-70.

76. Caballero-George, C., Vanderheyden, P.M.L., Okamoto, Y., Masaki, T., Mbwambo, Z., Apers, S., Gupta, M.P., Pieters, L., Vauquelin, G. and Vlietinck, A. (2004). Evaluation of bioactive saponins and triterpenoidal aglycons for the binding properties on human endothelin ET_A and angiotensin AT_1 receptors. Phytotherapy Research, 18: 729-736.

77. Gulavita, N.K., Wright, A.E., McCarthy, P.J., Pomponi, S.A., Kelly-Borges, M., Chin, M. and Sills, M.A. (1993). Isolation and structure elucidation of *34*-Sulfatobastadin 13, an inhibitor of the endothelin A receptor, from a marine sponge of the genus *Ianthella.* Journal of Natural Products, 56: 1613-1617.

78. Gao, J., Caballero-George, C., Wang, B., Rao, K.V., Shilabin, A.G. and Hamann, M.T. (2009). 5-OHKF and NorKA, depsipeptides from a Hawaiian collection of *Bryopsis pennata*: binding properties for

NorKA to the human neuropeptide Y Y_1 receptor. J Nat Prod., 72: 2172-2176.

79. Corder, R., Douthwaite, J.A., Lees, D.M., Khan, N.Q., Viseu dos Santos, A., Wood, E., Carrier, M. (2000). Endothelin-1 synthesis reduced by red wine. Nature, 414: 863-864

80. Kim-Park, S., Ku, D.D. (2000). Garlic elicits a nitric oxide-dependent relaxation and inhibits hypoxic pulmonary vasoconstriction in rats. Clinical and Experimental Pharmacology and Physiology. 27: 780-786

81. Zhang, T., Cui, B., Dai, D., Su, W. (2005). CPU 86017, *p*-chlorobenzyltetrahydroberberine chloride, attenuates monocrotaline-induced pulmonary hypertension by suppressing endothelin pathway. Acta Pharmacologica Sinica, 26: 1309–1316.

82. Taylor, M.W., Radax, R., Steger, D. and Wagner, M. (2007). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology Reviews, 71: 295-347.

83. Del Sol Jiménez, M., Garzón, S.P. and Rodríguez, A.D. (2003). Plakortides M and N, bioactive polyketide endoperoxides from the Caribbean marine sponge *Plakortis halichondrioides*. J Nat Prod, 66: 655-661.

84. Rumalla, C.S., Ali, Z., Weerasooriya, A.D., Smillie, T.J. and Khan, I.A. (2010). Two new triterpene glycosides from *Centella asiatica*. Planta Med, 76: 1018-1021.

85. Laszczyk, M., Jäger, S., Simon-Haarhaus, B., Scheffler, A. and Schempp, C.M. (2006). Physical, chemical and pharmacological characterization of a new oleogel-forming triterpene extract from the outer bark of birch (Betulae cortex). Planta Med., 72: 1389-1395.

86. Lin, K.W., Huang, A.M., Tu, H.Y., Lee, L.Y., Wu, C.C., Hour, T.C., Yang, S.C., Pu, Y.S. and Lin, C.N. (2011). Xanthine oxidase inhibitory triterpenoid and phloroglucinol from guttiferaceous plants inhibit growth and induced apoptosis in human NTUB1 cells through a ROS-dependent mechanism. J Agric Food Chem, 59: 407-414.

87. Csapi, B., Hajdú, Z., Zupkó, I., Berényi, A., Forgo, P., Szabó, P. and Hohmann, J. (2010). Bioactivity-guided isolation of antiproliferative compounds from *Centaurea arenaria*. Phytother Res, 24: 1664-1669.

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Simple Approach to Reactive Dye Decolorization Using *Trichosanthes dioica* Proteins at Low Concentration of 1-hydroxybenzotriazole

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Abstract

Enzymatic catalysis in the presence of redox mediators has emerged as an effective and feasible technique for degradation of complex structural compounds. We investigated peroxidase from *Trichosanthes dioica* to study decolorization of reactive dyes namely Reactive Blue15 (RB15) and Reactive Red4 (RR4) under different experimental conditions like pH, temperature, time interval, enzyme concentration and in the presence of redox mediators. Six different redox mediators; syringaldehyde, guaiacol, 1-hydroxy-benzotriazole (HOBT), vanillin, bromophenol and quinol were simultaneously evaluated. *T. dioica* peroxidase showed remarkable decolorization of reactive dye in the presence of 1-hydroxybenzotriazole. At an enzyme concentration of 0.45 $EUmL^{-1}$ the peroxidase decolorized Reactive Red15 almost completely up to a maximum of 98.6% whereas Reactive Red4 decolorized upto 68.2% with 1.0 mM 1-hydroxybenzotriazole. Maximum decolorization was recorded at a temperature range of 40°C to 50°C at pH 5.0. Time activity plot exhibited maximum decolorization at 90 min and 180min for RB15 and RR4 respectively. It can be concluded that *T. dioica* peroxidase could be a potential source for developing an inexpensive and efficient method for the treatment of recalcitrant reactive dyes that are potentially toxic or even carcinogenic.

Key words: Reactive Dyes; 1-hydroxybenzotriazole; Decolorization; *Trichosanthes dioica* Peroxidase

Introduction

Commercially available synthetic dyes are not only growing in number but their application spectrum is also widening. Dyes released from textile industries pose a serious threat to all forms of life. Thus, there is a great environmental concern about the fate of these unbound compounds (1). These discharged dyes form toxic products, while their strong color causes turbidity which even at very low concentrations alters the aquatic environment. Effluents from the textile industries containing dye are highly coloured and are therefore visually identifiable (2). These synthetic reactive dyes bond covalently with fabric and contain chromophoric groups like anthraquinone, azo, triarylmethane etc. along with reactive group's viz., vinyl sulphone, chlorotriazine, trichloropyrimidine etc.(3,4).

The complex aromatic structure of the dyes is resistant to light, biological activity, ozone and other degradative environmental conditions. This renders conventional waste water treatment ineffective. Anionic and non-ionic azo dyes release toxic amines due to the reactive cleavage of azo groups (5). Till date scientists have been trying to develop a single and economical method

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for the treatment of dyes in the textile waste water but it still remains a big challenge (6).Various physico-chemical methods pertaining to treatment of textile wastewater for removal of dye has been studied (7,8,9). The major disadvantage associated are their high cost, low efficiency, limited versatility, interference by other wastewater constituents and handling of the waste generated.

Decolorization of dye wastewater is an area where innovative treatment technologies should be investigated. The focus in recent times has shifted towards enzyme based treatment of colored wastewater/industrial effluents. Bioremediation is a viable tool for restoration of contaminated subsurface environments. It is gaining importance due to its cost effectiveness, environmental friendliness and production of less sludge as compared to chemical and physical decomposition processes. The redox mediated enzyme catalysis has wide application in degradation of polycyclic aromatic hydrocarbons which includes phenols, biphenyls, pesticides, insecticides etc. (10, 11).

The plant of our interest *Trichosanthes dioica* commonly known as pointed gourd is widely planted and abundantly available in tropical areas. The aim of the present study was to evaluate the effectiveness of peroxidase from *Trichosanthes dioica* in decolorizing industrially important reactive dyes under varying experimental conditions of pH, temperature, time interval and enzyme concentration.

Materials and methods

Dyes and Chemicals : The reactive dyes namely Reactive Blue 15 (RB15) Reactive Red 4 (RR 4), ammonium sulphate, and Tween -20 were procured from Sigma Chemical Co. (St. Louis, MO, USA) and all other chemicals were of analytical grade. Redox mediator's viz., syringaldehyde, guaiacol, 1-hydroxybenzotriazole (HOBT), vanillin, bromophenol, and quinol were obtained from SRL Chemicals (Mumbai, India). The pointed gourds were purchased from the local market.

Partial purification of** T. dioica **by ammonium sulphate precipitation : A 100 g of *T. dioica* fruit pulp was homogenized in 200 mL of 100mM sodium acetate buffer, pH 5.6. The homogenate was filtered through four layers of cheesecloth and then centrifuged at the speed of 10,000 × g on a Remi C-24 Cooling Centrifuge for 25 min at 4°C. By adding 20-80% (w/v) of ammonium sulphate, salt fractionation was carried out with the clear supernatant. The content was stirred overnight to get maximum precipitate at 4°C. The precipitate was collected by centrifugation at 10,000 × g on a Remi C-24 Cooling Centrifuge, dissolved in 100mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer (12).

***T. dioica peroxidase activity assay* :** Protein concentration was estimated by taking BSA as a standard protein and following the procedure of Lowry et al (1951) (13). Peroxidase activity was determined by a change in the optical density (A_{460} nm) at 37°C by measuring the initial rate of oxidation of 6.0 mM o-dianisidine HCl in the presence of 18.0 mM H_2O_2 in 0.1 M glycine-HCl buffer, pH 4.0, for 20 min at 37°C. One unit of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of o-dianisidine-HCl in the presence of H_2O_2 into 1.0 µmol of chromophoric complex (ε_m = 30,000 M^{-1} cm^{-1}) per min at 37 °C (12).

Preparation and treatment of reactive dye solution : The reactive dyes were prepared in 0.1 M glycine HCl buffer, pH 4.0. RB15 and RR4 were independently incubated with pointed gourd peroxidase (PGP) (0.45 $EUmL^{-1}$) in 0.1M glycine HCl buffer, pH 4.0 in the presence of 0.8 mM

H_2O_2 for varying times at 37°C. The reaction was terminated by boiling at 100°C for 10 min. Dye decolorization was monitored by measuring the difference at the maximum absorbance for each dye (λ_{max} for RB 15 and RR 4 are 675nm & 517nm respectively) as compared with control experiments without enzyme on UV-visible spectrophotometer (JASCO V-550, Japan). Untreated dye solution (excluding the enzyme) was used as control (100%) for the calculation of percent decolorization. The dye decolorization was calculated as the ratio of the difference of absorbance of treated and untreated dye to that of treated dye and converted in terms of percentage (14).

$$\% \text{ decolorization} = \frac{\text{Initial absorbance - Observed absorbance}}{\text{Initial absorbance x 100}}$$

Five independent experiments were carried out in duplicate and the mean was calculated.

Effect of redox mediator on T. dioica peroxidase mediated reactive dye decolorization : Each of the two reactive dyes (5.0 mL) were incubated with (PGP) (0.45 $EUmL^{-1}$) in the presence of each redox mediators viz., syringaldehyde, guaiacol, 1-hydroxybenzo-triazole (HOBT), vanillin, bromophenol and quinol (0.5 mM) and 0.75 mM H_2O_2 in 0.1 M glycine HCl buffer, pH 4.0 for 1 h at 37°C. The reaction was terminated by boiling the sample at 100°C for 10 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%).

To find out the optimum concentration of HOBT a similar set of experiment as mentioned above was performed in the presence of varying concentrations of HOBT (0.05 to 2.0 mM). The reaction was terminated by boiling the sample at 100°C for 10 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%) in all the subsequent sets of experiments.

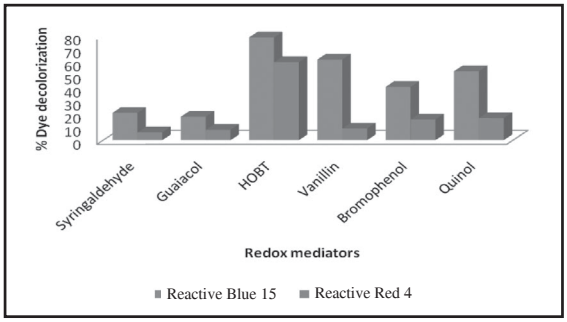
Reactive dye decolorization with varying concentration of Enzyme (PGP) and H_2O_2 : Each of the two reactive dyes were incubated with increasing concentrations of PGP (0.065 to 0.50 $EUmL^{-1}$) and H_2O_2 (0.2 to 1.8 mM) in 0.1 M glycine HCl buffer, pH 4.0 in the presence of 0.75 mM H_2O_2 for 1 h at 37°C. HOBT used as a redox mediator at concentrations of 1.0 mM for both the dyes. The reaction was stopped by boiling the sample at 100°C for 10 min.

Reactive dye decolorization as a function of temperature and pH : Each of the two reactive dyes was incubated with PGP (0.45 $EUmL^{-1}$) at different temperatures (20°C to 90°C). Other reaction conditions were common. The reaction was stopped by boiling the sample at 100°C for 10 min. The reactive dye solutions were made in different buffers each of 100mM and in the range of pH 2.0 to pH 10.0. The buffers were glycine-HCl (pH 2.0, 3.0 and 4.0), sodium acetate (pH 5.0), sodium phosphate (pH 6.0, 7.0 and 8.0), and Tris-HCl (pH 9.0 and 10.0). Each of the two dye was treated with PGP (0.45 $EUmL^{-1}$) in buffers of varying pH and in the presence of 1.0 mM H_2O_2 for 1 h at 37°C. The decolorization of each dye RB15 and RR4 was also performed in the presence of 1.0 mM HOBT. The reaction was stopped by boiling the sample at 100°C for 10 min.

***T. dioica* mediated reactive dye decolorization as a function of time :** The individual reactive dye was treated with PGP (0.45 $EUmL^{-1}$) in the presence of 1.0 mM H_2O_2 in 0.1 M glycine HCl buffer, pH 4.0 at 37°C for varying time intervals. HOBT was used as a redox mediator at concentrations of 1.0 mM. The reaction was terminated by boiling the sample at 100°C for 10 min.

Results

Effect of different redox mediators on the extent of reactive dye decolorization : The effect of different redox mediators on dye decolorization by PGP is shown in Fig. Ia. Among the six different redox mediators studied for dye decolorization, HOBT was most effective in decolorizing the dyes. The extent of decolorization was 98.6% and 68.2% for RB15 and RR4 respectively in the presence of HOBT. The other redox mediators were relatively less effective. Fig.1b shows the effect of increasing concentrations of HOBT (0.05 to 2.0 mM) on RB15 and RR4. With increasing concentration of HOBT there was an increase in the extent of decolorization of both dyes. Maximum decolorization was achieved in the presence of 1.0 mM HOBT. At concentration above 1.0mM there was insignificant change in percent decolorization of the dyes.



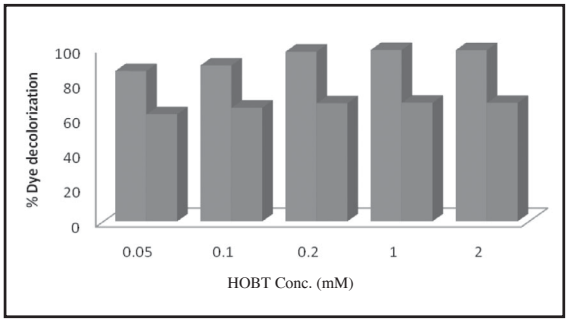


Fig.1a. Reactive dye decolorization as a function of different redox mediators. The concentration of each redox mediator viz., syringaldehyde, guaiacol, 1-hydroxybenzotriazole (HOBT), vanillin, bromophenol, and quinol was 0.5mM; other conditions were 0.8mM H_2O_2, 100mM glycine HCl buffer, pH 4.0 for 60 min at 37°C. (λ_{max} for RB 15 and RR 4 are 675nm & 517nm respectively).

Fig.1b. Reactive dye decolorization as a function of different concentrations of HOBT (0.05 to 2.0 mM).

Effect of enzyme concentration and pH on the extent of reactive dye decolorization : Fig.2 shows the extent of decolorization of RB15 and RR4 with varying concentrations of PGP. The maximal decolorization for these two dyes was observed at PGP concentration of 0.45EUmL^{-1}. At concentrations above this, there was no remarkable change in dye decolorization. Fig.3 shows the percent decolorization improved with

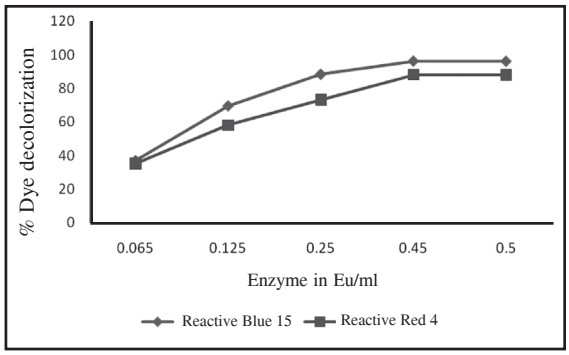


Fig. 2. Enzyme activity plot. Reactive dye decolorization as a function of different enzyme (PGP) concentrations (0.065 to 0.50 EUmL^{-1}).

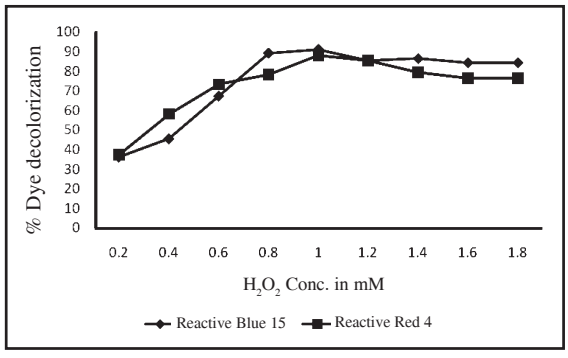


Fig. 3. Hydrogen peroxide activity plot. Reactive dye decolorization at different concentrations of H_2O_2 (0.2 to 1.8 mM).

Vol. 5 (3) 1273 -1281 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

the increasing concentration of H_2O_2 and maximum decolorization was observed at a concentration of 1.0 mM H_2O_2 that remained substantially unaffected till 1.2 mM H_2O_2. At concentrations of hydrogen peroxide beyond 1.2 mM there was decrease in decolorization of both RB15 and RR4, although on comparison RR4 showed more reduction in decolorization than RB15.

Effect of temperature and pH on the extent of reactive dye decolorization : The results of temperature activity plot are shown in Fig.4. Both dyes (RB15 and RR4) showed sufficient decolorization in temperature range of 40°C to 50°C. Buffers (pH 2.0 to pH 10.0) were used to find out the range of pH in which significant decolorization was observed. The results of pH activity plot are shown in Fig.5. An acidic range of pH (4.0 to 5.0) was better suited for dye decolorization. A pH optimum recorded for both the dyes was pH 5.0. The extent of decolorization significantly decreased in alkaline medium.

Effect of time on the extent of reactive dye decolorization : The extent of decolorization of both dyes RB15 and RR4 as a function of time is shown in Fig.6. RB15 showed maximum decolorization in 90min whereas RR4 decolorized maximally after 3h of incubation at 40°C. However, no effective increase was observed when the dyes were further incubated for longer durations. Sufficient amount of RB15 was decolorized within 60 min while the decolorization of RR4 was slow in the same time interval.

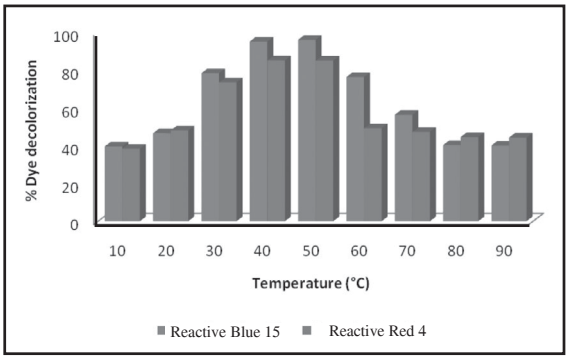


Fig.4: Temperature activity plot**.** Reactive dye decolorization as a function of temperature (20°C to 90°C).

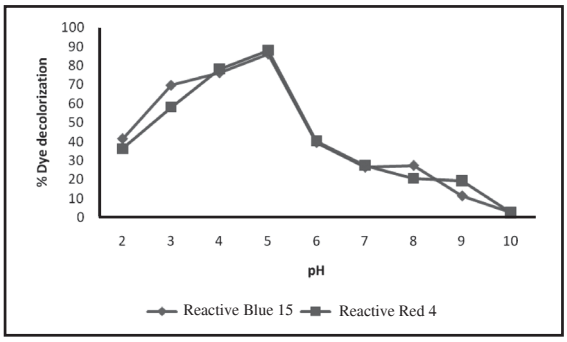


Fig.5: pH activity plot. Reactive dye decolorization as a function of pH (pH 2.0 to pH 10.0). The buffers were glycine-HCl (pH 2.0, 3.0 and 4.0), sodium acetate (pH 5.0), sodium phosphate (pH 6.0, 7.0 and 8.0), and Tris-HCl (pH 9.0 and 10.0).

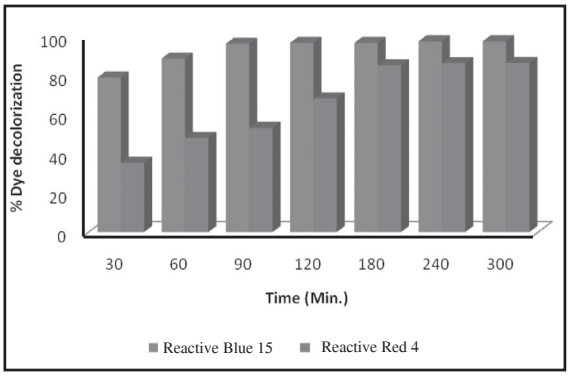


Fig.6: Time activity plot. Reactive dye decolorization as a function of time (30 min to 300 min).

Discussion

The reactive dye decolorization ability of peroxidase isolated from *Trichosanthes dioica* has been evaluated in the presence of redox mediators under different sets of conditions. The enzyme pointed gourd peroxidase (PGP) has been partially purified and used for studying decolorization of reactive dyes using simple techniques. The emphasis on enzyme purification

Vol. 5 (3) 1273 -1281 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

has been ignored due to its enormous cost. Findings supportive to PGP has been demonstrated with bitter gourd peroxidase (15). The reactive dye solutions were recalcitrant to HOBT, H_2O_2 or to enzyme alone but in the presence of redox mediator PGP showed higher efficiency in accomplishing decolorization of the reactive dyes, implying that dye decolorization was a redox mediated H_2O_2-dependent enzymatic interaction.

That redox mediator has the potential to mediate an oxidation reaction between a substrate and an enzyme (16). The mediation efficiency is governed by redox potential of redox mediator and the oxidation mechanism of substrate. The reactive dyes underwent decolorization by the formation of precipitate which disappeared in the presence of 1.0 mM HOBT. This finding supports earlier reports that treatment of phenols and aromatic amines by peroxidases resulted in formation of large insoluble aggregates (17, 18, 19, 20). Oxidation of substrate occurs by free radical formation by the mediator. The free radicals can be formed either by one-electron oxidation of substrate or by abstraction of a proton from the substrate (21). In this study, redox-mediating property of six different compounds as peroxidase mediators was examined to identify the most suitable redox mediator for *T. dioica* peroxidase system (Fig.Ia). Among six compounds investigated, HOBT was found to have the best mediating property for the decolorization of RB15 and RR4. This observation was in agreement with earlier reports where HOBT was found to enhance decolorization of reactive and direct dyes drastically (22). Also, it is documented that the redox potential of enzymes varies depending upon the source of the enzyme.

The pointed gourd peroxidase was effective in decolorizing the reactive dyes at low concentrations of HOBT (Fig.1b). Although the extent of decolorization of RB15 and RR4 increased with increasing concentrations of HOBT, the maximum decolorization was observed to be 98.6% for Reactive Blue15, whereas Reactive Red4 decolorized upto 68.2% with 1.0 mM 1-hydroxybenzotriazole, respectively. Further addition of HOBT resulted in a slow and insignificant decrease in decolorization of both the dyes. This inhibition probably is due to the high reactivity of HOBT radical, which might undergo chemical reactions with reactive side groups of aromatic amino acid by enzyme leading to inactivation (23, 16). Thus, the dosage of redox mediator is an important factor contributing to enzyme-mediated decolorization under the given set of conditions (24).

The enzyme reacted well to decolorize both the reactive dyes in the presence of 0.8 mM H_2O_2 (Fig.2). Maximum decolorization was obtained at 1.0 mM H_2O_2 although at 0.8 mM H_2O_2 concentration RB15 was more efficiently decolorized than RR4 (Fig.3). However, disperse red19 and disperse black9 exhibited maximum decolorization at lower concentration of hydrogen peroxide with this enzyme (15). Dye decolorization at similar concentrations has been reported for soybean peroxidase, bitter gourd peroxidase (BGP) and turnip peroxidase (25). Higher concentrations of H_2O_2 irreversibly oxidized the enzyme ferri-heme group essential for peroxidase activity consequently inhibiting peroxidase activity but our results are consistent and very near to values reported earlier for maximum functional concentration of H_2O_2 (26). The decolorization of reactive dyes is influenced by temperature (Fig.4). The maximum decolorization for both the dyes RB15 and RR4 was in the range of 40°C to 50°C. In this range there was an insignificant change on the overall decolorization maxima supporting that dye decolorization is generally effective at 40°C (15).

Vol. 5 (3) 1273 -1281 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Decolorization to a large extent was observed at an acidic range of pH 4.0 to pH 5.0 but maximum decolorization of RB15 and RR4 was achieved at pH 5.0 (Fig.5). It has earlier been reported that the degradation of industrially important dyes by peroxidases from different sources operates to a maximum level in the buffers of acidic pH. The incubation period is an important parameter to study the extent of decolorization (12, 24). Time activity plot exhibited maximum decolorization at 90 min and 180min for RB15 and RR4 respectively (Fig.6). The decolorization profile was unaffected or slightly decreased over prolonged incubation upto 5h. It was also evident from the observation that RB15was decolorized to a greater extent within 30 min in the presence of only 1.0 mM HOBT, but decolorization rate was slow for RR4 under the same set of conditions. This data supports the earlier view that decolorization rate varies, depending upon the type of dye to be treated (27).

The rate of decolorization of dye mixture was also studied which was slower in comparison to that of individual dyes both in the presence and absence of HOBT (data not shown). However, the HOBT mediated dye decolorization was more effective. This finding is consistent with the earlier observation that the biodegradation of various phenols in the form of mixtures was quite slow compared to the independent phenol (28). Several workers have demonstrated that the use of redox mediator system enhanced the rate of dye decolorization by several folds but these mediators were required in very high concentrations (29, 30).

The salt fractionated peroxidase from *T. dioica* significantly catalyzed the decolorization/ degradation of reactive textile dyes. Decolorization enhanced remarkably in the presence of redox mediator HOBT which was effective at low concentration. Traditional wastewater treatment technologies have proven to be markedly ineffective for handling wastewater of synthetic textile dyes due to the chemical stability of these pollutants. Therefore, establishment of result oriented enzyme based cheap and ecofriendly techniques holds considerable promise. Thus, this study demonstrated that *T. dioica* peroxidase in its salt fractionated state can be coupled with low concentration of redox mediators to cause effective decolorization of synthetic and recalcitrant reactive dyes.

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References

1. Pierce, J. (1994). Colour in textile effluents—the origins of the problem. J. Soc. Dyers Colour, 110: 131–134.
2. Kilic, NK, Nielson, JP, Yuce, M. and Donmez, G. (2007). Characterization of a simple bacterial consortium for effective treatment of wastewaters with reactive dyes and CrVI. Chemosphere, 67: 826–831.
3. Sumathi, S. and Manju, B.S. (2000). Uptake of reactive textile dyes by *Aspergillus foetidus*. Enzyme Microbial Technology, 27: 347–355.
4. Keharia, H. and Madamvar, D. (2003). Bioremediation concept for treatment of dye containing wastewater: a review. I J Exp Biology, 41:1068–1075.

5. Joshi, M., Bansal, R. and Purwar, R.(2004). Colour removal from textile effluents. Ind. J Fibre Textile Research, 29: 239–259.

6. Santos, A.B., Cervantes, F.J. and Lier, J.B. (2007). Review paper on current technologies for decolourization of textile wastewaters: perspectives for anaerobic biotechnology. Bioresource Technology, 98: 2369–2385.

7. Hao, O.J., Kim, H. and Chiang, P.C. (2000). Decolourization of wastewater. Crit Rev Environ Sci Technology, 30: 449–505.

8. Robinson, T., McMullan, G., Marchant, R. and Nigam P. (2001). Remediation of dyes in textile effluent: a critical reviewon current treatment technologies with a proposed alternative 2001. Bioresource Technology, 77: 247–255.

9. Forgacs E, Cserhati, T.and Oros G. (2004). Removal of synthetic dyes from wastewaters: a review. Environment International, 30: 953–971.

10. Calcaterra, A., Galli, C. and Gentili, P. (2008). Phenolic compounds as likely natural mediators of laccase : A mechanistic assessment. J Mol Catal B: Enzymatic, 51:118-120.

11. Husain, M. and Husain, Q. (2008). Applications of redox mediators in the treatment of organic pollutants by using oxidoreductive enzymes. Crit Rev Environ Sci Technology, 38:1-42.

12. Akhtar, S., Khan, A.A. and Husain, Q. (2005). Partially purified bitter gourd *Momordica charantia* peroxidase catalyzed decolorization of textile and other industrially important dyes Bioresource Technology, 96:1804–1811.

13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin–phenol reagent. J Biological Chemistry, 193: 265–275.

14. Jamal, F., Pandey, P.K. and Qidwai, T. (2010). Potential of Peroxidase Enzyme from *Trichosanthes dioica* to mediate Disperse Dye Decolorization in conjunction with Redox Mediators. J Mol Catal B: Enzymatic, 66:177-181.

15. Satar, R. and Husain, Q. (2009). Use of Bitter Gourd *Momordica charantia* peroxidase together with redox mediators to decolorize disperse dyes. Biotechnol Bioprocess Engineering, 14: 213–219.

16. Baiocco, P., Barreca, A. M., Fabbrini, Galli, M.C. and Gentili, P. (2003). Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase-mediator systems. Organic and Bimolecular Chemistry, 1:191–197.

17. Wada, S., Ichikawa, H. and Tatsumi, K. (1995). Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and a coagulant. Biotechnol Bioengineering, 45: 304–309.

18. Tatsumi, K., Wada, S. and Ichikawa, H. (1996). Removal of chlorophenols from wastewater by immobilized horseradish peroxidase. Biotechnol Bioengineering, 51:126–130.

19. Husain, Q. and Jan, U. (2000). Detoxification of phenols and aromatic amines from polluted wastewater by using phenol oxidases. J Sci Ind Research, 59: 286–293.

20. Duran, N., Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review.

Appl Catalysis B: Environmental, 28:83–99.

21. Fabbrini, M., Galli, C. and Gentili, P. (2002). Comparing the catalytic efficiency of some mediators of laccase. J Mol Cat B: Enzymatic, 16: 231–240.

22. Matto, M. and Hussain, Q. (2007). Decolorization of direct dyes by salt fractionated turnip proteins enhanced in the presence of hydrogen peroxide and redox mediators. Chemosphere, 69: 388–345.

23. d'Acunzo, F. and Galli, C. (2003). First evidence of catalytic mediation by phenolic compounds in the laccase-induced oxidation of lignin models. European J Biochemistry, 270: 3634–3640

24. Murugesan, K., Nam, I. H., Kim, Y. M. and Chang, Y. S. (2007).Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture. Enzyme and Microbial Technology, 40:1662–1672.

25. Kulshrestha, Y. and Husain, Q. (2007). Decolorization and degradation of acid dyes mediated by salt fractionated turnip *Brassica rapa* peroxidases. Toxicological and Environmental Chemistry, 89: 255–267.

26. Vazquez-Duarte, H., Garcia-Almendarez, B., Regalado, C. and Whitaker, J. R. (2001). Purification and properties of a neutral peroxidase from turnip Brassica napus L varpurple top white globe roots. Journal of Agricultural and Food Chemistry, 49:4450–4456.

27. Camarero, S., Ibarra, D., Martinez, M.J. and Martinez, A.J. (2005). Lignin derived compounds as efficient laccase mediators or decolorization of different types of recalcitrant dyes. Applied Environmental Microbiology, 71: 1775–1784.

28. Kahru, A., Pollumaa, L., Reiman, R., Ratsep, A., Liiders, M. and Maloveryan, A. (2000). The toxicity and biodegradability of eight main phenolic compounds characteristic to the oil-shale industry wastewater: a test battery approach. Environmental Toxicology, 15:431–442.

29. Soares, G.M., De Amorim, M.T. and Costa-Ferreira, M. (2001). Use of laccase together with redox mediators to decolorize Remazol Brilliant Blue R. J Biotechnology, 89: 123–129.

30. Claus, H., Faber, G. and Konig, H. (2002). Redox-mediated decolorization of synthetic dyes by fungal laccases. Applied Microbiology and Biotechnology, 59: 672–678.

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Studies on the Production of Actinomycin by *Nocardioides luteus*, a Novel Source

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Abstract

Efforts have been devoted to explore the antimicrobial activity of local egyptian actinomycetes isolates. 127 pure actinomycetes isolates were recovered from the stones at the location of the tomb of Tell Basta (the site of the ruins of the old temple of Bast) which is south-east of the Nile Delta at the township of Zagazig in Egypt. The most competent isolate showed promising activity against some pathogenic yeasts, fungi and bacteria. It was identified as *Nocardioides luteus*. Using 2 ml of 2 days old inoculum of *Nocardioides luteus* in fermentation medium consisting of (g/l): starch, 10; yeast extract, 4; NaCl, 10; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02 and $FeSO_4.7H_2O$, 0.01, adjusted before autoclaving at pH 7 and incubated for 7 days with shaking rate 200 rpm resulted in maximum antimicrobial activity against *Candida albicans* ATCC 10231. The minimum inhibitory concentration (MIC) of the culture was determined to be 0.4 µg/ml. Trials have been conducted to separate and identify the active compound(s) responsible for the anticandidal activity. Based on the spectroscopic data from UV, IR, ^{1}H-NMR and ^{13}C-NMR spectra, the isolated compounds were identified as derivatives of actinomycins class. To our knowledge, this is the first time not only to study systematically the bioactivities of *Nocardioides luteus*; but also to report actinomycin production by a *Nocardioides* spp. Cytotoxicity of both the culture filtrate extract and the impure compound were tested against different tumor cell lines where they showed remarkable cytotoxicity against all challenged cell lines.

Keywords: actinomycetes, antimicrobial, actinomycin, cytotoxicity.

Introduction

Actinomycetes are a group of bacteria, whose members are famous for producing antibiotics for medicinal use. They provid us with a large database of compounds with different structures; including aminoglycosides, anthracyclines, glycopeptides, â-lactams, macrolides, nucleosides, peptides, polyenes, polyketides, quinones, actinomycins, tetracyclines and even alkaloids. Only a very minute quantity of terrestrial and marine actinomycetes were actually isolated and tested for antibacterial activity. In the time interval between 1981-2006, 19 out of 24 naturally originating compounds acting as the starting material of marketed drugs, were isolated from soil actinomycetes (1,2,3).

Within the actinomycetes, *Streptomyces* is the most abundant genus that accounts for 70-80% of secondary metabolites, with smaller contributions from genera such as *Saccharopolyspora, Amycolatopsis,*

Micromonospora and *Actinoplanes* (4). Although *Candida* spp. occur among normal body flora in mouth and gastrointestinal tract, they can be responsible for many types of diseases in both humans and animals ranging from superficial mucocutaneous illness to deep life-threatening invasive, systemic diseases that can occur in different organs thus, causing different signs and symptoms. There are more than 20 species of *Candida*, of which *Candida albicans* is the most common. Yet, all *Candida* spp. cause the same spectrum of diseases however, differences in disease severity and susceptibility to antifungal agents were reported.

Among the increase in the incidence of nosocomial fungal infections, those caused by *Candida albicans*, has increased proportionally. This problem appears to be a global one; in large, university-affiliated hospitals (5).

Therefore, new sources and strategies are required to find new broad-spectrum antifungal molecules to combat these pathogens. For this reason, intensive program searching for new antibiotics is running worldwide mainly by screening for members of the clade actinomycetes as being a rich, unrivaled source of antibiotics.

Impressed by these facts, our work aims to investigate the potentialities of some locally isolated actinomycetes to produce bioactive compounds acting against *Candida albicans* pathogen. The optimal fermentation conditions for bioactive compound production by the most active actinomycetes isolate have been investigated. In addition, trials to identify the active substances were reported.

Materials and Methods

Isolation of actinomycetes : Stone samples were crushed to a fine powder. Stone powder was dispersed in sterile 1/4 strength Ringer's solution supplemented with 0.001 % Tween 80 and shaken vigorously for up to one hour (6). Suspensions were then serially diluted in phosphate buffer. Spread plate method was applied for actinomycetes isolation; 0.1 ml of the appropriate dilution was spread evenly over the corresponding overnight dried medium plates. Five different isolation media known to support the growth of actinomycetes were used namely; starch casein medium (7), arginine glycerol salts medium (9), starch nitrate medium, M3 medium (9) and ISP medium 5 (Glycerol-Asparagine Agar) (10).

After incubation, representatives of each colony form were picked off and streaked to obtain a pure culture. They were finally stored on starch casein slopes at 4 °C, and as spore suspensions in 20 % glycerol at 20 °C (11).

Target organisms : The isolated strain activities were tested against*: Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27953 and *Alcaligenes faecalis* NRRL B-170 as examples for Gram negative pathogens, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* NRRL B-4219 and *Micrococcus luteus* NRRL B-287 as examples for Gram positive pathogens, *Aspergillus niger* NRRL 599, *Aspergillus flavus* NRRL 1957 and *Trichophyton mentagrophytes* ATCC 9533 as examples for fungal pathogens and *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 10275 as examples for yeasts.

The challenged fungi were maintained and cultured on Dox's agar medium that has the following composition (g/l): sucrose, 20; $NaNO_3$, 2; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.1; agar, 20. The pH of the medium was adjusted to 7.0-7.2 before sterilization.

The tested yeasts were maintained and cultured on malt yeast medium that has the following composition (g/l): glucose, 4; yeast extract, 4; malt extract, 20; agar, 20. The pH of

the medium was adjusted to 7.0-7.2 before sterilization. The used bacteria were maintained and cultured on nutrient agar medium that has the following composition (g/l): glucose, 20; peptone, 10 and agar, 20. The pH of the medium was adjusted to 7.0-7.2 before sterilization.

Screening of actinomycetes isolates for antimicrobial activity : Five days old slants were harvested by scratching with sterile distilled water and poured into 250 ml Erlenmeyer flasks containing 50 ml sterile starch casein nitrate broth. The production flasks were incubated in a rotary shaker at 150 rpm and 30 °C ± 2 °C for 7 days. At the end of the incubation period, the cultured broths were filtered and assayed for their activity against the target organisms using agar well diffusion method. 0.1 ml of each culture filtrate was placed in holes (0.9 cm diameter) made in seeded agar plates. The agar plates were incubated at 30 °C for 24 hours for bacteria and for 48 hours for fungi and yeasts. The diameters of the resulting inhibition zones were then measured and considered to estimate the isolates antimicrobial potentialities.

Characterization of actinomycete isolate of choice : Only the actinomycetes isolate of the highest activity was subjected to identification at the genus and species level. A wide range of morphological, physiological and biochemical criteria were used to characterize this isolate. These criteria include morphological features, pigmentation, utilization of organic compounds and resistance to antibiotics as well as diaminopimelic acid in whole cell hydrolysate.

Cultivation factors affecting the production of antifungal compound(s) : Unless otherwise stated, the basal fermentation conditions were: 4 ml of distilled water was added to a 5 day old slant and a spore suspension in 250 ml Erlenmeyer flask containing 50 ml of starch casein nitrate medium. The flask was incubated in a rotary shaker at 150 rpm, 30 °C± 2 for 2 days. 2 ml was used to inoculate 50 ml of starch casein nitrate medium in 250 ml Erlenmeyer flasks. The flasks were incubated on an incubatory shaker at 30 °C at 150 rpm for 7 days.

At the end of the fermentation process, the antifungal activity was tested against *Candida albicans* using agar well diffusion method. Also, the cell dry weight in the fermentation broth was determined by filtering the flask content on a pre-weighed filter paper (Whatman no.1). The filter papers were dried at 60-70 °C until a constant weight. The difference between the weights of the filter papers after and before filtration should correspond to the cell dry weight.

Effect of fermentation period, inoculum age, size : The effect of prolongation of incubation for maximum growth and antibiotic production was observed up to 12 days of incubation. To determine the effect of the age of inocula, 2 ml of the inoculum broth were taken at time intervals 2, 3, 4, 5, 6, 7 days to inoculate separate flasks of the fermentation media. In addition, different inoculum volumes (ranging from 2-8 % v/v) were used to inoculate the fermentation media to determine the most suitable volume for producing antifungal compound(s).

Carbon and nitrogen supplements : Six carbon sources namely: starch, fructose, maltose, glucose, mannose, and sucrose were tested; each at 1 % level. After selection of the best source, different concentrations (5, 10, 15, 20 gm/l) were tested.

Different organic and inorganic nitrogen sources were individually added as sole nitrogen sources in fermentation media to replace KNO_3 and casein. These are: peptone, yeast extract, corn steep liquor, sodium nitrate and ammonium chloride. They were added according to nitrogen

Vol. 5 (3) 1282-1297 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

equivalent basis. After selection of the best source, different concentrations 2.5, 3, 3.76, 4, 4.5 g/l were investigated.

***Mineral* salts :** Different concentrations (0-4 %) of NaCl were used in the cultivations to determine the best for antifungal production. Different concentrations (0-1.6 %) of K_2HPO_4 in the culture medium were tested.

Different pH and shaking rate : Varied initial pH values were tested using different buffering solutions. A control flask was done by adjusting the pH at 7 by 1M HCl and 1M NaOH. The buffer solutions used were: Citrate phosphate buffer at pH 6, 6.5 and 7, Phosphate buffer at pH 7, 7.5 and 8 and Tris-maleate buffer at pH 8 and 8.5. Different rpm were investigated (100, 150, 200) as well as static conditions were maintained.

Extraction of the antifungal bioactive compound (s) : After fermentation of the media, the culture broth was filtered in vacuo and the filtrate was extracted with equal volume of ethyl acetate. The latter was dried under reduced pressure using a rotary evaporator at a temperature not exceeding 50 °C, yielding an orange-yellow residue (270 mg).

Thin Layer chromatography (TLC) : Ethyl acetate extract of the culture filtrate was developed on TLC plate using solvent system consisting of Benzene: Ethyl acetate: Acetic acid (1: 3.5: 0.5 v/v) to evaluate the secondary metabolite constituents of *Nocardioides luteus*.

The chromatogram was detected under a UV lamp and each spot was scratched from the TLC plate and dissolved in ethyl acetate. Silica gel was removed by centrifugation at 2000 rpm for 10 min. The supernatant was air-dried and the constituents were tested by paper disc assay method (12).

Isolation of the active compound : The residue (270 mg) was dissolved in 5 ml ethyl acetate and adsorbed on silica gel G for column chromatography. The mixture was added at the top of a column chromatography (45 cm long × 4 cm wide) using silica gel G as an adsorbent, benzene/ ethyl acetate (1:1) was used as an eluent. Fractions of each equal to 15 ml, were collected individually. Then, the polarity was increased after 53 fractions to benzene/ ethyl acetate (1:2) for 97 fractions followed by ethyl acetate for 25 fractions then, 5 % methanol in ethyl acetate for 11 fractions and finally methanol for 39 fractions.

Fractions were grouped in accordance to their TLC profiles and tested for their antimicrobial activity (Bioassay- guided fractionation). The TLC plates revealed that the fractions were to be pooled into 8 fractions, only 5 of which were active against *C.albicans*. The most active was subjected to further purification by re-fractionation using column chromatography.

The elution with benzene/ ethyl acetate (1:2) afforded 97 fractions. The fractions from 45 to 70 were collected together to give rise to 80 mg of an orange - yellow mixture. It was re-fractionated on flash silica gel column (45 cm long × 4 cm wide) using chloroform/ methanol (9:1) as eluent. Fractions, each equal to 10 ml, were collected individually. The fractions from 27 to 34 were collected together once again and purified on RP-18 column (30 cm long × 4 cm wide) using 20 % water in methanol to afford 10 mg of semi-pure compound. Further purification failed due to the instability of the compound.

The partially purified active material obtained was subjected to various examinations by ultraviolet, infrared, ^{1}H-NMR 500MHz and ^{13}C-NMR.

Cytotoxicity assay to human tumor cell lines: Sulphorhodamine-B (SRB) assay of cytotoxic activity (13):

These tests were done at National Cancer Institute, Pharmacology Unit- Tissue Culture Department.

Cell lines: HEPG2 (liver carcinoma), HeLa (cervix carcinoma), MCF7 (breast carcinoma), HCT116 (colon carcinoma), HEP2 (larynx carcinoma), CACO (colon carcinoma and HFB4 (normal melanocytes) were maintained and cultured on RPMI-1640 medium supplemented with penicillin/streptomycin (100 units/ml Penicillin and 2 mg/ml Streptomycin) and 10 % fetal bovine serum (heat inactivated at 56 °C for 30 min).

Cells at exponential phase cultures were harvested by trypsinization and seeded in 96-well microtiter plates at a concentration of $5x10^4$-10^5 cell/well for 24 hrs. Cells were then incubated with different concentrations (0, 1, 2.5, 5 and 10 ìg/ml in DMSO) of both the culture filtrate and the partially-purified compound. The volume was completed to total volume of 200 ìl/well using fresh medium and incubated for 24, 48 and 72 hrs in triplicate. Control cells were treated with vehicle alone. After incubation, the cells were fixed with 50 ìl cold 50 % trichloroacetic acid for 1 hr at 4 °C, washed with distilled water and then stained for 30 min at room temperature with 50 ìl 0.4 % SRB dissolved in 1 % acetic acid. The wells were washed with 1 % acetic acid to dissolve unbound SRB, the plates were air-dried and the dye was solubilized with 100 ìl/well of 10 mM tris base (pH 10.5) for 5 min on a shaker at 1600 rpm. The optical density (O.D.) of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader. The relation between survival fraction and extract concentration was plotted to give the survival curve of each tumor cell line, and the IC_{50} was calculated.

Survival fraction = O.D. (treated cells)/ O.D. (control cells).

Results and Discussion

In the current study, a total of 127 pure actinomycetes isolates were isolated from the stone of monuments in Tell Basta Tombs, Zagazig city. Bacteria isolated from weathered rock samples in the Mediterranean basin were, in the majority of cases, actinomycetes and other Gram-positive bacteria. The other possible reason for these high counts was the ability of actinomycetes to cope with dry habitats. They were frequently isolated from stone environments and many of their members existed for extended periods as resting arthrospores that germinated in the occasional presence of exogenous nutrients (14,15).

In view of the decline in the discovery of new antifungal compounds, the isolated actinomycetes were assayed for their antimicrobial activities, specially their anticandidal activity.

Among the isolated 127 actinomycetes, only 10 isolates showed promising activity. However, relatively higher anticandidal activity was exhibited by a culture referred to as isolate no.9. The antimicrobial activities offered by this isolate are recorded in Table 1.

According to the plan of this work, the isolate with highest activity was only considered for identification. The criteria used in the identification were micromorphology, detection of diaminopimelic acid (DAP) isomers in the whole cell hydrolysate, whole cell diagnostic sugars and presence of mycolic acids (16). Based on these criteria, the isolate was found to belong to the genus *Nocardioides*. The isolate was further classified to the species level by morphological, physiological and chemotaxonomic features. This identification was according to Bergey's Manual of Systematic Bacteriology (17) and Bergey's Manual of Determinative Bacteriology (18). The criteria

Table 1 The antimicrobial activities of actinomycetes isolate No.9

Tested microbial pathogen	Diameter of inhibition zone (mm)
Escherichia coli ATCC 25922	20
Bacillus subtilis NRRL B-4219	30
Pseudomonas aeruginosae ATCC 27953	-
Staphylococcus aureus ATCC 29213	20
Candida albicans ATCC 10231	30
Saccharomyces cerevisiae ATCC 10275	25
Aspergillus niger NRRL 599	30
Aspergillus flavus NRRL 1957	-
Alcaligenes faecalis NRRL B-170	30
Micrococcus luteus NRRL B-287	10
Trycophyton mentagrophytes ATCC 9533	14

Table 2 Identification criteria of actinomycetes isolate no. 9 (*Nocardioides luteus*)

Experimental Parameter	Character	Experimental Parameter	Character
Aerial mycelia color	Grey	L-diaminopimelic acid in whole cell hydrolysate	+
Vegetative mycelia color	Yellow-Brown	Growth on sole carbon sources (1.0 % w/v)	
Melanin on tyrosine agar	+	Sucrose	+
Degradation of	-	Lactose	+
Casein	+	Salicin	+
Xanthene	-	D-Fructose	+
Hypoxanthene-	Galactose	+	
Urea	+	D-Mannose	+
Growth at		D-Mannitol	+
45°C	-	Rafinose	+
10°C	+	D-Xylose	+
pH 4.3	+	L-Arabinose	+
Growth in the presence of (%w/v)		Cellobiosem	
NaCl (4)	+	Inositol	+
NaCl (7)	+	Rhamnose	+
NaCl (10)	+	Glucose	+
Crystal violet (0.0001)	-	Sodium acetate (0.1 %)	+
Phenol (0.1)		Sodium citrate (0.1%)	+
Resistance to antibiotics		Sodium propionate (0.1 %)	+
Gentamicin (10 µg)		Growth on sole nitrogen source (0.1 %)	
Streptomycin (10 µg)	-	L-Arginine	+
Neomycin (10 µg)	-	L-Histidine	+
Penicillin G (10 units)	-	L-Cysteine	+
Rifampicin (5 µg)	-	L-Valine	+
Tetracycline (30 µg)	-	L-Phenyl alanine	+
Tobramycin (10 µg)	+	Potassium nitrate	+
Erythromycin (15 µg)	-	L-Threonine	+
Bacitracin (10 µg)	-	L-Serine	+
Chloramphenicol (30 µg)	-	L-Methionine	+
Novobiocin (5 µg)	-		

used in the identification were listed in materials and methods, results of which are given in Table 2.

The genus *Nocardioides* is easily differentiated from all other actinomycetes on the basis of the diaminopimelic acid type in the cell wall peptidoglycan, menaquinone profile, and the cellular fatty acid profile (19,20).

The genus *Nocardioides* is one of a few genera that have LL-2,6-diaminopimelic acid (LL-DAP) as the diagnostic diamino acid in the cell wall peptidoglycan layer(21). This feature, together with glycine in the cell wall peptidoglycan, indicates that this genus is wall chemotype I (22). This chemical marker distinguishes the genus *Nocardioides* from many other actinomycetes genera that possess other types of diamino acids in the cell wall peptidoglycan.

Regarding the bioactivities of the *Nocardioides* spp., only a few *Nocardioides* strains were found to produce antibiotics (23,24,25). Strain ATCC 39419, which produced a novel anti-tumor antibiotic, sandramycin, was originally assigned to *Nocardioides* (26) but has later been classified as a member of a new genus *Kribbella* (27).

However, to our knowledge, this is the first study to investigate the ability of a *Nocardioides* sp. to produce antibiotics in detail; regarding the physiological parameters that affect the production followed by an attempt to isolate and identify the active compound(s).

Cultivation parameters : Since antibiotics are secondary metabolites synthesized by pathways, which are often connected and influenced by primary metabolism, thus, frequently an intermediate metabolite from primary metabolism serves as precursor for the biosynthesis of the antibiotic. Therefore, the composition of the culture medium, closely connected with the metabolic capacities of the producing organism, greatly influences the biosynthesis of antibiotics. Changes in the nature and concentration of carbon and nitrogen sources, phosphorus concentration and trace elements have been reported to affect antibiotic biosynthesis in different organisms. Results (Fig. 1) showed that activity and growth gradually increased with increasing the incubation period till the activity reached maximum after 7 days

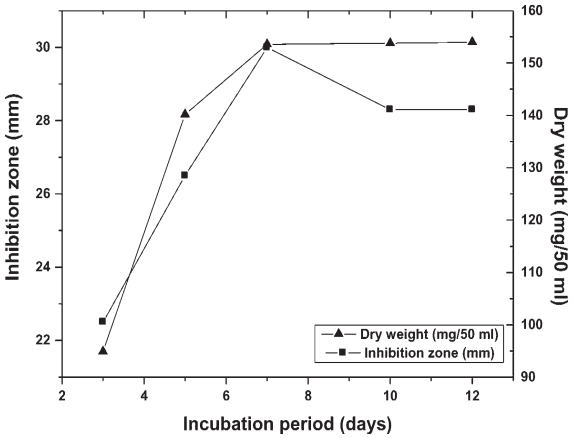

Fig. 1. Effect of different fermentation periods on anticandidal activity of *Nocardioides luteus*

of incubation then it began to decline with time while the growth nearly remained constant after 7 days. These results revealed that the anticandidal metabolite was early produced and reached maximum at the stationary phase of growth. The cessation of growth in the stationary phase is most commonly caused by the exhaustion of the essential nutrients of the medium as well as the accumulation of undesirable metabolites. Similarly, the optimal incubation period required for maximum growth and antibiotic yields by *Streptomyces sp.* 201 was six days (28). On the other hand, the highest biomass and antibiotic production of *Streptomyces sp.* KEH23 is observed after 96 h of incubation. However, after 108 h, the diameter of the inhibition zones dropped gradually (29).

The performance of a microbial culture can be strongly influenced by the type of the inoculum as shown in Fig. 2. Thus, the age and size of the inoculum should be optimized to improve the performance of the fermentation process. Our results showed that maximum inhibition zone, corresponding to maximum anticandidal activity, was observed at inoculum age 48-72 hrs, while both the biomass yield remained unaffected. On the other hand, the cell growth increased by increasing the inoculum size, increase in anticandidal activity was not observed on using more than 2 ml inoculum/50 ml production medium.

The anticandidal activity production (Fig.3A) seems to be no way correlated to the biomass yield. Starch showed maximum inhibition zone, whereas maximum growth was produced by maltose. Minimum inhibition zone was produced by fructose while minimum biomass was recorded by sucrose. These findings were in agreement with the fact that secondary metabolite production in actinomycetes is often stimulated by slowly assimilated complex carbohydrates or oils in the production media, while it decreased when more rapidly utilized monosaccharide such as glucose were present (30). A possible explanation of this phenomenon

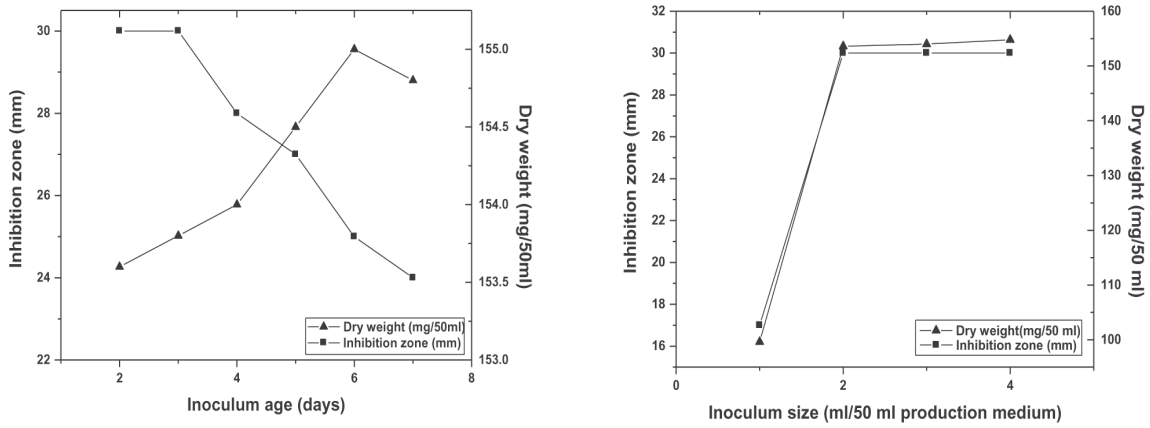

Fig. 2. The growth and anticandidal activity as affected by *N.luteus* inoculum

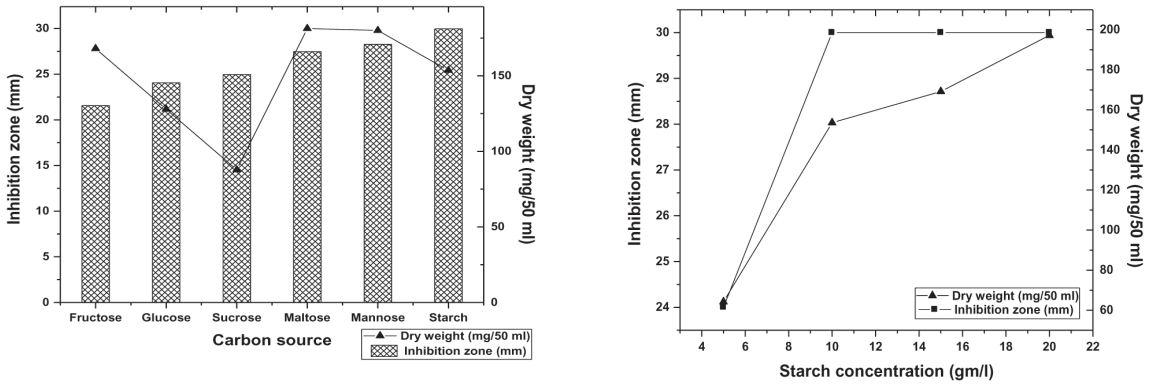

Fig. 3. Role of carbon nutrition on the growth and anticandidal activities of *N.luteus*
A. Effect of carbon source **B**. Effect of starch concentration

is that glucose or other simple carbon sources cause catabolite repression in which the production of an enzyme of secondary metabolite biosynthesis is inhibited.

The growth profile and antibacterial biosynthesis by the tested organism was not only affected by the nature of the used carbon source, but also with its level. Results (Fig. 3B) showed that the activity increased with increasing the starch concentration till it reached a maximum of 30 mm at 10 gm/l after which it remained constant while the dry weight values kept increasing with increasing starch concentrations. Antibiotic production from alkaliphilic actinomycetes isolate A2D, identified as *S. tanashiensis* strain A2D, was higher in medium having glucose (1 %) as carbon source while highest biomass resulted on using 1.5 %(w/v) glucose concentration (31). However, maximum growth and antibiotic production by *Streptomyces* sp. 201, were obtained in medium supplemented with mannitol as a sole carbon source followed by sucrose and glycerol (32).

Our results showed that organic nitrogen sources such as peptone, corn steep liquor and specially yeast extract were highly favorable for anticandidal activity although this doesn't necessarily correlate to the growth of *Nocardioides luteus*, as shown in Fig. 4A. On the other hand, the tested inorganic nitrogen sources resulted in a decreased anticandidal production that also doesn't correlate with growth. Maximum inhibition zone was observed on using yeast extract. Therefore, it was chosen as a sole nitrogen source instead of casein and KNO_3 in the proceeding work. The activity increased with increasing the nitrogen source concentration till it reached a maximum at the concentration 3.76 gm/l, then constant through the concentration 4 gm/l. Thereafter, it began to decrease. The dry weight increased gradually as well by increasing the yeast extract concentrations until reaching 4 gm/l, after which it started to decrease, Fig. 4B. The superiority of the organic nitrogen sources has been reported by many investigators (30-33).

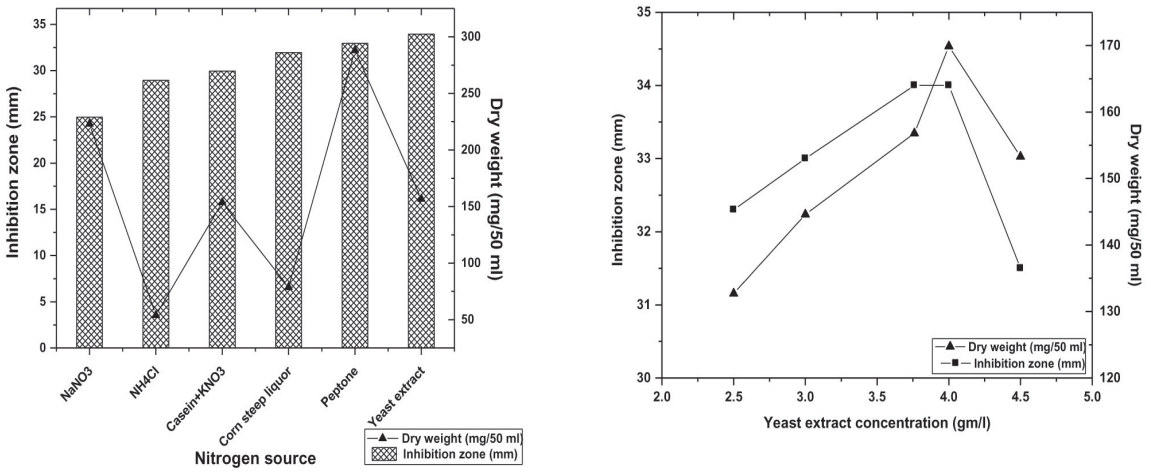

Fig. 4. Role of nitrogen nutrition on the growth and anticandidal activity of *N.luteus*
A. Effect of nitrogen source **B.** Effect of nitrogen concentration

Evidently, the activity was maximal, with an inhibition zone diameter of 37.7 mm, on using sodium chloride at concentration 1 gm %. The antifungal activity at a greater NaCl concentration decreased to reach 30 mm at 4 gm% concentration (Fig. 5).

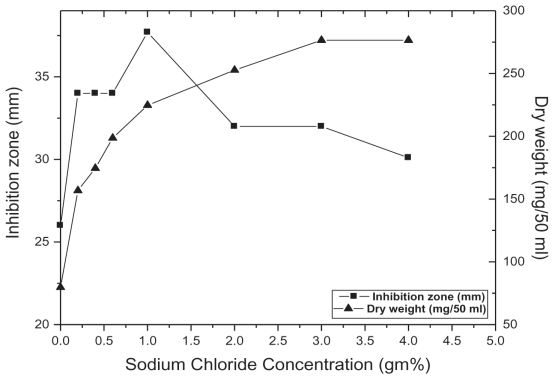

Fig. 5 Effect of NaCl concentration

Antibiotic biosynthesis by certain halophytic actinomycetes species was found to be optimized at specific NaCl levels (34,35). Similarly, the optimum salt requirement of the strain *Streptomyces tanashiensis* strain A2D for antibiotic production in medium containing carbon source (glucose) and nitrogen source (soybean). They found that 2 % NaCl was optimum for maximum growth (4.9 mg/ml) as well as antimicrobial compound production (20.9 mg/ml). Further increase in salt concentration reduced the antimicrobial agent biosynthesis (36).

Similarly, the role of K_2HPO_4 on the growth and anticandidal activity was investigated. The productivity of the experimental organism was optimized at 0.1 gm% (Fig. 6). However the depletion of the tested salt from the fermentation medium resulted in a decrease in the antifungal activity. The dry weight yield increased gradually with increasing K_2HPO_4 concentration until 1.2 gm% after which it remained constant. It was

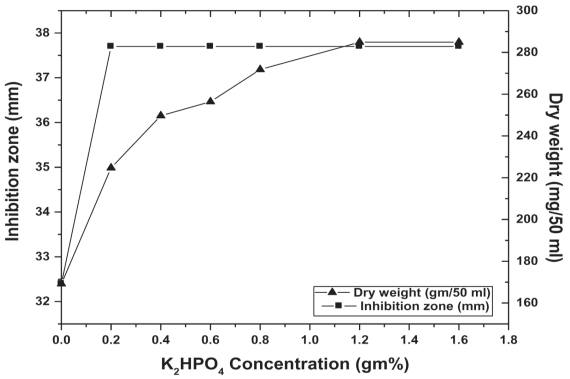

Fig. 6. Growth and anticandidal activity of *N.luteus* as affected by K_2HPO_4

reported that phosphate ions seemed to stimulate the process of primary metabolite production at the expense of secondary metabolite. Also, they reported that the highest dipotassium hydrogen phosphate concentrations studied at 1.6 g/l proved to provide non limiting growth conditions without affecting the antibiotic production (33). It was also noted that the excess PO_4 altered the biochemical composition of the mycelium protoplasm and affected the physiological function of the cell and decreases the biosynthesis of the antibiotic (37). Conversely, phosphate at all concentrations inhibited the production of inhibitory substance(s) by *Streptomyces* (Ds-104) strain (36).

The pH value of the cultivation medium is very important for growth of the microorganism, characteristic of their metabolism and hence for biosynthesis of metabolites. The hydrogen ion concentration may have direct effect on the cell or it may indirectly affect it by varying the dissociation degree of the medium components. Results showed that both production and growth were maximal in the neutral medium either on using buffers or by initial adjustment with 1M HCl and 1M NaOH. It is also noted that tris-maleate buffer, although not causing a

Table 3. The TLC profile of the different constituents responsible for the anticandidal activity of *N.luteus*

Band	Retention factor (Rf)	Inhibition zone (mm)
1	0.07	20
2	0.20	21.5
3	0.47	26
4	0.6	19
5	0.68	13.5
6	0.82	17.5

Table 4. The antitumour activities exhibited by *N. luteus* bioactive extract

Doxorubicin	Crude Extract	Semi-pure compound	Tested cell line
3.96 µg/ml	3.43 µg/ml	2.82 µg/ml	HFB4 (normal melanocytes)
5.5 µg/ml	2.51 µg/ml	2.99 µg/ml	HEPG2 (liver carcinoma cell line)
2.97 µg/ml	3.28 µg/ml	2.86 µg/ml	MCF7 (breast carcinoma cell line)
4.89 µg/ml	3.13 µg/ml	2.4 µg/ml	HEP2 (larynx carcinoma cell line)
3.64 µg/ml	2.67 µg/ml	2.82 µg/ml	HELA (cervical carcinoma cell line)
3.43 µg/ml	2.82 µg/ml	2.97 µg/ml	CACO (colon carcinoma cell line)
3.743 µg/ml	3.74 µg/ml	2.97 µg/ml	HCT116 (colon carcinoma cell line)

remarkable decrease in activity yet, it resulted in a great decrease in the cell biomass (Data not shown). Increasing the pH value of the culture media of *Streptomyces* spp. led to an increase in the antifungal production up to certain limit (pH 7.0) above which any increase in the pH value was accompanied by a decrease in the antifungal production and consequently the antagonistic activity. Maximum antifungal activity recorded by *Streptomyces* strain Ds-104 occurred on using fermentation medium at pH range 7.0-7.5. It afforded an inhibition zone diameter range between 12 and 18 mm. The results obtained by other scientists also assured that the neutral range of pH value enhanced the formation of anticandidal agents from different *Streptomyces* spp. (36-39).

With respect to the role of the agitation rate, the obtained results revealed that the maximum activity was obtained at 200 rpm, though cell biomass yields decreased with increasing the agitation speed being maximal at static state (Fig. 7). Antifungal antibiotic production by the strain *Streptomyces rimosus*_MY02 increased slightly corresponding to agitation rate. But antifungal antibiotic production had no significant differences at 150–200 rpm. Finally, they concluded that the best agitation rate was 180 rpm (40).

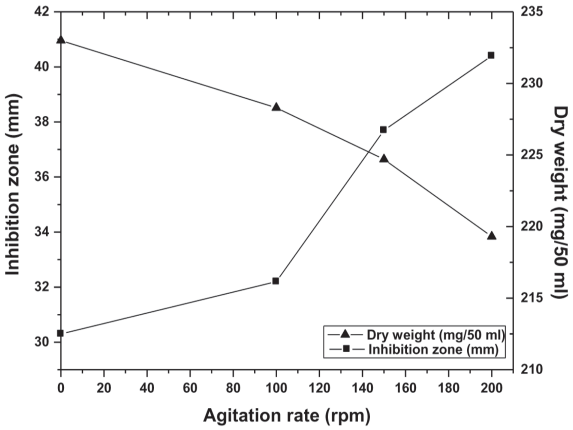

Fig. 7. Growth and anticandidal activity of *N.luteus* as affected by the agitation rate.

Isolation, purification and identification of the anticandidal component (s) : The TLC chromatogram for ethyl acetate extract of the culture filtrate of *Nocardioides luteus* developed as 6 major bands. By testing their activity against *C.albicans* using paper disc assay method, they all showed considerable activity as shown in Table 3.

The compound was subjected several times to chromatographic purification but it could not be obtained as a pure sample. The semi-pure compound was identified from its UV, IR, ^{1}H-NMR and ^{13}C-NMR spectra and comparing these spectra with published data (41) as derivative of the actinomycins class of compounds. Its UV spectrum showed maximum absorption ($ë_{max}$ in CH_3OH) at 224, 427 and 444 nm.

Its IR spectrum showed the characteristic peaks of NH group at 3433 cm^{-1}, aliphatic hydrogens (CH, CH_2 and CH_3) at 2966, 2924 and 2855 cm^{-1}. The amide group (CONH) signal appeared at 1648 cm^{-1}.

The ^{1}H-NMR measurement showed the characteristic signals of the phenoxazone chromophore at δ 2.21 (CH_3 at position 4), 2.52 (CH_3 at position 6), 7.34 (d, H-7) and 7.61 (d, H-8). The presence of two peptide rings in the actinomycin structure was established by the presence of 3 amide signals at δ 8.19, 8.01 and 7.73. Also, the methyl groups of valine and sarcosine amino acids gave the signals at δ 2.9 (one methyl) and 2.86 (two methyl groups).

The ^{13}C-NMR measurement showed the characteristic signals of the phenoxazone chromophore at δ 7.8 (CH_3 at position 4) and δ 15.11 (CH_3 at position 6), in addition to the carbonyl group at δ 173.39 (C-3). The carbonyl signals in between δ110-140 were attributed to the other carbons of the phenoxazone group. The signals corresponding to the amide and lactone groups of the peptide system were detected in between δ 160-172

The actinomycins are a family of chromopeptide antibiotics, differing only in the amino acids present in the pentapeptide chains, which are elaborated by certain members of order *Actinomycetales*. Actinomycin was first isolated from a culture of *Streptomyces antibioticus* by Waksman and Woodruff (40,41). It was the first antibiotic to be produced after penicillin. The various products have been designated A, B, C, D, I, J, and X(42,43,44). It has been shown, however, that these actinomycins were not homogeneous substances but consisted of a number of closely related biologically active components. Evidence also suggested that the A, B, D, and X complexes might consist of identical components but differ in the relative amount of each component present. Structural studies have revealed that the actinomycin molecule is composed of two polypeptides linked to a chromophoric quinonoid moiety which is the same for all components. Differences in the components of an actinomycin complex or of different complexes are due to variations in the number, arrangement, and kinds of amino acids present in the peptides.

Since its discovery by Waksman and Woodruff in 1940, actinomycin was obtained from the culture media of a number of actinomycetes species; *Streptomyces, Actinoplanes* and *Micromonospora* by various investigators and was isolated in crystalline form. Yet, this is the first time it was reported to be isolated from a *Nocardioides* sp not to mention, *Nocardioides luteus*.

Cytotoxicity assay : Due to the broad spectrum of the activity of the culture filtrate extract and its activity against *Saccharomyces cerevisiae*, which is used as biological model to establish the cytotoxicity and cytostaticity of natural and/or synthetic chemical compounds (43,44) as well as our discovery that it belonged to actinomycins class, we carried out cytotoxicity tests against different human tumor cell lines to support the previous data. Knowing that antitumor antibiotics produced by actinomycetes are among the most important cancer chemotherapeutic agents including members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families (45,46), we extended our scheme to test the antitumor activity of the extract produced by the experimental *Nocardioides luteus*. It showed significant antitumor activity against the tested human cancer cell lines surpassing doxorubicin's activity in most cases. The IC_{50} of crude extract, semi-pure compound on different human tumor cell lines compared to doxorubicin is illustrated in Table 4.

References

1. Baltz, R.H. (2008). Renaissance in antibacterial discovery from actinomycetes. Current Opinion in Pharmacology. 8:557-563.

2. Ganesan, A. (2008). The impact of natural products upon modern drug discovery. Current opinion in chemical biology, 12:306-317.

3. Lam, K.S. (2007). New aspects of natural products in drug discovery. Trends in Microbiology, 15(6):279-289.

4. Challis, G.L. and Hopwood, D.A. (2003). Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. Proc Natl Acad Sci. USA. 25:14555-14561.

5. Ruan, S. and Hsueh, P. (2009). Invasive Candidiasis: An Overview from Taiwan. J Formos Med Assoc. 108(6):443-451.

6. Lewis, F.J., May, E., Bravery, A.F. (1985). Isolation and enumeration of autotrophic and heterotrophic bacteria from decayed stone. In Felix, G. (ed.), Proceedings of the 5th international congress on deterioration and conservation of stone. Lausanne, Switzerland. Press Polytechniques Romandes, pp. 633-641.

7. Kuster, E. and Williams, S.T. (1964). Selection of media for isolation of *Streptomycetes*. Nature, 202:9928-9929.

8. El-Nakeeb, M.A. and Lechavalier, H.A. (1962). Selective isolation of aerobic actinomycetes. J Appl Microbiol. 2:75-77.

9. Williams, S.T. and Wellington, E.M.H. (1982). Actinomycetes. In Page, A.L., *et al*. (eds.), Methods of soil analysis, Part 2, Chemical and Microbiological Properties (2nd edition), American Society of Agronomy/Soil Science Society of America, Madison, pp. 969-987.

10. Pridham, T.G. and Lyons Jr., A.T. (1961). *Streptomyces albus* (Rossi Doria) Waksman and Henrici: Taxonomic study of strains labeled *Streptomyces albus*. J Bacteriol. 81:431-441.

11. Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H. (1985). Genetic manipulation of *Streptomyces*: A laboratory manual. John Innes Foundation, Norwick, UK.

12. De Beer, E.J. and Sherwood, M.B. (1945). The paper-disc agar-plate method for the assay of antibiotic substances. J Bacteriol. 50(4):459-467.

13. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S. and Boyd, M.R. (1990). New colorimetric cytotoxicity assay for anti-cancer drug screening. J Natl Cancer Inst. 82:1107-1112.

14. Urzi, C.P., Salamone, P., Schumann, P. and Stackebrandt, E. (2000). *Marmoricola aurantiacus* gen. nov., sp. nov., a coccoid member of the family *Nocardioidaceae* isolated from a marble statue. Int J Syst Evol Microbiol. 50:529-536.

15. Abdulla, H., May, E., Bahgat, M. and Dewedar, A. (2008). Characterisation of actinomycetes isolated from ancient stone and their potential for deterioration. Polish J Microbiol. 57(3):213-220.

16. Dietz, A. and Thayer, D.W. (1980). Actinomycete taxonomy. Society for Industrial Microbiology, special publication no.6, Arlington, Virginia, USA.

17. Holt J.G., Ed. (1989). Bergey's Manual of Systematic Bacteriology, Vol. 4, 1st edition, Williams and Wilkins, Baltimore.

18. Holt, J.G. Ed. (1994). Bergey's Manual of Determinative Bacteriology, 9th edition, Williams and Wilkins, Baltimore.

19. Park, Y.H., Yoon, J.H., Shin, Y.K., Suzuki, K.I., Kudo, T., Seino, A., Kim, H.J., Lee, J.S. and Lee, S.T. (1999). Classification of "*Nocardioides fulvus*" IFO 14399 and *Nocardioides* sp. ATCC 39419 in *Kribbella* gen. nov., as *Kribbella flavida* sp. nov. and *Kribbella sandramycini* sp. nov. Int J Syst Bacteriol. 49:743-752.

20. Wang, Y.M., Zhang, Z.S., Xu, X.L., Ruan, J.S. and Wang, Y. (2001). *Actinopolymorpha singaporensis* gen. nov., sp. nov., a novel actinomycetes from the tropical rainforest of Singapore. Int J Syst Evol Microbiol. 51:467-473.

21. Prauser, H. (1976). *Nocardioides*, a new genus of the order *Actinomycetales*. Int J Syst Bacteriol. 26:58-65.

22. Lechevalier, M.P. and Lechevalier, H.A. (1970). A critical evaluation of the genera of aerobic actinomycetes. In Prauser, H. The *Actinomycetales*. Gustav Fischer-Verlag, Jena, Germany, pp.393-405.

23. Loppinet, V., Hilali, L., Youssef, N., Bonaly, R. and Finance, C. (1997). Isolation of an antifungal macrolide from soil sample *Nocardioides* strain: Production and structure elucidation. In Zinner, S.H., Young, L.S., Acar, J.F. and Neu, H.C. (eds.), Expanding indications for the new macrolides, azalides and streptogramins. 1st Ed., Informa Healthcare, pp. 286-292.

24. Coombs, J.T., Michelsen, P.P. and Franco, C.M.M. (2004). Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. *tritici* in wheat. Biological Control, 29:359-366.

25. Romanenko, L.A., Tanaka, N., Uchino, M., Kalinovskaya, N.I. and Mikhailov, V.V. (2008). Diversity and antagonistic activity

of sea ice bacteria isolated from the sea of Japan. Microbes Environ. 23(3):209-214.

26. Matson, J.A. and Bush, J.A. (1986). US Patent 4,582,639.

27. Thakur, D., Bora, T.C., Bordoloi, G.N. and Mazumdar, S. (2009). Influence of nutrition and culturing conditions for optimum growth and antimicrobial metabolite production by *Streptomyces* sp. 201. Journal de Mycologie Médicale, 19:161-167.

28. Oskay, M. (2009). Antifungal and antibacterial compounds from *Streptomyces* strains. African Journal of Biotechnology, 8(13):3007-3017.

29. Bertasso, M., Hulzenkampfer, M., Zeexk, A., Doll Antonia, F. and Fiedler, H. (2001). Bahremycin A and B, novel antibiotics from *Streptomyces* sp.TU. 4128. J Antibiot. 54:730-736.

30. Singh, L.S., Mazumder, S. and Bora, T.C. (2009). Optimization of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete, *Streptomyces tanashiensis* strain A2D. Journal de Mycologie Médicale, 19:225-233.

31. Suthindhiran, K. and Kannabiran, K. (2009). Cytotoxic and Antimicrobial Potential of Actinomycete Species *Saccharopolyspora salina* VITSDK4 isolated from the Bay of Bengal Coast of India. American Journal of Infectious Diseases, 5(2):90-98.

32. Saadoun, I. and Muhana, A. (2008). Optimal production conditions, extraction, partial purification and characterization of inhibitory compound(s) produced by *Streptomyces* Ds-104 isolate against multi-drug resistant *Candida albicans*. Current Trends in Biotechnology and Pharmacy, 2(2):402-420.

33. Singh, M.P., Mandal, S.K. and Roy, D.K. (1981). Studies on erythromycin fermentation: Nutritional requirements of *Streptomyces erythreus* for erythromycin production. Ind J Pharm Sci. 43(4):137-141.

34. Egorov, N.S. (1985). Antibiotics: A Scientific Approach. Translated from Russian by Alexander Rosinkin. 1st edition, Mir Publishers, Moscow.

35. El-Mehalawy, A.A., Abd-Allah, N.A., Mohamed, R.M. and Abu-Shady, M.R. (2005). Actinomycetes antagonizing plant and human pathogenic fungi. II: Factors affecting antifungal production and chemical characterization of the active components. International journal of agriculture and biology, 7(2):188-196.

36. Arasu, M.V., Duraipandiyan, V., Agastian, P. and Ignacimuthu, S. (2009). In vitro antimicrobial activity of *Streptomyces* spp. ERI-3 isolated from Western Ghats rock soil (India). Journal de Mycologie Médicale, 19:22-28.

37. Yu, J., Liu, Q., Liu, Q., Liu, X., Sun, Q., Yan, J., Qi, Q. and Fan, S. (2008). Effect of liquid culture requirements on antifungal antibiotic production by *Streptomyces rimosus* MY02. Bioresource Technology, 99:2087-2091.

38. Lackner, H. (1975). Three dimensional structure of the actinomycins. Angewandte Chemie, 14(6):375-386.

39. Goss, W.A. and Katz, E. (1957). Actinomycin Formation by *Streptomyces* Cultures. Appl Microbiol. 5(2):95-102.

40. Waksman, S.A. and Woodruff, H.B. (1940). Bacteriostatic and bactericidal substances produced by a soil *actinomyces*. Proc Soc Expt Biol Med. 45:609-614.

41. Waksman, S.A. (1954). The Actinomycetes. Their nature, occurrence, activities, and importance. In Stackebrandt, E. and Schumann, P. (2006). Introduction to the Taxonomy of Actinobacteria. In Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. (eds.), The Prokaryotes: a handbook on the biology of bacteria. Vol. 3, 3rd edition, Springer-Verlag, pp. 297-322.

42. Hixon, S.C., Ocak, A., Thomas, J.E. and Daugherty, J.P. (1980). Resistance to adriamycin cytotoxicity among respiratory-deficient mutants in yeast. Antimicrobial Agents and Chemotherapy, 17(3):443-449.

43. Elsea, S.H., Osheroff, N. and Nitiss, J.L. (1992). Cytotoxicity of quinolones toward eukaryotic cells. J Biological Chemistry, 267(19):13150-13153.

44. Castro, F.A.V., Mariani, D., Panek, A.D., Eleutherio, E.C.A. and Pereira, M.D. (2008). Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. PLoS One, 3(12):e3999.

45. El-Shatoury, S.A., El-Shenawy, N.S. and Abd El-Salam, I.M. (2009). Antimicrobial, antitumor and in vivo cytotoxicity of actinomycetes inhabiting marine shellfish. World J Microbiol Biotechnol. 25:1547-1555.

46. Kavitha, A., Vijayalakshmi, M., Sudhakar, P. and Narasimha, G. (2010). Screening of Actinomycete strains for the production of antifungal metabolites. African Journal of Microbiology Research, 4(1):27-32.

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Improvement of rCHO Cell Density and Heterologous Protein Production: Effect of Culture Conditions

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Abstract

Physico-chemical parameters have a great impact on mammalian cell growth. In this study, the effects of a number of amino acids, precursors, serum, glucose, osmolality and temperature on the recombinant Chinese hamster ovary (rCHO) cells producing the recombinant human coagulation factor VIII (rhFVIII) were investigated, so as to improve the rCHO cell density in the packed-bed bioreactor. The results showed that while the concentration of ammonium, as the main by-product, increased by more than 6mM, the rCHO cell density decreased significantly. On the other hand, adding certain amino acids and precursors mitigated the stress caused by ammonium and consequently increased cell density. Proline and glycine increased cell density up to approximately 28% in comparison with the negative control. Nucleic acids, as the main precursors, had a significant impact on rCHO cell growth, which increased by up to 20%. Higher amounts of glucose (3.5 g/l) supplied the carbon and energy requirements of the cells, inhibited by-products formation and sequentially increased heterologous protein production. The precursors allowed a 14% improvement in rhFVIII activity at high glucose concentrations, which was 1.5 fold greater than the negative control. Both hyperosmotic pressure and temperature downshifts had remarkable adverse effects on the rCHO cell density,; being reduced to 2.7×10^6 cells/ml. The acquired suitable culture conditions were applied to the packed-bed bioreactor, and consequently an rCHO cell density of 18×10^8 cells/l was achieved during 120 h of cultivation. The results of this study indicated that the rCHO cells essentially need supplementary materials to deal with inappropriate culture conditions; especially in a serum-free medium.

Keywords: Factor VIII, Packed-bed bioreactor, Recombinant CHO cells, Serum-free medium.

Introduction

Mammalian cell cultures are necessary for production of glycosylated recombinant pharmaceutical proteins (1). Generally, mammalian cells have low productivity,

Vol. 5 (3) 1298 -1308 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

therefore, large amounts of culture are needed per clinical dose of the final product. High cell density cultures can overcome this problem by increasing the volumetric productivity, which is directly related to both cell density and specific productivity (2,3). Packed-bed bioreactors enable the achievement of high-volume cell density and productivity under low-volume media conditions. Cong et al. (2001) managed to achieve a CHO cell density of 2×10^7 cells/ml after fifteen days cultivation in a packed-bed perfusion bioreactor (4).

Culture compositions have a great impact on cell viability, density and productivity, and also influence cell attachment to microcarriers. Reduction in glutamine concentrations decreases cell growth and viability (5,6). Whereas glucose and glutamine are vital for rCHO cells, glutamine cannot support cell maintenance in the absence of glucose. Meanwhile, glutamine metabolism represents the main source of ammonium as a toxic by-product (5,7,8). Elevated ammonium concentrations significantly inhibit cell growth and final cell density and consequently decrease volumetric productivity of recombinant proteins. The adverse effects of ammonium on gene expression and protein glycosylation in rCHO cells have also been previously reported (9-12). Lactate is another toxic by-product, which is formed in the presence of excess glucose. Glutamine metabolism and ammonium buildup increase intracellular pH, while lactate formation decreases intracellular pH in mammalian cell cultures. Various strategies have been developed to alleviate the detrimental effects of both by-products in CHO cells (13). Addition of higher concentrations of certain amino acids has been reported to diminish the negative effects of ammonium, especially cell growth and productivity in CHO cells (14). Besides, certain amino acids can protect rCHO cells from elevated osmolality and carbon dioxide pressure which inhibit both cell growth and recombinant protein production (15,16). Shift of cultivation temperature from 37°C to 30°C has been shown to cause a growth arrest and improvement in the productivity of rCHO cells producing alkaline phosphatase. Since temperature shifts have been found to have different effects on mammalian cell growth and recombinant protein production that is dependent on the cell line and expression system, optimization of temperature has been proposed for each cell line (17,18).

Human Coagulation factor VIII is a large plasma glycoprotein that is necessary for prophylactic treatment of hemophilia A patients. Increasing demand for rhFVIII has resulted in the development and improvement of rhFVIII production, in order to decrease the cost of rhFVIII (19,20). However, there are many problems with regard to rhFVIII production, such as interaction with protein chaperones and its stability in medium (21,22). Secretion of rhFVIII has been found to increase in culture medium supplemented with different concentrations of propionic and butyric acids (23). Implementing different cell lines and improving glycosylation sites have also been effective methods with regard to increasing rhFVIII production (24,25). It is commonly accepted that all mammalian cell lines have the same basic nutrient requirements; however their individual metabolisms, e.g. specific nutrient consumption and metabolite production rates often differ based on the cell line and expression system. Consequently, the main objective of this study was to investigate the physico-chemical culture conditions of the rCHO cells that affect their growth behaviors, in order to improve the cell density and recombinant protein production in a packed-bed bioreactor.

Materials and methods

Materials: Dulbecco's modified Eagle's medium (DMEM), Ham's F12, fetal bovine serum (FBS),

Vol. 5 (3) 1298 -1308 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

and trypsin were purchased from Gibco. All chemicals were of analytical grade, and were purchased from Sigma-Aldrich (St. Louis, MO).

Cell line: The rhFVIII expressing CHO cell line obtained from the National Institute of Genetic Engineering and Biotechnology (26) was used as the rCHO cell line.

Culture conditions: The rCHO cells were cultured in a basal medium containing DMEM and Ham's F12 nutrient mixture at a ratio of 1:1 (v/v), supplemented with 10% FBS. The rCHO cells were grown at different concentrations of glucose (1.2, 3.5, 7 g/l) in a T-75 flask so as to obtain an appropriate glucose concentration in the basal medium, for the purpose of obtaining higher amounts of cell density.

The basal medium was prepared at different concentrations of NH_4Cl (2, 4, 6, 8, 10, 12 mM) to determine the effect of ammonium stress on rCHO cell growth and rhFVIII production. The control flask contained basal medium without excess ammonium.

The amino acid-supplemented T-flasks contained basal medium plus 20 mM of each of the appropriate amino acids and 10 mM NH_4Cl. The negative control flask contained the basal medium plus 10 mM NH_4Cl, and the positive control flask contained only basal medium. This experiment was carried out at low and high glucose concentrations (1.2, 3.5 g/l). The precursor-supplemented T-flasks contained basal medium plus 10^{-3} M of each of the dNTPs (dATP, dCTP, dTTP, dGTP). The control flask did not contain any precursors.

For studying effects of temperature on cell growth and protein production, the rCHO cells were cultivated at 37°C until the culture reached the exponential phase of growth. The temperature was then reduced to 30°C., but the control flask was maintained at 37°C. The osmolality of the medium supplemented with 10% FBS was 320 mOsmol/kg. To determine the effects of osmolality on cell growth and protein production, NaCl stock solution was added to the basal medium increasing osmolality by up to 410 mOsmol/kg (1 mg NaCl/ml = 32 mOsm increase). The control flask had no excess sodium chloride.

In order to investigate the effects of certain additives on cell growth, the rCHO cells were cultivated in basal medium with 10% FBS. After 48 h of cultivation, the medium was replaced with fresh medium without FBS. Appropriate amino acids and biotin were added to the serum-free medium. Basal medium was supplemented with 10 mg/l biotin. The serum-free control flask had no additives.

Bioreactor: The batch bioreactor experiment was performed in a 5 l (3.5 l working volume) Celligen bioreactor (New Brunswick Scientific, USA) maintained at 37°C, pH 7.2, and stirred at 50-100 rpm. The pH level was controlled by acidic and basic solutions (2N HCl and 80g/l $NaHCO_3$). Dissolved oxygen (DO) was adjusted to 50% of air saturation, as manipulated by the bioreactor control program. The polyester disks (134 g) purchased from New Brunswick Scientific Co., were used as microcarriers for the attachment of the rCHO cells in the packed-bed bioreactor. The appropriate conditions obtained in the T-flask were also employed in packed-bed bioreactor. Glutamine was added after 48 h of cultivation at 20 mM and other additives (proline, glycine, threonine, glutamine, biotin, dNTP and glucose) were prepared in the basal medium. The concentrations of the additives were; amino acids (proline, glycine, threonine) 20 mM, biotin 2 mg/l, dNTP 10^{-3} M, and glucose 3.5 g/l. After 120 h of cultivation, the polyester microcarriers were removed from the bioreactor and the rCHO cells were separated by the EDTA-Trypsin solution.

Vol. 5 (3) 1298 -1308 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Analysis: All T-flask samples were cultivated in a humidified incubator at 37°C and 5% CO_2. After proliferation and cell growth, the medium was removed from the flasks and eluted with phosphate buffer solution (PBS). The cells were removed from the surface of the flasks by trypsin-EDTA solution (0.25%). After the incubation period (ca. 5 min), trypsin was inhibited by adding FBS. The cell suspensions were centrifuged at 1000 rpm for 5 min at 4°C. The supernatants were then removed and the cell pellets were resuspended in 1 ml of medium and used for subsequent counting of cells. Cell viability was determined by the trypan blue exclusion method.

Deficient human coagulation factor VIII plasma was purchased from Diagnostica Stago,France. Biological activity of rhFVIII was determined using a one-stage activated partial thromboplastein time (aPTT) assay. Briefly, the samples were mixed with deficient plasma as substrate. The clotting time was measured, following addition of $CaCl_2$ (0.025 M). The activity of rhFVIII was determined from a standard curve constructed by the pooled plasma. Data was presented as relative specific activity, and computed as the specific activity of samples against the specific activity of the pooled plasma.

Glucose, ammonium and lactate concentrations were enzymatically measured by glucose, ammonium and lactate assay kits (ChemEnzyme co., Iran)

Results and Discussion

Effect of glucose on cell density and protein production: Figure 1 shows that the rCHO cell density significantly decreased by 17% in medium containing 7 g/l glucose (ANOVA, $p < 0.05$) in comparison with the medium consisting 3.5 g/l glucose in which the cell density reached 3.5×10^6 cells/ml after 96 h of cultivation. It has been reported that glucose is vital for cell growth and productivity, but the excess glucose concentrations inhibit cell growth in the rCHO cells which are cell line- and culture mode-dependent. On the other hand, decreasing glucose concentrations to 1.2 g/l led to a reduction in cell density by more than 20% (ANOVA, $p < 0.05$) when compared to 3.5 g/l glucose concentration. High glucose concentrations reduce cyclic AMP levels both in prokaryotes and eukaryotes (27). Furthermore, formation of ammonium and lactate as the toxic by-products in the mammalian cell's metabolic pathway depends on glucose concentration. To explain the effects of glucose on cell density, both by-products were measured simultaneously. While glucose concentration was elevated from 1.2 to 3.5 g/l, ammonium formation was reduced from 37 mg/l to 29 mg/l after 96h. But ammonium formation was no longer decreased by increasing glucose concentration. Furthermore, higher amounts of ammonium were formed at earlier time of culture at the low glucose concentration (Figure 2). It can be due to the glucose depletion at 48 h when glucose concentration was 1.2 g/l (data not shown).

Ammonium formation in rCHO cells was increased under glucose limitation. The main source of ammonium formation is glutamine.

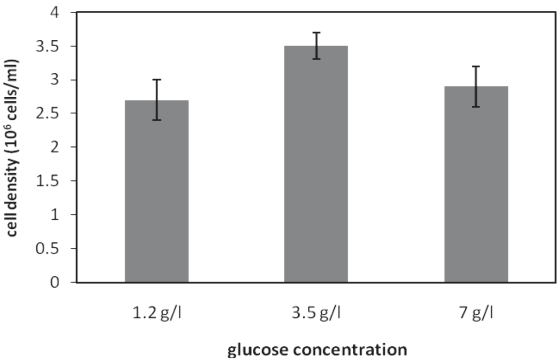

Fig. 1. The rCHO cell density at different concentrations of glucose after 96 h of cultivation

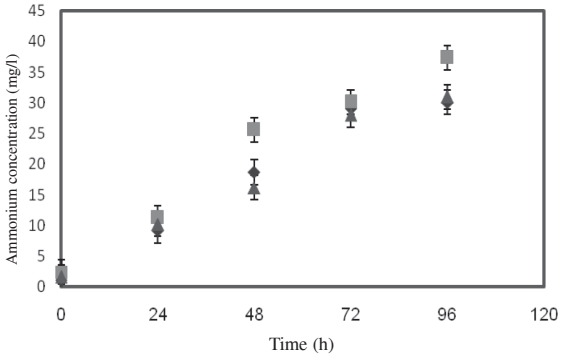

Fig.2. Time profile of ammonium formation at different glucose concentrations; 1.2 g/l (▪), 3.5 g/l (♦) and 7 g/l (▲)

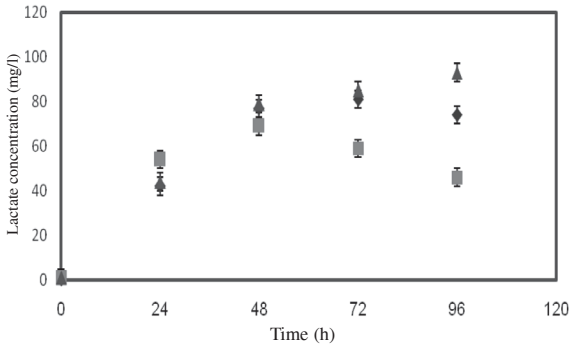

Fig. 3. Time profile of lactate formation at different glucose concentrations; 1.2 g/l (▪), 3.5 g/l (♦) and 7 g/l (▲)

Glutamine is another carbon and main nitrogen source for rCHO cell metabolism. Glutamine is not essential for rCHO cells, which have glutamine synthetase that catalyzes the following reaction (28):

L-glutamate + NH_4^+ + ATP <-> L-glutamine + ADP + Pi + H^+

ATP can be generated if glutamine is consumed, which commonly occurs at low glucose concentrations in order to replenish the energy deficit and consequently produce ammonium. As shown in Figure 3, there is no significant difference in lactate formation at various glucose concentrations up to 48 h. The rCHO cells then consume lactate as carbon source at the lower glucose concentration. So, detoxification of lactate can occur under glucose limitation. While the glucose concentration increased, there was no need to consume lactate for replenishing the energy deficit and the lactate concentration was not highly decreased.

The effect of glucose concentration on recombinant protein production is shown in Table 1. Activity of the recombinant hFVIII was increased to 8.4%when glucose concentrations were increased to 3.5 g/l, and then dropped off to 4.2 % when glucose concentration reached 7 g/l. The increase in activity may be due to the increased cell density at 3.5 g/l of glucose in culture medium. Another possible reason that can be attributed to the glucose concentration effect is ATP content of the cell which increases when glucose levels are appropriately high. In fact the chaperon immunoglobulin-binding protein (BiP) has been introduced as the main barrier in the secretion of rhFVIII. Release of BiP from rhFVIII depends on the intracellular content of ATP (29,30).

Table 1. Activity of rhFVIII at different concentrations of glucose

Glucose concentration (g/l).	rhFVIII activity (%)
1.2	6.1
3.5	8.4
7.0	4.2

Mitigation of ammonium stress by amino acid addition: Ammonium inhibits cell growth and protein production as mentioned above. The critical level of ammonium inhibiting cell growth has been reported as 10 mM (14). Figure 4 indicates that the rCHO cell density significantly decreased to 3.4×10^6 cells/ml at 4-6 mM NH_4Cl,

Vol. 5 (3) 1298 -1308 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

while it was 5.3×10^6 cells/ml in the control (culture medium without excess ammonium). Therefore, the threshold concentration of ammonium for the rCHO cells is 6 mM. The effect of ammonium stress on the activity of rhFVIII is shown in Table 2. Recombinant protein production was completely stopped at 11 mM NH_4Cl.

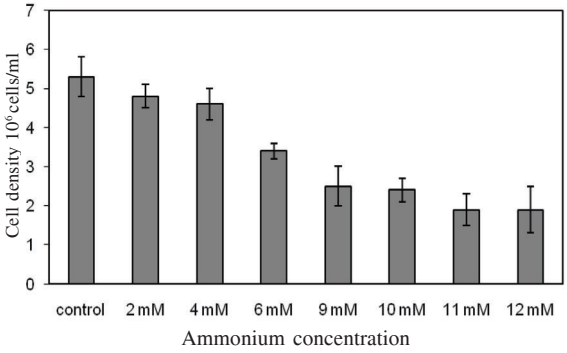

Fig. 4. The effect of ammonium concentration on the rCHO cell density

Table 2. Activity of rhFVIII under ammonium stress

Ammonium concentration (mM)	rhFVIII activity (%)	Ammonium concentration (mM)	rhFVIII activity (%)
Control	6.4	9	2.6
2	4.4	10	2.1
4	4.1	11	0
6	3.2	12	0

To mitigate the adverse effects of ammonium, some amino acids were selected and then added to the culture medium. According to the isoelectric point of amino acids and their role in metabolic pathways related to ammonium formation (14), four amino acids consisting of proline, threonine, glycine and asparagine were selected. As it is shown in Figure 5, proline and glycine increased the ammonium-stressed rCHO cell density by up to 24% and 28 % (ANOVA, $p<0.05$) at high glucose concentrations, respectively, as compared to the negative control. Interestingly, increasing glucose concentrations from 1.2 to 3.5 g/l had a positive impact on alleviation of ammonium-induced stress and a higher cell density was obtained, whereas no remarkable difference in connection with the addition of amino acids was observed at low glucose concentrations (Figure 5). Amino acids have potential to act as buffer, because of their zwitterionic properties at the physiological pH. Free amino acids are directly taken up by rCHO cells to be used in the formation of proteins and as nucleotide precursors. The Golgi apparatus plays an important role in the synthesis of glycoproteins, representing the major site o f carbohydrate biosynthesis, which is very sensitive to the pH of the Golgi apparatus (i.e. 6.5) (31). Using amino acids with isoelectric points close to the Golgi pH, e.g. proline (pI=6.3) and glycine (pI=6.0), can provide an appropriate buffering system for controlling intracellular pH and may overcome elevation in the intracellular pH caused by glutamine metabolism and ammonium formation.

Threonine and aspargine could not lessen the adverse effects of ammonium on the rCHO cell density. Asparagine metabolism produces as much ammonium as glutamine does. In fact, in order to alleviate the adverse effects of ammonium, aspargine has been proposed to be added to the medium, instead of glutamine, at the beginning of the cultivation because of its longer half life (28). Although glycine and threonine are converted to pyruvate via the same pathway, they do not show the same effect on the cell density of this recombinant cell line. From the observations made in this study, it seems that threonine degradation raises pyruvate levels and improves cell energetics, but it cannot diminish the detrimental effects of ammonium on cell growth through in an effective way, as mentioned above for glycine.

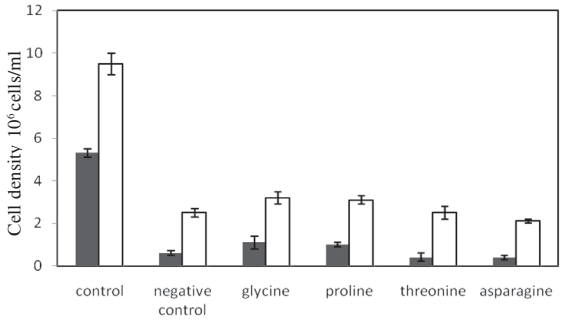

Fig. 5. The rCHO cell density under ammonium stress at low glucose concentrations, 1.2 g/l (black bars) and high glucose concentrations, 3.5 g/l (white bars)

Effect of precursors on the rCHO cell density and protein production: The rCHO cell growth was promoted in the presence of the precursors. Cell density increased by up to 5% and 20% (ANOVA, $p< 0.05$) in comparison with the control cultures, at low and high glucose concentrations, respectively. The maximum cell density of 1.2×10^7 cells/ml was achieved in the presence of the precursors at high glucose concentration. Ammonium formation also decreased, when precursors were added to the medium (Figure 6). The rhFVIII activities were obtained as 10.58% and 14.02% at low and high glucose concentration, as compared to the negative control showing activities of 6.4% and 9.19%, respectively. dNTPs as precursors are converted to glutamate in the metabolic pathways. The consumption of glutamate forms lesser amounts of ammonium than that of glutamine. Furthermore, the precursors are imported into the TCA cycle and improve cell energetics. Another possible reason that can be attributed to the nucleic acids is stoping the elongation of RNA polymerase while the interacellular concentration of nucleic acids decreases (32).

Effect of temperature and osmolality on cell density and rFVIII production: The rCHO cell densities were 2.7×10^6 and 3.7×10^6 cells/ml at 30°C and 37°C, respectively. The activity of

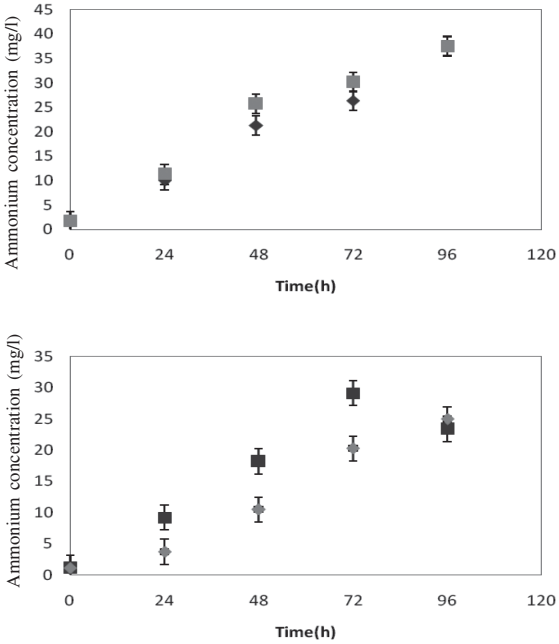

Fig. 6. Time profile of ammonium in the presence (◆) and absence (▪) of precursors at (A) low glucose concentrations; 1.2 g/l and (B) high glucose concentrations; 3.5 g/l

rhFVIII increased 2.2-fold when temperature was increased from 30°C to 37°C. Different cell lines exhibit various reactions during their growth and heterologous protein production in response to temperature up- or down-shift. Since rhFVIII is constitutively expressed in the rCHO cell line, its production depends directly on cell density. Figure 7 depicts the morphology of the rCHO cells in response to a temperature downshift. The proliferation of the rCHO cells was reduced at 30°C and cell's morphology changed to triangular shapes, while the CHO cells at 37°C are usually spindle shaped. It has been reported that when temperature is downshifted to 30°C, a cell cycle inhibitory protein named p27/KIP is over-expressed, which has a negatively regulates cyclin dependent kinases (17,18).

Hyperosmolality had negative effects on the rCHO cell density, which decreased from

4.3×10^6 cells/ml in the control to 2.7×10^6 cells/ml. The activity of the rhFVIII was estimated to be 5.2% at hyperosmotic conditions when compared to 11.6% in the control. Accumulation of intracellular osmolytes such as Na^+ and Cl^- ions cause instability of enzymes and RNA, and consequently decrease cell density and productivity (16).

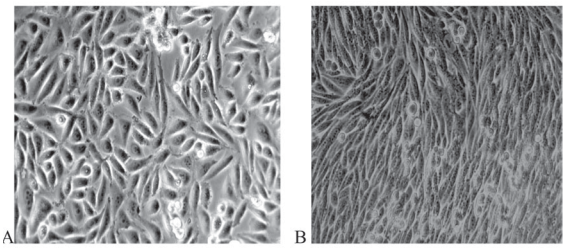

Fig. 7. The rCHO cell morphology at different temperatures; (A) 30°C and (B) 37°C

Effect of serum elimination on the rCHO density: Serum is normally enriched with precursors, growth factors, vitamins, andamino acids, which can remarkably increase mammalian cell proliferation. On the other hand, these ingredients cause problems in downstream processing of recombinant proteins. In addition, serum composition is variable resulting in a process with non-reproducibility. However, elimination of serum should be accompanied by adding appropriate substances, such as amino acids and vitamins. To eliminate serum from the culture medium, four amino acids as well as biotin were added and their effects on the rCHO cell viability were investigated. Figure 8 shows that approximately 80% of the rCHO cells were viable in presence of amino acids and biotin in serum-free medium, after 48 h of cultivation, whereas the rCHO cell viability was around 60% in the absence of serum and additives. So, these additives can be considered as suitable substitutes for serum in the rCHO cell culture medium. Since, biotin is a cofactor of a number of vital enzymes, e.g. pyruvate carboxylase and acetyl-CoA carboxylase involving gluconeogenesis and fatty acid biosynthesis; it can be considered as an effective parameter with regard to cell proliferation in serum-free media.

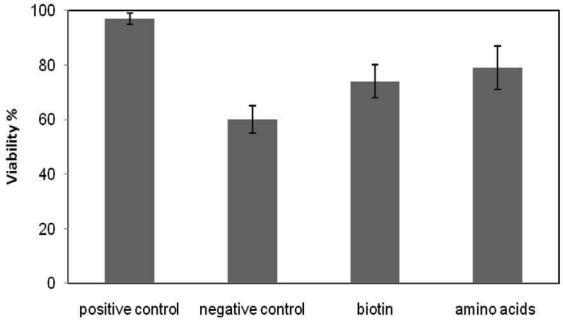

Fig. 8. The effect of amino acids and biotin as additives on the rCHO cell density in serum-free medium

Packed-bed bioreactor culture: Cell density in the packed-bed bioreactor reached 18×10^8 cells/l after 120 h of cultivation. The glucose concentration was depleted to 0.3 g/l at the end of cultivation. The time profile of glucose consumption indicated that the lag phase in the bioreactor was approximately 18 h (Figure 9). Ammonium and lactate were also measured daily. The results showed that the ammonium and lactate concentrations were at the inhibitory levels of 1.05 and 0.072 g/l at 120 h of cultivation, respectively (Figure 9).

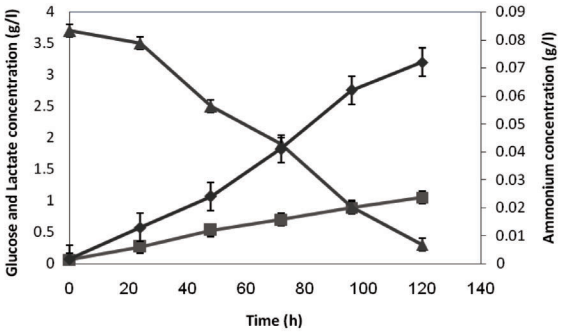

Fig. 9. Time profile in the packed-bed bioreactor culture, (▲) glucose consumption, (◆) ammonium formation, (■) lactate formation

Activity of rhFVIII reached approximately 7% after 120 h of cultivation when compared to that of the pooled plasma, while this amount was 0% and 1.2% at 48 h and 96 h of cultivation, respectively. These results demonstrated that there is a direct relationship between protein production and cell density. Although there is no report on rhFVIII production in packed-bed bioreactors in the literature, but they have been employed for production of some recombinant pharmaceuticals (4). Trombopoietin was produced by the rCHO cells attached to polyester microcarriers in a packed-bed bioreactor. The maximum cell density and productivity of 2×10^7cells/ml and 1.3-1.8 mg/l.d were obtained after 15 days, respectively (Cong et al. 2001). Because of the low stability of rhFVIII, the culture time period should be decreased. The results show that rhFVIII is biologically active during 120 h of cultivation when using enriched medium, in the packed bed bioreactor.

Conclusion

To our knowledge, no reports have been found in the literature on the physico-chemical culture conditions of recombinant CHO cells for improvement of rhFVIII production. The results of this study have demonstrated that medium supplemented with amino acids, precursors and glucose has a significant positive effect on the rCHO cell density and rhFVIII production, mainly because of the reduction of by-products formation. These data have provided useful information for the process development. Since rhFVIII is very sensitive to proteolytic degradation, culture time is an important factor in achieving an appropriate rhFVIII activity. Therefore, continuous packed-bed or perfusion cultures are suggested as suitable systems for production of rhFVIII in large-scale cultivation.

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References

1. Wurm, F.M. (2004). Production of recombinant therapeutics in cultivated mammalian cells. Nat Biotechnol, 22:1393-1398.
2. Oh, D.J., Choi, S.K. and Changt, H.N. (1994). High density continuous cultures of hybridoma cell in a depth filter prefusion system. Biotechnol Bioengin, 44:895-901.
3. Kim, DY., Lee, J.C, Chang, H.N. and OH, D.J. (2005) Effects of supplementation of various medium components on Chinese Hamster Ovary cell cultures producing recombinant antibody. Cytotechnology, 47:37–49.
4. Cong, C., Chang, Y., Deng, J., Xiao, C. and Su, Z. (2001). A novel scale-up method for mammalian cell culture in packed-bed bioreactor. Biotechnol Lett, 23:881-885.
5. Sanfeliu, A. and Stephanopoulos, G. (1999). Effect of glutamine limitation on the death of attached Chinese Hamster Ovary cells. Biotechnol Bioengin, 64:47-53.
6. Landauer, K., Durrschmid, M., Klug, H., Wiederkum, S., Bluml, G. and Doblhoff-Dier,O. (2002). Detachment factors for enhanced carrier to carrier transfer of CHO cell lines on macroporous microcarriers. Cytotechnology, 39:37-45.
7. Lu, S., Sun, X. and Zhang, Y. (2005). Insight into metabolism of CHO cells at low glucose concentration on the basis of the determination of intracellular metabolites. Process Biochem, 40:1917–1921.
8. Sun, X. and Zhang, Y.(2004). Glutamine cannot support recombinant CHO cell

growth and maintenance in the absence of glucose. Process Biochem, 39:717–720.

9. Fang, Z., Xiao-Ping, Y., Xiang-Ming, S. and Yuan-Xing, Z. (2006). Reduction of toxic effect of ammonia on metabolism of recombinant CHO-GS cell. Chin J Biotech, 22:94-100.

10. Hansen, H.A. and Emborg, C. (1994). Influence of ammonium on growth, metabolism, and productivity of a continuous suspension Chinese Hamster Ovary cell culture. Biotechnol Prog, 70:121-124.

11. Yang, M. and Butler, M. (2000). Effects of ammonia on CHO cell growth,erythropoietin production,and glycosylation. Biotechnol Bioengin, 68:370-380.

12. Chen, P. and Harcum, S.W. (2006). Effects of elevated ammonium on glycosylation gene expression in CHO cells. Metab Eng, 8:123-132.

13. Altamirano, C., Illanes, A., Becerra, S., Cairo, J.J. and Godia, F. (2006). Considerations on the lactate consumption by CHO cells in the presence of galactose. Biotechnol, 125:547–556.

14. Chen, P. and Harcum, S.W. (2005). Effects of amino acid additions on ammonium stressed CHO cells. Biotechnol, 117:277–286.

15. Takuma, S., Hirashima, C. and Piret, J.M. (2007). Dependence on glucose limitation of the pCO2 influences on CHO cell growth, metabolism and IgG production. Biotechnol Bioengin, 97:1479-1488.

16. Dezengotit, V.M., Kimur, R. and Miller, W.M. (1998). Effects of CO2 and osmolality on hybridoma cells: growth, metabolism and monoclonal antibody production. Cytotechnology, 28:213–227.

17. Fogolin, M.B., Wagner, R., Etcheverrigaray, M. and Kratje, R. (2004). Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. Biotechnol, 109:179–191.

18. Przybytkowski, E., Bates, J.T., Bates, D.A. and Mackillopp, W.J. (1986). Thermal Adaptation in CHO Cells at 40 C: The Influence of Growth Conditions and the Role of Heat Shock Proteins. Radiat Res, 107:317-331.

19. Fay, P.J. (2006). Factor VIII Structure and Function. International. J Hematol, 83:103-108.

20. Shima, M. (2006). Characterization of Factor VIII Inhibitors. Int J Hematol, 83:109-118.

21. Shi, Q., Wilcox, D.A., Fahs, S.A., Kroner, P.A. and Montgomery, R.R. (2003). Expression of human factor VIII under control of the platelet-specific aIIb promoter in megakaryocytic cell line as well as storage together with VWF. Mol Gen Metab, 79:25-33.

22. Pipe, S.W., Morris, J.A., Shah, J. and Kaufman, R.J. (1998). Differential Interaction of Coagulation Factor VIII and Factor V with Protein Chaperones Calnexin and Calreticulin. J Biol Chem, 273:8537-8544.

23. Chun, B.-H., Park, S.Y, Chung, N. and Bang, W.G. (2003). Enhanced production of recombinant B-domain deleted factor VIII from Chinese hamster ovary cells by propionic and butyric acids. Biotechnol Lett, 25:315–319.

24. Campos-da-Paz, M., Costa, C.S., Quilici, L.S., Simoes, I.D.C., Kyaw, C.M., Queiroz Maranhao, A. and Brigido, M.M. (2008). Production of Recombinant Human Factor VIII in Different Cell Lines and the Effect of Human XBP1 Co-Expression. Mol Biotechnol, 39:155-158.

25. Dorner, A.J, Bole, D.G. and Kaufman, R.J. (1987). The Relationship of N-linked Glycosylation and Heavy Chain-binding Protein Association with the Secretion of Glycoproteins. J Cell Biol, 105:2665-2674.

26. Amirizadeh, N., Zomorodipour, A., Deezagi, A., Pourfathollah, A.A., Khodabandeh,M., Rastegar Lari, G., Lenjannejadian, H., Ataie, F. and Salek, M. (2005). Expression of Biologically Active Recombinant B-Domain-Deleted Human Factor VIII in Mamalian Cells. J Sci IR Iran, 16:103-112.

27. Kurano. N., Leist, C., Messi, F., Kurano, S. and Fiechter, A. (1990). Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. Biotechnol, 15:113-128.

28. Dyring, C., Hansen, H.A. and Emborg. C. (1994). Observations on the influence of glutamine, asparagine and peptone on growth and t-PA production of Chinese hamster ovary (CHO) cells. Cytotechnology, 16:37-42.

29 . Dorner, A. and Wasley, C. (1990). Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. J Cell boil, 87:7429-7432.

30. Lenting, P.J., Neels, J.G.,Van, den Berg, B.M.M., Clijsters P.P.F.M., Meijerman, D.W.E., Pannekoek, H., Mourik, J.A.V., Mertens, K. and Zonneveld, A-J.V. (1999). The Light Chain of Factor VIII Comprises a Binding Site for Low Density Lipoprotein Receptor-related Protein. Biol Chem, 274:23734–23739.

31. Andersen, D.C. and Goochee, C.F. (1995). The effect of ammonia on the O-linked glycosylation of granulocyte-colony-stimulating factor produced by Chinese-hamster ovary cells. Biotechnol Bioeng, 47:96-105.

32. Wait, G.N., Wait, L.R. (2007). Molecular Biology for Engineering. Mcgraw-Hill Companies, Inc.

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Cloning and Expression of Codon Optimized Minor Capsid Protein (L2) of Human *Papilloma* Virus type 16 in *Pichia pastoris*

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Abstract

Cancer of uterine cervix is a significant public health burden across the world. Mortality of women from cervical cancer is reported to be highest in developing countries. Prophylactic vaccine made of theVirus like particles (VLPs) of Human papilloma virus (HPV), though expensive, is effective in preventing onset of homologous infection in vaccinated women. Preparation of cross protective vaccine containing lesser number of recombinant proteins derived from various genotypes of HPV would help reduce the vaccine cost. We report the expression of codon optimized HPV 16 L2 in *Pichia pastoris*. The expression of HPV L2 protein was verified using RT-PCR and immunoblotting. This manuscript describes initial effort in expressing full length minor capsid protein L2 of HPV16 aimed at developing a broad spectrum prophylactic vaccine for cervical cancer.

Key Words: HPV, Cervical Cancer, Minor capsid protein (L2), Cross Neutralizing Epitopes, Prophylaxis, Vaccine

Introduction

Cancer of the uterine cervix is the second most common cancer in women. Annual incidence of approximately 520,000 new cases and 274,000 deaths have been reported worldwide (1). India alone accounts for approximately 132,000 new cases and 73,000 deaths each year (1) and holds the dubious distinction of accounting for nearly a quarter of all cervical cancer death recorded globally (2). Presently nearly half of all cervical cancer incidences occur in the developing countries; and it is estimated that by the year 2025 the incidence rates in the developing world would increase to nearly 80% of the global rates (1).

Human Papillomaviruses (HPV) are highly epitheliotropic; they establish productive infections only within the stratified squamous epithelia of the skin or the anogenital tract of the humans (3). HPV genome consists of a circular double-stranded DNA of approximately 8kb and encodes for non-structural-proteins (the early genes) and structural proteins (the late genes) (4). Over 200 papillomavirus types have been described, of which around 100 infect humans and are therefore classified as HPV (4, 5 and 6). Nearly 35 different HPV types are found to be associated with cervical cancer in women and these are referred to as 'high-risk' HPV types. Persistent infection by high-risk HPV types is the single most common cause for the establishment of the cervical cancer (7).

The HPV major capsid protein L1 and minor capsid protein L2 are synthesized late in infection cycle, whose function is to encapsidate the closed circular double-stranded DNA (8).

Recombinant major capsid protein (L1) self assembles into a structure that mimics the native virus, both structurally and immunologically. Two recombinant vaccines have been commercialized that contain the HPV L1 based VLPs of HPV16 and 18, which cause 70% of all cervical cancer. One of the prophylactic vaccines also contains two additional HPV types that cause benign proliferation (warts) in the genital area of men and women. Clinical studies indicate that these vaccines protect vaccinees from the infection of cognate type HPVs. Given that the available vaccine has a limited spectrum, multivalent VLP vaccines would be required to improve prophylactic efficacy (9). Several other investigators have explored the possibility of using alternate expression platform such as plant (10, 11 and 12), Salmonella (13) vaccinia virus (14) etc. All of these are aimed at reducing the cost of vaccine production or ease of administration, thereby increasing the vaccine coverage in the population.

The HPV L2 is also referred to as minor capsid protein. It plays an important role in the infectivity of HPV (15, 16). The HPV L2 protein contributes to the binding of the virions to the host cell receptor(s), disrupt endosomal membranes and facilitate sub-cellular trafficking of incoming viral genome. HPV L2 also plays a crucial role in the invasion and injection of the viral genome into the host cell. Studies performed in animal PVs demonstrated generation of cross neutralizing antibodies after immunization with the amino terminal peptide of L2 protein (17). These antibodies protected the animals against challenge with cognate papilloma virus types (18, 19). Other investigators have demonstrated that L2 neutralizing epitopes are common among Papillomaviruses, which leads to the possibility of its use in cross protective HPV vaccines (20).

In this manuscript, we describe cloning, integration and expression of codon optimized HPV 16 L2 gene in *Pichia pastoris strain* GS115. The expression of HPV 16L2 was confirmed using RT-PCR and immunoblotting.

Materials and Methods

2.1 Pichia strain and expression vector : Methylotropic yeast *Pichia pastoris* strain GS115 and *Pichia* expression vector pPICZB were procured from Invitrogen, USA.

2.2 Yeast growth and expression media: Components for preparing *Pichia pastoris* growth and induction media were procured from Hi-Media Labs, India. All other chemicals and fine-chemicals were either sourced from Sigma Chemical Company, USA or Merck, India.

2.3 Codon optimization of HPV 16 L2 gene for expression in Pichia pastoris: DNA sequence coding for the minor capsid protein encoding gene (L2) of HPV 16 was codon optimized for expression in *Pichia pastoris.* Synthetic gene construct for this purpose was procured from GeneArt (Regensburg, Germany).

2.4 Cloning of the minor capsid protein gene of HPV 16 : The codon optimized L2 gene (1.5 kb) was PCR amplified from the synthetic construct using *Pfu* DNA polymerase (Qiagen, Germany). The PCR was set-up with the following oligonucleotide sequence and reaction conditions.

16L2 For: 5' TACCGAATTCATGAGAC ACAAGAGATCCGCT3'; and
16L2 Rev: 5'GCTGGAGCTCGAGCTATT AAGCAGCCAAGGA3'.

The PCR reaction mixture contained 100 ng of codon optimized HPV 16 L2 plasmid, 1× PCR reaction buffer (Qiagen, Germany), 200μM of each dNTPs, 50 pico moles each of forward and reverse gene specific primers, three units of *Pfu* DNA polymerase enzyme. The reaction volume was made to 50 μl using nuclease free water.

PCR was performed using the following temperature cycling conditions: initial denaturation of template was carried out at 94ºC for 5 minutes; 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 40 seconds and extension at 72ºC for 90 seconds were used for PCR amplification; the final extension was performed at 72ºC for 10 minutes. A control reaction using DNA extracted from untransformed *Pichia pastoris* cells were used as negative control. The PCR products were resolved on 1% (w/v) Agarose gel and stained using ethedium bromide.

The amplified HPV 16 L2 PCR product and *Pichia* expression vector (pPICZB) were digested using restriction enzymes *EcoR*I and *Xho*I (New England Bio-labs, USA). These restriction enzyme digested 16 L2 gene and the vector were resolved using agarose gel electrophoresis and purified using gel extraction kit (Qiagen, Germany). The insert and vector were ligated using T4 DNA ligase (Genei, India) by incubating at 16ºC for 60 minutes. The ligated product was transformed into Top10 *E. coli* competent cells and were selected for Zeocin (100µg/ml; Invitrogen, USA) resistance. The HPV 16L2 gene cloned into pPICZB was verified using restriction analysis and DNA sequencing.

2.5 Integration of the HPV 16L2 expression cassette into Pichia pastoris Chromosomal DNA: The pPICZB plasmid clone containing HPV L2 gene insert was used to transform *Pichia pastoris* GS115 (Invitrogen, USA) using *Pichia* EasyComp™ Kit (Invitrogen, USA). Transformants harbouring HPV L2 gene was selected on Yeast-extract Peptone Dextrose (YPD) plates containing 200µg/ml Zeocin (Invitrogen, USA). Integration of HPV L2 gene into *Pichia pastoris* was PCR verified using the promoter specific primers (Alcohol Oxidase primers; AOX) and the 16L2 gene specific primers. AOX primer sequences are mentioned below and 16L2 gene specific sequences were mentioned earlier in Section 2.4.

AOX For: 5'GACTGGTTCCAA TTGA CAAGAC 3';

AOX Rev: 5'GCAAATGGCATTC TGAC ATCC3';

PCR reaction was set-up and using 50 ng of codon optimized HPV 16 L2 plasmid, 1× PCR reaction buffer (Bangalore Genie, India), 200µM of each dNTPs, 50 picomoles each of AOX forward and reverse primers, three units of *Taq* DNA polymerase enzyme. The reaction volume was made to 50 µl using nuclease free water.

Thermal cycling condition used for PCR amplification were same as mentioned earlier in the methods (section 2.4)

2.6 Expression of HPV Minor Capsid protein (L2) in Pichia pastoris: *Pichia pastoris* transformants containing HPV 16L2 gene was grown overnight in a shake-flask with YPD medium supplemented with 100µg/ml Zeocin and the cells were transferred into freshly prepared Buffered Minimal Glycerol (BMGH) medium containing 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 0.02% (w/v) biotin, 1% (v/v) glycerol, 100µg/ml Zeocin and 0.004% (w/v) L-Histidine. After an overnight growth recombinant *Pichia pastoris* cells were induced for expression using Buffered Minimal Methanol (BMMH) medium containing 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 0.02% (w/v) biotin, 0.5% (v/v) methanol and 100µg/ml Zeocin supplemented with 0.004% (w/v) L-Histidine. To induce expression of the recombinant HPV L2 from *Pichia pastoris*, 0.5% (v/v) of methanol was added every 24 hours to the culture until 72 hours of growth. The cells were harvested after 96 hours of growth and stored at -80°C until further use.

2.7 Reverse Transcriptase PCR: Methanol induced recombinant *Pichia pastoris* clones expressing HPV16 L2 protein was sampled every 24 hours until 72 hours. Total RNA was extracted from the samples using RNAEasy™ kit (Qiagen, Germany). RT-PCR used to detect the presence of HPV L2 transcripts in the induced *Pichia pastoris* cells using One-Step RT-PCR kit (Qiagen, Germany) and HPV16 L2 gene specific primers. RNA samples from 'untransformed' *Pichia pastoris* strain GS115 was used as negative control. The RT-PCR was performed using the following temperature cycle: cDNA synthesis was performed at 50ºC for 50 minutes; Reverse Transcriptase inactivation and *Taq* polymerase activation was performed by heating the reaction at 95ºC for 10 minutes; 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 40 seconds and extension at 72ºC for 40 seconds were used for PCR amplification; the final extension was performed at 72ºC for 10 minutes. A control PCR reaction using identical RNA samples isolated from induced *Pichia pastoris* cells was also set-up to rule out the possibility of genomic DNA contamination of the RNA preparation.

2.8 Immuno Blotting: HPV 16 L2 proteins from *Pichia pastoris* crude cell lysate was resolved on 12% SDS-PAGE gel were transferred to PVDF membrane (GE Healthcare, USA) using Bio-Rad semi-dry protein transfer apparatus. Unused active surface on the PVDF membrane was blocked using PBS containing 2% (w/v) skim milk, 0.1% (v/v) Tween-20. The blot was probed using 1:200 dilution of HPV16 L2 mouse monoclonal antibody produced against amino acids 40-150 of HPV16 L2 (2JGmab#5; Santa Cruz, USA). Goat anti-mouse HRP monoclonal antibody (1:5000 dilution; Sigma, USA) was used as detection antibody and the bands were stained using 3,3'-Diaminobenzidine tetrahydrochloride (DAB) as chromogenic substrate.

Results and Discussion

In the present study we used *Pichia pastoris* as a heterologous expression system for expressing recombinant HPV16 L2 protein. Yeasts with the heterologous gene integrated into the chromosome provide an alternate platform for recombinant gene expression. Integration of genes for expression under the Alcohol oxidase (AOX) promoter is known to induce high level of expression in *Pichia pastoris* (21). The necessity of codon optimization for HPV capsid proteins expression in heterologous systems is also well documented (21). HPV 16 L2 gene (Genbank accession number: U34164) was codon optimized and synthesized *in-vitro*.

Codon optimization was performed to improve the transcription efficiency and transcript stability. This was achieved by improving the overall GC content of the gene, distribution of preferred codon usage along the entire length of the coding sequence (codon adaptation index and codon frequency distribution) and removing negative elements that may for unfavourable secondary structures on mRNA.

The codon adaptation index is graded on the scale of 0.0 to 1.0, with 1.0 indicating the ideal codon usage in a given host. Codon adaptation index on the native HPV 16 L2 was 0.67, while the optimized HPV L2 gene sequence had a codon adaptation index of 0.90 (Fig. 1), indicating that the optimized gene sequence could express well in *Pichia pastoris*. Percentage of codon having a frequency distribution of 91-100 in the native L2 gene was 39% which was significantly improved to 72% in the optimized gene sequence (Fig. 1). The overall GC content which is a measure of transcriptional and translational efficiency was improved from 37.63% to 45.7% upon codon optimization. There were 11 negative CIS elements in the native L2 gene sequence which were reduced to

Vol. 5 (3) 1309-1317 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

5 after codon optimization (Fig. 1). Taken together the codon optimized gene sequence of HPV L2 was favourable for expression in *Pichia pastoris.*

3.1 Cloning and Integration of HPV16L2 gene into Pichia pastoris: Human papilloma virus 16 L2 gene codon optimized for expression in *Pichia pastoris* was cloned into expression vector pPICZB (Fig. 2). Cloning of the minor capsid protein 16 L2 gene into pPICZB was verified by restriction enzyme analysis using *EcoRI* and *XhoI*. A band of approximately 1.5 kb size was observed on agarose gel indicating the presence of L2 gene (Fig. 3A). The clone was confirmed by restriction analysis using *XbaI* which is located in 16L2 gene sequence as well as pPICZB vector backbone. The restriction digested plasmid revealed a band of approximately 800bp on agarose gel confirming the presence of HPV 16 L2 gene (Fig. 3B). Further, DNA sequencing of the PCR amplicons using Alcohol Oxidase (AOX) primers confirmed the identity of the integrated gene upon BLAST search (data not shown).

HPV 16 L2 gene was integrated into *Pichia pastoris* genome by transforming the chemically competent *Pichia pastoris* cells using the recombinant plasmids. The integration was PCR verified using primers that either bind at AOX promoter site or HPV L2 gene. The PCR products showed the bands size of approximately 1.8 kb and 1.5kb with AOX primers and HPV 16 L2 gene specific primers respectively (Fig. 4A & 4B).

3.2 Reverse Transcriptase PCR: Recombinant *Pichia pastoris* clones expressing HPV16 L2 were induced for protein expression using 0.5% (v/v) methanol. An aliquot of cells were withdrawn after 24, 48 and 72 hours of induction. Total RNA were extracted from these induced *Pichia pastoris*, produced an amplification

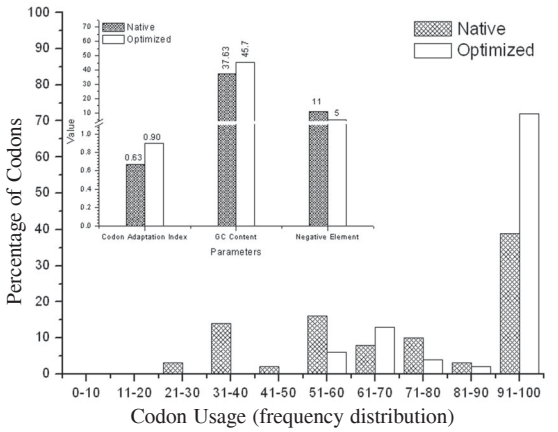

Fig. 1. Comparative Analysis of various parameters employed for codon optimization. The bar graph shows frequency distribution of codon usage. The native HPV 16L2 had 39% of codons that were most preferred in *Pichia pastoris*, while the optimized gene sequence had 72% codons that were most preferred in *Pichia pastoris*. The graph in the inset shows Codon adaptation index, Overall GC content and CIS negative elements present in the native and codon optimized HPV 16 L2 DNA sequences. All the parameters show a significant improvement over the native L2 DNA sequence.

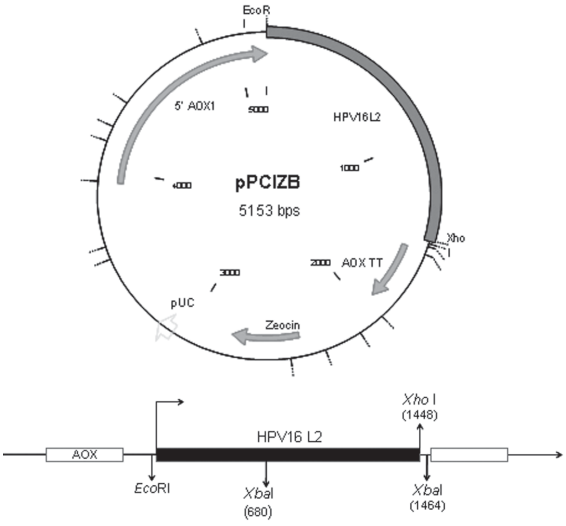

Fig. 2. Schematic diagram of HPV16 L2 constructs. The filled box represent HPV16 L2 gene. Unique restriction enzyme sites present within HPV 16L2 are marked with nucleotide position in parenthesis. HPV16 L2 gene was cloned downstream of AOX promoter between *Eco*RI and *Xho*I enzyme sites.

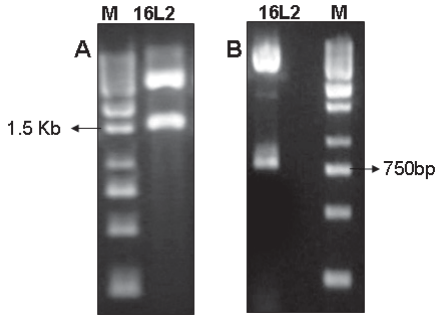

Fig. 3. Restriction enzyme analysis of HPV 16L2 cloned in pPICZB. The DNA fragments were resolved on 1% Agarose gel and visualized by Ethedium Bromide staining. A. HPV 16 L2 clone digested with *EcoRI & XhoI* Lane M-Gene ruler, 1Kbp ladder (Fermentas), Lane '16L2' indicates *E. coli* clone with the released 16 L2 gene of approximately 1500 bp in size.
B. HPV 16 L2 clone digested with restriction enzyme *XbaI*, 1Kbp ladder (Fermentas), Lane '16L2' indicates clone showing the released 16 L2 gene of approximately 800 bp in size.

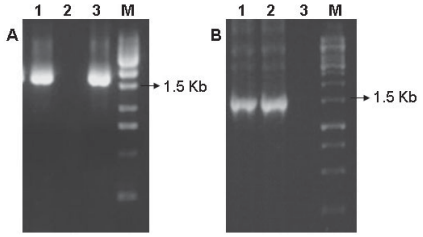

Fig. 4. A. PCR amplification of HPV 16L2 clone using AOX promoter specific primers. The PCR products were resolved on 1% agarose gel. Lane 1 16 L2 *Pichia* clone, Lane 2 negative control, Lane 3 positive control (pPICZB 16L2 plasmid used for transforming the *Pichia pastoris* genome) and Lane M 1 Kbp ladder (Fermentas). The amplified bands corresponds approximately 1.8 Kbp size B. PCR amplification HPV 16 L2 region using Gene specific primers .The PCR products were resolved on 1% agarose gel. Lane 1 positive control (pPICZB 16L2 plasmid used for transforming the *Pichia pastoris* genome), Lane M 1Kbp ladder (Fermentas), Lane 2 HPV 16L2 *Pichia* clone and Lane 3 negative control.

product of approximately 1.5kb as seen on agarose gel. RT-PCR analysis was performed for recombinant HPV16 L2 *Pichia* clone and the assay confirmed that the gene is transcribed on induction with methanol (Fig. 5). The HPV L2 gene amplification from possible DNA contamination in extracted RNA was ruled out in a PCR reaction that employed identical RNA samples isolated from induced *Pichia pastoris* cells.

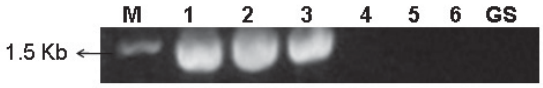

Fig. 5. RT PCR analysis of methanol induced HPV 16 L2 *Pichia pastoris* clone: RT-PCR of methanol induced *Pichia pastoris* clone expressing HPV 16 minor capsid protein after 24 hr (lane 1), 48 hr (lane 2) & 72 hours (lane 3) post induction.; PCR (without reverse transcription reaction) of RNA samples obtained from induced *Pichia pastoris* clones expressing HPV 16 major capsid protein after 24 hr (lane 4), 48hr (lane 5) and 72 hours (lane 6). GS: Naïve *Pichia pastoris* strain GS115. Lane M 1Kbp ladder (Fermentas). The amplified 16 L2 product corresponds to the approximately 1.5kb in size.

3.3 Immuno Blotting : The cell lysates of recombinant *Pichia pastoris* were analyzed in Western blot using HPV L2 peptide specific mouse monoclonal antibody, which showed the staining of band size of approximately 55KDa. This experiment proved beyond a reasonable doubt, the expression of HPV16 L2 in *Pichia pastoris*. The mouse monoclonal antibody did not react with naïve *Pichia* cell lysate used as negative control (Fig.6).

In the Western blot apart from the 55KDa band, which is the expected size of L2 a higher molecular weight protein was also observed. There are reports indicating the frequent association of Hsp 70 family proteins with HPV L2. The Hsp70 proteins are thought to act like chaperon that help protein fold or unfold (23). Some investigators observed that apart from HSPs the sumoylation proteins are also tightly associated with HPV L2 (24). The higher size protein band corresponding to HPV L2 observed in this study might be a consequence of the association of any of these chaperones. Further

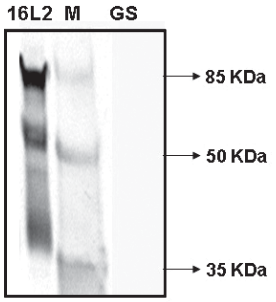

Fig. 6. Western blot analysis demonstrating specific reactivity of *Pichia pastoris* expressed HPV 16 L2 protein with anti-HPV 16L2 peptide (40-150 amino acids) monoclonal antibody showing protein band at approximately 55 KDa. Lane marked "GS" was loaded with non-recombinant *Pichia pastoris* strain GS115 cell lysate.

investigations are required to ascertain these observations in *Pichia pastoris*.

Conclusion

The available HPV L1 Virus like particle based vaccines provide type specific protection against the HPV genotypes associated with the cervical cancer. Although there are reports of the VLP based vaccines to provide cross protection to the HPV types that are phylogenetically similar to the vaccine types, these are limited both in spectrum and levels of protection. Development of a vaccine offering cross protection to a wide variety of HPV types that cause cervical cancer remains a challenging task. Some investigators have reported that cross-reactive antigen derived from the minor capsid protein L2 may offer a possible alternative to multivalent L1 VLP vaccines for protection against infection with high-risk HPV types (25). A multimeric L2 peptides derived from different HPV types is under clinical evaluation (26). This work is a preliminary step towards exploring the utility of full length HPV L2 as prophylactic vaccine for cervical cancer.

References

1. Human Papillomavirus and Related Cancers in India. (2010). WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre) Summary Report: 4-5

2. Parkin, D.M., Louie, K.S. and Clifford, G. (2008). Burden and trends of type-specific human papillomavirus infections and related diseases in the Asia Pacific region. Vaccine. S26: M1-16.

3. Doorbar, J. (2005). The papillomavirus life cycle. J. Clin. Virol., 32 (Suppl 1):S7–S15.

4. IARC monographs on the evaluation of carcinogenic risks to humans. Human papillomaviruses. (2007). IARC: Lyon: IARC Press. 90: 179-327.

5. Burd, E.M.(2003). Human papillomavirus and cervical cancer. Clin.Microbiol. Rev., 16: 1-17

6. De Villier, E.M., Fauquet, C., Broker, T.R., Bernard, H.U. and zur Hausen, H.(2004). Classification of papillomaviruses. Virology. 324: 17–27

7. Harper, D.M., Franco, E.L., Wheeler, C., Ferris, D.G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P., De Carvalho, N.S., Roteli-Martins, C.M., Teixeira, J., Blatter, M.M., Korn, A.P., Quint, W. and Dubin, G. (2004). Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet. 364 (9447):1757-65.

8. Hagensee, M.E., Yaegashi, N. and Galloway, D.A. (1993). Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by

coexpression of the L1 and L2 capsid proteins. J. Virol., 67: 315–322

9. Villa LL. (2010). HPV prophylactic vaccination: The first years and what to expect from now. Cancer letters.

10. Fernandez- San Millan A, Ortogosa SM, Herras- Stubbs S Corral- Martinez P, Sequi-Simmaro JM, Gactan J, Coursaget P, Veramendi J. (2008). Human papillomavirus L1 protein expressed in tobacco chloroplasts self- assembles into virus-like particles that are highly immunogenic. Plant Biotechnol J. 6(5): 427-41

11. Bimelt S, Sonnewald U, Galmbachar P, Willmitzer L, Muller M . (2003) Production of human papillomaviruses type 16 virus-like particles in transgenic plants. J Virol. 77: 9211-9220.

12. Varsani A, Williamson AL, Rose RC, Jaffer M, Rybicki EP. (2003). Expression of human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum cv. Xanthi*. Arch Virol. 148: 1771-1786.

13. Nardelli-Haefliger D, Roden RB, Benyacoub J, Sahli R, Kraehenbuhl JP, Schiller JT, Lachat P, Potts A, De Grandi P. (1997). Human papillomavirus type 16 virus-like particles expressed in attenuated *Salmonella typhimurium* elicit mucosal and systemic neutralizing antibodies in mice. Infect Immun. 65(8):3328-36.

14. Zhou J, Sun XY, Stenzel DJ, Frazer IH. (1991). Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology. 185(1):251-7

15. Roberts, J.N., Buck, C.B., Thompson, C.D., Kines, R. and Bernardo, M. (2007). Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. Nat. Med. 13(7): 857–861.

16. Holmgren, S.C., Patterson, N.A., Ozbun, M.A. and Lambert, P.F. (2005). The minor capsid protein L2 contributes to two steps in the human papillomavirus type 31 life cycle. J. Virol. 79(7): 3938–3948

17. Gambhira, R., S. Jagu, B. Karanam, P. E. Gravitt, T. D. Culp, N. D. Christensen. and R. B. S. Roden. (2007). Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. J. Virol. 81:11585–11592

18. Embers, M. E., L. R. Budgeon, M. Pickel. and N. D. Christensen. (2002). Protective immunity to rabbit oral and cutaneous papillomaviruses by immunization with short peptides of L2, the minor capsid protein. J. Virol. 76:9798–9805

19. Gaukroger, J. M., L. M. Chandrachud, B. W. O'Neil, G. J. Grindlay, G. Knowles. and M. S. Campo.(1996). Vaccination of cattle with bovine papillomavirus type 4 L2 elicits the production of virus-neutralizing antibodies. J. Gen. Virol. 77:1577–1583

20. Kawana, K., Yasugi, T., Kanda, T., Kino, N., Oda, K., Okada, S., Kawana, Y., Nei, T., Takada, T., Toyoshima, S., Tsuchiya, A., Kondo, K., Yoshikawa, H., Tsutsumi, O. and Taketani, Y. (2003). Safety and immunogenicity of a peptide containing the cross-neutralization epitope of HPV16 L2 administered nasally in healthy volunteers. Vaccine. 21: 4256–4260

21. James, C.C., Joseph, G. J., Hugh, A.G., Loren, D.S., William, M.H., Kathrin, U. J., Robert, W. H., Charlotte, I.P., Robert, S. L., Paul M. K. and E Dale, L. (1999). Purification of Virus-like Particles of Recombinant Human papillomavirus Type 11 Major Capsid Protein L1fr'om *Saccharomyces cerevisiae*. Prot. Expr. Pur. 17: 477–484

22. Hindmarsh, P.L. and Laimins, L.A. (2007). Mechanisms regulating expression of the HPV 31L1 and L2 capsid proteins and pseudovirion entry. Virol. J., 4:19

23. Reading, D.S., Hallberg, R.L. and Myers, A.M. (1989). Characterization of the yeast HSP60 gene coding for a mitochondrial assembly factor. Nature, 337(6208): 655-659

24. Martina, B.M., Nina, M., Mia L., Lawrence B. and Helena, S.G. (2010). Modification of Human Papillomavirus Minor Capsid Protein L2 by Sumoylation. J. Virol., 84: 11585-11589

25. Roden, R. and T. C. Wu. (2006). How will HPV vaccines affect cervical cancer? Nat. Rev. Cancer. 6: 753–763

26. B Karanam, S Jagu, Huh WK and Roden R (2009). Developing vaccines against minor capsid antigen L2 to prevent papillomavirus infection. Immunol Cell Biol., 87(4): 287–299.

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Vol. 5 (3) 1318 -1324 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Docking and Molecular Modelling of the Target - Penicillin Binding Protein -1A of *Haemophilus influenzae*

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Abstract

Penicillin binding protein-1A (PBP-1A; IPR011816) plays a pivotal role in the biogenesis of cell-wall and biosynthesis of peptidoglycan in bacteria. It is considered to be a novel target for pneumonia. The present study is to deduce the structure of the PBP-1A using ICM Molsoft and to validate the same with Ramachandran plot. These bioinformatics tools have been used to identify a lead molecule against PBP-1A of *Haemophilus influenza*. The proposed model structure is further explored for *in silico* docking studies with suitable inhibitors. The docking scores indicated that the ampicillin could be a better inhibitor among the seven antibiotics chosen in the present investigation.

Key words: *Haemophilus influenzae* strain 86-028NP, PBP protein, ICM Molsoft, Argus lab 4.0.1, Gold.

Introduction

Pneumonia is an inflammation of lungs, usually caused by a pathogen. Three common causative groups of pathogens that cause pneumonia are bacteria, viruses and fungi (1). The people at risk are older than 65 or younger than 2 years of age, or those with impaired immunity. Some of the gram-negative bacteria that cause pneumonia include *Haemophilus influenzae* (HI), *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Moraxella catarrhalis*.

HI is a non-motile gram-negative bacterium belonging to the family *Pasteurellaceae*. Pneumonia caused by HI seems to occur exclusively among humans. HI was first isolated by Pfeiffer during the influenza pandemic of 1890. This pathogen is present in the nasopharynx of approximately 75 percent of healthy children and adults. It is rarely encountered in the oral cavity and has not been detected in any other animal species (2). HI was the first pathogenic organism to have its entire genome sequenced during the year 1995 (3).

Bacterial cell wall and PBPs are the targets for drugs of selective toxicity because the related metabolic pathways and enzymes are unique to prokaryotes (4). Whereas, mitogen-activated protein kinase and trypanothione reductase are considered as targets in a eukaryotic protozoan parasite viz., *Leishmania infantum* (5, 6). However, in bacterial system, PBPs have been shown to catalyse a variety of reactions involved in the process of synthesizing cross-linked peptidoglycan from lipid intermediates and mediate the removal of D-alanine from the precursor of peptidoglycan (7). PBP-1A in HI strain 86-028np is coded by mrcA gene. This possesses two domains *viz.,* penicillin-insensitive transglycosylase EC=2.4.2.- and penicillin-

Vol. 5 (3) 1318 -1324 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

sensitive transpeptidase EC=3.4.-.- Its location is in the inner cell membrane as type II membrane protein and plays a key role in the biogenesis of cell wall and peptidoglycan (4). The present article aims at the screening of a few chosen antibiotics against PBP-1A with the intention of identifying a lead molecule.

Materials and Methods

The amino acid sequence of PBP-1A of HI strain 86-028NP was extracted from SWISS Prot database with ID: Q4QNA5. Later, submitted to NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST), searched against protein data bank (http://www.rcsb.org/pdb/), extracted suitable templates for the query sequence and found one of the best templates *viz*., peptidoglycan glycosyltransferase (PDB ID:2OQOa) with 52 % identities (8). The length of PBP-1A was found to be 864 amino acids and the source of the template is *Aquifex aeolicus* (http://www.rcsb.org/pdb/). The primary properties of protein sequence was analysed by using Bioedit and the protein secondary structure prediction was carried out using SOPMA (Self Optimized Prediction Method). Both the sequences were aligned using ICM Molsoft and the same was employed in the construction of three dimensional structure of PBP-1A. The alignment of the sequence to be modeled was done with known related structure in PDB format. ICM Molsoft further generated a model structure by minimizing the side chains. To correct the geometric inaccuracies the theoretical model was subjected to Procheck. The program, Procheck concentrates on the parameters such as bond length, bond angle, main chain and side chains parameters, residue properties, rms distance from planarity and distorted geometry plots. The Protein ligand interaction was performed using Argus lab engine with flexible docking (9). Docking analysis was carried out using on-line GOLD programme.

Results

The *in silico* tools as indicated in the previous section, *viz*., homology modelling, active site analysis and docking were employed in the present study for screening the chosen drugs to bind to the active site of PBP-1A.

The homology modelling of PBP-1A from HI was obtained from the protein data bank with ICM Molsoft software. The final model was evaluated using Procheck. Further validation analysis employing Ramachandran plot, revealed that the PBP-1A model showed 92.0% of residues lie in the most favoured regions and the remaining 6.3% in the additional allowed regions. Thus, 98.3% residues are in the allowed portions of the plot. Hence, PBP-1A is a potential target to exploit for drug designing, energy minimization and molecular dynamic simulations by means of refining loops and rotomers, checking bonds and adding hydrogen atoms. The refined model structure thus obtained after energy minimization and molecular

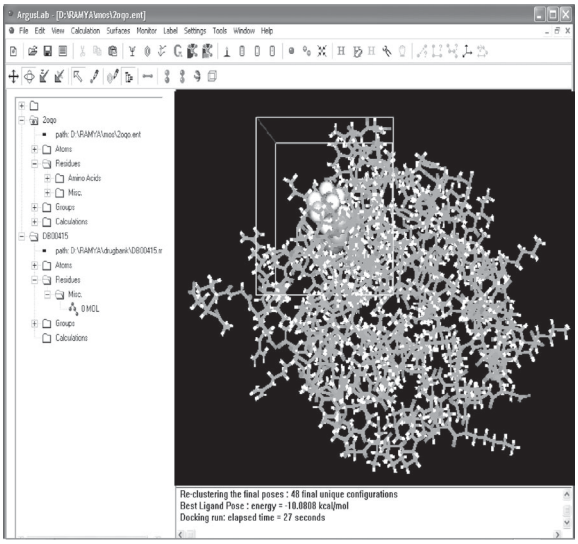

Fig. 1. Docking in Argus Lab. Docking studies with Argus lab engine showed that the active site residue is at 159 LEU in PBP-1A. This made a strong hydrogen bond interaction with ampicillin and yielded least energy -10.2333.

dynamic simulations was subjected for docking studies.

Docking studies with Argus lab engine gave an insight into the binding modes of the various inhibitors. Out of the seven drugs that were selected in the present study for screening, it was found that the active site residue *viz*., 159 LEU of PBP-1A forms a strong hydrogen bond interaction with ampicillin and yielded least energy -10.2333 (Table-1; Fig.1). GOLD program uses a genetic algorithm to explore the full range of ligand conformational and the rotational flexibilities of selected receptor hydrogen atoms. The mechanism for ligand placement is based on fitting points. The GOLD score fitness was found to be -16.596 (Fig.2). Amino acid primary structure analysis was done

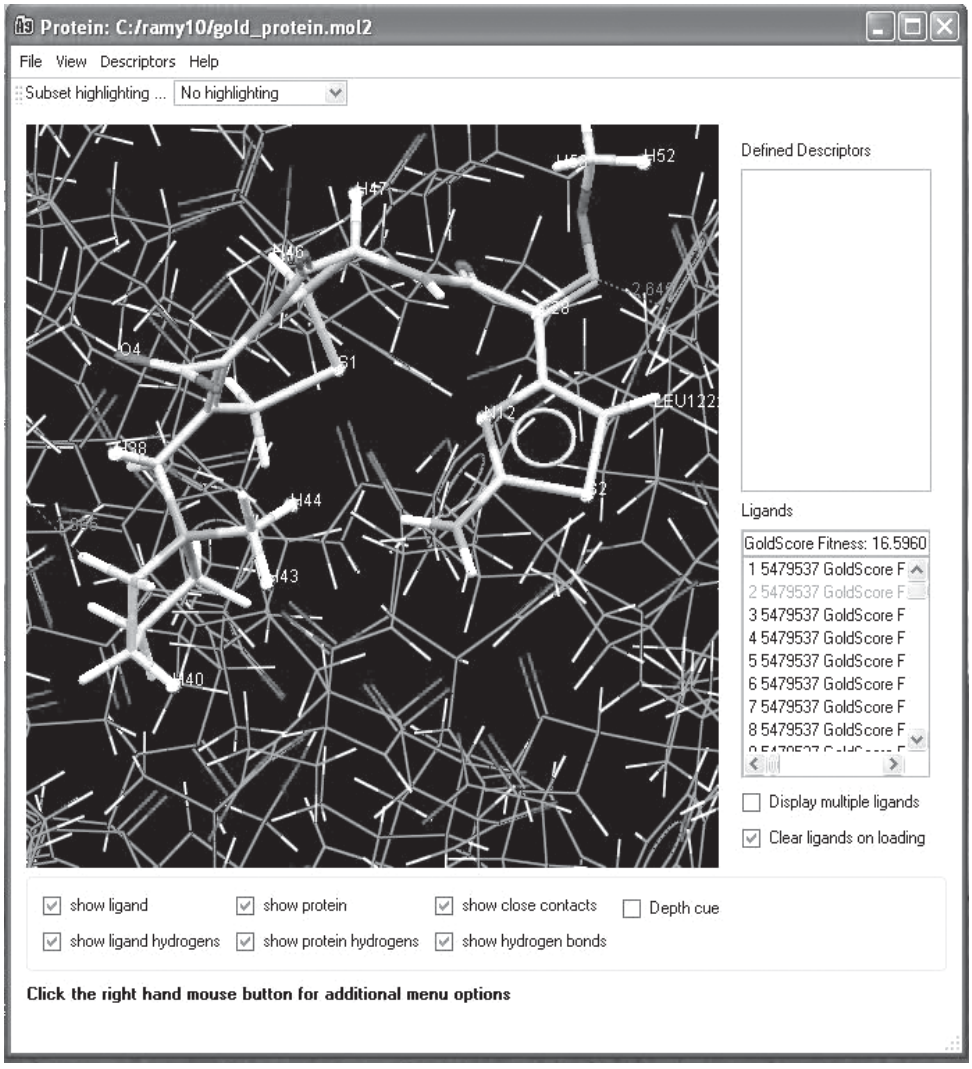

Fig. 2. Protein-Ligand interactions in GOLD**.** The mechanism for ligand placement is based on fitting points. The GOLD score fitness was found to be -16.569.

Vol. 5 (3) 1318 -1324 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

by using Bioedit (Fig.3). Pre-ADME properties envisaged that among the chosen ligand molecules, ampicillin fulfilled the requirements for the drug solubility, distribution and elimination (Table- 2).

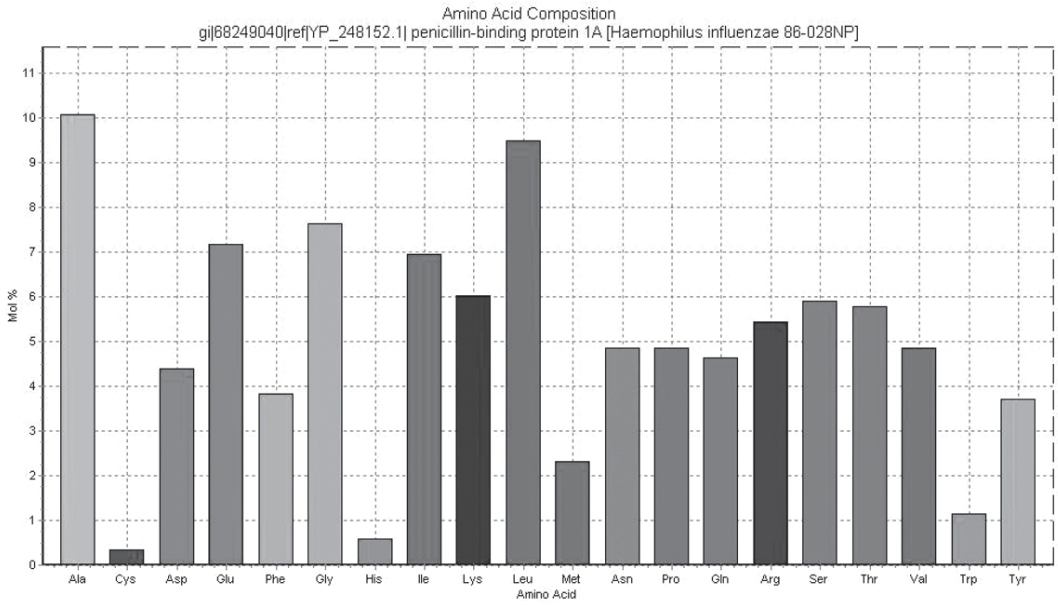

Fig. 3. Amino Acid Composition by Bioedit**.** Protein: gi|68249040|ref|YP_248152.1| penicillin-binding protein 1A [Haemophilus influenzae 86-028NP]. Length = 864 amino acids. Molecular Weight = 95630.89 Daltons

Discussion

The proteomic analysis of PBP-1A revealed its primary properties and secondary structural information and they are essential to understand its structure, function and nature of interaction. The stereochemistry evaluation of predicted 3D structure of the drug target suggested that the proposed model is of a good quality. The most common goal of protein-drug (small molecule) docking relates to drug design (5, 10). Moreover, the interaction between the target and the ligand proposed in this study (Fig.2) is useful for understanding the potential mechanism of enzyme and the substrate binding (11). Hydrogen bonds play important role for the structure and function of biological molecules, especially for the enzyme catalysis (12). In the present study, it was found that the active site residue of PBP-1A *viz.*, LEU at the position 159 yielded least energy viz., -10.233 (Table-1), and formed a strong hydrogen bond interaction with ampicillin upon comparison with the remaining six ligands. GOLD, binding site–dependent software, showed energy minimization and re-ranking of the top N poses. The GOLD score fitness was found to be -16.5690.

PBPs have been shown to catalyse a number of reactions involved in the process of synthesising cross-linked peptidoglycan from lipid intermediates and mediate the removal of D-alanine from the precursor of peptidoglycan (13). PBPs are reported to have a penicillin-insensitive transglycosylase N-terminal domain involved in the formation of linear glycan strands and a penicillin-sensitive transpeptidase C-terminal domain involved in cross-linking of the peptide subunits and the serine at the active site

Table 1. Docking score obtained through GOLD programme

Aminoacid	Residue No	*Ampicillin*	*Meropenem*	*Calvulanic acid*	*Ceftriaxone*	*Cefepime*	*Amoxicillin*	*Cefotaxime*
LEU	159	-10.2333	-7.3479	-5.5314	-7.6496	-6.4803	-9.3322	-7.4691
ASN	162	-8.8721	-7.9781	-5.4862	-7.5311	-7.7525	-9.1216	-6.8802
LYS	163	-9.55741	-7.3903	-5.5597	-7.7845	-7.4571	-8.6496	
TYR	171	-8.93631		-5.4171	-6.8628		-8.7681	
PHE	126	-9.78251	-8.0307	-5.5429	-7.4848	-6.8116	-8.9479	
PHE	127	-8.8829	-7.9319	-5.1586	-6.7787	-6.9444	-9.405	-8.0038
LEU	128	-8.6724	-7.5348	-5.3248	-7.1164	-6.0453	-7.8047	-7.681
THR	129	-8.2623	-7.3276	-5.3279	-7.4413		-7.3466	
LYS	132	-7.0311	-6.6989	-5.2153	-6.161	-6.411	-7.1245	
ARG	136	-9.0483	-7.0893	-5.2048	-6.3208	-6.2259	-8.4738	
GLU	140	-9.6544	-7.9182	-5.4616	-6.7677	-7.3372	-7.4998	
GLN	61	-8.3597	-7.4121	-5.4106	-7.6063	-7.0948	-8.3251	
ARG	62	-9.3446	-7.1034	-5.4239	-7.3805	-7.1783	-8.9781	
ARG	63	-7.8562	-6.2306	-5.0896	-6.3539	-6.0002	-6.702	
ILE	64	-7.2376	-6.7924	-5.0286	-7.0368	-5.9156	-7.1915	-6.0871
LEU	122	-9.9338	-7.1636	-5.2466	-6.8293	-7.7662	-8.2897	
ARG	124	-9.1766	-7.3872	-5.6417	-7.3239	-7.849	-9.3103	-7.3105
ASN	125	-9.3158	-7.7364	-5.7181	-7.3453	-7.9354	-8.9336	

Table 2. Pre-ADME properties derived through Accelrys discovery studio 2.1.

Ligand Molecule	Absorption level	solubility	Solubility level	Hepato-toxicity	CYP 2D6	CYP 2D6 Probability	Ppb level	A log P98	PSA-2D
Ampicillin	0	-2.502	3	0	0	0.495	0	0.182	115.42
Meropenem	1	-0.929	4	0	0	0.386	0	-0.932	113.04
Ceftriaxone	3	-4.547	2	1	0	0.267	0	-0.576	209.02
Cefepime	2	-2.194	3	0	0	0.396	0	-1.125	146.93
Amoxicillin	1	-2.523	3	0	0	0.445	0	-0.06	136.23
Cefotaxime	3	-3.124	3	1	0	0.425	0	-0.53	173.16

is conserved in all members of the PBP family (14, 15).

Both bacterial and protozoan parasites are wide spread around the world and more so in the tropical region causing an impediment in the health of humans and livestock. In the recent years, there is an upsurge in the biotechnological and *in silico* approaches to identify the possible pathogen-directed targets (5, 6). Interestingly, the biochemical mechanisms such as cell membrane receptors, metabolic pathways, cellular protein synthetic machinery and growth and

Vol. 5 (3) 1318 -1324 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

differentiation of cellular systems of parasites which are nonetheless unique for their survival and hence they are considered as avenues for drug/ligand targets. Furthermore, the software tools for homology modelling, simulations and structure-based virtual screening and on-line databases are in turn enhancing the rapid developments in building the repertoire of the receptor and ligand libraries. Therefore, in the present study, the chosen inhibitors *viz.*, ampicillin, amoxicillin, ceftriaxone, cefotaxime, cefepime, meropenem, and calvulanic acid were made to bind in the active site of PBP-1A. Of which, ampicillin has a good docking score (Table-1) and thus, ampicillin is considered as the best inhibitor for PBP-1A of HI (Francis et al., 2003). The Pre-ADME properties of ampicillin (Table-2) are coinciding with the results of Sarah et al., (16).

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References

1. Jeffery Errington, Richard, A. Daniel and Dirk-Jan Scheffers. (2003). Cytokinesis in Bacteria. Microbiol Mol Biol Rev.67(1); 52-65.

2. Jothi, R., Przytycka, T.M. and Aravind L. (2007). Discovering functional linkages and uncharacterized cellular pathways using phylogenetic profile comparisons: a comprehensive assessment. BMC Bioinformatics, 8:173.

3. Robert D. Fleischmann, Mark D. Adams, Owen White and Rebecca A. Clayton. (1995). Whole-Genome Random Sequencing and Assembly of *Haemophilus influenzae Rd*. Science. 269: 496-512.

4. Chambers, H. F. (1999) Penicillin-binding protein-mediated resistance in *pneumococci* and *staphylococci*. The Journal of Infectious Diseases. 179: S353-S359.

5. Venkatesan, S. K., Shukla, A. K. and Dubey, V. K. (2010). Molecular docking studies of selected tricyclic and quinine derivatives on trypanothione reductase of *Leishmania infantum*. J. Comp. Chem. 31 (13):2463-2474.

6. Saravanan, P., Venkatesan, S. K., Mohan, C. G., Patra, S. and Dubey V. K. (2010) Mitogen-activated protein kinase 4 of Leishmania parasite as a therapeutic target. Eur. J. Med. Chem. 45, 5662-5670.

7. Jean van Heijenoort. (2007). Lipid Intermediates in the Biosynthesis of Bacterial Peptidoglycan. Microbiol Mol Biol Rev. 71(4): 620–635.

8. Adeline Derouaux, Benoît Wolf, Claudine Fraipont, Eefjan Breukink, Martine Nguyen-Distèche and Mohammed Terrak. (2008). The Monofunctional Glycosyltransferase of Escherichia coli Localizes to the Cell Division Site and Interacts with Penicillin-Binding Protein. J Bacteriol. 190(5): 1831–1834.

9. Shide Liang, Samy O. Meroueh, Guangce Wang, Chao Qiu and Yaoqi Zhou. (2010). Consensus scoring for enriching near-native structures from protein-protein docking decoys. Proteins. 1; 75(2): 397–403.

10. Pamela J. Yeh, Matthew J. Hegreness, Aviva Presser Aiden and Roy Kishony. (2009). Drug interactions and the evolution of antibiotic resistance. Nature Reviews Microbiology 7: 460-466.

11. Koch, B., Liljefors, T., Persson, T., Nielsen, J., Kjelleberg, S. and Givskov, M. (2005). The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors Microbiology. 151: 3589-3602.

12. Shih-Long Tu, Hsiu-Chen Chen and Li-Wen Ku. (2008). Mechanistic Studies of the Phytochromobilin Synthase HY2 from Arabidopsis. The Journal of Biological Chemistry, 283: 27555-27564.

13. Frank, P.Y. Lin, Enrico Coiera, Ruiting lan and Vitali Sintchenko. (2009). In Silico Priortisation of candidate genes for prokaryaotic gene function discovery: an application of phylogenetic profiles. BMC Bioinformatics, 10:86.

14. Spratt, B. G. (1977) Properties of the penicillin binding proteins of Escherichia coli K12. Journal of Biochem. 14: 342-352.

15. Wu, J., Hu, Z. and DeLisi, C. (2006). Gene annotation and network inference by phylogenetic profiling. BMC Bioinformatics, 7:80.

16. Francis C. Neuhaus and James Baddiley. (2003). A Continuum of Anionic Charge: Structures and Functions of d-Alanyl-Teichoic Acids in Gram-Positive Bacteria. Microbiol Mol Biol Rev 67(4): 686–723.

17. Sarah Lebeer, Jos Vanderleyden and Sigrid C. J. De Keersmaecker.(2008). Genes and Molecules of Lactobacilli Supporting Probiotic Action. Microbiol Mol Biol Rev. 72(4): 728–764.

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Sequential Optimization of Production of Cephamycin C Using *Nocardia lactamdurans*: Effect of Nutritional Supplements, Metabolic Precursors and Inducers

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Abstract

In this study, *Nocardia lactamdurans* NRRL 3802 was optimized for the first time for production of cephamycin C by using submerged fermentation. The effects of various carbon source, nitrogen source, inoculum size, initial pH of culture medium, minerals and amino acids were investigated for the maximum production of cephamycin C by *N. lactamdurans* NRRL 3802 in submerged fermentation. Subsequently, selected fermentation parameters were further optimized by Taguchi orthogonal array method and response surface methodology (RSM). Glycerol and yeast extract as carbon and nitrogen sources with initial pH of culture medium of 5.5 and inoculum size of 10^9 CFU/ml at 28 ± 2 °C after 3 days gave maximum production of 3042.19 ± 62.36 mg/l of cephamycin C as compared to 243.03 ± 9.43 mg/l before optimization. Incorporation of 1,3-diaminopropane at 1.2 % v/v in RSM optimized medium further increased the yield to 3973.20 ± 57.96 mg/l.

Keywords: Cephamycin C, *Nocardia lactamdurans,* Inducer, Taguchi, RSM

Introduction

Since the discovery of penicillin, a number of microbial species producing a variety of related antibiotic products have been isolated. In all, over 1,000 naturally occurring or chemically modified antibiotic products have been reported, of which over 100 are commercially produced. One important class of microbially produced compounds, the cephamycins, was separately isolated in the early 1970s by Merck and Co., Inc., Germany and by Eli Lilly and Co., IN, USA. Cephamycin C is one of the extracellularly produced â-lactam antibiotics having hydrophilic properties. They are produced by actinomycetes such as *Nocardia lactamdurans* (1) and several species of *Streptomyces viz. Streptomyces cattleya* and *Streptomyces clavuligerus* (2). The increasing occurrence of bacterial resistance to â-lactam antibiotics is through synthesis of the enzyme â-lactamases that hydrolyze the â-lactam ring. Cephamycins are substituted with a methoxy group at the 7á-position of the cephalosporin nucleus, a modification that considerably reduces inactivation by â-lactamases. It is currently being used as an intermediate for semi-synthetic antibiotics such as cefoxitin, cefametazole and cefotetan having better pharmacokinetic and pharmacodynamic properties.

Development of economical medium requires selection of carbon, nitrogen, inorganic salts, and trace element sources. The optimization of nutritional and environmental conditions plays an important role in developing bioprocesses and improving their performance. Nutritional

Vol. 5 (3) 1325 -1337 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

requirement can be manipulated by the conventional or statistical methods (3). The conventional method involves changing one independent variable at a time, while keeping other variables at a fixed level. Statistical methods, on the other hand, (a) shortlist significant variables, (b) help in understanding the interactions among the variables at different levels, and (c) reduce the total number of experiments resulting in saving time and resources. Hence these methods are more rapid and reliable (4).

There are very few reports on production of cephamycin C from *Nocardia lactamdurans* using submerged fermentation (SmF) (5,6). To the best of our knowledge, no report on optimization of the fermentation media composition and conditions for cephamycin C production are available, which prompted us to undertake this study. This paper reports a detailed study on the optimization of cephamycin C production from *Nocardia lactamdurans* NRRL 3802 using SmF, initially by the conventional one factor at-a-time method and subsequently by statistical methods such as Taguchi L_{16}-orthogonal array design and central composite rotatable design. Furthermore, the effects of precursors and inducer on the production of cephamycin C were also undertaken.

Materials and Methods

***Materials*:** Nutrient broth, yeast extract, malt extract, peptone, L-glutamic acid, sucrose, fructose, lactose, maltose, galactose, myo-inositol and 1,3-diaminopropane were purchased from Himedia Limited, Mumbai, India. Glucose, glycerol, starch, ammonium chloride, calcium carbonate, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium chloride, magnesium sulphate, ferrous sulphate, manganese sulphate, zinc sulphate, sodium thiosulphate, orthophosphoric acid, methanol, L-cysteine, L-valine, DL-methionine, L-lysine hydrochloride, heptanesulphonic acid sodium salt for HPLC and HPLC grade acetonitrile were purchased from s.d. Fine-Chem Limited, Mumbai, India. Soybean oil, sesame oil, coconut oil, groundnut oil and mustard oil were procured from local market of Matunga, Mumbai, India. All solvents used were of AR grade unless mentioned. Standard cephamycin C (authentic sample) was a gift sample through kind courtesy of Merck Research Laboratories, Rahway, NJ, USA.

A cephamycin C producing strain, *Nocardia lactamdurans* NRRL 3802, was a gift sample from Northern Regional Research Laboratory, Peoria, Illinois, USA.

***Preparation of seed inoculums*:** The strain was maintained as glycerol stock by adding 1 ml of culture solution, prepared by growing 1 ml (1 x 10^9 CFU/ml) of the organism in 50 ml NYG medium [(g/l) nutrient broth 8, yeast extract 2, glucose 10, $MgCl_2$ 4, pH 6.8) for 48 h (7)], to preautoclaved 1.5 ml microcentrifuge tube containing 0.5 ml of 50% glycerol solution and stored at -20°C. For seed inoculum, the glycerol stock (1 x 10^9 CFU/ml) prepared earlier was inoculated into 50 ml of sterilized NYG media and kept on orbital shaker at 180 rpm for 48 h.

***Fermentative production of cephamycin C*:** The production medium for cephamycin C contained (g/l) glucose, 10.0; L-glutamic acid, 4.25; NH_4Cl, 1.0; $CaCO_3$, 0.25; K_2HPO_4, 2.0; myo-inositol, 0.2; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.025; $MnSO_4.H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.01; para-amino benzoic acid (PABA), 0.0001 and $Na_2S_2O_3.5H_2O$, 0.36. The pH of the production medium was adjusted to 6.8 ± 0.05 (5). 50 ml of production medium was taken in 250 ml Erlenmeyer flask and sterilized by autoclaving at 121°C for 20 min. After cooling, the media was inoculated with 1 ml of 48 h old seed culture

Vol. 5 (3) 1325 -1337 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

and incubated in an orbital incubator shaker at 28 ± 2ÚC for 88 h. All experiments were carried out in triplicates.

Optimization of fermentation medium using one factor at-a-time method

Using the production medium selected for the optimization study, the following parameters were optimized

***Effect of various carbon sources*:** The effect of various carbon sources on production of cephamycin C was studied by substituting glucose in the production media with other carbon sources such as fructose, galactose, glycerol, lactose, maltose, sucrose, starch. Several oils such as soybean oil, groundnut oil, mustard oil, coconut oil and sesame oil were also evaluated for their effect on cephamycin C production. Each carbon source was used at 10 g/l. Reducing sugars were autoclaved separately and added before inoculation. The carbon source which supported maximum production of cephamycin C was used for further studies.

***Effect of inorganic and organic nitrogen sources*:** Here ammonium chloride was substituted with other nitrogen sources such as yeast extract, peptone, malt extract, ammonium nitrate, urea, ammonium sulphate, $(NH_4)_2HPO_4$ and $(NH_4)H_2PO_4$ to check their suitability for cephamycin C production. Each nitrogen source was used at 1 g/l. The nitrogen source which supported maximum production of cephamycin C was used for further studies.

***Production profile of cephamycin C and growth curve of N. lactamdurans NRRL 3802*:** To obtain production profile for cephamycin C and growth curve of *N. lactamdurans* NRRL 3802, three flasks were taken out each day for a period of 5 days and processed to determine cephamycin C content and dry cell weight. All other conditions were maintained as previously described. The incubation period that gave maximum production of cephamycin C was chosen for subsequent experiments.

***Effect of initial pH*:** In order to investigate the effect of initial pH of the medium on cephamycin C production, *N. lactamdurans* was cultivated at different initial pH of medium (pH 5.0 - 8.0). The pH was adjusted using 1N hydrochloric acid or 1N sodium hydroxide. The fermentation was carried out at 28 ± 2°C for 72 h. The optimum pH was used for further studies.

***Effect of inoculum size*:** To study the effect of inoculum size on cephamycin C production, by *N. lactamdurans*, the production media was inoculated with 1 ml of 10^9,10^8,10^7 and 10^6 CFU/ml in 50 ml of autoclaved medium and studied for production of cephamycin C.

***Effect of various minerals*:** Here each component from the production medium was removed one at-a-time (except glycerol, yeast extract and L-glutamic acid) to check its effect on cephamycin C production.

Optimization of fermentation medium using statistical methods

***Optimization using the L_{16}-orthogonal array*:** Taguchi design was used to determine the most significant factors which affect the production of cephamycin C. The design for the L_{16}-orthogonal array with 8 factors at two levels to give a total of 16 experiments was developed and analyzed using "MINITAB 13.32" software. The factors selected were glycerol, yeast extract, L-glutamic acid, K_2HPO_4, NaCl, $MgSO_4$, $FeSO_4$ and $Na_2S_2O_3$. Table 1 depicted L_{16}-orthogonal array design, which was used in the present study. All experiments were performed in triplicates.

***Optimization of concentrations of the selected medium components by RSM*:** To examine the

combined effect of four independent variables identified by L_{16}-orthogonal array design (A**:** $Na_2S_2O_3$ (g/l); B**:** yeast extract (g/l); C**:** K_2HPO_4 (g/l); D**:** L-glutamic acid (g/l)) on maximum production of cephamycin C, media optimized by one factor at-a-time method was used. RSM using central composite rotatable design (CCRD) was applied. Each variable in the design was studied at five different levels, with all variables taken at a central coded value of zero. The experiments were designed using the software, Design Expert Version 6.0.10 version (Stat Ease, Minneapolis, MN) which gave (factorial portion $2^4 = 16$ with 8 star points where á is equal to square root of k and k is 4) and 24 plus 6 centre points leading to 30 experiments. The CCRD design matrix in terms of coded and actual values of independent variable is given in Table 2.

The second order polynomial coefficients were calculated using the software package Design Expert Version 6.0.10 to estimate the responses of the dependent variable. Response surface plots were also obtained using Design Expert Version 6.0.10. The quadratic model suggested by RSM was validated by using the optimized medium composition shown in Table 3.

***The effect of metabolic precursors on cephamycin C production*:** The effects of amino acids on the production of cephamycin C was studied by supplementing the medium with different amino acids *viz.* L-lysine hydrochloride, L-valine, L-cysteine and DL-methionine. Here amino acids were added at 0.5% - 1.5% in RSM optimized media.

***The effect of inducer on cephamycin C production*:** Diamines are known to increase the production of cephamycin C by inducing the enzymes responsible for conversion of lysine to á-aminoadipic acid (8,9). 1,3-Diaminopropane (a three carbon diamine) was used as an inducer for the production of cephamycin C. It was varied from 0.4-1.6% v/v to the RSM optimized media supplemented with amino acids.

Analytical methods

***Cephamycin C assay by HPLC*:** The fermentation broth was centrifuged at 4000 x g for 10 min to remove biomass and 1 ml of supernatant was extracted with 4 ml of methanol to precipitate the proteins. The precipitated proteins were removed by centrifugation at 4000 x g for 10 min and the supernatant was filtered through 0.45 µm membrane filter (Millex®-HV, Millipore, USA). Cephamycin C was estimated by HPLC method reported by Kagliwal *et al.* (10). Jasco HPLC system fitted with a reverse phase column Thermo ODS-2 Hypersil (C_{18} octadecylsilane, 250 x 4.6 mm ID) was used. The mobile phase was 50 mM KH_2PO_4 with 20 mM heptanesulphonic acid (HSA) adjusted to pH 4.0 with concentrated phosphoric acid. A 90% 50 mM KH_2PO_4 with 20 mM HSA (pH 4.0) and 10% acetonitrile mixture was used for fine resolution of peaks. 20-ìl fermentation broth (the centrifuged and deproteinized extract) was injected and eluted at a flow rate of 0.8 ml/min. Cephamycin C was detected at 253 nm.

***Dry cell weight determination*:** To determine the biomass produced, 1 ml of fermentation media was taken in microcentrifuge tube and centrifuged at 10,000 x g for 10 min. The supernatant was decanted and settled biomass was dried at 60°C for 24 h.

Results and Discussion

Optimization of fermentation medium using one factor at-a-time method

***Effect of carbon sources*:** During the microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but is also used as energy source. Glycerol supports the production of antibiotics, and glucose supports the growth of the organism

(11). Fig. 1 shows the effect of different carbon sources on cephamycin C production. The medium was supplemented with monosaccharide, disaccharide, polysaccharide and oils as carbon source. Only glycerol and soybean oil was found to be promising. Glycerol supported maximum production of 324.67 ± 7.13 mg/l, while soybean oil supported 313.26 ± 11.09 mg/l of cephamycin C after 88 h of fermentation. Hence, for all further studies, glycerol was used as a carbon source. Addition of fructose, maltose, galactose and coconut oil as carbon source did not have any major effect on cephamycin C production. The production of cephamycin C decreased with sucrose, starch and lactose to as low as 201.4 ± 4.03 mg/l as compared to glucose. This inverse relationship between specific productivity and lactose concentration suggested the presence of some type of carbon catabolite regulation (12). Park *et al.* (13) reported the maximum production of 525.4 mg/l of

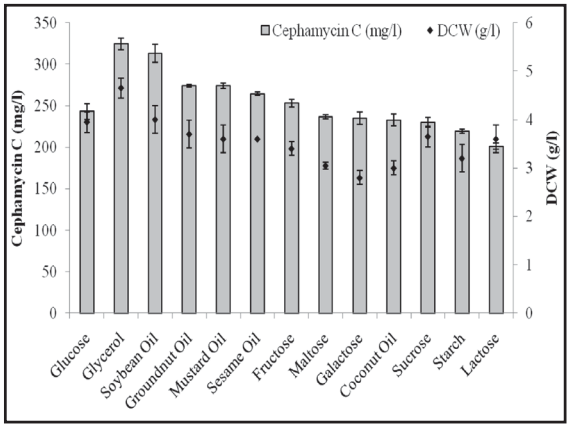

Fig. 1. Effect of carbon source on cephamycin C production by *N. lactamdurans* NRRL 3802 in SmF

cephamycin C using *Streptomyces* sp. p6621 when soybean oil was used alone as carbon source at 30g/l.

***Effect of inorganic and organic nitrogen sources*:** Nitrogen compounds also play an important role in determining both the growth and product yield in antibiotic fermentations. Nitrogen must be introduced to the culture in a manner which satisfy both the growth and antibiotic synthesis, but which restricts neither. Nitrogen sources such as yeast extract, peptone, malt extract, urea, NH_4Cl, $(NH_4)_2HPO_4$, $(NH_4)H_2PO_4$, NH_4SO_4 and NH_4NO_3 were added at 1 g/l to select a suitable nitrogen source for cephamycin C production (data not shown). Of the selected inorganic and organic nitrogen sources, yeast extract gave a maximum yield of 348.03 ± 14.62 mg/l of cephamycin C and $(NH_4)H_2PO_4$ gave the least (250.10 ± 22.61 mg/l). Hence, further studies were carried out using yeast extract as nitrogen source.

Ammonia regulation of antibiotic synthesis has been reported for several other secondary metabolite producing organisms (14). Aharonowitz and Demain (15) have observed a strong suppressive effect of ammonia on antibiotic production in the cephamycin producer *Streptomyces clavuligerus*. Brana *et al.* (16) have also reported effect of ammonium on cephamycin C production. When increasing concentration of NH_4Cl were added to the standard medium containing 15 mM asparagines, there was a progressive and strong decrease in the production of cephalosporins. Ammonium chloride depressed cephalosporin production (by about 75%), presumably as a result of repression of cyclase and expandase formation, but not of epimerase. Khaoua *et al.* (17) studied the effect of nitrogen source on production of cephamycin C by *S. cattleya* and found the production to vary with the use of asparagines, glutamine or ammonium as nitrogen sources.

This hypothesis is supported by studies done with cephamycin producing organism, *N. lactamdurans*. Ginther (7) observed that conditions which led to such secondary processes

as sporulation and protease formation also favoured the production of cephamycin C. Onset of sporulation is associated with the presence of metabolic starvation conditions, typically loss of available assimilable nitrogen. Similarly, excretion of extracellular proteolytic enzymes would be consistent with a run out of soluble amino nitrogen in the fermentation medium (7). This study is consistent with the idea that starvation conditions imposed by a nitrogen or carbohydrate limitation could induce sporulation, protease excretion and antibiotic synthesis in *N. lactamdurans*. However, this synthesis has by no means been quantitatively proved.

***Production profile of cephamycin C and growth curve of N. lactamdurans NRRL 3802*:** The production profile of the cephamycin C and growth curve of *N. lactamdurans* NRRL 3802 was carried out with respect to time (Fig. 2). The production of cephamycin C started on the first day itself, reached a maximum on the third day of fermentation (347.37 ± 4.80 mg/l), and there

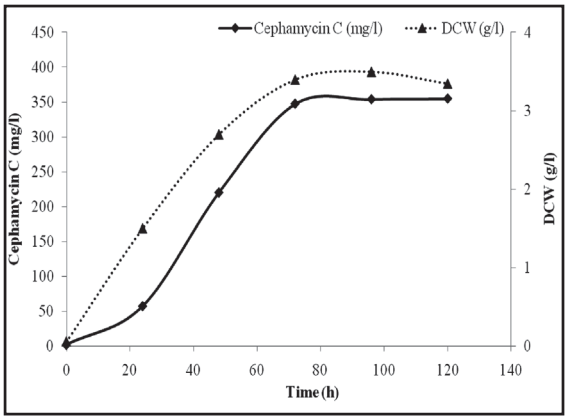

Fig. 2. Production profile of cephamycin C and growth curve of *N. lactamdurans* NRRL 3802 in SmF

was no further increase in cephamycin C until day 5. All the subsequent studies were carried out with fermentation for 3 days. Kirpekar *et al.* (5) reported the production of cephamycin C to reach a maximum after 88 h, and then decrease slightly up to 120 h of fermentation.

***Effect of initial pH*:** An initial pH of 5.5 supported maximum production of 351.75 ± 7.61 mg/l of cephamycin C (data not shown). Hence, all further studies were carried out at pH 5.5. At higher pH, the production decreased, probably due to repression of the enzymes cyclase and expandase due to release of ammonia (16). An initial pH of 6.0 to 7.0 has been reported to be optimum for cephamycin C production by many investigators (11,18,19), but these studies were performed with pH control. At shake flask level, without pH control, the organism would probably be getting more time for production at pH 5.5

***Optimization of inoculum size*:** An inoculum size range of 10^9 - 10^6 CFU/ml was used. Maximum production of 352.45 ± 1.85 mg/l of cephamycin C was obtained with 10^9 CFU/ml in a 50 ml media (data not shown). This inoculum size was used for further studies. Lower inoculum size resulted in poor cephamycin C production which could be due to insufficient biomass produced. Sanchez and Brana (20) studied the effects of cell density on cephamycin C production and observed the production of cephamycin C and clavulanic acid by *Streptomyces clavuligerus* to take place during the exponential phase of growth in a defined medium.

***Effect of various minerals*:** Shake flask experiments were carried out using variations of the media. Here each component of media was removed one at a time (except glycerol, yeast extract and L-glutamic acid) to check their effect on cephamycin C production. Fig. 3 shows the effect of removal of various minerals on the cephamycin C production. Removal of $CaCO_3$, PABA, $MnSO_4$, myo-inositol and $ZnSO_4$ resulted in increase in production of cephamycin C as compared to control suggesting that these could

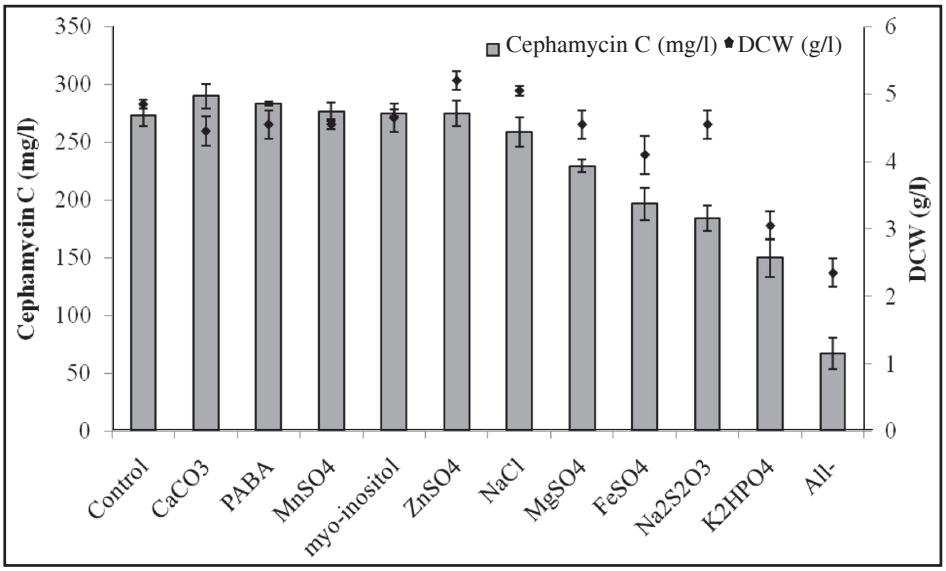

Fig. 3. Effect of minerals on cephamycin C production by *N. lactamdurans* NRRL 3802 by SmF

have inhibitory effect on the production of cephamycin C. Whereas, removal of NaCl, $MgSO_4$, $FeSO_4$, $Na_2S_2O_3$ and K_2HPO_4 decreased the production of cephamycin C suggesting them to play an important role in the production of cephamycin C.

Calcium has been identified to be inhibitory to the biosynthesis of cephamycin C in resting cells of *N. lactamdurans* (21). Therefore, calcium probably does not directly interfere with the biosynthetic enzymes for either cephamycin C or efrotomycin. Inhibition is more likely to be caused by calcium interfering with an enzyme or cellular process, which is generally the case in secondary metabolism (22). $MgSO_4$ is a cofactor for the enzyme ACV synthetase, and $FeSO_4$ for isopenicillin N synthase (IPN synthase) and deacetoxycephalosporin C synthase (DAOC synthase). Hence they are essentially required by these enzymes for the biosynthesis of cephamycin C. Sulphur from sodium thiosulphate gets incorporated into cysteine by reduction, and ultimately into â-lactam products. Phosphate, despite not being incorporated directly into the â-lactam nucleus, affects secondary metabolism in â-lactam producing organisms. They can independently inhibit enzyme action, regulate protein expression and limit cell growth.

Optimization of fermentation medium using statistical methods

***Optimization using the L_{16}-orthogonal array*:** Taguchi design was used to determine most significant factors which affect the production of cephamycin C. The 8 factors selected were glycerol, yeast extract, L-glutamic acid, K_2HPO_4, NaCl, $MgSO_4$, $FeSO_4$ and $Na_2S_2O_3$. The concentrations of the medium components were selected based on the production medium composition that was optimized using one factor at a time.

Table 4 represents the response table for means (larger is better) and for signal to noise ratio obtained with L_{16}-orthogonal array. The last two columns in the Table 4 document the delta

values and ranks for the system. Rank and delta values help to assess which factors have the greatest effect on the response characteristic of interest. Delta measures the size of the effect by taking the difference between the highest and lowest characteristic average for a factor. A higher delta value indicates greater effect of that component. 'Rank' orders the factors from the greatest effect (based on the delta values) to the least effect on the response characteristic. The order in which the individual components selected in the present study effect the fermentation process can be ranked as $Na_2S_2O_3$ > yeast extract > K_2HPO_4 > L-glutamic acid > $MgSO_4$ > $FeSO_4$ > glycerol > NaCl suggesting that $Na_2S_2O_3$ had a major effect, and NaCl has the least effect on cephamycin C production by *N. lactamdurans.*

Fig 4 represents the main effects plot for the system. Main effects plot show how each factor affects the response characteristic. A main effect is said to be present when different levels of a factor affect the characteristic differently. MINITAB creates the main effects plot by plotting the characteristic average for each factor level. These averages are the same as those displayed in the response Table 4. A line connects the points for each factor. A horizontal line (parallel to the x-axis) indicates absence of main effect. Each level of the factor affects the characteristic in the same way and the characteristic average is the same across all factor levels. A main effect is present when the line is not horizontal (not parallel to the x-axis). Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points (the more the line is not parallel to the x-axis), the greater is the magnitude of the main effect.

In the present study it was seen that among the 8 variables at 2 levels, one level increased the mean compared to the second level. This difference is a main effect. Variables like glycerol, NaCl and $MgSO_4$ showed greater mean at level one whereas yeast extract, L-glutamic acid, K_2HPO_4, $FeSO_4$ and $Na_2S_2O_3$ showed greater mean at level two.

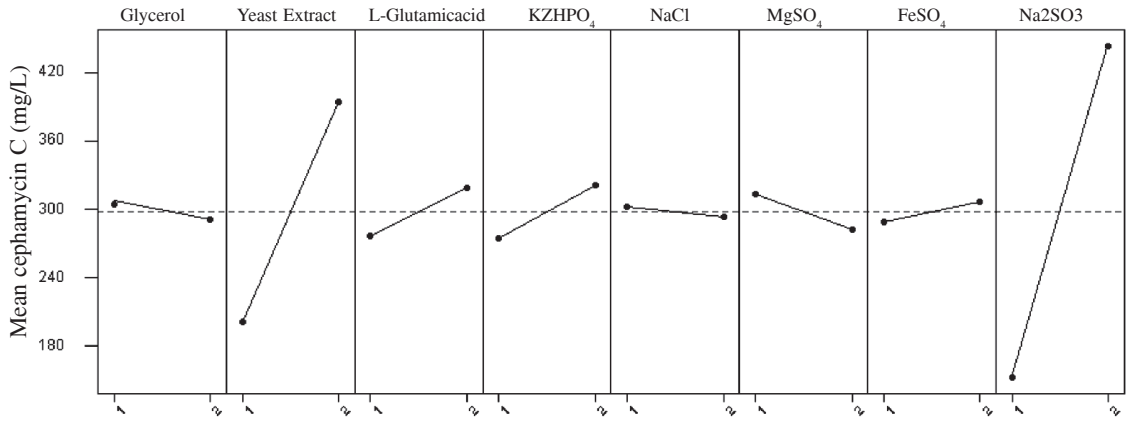

Fig. 4. Main effects plot for means

***Optimization of concentrations of the selected medium components by RSM*:** The combined effect of four independent variables identified by Taguchi L_{16}-orthogonal array design (A: $Na_2S_2O_3$ (g/l); B: yeast extract (g/l); C: KH_2PO_4 (g/l); D: L-glutamic acid (g/l)) for production of cephamycin C was examined using RSM. The CCRD gave quadratic model for the given set of experimental results. Eq. (1) represents the mathematical model relating the production of cephamycin C with the independent process variables, A to D and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert 6.0.10. The experimental and predicted values of yields of cephamycin C are given in Table 2.

The results were analyzed by using ANOVA *i.e.* analysis of variance suitable for the experimental design used. The ANOVA of the quadratic model indicates that the model is significant. The Model *F*-value of 37.48 implies the model to be significant and is calculated as ratio of mean square regression and mean square residual. Model *P*-value (Prob > *F*) is very low (<0.0001), again signifying the model to be significant.

The *P* values were used as a tool to check the significance of each of the coefficients, which, in turn are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.050 indicate the model terms to be significant. The coefficient estimates and the corresponding *P* values suggests that, among the test variables used in the study, A, B, C, B^2, C^2, AD, and BD (where A = $Na_2S_2O_3$ B = yeast extract, C = K_2HPO_4 & D = L-glutamic acid) are significant model terms whereas the mutual interactions between any of the components (AC, BC, CD) were not found to be significant. The second order response model found after analysis for the regression was:

Cephamycin C (mg/l) = -214.78 + 124.78 x $Na_2S_2O_3$ + 116.55 x Yeast extract + 147.97 x K_2HPO_4 + 12.76 x Yeast extract^2 - 13.26 x $K_2HPO_4^2$ + 38.42 x $Na_2S_2O_3$ x L-glutamic acid - 21.78 x Yeast extract x L-glutamic acid (1).

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.972, indicating that 97.2% of the confidence level of the model to predict the yield of cephamycin C. The "Pred R-Squared" of 0.843 is in reasonable agreement with the "Adj R-Squared" of 0.946. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 22.35 indicates an adequate signal.

The special features of the RSM tool, "contour plot generation" and "point prediction" were also studied to find optimum value of the combination of the four media constitutes for the maximum production of cephamycin C. These predicted values were experimentally verified. The maximum production of cephamycin C obtained using the optimized medium was 3042.19 ± 62.36 mg/l. Table 3 documents the yields of cephamycin C by various predicted media combination. The cephamycin C yield (3042.19 ± 62.36 mg/l) in the present study is quite high as compared to that reported in the literature. The maximum cephamycin C production reported till date by using wild type strain *N. lactamdurans* NRRL 3802 is 1291 mg/l (6).

Accordingly, three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two at their center point levels. Graphs are given here to highlight the roles played by various factors

(Fig 5). From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified.

***The effect of metabolic precursors on cephamycin C production*:** The first step in the biosynthesis of cephamycin C is the condensation of three amino acid precursors, viz. á-aminoadipate, L-cysteine and L-valine to form ACV tripeptide. L-lysine is biotransformed to á-aminoadipate by two sequential enzymatic steps. The first step, considered to be one of the rate-limiting steps, involves lysine-6-aminotransferase activity (LAT). A methyl group for methoxy substitution at 7á carbon of cephamycins is provided by methionine. It is this characteristic, which imparts â-lactamase resistance to cephamycins (23). Amino acids viz. lysine, valine, cysteine and methionine at three

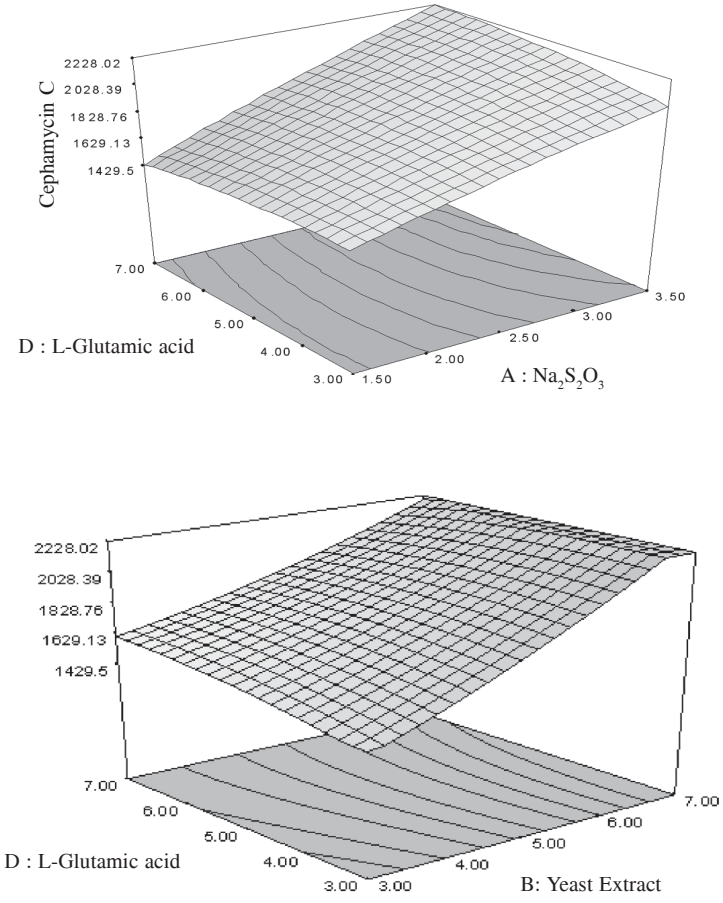

Fig. 5. 3D – surface plot for cephamycin C: **(A)** Effect of $Na_2S_2O_3$ and L-glutamic acid when other variables were held at zero level **(B)** Effect of yeast extract and L-glutamic acid when other variables were held at zero level

Vol. 5 (3) 1325 -1337 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

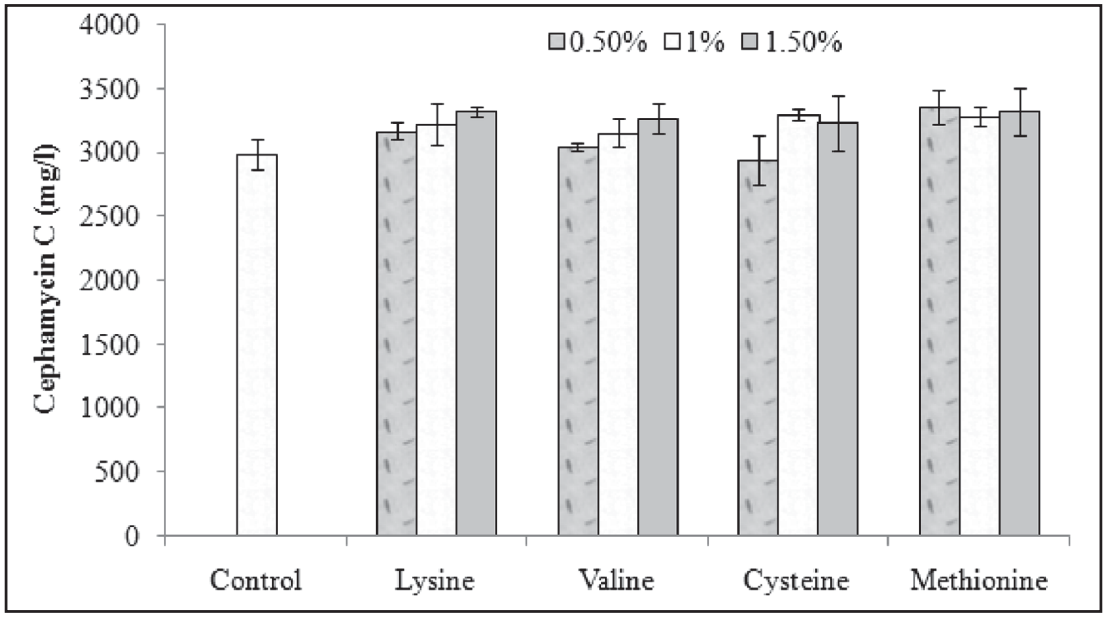

Fig. 6. Effect of metabolic precursors on cephamycin C production by *N. lactamdurans* NRRL 3802 in SmF

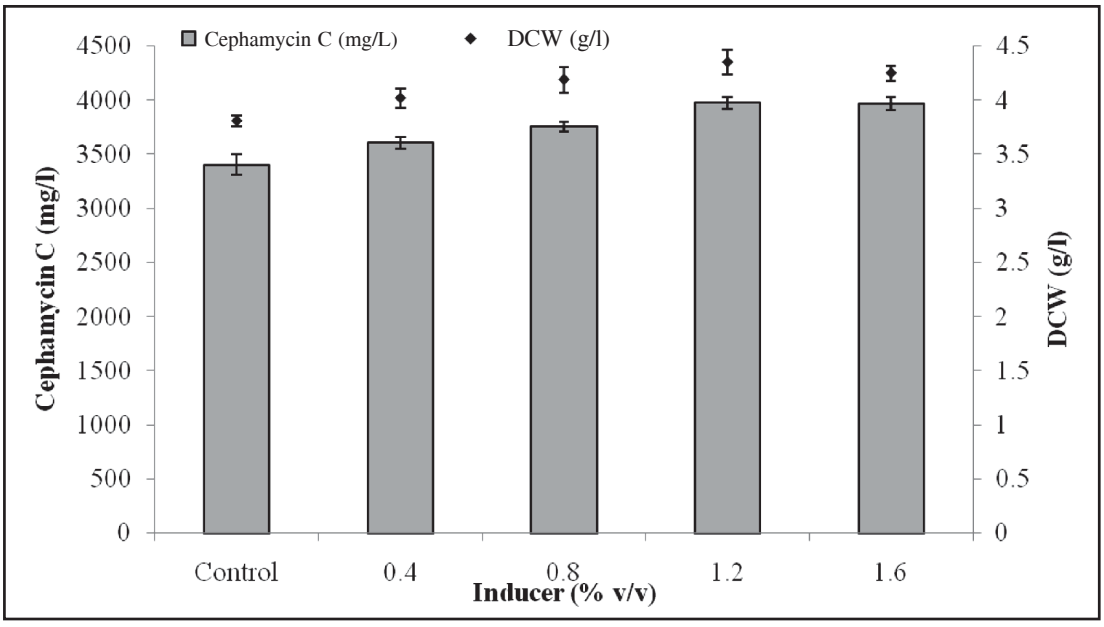

Fig. 7. Effect of inducer (1,3-diaminopropane) on cephamycin C production by *N. lactamdurans* NRRL 3802 in SmF

different concentrations were screened, and were found to increase the production of cephamycin C as compared to control (Fig. 6).

***The effect of inducer on cephamycin C production*:** Diamines are known to increase the production of cephamycin C (8,9). 1,3-Diaminopropane (a three carbon diamine) was used as an inducer for the production of cephamycin C. It was added at 0.4-1.6% v/v to the RSM optimized media supplemented with amino acids. Fig. 7 shows the effect of 1,3-diaminopropane on production of cephamycin C. The optimum production of cephamycin C obtained was 3973.20 ± 57.96 mg/l at 1.2% v/v of 1,3-diaminopropane. 1,3-Diaminopropane increases the enzyme levels of lysine-6-aminotransferase and P6C dehydrogenase (the first and second enzymes of the cephamycin biosynthetic pathway) responsible for the conversion of lysine to á-aminoadipic acid (a rate limiting step) at transcriptional level (9).

Conclusion

Glycerol and yeast extract were found to be suitable carbon and nitrogen source for the production of cephamycin C from *N. lactamdurans* NRRL 3802. Taguchi L_{16}-orthogonal array design demonstrated the effect of $Na_2S_2O_3$, yeast extract, K_2HPO_4 and L-glutamic acid in the production medium to be significant. Further optimization of the medium using RSM showed complex interaction between them and also increased the production of cephamycin C to 3042.19 ± 62.36 mg/l. There was a 12.52 fold increase in production of cephamycin C after RSM and 16.35 fold increase after addition of metabolic precursors and inducer.

References

1. Stapley, E.O., Jackson, M., Hernandez, S., Zimmerman, S.B., Currie, S.A., Mochale, S., Mata, J.M., Woodruff, H.B. and Henlin, D. (1972). Cephamycins, a new family of â-lactam antibiotics. Antimicrobial Agents and Chemotherapy, 2: 122-131.

2. Nagarajan, R., Boeck, L.D., Gorman, M., Hamill, R.L., Higgens, C.E., Hoehn, M.M., Starck, W.M. and Whitney, J.G. (1971). â-Lactam antibiotics from *Streptomyces*. Journal of the American Chemical Society, 93: 2308–2310.

3. Kumar, P. and Satyanarayana, T. (2007). Optimization of culture variables for improving glucoamylase production by alginate entrapped *Thermomucor indicae-seudaticae* using statistical methods. Bioresource Technology, 98: 1252-1259.

4. Survase, S.A., Annapure, U.S. and Singhal, R.S. (2009). Statistical optimization for improved production of cyclosporin A in solid-state fermentation. Journal of Microbiology and Biotechnology 19: 1385-1392.

5. Kirpekar, A.C., Kirwant, D.J. and Stieber, R.W. (1991). Effects of glutamate, glucose, phosphate, and alkali metal ions on cephamycin C production by *Nocardia lactamdurans* in defined media. Biotechnology and Bioengineering, 38: 1100-1109.

6. Inamine, E. and Birnbaum, J. (1973). Fermentation process. US patent 3,770,590.

7. Ginther, C.L. (1979). Sporulation and serine protease production by *Streptomyces lactamdurans*. Antimicrobial Agents and Chemotherapy, 15: 522–526.

8. Inamine, E. and Birnbaum, J. (1976). Fermentation of cephamycin C. US patent 3,977,942.

9. Leitao, A.L., Enguita, F.J., De la Fuente, J.L., Liras, P. and Martin, J.F. (1999). Inducing effect of diamines on transcription of the cephamycin C genes from the *lat* and *pcb*AB promoters in *Nocardia lactamdurans*. Journal of Bacteriology, 181(8): 2379-2384.

10. Kagliwal, L.D., Survase, S.A. and Singhal, R.S. (2009). A novel medium for the production of cephamycin C by *Nocardia lactamdurans* using solid-state fermentation. Bioresource Technology, 100: 2600-2606.

11. Gorman, M., Higgens, C.E. and Nagajarian, R. (1979). Antibiotic A 16886II. US Patent 4,132,790.

12. Omstead, D.R., Hunt, G.R. and Buckland, B.C. (1985). In: Moo-Young M. (ed.). Comprehensive Biotechnology: The principles, applications and regulations in industry, agriculture and medicine. Pergamon press, Oxford.

13. Park, S., Momose, I., Tsunoda, K. and Okabe, M. (1994). Enhancement of cephamycin C production using soybean oil as a sole carbon source. Applied Microbiology and Biotechnology, 40: 773-779.

14. Martin, J.F. and Demain, A.L. (1980). Control of antibiotic synthesis. Microbiological Reviews, 44: 230-251.

15. Aharonowitz, Y. and Demain, A.L. (1979). Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerus*. Canadian Journal of Microbiology, 25: 61-67.

16. Brana, A.F., Wolfe, S. and Demain, A.L. (1985). Ammonium repression of cephalosporin production by *Streptomyces clavuligerus*. Canadian Journal of Microbiology, 31: 736-743.

17. Khaoua, S., Lebrihi, A., Germain, P. and Lefebvre, G. (1991). Cephamycin C biosynthesis in *Streptomyces cattleya*: nitrogen source regulation. Applied Microbiology and Biotechnology, 35: 253-257.

18. Hasegawa, T., Hatano, K., Fukase, H. and Iwasaki, H. (1977). 7-Methoxy cephalosporin derivatives. US Patent 4,017,485.

19. Kamogashira, T., Nishida, T., Nihno, M. and Takegata, S. (1982). Process for production of antibiotic cephamycin C. US Patent 4,322,891.

20. Sanchez, L. and Brana, A.F. (1996). Cell density influences antibiotic biosynthesis in *Streptomyces clavuligerus*. Microbiology, 142: 1209-1220.

21. Chimel, A., Brzeszczynska, A. and Kabza, B. (1986). Biosynthesis of cephamycin by resting cells of *Streptomyces lactamdurans.* Acta Microbiologica Polonica, 35: 251-257.

22. Cover, W.H., Kirpekar, A.C., George, H. and Stieber, R.W. (1991). Calcium inhibition of efrotomycin production by *Nocardia lactamdurans*. Journal of Industrial Microbiology 7: 41-44.

23. Bussari, B., Survase, S.A., Saudagar, P.S. and Singhal, R.S. (2009). An integrated approach for production of cephamycin C using *Streptomyces clavuligerus* NT4: Sequential optimization of production medium and effect of amino acids. Current Trends in Biotechnology and Pharmacy 3: 372-384

Current Trends in Biotechnology and Pharmacy
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In-vitro Evaluation of Rat Small Intestine as a Gluconeogenic Organ During Fasting

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Abstract

The liver and kidneys maintain the blood glucose levels in periods of fasting, starvation, low-carbohydrate diets, or intense exercise by gluconeogenesis. The present study was designed to evaluate whether small intestine provides substrates for hepatic gluconeogenesis during fasting in an in-vitro model using rat everted sac technique. Healthy adult male albino Swiss rats were chosen for this study and divided into three groups consisting of control as well as 4 and 6 days of fasting animals respectively. The parameters studied were animal body weight, jejunal intestinal weight, and protein content of the jejunum, estimation of glucose, pyurvate and lactate dehydrogenase levels. Data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison. The significant level was fixed at $p<0.05$. There was significant increase in glucose, pyurvate and lactate dehydrogenase levels in the fluid medium with a significant decrease in body weight, jejunal intestinal weight and protein levels after 4 and 6 days of fasting when compared with their control animals. It has become clear that as a result of fasting i.e. in phase III fasting state there is a strong increase in protein utilization as a substitute fuel for lipids and a rise in plasma corticosterone level which favors gluconeogenesis. This suggests that the conversion of glucose to lactic acid may play an important part in the absorption of glucose by rat intestine. These results establish that small intestine could serve as a gluconeogenic organ by providing substrates needed for hepatic gluconeogenesis.

Keywords: Gluconeogenesis, everted intestine sac, pyruvate and lactate dehydrogenase.

Introduction

Gluconeogenesis is a ubiquitous process, present in plants, animals, fungi, bacteria and other microorganisms (1). Gluconeogenesis is the biosynthesis of glucose from nonhexose or non-carbohydrate precursors, such as lactate, pyruvate, glycerol and the amino acids alanine and glutamine. The production of glucose from other metabolites is necessary for use as a fuel source by the brain, testes and erythrocytes, since glucose is the sole energy source for these organs. For fasting periods longer than one day, or during periods of intense exercise, glucose must be synthesized from non carbohydrate precursors in order to maintain the blood glucose levels. The non carbohydrate precursors enter the gluconeogenic pathway in the forms of pyruvate, oxaloacetate and dihydroxyacetone phosphate.

There are two major sites for gluconeogenesis, the liver and the kidneys. The liver accounts for 90% of gluconeogenesis in the body and the kidney produces the other 10%. The liver and kidneys maintain the glucose level

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in the blood so that the brain, muscle and red blood cells have sufficient glucose to meet their metabolic demands. Gluconeogenesis is often associated with ketosis. Gluconeogenesis is also a target of therapy for type II diabetes, such as metformin, which inhibits glucose formation and stimulates glucose uptake by cells (2).

During the development of drugs and formulations (3) intended for oral administration, it is of considerable value to have reliable and predictive *in vitro* methods to quantify drug transport across the intestinal epithelium. Several *in vitro* models are available to determine permeability, such as human Colon Carcinoma (Caco-2) cells, Madin Darby Canine Kidney (MDCK) cells, Immobilized Artificial Membrane (IAM) columns, Parallel Artificial Membrane Permeation Assay (PAMPA), excised animal tissues in using chambers and everted gut sacs.

The everted gut sac model, especially the improved technique offers advantages as it is easy and inexpensive to perform and offers the possibility of conducting regional and mechanistic studies. The everted gut sac of the rat small intestine can be used to determine various aspects of drug absorption with high reliability and reproducibility in early stages of drug discovery. It was suggested that fasting intestine contributes in two ways to maintain blood glucose level, directly by absorbing more glucose and indirectly by providing lactate and alanine (4). In catheterized rat model, portal vein draining the gut did not show any increase in the substrates of gluconeogenesis (5). An increase in the content of gluconeogenic enzymes and glucose transporter levels in the rat intestine after prolonged fasting was observed (6). Glucose-6-phosphatase gene is expressed in the small intestine of rats and humans and that it is induced in insulinopenic states such as fasting and diabetes (7). In the small intestine, glutamine and, to a much lesser extent, glycerol is the precursor of glucose, whereas alanine and lactate are the main precursors in liver. A recent report suggested that glucose-6-phosphatase and Phosphoenolpyruvate carboxykinase genes are expressed in rat small intestine and are strongly induced in fasted and diabetic states (8, 9). This contributes to 20–25% of systemic endogenous glucose production in insulinopenic rats. In addition, glucose production in small intestine may be suppressed by insulin. This identifies small intestine as a new insulin-sensitive tissue. Recent work has clearly indicated that glucose uptake and lactic acid output by the everted jejunal sacs of 4 days fasted rats was significantly increased indicating that the glycolytic pathway of metabolism was enhanced (10). However, the provision of intestinal substrates for hepatic gluconeogenesis is less documented and hence this lacuna forms the focus of this study.

Materials and Methods

The study was approved by the Institute's Animal Ethical Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Healthy adult male Swiss albino rats weighing about 180- 200g have been used for this study and allowed to have food and water *ad libitum.* Fasting rats were deprived of food for 4 and 6 days before experiments and were allowed to have free access to water only. Animals were divided into three groups, each group consisting of six animals. Group 1 serves as control animals, Group 2 as 4 days fasted animals and Group 3 acts as 6 days fasted animals.

Surgical procedure: On the day of experiment, rats were sacrificed by cervical dislocation. The abdomen was opened by a midline incision. The entire small intestine was removed quickly by cutting across the upper end of the duodenum and the lower end of the ileum, and by stripping

the mesentery manually (11). Next, the entire intestine is transferred to a Petri dish containing Krebs-Ringer saline, and quickly extended to its full length. At this point it may be cut into segments of convenient length i.e. 5cm of jejunum segment was taken and washed out with normal saline solution (0.9% w/v NaCl) using a syringe equipped with blunt end.

Preparation of Everted intestinal sac: Intestinal segments (5cm) were everted according to the method described earlier (12). A narrow glass rod was inserted into one end of the intestine. A ligature was tied over the thickened part of the glass rod and the sac was everted by gently pushing the rod through the whole length of the intestine (Fig. 1). Krebs-Ringer saline with the pH 7.3 was used as the incubation medium. The loose ligature over the needle was tightened and 2 ml of the Krebs Ringer Saline (in mM: 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 11.1 glucose, pH 7.3) was injected into the sac (Fig. 2). All the ligatures have to be firm enough to prevent leaks but not too tight so as damage the tissue.

The compartment containing the buffer in the sac was named serosal fluid compartment and the buffer in the petridish was named as mucosal fluid compartment respectively. The distended sacs was placed in petri dish (13) with 10 ml of the same medium and incubated for a suitable period of 45 minutes at 37°C, with oxygen entering through a plastic tube. The loss of substrate from the medium was taken as uptake by the sac while gain in substrate was taken as the release of the metabolite by the sac (Fig. 3).

***Sample collection*:** After incubation**,** the sacs were removed from the petridish and the serosal fluid from the sac was collected through a small incision and transferred into a test tube. The emptied sac was shaken gently to remove the adhered fluid whereas the mucosal fluid was

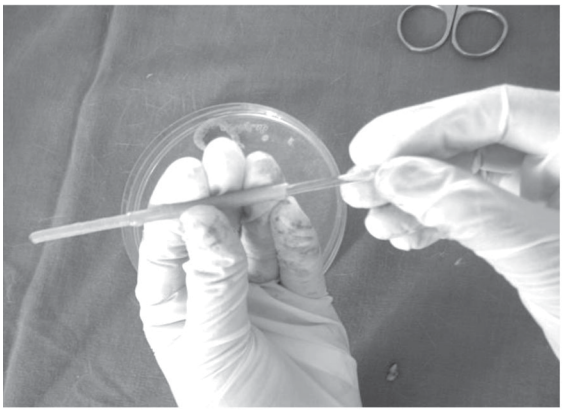

Fig. 1. Everting the rat intestine using a glass rod

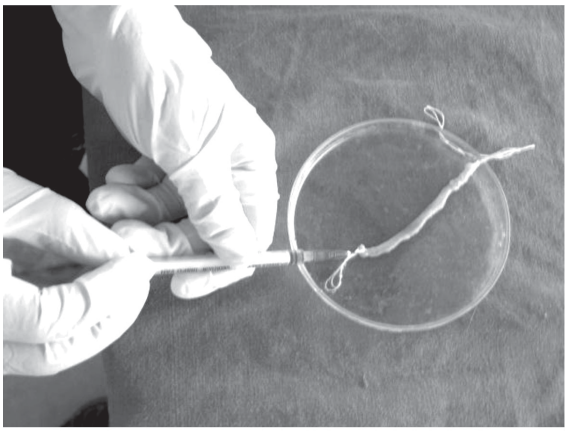

Fig. 2. Injection of buffer into the everted intestinal sac

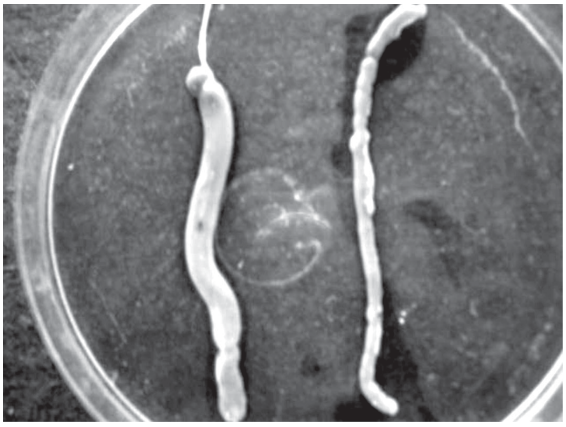

Fig. 3. Everted intestinal sac

Vol. 5 (3) 1338 -1345 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

directly collected from the mucosal compartment.

Biochemical analysis

***Estimation of protein*:** To 0.1 ml of homogenized jejunal sac, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added and kept at room temperature for 10 minutes, then 0.5 ml of Folin's reagent was added and the color developed was read after 20 minutes at 640 nm (14). The level of protein was expressed as mg/5 cm of jejunal segment.

***Estimation of glucose*:** Glucose was estimated in the serosal fluid of the everted intestinal sac by the glucose oxidase/peroxidase (GOD/POD) method (15) by the AGAPPE reagent kit, India. Glucose is oxidized by glucose oxidase into gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of peroxidase (POD) oxidizes the chromogen 4-aminoantipyrine compound to a red colored compound. The intensity of the red colored compound is proportional to the glucose concentration and is measured at 505 nm. The final color is stable for 2 hours.

***Estimation of Pyruvate*:** Pyurvate levels was estimated in the serosal fluid of the everted intestinal sac by DNPH method (16) based on reaction of pyruvate with DNPH which produces a color and the color was read at 520 nm.

***Estimation of lactate dehydrogenase*:** Lactate dehydrogenase was estimated in the serosal fluid of the everted intestinal sac by using UV kinetic method by commercially available AUTOPAK kit, India. Lactate dehydrogenase catalyzes the conversion of pyruvate to lactate and NADH to NAD. Lactate dehydrogenase activity in sample is directly proportional to the rate of decrease in the absorbance of NADH at 340 nm.

Statistical Analysis: Data was analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison. The significant level was fixed at $p<0.05$. Data was expressed as Mean ± SD in bar diagram.

Results and Discussion

The three phases of fasting were first observed in penguins and rats (17,18). The three distinct phases of fasting (17,19) are after a rapid period of adaptation marked by the depletion of glycogen reserves (phase-I), lipid stores are progressively oxidized whereas body proteins are efficiently spared (phase-II) and later fasting phase is characterized by both a strong increase in protein utilization as a substitute fuel for lipids (phase III) and a concomitant rise in plasma corticosterone level. The changing hormone levels during fasting may play a role in the regulation of gluconeogenic enzymes in the small intestine and on intestinal glucose transporters. Fasting also induces an increase in gluconeogenesis in the small intestine which produces up to one-third of endogenous glucose after 72 h of fasting (4). The main precursors of gluconeogenesis in the small intestine are glutamine and glycerol, the latter to a much lesser extent (7). There was no mortality observed in fasting groups studied. Only jejunal segments were taken up for the study as reported earlier (10). On the other hand, the jejunum of the rat has a thicker mucosa and also a greater mucosal area/cm of serosal length (20) than the ileum. In our study, there was a significant decrease in body weight as well as intestinal segment weight in both 4 days fasted as well as 6 days fasted rats when compared with control animals (Figs. 4 and 5). This is quite contrary to earlier reports (10) where a significant loss in the body weight was observed in 6 days fasted rats.

The jejunal weights of all fasted animals decreased significantly when compared to those

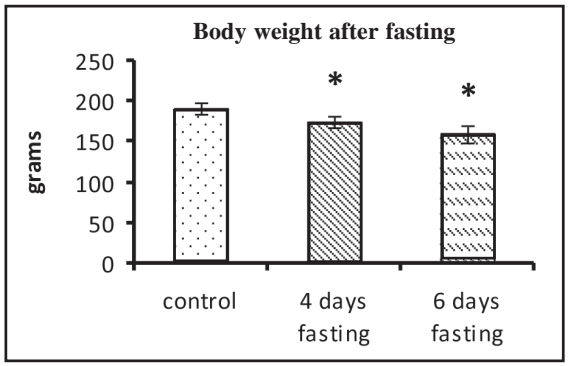

Fig. 4. Body weight of the control, 4 days and 6 days fasting rats. Results were shown as mean ± SD. P<0.05 was considered significant. *Indicates significant compared with control.

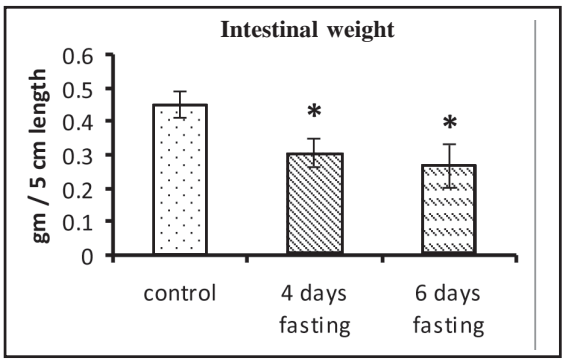

Fig.5. Intestinal weight of the control, 4 days and 6 days fasting rats. Results were shown as mean ± SD. P<0.05 was considered significant. * indicates significant compared with control.

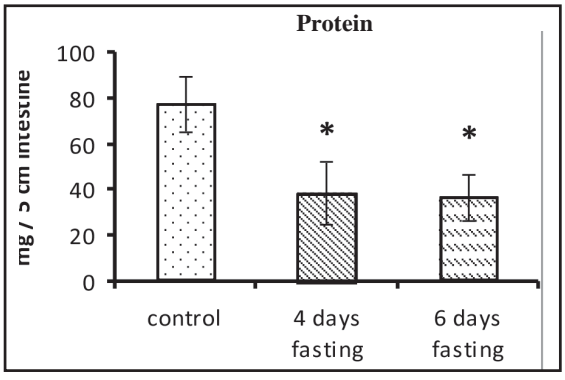

Fig.6. Protein content of the intestine of the control, 4 days and 6 days fasting rats. Results were shown as mean ± SD. P<0.05 was considered significant. * indicates significant compared with control.

from the non-fasting controls. This observation indicates the possibility that protein breakdown in the gut occurs earlier (6) so that thinning of the gut during fasting facilitates absorption of nutrients which increase by one and half times. These changes may have a role in promotion of survival mechanisms during short term fasting (21).

In our study there is a significant decrease in protein level of the jejunal sac in both 4 and 6 days of fasting when compared with their controls (Fig. 6). The study also shows an increase in intestinal gluconeogenesis during the phase III fasting period, during which the gluconeogenic precursors may be amino acids coming from protein catabolism, compared to the phase II fasting period, i.e. when glycerol may be the main gluconeogenic precursor. The protein and gene expressions of the facilitative glucose transporter GLUT2 were down-regulated during phase II and phase III fasting and up-regulated by refeeding. These changes may be linked to plasma corticosterone, which shows to inhibit this transporter in stressed rats (22). It appeared that during protein catabolism (phase-III fasting), the active process of glucose absorption is induced by an increase in SGLT1 in the brush-border membrane, whereas the facilitated component of intestinal glucose transport is triggered at refeeding by an increase in GLUT2 translocated to the apical membrane.

In our study, glucose uptake and pyruvate release in serosal fluid were significantly increased in 4 days fasted as well as in 6 days fasted rats when compared with their control rats (Fig. 7). The increase in glucose uptake by jejunal segments of 4 days fasted rats in our study is in agreement with the earlier reports (10, 21). However in 6 days fasted rats, the significant increase in glucose uptake was also observed (6) which reported significant increase in glucose uptake in rats fasted for more than 6 days. The

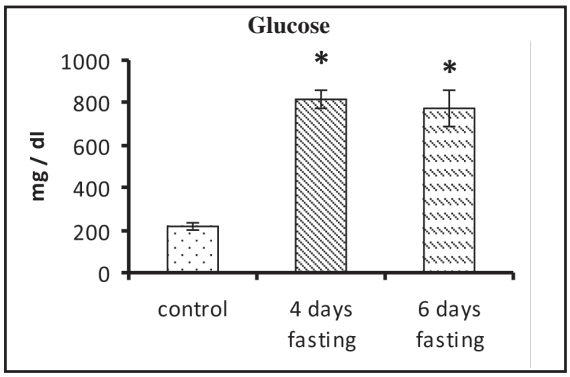

Fig.7. Glucose content of the control, 4 days and 6 days fasting rats. Results were shown as mean ± SD. P<0.05 was considered significant. * indicates significant compared with control.

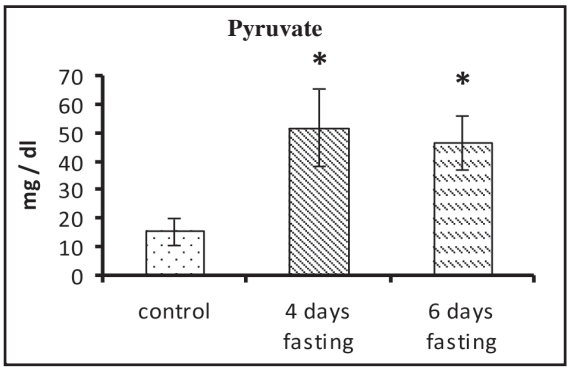

Fig.8. Pyruvate level of the control, 4 days and 6 days fasting rats. Results were shown as mean ± SD. P<0.05 was considered significant. * indicates significant compared with control.

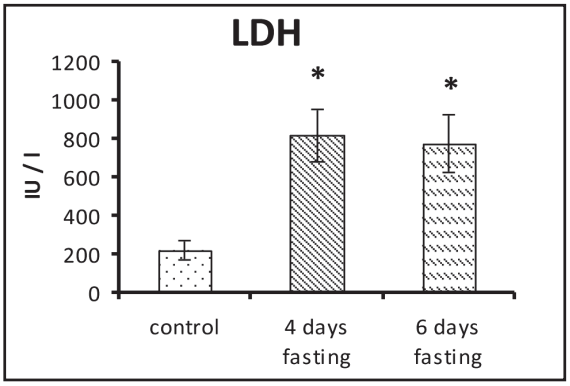

Fig.9 Lactate dehydrogenase level of the control, 4 days and 6 days fasting rats. Results were shown as mean ± SD. P<0.05 was considered significant. * indicates significant compared with control.

eversion exposes the highly active mucosa to the well-oxygenated suspending medium, while the distension increases the surface area of the sac and reduces the thickness of the sac wall. The oxygenation of the inner layer of the serosal surface is facilitated by an oxygen bubble which is introduced into the sac along with the inner fluid. The relatively small volume of fluid contained in the sac (serosal side) allows a rapid rise in concentration of transferred substances.

The amounts of glucose which disappear in the rat intestine are considerably greater than the amounts which are transferred across the wall. It is very remarkable that about half of the glucose which disappears from the mucosal side is converted into lactic acid and that this substance appears in much higher concentration on the serosal side than on the mucosal side. A similar lactate concentration gradient was observed (12). This suggests that the conversion of glucose to lactic acid may play an important part in the absorption of glucose by rat intestine.

In our study there is a significant increase in pyruvate level and lactate dehydrogenase levels (Figs. 8 and 9) in 4 days as well as 6 days fasted rats. They showed a significant increase in serosal fluid when compared with their respective control fed rats. One of the most important pathways of pyruvate utilization is its conversion to lactate by lactic dehydrogenase activity (23). It has become clear that, as a result of fasting, some of the enzymes decrease parallel with the depletion of liver nitrogen, whereas others are preferentially maintained or preferentially decreased. Pyruvate dehydrogenase is a rate-limiting enzyme for pyruvate entry into the tricarboxylic acid cycle (24); its catalytic activity influences both pyruvate oxidation and lactate production. Since lactate dehydrogenase is an equilibrium enzyme, increased lactate production will be due to a mass-action effect exerted by increases in

pyruvate concentrations. Because the equilibrium constant of the lactate dehydrogenase reaction markedly favors lactate over pyruvate, small increases in pyruvate concentration will result in large increases in lactate concentration.

Conclusion

In the light of the results obtained, it appears that energy depletion in relation to body fuel uses and gluconeogenic precursor availability increases the ability of the intestine to absorb glucose from its lumen and stimulates intestinal gluconeogenesis. In phase III fasting, there is an increased catabolism of proteins and in the absence of GLUT2, Glucose 6 Phosphsatase could also play a role in glucose transport through the cell and thereby, in its secretion into the bloodstream. From the above mentioned data, it is clear that small intestine serves as a gluconeogenic organ by providing substrates needed for hepatic gluconeogenesis.

Acknowledgement

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References

1. David, L.N. and Michael, M.C. (2000). Lehninger Principles of Biochemistry. 4th ed. Worth Publishers. USA, p. 724.
2. Hundal, R., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S., Schumann, W., Petersen, K., Landau, B. and Shulman, G. (2000). Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes, 49: 2063-2069.
3. Anand Patil., Varun Raheja. and Anagha Damre. (2010). Simultaneous analysis of intestinal permeability markers, caffeine, paracetamol and sulfasalazine by reverse phase liquid chromatography: a tool for the standardization of rat everted gut sac model. Asian J. Pharm.Clin. Res., 3: 204-207.
4. Mithieux, G., Gautier-Stein, A., Rojas, F. and Zitoun, C. (2006). Contribution of intestine and kidney to glucose fluxes in different nutritional states in rat. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 143: 195-200.
5. Kimura, R.E., La pine, T.R., Johnston, J. and Ilich, J.Z. (1988). Effect of fasting on rat portal venous and aortic blood glucose, lactate, alanine and glutamate. Pediatr. Res., 23: 241-244.
6. Habold, C., Foltzer-Jourdainne, C., Le Maho, Y., Ligurt, J. and Oudart, H. (2005). Intestinal gluconeogenesis and glucose transport according to body fuel availability in rats. J. Physiol., 566: 574-586.
7. Martine, C., Fabienne, R., Carine, Z., Jean-Marc, H., Sandrine, M., and Gilles, M. (2001). Rat Small Intestine Is an Insulin-Sensitive Gluconeogenic Organ. Diabetes, 50: 740-746
8. Rajas, F., Bruni, N., Montano, S., Zitoun, C. and Mithieux, G. (1999). The glucose-6 phosphatase gene is expressed in human andrat small intestine: regulation of expression in fasted and diabetic rats. Gastroenterology, 117: 132–139.
9. Rajas, F., Croset, M., Zitoun, C., Montano, S. and Mithieux G. (2000). Induction of PEPCK gene expression in insulinopenia in ratsmall intestine. Diabetes, 49: 1165–1168.
10. Kirtana Pai, M., Sareesh, N.N., Prakasa Rao, J., Gupta, P.D., Vivekananda Kedage,

Manjunatha Muttigi, S., Lakshmi Prabhu, S. and Mungli Prakash. (2010). Rat everted intestinal segments demonstrate that Fasting is a regulator of uptake and metabolism of glucose. Indian J. Physiol. Pharmacol., 54; 89-91.

11. Barthe, L., Woodley, J.F., Kenworthy, S. and Houin, G. (1998). An improved everted gut sac as a simple and accurate technique to measure paracellular transport across the small intestine. Eur. J. Drug Metab. Pharmacokinet., 23: 313-323.

12. Wilson, T.H. and Wiseman, G. (1954). The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J Physiol, 123, 116-125.

13. Crane, R.K. and Wilson, T.H. (1958). In vitro method for the study of the rate of intestinal absorption of sugars. J. Appl. Physiol., 12: 145-146.

14. Lowry, O.H., Rosenborough, N.J., Farr, A.I. and Randall, R.T. (1951). Protein measurement with the Folin-phenol reagent. J. Biol. Chem., 193: 265-275.

15. Trinder, P. (1969). Determination of blood glucose using an oxidase peroxidase system with a non-carcinogenic chromogen. J. Clin. Path., 22: 158–161.

16. Friedemann, T.E. and Haugen, G.E. (1943). Pyruvic acid: II. The determination of keto acids in blood and urine. J. Biol. Chem., 147: 415-442.

17. Le Maho, Y., Vu Van Kha, H., Koubi, H., Dewasmes, G., Girard, J., Ferre, P. and Cagnard, M. (1981). Body composition, energy expenditure, and plasma metabolites in long-term fasting geese. Am. J. Physiol., 41; E342–E354.

18. Cherel, Y. and Le Maho, Y. (1991). Refeeding after the late increase in nitrogen excretion during prolonged fasting in the rat. Physiol. Behav., 50: 345–349.

19. Goodman, M.N., Larsen, P.R., Kaplan, M.N., Aoki, T.T., Young, V.R. and Ruderman, N.B. (1980). Starvation in the rat. II. Effect of age and obesity on protein sparing and fuel metabolism. Am. J. Physiol., 239: E277–E286.

20. Fisher, R.B. and Parsons D.S. (1950). The gradient of mucosal surface area in the small intestine of the rat. J. Anat. Lond., 84: 272-282.

21. Gupta, P.D. and Waheed, A.A. (1992). Effect of starvation on glucose transport and membrane fluidity in rat intestinal epithelial cells. FEBS Lett., 300: 263-267.

22. Shepherd, E.J., Helliwell, P.A., Mace, O.J., Morgan, E.L., Patel, N. and Kellett, G.L. (2004). Stress and glucocorticoid inhibit apical GLUT2-trafficking and intestinal glucose absorption in rat small intestine. J. Physiol., 60: 281-290. .

23. George Weber. (1971). Effect of six-day starvation on rat liver lactic dehydrogenase activity. J. Nutrition, 71: 156-158.

24. Heigenhauser, G.J. and Parolin, M.L. (1999). Role of pyruvate dehydrogenase in lactate production in exercising human skeletal muscle. Adv. Exp. Med. Biol., 474: 205-218.

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Effect of NPK and Farm Yard Manure on the Accumulation of Forskolin, an Anti-Obesity Diterpenoid Molecule in *Coleus forskohlii*

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Abstract

Medicinal plants growing in wild cannot ensure consistent quality due to genetic variability found in the natural populations, geographical variations and season of collection. Therefore, there is a need to cultivate medicinal plants using known varieties for extraction of high yields of active principles. This can be achieved only with organic farming practices and post harvest technologies. The research conducted by institutes like CIMAP and other CSIR institutes, ICAR and Universities has led to the release of a number of high yielding medicinal plant cultivars. *Coleus forskohli* is a herb with fleshy, fibrous roots that grows in wild in the warm, subtropical areas like in India, Burma, and Thailand. *Coleus forskohlii* is being grown in several parts of the country as a commercial crop. In the present investigation the effect of nitrogen, phosphorus, potassium (NPK) and farm yard manure amendments on the growth of roots and accumulation of forskolin was studied. The results revealed that 50% of NPK and 10 tonnes of farm yard manure per hectare at a harvesting time 150 days are ideal for maximum yield of forskolin.

Key words: Forskolin, Farm Yard Manure, NPK, *Coleus forskohlii*

Introduction

Cultivation of medicinal plants appears to be an important strategy for research and development. More than 400 plant species used for production of medicines by the Indian Industry. Cultivation of various varieties of medicinal plants is important to isolate the active principles. The application of organic farming practices and post harvest technologies can enhance their yields so that it becomes profitable to the farmers. The potential returns to the farmer from cultivation of medicinal plants are reported to be quite high if fertilizers are used at a proper dose and time. The application of bio-fertilizers in combination with the inorganic fertilizers substitutes the required quantity of the fertilizers needed by the medicinal and aromatic crops. The bio-fertilizers due to its eco friendliness will change the phase of medicinal and aromatic crops production in the years to come (1). In *Chlorophytum borivallianum,* the number of tubers, fresh and dry tuber yields and steroid content increased with the application of vermicompost of 5 tonnes ha^{-1} (2).

When *Coleus forskohli* was grown in FYM amended soil with *Paecilomyces lilacinus*, there was better root development due to control of

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nematodes (3). Earlier studies on *Coleus forskohlii*, with a combination of 40 kg N, 60 kg P_2O_5 and 50 kg K_2O ha^{-1} resulted in optimum fresh and dry tuber yields of 13.12 tonnes ha^{-1} (4). Raising the level beyond this was found to reduce the tuber yield significantly. Nitrogen at 60 kg ha^{-1} produced higher tuber yield, high dry matter yield but maximum bulking rate along with 120 kg K_2O ha^{-1} in *Coleus parviflorus* (5). Somanath *et al*. (6), in their study on influence of FYM and NPK on uptake of nutrients and growth of *Coleus forskohlii* found that maximum increase in fresh weight of roots was recorded in all the stages of growth (at 30 DAP 0.135 kg $plant^{-1}$, at 90 DAP 0.347 kg $plant^{-1}$ and at 150 DAP 0.600 kg $plant^{-1}$).

Application of N at 100 kg ha^{-1} (half basal + half one month after transplanting) along with a basal application of 100 kg ha^{-1} P2O5 and 20 tonnes FYM ha^{-1} increased the fresh weight of rhizomes in *Acorus calamus* (7). In Aswagandha (*Withania somnifera),* high dry weight of roots (1.297 g $plant^{-1}$) were obtained with 15 kg N ha^{-1} (8). In *Catharanthus roseus*, 100 kg N, 40 kg P_2O_5 and 50 kg K ha^{-1} gave maximum root yields (9). Application of N (30kg N ha^{-1}) increased the root yields (2702 kg ha^{-1}) in *Rauwolfia serpentina* (10). Highest root yield of Aswagandha was obtained with the application of 20 kg N and 40 kg P_2O_5 ha^{-1} (11).

But there are no reports on the influence of nutrients (inorganic and bio- fertilizers) on the content of forskolin, the main chemical constituent of *Coleus forskohlii*. Keeping this view in mind, the present investigation was taken up to enhance the forskolin levels under different fertilizer amendments. The aim of the present investigation is to increase the yields of roots and forskolin content under different fertilizer amendments so as to make it profitable to the farming community.

Materials and methods

Preparation of soil bed :The experimental area was repeatedly ploughed; finally, it was leveled and divided into plots as per the layout. The experiment was laid out in a randomized block design with 6 treatments and replicated thrice. A plot size of 3 X 2.5 m was taken for experimental work.

Treatments of fertilizers and farm yard to the soil for cultivation of *Coleus forskohlii*

The details of the treatments are:

T_1 : Control – No manures
T_2 : 100% NPK recommended dose
T_3 : 50% NPK + 10 tonnes of farm yard manure / ha
T_4 : 50% NPK + 5 tonnes of vermicompost/ ha
T_5 : 20 tonnes of farm yard manure / ha
T_6 : Vermicompost 10 tonnes/ha

Extraction of forskolin : *Coleus forskohlii* roots (10 kg) were extracted with methanol (25 x 3) at room temperature. After this, methanol was distilled out to give a residue of 1.5 kg. This residue was further extracted with Petroleum-Ether (5 x 5) to give another residue of 230 g containing waxes and fats. After this, defatted residue was extracted with ethyl acetate to give a residue of 270 g, which contains 27% forskolin.

Fresh and dry weights of the root : The influence of different fertilizer amendments on fresh weight and dry weights of the root [kg plant $^{-1}$] at 30, 90 and 150 Days after Planting (DAP) of growth in *Coleus forskohlii* was recorded.

HPLC analysis of forskolin

Conditions:

Mobile phase	:	Mehanol :Water (65:35 v/v)
Column	:	Hypersil C-18 (250 mm* 4.6 mm)
Detection	:	Refractive index
Flow rate	:	1 µl/min
Injection volume	:	20 micro lit.

Coleus forskohlii (roots) was cultivated under different treatment conditions, and

Vol. 5 (3) 1346 -1352 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

forskolin was extracted in a soxhlett apparatus with methanol and concentrated. TLC solvent system (Rf 0.45, 20% EtOAc + petroleum ether) showed the presence of forskolin. The extracts were subjected to HPLC analysis.

Results

Fresh weight of the root : The crop was harvested at 30, 90 and 150 days after transplantation. Light irrigation was given one day before harvesting for easy lifting up of roots. Harvesting was carried out manually by digging and uprooting the individual plants. The roots were separated and washed to remove the soil. Forskolin content was determined in roots 150 days after transplantation. The influence of different fertilizer amendments on fresh weight of the root [kg plant $^{-1}$] at 30, 90 and 150 DAP of growth in *Coleus forskohlii* is shown in table 1. Maximum increase in fresh weight of roots was recorded in treatment number 3 in all the stages (at 30 DAP 0.135 kg plant^{-1}, at 90 DAP 0.347 kg plant^{-1} at 150 DAP 0.600 kg plant^{-1}) of growth.

Dry weight of the root and content of forskolin **:** Influence of various fertilizer amendments on dry weight of the root [kg plant $^{-1}$] at 30, 90 and 150 DAP of growth in *Coleus forskohlii* is shown in Table 2. In treatment number 3, maximum fresh weight was observed i.e., at 30 DAP 9.1 g plant^{-1}, at 90 DAP 18.9 g plant^{-1} at 150 DAP 60.0 g plant^{-1}. The fresh and dry weights of roots were measured under different nitrogen regimes and are shown in Table 3. The treatment 50% NPK + 10 tonnes of farm yard manure gave the best dry root weight among the different treatments (Table 3). Samples were collected under different treatments for quantification of forskolin. Forskolin was extracted in a soxhlett apparatus, concentrated and used for HPLC analysis (Fig. 1). HPLC analysis revealed that application of 50% NPK + 10 tonnes of farm yard manure ha^{-1} gave maximum yields of forskolin after 150 days of harvest.

Discussion

Somanath *et al*. (6), in their study on influence of FYM and NPK on uptake of nutrients and growth of *Coleus forskohlii* observed maximum increase in fresh weight of roots in treatment number 3 (at 30 DAP 0.135 kg plant^{-1}, at 90 DAP 0.347 kg plant^{-1} at 150 DAP 0.600 kg plant^{-1}). Application of N at 100 kg ha^{-1} (half basal + half one month after transplanting) along with a basal application of

Table 1. Effect of various fertilizer amendments on fresh weight of root (g per plant) at different stages of growth in *Coleus forskohlii*

Treatments	Days after planting		
	30	90	150
T_0 : Control – No manures	58	120	267
T_1 : 100% NPK recommended dose (N- 40 kg ha^{-1})	83	340	490
T_2 : 50% NPK + 10 tonnes of farm yard manure/ha	149	468	600
T_3 : 50% NPK + 5 tonnes of vermicompost/ha	100	375	510
T_4 : 20 tonnes of farm yard manure/ha	69	160	310
T_5 : Vermicompost 10 tonnes/ha	73	190	385
T_6			

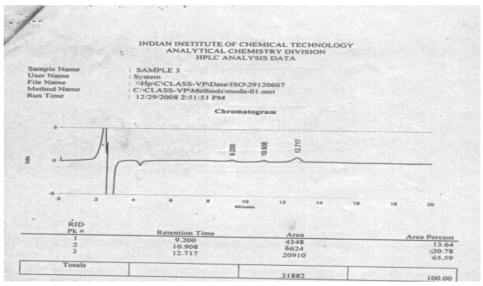
Vol. 5 (3) 1346 -1352 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 2. Effect of various fertilizer amendments on dry weight of root (g per plant) at different stages of growth in *Coleus forskohlii*

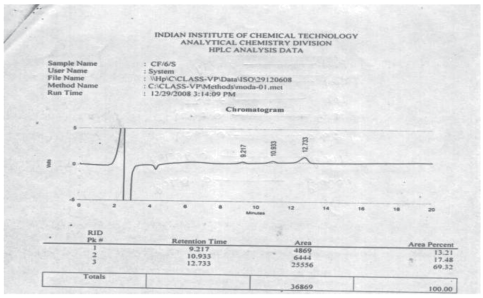
Treatments	Days after planting		
	30	90	150
T_1 : Control – No manures	3.8	8.2	26.8
T_2 : 100% NPK recommended dose (N- 40 kg ha^{-1})	7.3	16.5	47.5
T_3 : 50% NPK + 10 tonnes of farm yard manure/ha	9.1	18.9	60
T_4 : 50% NPK + 5 tonnes of vermicompost/ha	6.2	16.7	55
T_5 : 20 tonnes of farm yard manure/ha	4.5	11.7	29.4
T_6 : Vermicompost 10 tonnes/ha	5.1	14.9	44

Figure 3. HPLC Analysis

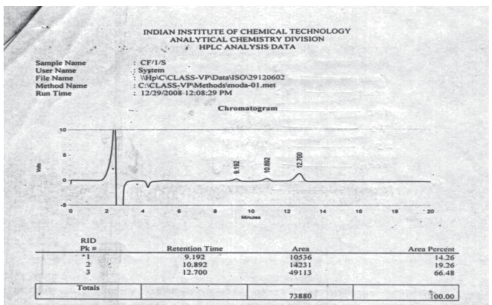
Treatment No. 1 Forskolin % W/W-1.60



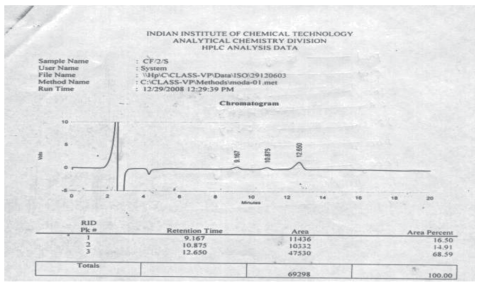
Treatment No.2 Forskolin % W/W- 3.52



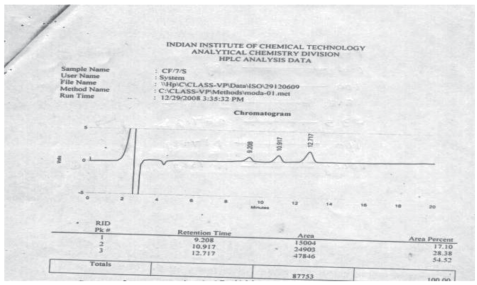
Treatment No. 3 Forskolin % W/W-4.78



Treatment No. 4 Forskolin % W/W-3.86



Treatment No. 5 Forskolin % W/W-3.14



Treatment No. 6 Forskolin % W/W-2.69

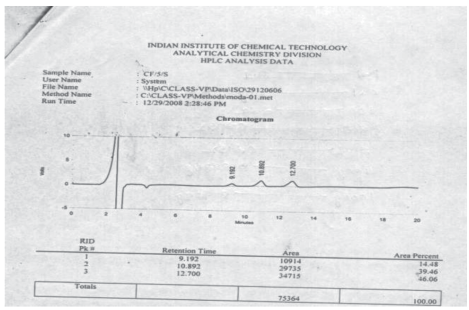


Table 3. Fresh and weights of roots grown under different nitrogen regimes. N = Replicates, M = Mean, SE = Standard error

Treatment			30 days		90 days		150 days	
		N	M-SE	M+SE	M-SE	M+SE	M-SE	M+SE
T1	Fresh weight of root	3	58	59.33	120	121.33	267	267.67
	Dry weight of root	3	3.73	3.8	8.2	8.27	26.8	27.27
T2	Fresh weight of root	3	83	84.33	340	340.67	490	490.67
	Dry weight of root	3	7.44	7.96	16.6	17.4	47.67	48.13
T3	Fresh weight of root	3	149	150.33	468	468.67	600	601.33
	Dry weight of root	3	9.24	10.03	18.85	19.09	60	60.67
T4	Fresh weight of root	3	100	100.67	374.33	375	510	510.67
	Dry weight of root	3	6.29	6.84	16.79	17.08	55	55.67
T5	Fresh weight of root	3	69	69.67	160	160.67	309.33	310
	Dry weight of root	3	4.59	5.01	11.79	12.14	29.49	29.97
T6	Fresh weight of root	3	73	73.67	190	190.67	385	385.67
	Dry weight of root	3	5.1	5.23	14.85	15.09	44	44.67

T1 = control; T2 = 100% NPK; T3 = 50% NPK + 10 tonnes of farm yard manure; T4 = 50% NPK + 5 tonnes of vermicompost; T5 = 20 tonnes of farm yard manure; T6 = 10 tonnes of vermicompost.

100 kg ha^{-1} P2O5 and 20 tonnes FYM ha^{-1} increased the fresh weight of rhizomes in *Acorus calamus* (7). Sheu *et al*. (12) also observed higher yield (23 tonnes ha^{-1}) in *Dioscorea doryophora* with organic and inorganic fertilizer amendments. In treatment number 3, maximum fresh weights were recorded (Table 3 and Fig. 4.). In Aswagandha (*Withania somnifera),* dry weight of roots (1.297 g $plant^{-1}$) were obtained with 15 kg N ha^{-1} (Maryada *et al*., 2001). In *Catharanthus roseus*, 100 kg N, 40 kg P_2O_5 and 50 kg K_2O) ha^{-1} gave maximum root yields (Hegde, 1988). Highest root yield of aswagandha was obtained with the application of 20 kg N and 40 kg P_2O_5 ha^{-1} (11). In aswagandha, application of 40 kg N ha^{-1} and 60 kg P_2O_5 ha^{-1} and 20 kg K_2O ha^{-1} gave the highest dry root yields of 770.37 kg ha^{-1} (13).

Root characters like number of roots per plant and length of roots increased in *Chlorophytum borivallianum,* along with the number of tubers, fresh and dry weights of tuber yield and steroidal content with the application of vermicompost of 5 tonnes ha^{-1}. The steroidal content was the highest with vermicompost application (2). Maheshwari *et al*. (1988) also observed increase in the root yield in *Rauwolfia serpentaina* with the application of N (30 kg N ha^{-1}). Application of NPK at 30:30:0 kg ha^{-1} recorded the highest dry root yields of Aswagandha but further increasing the levels of

Vol. 5 (3) 1346 -1352 July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

fertilizers had no effect on the yields of dry roots (14). Highest root yield of aswagandha was obtained with the application of 20 kg N and 40 kg P_2O_5 ha^{-1} (11). HPLC analysis revealed that application of 50% NPK + 10 tonnes of farm yard manure ha^{-1} gave maximum yield of forskolin. The statistical analysis revealed that fresh and dry weights of roots, number of roots $plant^{-1}$ and root length were significant compared to controls and positively correlated with each other. It is suggested that appropriate dose of application of fertilizers at appropriate time will result in higher tuber yield and forskolin content. Our results also suggest that time of harvest for optimum yields of tuber is also important.

References

1. Srinivasappa, K.N., Anuradha, M.N., Farooqi, A.A., Kathiresan, C., Suresh, H.C., and Shivananda, T.N. (2007). Use of bio-fertilizers in cultivation of medicinal and aromatic plants - a review. BIOMED, 2: 235-256.

2. Paturde, J.K., Wankhade, S.G., Khode P.P, Chatol, P.U, Deo, D.D. and Bhuyar, S.A. (2002). Effect of organic manures and plant population on yield of safed musli (*Chlorophytum borivallianum*). Agricultural Science Digest, 22: 51-52.

3. Sivakumar, C.V., and Vidhyasekaran, P. (1990). Control of Meloidogyne incognita on *Coleus Forskohlii* with *Paecilomyces lilacinus* in farm yard manure amended and non-amended soil. Journal of Biological Control, 4: 68-69.

4. Veeraraghavathatham, D, Venkatachalam, R., and Sundararajan., S. (1988). Effect of various levels of N, P and K on the tuber Yield of *Coleus forskohlii*. South Indian Horticulture, 36: 252-257.

5. Geetha, K., and Madhavan Nair., K.P. (1993). Effect of N and K in *Coleus*. Journal of Tropical Agricultural, 31: 198-203.

6. Somanath, I., Bhaskar, S., and Sreenivasmurthy C.A. (2007). Influence of FYM and NPK on uptake of nutrients and growth of *Coleus forskohlii*. BIOMED, 2: 265-271.

7. Tiwari, J.P., Tiwari, A.B., Tiwari, G., and Singh A.K. (2000). Effect of nitrogen application on growth and yield of *Acorus calamus*. Proceedings of the National Seminar on the Research and Development in Aromatic Plants. Current trends in biology, uses, production and marketing of essential oils, Lucknow, India, 30-31 July 1999. Journal of Medicinal and Aromatic Plant Sciences, 22: 636-638.

8. Maryada, M, Kaushal, S.K and Kaistha, B.P. (2001). Response of aswagandha (*Withania somnifera*) to nitrogen fertilization under mid hill conditions of Himachal Pradesh. Himachal Journal of Agriculture Research, 27: 77-80.

9. Hedge, D.M. (1988). Response of periwinkle (*Catharanthus roseus* L.) to nitrogen, phosphorus and potassium fertilization. Agricultural Research Journal of Kerala, 26: 227-233.

10. Maheshwari, S.K., Yadav, S, Gangrade, S.K and Trivedi K.C. (1988). Effect of fertilizers on growth, root and alkaloid yield of rauwolfia (*Rauwolfia serpentina*). Indian Journal of Agricultural Sciences, 58: 87-488.

11. Maheshwari, S.K. and Yadav, S. (1981). Response of *Withania somnifera* to variable levels of N and P rainfed. Paper presented at the IV All India Workshop on Medicinal

and Aromatic Plants, Madhurai, 31st August-September 3, pp: 11-12.

12. Sheu, B.W., Juan Y.H. and Yan H.F. (2000). The effects of organic and inorganic fertilizers on the growth and yield of Herng-Chuen Yams, *Dioscorea doryophora*. Bulletin of National Pingtung University of Science and Technology, 8: 103-107.

13. Muthumanickam, D and Balakrishna-murthy G. (1999). Studies on nutritional requirement for aswagandha (*Withania somnifera*) in sheuroy hills of Tamilnadu, India. Journal of species and Aromatic Crops, 8: 179-183.

14. Nigam K.B., Rawat G.S. and Prasad B. (1984). Effect of methods of sowing, plant density and fertility level on aswagandha (*Withania somnifera* Dunal.). South Indian Horticulture 32: 356-359.

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Information Technology Consolidation with Virtualization in a Contract Research Organization

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Abstract

The enterprise server and storage products from most of OEMs like IBM, HP etc support virtualization and continuity, enabling Contract Research Organizations (CRO) to consolidate workloads across diverse operating environments and to deploy new solutions quickly, reliably and with a significantly lower total cost of ownership. The key requirement of the CRO at Hyderabad is that the existing application and database servers be replaced with HP blade and integrity servers, storage and backup solution to be provided for the servers; and replication is setup between primary and secondary sites for database (db) and non-db data. HP solution achieved the disaster tolerence by implementing Metro Cluster for databases and applications. The Lab requires 99.9% high availability for all servers and scientific applications to be migrated from the old platform to the new HP platform.

Keywords: Server virtualization, high availability, content management, laboratory information management, database management.

Introduction

A Contract Research Organization (CRO) is a company or organization that offers a wide range of pharmaceutical services primarily to the pharmaceutical and biotechnology industries. The government defines a CRO as a corporation that assumes, as an independent contractor with the sponsor, one or more of the obligations of a sponsor to design, select, monitor, evaluate, and/or prepare materials to be submitted to the Food and Drug Administration [21 CFR 312.2(b)]. Services can include but are not limited to: product development, formulation activities, phase I through IV activities, data entry and management, project management, Institutional Review Board (IRB) approval, statistical analysis, and other required activities necessary to apply for a New Drug Application (NDA) or an Abbreviated New Drug Application (ANDA).

Contract work in basic research has evolved from low-end research activities to more value-added high-end research. Reputation for research quality and thoroughness, speed to project completion, and strong client relationships are key to success in research partnering. Domain expertise as an entry barrier is restraining contract research partnering opportunities. Availability of top of the line infrastructure and manpower is a defining factor for success in drug discovery contract research.

A host of players from Andhra Pradesh (India) are competing for CRO role such as Vimta Labs, Divi's Laboratories, Ocimum Biosolutions, Actimus Biosciences, Asian Clinical Trials Pvt Ltd, GVK Biosciences Pvt Ltd, iGate Clinical Research International, Sipra Labs Pvt Ltd, etc.

These leading CROs are performing cost-effective bio-equivalence / bioavailability (BE/BA) studies on various dosage forms of pharmaceuticals and to conduct testing services in the areas of clinical research, pre-clinical (animal) studies, clinical reference lab services, agriculture sector, bioscience, environmental assessments and analytical testing of a wide variety of products (1). One of the goals of the CROs is to help its customers reduce the cycle times of new drug discovery by connecting different stakeholders who are geographically distributed using automated clinical trial processes by leveraging technology solutions available on different platforms.

In general, the clinical data are collected through a variety of data acquisition systems like Web Forms, telephone interactive voice response (IVR), fax, clinical devices, hospital information and management systems/labs and provide a central storage for the collected data. The data are critical and sensitive and hence the security and privacy needs are high. The data collection and management is a process that is regulated by regulatory authorities and goes through regular audit checks. Life expectancy of the data is driven by customers as well as regulatory authorities. It is traditionally kept for fairly long periods. Over the past several years, however, economic pressures on drug developers have grown and CRO are now seen as a key means to contain ever-expanding R&D costs and shorten time to market by virtue of their ability to achieve economies of scope and scale in the testing process. Therefore, an early adoption of leading edge technologies is one of the key enablers for delivering high quality services and the CROs should have a policy to protect themselves from technological obsolescence by duly validating all technologies deployed. Leveraging new technologies enable to reduce the cost of services and improve the effectiveness of the solutions / services offered to its customers and to help business re-design/re-model its operational processes to take full advantage of the new technologies. The CRO must make sure that the data is protected and secured with a key principle. The aim of all CROs is to see that the objectives mentioned below are met:

1. Seamless access of the applications at Data Center (DC) or Disaster Recovery Center (DRC)
2. Improved infrastructure efficiency
3. More stable environment
4. Flexibility and ease of maintenance
5. Improve the data quality and security

Results and Discussion

"Data are provided to people on a need-to-know' basis". The problems for the CRO under study with the existing obsolete infrastructure architecture are agility and gaining competitive advantage and therefore, an adaptive infrastructure solution is necessary to accelerate growth and to reduce costs. The solution proposed for the CRO under study on HP platform can address this problem by providing powerful servers, sophisticated storage systems, and flexible consulting and support services.

There are a variety of factors that make the CRO business more challenging than before. Regulators around the world are increasingly asking for more detailed safety and security assessments not only of data in the data center but also on drug candidates. Given below some of the challenges in CRO:

Challenge: Improving performance, business continuity, data security and data integrity: To have a consolidated infrastructure model that can deliver applications to its disaster recovery (DR) and services to all its satellite centers while at the same time enabling VoIP and video

conferencing facilities between primary and secondary sites. With existing servers being saturated, and with limited bandwidth, high utilization and large data transfers, the challenges are in terms of avoiding costly upgrades and want to see the application performance not being compromised as well the future application deployment requirements are taken care of.

Challenge: Enable accelerated replication of data and virtual server infrastructure to enable disaster recovery (DR): To have disaster recovery replication strategy for business continuity and regulatory compliance reasons. Every CRO that is implementing DC/DR strategy should have two facilities geographically separated. One facility is designated as primary data center and the second one as secondary data center or disaster recovery site.

To address these challenges and pave the way for continued growth, CROs need to initiate steps like continued geographical expansion, deepening of service offerings and enhancement of technological expertise.

The consolidated IT environment will enable all its users globally many services like e-mail, file transfer protocol (FTP), network file transfers etc. The migration to leading edge technologies is expected to benefit CRO enormously in implementing scalable infrastructure besides enhancing customer satisfaction, increased employee productivity and reduced time to market its products and services.

The solution for the CRO under study: The demand for data quality, security and privacy continues to grow as clinical trials evolve with technological trends. The data acquisition has shifted from paper to electronic means. The role of data center and disaster recovery center are more important than before as the way the data being captured and managed are changing.

Infrastructure: Table 1 describes the new infrastructure proposed to replace the existing IBM systems at the data center.

The new infrastructure has met the following goals to fulfil the uptime commitments of CRO.

- High availability (24X7)
- No single point failure
- Security
- Manageability

System perspective

Figure 1 describes the IT environment of the CRO under study that comprises of physical servers, Local Area Network (LAN) switches and firewalls in addition to the servers that are being virtualized catering to varied business needs and scientific functions.The primary data centre has built-in redundancy in terms of core switches and servers, therefore, all resources are highly available on 24 X 7 scale. The data replication is carried over the metro link and the disaster tolerance is provided using clustering of servers within a site and between sites as well.

VMware

One of the primary benefits of server virtualization with VMware is that it allows IT organizations to consolidate the servers. The (Table 1) describes the virtualized applications that are made available in a single physical server which means a single physical server can support multiple virtual machines (VMs) and Fig 2 describes VMs specific to each application /or function within the data center. The applications that would normally require a dedicated server can now share a single physical server. The server virtualization results in a reduction in the number of servers in a data center, which leads to

Table 1 - New infrastructure at data center

Itanium			Intel		
Server Type	Function	Server Model/OS	Server Type	Server Model/Os	Function
Oracle	1. Database 2. Database Failover 3. Application 4. Application Failover	BL 870 / HP-UX	Web	BL460 / RHEL	1.Vimta Access 2. File server 3. Domain Controller
SAP	1. Database 2. Application 3. Failover Server	BL 870 / HP-UX	Application	BL460/ Win 2008	1. SDMS 2. LIMS 3. Empower 4. Trim Doc 5. Trim Event
Metro Cluster	Arbitration Server	rx2660/ HP-UX	VMware	BL460/ Win 2008	1. vCenter 2. VMware - Heartbeat server 3. VMware-SRM Server
Backup	1. Backup 2. Backup Failover 3. SAN Array Manager	BL460/ Win 2008	Infrastructure	BL460/ RHEL	1. Proxy 2. DHCP 3. DNS 4. Addl. Domain Controller
				BL460/ Win 2008	1. HPSAM 2. HP Client Automation 3. HP Site Scope
Non Production	Test and Development	BL460c / RHEL/Win 2008	Mail	BL460/ Win 2008	1.Mdaemon 2.Notes

significant savings in Capital Expenditure (CapEx) and Operational Expenditure (OpEx). ESX server that supports full virtualization has been implemented at the CRO under study to

create virtual machines. A virtual network is created between VMs hosted on the same physical system with no virtual network traffic consuming bandwidth on the physical network. The solution that has been implemented at the CRO under study has the virtual infrastructure containing VMware VMotion, VMware® Distributed Resource Scheduler (DRS) and VMware® High Availability (HA). VMotion allows VMs to be seamlessly relocated to different systems while keeping their MAC addresses (1). There is no downtime required to move VMs within compatible pools of servers. The CRO has deployed 90% of Windows and Linux servers running inside of VMware ESX server as virtual machines and the complex applications like Lotus Notes and HP Trim (Content Management Software) have been implemented inside virtual machines and found to be running very smoothly.

Server virtualization helps :

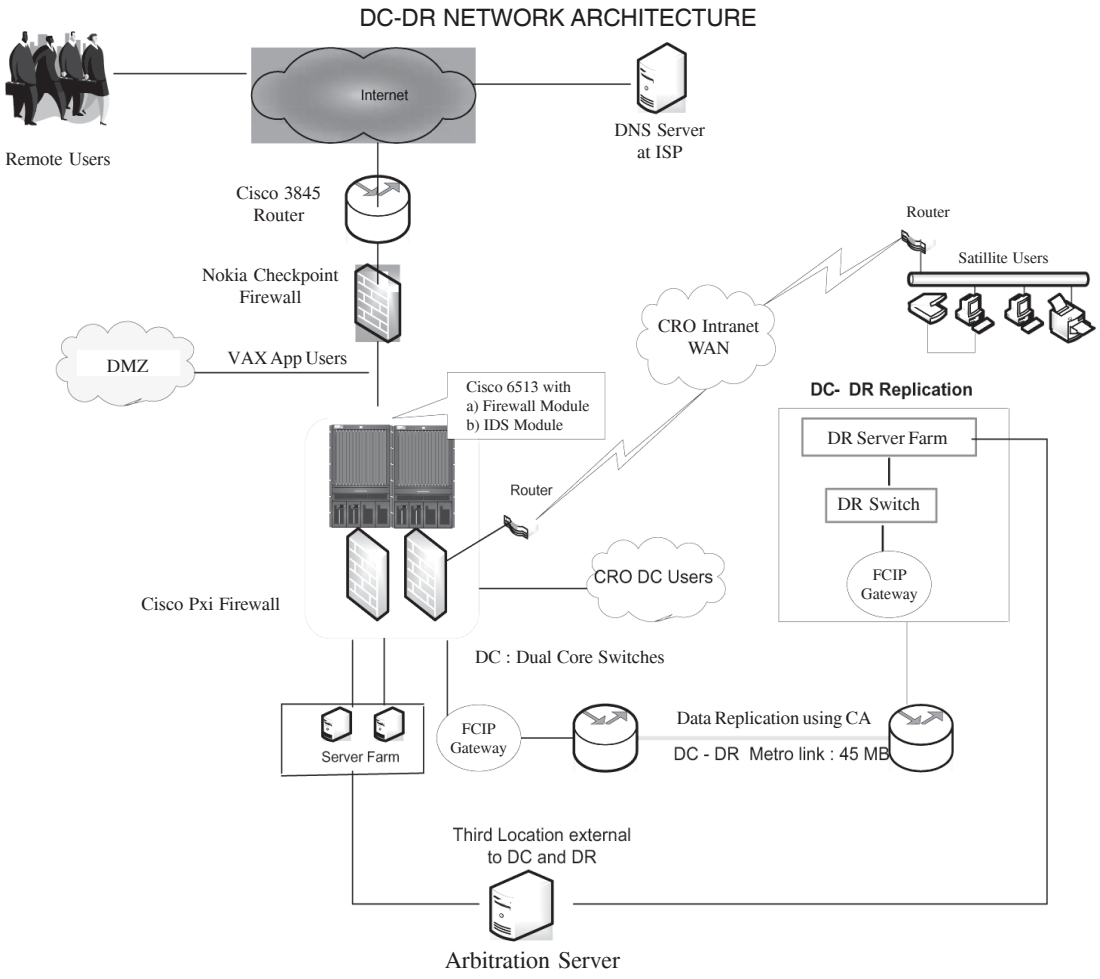

Fig. 1: The network architecture of CRO under study

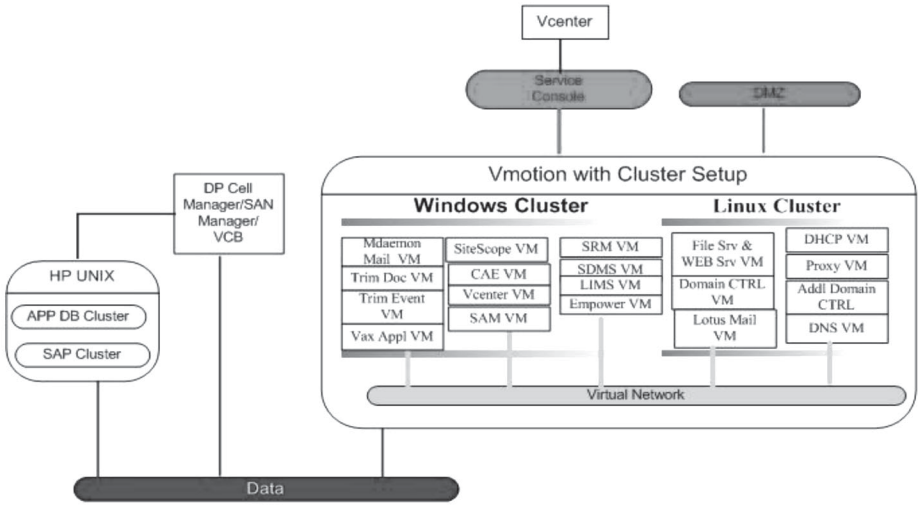

Fig. 2. Data center with virtualization

1. To increase hardware utilization and reduces hardware requirements with server consolidation (also known as Physical-to-Virtual or P2V transformation).
2. To reduce required data-center rack space, power cooling, cabling, storage and network components by reducing the sheer number of physical machines.
3. To improve application availability and business continuity independent of hardware and operating systems.
4. To improve responsiveness to business needs with instant provisioning and dynamic optimization of application environment.

Virtualization has brought an advantage to make data center more dynamic and providing performance, flexibility and capacity at a much lower cost, enabled the automatic and dynamic allocation of resources depending on workloads and business requirements. VMware technology has helped to replicate data from primary to secondary with Site Recovery Manager (SRM) thereby creating resiliency from a disaster recovery perspective as well as creating the snapshot of these virtual machines to replicate them to other sites (2).

Data center applications: The CRO under study has a variety of applications deployed at its data center. Some of the key systems deployed have been described below.

Enterprise management system: As the virtual IT infrastructure becomes more dynamic in order to meet on-demand application delivery, the Enterprise Management Software (EMS) tools given below are used to control and manage the infrastructure at the CRO.

SiteScope: At CRO, SiteScope (3) has been implemented that helps to monitor the availability of hardware along with operating system as well as the status of all applications deployed at DC and DR SiteScope is a Web-based agent-less monitoring solution designed to ensure the availability and performance of distributed IT infrastructure. The real time data collected from SiteScope by monitoring the total infrastructure would be used for trend analysis

and for generating performance reports. The solution presents a single view of the total infrastructure of CRO i.e. single view of system, network and applications metrics.

Client automation enterprise (CAE) : Client Automation Enterprise (3) has been implemented to collect the hardware and software inventory details of each desktop in the CRO besides distributing software simultaneously to all these desktops from centrally located server. By deploying this tool, the CRO will derive the following benefits.

1. Monitoring the application usage pattern on individual desktops and hence helps to strategize the application distribution across the desktops.
2. Monitoring and managing the licenses installed on each desktop.
3. To have a homogenous baseline desktop environment.
4. To keep all desktops in synchronization with latest software releases from application or Operating Systems (OS) specific vendors.

Content management : The CRO has been using Lotus Domino document manger for managing documents and its workflows. However, it has been decided to phase out the existing Domino Document Manager because of the following reasons.

1. It has reached end of life with no support from IBM which is a major concern for some organization like Vimta.
2. It does not meet specific regulatory requirements such as 21 Code for Federal Regulation (CFR) Part 11.
3. It has performance problems due to resource constraints.
4. Growing licensing requirements because of tight integration with Lotus mail.

HP TRIM (Transparent Records & Information Management) document management system, shown in (Fig 3), is a transparent records and information management system that can capture, share and manage information by applying automated rules, classifications and workflows. It adheres to ISO 15489 quality standards and complies with US FDA (Federal Drug Agency) 21 CFR Part 11 guidelines (2). TRIM document solution implemented at the Labs under study addresses the above problems besides replacing the current document management with a leading edge solution from HP. All the existing data and customization has been exported to TRIM using tools and techniques provided by HP. HP TRIM depends on Lightweight Directory Access Protocol (LDAP) for user authentication and it minimizes the cost impact by alleviating the earlier licensing requirements.

Scientific applications: A clinical trial is sponsored by an organization and various

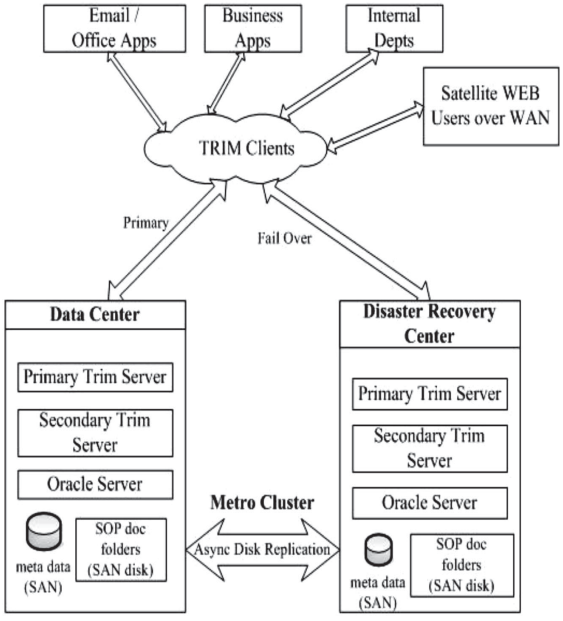

Fig 3. TRIM high level view

trial process. The CRO's role under study in trial process is Contract Research and Testing Organization (CRTO) and site management. Each role has different service requirements which are met by various scientific applications that the organization has deployed at its data center. The major applications in the laboratory are described below.

Laboratory Information Management System (LIMS) : LIMS is a full-featured client-server application on Microsoft Windows that meets all Good Laboratory Practices (GLP) requirements by providing full sample tracking, user certification, instrument management, standard and reagent management, full auditing, report and sample scheduling, on-line help, and many other functions. LIMS was designed to conform to the laboratories environment through configuration; not by custom programming. While most customers' needs are met by off the shelf LIMS product, there are times that extensions to the system are desirable. To meet these extensions, LIMS open architecture allows configuration changes and customization using basics (4).

Empower: Empower 2 is Waters' flagship chromatography data software (CDS) package for advanced data acquisition, management, processing, reporting and distribution. Designed for a data-secured regulatory lab environment, Empower 2 will help labs to perform more efficiently and securely (4). It can help:

- Store and retrieve chromatography data using an embedded, relational database.
- Calculate more precise peak integrations with ApexTrack™ algorithm. .
- Achieve more enhanced detection capabilities when using Waters mass detectors or the Waters Photodiode Array (PDA) detector
- Grow seamlessly from a single workstation to an enterprise-wide system with Empower 2's scalable, modular architecture

SDMS: SDMS is a Scientific Data Management System (SDMS) is used to create a common electronic repository for all types of scientific data in the lab environment. SDMS allows the scientist in the lab to focus on the core work by eliminating the burden of paper work. SDMS improves the value of information by enabling scientist to share and utilize the data generated from a range of laboratory applications. The workflow in SDMS ensures that the documents can be viewed by analysts, group leader, quality control (QC) and QA to review and approve on time. SDMS is used for Project Management and storage and retrieval of instrumental and non-instrumental data with compliance to 21CFR Part11 of USFDA (4).

Analysis: (Fig 4, Fig 5) are drawn using the data collected from two different implementations i.e. old and new database servers.

The old database server is hosted on IBM AIX version 5.3. From figure 4, it is obvious

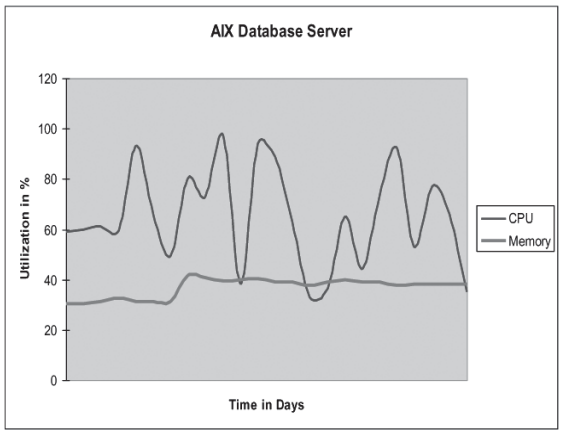

Fig. 4. CPU and memory utilization of the old database server

that central processing unit (CPU) utilization is found to be varying between 97 to 32 % with an average of 65% consumption where as memory utilization is very steady with an average of 37% consumption.

The new database server is hosted on HP UNIX. From figure 5, it is clear that the average CPU utilization has dropped to 17.5% and average memory utilization to 5% even after

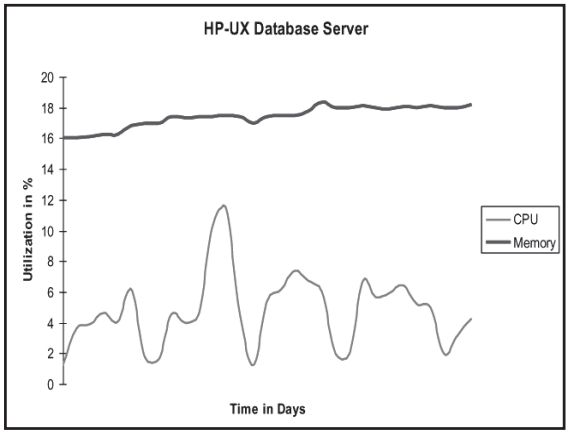

Fig. 5. CPU and Memory Utilization of the new db server

adding additional databases for new applications like SiteScope, CAE and HP TRIM. Therefore, the server on the new platform offers more scalability and manageability besides taking care of all future requirements of the Labs under study for the next decade.

Conclusion

The ability to replicate the most critical systems on a continuous basis has been a huge win for the business. Majority of the disaster recovery systems are available as hot backups to the production system at a primary data center. The primary goal of CRO under study is addressed i.e. data storage and retrieval as stored and making systems highly available for data access. The new implementation will benefit the customers as the data access continues to be available even if the entire site goes down. The new implementation with leading edge technologies will help CRO under study to attract more customers, which benefits its investors ultimately.

Acknowledgement

We gratefully acknowledge the support of all members of Vimta Labs especially Mr. Harriman, Director (Technical) for his advice and encouragement in preparing this paper. We are thankful to all other websites for providing the valuable information on data center virtualization.

References

1. Source: for Vimta Labs information – www.vimta.com
2. Source: for VMware - www.vmware.com
3. Source: for HP products - www.hp.com
4. Source: for Waters products - www.waters.com

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In-vitro Antioxidant and Antilipidemic Activities of *Xanthium strumarium* L.

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Abstract

In the present study Chloroform and alcohol extracts of *Xanthium strumarium* L. were evaluated for *in vitro* antioxidant and anti-lipidemic activity by various methods namely total reducing power, scavenging of free radicals such as 1,2-diphenyl-2-picrylhydrazyl (DPPH), super oxide, nitric oxide, and hydrogen peroxide. The percentage scavenging of various free radicals were compared with standard antioxidants such as ascorbic acid and Butylated hydroxyl toluene (BHT). The extracts were also evaluated for anti-lipidemic activity in Triton WR-1339 (iso-octyl polyoxyethylene phenol)-induced hyperlipidemia in Swiss albino rats by estimating serum triglyceride, very low density lipids (VLDL), cholesterol, low-density lipids (LDL), and high-density lipid (HDL) levels. Significant antioxidant activity was observed in all the methods, significant reduction ($P < 0.01$) in cholesterol at 6 and 24 h and ($P < 0.05$) at 48 h. There was significant reduction ($P < 0.01$) in triglyceride level at 6, 24, and 48 h. The VLDL level was also significantly ($P < 0.05$) reduced from 24 h and maximum reduction ($P < 0.01$) was seen at 48 h. There was significant increase ($P < 0.01$) in HDL at 6, 24, and 48 h. From the results; it is evident that alcohol and chloroform extracts of *X. strumarium* can effectively decrease plasma cholesterol, triglyceride, LDL, and VLDL and increase plasma HDL levels.

Keywords: Anti-lipidemic, antioxidant, *X. strumarium*, triton

Introduction

Oxidation is one of the destructive processes, wherein it breaks down and damages various molecules. Oxygen via its transformation produces reactive oxygen species (ROS) such as super oxide, hydroxyl radicals, and hydrogen peroxide. They provoke uncontrolled reactions (1). Molecular oxygen is an essential component for all living organisms, but all aerobic species suffer from injury if exposed to concentration more than 21% (2).

Free radicals attack and induce oxidative damage to various bio-molecules including proteins, lipids, lipoproteins, and DNA. The body possesses several defense systems comprising enzymes and radical scavengers. Some of them constitute the repair systems for bio-molecules that are damaged by the attack of free radicals (2, 3).

An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer, diabetes mellitus,

atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive sleep, apnea, and other diseases. The process of aging may result, at least in part, from radical-mediated oxidative damage was very well explained long ago (4).

Antioxidants are compounds that act as inhibitors of the oxidation process and are found to inhibit oxidant chain reactions at small concentrations and thereby eliminate the threat of pathological processes (1). Phenolic compounds present in medicinal plants have been reported to possess powerful antioxidant activity (2). Flavonoids are a major class of phenolic compounds present in medicinal plants and are found to have a potential role in prevention of various diseases through their antioxidant activity (5).

X . strumarium (Asteraceae), is a shrub found throughout India and warmer parts of the world. This plant has been widely reported to have several medicinal properties in traditional form of medicine. The beneficial properties are diuretic, astringent, sedative, demulcent, diaphoretic, analgesic, sialagogue, styptic, sudorific, tonic, anodyne, antibacterial, antifungal, antispasmodic, bactericide, bitter, depressant, hemostat, laxative, refrigerant and anti- rheumatic. The leaves and roots are often used as anodyne, anti-rheumatic, appetizer, diaphoretic, diuretic, emollient, laxative and sedative, anti-malaria, bitter tonic, febrifuge and the treatment of scrofulous tumors. A decoction of the root has been used in the treatment of high fevers and oxytocic. Similarly, the seed's decoction is used in the treatment of bladder complaints and poultice of the powdered seed has been applied to treat open sores. The fruits are also reported for their use in the treatment of lesions, hypoglycemia, scrofula, fever, herpes, and cancer. *X. strumarium* is also used for many domestic purposes. The fruits are boiled in water and still drank as a tea in some parts of Asia. Cocklebur is also used in the manufacture of dye, repellent, and tannin. The dried plant repels weeevils from stored wheat grain (6). There are also very few reports available on the phytochemical and pharmacological activities of *Xanthium strumarium* L. and other species related to the genus *Xanthium.*

Earlier, workers on this plant have extensively used the aerial parts of the plant, whereas the root being one of the important parts of the plant where in most of the active constituents are stored has not been subjected for systematic investigation. They are no reports to show the use of roots of *X. strumarium* for the screening of phytochemical analysis and pharmacological investigations. Hence, the root served as the core material for all the investigations carried out in the present study. Since polyphenolic compounds are present in the ethanol and chloroform extracts of root of *X. strumarium.*, it was thought that it would be worth while to evaluate the plant for antioxidant activity. Lipids are one of the most susceptible targets of free radicals. This oxidative destruction is known as lipid per oxidation and may induce many pathological events. Apart from antioxidant studies, the present study therefore also involves evaluation of anti-lipidemic activity.

Materials and Methods

***Plant material and extraction*:** The root of *X. strumarium* were procured and authenticated from Dr. Krishna, Professor and Taxonomist, Department of Bio-technology, Kuvempu University, Shimoga. The authenticated root was dried in shade and powdered coarsely. Extraction was done according to standard procedures using analytical grade solvents. Coarse powder of the root was (1 kg) Soxhlet extracted with 90%

ethanol. The chloroform extract was prepared using the same marc by the process of maceration. The extracts obtained were concentrated under reduced pressure to yield the ethanol extract and chloroform extract of root (4.3 and 4.2%, respectively).

Preparation of test solution: The various extracts of *X. strumarium* root such as alcohol and chloroform were used for *in vitro* antioxidant studies. Pilot studies were carried out for the ethanol and chloroform extracts of root *X. strumarium* for *in vitro* antioxidant studies and the concentrations at which the extracts gave good antioxidant activity was selected.

Animals: Albino male Wister rats weighing between 150 and 200 g were procured from registered breeders. The animals were housed under standard conditions of temperature (25 ±2°C) and relative humidity (30-70%) with a 12:12 light-dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. Approval of the Institutional Animal Ethics Committee (IAEC) of SJM College of Pharmacy, Chitradurga, was obtained.

***Acute toxicity studies*:** Acute toxicity studies for chloroform and ethanol extracts of *X. strumarium* were conducted as per OECD guidelines 423(7) using albino Wister rats. Each animal was administered the aqueous solution of the extract by oral route. The animals were observed for any changes continuously for the first 2 h and up to 24 h for mortality.

***Antioxidant studies*:** The ability of the extracts to scavenge hydrogen peroxide, DPPH (1, 2-diphenyl-2-picrylhydrazyl) radical (8), nitric oxide (9,10), superoxide radical (4), and its reducing power (8) was determined at different concentrations. Butylated hydroxy toluene (BHT) and ascorbic acid were used as standards for the various *in vitro* antioxidant studies. The percentage scavenging of various radicals were calculated using the following formula:

$$\% \text{ Radical scavenged} = \frac{A_0 - A_1}{A_0}$$

Where A_0 is absorbance of the free radical alone and A_1 is absorbance of free radical in the presence of extract/standard. All the experiments were performed in triplicate.

***Antilipidemic activity*:** The method of Tamasi *et al* (11). was used for evaluation of anti-lipidemic activity. Albino Wister rats weighing between 150 and 250 g were assigned to various groups of six animals each. Animals were fasted for 16 h prior to the experiment with water *ad libitum*. The *X. strumarium* ethanol and chloroform extract each at doses of 200 and 400 mg/kg body weight, Simvastatin at 4 mg/kg and Fenofibrate at 20 mg/kg, were administered p.o. Group I served as control. On the day of the experiment, the animals of the groups II-IV received the respective drugs by oral route. Simultaneously, all the animals received Triton WR-1339 at 100 mg/kg body weight by intraperitoneal route. The control animals were given only Triton WR-1339 at 100 mg/kg body weight. Serum cholesterol, triglyceride, and HDL were estimated at 6, 24, and 48 h using AGAPPE diagnostic kits. Blood samples were withdrawn by retro orbital puncture. Total cholesterol was estimated by CHOD-PAP methodology, Triglycerides by GPO-PAP methodology, and HDL by the precipitation method using phosphotungstate magnesium acetate reagent.

$$\text{VLDL was calculated using the formula, VLDL} = \frac{\text{Triglycerides}}{5}$$

LDL cholesterol was calculated as

$$\text{LDL} = \text{Total Cholesterol} - \text{HDL} - \frac{\text{Triglycrides)}}{5}.$$

Chemicals: The chemicals DPPH (1,2-diphenyl-2-picrylhydrazyl), N-(1-Naphthyl) thylenediamine dihydrochloride, Triton WR-1339, NADH, SNP, phenazine methosulphate, trichloro acetic acid, and potassium ferricyanide were purchased from Sigma Chemicals, St Louis, MO, USA. All other chemicals and reagents used were of analytical grade. UV-1700 Shimadzu UV-Vis spectrophotometer was used for *in vitro* anti-oxidant studies.

Statistical analysis: All the values are presented as mean SD. Data were statistically analyzed by one-way ANOVA followed by post hoc test; P values < 0.05 were considered as statistically significant. Linear regression analysis was used for calculation of IC_{50}.

Acute toxicity studies: There was no mortality and noticeable behavioral changes in all the groups tested. The chloroform and ethanol extracts of root of *X.strumarium* were found to be safe up to 2000 mg/kg body weight.

Results and Discussions

***Hydrogen peroxide scavenging activity*:** At 125 µg/ml concentration, the chloroform extract and ethanol extract 100 µg/ml produced H_2O_2 scavenging activity comparable ($P < 0.05$) to that of the standards BHT and ascorbic acid and were found to have IC_{50} (mean ± SD) of 8.89±1.10,9.15±1.21,10.18±1.50,9.23±0.91, respectively (Fig. 1; Table-1).

DPPH radical scavenging activity: The chloroform and ethanol extract produced significant DPPH radical scavenging activity. The IC_{50} (mean ± SD) of ascorbic acid, BHT and other two extracts were found to be 27.35±1.20, 26.01±0.93, 29.81±0.95, 24.85±2.49 respectively (Table-1; Fig.1)

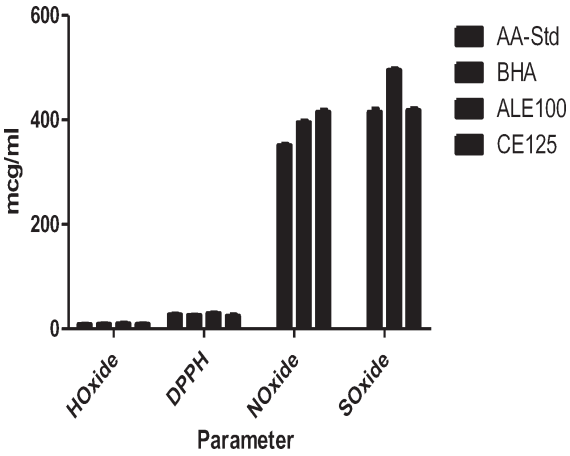

Fig. 1. Free radical activity of different extracts of *X. strumarium*

Table 1. Free radical scavenging activity of different activities of *X. Strumanium*

Test/Std	IC50 Values ± SD(µg/ml)for free radical scavenging activity			
	Hydrogen peroxide	DPPH	Nitric oxide	Superoxide
Ascorbic acid	8.89±1.10	27.35±1.20	--	--
BHA	9.15±1.21	26.01±0.93	351.01±2.72	415.14±5.98
Alcoholic extract 100	10.18±1.50	29.81±0.95	395.20±3.18	495.30±3.09
Chloroform extract 125	9.23±0.91	24.85±2.49	415.18±3.83	418.30±4.15

***Nitric oxide radical scavenging activity*:** Scavenging of nitric oxide by various extracts was found to be concentration dependent. Maximum inhibition of nitric oxide formation was produced by alcoholic extract and had IC_{50} of 495.30±3.09 as against 415.14± 5.98 for BHA (Fig. 1; Table-2).

Total reducing power: From 100 µg/ml, chloroform and ethanol extracts and standard-ascorbic acid and BHA showed reductive capabilities. At 500 µg/ ml, the reducing power of standards and extracts showed the following order: BHA > ethanol extract > Ascorbic acid > chloroform. Reducing power of ethanol and chloroform extracts at 500µg/ml was comparable ($P < 0.05$) to that of ascorbic acid (Table-1).

***Superoxide anion radical scavenging activity*:** Superoxide anion radical generation was

Table 2. Total reducing power, nitric oxide and super oxide scavenging activity of different concentrations of AA.BHA.XSCA,XSAE

Group(µg/ml)	Reducing power(Abs)	Nitric oxide%	Superoxide scavenged %
AA			
100	0.08±0.01	--	--
200	0.16±0.01	--	--
300	0.46±0.02	--	--
400	0.66±0.03	--	--
500	0.87±0.02	--	--
BHA			
100	1.11±0.01	11.34±0.41	18.40±0.33
200	0.22±0.02	18.36±0.45	27.34±0.32
300	0.53±0.04	32.45±0.21	37.32±0.21
400	1.21±0.03	39.51±0.23	57.43±0.12
500	1.57±0.04	59.87±0.23	64.34±0.14
CE			
100	0.04±0.01	9.31±0.21	12.31±0.21
200	0.07±0.01	14.45±0.25	25.45±0.14
300	0.15±0.02	29.32±0.15	34.31±0.11
400	0.41±0.02	35.23±0.14	54.43±0.24
500	0.69±0.03	54.53±0.23	61.76±0.14
AE			
100	0.10±0.02	10.13±0.21	16.24±0.23
200	0.18±0.03	16.34±0.25	23.21±0.12
300	0.49±0.02	22.45±0.11	34.02±0.10
400	0.72±0.04	35.21±0.21	55.13±0.12
500	0.91±0.02	52.87±0.12	61.14±0.11
Values are mean ± SD [1]P<0.05, [2]P<0.01, [3]P<0.001as compared to AA, BHA, CE and AE			

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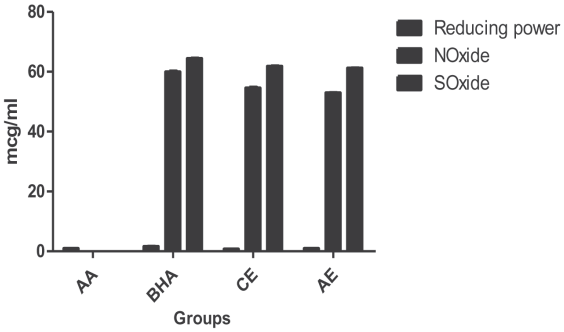

Fig. 2. Comparison of Total reducing power, Nitric Oxide and Super-oxide scavenging activity of 100mcg/ml of AA, BHA, Alcohol and Chloroform Extract of the root of *X.*

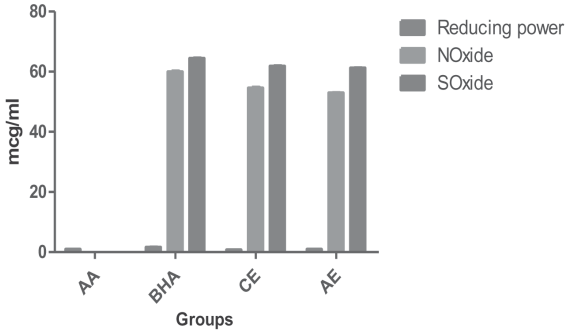

Fig. 3. Comparison of Total reducing power, Nitric Oxide and Super-oxide scavenging activity of 500mcg/ml of AA, BHA, Alcohol and Chloroform Extract of the root of *X. strumarium*

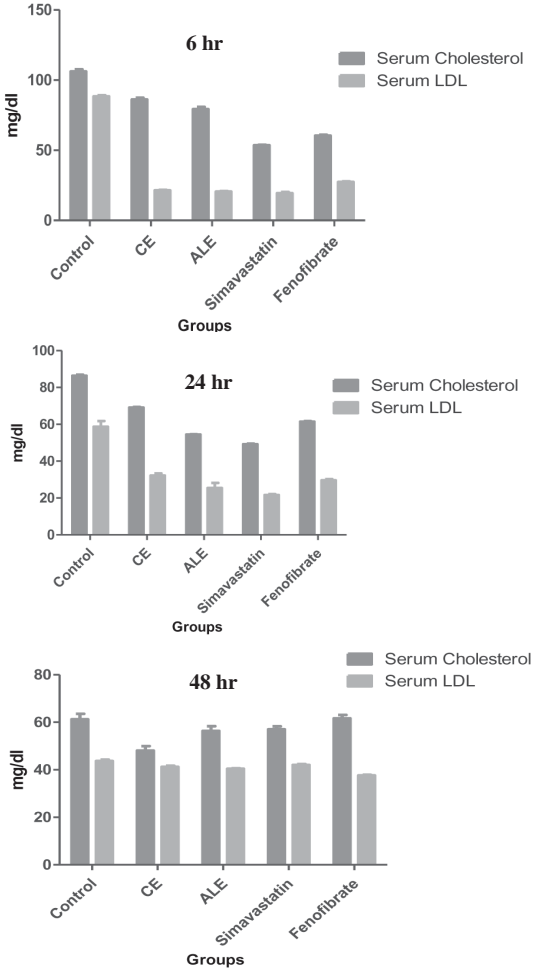

Fig. 4. Effects of Chloroform and ethanol extracts of root of *X.strumarium* on total cholesterol and low density lipids levels in triton-induced hyperlipidemic rats

inhibited by BHA (standard) and extracts from 100µg/ml. Both the extracts produced significant superoxide radical scavenging activity in a concentration-dependent manner. Chloroform extract showed the lowest IC_{50} value (418 ± 4.15) which is better than BHA (415.14 ± 5.98).

***Antihyperlipidemic activity*:** The non ionic detergent, Triton WR-1339, has been widely used to block the uptake of triacyl glycerol rich lipoprotein from plasma by peripheral tissues in order to produce acute hyperlipidemia in animal models which are often used for a number of objectives. The parenteral administration of Triton induces hyperlipidemia in adult rats, where the blood cholesterol and triglycerides levels were reached maximum in 20hrs, followed by a decline to normal values (12-14). Administration of Triton resulted in increase in serum levels of cholesterol, triglycerides, VLDL, and LDL. A significant reversal in serum levels of cholesterol, triglycerides, VLDL, and LDL levels was noticed in the animals treated with *X. strumarium* root extracts when compared with the control group (Table-3). Simvastatin (standard) produced maximum cholesterol- and LDL-lowering effect at both 6 h and 24 h. ethanol extract 200 and Chloroform extract 400 produced a significant decrease in serum cholesterol and

Table 3. Effects of chloroform and ethanol extracts of root of *X. strumarium,* total cholesterol and low density lipids levels in triton-induced hyperlipidemic rats.

Group	6 hr		24 hr		48 hr	
	Serum Cholesterol (mg/dl)	Serum LDL (mg/dl)	Serum Cholesterol (mg/dl)	Serum LDL (mg/dl)	Serum Cholesterol (mg/dl)	Serum LDL (mg/dl)
Control	106±1.56	88.32±0.73	86.34±0.65	58.67±3.14	61.23±2.34	43.67±0.64
CE	86.12±1.20	21.35±0.32	69.12±0.37	32.21±1.18	48.12±1.87	41.24±0.46
ALE	79.23±1.56	20.43±0.41	54.43±0.19	25.45±2.76	56.34±2.02	40.42±0.19
Simvastatin	53.47±0.34	19.32±0.87	49.20±0.32	21.65±0.45	57.00±1.27	42.00±0.42
Fenofibrate	60.34±0.54	27.32±0.36	61.45±0.36	29.65±.67	61.65±1.43	37.63±0.25

Table 4. Effects of chloroform and ethanol extracts of root of *X. strumarium,* on total triglyceride and low density lipids levels in triton-induced hyperlipidemic rats.

Group	6 hr		24 hr		48 hr	
	Total TG (mg/dl)	Serum VLDL (mg/dl)	Total TG (mg/dl)	Serum VLDL (mg/dl)	Total TG (mg/dl)	Serum VLDL (mg/dl)
Control	67.43±0.23	12.78±0.25	52.34±0.54	11.12±0.31	72.21±0.18	14.32±1.03
CE	61.21±0.43	11.26±0.21	62.45±0.23	11.34±0.12	62.67±0.31	11.98±1.10
ALE	60.34±0.71	10.43±0.17	59.98±0.31	10.32±0.16	59.34±0.51	11.42±1.08
Simvastatin	62.31±1.21	11.82±0.34	62.14±0.46	11.45±0.23	64.26±0.43	12.78±0.65
Fenofibrate	54.38±0.35	10.97±0.32	53.65±0.54	9.32±0.43	56.98±0.45	11.46±0.21

LDL levels, which was found to be significantly greater than the effects of fenofibrate (at 6 h, 24 h and 48 h) and simvastatin (at 48 h) (Table-3; Fig. 3). Maximum reduction of triglyceride and VLDL levels was produced by fenofibrate at 6 h. At 24 h and 48 h, Chloroform and ethanol extracts of *X. strumarium* root produced significant triglyceride- and VLDL-lowering effect which was comparable to that of fenofibrate and significantly greater than that of Simvastatin (Table-4, Fig-4). Simvastatin, fenofibrate, chloroform 400 and ethanol 200 extracts 200 produced significant ($P < 0.01$) increases in serum HDL level at 6, 24, and 48 h when compared to control. At 6 h and 24 h ethanol 200 and chloroform400 extracts produced a significant ($P < 0.01$) increase in HDL level, which was significantly greater than that of simvastatin and fenofibrate. At 48 h ethanol 200 and chloroform 400, produced significant ($P < 0.01$) increase in HDL level, which was significantly greater than that of simvastatin and fenofibrate (Table-5; Fig-5).

The potentially reactive species of oxygen ascribed as ROS such as superoxide radical, hydroxyl radical, and hydrogen peroxide are continuously generated inside the human body as a consequence of exposure to exogenous chemicals and/or a number of endogenous

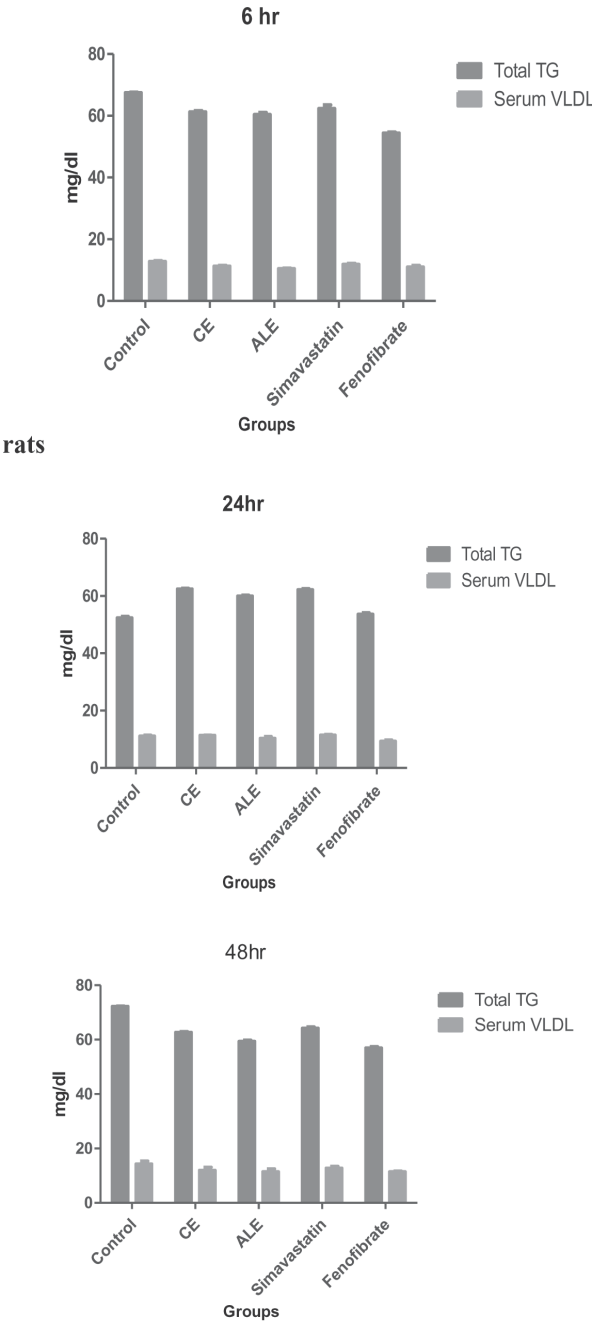

Fig. 5. Effects of Chloroform and ethanol extracts of root of *X. strumarium* on total triglyceride and very low density lipids levels in triton- induced hyperlipidemic rats

metabolic processes involving redox enzymes and bioenergetics electron transfer .Owing to the ROS overproduction and/or inadequate antioxidant defense, there is upsurge of ROS and this culminates in oxidative stress. It is quite interesting to note that plants have good antioxidant ability and are safer than the synthetic antioxidants . The antioxidant activity can be attributed to various mechanisms like prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity, and radical scavenging activity (4).

In the present study, five different antioxidant methods for evaluation of antioxidant activity have been used. Ethanol and chloroform extracts of *X. Strumarium* root produced significant antioxidant activity. This can be attributed to the flavonoids and other phyto-constituents present in the extracts, similar lipid lowering activity was also observed in the leaf extracts of *A.marmelos* which may be attributed to the phyto-chemical constituents present in it, such as flavonoids, saponins and phenolic ingredients as reported (15-16). Hyperlipidemia is one of the important risk factors involved in the development of cardiovascular diseases (17). Increase HDL levels and reduction in LDL shows the intensive conversion of LDL to HDL and clearance of circulating lipids. Total cholesterol/ HDL-C ratio of >4.5 is associated with increased coronary heart disease (CHD) risk and the ideal ratio is <3.5(15, 16). Atherosclerosis and congestive heart diseases are strongly associated with disorders of lipid metabolism and plasma lipoproteins. Triton WR-1339-treated rats are considered to be a useful acute hyperlipidemic model associated with inactive lipoprotein lipase (18). Triton WR-1339 acts as a surfactant to block the uptake of lipoprotein from the circulation by extra hepatic tissues resulting in an increase in the level of circulatory lipoproteins (19).

Ethanol and chloroform extracts of *X. Strumarium* root produced significant cholesterol

Table 5. Effects of chloroform and ethanol extracts of root of *X. strumarium* on high density lipid levels in triton-induced hyperlipidemic rats

Group	Serum HDL(mg/dl)		
	6 hr	**24 hr**	**48 hr**
Control	36.23±1.87	35.21±1.10	32.45±0.32
CE	42.67±1.34	41.78±1.35	40.12±0.42
ALE	43.32±1.28	42.56±1.10	40.31±0.18
Simvastatin	44.13±0.43	41.32±0.65	39.25±0.36
Finofibrate	44.34±0.26	40.39±0.87	40.46±0.54

and LDL lowering effect at 6, 24, and 48 h. This indicates that *X. strumarium* not only reduces the synthesis of cholesterol, but may also reduce its metabolism. The extracts were found to be enriched in flavonoids and phenolic compounds, it is reported that flavonoids are found to inhibit HMG-CoA reductase activity (18). It may be concluded that the cholesterol-lowering effect of *X. Strumarium* and root extracts may be due to inhibition of HMG-CoA reductase activity. Simvastatin being a specific HMG-CoA inhibitor produces its hypocholesterolemic activity by reducing cholesterol synthesis.

Increase in triglyceride level was evident in control animals due to inhibition of lipoprotein lipase (LPL) by Triton. Treatment with ethanol and chloroform extracts of *X. strumarium* resulted in reduction of triglyceride levels. It is likely that treatment with *X. strumarium* might have lowered the serum triglyceride level by activating LPL. LPL is a prime enzyme related to triglyceride metabolism. Further VLDL levels were reduced significantly at 24 and 48 h. Both the extracts also showed protective action by increasing serum HDL level. The increased HDL facilitates the transport of triglyceride or cholesterol from serum to liver where it is catabolised and excreted out of the body. Significantly greater increase in HDL levels was produced by chloroform extract than ethanol extract.

Conclusion

The chloroform and ethanol extracts of *X. strumarium* Linn. have shown significant antioxidant activity. From the preliminary studies, it was found out that the chloroform and ethanol extracts of *X. strumarium* have shown promising anti-hyperlipidemic activity. The antihyperlipedimic may be due their significant antioxidant activity.

References

1. Nasik, S.R. (2003). Antioxidant and their role in biological functions: An overview. Indian Drugs. 40:501-515.
2. Shetgiri, P.P. and D'Mello, P.M. (2003). Antioxidant activity of flavanoids-A comparative a study. Indian Drugs. 40:567-569.
3. Gopinathan, N., Srinivasan, K.K. and Mathew, J.E. (2004). Free radical scavenging properties of ethanol extract of *Saccharum spotaneum*. Indian Drugs. 41:633-635.
4. Gurpreet Kaur, Saqrwar, A.M., Zoobi, J., Kaleem, J and Mohemmad, A. (2006).

Evaluation of antioxidant activity of *Cassiaa siamea* flower. J, Ethanopharmacol. 108:340-348.

5. Droge, W. (2002). Free radicals in the physiological control of cell function. Physiol Rev. 82: 47-95.
6. Agharkar, S.P. (1991). Medicinal plants of Bombay presidency. Scientific Publishers, Jodhpur. (India). 230.
7. Available from:www.oecd.org.chs.
8. llhami, G.A., Haci, A.A and Mehmet, C. (2005). Determination of in vitro antioxidant and 15 radical scavenging activities of propofol. Chem Pharma Bull. 53:281-285.
9. Yaowared, S., Yukihisa, M., Michihisa, T., Opa, V., Kinzo, M., Hiroshi, W. (2004). Evaluation of the nitric oxide radical scavenging activity of Manganese Complex of curcumin and its derivative. Biol Pharma Bull. 271:70-3.
10. Kumar, R.S., Sivakumar,T., Sunderm, R.S., Gupta, M., Mazumdar, U.K., Gomthi, P. (2005). Antioxidant and antimicrobial activities of *Bauhinia racemosa* L.stem bark. Braz J Med Biol Res. 38:1015-24.
11. Tamasi, G., Borsy, J. and Patthy, A. (1968). Effect of 3-carboxyl-5-methylpyrazole (CMP) on serum and liver enzymes. Biochem Pharmacol. 7:1789-794.
12. Sarvanakumar, A., Vanitha, S., Ganesh, M., Jayaprakash, J., Ramaswamy, N.M. (2010). Hypolipidemic activity of *Sesbania grandiflora* in Triton WR-1339 induced hyperlipidemic rats. International Journal of Phytomedicine. 2:52-58.
13. Arunkumar Dubey, Ahalya devi, Gopalan Kutty and Ravi Pathiyil Kutty. (2005). Hypolipidemic activity of Ginkobiloba Extract, EGb 761 in hypercholesrolemia Wiistar Rats. Indian Journal of Pharmaceutical Technology. 41:9-12.
14. Firdous S.M., Banerjee Subhasis and Koneri R. (2010). Antihyperlipidemic activity of *Momordica dioica* Roxb. Int. J. Drug Dev & Res.2 (1): 108-112.
15. Pengelly Andrew (2004). Triterpenoids and saponins. The constituents of medicinal plants (CABI publishing, USA), 74.
16. Regisusan, D.M.C., Devi, K. S. and Augusti K. T. (1998). Antioxidant effect of two flavonoids from the bark of *Ficus bengalensis* Linn in hyperlipdaemic rats. Indian J Exp Biol. 36: 902.
17. Castelli, W. P., Garrison, R. J., Wilson, P. W. F., Abbott, R.D., Kaloudian, S. and Kannel, W.B. (1986). Incidence of coronary heart disease and lipoprotein cholesterol levels-The Firmingham Study. JAMA, 256: 2835.
18. Xie, W., Wang, W., Su, H., Xing, D., Cal, G and Du, L. (2007). Hypolipidemic mechanisms of *Ananas comosus* L Leaves in mice: Different from fibrates but similar to statins. J Pharmacol Sci. 103:267-74.
19. Umesh, K.P., Saraf S. and Dixit, V.K. (2004). Hypolipidemic activity of seeds *Cassia tora*. J Ethanopharmacol. 90:249-52.

Vol. 5 (3) i - ii July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

NEWS ITEM

India lauds relationship with Canada at summit

The first-ever Education Summit between India and Canada held at Ottawa organized jointly by the Indian High Commission, Canada's Department of Foreign Affairs and International Trade and the Shastri Indo-Canadian Institute. The minister for state for HRD, Daggubati Purandeswari and Canadian Minister of Foreign Affairs John Baird reaffirmed the Canada's relationship with India and said Canada sees the Summit as an important step in developing bilateral academic relations after the signing of the bilateral Memorandum of Understanding on Education during PM Manmohan Singh's visit. Referring to education reforms in India, Purandeswari said that the reforms were being implemented to improve India's education system, emphasizing the importance of access for people in poverty and living in less-served rural areas.

Educating the nation on cancer is the need of the hour: President of India

President of India Smt. Pratibha Patil said that India needs education about cancer, and its various aspects of health complications. While addressing at the inauguration ceremony of an integrated cancer treatment and research centre of the Bharatiya Sancskriti Darshan Trust at Wagholi, Pune, the President said that support is needed for earlier detection, latest and advanced treatment and cure. She added that cancer is the sixth major cause among the death occurs in India and Indian Council of Medical Research has reported that about 10 lakh new cases of cancer are registered every year in India. Smt. Pratibha Patil, said that delayed cancer detection in India causes complications and becomes fatal. It is curable when detected earlier and for which our nation should be educated in such a way that cancer should be detected earlier. She told that there is a need for discussion among scholars of allopathy, ayurvedha and homeopathy on the effective use of drugs to cure various types of cancer.

Government to overcome skill shortage, develop integrated education system

Mr. Kapil Sibal, Honourable Union Minister for HRD, Government of India, speaking at the inaugural session of The TimesJobs.com Annual Skills Summit 2011 thanked TimesJobs.com for inviting him to the summit and drew the rapt audience's attention to the 'gargantuan task' of solving the problem of skills shortage in the country. The minister explained that there are 40 million children who enter high school every year despite a 56% dropout rate. With the government's envisioning a zero dropout rate is expected to grow exponentially. Mr. Sibal envisaged that by 2022 with India's population at 1.3 billion, the country need 500 million skilled people and 250 million graduates to sustain double-digit growth. The honourable minister asserted to overcome the skill shortage, development must be integrated with the education system.

India, Slovenia discuss international issues to enhance cooperation in the field of Research and Education

India and Slovenia held wide ranging talks on international, regional and bilateral issues apart from inking three pacts to promote investment and enhance cooperation in the field of Research and education. Prime Minister Manmohan Singh and his Slovenian counterpart Borut Pahor discussed ways to strengthen relations in diverse areas including trade, investment and education. Both sides signed an agreement on mutual promotion and protection of investments. Finance Minister Pranab Mukherjee signed the agreement from Indian side while Darja Radic, Minister of Economy of Slovenia, signed it for the European country. A memorandum of understanding was signed between the Bureau of Indian Standards and Slovenian Institute for Standardization for mutual cooperation in the areas of their interests. A third MoU was signed between Indian Institute of Science, Bangalore, and Slovenia's University of Nova Gorica for Cooperation in the field of research and education.

OPPORTUNITIES

Centre for Cellular and Molecular Biology (Council of Scientific & Industrial Research), Uppal Road, Hyderabad - 500 007, India. Applications are invited from eligible candidates to work as Project Assistant-IV / Project RA / PDF and Project Assistant / Project JRF in research projects funded by National and International agencies like DST/ DBT/ ICMR/ CSIR etc. Candidates with Ph.D. in any area of Life Sciences preferably Molecular & Cell Biology, Microbiology, Biochemistry, Animal Genetics & Breeding, Biotechnology and Bioinformatics for Project Assistant-IV / Project RA / PDF and 1st Class M.Sc in any area of Life Sciences / Biotechnology / Bioinformatics OR 1st Class BE/BTech. in Biotechnology / Bioinformatics for Project Assistant-II / Project JRF are considered. GATE/ NET qualification is desirable. Applications can be sent to Controller of Administration, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad – 500 007 on or before 30th June 2011. Date of Walk-in-Interview: 11th July, 2011. For further details visit the website: www.ccmb.res.in.

Vol. 5 (3) i - ii July 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Centre for DNA FingerPrinting and Diagnostics (CDFD), Bldg. 7, Gruhakalpa, 5 - 4 - 399 / B, Nampally, Hyderabad – 500 001, India. Applications are invited from eligible candidates to work as Project Associate/ Research Associate and Project Assistant / Project JRF in sponsored/ network projects at Centre for Cellular and Molecular Biology (Council of Scientific & Industrial Research), Uppal Road, Hyderabad - 500 007, India. Candidates with Ph.D. in any area of Physics / Chemistry / Life Science / Biotechnology / Genetics / Biochemistry / Microbiology / Bioinformatics from recognized University / Institute for Project Associate/ Research Associate and M.Sc. in Physics / Chemistry / Life Science / Biotechnology / Genetics / Biochemistry / Microbiology / Bioinformatics / Medicinal Chemistry from recognized University / Institute for Project Assistant / Project JRF are considered. Interested candidates may register their names by applying in the prescribed application form available online at www.cdfd.org.in. The printout of the completed application form along with the certificates in support of date of birth, educational qualifications, caste and NET fellowship etc. and fee of Rs. 200/- (Rupees two hundred only) by way of Demand draft / IPO drawn in favour of "The Director, CDFD" payable at Hyderabad should be sent to "The Head – Administration, Centre for DNA Fingerprinting and Diagnostics (CDFD), Office Block: 2nd Floor, Bldg. 7, Gruhakalpa, 5-4-399/B, M. J. Raod, Nampally, Hyderabad – 500 001". The online facility of application will open on 10.06.2011 and will be closed on 11.07.2011. The last date for receipt of the printout of the applications is 14.07.2011. For further details visit the website: www.cdfd.org.in.

SEMINARS/WORKSHOPS/CONFERENCES

National Conference on Emerging Trends in Biotechnology & Annual Meeting of Society for Biotechnologists (India): A National conference on Emerging Trends in Biotechnology & Annual Meeting of Society for Biotechnologists (India) will be held during September 24-26, 2011 at Acharya Nagarjuna University organized by Society for Biotechnologists (India) & Department of Biotechnology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India. The young researchers are provided with a good platform for presenting their research paper presentations and six awards have been instituted. Abstract can be submitted online through E-mail: raokrss@yahoo.in. Registration Fee For delegates - Rs.500/- For students and accompanying persons - Rs.300/- (DD from any nationalized bank drawn on Director of the National Conference and payable at Guntur). For further details contact: Prof. K.R.S. Sambasiva Rao, Director of the Seminar & Head, Department of Biotechnology, Acharya Nagarjuna University, Guntur, A.P., India – 522 510. Phone – 0863-2346355. Email – raokrss@yahoo.in

International Conference on Human Infectious Diseases and Immunity: An International conference on Human Infectious Diseases and Immunity will be held during 27th-29th August, 2011 at Karunya University, Karunya Nagar, Coimbatore - 641114 Tamil Nadu, India organized by School of Biotechnology and Health Sciences, Karunya University, Karunya Nagar, Coimbatore - 641114 Tamil Nadu, India. A copy of abstract can be submitted online through E-mail: ichidi@rediffmail.com. Registration fee for students is Rs.500 and for others it is Rs.1000. For further details contact: Dr.E.Rajasekaran, Organising Secretary, International Conference on Human Infectious Diseases and Immunity, Department of Bioinformatics, School of Biotechnology and Health Sciences, Karunya University, Karunya Nagar, Coimbatore - 641114 Tamil Nadu. Tel: 0422 2614472, Mobile: +91 9524825876, Email: sekaran@karunya.edu and Dr.Rajiv Kant, Organising Secretary, International Conference on Human Infectious Diseases and Immunity, Department of Biotechnology, School of Biotechnology and Health Sciences, Karunya University, Karunya Nagar, Coimbatore - 641114 Tamil Nadu. Tel: 0422 2614472, Mobile: 9003828003, Email: rajivkant@karunya.edu.

National Conference on Recent Advances in Plant Sciences: A National conference on Recent Advances in Plant Sciences will be held on October 15-16, 2011 at Dharm Samaj College, Aligarh (U.P.), India by P.G. Department of Botany, D.S. College, Aligarh, Uttar Pradesh, India. Young scientists below the age of 30 years as on 31st July 2011 will be given the award of 'Certificate of Achievement' to the best Paper presented in each section. Prizes are awarded for three best papers presented in oral presentations. For the best poster presentation, Rai Badri Prasad gold medal will be awarded. Smt. Shanti Devi gold medal will be awarded to the best paper presented by women participant. A copy of abstract can be sent to Dr. Prabodh Srivastava, Organizing Secretary, Department of Botany, D.S. College, Aligarh (U.P.) or submitted online through E-mail: praboodh23@yahoo.co.uk. Deadline for abstracts/proposals: 15 July 2011. For further details contact: Organizing Secretary, Department of Botany, D.S. College, Aligarh (U.P.), India. Phone – 09412273280. Email – praboodh23@yahoo.co.uk, dsbotany@gmail.com.

International Symposium on Innovations in Free Radical Research and Experimental Therapeutics
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Immunomodulators and Radioprotectors
Immunopharmacology
Toxicology
Translation Research
Pharmaceutical Biology
Drug Metabolism and Drug Interactions
Complementary and Alternative Medicines

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