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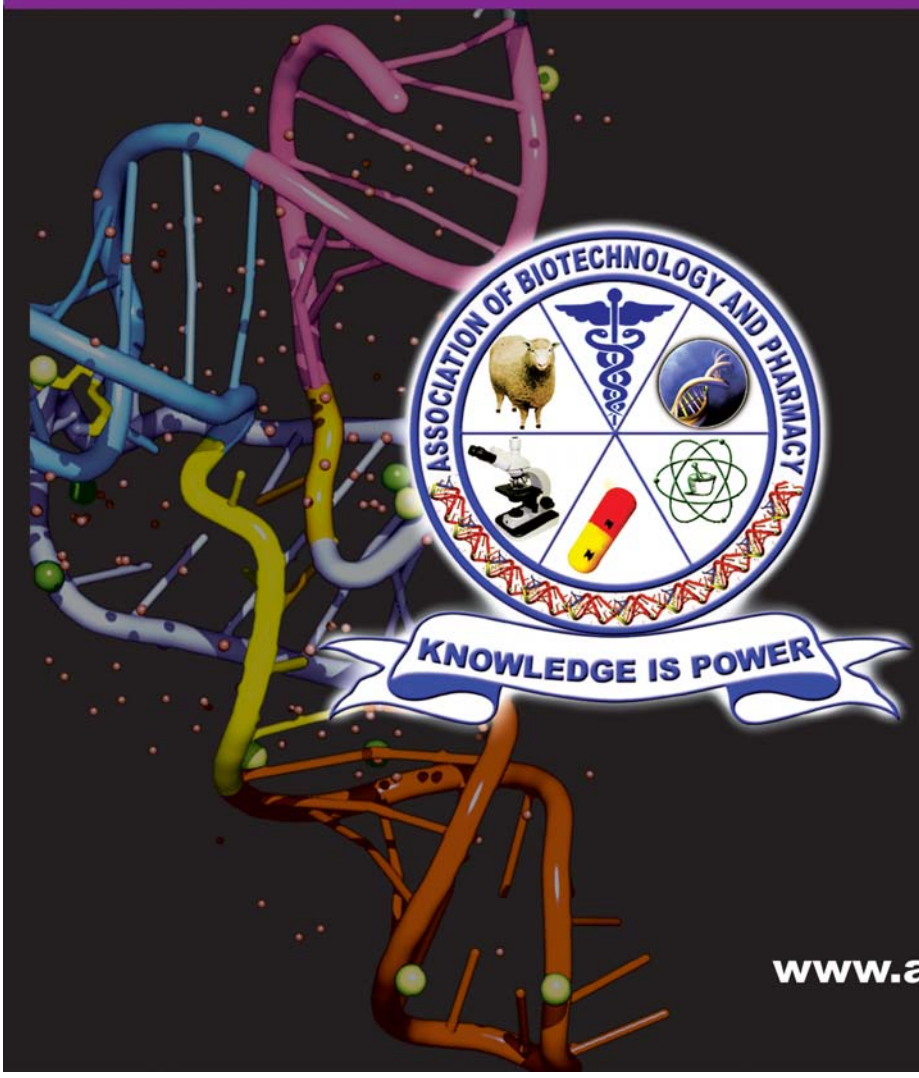

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Editors

Prof. K.R.S. Sambasiva Rao, India
krssrao@abap.co.in

Prof. Karnam S. Murthy, USA
skarnam@vcu.edu

Editorial Board

Assistant Editors

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(Regn. No. 28 OF 2007)

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Genetic Engineering - Trends in Drug Development

Basavaraj K. Nanjawade*, Kiran Suryadevara, Sai Sushmitha and Mac Kella
Dept of Pharmaceutics, Jawaharlal Nehru Medical College, KLE University,
Belgaum, Karnataka, India
*For Correspondence - bknanjwade@yahoo.co.in

Abstract

This review on the gene therapy describes about DNA and it's function. It further contains the description on gene therapy along with it's definition. Various approaches for gene therapy have been described in this paper. The genetic disorders and the use of gene therapy for these diseases have been discussed along with the methods that can be used for gene delivery. Vector and non-Vector methods can be used for gene therapy. For vector method retroviral method, herpes simplex vectors can be used for gene therapy. Whereas for non-vector method molecular conjugates, naked DNA can be used for the same purpose. Further the paper contains a note on the gene therapy for curing disorders like Ischemic stroke, cancer. Few case studies of setbacks and achievements for gene therapy have been discussed at last.

Key Words: DNA, Gene therapy, Vectors and non-vectors.

Introduction

Genes are the blueprint of our bodies, governing factors such as growth, development and functioning. DNA, the main constituent of genes is made up of four bases: adenosine, cytosine, guanine and thymine. Every individual has different genetic constitution, which determines the difference in the individuals (1).

***DNA has two main functions*:** 1) The 'template function' of DNA is to pass on genes through generations and allow different traits to be inherited. 2) The 'transcriptional function' of DNA is to allow these genes to be expressed at appropriate times and places (and not expressed at others) to allow the cell to do its work. Figure 1 shows the DNA strand with the combinations of it's four bases (2).

Gene therapy: Gene therapy is defined as the expression of gene that has been introduced into a target cell or a target tissue in order to cure a patient of the specific disorder or disease (3). In

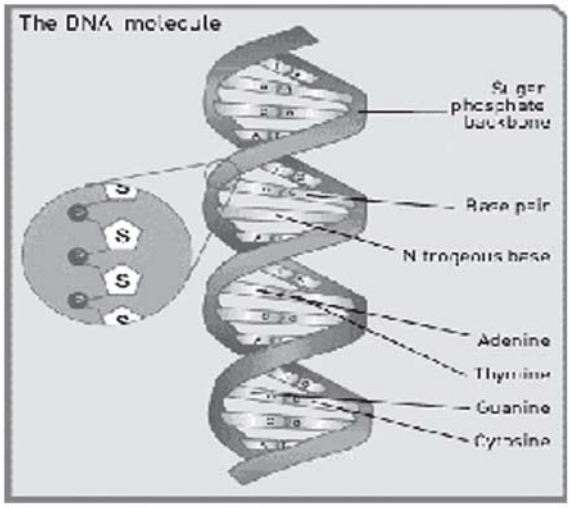

Fig. 1 DNA Strand

other words, genes act like 'drugs', with a specific pharmacological effect. Gene therapy involves the insertion of genetic material into a patient's cell(s) so that they are capable of producing therapeutic proteins. Gene therapy has an opportunity to fight the cause of a disease rather than its symptoms (4). Gene therapy works by replacing the missing or defective gene at the origin or by arresting undesired gene expression at the origin (5). Gene medicines are based on gene expression system which contain a therapeutic gene and a delivery system. This system controls the distribution and access of a gene expression unit to the target tissue, it's recognition by cell-receptors and it's intracellular trafficking (6). The introduction of genes into cells of various origins has been a major technique in cell biology research for more than decade.

Approaches for Gene Therapy

- A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common one (7).
- Another technique involves swapping of an abnormal gene with a normal gene through homologous recombination.
- The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function.
- The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.

Somatic gene therapy: Somatic gene therapy is the transfer of genes into the somatic cells of the patient, such as cells of the bone marrow, and hence the new DNA does not enter the eggs or sperm. The genes transferred are usually normal alleles that could 'correct' the mutant or disease alleles of the recipient (7).

***The technique of somatic gene therapy*:** The technique of somatic gene therapy involves inserting a normal gene into the appropriate cells of an individual affected with a genetic disease, thereby permanently correcting the disorder. Figure 2 explains the simple methods of getting genes into the person's cells using either viruses or liposomes. In some cells, the gene or genes become inserted into a chromosome in the nucleus.

There are three major obstacles that have to be overcome before somatic gene therapy is likely to work.

- First is to get the human gene into the patient's cells
- The second obstacle is getting the gene into the right cells.
- The final obstacle is making sure the gene is active, that is, switched on in the cell to produce the protein that the patient needs. This means it must be under the control of the sequence of DNA that is responsible for switching the gene on.

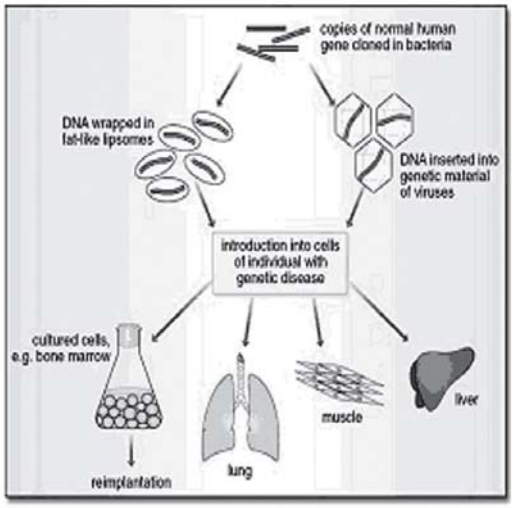

Fig 2. The technique of somatic gene therapy

Germline gene therapy : In germline gene therapy the genes are altered in eggs or sperm, or in a very early embryonic stage (8). The advantages of germline gene therapy are: The cells are accessible as they are outside the body; hence the gene delivery is less cumbersome than it tends to be with somatic cells. The inserted gene (or genes) would be present in all the cells of the person so treated because it would be transmitted to progeny cells during growth and development.

Genetic Disorders and Gene Therapy

A genetic disorder is an illness caused by abnormalities in genes or chromosomes (9,10,11). Disorders in which genes play an important role (genetic diseases) can be classified as: Single-gene defects, Chromosomal disorders and Multifactorial

Single gene disorder: A single mutated gene results in single gene disorder. Occurrence of Single gene defects is rare. Single gene disorders can be passed on to subsequent generations in several ways.

There are six basic patterns of single gene inheritance: a) Autosomal dominant, b) Autosomal recessive, c) X-linked dominant, d) X-linked recessive, e) Y-linked inheritance and f) Maternal (mitochondrial) inheritance

***Autosomal Dominant*:** Only one mutated copy of the gene is necessary for a person to be affected by an autosomal dominant disorder. There are 50% chances that a child will inherit the mutated gene.

***Autosomal Recessive*:** Two copies of the gene are required to be mutated for a person to be affected by an autosomal recessive disorder. Examples of this type of disorder: ADA deficiency (sometimes called the "boy in a bubble" disease), Alpha-1-antitrypsin (AAT) deficiency, Cystic fibrosis (CF), Phenylketonuria (PKU), Sickle cell anemia

***X-linked dominant*:**X-linked dominant disorders are caused by mutations in genes on the X chromosome. Only a few disorders have this inheritance pattern. One of these is hypophosphatemic rickets, also called vitamin D -resistant rickets.

X-linked recessive: X-linked recessive disorders too are caused by the mutations in genes of X chromosome. The male child of a man with an X-linked recessive disorder will not be affected, but his daughters will carry one copy of the mutated gene. Examples of this type of disorder: Duchenne muscular dystrophy, Hemophilia A, Color blindness, Muscular dystrophy and Androgenetic alopecia.

***Y-Linked inheritance*:** Y-linked disorders are caused by mutations on the Y chromosomes. As males inherit a Y chromosome from their fathers, so every son of an affected father will be affected by this disorder. But females inherit an X chromosome from their fathers; hence female offspring of affected fathers are never affected. Examples of this type of disorder: Male infertility and Hypertrichosis pinnae

Mitochondrial

Mitochondrial disorders can be caused by mutations, acquired or inherited, in mitochondrial DNA (mDNA) or in nuclear genes that code for mitochondrial components. The distribution of the defective mitochondrial DNA may vary from organ to organ within the body, and each mutation is modulated by other genome variations, the mutation that in one individual may cause liver disease might in other person cause a brain disorder. This type of inheritance is also known as maternal inheritance. Examples of this type of disorder: Blindness, Developmental delay,

Gastrointestinal problems, Hearing loss, Heart rhythm problems, Metabolic disturbances, Short stature

Chromosomal Disorder

The chromosomal disorders, are due to an excess or lack of the genes contained in a whole chromosome or chromosome segment. Examples of this type of disorder: Down syndrome, Klinefelter syndrome, Turner syndrome

Multifactorial Disorders

Genetic disorders can also be multifactorial or polygenic, it means that they are likely associated with the effects of multiple genes in combination with lifestyle and environmental factors. Multifactorial disorders do not have a clear-cut pattern of inheritance. Complex disorders are also difficult to study and treat because the specific factors that cause most of these disorders have not yet been identified. Examples of this disorder: Cancer, Coronary heart disease, Hypertension, Stroke, Diabetes, Obesity

Drug delivery through gene

A vector is the vehicle generally used to introduce gene to the target cells. Vectors may be DNA or RNA viruses or may be non viral in nature. Viruses that have the capacity to introduce themselves in the host genome are used as vectors for gene therapy (12). These vectors encapsulate therapeutic gene for delivery into the cell (13). Gene therapy the drug can be delivered through two ways: Vector method and Non-Vector method

Vector method

Viruses serve as the best potential vectors in gene therapy. The viruses have much more efficient gene transfer rates than non-viral gene delivery methods. Viruses when used as vehicles for therapeutic genes, must meet the following criteria (14,15).

The virus itself should not possess undesirable properties. The viral genome must be able to accommodate the therapeutic gene. Commonly used virus vectors in gene drug delivery are: Retroviral vectors, Lentivirus vectors, Adenovirus vectors, Adeno-associated vectors, Herpes simplex virus

Retroviral vectors: Retroviruses contain a single stranded RNA molecule as the genome. After infection, the viral genome is transcribed into double stranded DNA, which integrates into the host genome and is expressed as proteins. The retroviral genome contains at least three genes: gag (coding for core proteins), pol (coding for reverse transcriptase) and env (coding for viral envelope protein). Long terminal repeats (LTRs) are present at the each end of genome. This LTRs include enhancer/promoters regions and sequences involved in integration (16).

The first human replacement gene therapy using a retrovirus vector was approved for the replacement of the defected, missing, ADA gene in the children suffering from severe combined immune deficiency (SCID) (17).

One of the problems of gene therapy using retroviruses is that the integrase enzyme can insert the genetic material of the virus into any arbitrary position in the genome of the host; it randomly shoves the genetic material into a chromosome (18).

Retroviral vectors have been successfully used to treat X-linked severe combined immunodeficiency (X-SCID) (18).

Lentivirus Vectors: They are special group of retroviral vectors capable of infecting both proliferating and non-proliferating cells. The lentiviral vectors are derived from the human immuno deficiency virus. They are capable of integrating the proviral DNA into the non-dividing cells.

The replication defective lentiviral vectors are constructed and are used to efficiently deliver genes directly into the brain, muscle, lung, liver and islets (19).

Efforts are being made to use lentiviruses for blocking the expression of a specific gene using RNA interference technology. The expression of short-hairpin RNA (shRNA) reduces the expression of a specific gene, thus allowing researchers to examine the necessity and effects of a given gene in a model system (20).

Adenovirus vectors: They are non-enveloped DNA viruses, the genome of which is a linear, double-stranded DNA molecule (15).

Patients with hereditary (like cystic fibrosis) or acquired (like cancer) diseases have been treated in a clinical trial. In the majority of trials, adenoviral mediated gene transfer was found to be safe (21).

Adenovirus is currently being explored for gene therapy of disorders of the lung (cystic fibrosis), muscle (Duchenne's muscular dystrophy), liver, central nervous system and heart (22).

Gendicine, an adenoviral p53-based gene therapy was approved by the Chinese FDA in 2003 for the treatment of head and neck cancer (18).

After death of Jesse Gelsinger, participating in a gene therapy trial (1999) questions were raised about the safety of this virus (18).

Adeno-associated vectors: (AAV): Adeno-associated viruses are non-pathogenic human pathoviruses, dependent on helper virus, usually adenovirus to proliferate. AAV vectors integrate into the host genome allowing prolonged transgene expression (23)

One of the applications of AAV vector is gene transfer to the lung cells. AAV – mediated gene transfer is also used for gene therapy for Hemophilia B, a genetic disease which leads to improper blood clotting. AAV-vector mediated gene transfer to hematopoietic stem cells is also under study for the treatment of thalessima and sickle cell anemia (22)

Herpes Simplex Virus (HSV) : Herpes simplex virus is a human neurotropic virus, so it is being used as a vector for the gene transfer to the nervous system. The viral genome is a linear double stranded molecule.

A number of neurological diseases could be amenable to gene therapy by HSV vectors (24)

There has been some success in parkison's disease by expressing tyrosine hydroxylase in striatal cells thus replacing the supply of Levodopa (25)

Non Viral methods for gene transfer

Viral vectors induce immunological response to some extent in the body and as well as they have created some safety concerns. Also their capacity is limited & large scale production may be difficult to achieve.

The non-viral methods of DNA transfer require only a small number of proteins, having an infinite capacity, with no infectious or mutagenic capability and also large scale production is possible using pharmaceutical techniques. There are mainly three methods of non-viral DNA transfer (14,15)

- Naked DNA
- Liposomes
- Molecular conjugates

Also Dendrimers are being used for the gene transfer.

Naked DNA : A naked DNA is a histone free DNA that gives a new phenotype to the recipient cell during transformation process. Gene therapy using naked DNA is simple, safe and efficient method (26)

Clinical trials carried out on intramuscular injection of a naked DNA plasmid have given some success; however, the expression has been very low in comparison to other methods of transfection (18)

Liposomes: Liposomes are one of the good vehicles for gene transfer as they are non-toxic, they do not react with the human immune system and as well they do not cause cancer (27)

Liposomes are lipid bilayers entrapping a fraction of aqueous fluid. DNA will spontaneously associate to the external surface of cationic liposomes and these liposomes interact with the cell membrane (28)

This is a common method of non-viral gene transfer. In this the cationic lipid associated DNA complex is being administered in the body. The DNA molecules have a negative charge at neutral pH and are complexed with cationic lipids to form colloidal structures. These liposome-DNA complexes bind to the cellular membranes and are internalized in the endosomes.

The most common use of liposomes has been gene transfer into cancer cells, where the supplied genes have activated tumor suppressor control genes in the cell and decrease the activity of oncogenes.

Liposomes have been widely used for gene delivery vectors but dose dependent toxicity of liposomes was also observed which could limit their therapeutic usages (18)

Molecular conjugates: The DNA molecular conjugates bind to specific receptor of the target cells. The molecular conjugate is engulfed by the cell membrane forming an endosome which helps in protecting the DNA from being degraded. The endosome releases the DNA which then enters the nucleus thus expressing the therapeutic gene. The DNA molecular conjugate is useful in targeting the large sized therapeutic DNA's to the specific tissues. Poly-L-lysine is the most commonly used synthetic conjugate (29).

Dendrimers: A dendrimer is a highly branched macromolecule with a spherical shape. It is possible to construct a cationic dendrimer, i.e. one with a positive surface charge. When in the presence of genetic material such as DNA or RNA, charge complimentarity leads to a temporary association of the nucleic acid with the cationic dendrimer. On reaching its destination the dendrimer-nucleic acid complex is then taken into the cell via endocytosis (18).

Dendrimers offer robust covalent construction and also an extreme control over molecule structure, and size. These give compelling advantages compared to existing approaches.

Major accomplishments/Landmarks in Gene Therapy

Gene therapy for Adenosine Deaminase Deficiency: Severe combined immunodeficiency (SCID) is an inherited immune disorder due to the dysfunction of T-lymphocytes and B-lymphocytes. More then half of the patients of SCID have a defect in the gene which encodes for adenosine deaminase. Due to the deficiency of adenosine deaminase, deoxyadenosine and it's metabolites accumulate and destroy T-lymphocytes. The T-Lymphocytes play a major role in body defense mechanism and they also promote the production of antibodies, a major function of B-Lymphocytes (30).

In the gene therapy for correcting this disorder a part of the proviral DNA from a plasmid vector is replaced by the ADA gene and a gene (G 418) coding for antibiotic resistance, and then cloned. The antibiotic resistance gene helps in selecting the desired clones with ADA gene. The circulating lymphocytes in the body of a patient suffering from ADA deficiency are removed and are exposed to billions of retroviruses containing ADA gene. Thus the genetically-modified lymphocytes obtained by transfection of ADA gene and lymphocytes are then grown in cultures for expression of ADA gene. The so obtained modified lymphocytes are injected in the patient's body and thus increasing their ability to produce antibodies.

Gene therapy for Cancer: Cancer is one of the leading causes of death all over the world. There are various strategies for the treatment of cancer like surgery, radiation therapy, chemotherapy. Gene therapy is the novel approach under development for the treatment of cancer (31).

TNFerade is one treatment option for treatment of tumour. This agent is a replication incompetent adenoviral vector that delivers the tumor necrosis factor-á (TNF-á) gene under the transcriptional control of a radiation inducible promoter. TNF-á is a cytokine having potent anticancer properties and high systemic toxicity. TNF-á gene therapy is a way to target this molecule only to the cancer cells through the use of intratumoral injections and a promoter that is activated by radiation therapy. After TNFerade is injected, the patient receives radiation therapy to the tumor to activate the gene. The activated gene then produces the TNF-á molecule which in combination with the radiation therapy promotes cell death in the affected cancer cells and surrounding cells.

Another gene therapy treatment agent is Rexin-G. It is used for the treatment of pancreatic cancer. Rexin-G contains a gene which interferes with the cyclin G1 gene. This gene is transferred with retroviral vector. The gene integrates into the cancer cell's DNA to disrupt the cyclin G1 gene and thus causes cell death or growth arrest.

Gene therapy for Ischemic stroke: Stroke is a leading cause of disability and is a major cause of death in the developed countries. Out of that 85% of the strokes are Ischemic (32).

Gene transfer is a great option for the treatment of Ischemic stroke. Viral systems like retrovirus, adeno-associated virus, Herpes Simplex virus have been used for the gene delivery to the Ischemic stroke (33)

Gene therapy with rAAV vectors has displayed evident curative effect in animal stroke models by increasing glucose uptake and neovascularization, inhibiting apoptosis, inflammation, excitotoxicity, and free radicals, reducing cytosolic calcium and protecting proliferating endogeneous precursor cells (33)

The Major setback for gene therapy : Jesse Gelsinger suffered from ornithine transcarbamylase deficiency, which is an X-linked genetic disease of the liver, the symptoms of which include an inability to metabolize ammonia - a byproduct of protein breakdown. He died during the clinical trials of gene therapy in the year 1999.

Gelsinger was injected with adenoviruses carrying a corrected gene. He died four days later, due to a massive immune response triggered by the use of the viral vector used to transport the gene into his cells. This led to multiple organ failure and brain death (34).

Encouraging signs for gene therapy: Ashanti

DeSilva was born with a rare genetic disease called severe combined immune deficiency (SCID), she lacked a healthy immune system, and was vulnerable to every passing germ.

Ashanti's gene therapy procedure included removing white blood cells from the child's body, allowing the cells to grow in the lab, inserting the missing gene into the cells, and then infusing the genetically modified blood cells back into the patient's bloodstream. Laboratory tests showed that the therapy strengthened Ashanti's immune system.

This procedure was not a cure; the white blood cells treated genetically work for a few months, and the process has repeated every few months (35).

Conclusion

Gene therapy involves the introduction of gene into a target cell or a target tissue in order to cure a patient of the specific disease. Thus acting as 'drugs', with a specific pharmacological effect. Means gene therapy involves insertion of genetic material into a patient's cell(s) so that they are capable of producing therapeutic proteins. Somatic gene therapy is the transfer of genes into the somatic cells of the patient, such as cells of the bone marrow, and hence the new DNA does not enter the eggs or sperm. In germline gene therapy the genes are altered in eggs or sperm, or in a very early embryonic stage. The gene transfer can be done by vector and non-vector methods. In the vector method retroviral vectors, adeno viral vectors, herpes simplex vectors can be used. Whereas for non-viral methods naked DNA, molecular conjugates can be used. The gene therapy can be used for curing various disorders and diseases like cancer, Ischemic stroke.

Table 1. Gene Transfer Methods (Vectors – Viral and non-viral/Delivery Systems) (14,15)

Delivery Systems	Description
Naked nucleic acid	Plasmid DNA, in the absence of transfection reagents
Nonviral vector	Plasmid DNA/transfection reagent mixture
Retroviral vector	Derived from murine leukemia virus
Lentiviral vector	Derived from HIV-1
Adenoviral vecto	Deletions in the virus genes E1, E3, or E4, E2ts, combinations thereof, or 'gutted'
Adeno-associated virus vector	Wild-type AAv-derived
Herpes –viral vector	Herpes simplex virus

Table 2. Gene therapy used in various disorders

Disease	Gene Therapy
Severe combined immunodeficiency	Adenosine deaminase
Cystic fibrosis	Cystic fibrosis transmembrane regulator
Familial hypercholesterolemia	Low density lipoprotein receptor
Emphysema	α_1- Antitrypsin
Hemophilia B	Factor 1X
Thalasssemia	α - or β-Globin
Sickle-cell anemia	β-Globin
Lesch-Nyhan syndrome	Hypoxanthine-guanine phosphoribosyltransferase
Gaucher's disease	Glucocerebrosidase
Peripheral artery disease	Vascular endothelial growth factor
Fanconi anemia	Fanconi anemia C
Melanoma	Tumor necrosis factor
Head and neck cancer	P^{53}
Breast cancer	Multidrug resistance 1
AIDS	Rev and env

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16S rRNA based identification and phylogenetic analysis of a novel dextran producing *Pediococcus pentosaceus* isolated from north-east Indian microbial diversity

Seema Patel and Arun Goyal
Department of Biotechnology
Indian Institute of Technology Guwahati
Guwahati- 781 039, Assam, India
*For correspondence - arungoyl@iitg.ernet.in

Abstract

Looking at the high application potential of dextrans in food, pharmaceutical and tissue engineering fields, a dextran producing strain of lactic acid bacterium was isolated from the sugarcane field soil sample from Assam, falling under north-east Indian microbial diversity. The full-length 16S rRNA gene of the isolate was amplified by Polymerase Chain Reaction (PCR) and sequenced by Sanger's method. The sequence was aligned with the reference sequences in the GenBank of National Centre for Biotechnological Information (NCBI) and Ribosomal Database Project (RDP) using BLAST programme. Distance matrix based phylogenetic analysis resulted in well-resolved trees with nodes supported with high bootstrap pseudoreplicate scores. The lactic acid bacterium isolate was identified as *Pediococcus pentosaceus* (GenBank Accession Number EU569832). This identification revealed the dextran production attribute of *Pediococcus* genus of lactic acid bacteria for the first time ever, heralding further rigorous investigation on this aspect. This investigation also unravelled the abundance of industrially valuable microbial flora in the soil of north-east India.

Key words: *Pediococcus pentosaceus*, dextran, 16S rRNA gene, phylogenetic analysis.

Introduction

Dextrans $(C_6H_{10}O_5)n$ are a class α-(1>6) linked glucans having branchings of α-(1>2), α-(1>3), α-(1>4) linkages (1). Dextrans have enormous industrial applications as food additives, chromatography column matrices, blood plasma substitutes, treatment of anaemia etc. (2). Recently, dextrans have found use in nanoconstructs as potential vectors for anti-cancer agents (3). *Lactobacillus*, *Leuconostoc, Streptococcus* and *Weisella* genera of lactic acid bacteria synthesize dextrans (4). There was not any concrete report on dextran production by *Pediococcus* genus, however, Smitinont *et al.* (1999) had emphasized on dextran synthesizing ability of this genus (5). Patel et al. (2010) reported the dextran production ability of *Pediococcus pentosaceus* for the first time ever (6). *Pediococci* are a heterogeneous group of homofermentative lactic acid bacteria (7). Currently ten species of *Pediococcus* are recognized, including *Pediococcus damnosus*, *P. parvulus*, *P. inopinatus*, *P. cellicola*, *P. ethanolidurans*, *P. claussenii*, *P. stilesii*, *P. acidilactici*, *P. pentosaceus* and *P. dextrinicus* (8, 9). The species of this genus are cosmopolitan in distribution as they have been isolated from soil (10), plants (11), wines (12), cheese (13), wheat kernels (14), meat (15) and sausages (16). Members of the *Pediococcus* genus have great

economic importance in the fermented food industry (17). *Pediococcus* genus has been known for its use as starter cultures in fermentation processes of milk, meat, vegetable products and sausages (17, 18). *P. acidilactici* has GRAS (Generally Recognized As Safe) status and is used as probiotic culture and nutritional enhancer in silage (18, 19). In addition to their contribution to fermented foods, several *Pediococcus* strains produce bacteriocins that inhibit the growth of major Gram-positive foodborne pathogens, as well as other food spoilage bacteria (20). Bacteriocinogenic strains of *Pediococcus* genus has been found active against lactic acid bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria ivanovii* and *Listeria monocytogenes* (21).

Till date, there have been several studies on pediocin production from *Pediococcus* genus (22). However, only sparse literature on exopolysaccharide production aspect of *Pediococcus* strains has been documented. Bacterial exopolysaccharides have attracted huge attention in recent years due to their widespread industrial potentials and they warrant studies in *Pediococcus* genus like other exopolysaccharide producing lactic acid bacteria. In *P. damnosus*, a plasmid associated with glucan-production has been reported in wine (23). Ropy *Pediococcus damnosus* (strain 2.6) was used for production of exopolysaccharide (EPS) in a semidefined medium (24). In some Argentinian wines, the ropiness inducing lactic acid bacteria include *Pediococcus pentosaceus* (25).

Traditionally, natural isolates have been identified by phenotypic methods, which are time consuming, potentially inaccurate and unreliable. The identification of microorganisms historically has relied on phenotypic methods that are often time-consuming, because of the inherent mutability of biochemical characteristics and subject to interpretive bias (26). Recent years have witnessed an explosion in the development and application of molecular tools for exploring the microbial diversity and identifying the isolates, owing to their superiority over the conventional approaches. Many of the modern molecular tools are based on 16S ribosomal RNA sequences, complete or partial genome determination and monitoring by specific fluorescent probes coupled to flow cytometry (27). Availability of large databases or the online resource offers powerful platform for rapid *in silico* specificity profiling. Classification and phylogenic studies of microorganisms based on these high throughput molecular approaches are increasingly applied to strains of lactic acid bacteria (LAB) (27). Sequence analysis of amplified 16S rRNA is recognized as a reliable technique for genus and species identification in different bacterial genera including *Lactobacillus*, *Weisella*, *Actinomyces*, *Bifidobacterium*, *Propionibacterium* and *Streptococcus* (28).

In the quest of a novel dextran, a bacterium, SPA (named after the author and the place of origin) was isolated from the soil sample of microbial diversity rich north-east India. Phenotypic characterization had already established the isolate SPA as a member of lactic acid bacteria family (10). In this work, the 16S rRNA gene sequencing based identification of the isolate was conducted to assign it the generic and species name, also to trace its taxonomic position by phylogenetic analysis.

Materials and Methods

Microorganism and maintenance conditions **:** The lactic acid bacterium isolate SPA screened from the sugarcane field soil of Assam was grown in enzyme production medium devised by Tsuchiya *et al.* (29) at 25°C. The isolate culture was propagated as stab in modified MRS medium

(30) at 28°C and stored at 4°C. For long term preservation, the isolate was kept in 20% (v/v) glycerol at –80°C (10).

Extraction of genomic DNA : The bacterial cell pellet was lysed using a solution containing guanidium thiocyanate (a chaotropic agent) and SDS (a detergent), to extract DNA (31). This lysis solution was used to disrupt the cell, remove proteins, polysaccharides and partial hydrolysis of RNA. The genomic DNA was extracted by GeNei™ Genomic DNA Extraction kit (Bangalore Genei Pvt. Ltd.). DNA was then precipitated using alcohol and washed with 70% alcohol to remove contaminants. DNA pellet was solubilized in an appropriate buffer (sodium acetate at pH 5.5 added to a final concentration of 0.3 M in isopropanol) at higher temperature (50-55°C), to increase the solubility of genomic DNA. The extracted DNA was purified using Ultra pure prep kit (KT83B, Bangalore Genei, India). Genomic DNA was purified in a gravity flow column using elution buffer, followed by treatment with lysozyme, proteinase K and RNase A supplied with the kit. DNA concentrations were determined in duplicate using a spectrophotometer.

PCR amplification : Genomic DNA of the isolate SPA was used for amplification of 16S rRNA gene. The universal 16S rDNA primers, forward primer BG395F (sequence hidden) and reverse primer BG396R (sequence hidden) were used for the polymerase chain reaction (PCR). The PCR amplification was carried out in a reaction mixture containing ~10ng genomic DNA as template, 1µl dNTP mix (2.5 mM each), 100 ng/µl each of forward and reverse primer, 1X *Taq* DNA polymerase assay buffer (10X), 3U *Taq* DNA polymerase enzyme (Bangalore Genei Ltd., Bangalore, India) and distilled water enough to make up the volume to 50 µl reaction mixture. Amplification conditions were: 5 min initial denaturation at 94°C, 30s denaturation at 94°C, 30s of primer annealing at 54°C, 1 min elongation at 72°C for 35 cycles and a final extension of 10 min at 72°C. The reactions were carried out in a Thermal Cycler (Applied Biosystems, model ABI 2720).

Electrophoresis : Fifteen µl of PCR amplification product was electrophoresed on 1% (w/v) low EEO; agarose gel in 1X TBE buffer (45 mM Tris-borate, pH 8.3 and 1 mM Na 2 EDTA) at 100V for 2h. The gel was stained with ethidium bromide in a final concentration of 0.5µg/ml, visualized and photographed under UV light. A low range DNA ladder (1 Kb) (Bangalore Genei Pvt Ltd, India) was used as a molecular weight marker.

Sequencing of the 16S rRNA gene : The 16S rRNA gene from the isolate was amplified as mentioned above. The amplification product was eluted and purified from the gel slice using the GeneiPure ™ Gel Extraction Kit (QIAGEN) and sequenced by Sanger's method using an automated genetic analyser (Make*:* ABI, Model*:* 3100) which uses the sequencing analysis software v.5.1 with the kb basecaller (32).

Sequence alignment of the 16S rRNA gene : The 16S rRNA sequence of the isolate SPA was compared with above 100 homologous sequences taken from the National Centre for Biotechnological Information (NCBI) genbank and Ribosomal Database Project (RDP) (33). Multiple sequence alignment of the sequence was conducted by ClustalW algorithm. Phylogenetic analysis was performed by applying distance matrix method. The isolate was assigned accession number.

Results and Discussion

16S rRNA gene sequence analysis after PCR amplification was performed for identifying

the isolate SPA. Profiles obtained by PCR amplification allowed identification of the isolate at both genus and species level. The Polymerase Chain Reaction of 16S rRNA revealed a 1,491bp amplicon by 1% agarose gel electrophoresis (Fig. 1). The full length sequencing of 1491bp 16S rRNA gene was identified by Sanger's method (Fig. 2). The 16S rRNA sequence was compared with that of reference bacteria obtained from National Centre for Biotechnological Information (NCBI) Genbank (http://www.ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP). Similarity searches were carried out using the BLAST algorithms available at (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments comparing the sequences were performed using ClustalW (http://www.ebi.ac.uk/clustalw/) (34). The alignment was checked visually and corrected manually using the sequence editor. The homology in sequences identified by above methods is used to find out common ancestry.

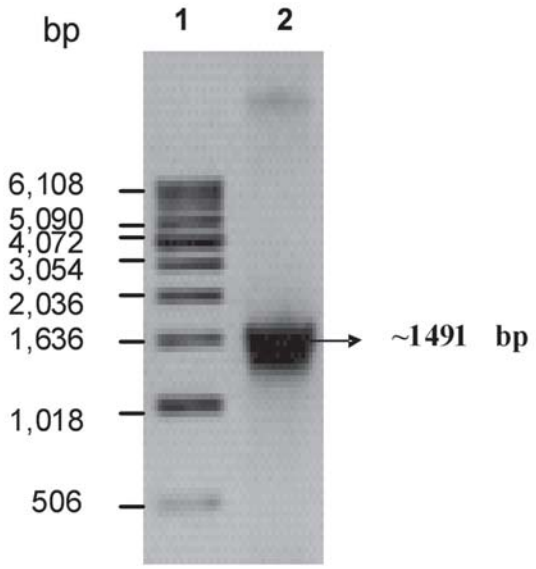

Fig. 1. Full length 16S rRNA gene (1491 bp) of the isolate SPA amplified with universal primers. The amplicon was electrophoretically resolved on a 1% agarose gel in 1X TBE buffer (A) Lane1: 1 eb plus. DNA ladder (B) Lane 2: Amplified product of full length 16S rRNA gene.

After the alignment the sequences were subjected to distance matrix based on nucleotide sequence homology using the Kimura 2 parameter (35). Pairwise distance between and within the genotypes at the nucleotide levels were calculated with Kimura 2 parameters. Kimura 2 parameter is used to correct the rates of transition and transversion and to remove the base bias. Substitutions in base occurs randomly and the transitions (C to T or A to G) are more frequent than transversions (A to T and C to G). This is the method of distance corrections in a phylogenetic analysis. The last step followed for identification was the phylogenetic tree based on nearest neighbour joining method, which actually identified the homology of the organism. Trees were drawn, the distance matrix for which was generated by MEGA 3.1 software (35) (Fig. 3). The stability of branching pattern and the statistical significance of the tree topology were confirmed by bootstrapping (32). Bootstrap values indicate the number of times a node was supported in 1000 sampling replications (35). A cut-off of 97-98% similarity in 16S rRNA sequence was recommended as a criterion for demarcating species (37).

The isolate SPA and the strain *Pediococcus pentosaceus* KC007 (Genbank Accession Number EU569832) clustered together (Fig. 3). The sequence similarity was calculated to be 96% (Table 1). Hence, the isolate SPA was identified as *Pediococcus pentosaceus* and assigned the Genbank Accession Number EU569832. The closest homolog of the isolate was *Pediococcus sp.* MMZ60A (EU157914).

TGATGACGCTGGCGGCGTGCCTATTACATGCAAGTCGAACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGAT
TGA GATTTTAACAC GAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGT AACCTGCCC AGAAGTAGGGG ATAAC ACC
TGGAAAC AGATGCTAATACCG TATAACA GAGAAAAC CGCATGGTTTTCTTTTAAAAGATGGCTC TGCTA TCACTTC
TGGATGGACCCGCGG CGTATTAG CTAGTTGGTG AGGTAAAGGCTCACCAA GGCAGTGA TACGTAGCC GACCTGA GAG
GGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG GGAAT CTTCC ACAATG GA
CGCA AGTCTGATGGAGCAAC GCCGCG TGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAA GAA CGTG
GGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC GCGGTAATA
CG TAGGTGGCAAGCGTTATCCGGATTT ATTGGGCGTTAGCGA GCGCAGGCGGTCTTT TAAGTC TAATG TGAAA GCC
TTCGGCTCAACCGA AGAAGTGCATTGGAAACTG GGAGACTTGAGTGC AGAAGAGGACAGTG GAACTCCATGTGT AGC
GGTGAAATG CGTAGAT ATATGG AAGAACACCAGTGGCGAAG GCGGCTG TCTGGTC TGCAACTGA CGCTGAGG CTCGA
AAGC ATGGGTAGCGAA CAGGATTA GATACC CTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGG GTTT
CCG CCCTTCAGTG CTGCAGCT AACGCATTA AGTAATCCGCCTGGG GAGTACGACCGCAAGG TTGAAACTCAAA
AGAATT GACGGGGGCCC GCACAATCG GTGG AGCATGTGGTTT AATTCGAAGCTACG CGAA GAA CCTTACCAGG TCT
TGACATCTT CTGACAGTCTAAGAGATTATAG GTTCCCTTCGGGGACA GAATGACA GGTGGTG CATGGTT GTCG TCA
GCTCGTGTCGTGAGATGTTGGGTT AAGTCCCGCAACGAGCGCAACCCTTATTACT AGTTGCCAGC ATTAAG TTGGGCA
CTCTAG TGAGACTGCCGGTGA CAAACCGGAGGA ATGTGGGGACG ACGTCAAAT CATCATGCC CCTTATG ACTTG GG
CTATCACGTGCTACGATGGATGGTACAACGAGTCGCG AGACGCG AGATTAGCTAAT CTCTTAAAAACATTCTCA GTT
CGG ACTGCAGG TGCACTCCCTA AACGAAGTCGGATCGCGTAG TAACGGGGATCACATGCCCCGTTGATA CCTTCCGG
GCCTTGTAC ACACCGCCCGTCA CACCATGAGAGTTTGTAACA CCCAAAGCCG GTGGGGTAAC CTTTTAGGAGC TAGC
CGTCT AAGG TG GGACAGAGATAGTGTTTCTGG

Fig. 2. 16S rRNA gene sequence (1491 bp full length) of the isolate SPA.

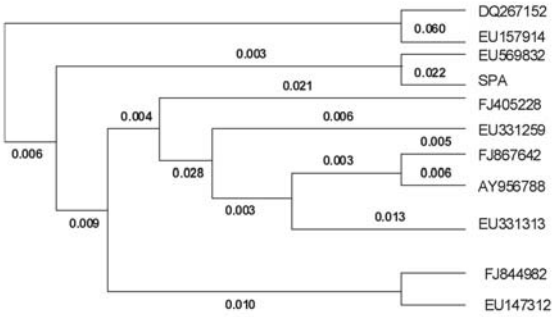

Fig. 3. Phylogenetic Tree made in MEGA 3.1 software using Neighbour Joining method. The Phylogenetic tree demonstrates the relationship of isolate SPA to other reference *Pediococcus* species from NCBI and RDP.

Table 1. Multiple sequence alignment results using combination of NCBI GenBank and RDP database

Identity	**Alignment Results**	**Sequence Description**
Isolate SPA	0.98	Studied sample
EU157914	0.94	*Pediococcus sp.*strain:MMZ60A
DQ267152	0.94	*Pediococcus pentosaceus*
EU569832	0.96	*Pediococcus pentosaceus* strain:KC007
FJ844982	0.97	*Pediococcus acidilactici* strain:IMAU20070
EU147312	0.96	*Pediococcus acidilactici* strain:BFE 8384
FJ405228	0.83	*Pediococcus claussenii strain* ZJ5
EU331259	0.96	*Pediococcus parvulus* strain Bpe301
FJ867642	0.91	*Pediococcus ethanolidurans* strain P2
AY956788	0.89	*Pediococcus cellicola* strain Bpe260
EU331313	0.95	*Pediococcus damnosus* strain Bpe260

Other close homologs of the isolate SPA clustering separately are *Pediococcus pentosaceus* (DQ267152), *Pediococcus calssenii* ZJ5 (FJ405228), *Pediococcus parvulus* Bpe301 (EU331259), *Pediococcus ethnolidurans* P2 (FJ867642), *Pediococcus cellicola* Z-8 (AY956788), *Pediococcus damnosus* Bpe260 (EU331313). This isolate is farthest from the strain *Pediococcus acidilactici* IMAU20070 (FJ844982) and *Pediococcus acidilactici* BFE 8384 (EU147312) (Fig. 3). Distance matrix based on Nucleotide sequence homology (using Kimura-2 parameter) corroborated the above finding. Phylogenetic analysis of the complete 16S rRNA dataset resulted in trees with much greater resolution and well supported with high bootstrap pseudoreplicate score (1000 pseudoreplicates).

Conclusion

The isolate SPA was identified to the species level by full length sequence analysis of its 16S rRNA gene. The sequence analysis followed by alignment revealed the identity of the isolate SPA as *Pediococcus pentosaceus* (Genbank Accession Number EU569832). The genetic relationships of this isolate with its neighbours were traced and a phylogenetic tree was constructed. Based on this finding, it is concluded that direct sequence analysis of amplified 16S rRNA gene is a promising rapid and accurate method for species determination of lactic acid bacteria. This study, revealed the dextran production attribute of *Pediococcus* genus of lactic acid bacteria for the first time ever, heralding further rigorous investigation on this aspect. This discovery is expected to be a major break-through in the history of dextran production. This unique isolate SPA discussed in this work represented an emerging group of dextran producing *Pediococcus*. This investigation also unravelled the abundance of industrially valuable microbial flora in soil. Screening of the microbial biodiversity enriched North Eastern region of India needs proper attention. The meteoric rise in the usage of dextrans in food, pharmaceutical and cosmetics industries emphasizes the importance of exploration of the new strains and characterization of their traits.

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Sequencing of partial cypD gene for screening of Bifunctional Cytochrome P450 Monooxygenases from *Bacillus cereus* group

P. Brahmaiah, T. Sathish, M. Hymavathi, K.Sreedhar Reddy and R. S. Prakasham*
Bioengineering and Environmental Centre
Indian Institute of Chemical Technology
Hyderabad – 500 607, India.
*For Correspondence - prakasam@iict.res.in

Abstract

Four different PCR primers were specifically designed to amplify a fragment of cypD gene from isolated *Bacillus cereus* group strains. Amplification was carried out using these primers. Among all isolates, strains designated as CYPPB-1, 2 and 3 possess cypD gene. Phylogenetic and comparative sequence analysis revealed differences among the sequences of isolated *B .cereus* group strains as well as those from the public databases. Sequences of all three *B. cereus* group isolates clustered with that of *B. cereus* G9842 and there is high similarity between sequences of CYPPB-1 and 3 where as CYPPB-2 showed a little variation. Further microscopic and 16S rDNA analysis indicated that CYPPB-1, 2 belongs to *B.cereus* while CYPPB-3 to *B.thuringiensis*. Comparative translated aminoacid sequence analysis with respect to *B.cereus* 14579 revealed that serine instead of proline at position 570 in all three isolated strains and histidine instead of glutamine at position 602 in CYPPB-1 and 3 strains. This diversity of CYP102A in *B. cereus* group may signify potential resource of different bifunctional cytochrome P450 monooxygenases.

Key words: cypD gene, Bifunctional Cytochrome P450 Monooxygenase, *B.cereus* group, primer design, amplification and sequence analysis.

Introduction

Cytochrome P450 (CYP450) monooxygenases (EC 1.14.x.y) constitute the largest super families of monooxygenase enzyme proteins (1). These heam-thiolate enzymes sparingly distributed in prokaryotes and widely in all eukaryotic organisms (2). They represent a valuable group of biocatalysts that are capable of introducing oxygen functionalities into non-activated carbons (3,4) and catalyze variety of reactions such as hydroxylations, epoxidations, N- or O-dealkylations, and Baeyer–Villiger oxidations in a regio and stereo selective manner (5). Nearly 7232 (excluding variants and pseudo genes) P450 genes have been sequenced (http://drnelson.utmem.edu/p450stats. Feb2008.htm) and genome sequencing projects continued to reveal many new P450 members.

CYP450 requires the successive delivery of two electrons to catalyze the scission of molecular oxygen to oxygenate its substrate during catalysis. Though the source of electrons is NAD(P)H in both mammalian and bacterial systems, the intermediate redox partner differ. In bacterial cells, cytosolic ferredoxin i.e.,

NAD(P)H-dependent ferredoxin reductase (an iron–sulfur protein) shuttles electrons to CYP450 (5) while in mammalian cells this role is often played by the flavoprotein cytochrome P450 reductase (CPR) (6). Unlike other CYP450s that require additional proteins as a redox partner, the CYP102 subfamily represents a unique group of bacterial cytochrome P450 monooxygenases with their bifunctional nature, hence called as Bifunctional cytochrome P450 monooxygenases (BiFCyP450). BiFCyP450 enzymes characterized by the presence of a heme domain fused to a diflavin reductase. They are natural fusion enzymes of approximately 117–119 kDa comprising the N-terminal monooxygenase domain and a FAD and FMN containing a diflavin reductase domain (7). To date, only five members of this subfamily (CYP102A1 from *Bacillus megaterium* (8), CYP102A2 and CYP102A3 from the *Bacillus subtilis* strain 168 (9,10), CYP102A5 from *Bacillus cereus* ATCC 14579T (11) and CYP102A7 from *Bacillus licheniformis* (12) have been reported for cloning, expression and characterisation. Overall, 15 different members of the CYP102A subfamily (CYP102A1–A15) gene sequences are available at http://drnelson.utmem.edu biblioE.html#102.

In view of bi-functional nature associated with extraordinary catalytic efficiency of these enzymes, BiFCyP450 play pivotal role in drug development, bioremediation and the synthesis of fine chemicals and other industrially useful compounds (13) in comparison to other CYP450 monooxygenases. Hence, several scientific communities are involved in exploring of microbial strains for novel BiFCyP450. In this paper, authors report isolation and characterization of *B. cereus group* strains from oil industrial soil and amplification of partial cypD gene by specially designed primers. In addition, comparative sequence analysis of partial sequence of the cypD gene with public database to evaluate the variations at nucleotide and amino acid sequence level.

Materials and Methods

Isolation of Bacillus cereus group strains from soil : Soil samples collected from Ramcharan oil-processing industry located at suburban of Hyderabad used for this study. *B. cereus* selective agar used for isolation of *B. cereus* group strains according to (14). All isolated strains were purified, sub cultured at regular intervals on selective agar slants and stored at 4^{o}C until further use.

Screening for strains with monooxygenase activity : Monooxygenase producing microbial strains were identified by performing solid phase NAD(P)H depletion assay according to (15) at 30^{o}C. In brief, nitrocellulose membranes soaked in 0.1 M phosphate buffer (pH 8.0) containing 5 mM lauric acid, 100ìM polymyxin B sulfate and 1.6mM NADPH, were placed directly on the top of the bacterial colonies grown LB agar plates for 15 minutes. One ml of colour reagent (0.1 M phosphate buffer, pH 8.0 containing 0.5 mg Nitroblue tetrazolium (NBT) containing 0.03 mg Phenazine methosulfate (PMS) was added on top of the nitrocellulose filter disc. Monooxygenase producing microbial strains were identified by colour change (purple to white) up on reduction of NBT by residual NAD(P)H in the presence of PMS.

DNA extraction : The bacterial cell pellet was suspended in 5ml of lysis buffer (pH 8.0) consisting of 10mM NaCl, 50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 20mM EDTA. The bacterial cells were then lysed using 0.5ml of 10mg/ml lysozyme and incubated at 37^{0}C for 30-60min. Then 2µl of RNase (500µg/ml) added and incubated for another 15min. To this, 18µl of 10mg/ml proteinase-k (final conc.50µg/ml) and 180µl of 10% SDS (final concentration 0.5%) added

and incubated at 37^{0}C for 60 minutes to remove the protein contamination. Then the lysate was subjected to phenol:chloroform (25:24) extraction followed by ethanol precipitation. The obtained DNA pellet then re-suspended in 1ml of DNase and RNase free water.

Design of primers and amplification of cypD gene : All sequences (Table 1) used for constructing the primers were edited using the gene tool lite software. The reference sequences obtained from NCBI (Gen bank) and subsequently aligned for conservative sequences using MEGA 4.1 program (16). Four different primers were designed based on conserved regions from reference sequences (Table 1 and 2). Subsequently all primer sequences were tested *in silico* for their suitability for amplification using gene tool lite software. Partial gene amplifications were performed in 25µl PCR reaction mixture containing 2.5ìL of 10x PCR buffer, 2µL of 25mM $MgCl_2$, 0.5µL of 10mM each dNTP, 10pM each primer, 1.25 U recombinant *Taq* DNA Polymerase and 20-40ng of bacterial genomic DNA. Amplification carried out using Eppendorff thermal cycler (Master Cycler EP Gradient S, Germany) with the following optimized conditions: 95°C for 2 minutes followed by thermocycled for 30 cycles 95°C for 1min, 54.5°C for 1.5min and 72°C for 3min with a final extension step at 72°C for 4min. The amplified products separated using 1.2% agarose gel and analyzed.

16S rRNA gene amplification : Amplification of 16S rDNA was performed as described by Weisburg et al. (17) using following conditions of 95°C (30 s), 52°C (40 s) and 68°C (1.30 minutes) plus one additional cycle with a final 7 min chain elongation.

Sequence analysis **:** Sequences of cypD obtained were deposited in the GenBank (Accession No: GQ385936-GQ385938). These sequences were edited with gene tool lite software and similarity was determined using ClustalW Alignment option and phylogenetic trees were constructed using the neighbour-joining method in MEGA 4.1 program (16). Bootstrap analysis

Table 1. Sequences used for constructing primers for the amplification of cypD gene from isolated strains*

	P450Ox	P450Hb	P450L	P450Rd
B. cereus ATCC 14579T	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPTEDKLKND—	KQNMWSDAMKAF—-
B. cereus ATCC 10987	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPTEEKLKNH—	KE SMWSDAMKAF—
B. thuringiensis serovar str. 97–27	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPTEEKLKNH—	KQS MWSDAMKAF—-
B. thuringiensis str. Al Hakam	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPTEEKLKNH—	KQRMWSDAMKAF—
B. anthracis str. 'Ames Ancestor'	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPTEEKLKNH—	KQRMWSDAMKVF—-
B. anthracis str. Sterne	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPTEEKLKNH—	KQRMWSDAMKVF—-
B. weihenstephanensis KBAB4	—YKPFGNGQRACIGMQF—	FLIAGHETTSG—	TVLAPI EEKLKND—	KQSMWSDAMKAF—-

Table 2. List of primers designed for the amplification of cypD gene from isolated strains

Primer	Conserved domain	Sequence of the primer
P450OxF	Oxygen activation site (P450 domain)	GGC ATG AGA CAA CAA GTG GAT
P450HbF	Heme binding loop (P450 domain)	GGT CAG CGA GCA TGT ATC
P450LF	Linker between P450 and reductase domains	GCG CCT ACA GAG GAG AAA C
P450RdR	Reductase domain	CTT CAT CGC ATC AGA CCA CA

was carried out of the neighbour-joining data, using 1000 resamplings, to evaluate the validity and reliability of the tree topology. The 16S rDNA tree was rooted using the sequence of *B. subtilis* DSM 10T (AJ276351) as an out group. The cypD gene tree was rooted using the sequence of *B. megaterium* (J04832) isolate as an out group.

Results

Isolation of B. cereus group strains and screening for monooxygenase activity : Oil industrial effluent dump soil samples selected for isolation of *B. cereus* group bacterial strains in view of the environment where microbial strains adopted to this habitat utilizes the long chain fatty acids as carbon sources for growth after their degradation. Soil sample (0.5 g) suspended in 50mL of sterile saline solution and mixed thoroughly for 30 min at room temperature for suspension of microbial strains. The supernatant solution separated and spread on sterilized *B. cereus* selective agar plates after dilution to 10^{-3} to 10^{-4} concentrations by serial dilution method. These plates incubated at 30°C for 48 hours for the growth of strains. Single peacock blue colonies (general character of *B. cereus* group strains) were re-streaked and tested for hemolysis as previously described (14). These isolated strains were further confirmed for monooxygenase activity by screening for hydroxylation of lauric acid in presence of NAD(P)H and the data revealed that among 16 isolates, five strains possess the monooxygenase activity and designated as CYPPB-1 to 5.

Amplification and comparative sequence analysis of cypD gene **:** Four different primers P450LF, P450HbF, P450OxF and P450RdR (Tables 1 and 2) were designed based on the conservative domain sequences of reference organisms, synthesized and evaluated the genomic DNA of isolated strains for the amplification of cypD gene and presence of BiFCyP450 enzyme. Figure 1, represent positions of the designed primers in the cypD gene. PCR using the pair of primers P450HbF-P450RdR was successful with ~720bp band for strains CYPPB-1 and 2. However, PCR product could not obtained with this primer pair for the CYPPB-3 genomic DNA (Fig 2a and 2b). When PCR performed using the P450OxF–P450RdR, it resulted in obtaining a band of ~1100bp in length for strain CYPPB-3, but was unsuccessful for strains CYPPB-1 and 2 (Fig 2c). While, PCR with the use of P450LF-P450RdR pair of primers, a faint band of ~500bp in length was successful for strains CYPPB-1 and 2 whereas, this pair of primer was unsuccessful for amplification with the genomic DNA of CYPPB-3 (Fig. 2d and 2e).

The amplified fragments of partial cypD gene of three isolates were sequenced. The obtained sequences were edited and subjected to comparative sequence and phylogenetic analysis.

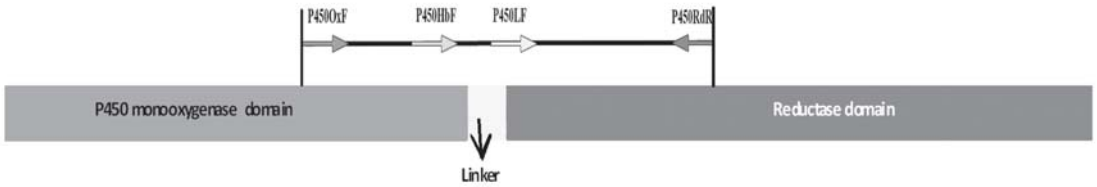

Fig. 1. Schematic representation of locations of primers used for amplification of cypD gene from isolated strains

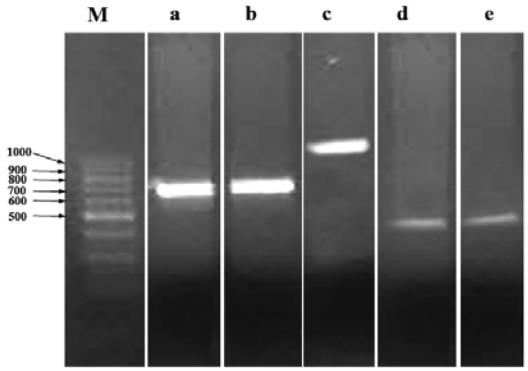

Fig. 2. Agarose gel electrophoresis of PCR products obtained using primers targeting cypD gene; M = Marker, a = CYPPB-1 PCR product amplified by P450HbF-P450RdR; b = CYPPB-2 PCR product amplified by P450HbF-P450RdR; c = CYPPB-3 PCR product amplified by P450OxF-P450RdR; d = CYPPB-1 PCR product amplified by P450LF-P450RdR; e = CYPPB-2 PCR product amplified by P450LF-P450RdR

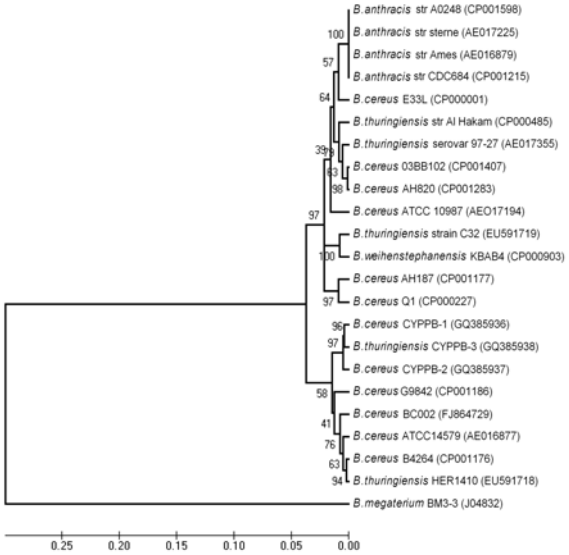

Fig. 3. Phylogenetic analysis of strains CYPPB-1, 2 and 3 on the basis of fragments of cypD gene sequences

Comparative analysis of data denoted that the sequence of isolate, CYPPB-2, revealed some variation from sequences of strains CYPPB-1 and 3 even though, all these three strains were more closely related at phylogenetic tree and formed a distinct cluster closer to *B. cereus* G9842 from public database (Fig. 3). Further comparative translated amino acid sequence analysis with CYP102A5 of *B. cereus* 14579 indicated variation at position 570 (serine instead of praline) in all three isolated strains and histidine instead of glutamine at 602 in CYPPB-1 and 3.

Characterization of cypD gene possessing B.cereus group strains : CYPPB-1, 2 and 3 strains were further characterized for morphological features. It was noticed that all these strains showed motility and produced endospores during a prolonged incubation on nutrient agar suggesting that they may belongs to *B.cereus* or *B. thuringiensis*. To confirm the same, the genomic DNA of these strains was amplified for 16S rDNA sequences using primers of FD1 and RD1 according to Weisburg et al., (1991). Agarose gel electrophpresis followed by determination of 16S rDNA sequences revealed 1054, 1430 and 1490bp length of 16S rDNA for CYPPB-1, CYPPB-2 and CYPPB-3, respectively and submitted to EMBL (Accession No: FN430421, FN430384 and FN430420). Blast analysis denoted that the sequence of CYPPB-1 and 2 were almost similar to that of *B. Cereus* PP13 (EF407813) while CYPPB-3 was similar to that of *B. thuringiensis* TSH67 (AB508858) indicating these isolates belong to *B. thuringiensis*. This was further confirmed based on the observation that CYPPB-3 produced endospores as well as crystals upon prolonged incubation (results not shown). The phylogenetic position of strains CYPPB-1, 2 and 3 with reference to public database shown in figure 4 thus, both the morphological examination and phylogenetic analysis indicated strain CYPPB-3 belongs to *B. thuringiensis* whereas CYPPB-1 and 2 belongs to *B. cereus.*

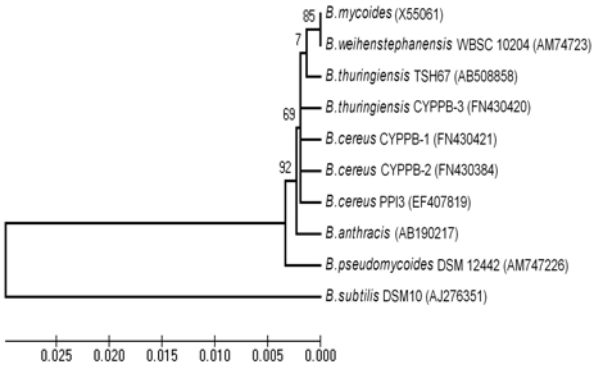

Fig. 4. Phylogenetic analysis of isolated strains of CYPPB-1,2 and 3 on the basis of 16S rRNA gene sequences

Discussion

Cytochrome P450 monooxygenases (CyP450) play a vital role in hydroxylation of different saturated, branched chain, and unsaturated fatty acids at ù-position, which subsequently enter into metabolic pool. Although, this pathway is a minor process (4–15%) (18) compared to cellular metabolism, its importance increases dramatically during starvation, by ethanol, hypolipidemic drugs, peroxisome proliferators, and in different metabolic diseases (19,20,21,22). In addition, CyP450s are also of industrial importance because of their high potential for the selective introduction of one atom of O_2 at even non-activated C–H bonds in a regio- and/or stereo specific manner (23). In spite of this unique property, their use for bio-industrial sectors is restricted due to low operational stability and the requirement of separate reductase (an electron transport protein) as well as a cofactor (NADP(H)) for electron donor (24). In view of the above, bifunctional or self-sufficient P450 monooxygenases are gaining importance for biotechnological applications. Such bifunctional cytchrome P450 monooxygenase was first isolated from *Bacillus megaterium* and reported that this monooxygenase consists of a bifunctional polypeptide with two domains; a heme-containing monooxygenase and a FAD- and FMN-containing reductase (7). However, only a few BiFCyP450 monooxygenases demonstrate activities, which are high enough for technical processes. For example, BiFCyP450 enzymes from the CYP116 family possess only very low activity (25,26) while CYP102A enzymes have higher catalytic activity (10).

The exact physiological role of CYP102A1 and similar P450s is still the subject of much debate. It has been proposed that efficient oxidation of branched chain fatty acids occur in presence of these enzymes (27,28). Palmer et al. (29) suggested that production of CYP102A1 may be a survival strategy as they protect its host (*B. megaterium* and related bacteria) from polyunsaturated fatty acids that are toxic. While Budde et al. (27) studies with CYP102A2 and CYP102A3 enzymes concluded that these enzymes help in regulation of membrane fluidity. Nevertheless, there is no information about physiological role of these enzymes produced by *B. cereus* group (pathogenic group of bacilli family). In view of the above, the present investigation of screening of *B. cereus* group strains for producing BiFCyP450 enzymes by using specially designed primers targeting partial cypD gene assumes importance as first step for future applications.

Generally, expression of BiFCyP450 is very low. This inturn causes difficulty to isolate and characterize BiFCyP450 producing microbial strains from different sources. Hence, traditional screening methods are not efficient for rapid screening of microbial strains containing BiFCyP450s. Consequently, PCR amplification with the specific primers, because of its rapidness and ease, could be effective for identification and distinguish them among other microbial strains.

In the present investigation, the cypD gene responsible for production of BiFCyP450 enzyme was targeted for screening BiFCyP450 producing *B. cereus* group isolates as well as to detect diversity of cypD gene using cypD gene targeted primers. It is evident from the data that among all three designed primer pairs, at least one suitable for amplification of cypD gene in either of isolated strains (CYPPB-1, 2 and 3). Further blast analysis of obtained sequences clearly confirmed that presence of cypD genes of BiFCyP450 in all isolated strains i.e., CYPPB-1, 2 and 3.

Microscopic and 16S rDNA analysis of these isolated strains further indicated that CYPPB-1, 2 belongs to *B. cereus* and CYPPB-3 to *B. thuringiensis*. This could be evidenced based on observation that CYPPB-1 and 2 strains showed motility which is the characteristic nature of *B. cereus* and CYPPB-3 revealed presence of crystals, which is the prominent phenomenon of *B. thuringiensis.*

According to P450 nomenclature, it is reported that cypD gene possessing *B. cereus* strains were designated as CYP102A5 while *B. thuringiensis* strains designated as CYP102A8 (http://drnelson.utmem.edu/biblioE.html#102). Based on above, the BiFCyP450 of CYPPB-1 and 3 strains should be identified as CYP102A5 while the other strain, CYPPB-2 identified as CYP102A8. In contrast, phylogenetic analysis revealed that CYPPB-1 and 2 belonging to *B. cereus* and CYPPB-3 belongings to *B. thuringiensis* (Fig. 3 and 4). This data suggest that one should reconsider at nomenclature of cypD gene possessing microbial strains owing to the observed diversity of cypD gene among *B. cereus* group. Overall, the data suggest that BiFCyP450 in *B. cereus* group represents an excellent potential resource of different BiFCyP450s.

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The Effect of Medium Supplementation with Second Carbon Source and Amino Acids for Enhanced Production of Cyclosporin A

Shrikant A. Survase*, Uday S. Annapure and Rekha S. Singhal
Food Engineering and Technology Department, Institute of Chemical Technology,
Matunga, Mumbai 400 019, India
*For Correspondence - shrikantraje1@rediffmail.com

Abstract

The effect of a second carbon source and amino acids on the production of cylcosporin A (CyA) in a medium optimized by one factor at a time and orthogonal array method was studied. The one factor at-a-time method was adopted to investigate the effects of medium components (i.e. carbon, and nitrogen) and environmental factors (i.e. initial pH) on biomass growth and CyA production. Subsequently, L_{16} orthogonal matrix was used to evaluate the significance of glucose, ammonium sulphate, K_2HPO_4 and inoculum size. The effects of media components were ranked according to their effects on the production of CyA as glucose > ammonium sulphate > K_2HPO_4 > inoculum size. The production increased from 58.35 mg/l to 148 mg/l when maltose (1 %) was added as a second carbon source after 8 days of fermentation. Evaluation of various amino showed that the addition of L-valine (6 g/l) and L-leucine (4 g/l) after 24 h fermentation enhanced the CyA production dramatically to 794 mg/l.

Key words: Cyclosporin A, submerged fermentation, orthogonal array method, *Tolypocladium inflatum*

Introduction

Cyclosporins represent a group of cyclic peptides composed of eleven amino acids. They are produced by the fungi of genus *Tolypocladium* in submerged (1) as well as solid state fermentation (2). Cyclosporin A (CyA) is a promising immunosuppressive drug which is being used extensively in organ transplantation and the treatment of autoimmune diseases (3, 4).

In a medium containing glucose and an alternate carbon source that is slowly utilized, glucose is usually used first. After glucose is depleted, the second carbon source is used for antibiotic biosynthesis (5). Polysaccharides or oligosaccharides are often found to be better than glucose as carbon sources for antibiotic production (6). Addition of specific amino acids to the culture media has been shown to alter the spectrum of secondary metabolite composition, favoring an increased production of CyA specified by incorporation of the surplus amino acid, preferably in position 2 (7).

Taguchi constructed a special set of general design guidelines for factorial experiments that cover many applications. The method uses a special set of arrays called orthogonal arrays, which stipulate the way of conducting the minimal number of experiments, and give the full information of all the factors that affect the performance parameter. Taguchi's orthogonal array provides an alternative to standard factorial

designs. While there are many standard orthogonal arrays available, each of the arrays is meant for a specific number of independent design variables and levels. The effect of one independent variable does not depend on the different level settings of any other independent variables and vice versa (8, 9). Survase et al. (10) and Chauhan et al. (11) used L_{16} and L_{12} orthogonal array designs to optimize fermentation media for scleroglucan and cholesterol oxidase, respectively.

To the best of our knowledge, there is scarcity of literature on the effect of supplementation of carbon source on the production of CyA in a submerged culture, although supplementation of amino acids has been studied by few researchers (12, 13). Accordingly, the objectives of the present study were initially to evaluate the significance of different media components and optimize their concentration to get the maximum production of CyA by *T. inflatum* MTCC 557 using L16 orthogonal array. Subsequently, effect of addition of secondary carbon source and different constituent amino acids and its time of addition was also evaluated further to increase the yields.

Materials and Methods

***Material*:** All the chemicals used in the present study were AR grade except acetonitrile which was of HPLC grade. Standard CyA (authentic sample) was a gift sample through the kind courtesy of RPG Life Sciences Ltd., Mumbai.

Microorganisms: Strains of *Tolypocladium inflatum* MTCC 989, *Tolypocladium inflatum* MTCC 557 (indicated as *Beauveria nivea* in the MTCC catalog), *Tolypocladium inflatum* NCIM 1283, were procured from MTCC, Chandigarh and NCIM, Pune. *Tolypocladium inflatum* NRRL 18950 was a gift sample from ARS Culture Collection, United States. The cultures were maintained on agar slants containing malt extract 2 % and yeast extract 0.4 % (MYA), pH 5.4 at 4 °C after growing it for 12 days at 24 °C. The strains were screened for maximum production of CyA.

***Preparation of the seed inoculum*:** The organism was subcultured onto a fresh MYA slant and incubated at 25 ± 2 °C. After 12 days, to a fully grown slant, 10 ml of sterile saline containing 0.1 % Tween 20 was added and mixed well. One milliliter of this saline containing approximately 10^8 -10^9 spores was added to 50 ml of medium composed of malt extract 2 %, yeast extract 0.4 %, pH 5.4 taken in a 250 ml flask and incubated at 180 rpm for 72 hours at 25 ± 2 °C.

***Fermentation*:** In the present study, medium developed by Kobel and Traber (7) was used for optimization of CyA production by *T. inflatum* MTCC 557. The composition of medium used was glucose (30 g/l), ammonium sulphate (10g/l) K_2HPO_4 (7.5 g/l) and trace element solution (1ml/l). Trace element solution contained (in mg/l) $ZnSO_4$ 4400, $FeSO_4$ 5000, $MnCl_2$ 180, Na_2MO_4 25, $CuSO_4$ 80, and H_2SO_4 2ml. pH was adjusted to 5.7 ± 0.2, and 5 ml seed culture was used to inoculate 50 ml of sterile production medium. The fermentation was carried out at 25 ± 2 °C for 14 days at 180 rpm.

***One Factor at-a-time*:** Four different *T. inflatum* strains were screened for the maximum production of CyA using the basal medium. To evaluate the effect of different carbon sources on the production of CyA, glucose in the basal medium was replaced with different carbon sources, *viz*., glycerol, sucrose, maltose, fructose, galactose, soluble starch, sorbitol, mannitol and xylose at 30 g/l. Different nitrogen sources at 10 g/l were tested for their effect on CyA production. They included sodium nitrate, ammonium chloride, ammonium sulphate, urea, diammonium hydrogen phosphate. To study the effect of pH on CyA production, fermentation runs were carried out at initial pH varying from 2.7 to 7.7.

***Orthogonal array method*:** The three medium components *viz.* glucose, ammonium sulphate, K_2HPO_4 and the inoculum's size were used for the design. Each variable was varied over four levels. The design for the L_{16}-orthogonal array was developed and analyzed using "MINITAB 13.30" software. Table 1 depicts the fermentation conditions and the L_{16}-orthogonal array, which was used in the present study. All experiments were performed in at least triplicates.

***Effect of supplementation of additional carbon source*:** Three different carbon sources viz. maltose, glycerol and maltodextrin were supplemented at three different concentrations (1 % , 2 %, 3 %) as a second carbon source and the effect was observed on biomass and CyA production. The effect of time of addition of the optimized concentration was also evaluated for the maximum production of CyA.

Effect of amino acids: The effect of different amino acid members of CyA molecule on drug production was evaluated by supplementing the fermentation medium. The amino acids tested were L-valine, L-leucine, DL-valine, L-methionine, L-aminobutyric acid and glycine, screened individually (4 g/l) as well as in combination. The time of addition of amino acids (0 to144 h) was also optimized to further increase the yield.

***CyA extraction and estimation*:** A 10 ml of culture broth was extracted with equal volume of n-butyl acetate. Before extracting the sample a concentrated solution of NaOH was added to reach the concentration of 1N and heated at 60 °C for 30 min. The mixed sample was kept on rotary shaker (180 rpm) for 24h. After centrifuging the extract was filtered using Whatman filter paper (No.1) and then using Pall 0.2 ìm membrane filter (Ultipor® N_{66}® Nylon 6, 6 membranes) to give a brown colour extract. One milliliter of the extract was evaporated under vacuum to dryness. The dried extract was dissolved in equal volume (1 ml) of HPLC grade acetonitrile. Twenty microliters of sample was analysed by the method described by Survase et al. (2). CyA content was analyzed using HPLC (JASKO System) fitted with a reverse-phase column Waters Sperisorb ODS (C_{18} octadecyl silane, 250×4.6 mm ID). The mobile phase consisted of acetonitrile and water in the ratio 70:30 with a flow rate of 1 ml/min. The temperature of the column was maintained at 70 ºC and the HPLC profile was monitored at 210 nm.

***Biomass measurement*:** A 10 ml of culture broth was filtered through a pre-weighed Whatman filter paper (No.1), washed twice with distilled water and dried at 80 °C to constant weight. This was reported as dry cell weight (DCW).

Results and Discussion

***Screening*:** Several strains were screened for the maximum production of CyA by using synthetic media reported by Kobel and Traber (7) with 10 % inoculum of 72 h old seed at 25 ± 2 °C for 14 days. *T. inflatum* MTCC 557 gave maximum production of 37.22 ± 1.13 mg/l, followed by 25.21 ± 1.10 mg/l and 22.28 ± 1.12 mg/l by *T. inflatum* NRRL 18950 and *T. inflatum* NCIM 1283, respectively. Hence *T. inflatum* MTCC 557 was used for further optimization. *T. inflatum* MTCC 989 gave lower titers of CyA.

Among the carbon sources screened for maximum production of CyA and biomass *T. inflatum* MTCC 557, glucose supported the maximum production of 37.8 ± 1.28 mg/l followed by sucrose and maltose (33.25 and 31.32 mg/l, respectively) (Fig. 1). It was also observed that glycerol, sorbitol, starch and galactose supported only biomass growth and were not able to create the physiological condition to produce CyA. Dreyfuss et al. (14) used glucose (40 g/l) as carbon source and reported to produce 180 mg/l of CyA using industrial strain of *T. inflatum*.

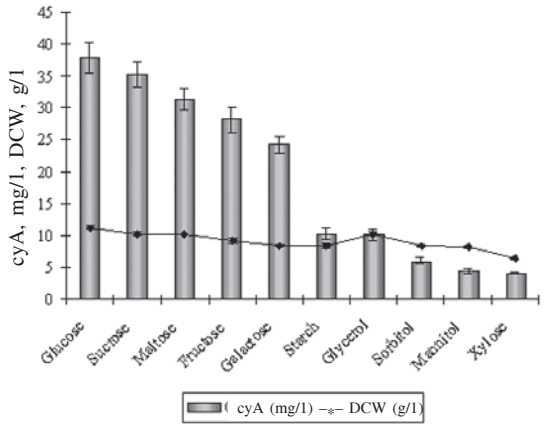

Fig. 1. Effect of carbon source on production of CyA using *T. inflatum* MTCC 557

Glucose has also been reported to be a better carbon source for CyA production by Balakrishnan and Pandey (12), Sallam et al. (15). Abdel-fattah et al. (1) used three carbon sources as glucose (10 g/l), sucrose (20 g/l) and starch (20 g/l) in combination to give maximum of 110 mg/l CyA production using *T. inflatum* DSMZ 915. Agathos et al. (16) reported use of sorbose (30 g/l) to produce a maximum of 105 mg/l CyA using *T. inflatum* ATCC 34921. Margaritis and Chahal (17) developed fructose based medium for the production of CyA using *Beauveria nivea.* They used fructose to minimize the catabolite repression and oxygen limitation in the pallets formed during the production stage to get maximum CyA yields.

Among the nitrogen sources studied, ammonium sulphate supported the maximum CyA production (38.56 mg/l) followed by $NaNO_3$ (33.60 mg/l) (data not shown). Ammonium sulphate supported the maximum biomass production of 11.35 g/l measured as DCW (dry cell weight). The production of CyA was least supported by urea. Abdel-fattah et al. (1) reported the use of ammonium sulphate as nitrogen source supporting maximum production. Dreyfus et al. (14) used combination of $NaNO_3$ and casein peptone as a nitrogen source for the production using industrial strain of *T. inflatum*.

An initial pH 5.7 supported maximum CyA (37 mg/l) and biomass (11.2 g/l as DCW) production. Most studies on fermentative production of CyA have been carried out at pH 5.7.

***L_{16} Orthogonal array*:** Once the best carbon and nitrogen sources were selected, the medium was optimized using L_{16} orthogonal array. The parameters optimized were concentrations of glucose, ammonium sulphate, KH_2PO_4 and inoculum size. Table 2 represents the response table for means (larger is better) and for signal to noise ratio obtained with L_{16} orthogonal array. Rank and delta values help to assess which factors have the greatest effect on the response characteristic of interest. The order in which the individual components selected in the present study effect the fermentation process can be ranked as glucose >ammonium sulphate > K_2HPO_4> inoculum size suggesting that sucrose had a major effect and inoculum size had least effect on CyA production using *T. inflatum* MTCC 557. Fig. 2 represents the main effect

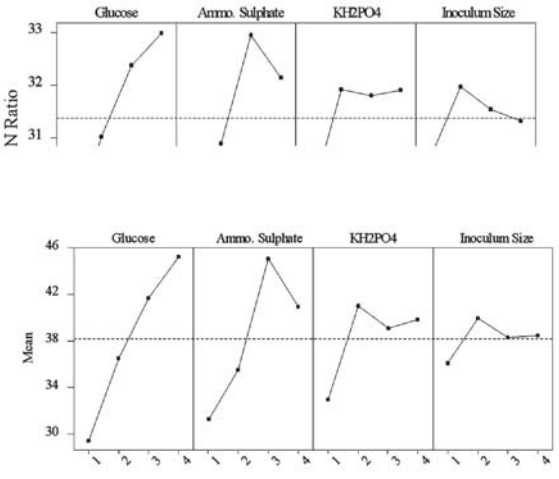

Fig. 2. Main effects plot for a) S/N ratios b) means

plots for the system. Main effects plots show how each factor affects the response characteristic. A main effect is 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 2. A line connects the points for each factor. When the line is horizontal (parallel to the x-axis), then there is no main effect present. When the line is not horizontal (parallel to the x-axis), then there is a main effect present. Different levels of the factor affect the characteristic differently. The greater the deviation from the parallel x-axis, the greater is the magnitude of the main effect. This difference is a main effect *i.e.* glucose at level 4, ammonium sulphate at level 3, K_2HPO_4 at level 4 and inoculum size at level 2 shows a main effect. These levels also represent the optimal concentrations of the individual components in the medium.

Response tables can also be used to predict the optimal levels of each component used in the study. The final medium for CyA production using *T. inflatum* MTCC 557 contained (in g/l) glucose 50, ammonium sulphate 15, K_2HPO_4 1.25 and inoculum size at 10 % v/v. To confirm these results, experiments were carried out using these nutrient concentrations and it was observed that the mean value obtained was 58.35 mg/l as compared to 57.32 mg/l predicted using MINITAB for the same composition. Fig. 3 presents kinetic data from batch fermentation of *T. inflatum* MTCC 557 using synthetic media optimized by orthogonal array method. It was observed that CyA production started from 3rd day of fermentation and reached a maximum on 14th day. After 10th day the biomass production was unchanged and the CyA production slowed down. This may be due to the very low levels of residual glucose in the medium after 10th day.

***Effect of additional carbon source*:** Table-3 shows the effect of addition of second carbon source at different concentrations, added at the beginning of the fermentation, on biomass and CyA production. It was observed that maltose at 1 % stimulated maximum CyA of 108 mg/l. An increase in maltose concentration did not show any further improvement. Addition of 1 % maltodextrin also improved CyA yield to 88 g/l. Glycerol addition did not show any promising effect on CyA production, although the biomass production was improved. Fig. 4 shows the effect

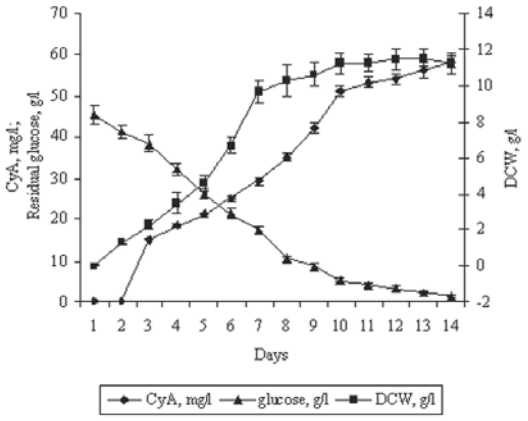

Fig. 3. Production profile, glucose utilization and biomass production from *T. inflatum* MTCC 557

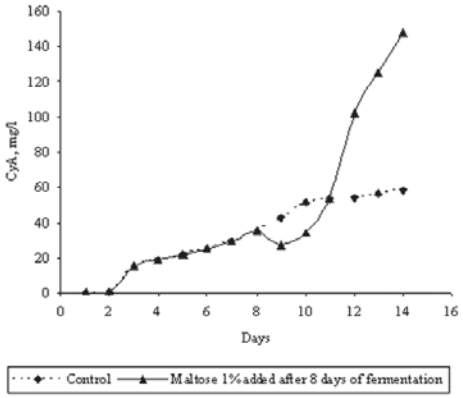

Fig. 4. Effect of maltose (1 %) addition on CyA production using *T. inflatum* MTCC 557. indicates point of addition of maltose.

of time of addition of 1 % maltose on CyA production. It was found that addition of maltose after 8 days of fermentation stimulated the production of CyA to 148 mg/l. A lag period was observed after addition of maltose which resulted in slight decrease in CyA production. The results are found to be in accordance with Agathos et al. (16). They reported the use of 2 % maltose as second carbon source and its addition after 8 days of fermentation to double the production of CyA. The observed decrease in CyA during lag phase may be due to some carbon catabolite regulation, where CyA levels were partially depleted either through degradation or metabolism.

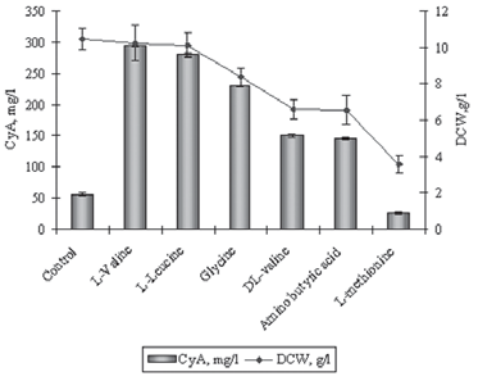

Fig. 5. Effect of different amino acids on production of CyA using *T. inflatum* MTCC 557

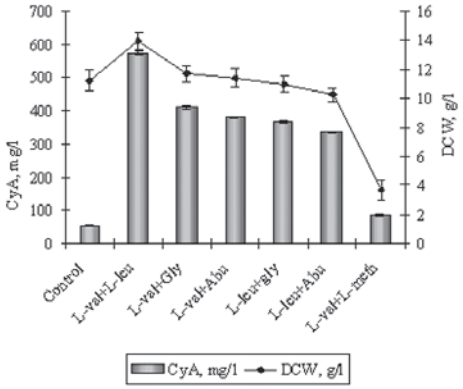

Fig. 6. Effect of combination of amino acids on production of CyA using *T. inflatum* MTCC 557 (Where, val - valine, leu - leucine, gly - glycine, meth - methionine, Abu - amino butyric acid)

***Effect of amino acids*:** Fig. 5 shows the effect of supplementation with different amino acids at 4 g/l on CyA production. Of all the amino acids tested, L-valine produced the maximum CyA of 295 mg/l followed by L-leucine and glycine (280 mg/l and 230 mg/l, respectively). These results were found to be in agreement with Lee and Agathos (13) and contrary to Kobel and Traber (7). Lee and Agathos (13) reported that L-valine supplementation of synthetic media did not support the production of cyclosporins C and D, and did not direct the synthesis preferentially to cyclosporin D. DL-valine did not increase the product titer as that of L-valine. We found a dramatic decrease in CyA production after supplementing the fermentation media with L-methionine. This may suggest that one or more methylation steps by L-methionine mediated through S-adenosyl-L-methionine might control the synthesis of CyA through a feedback mode. Even though the methylation step may not be rate limiting in a low production environment, it can be a bottleneck in physiological states involving large methionine pools.

When added together, L-valine and L-leucine (both at 4 g/l) increased the CyA production dramatically (574 mg/l) (Fig 6). These two amino acids seem to act independently and their mode of action is different. This effect was not observed with other amino acid combinations. A similar behavior was noticed in the synthesis of bacitracin, where the individually stimulatory amino acids, L-phenyl alanine and histidine, strongly increased antibiotic production when supplied together in a defined medium (18). Similar results were encountered by Lee and Agathos (13); Balakrishnan and Pandey (12) and Nisha et al. (19) in CyA biosynthesis. When L-methionine was added to a medium supplemented with L-valine, the stimulatory effect of L-valine was completely reversed. Zocher et al. (20) reported that methionine could not take part in

Table 1. L_{16} orthogonal array design for CyA production

Exp. No.	Glucose[a]	Ammonium Sulphate[a]		KH_2PO_4[a]	Inoculum Size[b] CyA[c], mg/l
1	1(20)	1(5)	1(0.5)	1(5)	18.30 ± 0.89
2	1(20)	2(10)	2(0.75)	2(10)	32.72 ± 1.12
3	1(20)	3(15)	3(1.0)	3(15)	36.36 ± 1.02
4	1(20)	4(20)	4(1.25)	4(20)	30.26 ± 0.98
5	2(30)	1(5)	2(0.75)	3(15)	28.70 ± 0.59
6	2(30)	2(10)	1(0.5)	4(20)	27.91 ± 1.02
7	2(30)	3(15)	4(1.25)	1(5)	44.36 ± 1.25
8	2(30)	4(20)	3(1.0)	2(10)	45.10 ± 0.97
9	3(40)	1(5)	3(1.0)	4(20)	37.28 ± 0.69
10	3(40)	2(10)	4(1.25)	3(15)	43.90 ± 1.13
11	3(40)	3(15)	1(0.5)	2(10)	41.26 ± 1.52
12	3(40)	4(20)	2(0.75)	1(5)	44.20 ± 1.25
13	4(50)	1(5)	4(1.25)	2(10)	40.73 ± 1.42
14	4(50)	2(10)	3(1.0)	1(5)	37.54 ± 1.23
15	4(50)	3(15)	2(0.75)	4(20)	58.37 ± 1.25
16	4(50)	4(20)	1(0.5)	3(15)	44.29 ± 1.45

[a] values in the parenthesis are real values in g/l
[b] values in the parenthesis are real values in %
[c] Values a re mean ± SD of three determinations

Table 2. Response table for means and S/N ratio

Level	**Glucose**		**Ammo. Sulphate**		**KH_2PO_4**		**Inoculum Size**	
	Mean	**S/N**	**Mean**	**S/N**	**Mean**	**S/N**	**Mean**	**S/N**
1	29.41	29.09	31.25	29.5	32.94	29.85	36.1	30.64
2	36.51	31.02	35.51	30.88	40.99	31.92	39.95	31.97
3	41.66	32.37	45.08	32.94	39.07	31.8	38.31	31.53
4	45.23	32.98	40.96	32.13	39.81	31.9	38.85	31.32
Delta	15.82	3.89	13.83	3.43	8.05	2.07	3.85	1.32
value								
Rank	1	2	3	4				

Table 3. Effect of second carbon source on CyA production and biomass formation (Second carbon source was added at 0 h of fermentation. Control is no second carbon source).

Second carbon source		CyA, mg/l	DCW, g/l
Control	58 ± 7	12.21 ± 0.58	
Maltose	1 %	108 ± 11	14.21 ± 0.48
	2 %	105 ± 8	14.02 ± 0.57
	3 %	96 ± 7	13.14 ± 0.68
Maltodextrin	1 %	88 ± 9	13.04 ± 0.57
	2 %	74 ± 6	12.56 ± 0.36
	3 %	62 ± 5	12.45 ± 0.24
Glycerol	1 %	61 ± 6	13.56 ± 0.34
	2 %	64 ± 5	13.98 ± 0.54
	3 %	68 ± 6	14.23 ± 0.45

the biosynthesis, as methylated amino acids interfere with the biosynthesis of cyclosporin in vivo.

The optimal amount and time of addition of L-valine was also investigated. It was observed that the precursor role of L-valine was consistent after reaching a saturation level at 6 g/l initial L-valine concentration. Maximum CyA production of 312 mg/l was observed at an initial L-valine concentration of 6 g/l. A further increase in concentration did not enhance the yield further. The optimum time for addition of L-valine and L-leucine in combination for maximum product titre was found to be 24 h (Data not shown). When added together after 24 h, CyA production of 794 mg/l was obtained. This may be due to the utilization of amino acids as nitrogen source in early phase of fermentation. These amino acids may have a role of inducer to increase the transcription of genes for CyA synthatase or other structural genes contributing to CyA synthesis which resulted in positive effect when added early in the fermentation.

Conclusion

The combination of conventional and statistical method could improve the CyA production successfully. The use of orthogonal array method gave most important nutrients for CyA production, and also increased the production of CyA to 58 ± 1.62 mg/l. Supplementation of medium with 1 % maltose after 8 days of fermentation supported maximum CyA production of 148 ± 1.12 mg/l. These results suggest that the production can further be improved by systematically studying the feeding of second carbon source. L-valine (6 g/l) and L-leucine (4 g/l) added after 24 h of fermentation gave maximum production of 794 mg/l.

Acknowledgement

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Anti-inflammatory and *in vitro* Antioxidant Property of *Zanthoxylum nitidum* Root

Sanjib Bhattacharya[1*], Pallab K Haldar[2] and Md. K Zaman[3]
[1]Bengal School of Technology (A College of Pharmacy), Delhi Road, Sugandha, Hooghly 712102, West Bengal, India.
[2]Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, West Bengal, India.
[3]Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh 786004, Assam, India.
*For Correspondence - sakkwai@yahoo.com

Abstract:

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), called *Tez-mui* or *Tejamool* in Assamese is a large prickly shrub occurring in North-East India and its roots are used traditionally for several medicinal purposes. In present study the methanol extract of roots from *Zanthoxylum nitidum* (ZNME) was evaluated for its anti-inflammatory activity in acute (carrageenan, histamine and serotonin induced rat paw oedema) and chronic models (cotton pellet induced granuloma). In all models the ZNME (75 and 150 mg/kg body wt. p.o.) exhibited significant anti-inflammatory activity ($p < 0.001$) in a dose dependent manner when compared with saline control. Indomethacin (10 mg/kg body wt. p.o.) was used as reference drug. The ZNME was evaluated for its antioxidant properties by 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay and *in vitro* lipid peroxidation induced by the Fe^{2+}- ascorbate system in rat liver homogenate. In DPPH radical scavenging assay, the ZNME demonstrated marked and dose dependent free radical scavenging effect and the mean inhibitory concentration (IC_{50}) of the ZNME was found to be 75.2 µg/ml, while the ascorbic acid (reference) exhibited 43.7 µg/ml. The ZNME effectively inhibited the lipid peroxidation in a dose related manner showing the IC_{50} value of 279.1 µg/ml, whereas the quercetin (reference) showed 46.6 µg/ml. These findings revealed that the *Z. nitidum* root had remarkable acute and chronic anti-inflammatory and *in vitro* antioxidant actions in the tested models validating its traditional uses.

Key words: *Zanthoxylum nitidum,* anti-inflammatory, radical scavenging, lipid peroxidation.

Introduction

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), called *Tez-mui* or *Tejamool* in Assamese, is a morphologically variable plant species occurring in South-East Asian countries and in Northern Australia (1). In India it grows as a large prickly shrub particularly in North-East India (Sikkim, Assam and Nagaland states). In India the plant has traditionally been used for various medicinal purposes. The root is used in toothache, stomachache, fever, rheumatism, paresis, boils and as an insecticide and piscicide. The fruit is used in the treatment of stomachache, cough, colic, vomiting, diarrhoea, and paresis and as an aromatic, stimulant and piscicide. The small branches, seeds and stem bark are prescribed in fever, diarrhoea and cholera (2-4). It has come to the author's notice that the rural people of upper Assam, India use the young stems of this plant as chewing stick in treatment of toothache and gingivitis. Previously the authors have reported

essential oil composition of fruits and leaves, pharmacognostic parameters of stem bark and root, antibacterial effects of stem bark and root, anti-nociceptive activity of stem bark of *Z. nitidum* from India (5-9). There are no reports of anti-inflammatory investigations carried out on *Z. nitidum* of Indian habitat. The present work therefore, attempts to report the preliminary results of studies on anti-inflammatory and *in vitro* antioxidant effects of *Z. nitidum* root in experimental models to justify the traditional and folkloric beliefs.

Materials and Methods

***Plant material*:** The mature entire plants of *Z. nitidum* were collected during the month of November 2007 from Dibrugarh district of Assam state, India. The species was identified by Dr. S. J. Phukan, taxonomist, from Botanical Survey of India, Eastern Circle, Shillong, India, and a voucher specimen (No. DUPS-06-003) was deposited in Department of Pharmaceutical Sciences, Dibrugarh University for future reference. The roots were separated from the aerial parts and cut into small pieces. Then the plant material was shade dried at temperature 21-24 °C and ground mechanically into a coarse powder and stored in an air-tight container.

Preparation of extract: Powdered plant material (150 g) was macerated with 400 ml of methanol at 21-24 °C temperature for 2 days with frequent shaking. After 2 days, the extracts were filtered and to the marc part 300 ml of the solvent was added and allowed to stand for next 2 days at same temperature for second time maceration (re-maceration) and after two days, again filtered similarly. The combined filtrates (macerates) were evaporated *in vacuo* at 40 °C and the dry extract obtained (ZNME, yield 11.6 % w/w) was stored in a vacuum desiccator for future use. Preliminary phytochemical studies indicated the presence of alkaloids, flavonoids, carbohydrates, reducing sugars and amino acids in ZNME (10).

Drugs and chemicals: λ-Carrageenan (type IV) was obtained from S. D. Fine Chemicals Ltd., Bombay; 5-hydroxytryptamine hydrochloride (serotonin), histamine sulphate, thiobarbituric acid were from Sigma Chemical Co., USA; indomethacin was from Recon, Bangalore, India; DPPH, L-ascorbic acid were from Sisco Research Laboratories Pvt. Ltd., India; quercetin was from Aldrich Chemical Co., UK. All other chemicals, reagents and solvents were of analytical grade available commercially.

Animals: Studies were carried out using adult male Wistar albino rats of weighing 150-180 g. They were obtained from the animal supplier (Ghosh & Co., Kolkata, India). The animals were grouped in polyacrylic cages (38 cm × 23 cm × 10 cm) with not more than four animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C, dark and light cycle 14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. All experimental methods were approved by University Animal Ethical Committee, Jadavpur University (Reg. no. 367001/C/CPCSEA).

***Acute toxicity*:** The oral LD_{50} value of ZNME in male Swiss albino mice were determined as per reported method (11).

Anti-inflammatory activity

***Carrageenan-induced rat paw oedema*:** The rats were divided into four groups (n = 6). The first group (which served as control) received normal saline (0.9 % w/v, 3 ml/kg body wt., p.o.). The second and third group received the test

extract ZNME (75 and 150 mg/kg body wt., p.o., respectively). The fourth group (which served as reference) received indomethacin (10 mg/kg body wt., p.o.). After 30 mins, acute inflammation was produced by the subplantar administration of 0.1 ml of 1 % (w/v) of freshly prepared suspension of carrageenan in the right hind paw of each rat. The paw volume was measured at 0 h and 3 h after carrageenan injection by using plethysmometer (Ugo Basile, Italy). The difference between the two readings was taken as the volume of oedema and the percentage of inhibition was calculated (12-14).

Mediator-induced inflammation: The paw oedema was induced in rats by subplantar injection of 0.1 ml of freshly prepared histamine (1 mg/ml) and serotonin (1 mg/ml) solutions respectively (15,16). Group division and treatment regime of the animals were same as the carrageenan induced rat paw oedema model and the paw oedema was measured as mentioned earlier.

***Cotton pellet-induced granuloma*:** The animals were divided into four groups (n = 6). The rats were anaesthetized and sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin region of each rat. The first group (which served as control) received normal saline (0.9 % w/v, 3 ml/kg body wt., p.o.). The second and third group received the test extract ZNME (75 and 150 mg/kg body wt., p.o., respectively). The fourth group (which served as reference) received indomethacin (10 mg/kg body wt., p.o.). All groups were treated in this way for seven consecutive days from the day of cotton pellet implantation (17). On 8th day the animals were anaesthetized and the pellets together with the granuloma tissues were carefully removed and made free from extraneous tissues. The wet pellets were then dried in an oven at 60 °C for 24 h to constant weight. Increment in the dry weight of the pellets was taken as a measure of granuloma formation (18).

***In vitro* antioxidant property**

Scavenging activity of DPPH: The antioxidant property of ZNME was determined on the basis of their scavenging activity of stable 1,1- diphenyl-2-picryl hydrazyl (DPPH) free radical (19, 20). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of ZNME suspension in water at different concentrations (25-200 ìg/ml). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using methanol as blank on UV-visible spectrophotometer Shimadzu, UV-1601. The scavenging activity was measured as the decrease in absorbance of the samples versus standard DPPH solution. Ascorbic acid was used as the reference. The results were expressed as percentage of inhibition at different concentrations and IC_{50} was determined. The IC_{50} (mean inhibitory concentration) value denotes the concentration of the sample (in µg/ml) required to scavenge 50 % of the DPPH free radicals. The percentage scavenging activity was calculated by using the following formula,

$$(A_{control} - A_{sample} / A_{control}) \times 100,$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test extract) and A_{sample} is the absorbance of the sample at different concentrations . All the tests were performed in triplicate and the results averaged.

Determination of inhibition of lipid peroxidation

***Tissue sample preparation*:** The liver of normal rat was excised and perfused *in vitro* with ice cold normal saline (0.9 % w/v). The tissues were then homogenized at a concentration of 10 % w/

v in 1.15 % w/v KCl solution and centrifuged at 1200 g at 4 ºC for 10 mins. The supernatant was collected which was again centrifuged at 10000 g at - 4ºC for 10 mins. The supernatant was taken and stored at -20ºC for use in the study (21).

Estimation method: Lipid peroxidation induced by Fe^{2+}- ascorbate system in rat liver homogenate was estimated as thiobarbituric acid reacting substance (TBARS) by the method of Ohkawa *et al* (22). The reaction mixture contained rat liver homogenate 0.1 ml in Tris-HCl buffer (40 mM, pH 7.0); KCl (30 mM); $FeSO_4(NH_4)_2SO_4.7H_2O$ (0.16 mM); sodium ascorbate (0.06 mM); and various concentrations of ZNME in a final volume of 0.5 ml. The reaction mixture was incubated at 37ºC for 1 h. After the incubation period, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS, 8.1%); 1.5 ml thiobarbituric acid (TBA, 0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of *n*-butanol and pyridine mixture (15: 1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The upper *n*-butanol-pyridine layer was removed and its absorbance at 532 nm was measured by using UV-visible spectrophotometer Shimadzu, UV-1601. Inhibition of lipid peroxidation was determined by comparing the absorbance of treatments with that of the control. Quercetin was used as the reference.

The inhibitory ratio of the test sample was evaluated by the following formula,

Percentage inhibition = $A_c - A_s/A_c \times 100$ %.

Where A_c is the absorbance of control (containing all reagents except the test extract) and A_s is the absorbance of the sample at different concentrations. All the tests were performed in triplicate and the results averaged.

***Statistical analysis*:** Except antioxidant studies the values were expressed as mean ± standard error of mean (SEM). The statistical significance was determined by using the Student's '*t*' test (23). Values of $p < 0.001$ were considered as statistically significant.

Results

The oral LD_{50} value of ZNME in mice was found to be 1500 mg/kg body weight.

The anti-inflammatory activity of *Z. ntidum* root extract (ZNME) was evaluated against carrageenan induced acute paw oedema in rats and the results are summarized in Table 1. The ZNME produced significant ($p < 0.001$) anti-inflammatory activity in a dose dependent manner.

Table 1. Effect of ZNME on carrageenan induced rat paw oedema

Treatment	Dose (mg/kg)	Increase in paw volume (ml) ± SEM	Percentage of inhibition
Control	-	0.774 ± 0.13	-
ZNME	75	0.483 ± 0.08*	43.67
ZNME	150	0.332 ± 0.04*	57.11
Indomethacin	10	0.219 ± 0.02*	71.71

Number of animals per group (*n*) = 6. SEM = Standard Error of Mean.
*$p < 0.001$, compared to control group.

The ZNME showed maximum inhibition of 57.11 % at the dose of 150 mg/kg body wt. after 3 h of treatment, whereas the reference drug indomethacin produced 71.71 % of inhibition.

In case of histamine and serotonin induced paw oedema, ZNME exhibited significant ($p < 0.001$) and dose dependent protection from oedema (Table 2 and 3). The ZNME produced 51.89 % inhibition in case of histamine and 59.66 % of inhibition in case of serotonin at the dose of 150 mg/kg body wt.; while the reference drug, indomethacin produced 62.37 and 70.63 % of inhibition of paw oedema respectively in above two mediators.

In chronic inflammatory model (cotton pellet induced granuloma) the ZNME significantly ($p < 0.001$) and dose dependently reduced the weight of cotton pellets as compared to the vehicle control (Table 4). The ZNME produced the maximum inhibition of 53.99 % at the dose of 150 mg/kg body wt. and the reference drug indomethacin produced 64.06 % of inhibition of granuloma formation.

The antioxidant property of ZNME was evaluated by DPPH radical scavenging assay and the results are summarized in Table 5. The percentage of inhibition was found to be 64.23 % at the concentration of 200 µg/ml and 9.18 % at the concentration of 25 µg/ml. The IC_{50} value of ZNME was 75.2 µg/ml. Ascorbic acid was used as reference and its IC_{50} value was found to be 43.7 µg/ml.

The antioxidant property of ZNME was also evaluated by the inhibition of

Table 2. Effect of ZNME on histamine induced rat paw oedema.

Treatment	Dose (mg/kg)	Increase in paw volume (ml) ± SEM	Percentage of inhibition
Control	-	0.582 ± 0.024	-
ZNME	75	0.341 ± 0.008*	41.41
ZNME	150	0.280 ± 0.015*	51.89
Indomethacin	10	0.219 ± 0.006*	62.37

Number of animals per group (n) = 6. SEM = Standard Error of Mean.
*$p < 0.001$, compared to control group.

Table 3. Effect of ZNME on serotonin induced rat paw oedema.

Treatment	Dose (mg/kg)	Increase in paw volume (ml) ± SEM	Percentage of inhibition
Control	-	0.647 ± 0.007	-
ZNME	75	0.389 ± 0.011*	39.88
ZNME	150	0.261 ± 0.005*	59.66
Indomethacin	10	0.190 ± 0.003*	70.63

Number of animals per group (n) = 6. SEM = Standard Error of Mean.
*$p < 0.001$, compared to control group.

Table 4. Effect of ZNME on cotton pouch induced granuloma in rats.

Treatment	Dose (mg/kg)	Increased wt of cotton pellet (mg) ± SEM	Percentage of inhibition
Control	-	39.23 ± 0.18	-
ZNME	75	25.72 ± 0.35*	34.44
ZNME	150	18.05 ± 0.27*	53.99
Indomethacin	10	14.10 ± 0.38*	64.06

Number of animals per group (*n*) = 6. SEM = Standard Error of Mean.
**p < 0.001,* compared to control group.

malondialdehyde formation generated by Fe^{2+}-ascorbate in rat liver homogenate and the results are summarized in Table 6. The percentage of inhibition was 66.98 % and 28.67 % at the concentrations of 1000 µg/ml and 10 µg/ml respectively. The IC_{50} value of ZNME was 279.1 µg/ml and that of quercetin (reference) was found to be 46.64 µg/ml.

Discussion

The present study establishes the significant anti-inflammatory activity of the methanol extract of the root of *Z. nitidum* (ZNME) in both acute and chronic models. Carrageenan-induced oedema has been commonly used as an experimental animal model for acute inflammation and it is believed to be a biphasic response. The early phase (1 – 2 h) of the carrageenan model is mainly mediated by histamine and serotonin (5-HT). The late phase is mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (24). The ZNME produced dose dependent and significant ($p < 0.001$) inhibition of carrageenan-induced paw oedema after a period of 3 h.

The ZNME also significantly ($p < 0.001$) suppressed the inflammation produced by the mediators viz. histamine and serotonin. It indicates that the ZNME inhibits the inflammation caused by carrageenan and mediators.

Table 5. Antioxidant property of ZNME on DPPH radical scavenging activity.

Test sample	Percentage of inhibition*	IC_{50} (µg/ml)
Control	-	
ZNME + DPPH		
25 µg/ml	9.18	
50 µg/ml	18.50	
100 µg/ml	38.38	75.2
200 µg/ml	64.23	
Ascorbic acid		
50 µg/ml	51.33	43.7
100 µg/ml	67.02	

* Values are means (*n* = 3).

Table 6. Antioxidant property of ZNME on Fe^{2+}-ascorbate induced lipid peroxidation.

Test sample	Percentage of inhibition*	IC_{50} (µg/ml)
Control	-	-
ZNME		
10 µg/ml	28.67	
100 µg/ml	37.08	
1000 µg/ml	66.98	279.1
Quercetin		
10 µg/ml	44.17	
100 µg/ml	51.15	46.6

* Values are means (*n* = 3).

The cotton pellet method is widely used to evaluate the exudative and proliferative components of the chronic inflammation (13). Chronic inflammation is a reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation. Chronic inflammation occurs by the development of proliferative cells. These cells can either spread or remain in granuloma form. The dry weight of the cotton pellets correlates with the amount of the granulomatous tissue formed (25, 26). The ZNME showed significant ($p < 0.001$) and dose dependent anti-inflammatory action in cotton pellet induced granuloma and hence found to be effective in chronic inflammatory conditions. Based on the results it can be concluded that the ZNME possesses anti-inflammatory potential in both acute and chronic phases of inflammation.

The results of the present study indicate that the ZNME has effective degrees of *in vitro* antioxidant activity by the methods employed. It is now well established that free radicals (e.g. superoxide, hydroxyl radical, nitric oxide) and other reactive species (e.g. hydrogen peroxide, singlet oxygen, peroxynitrite, hypochlorous acid) contribute to the pathology of many disorders including arthritis and connective tissue disorders, ageing, neurodegeneration, chronic inflammation and cancer (27). Free radicals may also be a contributory factor in the function of the immune system (28). Recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species (which act as pro-inflammatory agents) from phagocytes invading the inflammation sites (29, 30).

The DPPH test provides information on the reactivity of test extract with a stable free radical. DPPH is stable nitrogen centered free radical containing an odd electron in its structure that can accept an electron or hydrogen radical to become a stable diamagnetic molecule and usually utilized for detection of radical scavenging activity (19). Because of its odd electron DPPH gives a strong absorption at 517 nm in the visible region (deep violet colour). As the electron becomes paired off in presence of a free radical scavenger, the absorption diminishes, thus the resulting decrease in absorbance is stoichiometric with respect to the number of electrons taken up (20). The ZNME exhibited marked and dose dependent free radical scavenging effect in DPPH radical scavenging assay showing the IC_{50} value 75.2 µg/ml.

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids of cellular membranes undergo reaction with reactive oxygen species to yield lipid hydro-peroxides. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydro-peroxides into peroxyl and alkoxyl radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA). This lipid peroxidation can be prevented either by reducing the formation of free radicals or by supplying the competitive substrate for unsaturated lipids in the membrane or by accelerating the repair mechanisms of damaged cell membrane. Several natural and synthetic antioxidants are used to prevent the lipid peroxidation (31, 32).

The antioxidant activity of the ZNME was further confirmed by evaluating the inhibition in production of malondialdehyde (MDA) and related carbonyl products that are produced as by products of lipid peroxidation induced by Fe^{2+}-ascorbate system in the biomembranes of rat liver homogenate. These carbonyl products are responsible for DNA damage, carcinogenesis and aging related diseases (33). The MDA reacts with thiobarbituric acid in specific reaction medium to

produce a strong absorption at 532 nm. The ZNME effectively inhibited the lipid peroxidtion in a dose related manner exhibiting the IC_{50} value of 279.1 µg/ml. This activity is perhaps related to the H^+ ion donating capability of the extract, which can scavenge the peroxyl radical to inhibition or termination of the peroxidation chain (22).

Preliminary phytochemical analysis indicated the abundance of true alkaloids and flavonoids in ZNME. Its anti-inflammatory and antioxidant potential could be attributable to these putative constituents. Flavonoids are well known natural antioxidants due to their electron donating property which either scavenge the principal propagating free radicals or halt the radical chain (34). Thus the antioxidant activity of ZNME may be due to the presence of flavonoids which augmented the anti-inflammatory action.

Conclusion

Present investigation confirms significant acute and chronic anti-inflammatory and *in vitro* antioxidant properties of the ZNME in the tested models. It can be inferred that the anti-inflammatory activity of ZNME may be due to the inhibition of free radicals production that act as pro-inflammatory agents in acute and chronic inflammation. So the antioxidant property of ZNME can explain, in part, the mechanism of its anti-inflammatory activity. Present study therefore, substantiates the traditional uses of *Z. nitidum* root in pain and rheumatism in North-East India. Further studies are presently underway to confirm the identity of bioactive principles responsible for these actions by the root of *Z. nitidum.*

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Enhancing effect of cyclodextrins on *in vitro* skin permeation of hGH

Rogayyeh Shakory[1,2], Mahvash Khodabandeh[2*], Tayebeh Toliyat[3], Sadegh Azimzadeh[1,4], Zohreh-Azita Sadigh[5] and Leila Badiefar[2]

[1]Centeral Payam Noor of Tehran, Iran
[2]National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
[3]Tehran University of Medical Sciences, Faculty of Pharmacy, Tehran, Iran
[4]Zanjan University, Zanjan, Iran
[5]Razi Vaccine and Serum Research Institute, Iran
*For Correspondence - saba@nigeb.ac.ir

Abstract

Human growth hormone (hGH) is one of the most important large protein drugs, which is secreted by the pituitary gland. Deficiency in this hormone causes stunned growth and mental retardation. Recently, replacement therapies including hGH have become one of the most popular anti-aging treatments especially in cosmocauticals. In this study, the transdermal route as an alternative route for *in vitro* hGH hormone delivery was investigated. hGH was formulated using various cyclodextrins (CDs). The enhancing effect of CDs on hGH skin permeation was investigated *in vitro* using Franz diffusion cells. The results suggest the enhancing effect of CDs on in vitro transdermal delivery hGH.

Key words: hGH, Transdermal delivery, Chemical enhancers, Skin, Cyclodextrins.

Introduction

Transdermal delivery of therapeutic agents has been used successfully for decades. Transdermal systems for hormone replacement therapy, smoking, cessation and pain management are well accepted. However, there have been challenges in extending the use of the technology to the delivery of peptides, proteins and other macromolecules (4). In view of the above mentioned factors, this research aimed to develop the transdermal delivery of hGH. Hence, this drug was chosen for delivery via the transdermal route.

Transport of macromolecules into the skin is slow due to the resistance of the outer most layer of the skin, known as the stratum corneum (SC) (7). A variety of methods such as physical, biochemical and chemical have been studied in attempts to overcome this barrier. One of the most promising and the most extensively studied techniques is the use of chemical enhancers (8) . Many studies have investigated the mechanisms of the action of chemical enhancers and the following have been suggested as possible explanations for activity:

1. Interaction with intercellular lipids of the SC resulting in disorganization of the highly ordered structures thus enhancing paracellular diffusion through the SC.

2. Interaction with intracellular proteins of the corneocyte to increase transcellular permeation.

3. Increasing partitioning of the drug into the SC.

Several excipients are able to promote the transport of an active substance across the skin barrier. (1). Potential substances used for this purpose need to have both features, i.e, drug penetration-promoting effects and a low or no skin irritating potential. In fact pharmaceutical scientists are searching penetration enhancement techniques for transdermal penetration of drugs with no side effects. Complex of drugs with cyclodextrins has been used to enhance aqueous solubility and drug stability. Cyclodextrins of pharmaceutical relevance contain 6, 7 or 8 dextrose molecules (α-β-8 cyclodextrin) bound in a 1,4 configuration to form rings of various diameters, with a hydrophilic exterior and lipophilic core in which appropriately sized organic molecules can form non-covalent inclusion complexes resulting in increased aqueous solubility and chemical stability (6). Derivatives of â-cyclodextrin with increased water solubility (e.g. hydroxypropyl-β-cyclodextrin (hp-β-CD)) are most commonly used in pharmaceutical formulation.

Theoretically, cyclodextrin can enhance drug bioavailability by stabilizing drug molecules at the biomembrane surface. In general, drug stabilization associated with cyclodextrin complexation plays only a very minor role when it comes to drug delivery through biological membranes since it is their solubilizing effect that is usually related to improved drug delivery. Based on improvement in the quality of life that replacement therapy produces in patients with hGH-deficiency and based on clinical similarities between aging and hGH deficiency, researchers are now exploring possible additional therapeutic applications for hGH and using supplemental hGH in physiological doses to slow the normal catabolic changes of aging. In the 1990s, the FDA approved hGH therapy for adults who are deficient in the hormone, which includes most people who are over 40 years of age (5).

More readily available biosynthetic (recombinant) forms of hGH have helped to extend its clinical applications to adult patients who are not pathologically deficient in hGH. Because of the large size of the molecule and its labile structure, there is no acceptable delivery system for the human growth hormone other than by subcutaneous injection. There are no hGH products that bear labeling approval from the FDA for delivery by oral or transdermal routes. This absence of more appropriate dosage forms is the result of the fact that enzymatic degradation and poor absorption (across the skin) because of its large molecular weight presents access of the molecule to systemic circulation by non-parenteral administration. The objective of this paper was to contribute new experimental data in order to analyze and compare the effects of chemical enhancers on transdermal absorption of hGH. Chemical enhancers employed were á- â and hp-β cyclodextrin.

Materials and Methods

rhGH (4 IU/mg) was obtained from Novo Nordisk pharma Co (Tokyo, Japan). ELISA kits for determination of hGH concentration in the samples were purchased from PADTAN ELM (Iran). α-cyclodextrin, β- cyclodextrin and hp-β-CD were obtained from the Sigma Chemical Company. Male rats were obtained from the animal house of the Pasteur Institute (Tehran, Iran). All other solvents and reagents used in this study were procured from Merck and prepared with purified water.

Preparation of drugs: The Chemical penetration enhancers (CPEs) used in the permeation studies of the drug were α-CD, β-CD and hp-βCD. The hGH solution was prepared in the presence of CDs at weight ratios of 1:2 (hGH: CDs) and 1:4 (hGH: hp-βCD). For preparation of the 1:2 and 1:4 ratios, aqueous CD solutions were made by

dissolving 2.6 mg of CDs and 5.2 mg of hp-βCD in distilled water (2.6 ml). Then 1.3 mg of hGH was added to each aqueous CD solution and mixed with a magnetic stirrer at 300 rpm for 60s. A control solution of hGH in the absence of the enhancers was prepared at a concentration of 0.5 mg/ml. A 1.3 mg sample of hGH was dissolved in 2.6 ml of distilled water, then mixed with a magnetic stirrer at 300 rpm for 60 s. A 1 ml sample of the prepared solution was placed in the donor compartment of the skin equipped diffusion cells.

In vitro permeation studies: The drug diffusion studies were performed using the Franz diffusion cells (Fig.1). The total volume of the cell in the receptor compartment was 30 ml. The rat skin was placed between the donor and receptor compartments. The compartments were then clamped together. Drug (1 ml) was applied uniformly. The receptor compartment was filled with 30 ml of phosphate buffer (0.05 M, pH 7.5). The cell was placed in a water bath maintained at 37 ± 0.5°C. The receptor compartment was filled with 30 ml of degassed phosphate buffer solution (pH 7.2) under constant stirring with a magnetic stirrer. Samples (1 ml) were drawn from the receptor compartment periodically every 2, 4, 6, 8 and 24 h and then replaced by the same volume of receptor solution. The samples were analyzed for hGH content by using sandwich ELISA technique. The results were plotted, as the cumulative amount released (Q) versus time (T).

The parameters of the *in vitro* skin permeation study were calculated by plotting the cumulative drug amount permeated through the skin versus time. The slope of the linear portion of the permeation curve provided the flux value (μg $cm^{-2}h^{-1}$) at steady state. The lag time (T_{lag}) was determined by extrapolating the linear portion of the curve to the X-axis. The cumulative drug amount in the receptor compartment after 24 h was defined as Q24 (μg cm^{-2}). Enhancement ratio (ER) for flux was calculated using the following equation:

ER= Flux for skin treated with enhanser/Flux for control (skin without enhancer treatment)

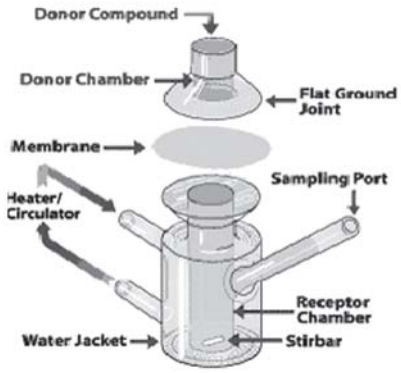

Fig. 1. Schematic representation of Franz diffusion cell.

***Preparation of rat abdominal skin*:** Because of the similarity between male rat and human skins, male rat skin (150-200g) was used as a biological model for *in vitro* skin penetration studies. A rat was killed by chloroform and hairs of the abdominal region were excised. Skin from the outer surface of the abdominal region was carefully dissected. Subcutaneous fat was carefully removed with a scalpel and rinsed with normal saline and stored at -20 °C until further use.

***Determination of hGH concentration by ELISA*:** The concentration of hGH in the receptor phase (an indicator of transdermal delivery) was analyzed by using the sandwich ELISA technique. The hGH quantitative test kit based on the solid phase enzyme immunoassay (EIA) used two mouse monoclonal antibodies directed against distinct antigenic determinants on the hGH

molecules. The GH present in the standards and samples was bound to the anti-GH antibodies, resulting in the development of a blue color. The intensity of the color produced was proportional to the amount of GH in the sample. The color intensity was determined in a microtiter plate by a spectrophotometer at 450 nm. Standard curves were constructed for each assay by plotting the absorbance value against the concentration of each standard. The GH concentrations of the samples were then obtained from the standard curve.

***Stability analysis*:** Stability studies of hGH in the enhancer solutions were performed under defined collection and processing conditions at room temperature (RT). The hGH solutions were prepared in the presence of CDs at weight ratios of 1:2 (hGH: CDs). The control solution of hGH in the absence of any enhancer was prepared at a concentration of 0.5 mg/ml. Samples were obtained periodically at 2, 4, 6, 8, 24 h and one week (168 h) post-incubation. Samples were then analyzed for hGH stability with 13% (w/v) SDS-PAGE and the ELISA assay.

***Data and statistical analysis*:** The amounts of hGH that permeated through the excised rat skin were plotted as a function of time. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA, α=0.05) the least significant different test (LSD) was followed if the ANOVA indicated that a difference existed. LogP value of the model drugs was determined using the ACD program. All the skin permeation experiments were repeated three times. The mean values and corresponding standard deviations are presented in the figures and tables. The student's t-test was performed to find any significant difference in the permeation between or among hGH containing CPEs and free enhancer formulation (control). A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

The skin permeation profiles of hGH in the control group and complexed with various CDs and comparison of the three CDs' effectiveness are shown in Fig. 2.

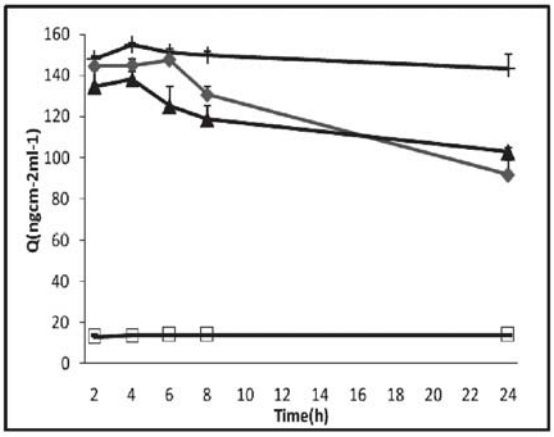

Fig. 2. Permeation profiles of hGH in the free state (control group) and complexed with CDs (+: hP-βCD, ♦: á-CD, ▲: β-CD, □ : control group).

The skin penetration of hGH without CDs was negligible because of its hydrophilic nature and also high molecular weight (20 KDa). As shown, CDs can enhance the penetration of hGH through the skin more effectively (Fig. 2). Among the various CDs, β-CD showed the lowest enhancing effects on hGH transdermal delivery. Probably, this is due to the limited aqueous solubility of β-CD in which the interaction of lipophilics with these CDs is poor, thus causing precipitation of the solid CD complexes from water and other aqueous system (9). Among the applied CDs, hp-βCD showed the highest enhancing effects on hGH transdermal delivery. In contrast to βCD, hp-βCD increases the solubility of the complexes

by approximately 10-100 times. By dividing the amount of drug released together with enhancer by the amount of released drug without enhancer, the percentage of enhanced drug penetration is achieved at different time courses (Fig. 3).

Different enhancers by interaction with intracellular lipids cause decomposition and increased fluidity of them. The results indicate that maximum permeation is related to hpCD and equals 15501 ng $ml^{-} cm^{-2}$ thus having the most effect on growth hormone permeation. Also, studying the stability of the growth hormone in the presence of hp-βCD by the ELISA test (table 1) and SDS- PAGE (Figs 4-a, b), indicated that the amount of the growth hormone remained constant after one week and was thus stable during this period of time.

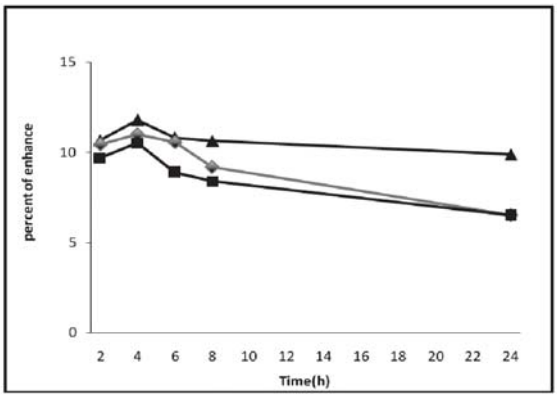

Fig. 3. Percent of enhanced for various CDS. ■:βCD, ◆: á-CD, ▲: hp-β-CD,

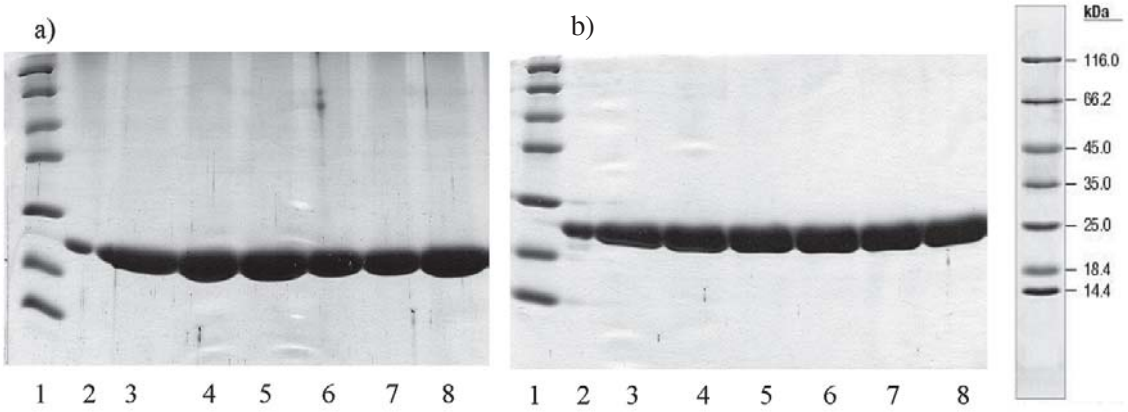

Fig. 4. a) SDS-PAGE for hGH (Nurditropin) standard. Lane 1: The low molecular size marker. Lanes 2-8: The samples were obtained after 2, 4, 6, 8, 24 h and one week. b) SDS-PAGE for hpβCD. Lane 1: The low molecular size marker. Lanes 2-8: The samples were obtained after 2, 4, 6, 8, 24 h and one week.

Table 1. Studying the stability of the growth hormone

Time (h)	**Control** (ng/ml)	**hpβCD** (ng/ml)
2	10.12	10.67
4	10.08	10.57
6	10.24	10.31
8	9.98	9.57
24	10.39	10.2
168	10.24	9.88

Among the three applied enhancers in this study, hp-βCD is the cheapest cyclodextrin, which can be obtained from a variety of sources. So the following studies were performed on the effect of the hp- βCD.

Effect of hp- βCD concentration on the skin permeation of hGH: The permeation multiplier effects of hp-βCD were studied at concentrations of 2 and 4 than growth hormone. As seen in fig. 5, the amount of the growth hormone permeation in the four fold concentration than twofold has indicated some deal increase to 6 h after sampling.

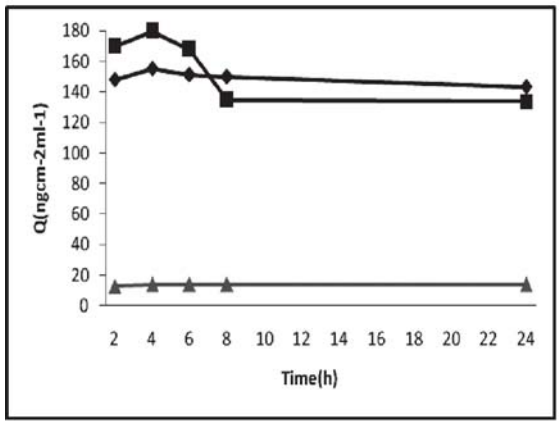

Fig. 5. Effect of hp-βCD concentration on the permeation of hGH through excised rat skin. ♦-hGH/hP-βCD = 1:2, ■ - hGH/hP-βCD = 1:4, ▲ - control group.

A probable justification for this observation is the increase in the amount of the drug contact with hpβ molecules that cause an increase in the levels of the drug complexed with hp-βCD.

According to former reports, existence of some interaction between the available hydrophobic side chains of the amino acids and hydrophilic CDs have been proved, examples of which are buserelin, insulin and α-chymotrypsin (2). In this regard cyclodextrins can be used to solubilize and stabilize various biomedically important peptides and proteins including growth hormones (3) by complexing with hp-βCD to become entrapped. The outer surface of the hp-βCD is very hydrophilic and interacts well with water to carry the guest into solution. It was found that large water soluble molecules with side chains capable of forming a complex react with cyclodextrins in aqueous solutions, resulting in modified solubility and stability (10). The objective of this paper was to contribute new experimental data in order to find any significant differences in the permeation the hGH containing CPE and free enhancer formulation (control). A value of $P < 0.05$ was considered statistically significant.

In conclusion cyclodextrins have significant potentials as drug enhancers in penetration of proteins and peptides. Therefore, growth in the number of cyclodextrin based commercial products can be expected in the future. Thus, it can be concluded that CDs (especially hp-βCD) are very suitable delivery systems for penetration of hGH.

Acnowledgement
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Rhinacanthus nasutus: A Plant with Potential activity in Radical Scavenging capacity

P. Visweswara Rao and M. Dhananjaya Naidu*
Department of Biotechnology, Sri Venkateswara University, Tirupati -517502, India
*For Correspondence - mdnbiotech@gmail.com

Abstract:

In-vitro antioxidant activity of methanolic extract of *Rhinacanthus nasutus* leaves was determined by DPPH free radical scavenging assay and Reducing Power assays. Ascorbic acid was used as standard and positive control for both the analysis. All the analysis was made with the use of UV-Visible Spectrophotometer. The methanolic extract of *Rhinacanthus nasutus* leaves had shown very significant DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging activity compared to standard antioxidant. The DPPH radical scavenging activity of the extract was increased with the increasing concentration. In DPPH free radical scavenging assay IC_{50} value of leaves extract of *Rhinacanthus nasutus* was found to be 34.4 ìg/mL. The reducing power activity of the leaf extract was moderately exhibited. The results concluded that the extract have a potential source of antioxidants of natural origin.

Key words: Antioxidant, *Rhinacanthus nasutus*, Free radical, DPPH.

Introduction

Rhinacanthus nastus (Linn) belongs to Acanthaceae family is a shrub with 1-2 meter height. It is commonly called as Nagamali, Kaligai, anichi in Tamil, Nagamalli in Telugu, doddapatika in kannda, jupani in Hindi, yuthikaparni in Sanskrit and Gajakarni in Marathi (1). The plant has long been used in Thai traditional medicine for treatment of tinea versicolor, ringworm, pruritic rash, abscess pain, and skin diseases. It has been reported that rhinacanthin-C, rhinacanthin-D and rhinacanthin-N isolated from R. nasutus possessed antimicrobial (fungal, antibacterial), anti proliferative, antiviral, anti-inflammatory, anti-allergic and cytotoxic activities. Various parts of this plant have been also used for the treatment in various other diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension, and various skin diseases, and the active components of this plant have been widely investigated [2-7]. Since the plant was used as antiseptic and anti parasitic, it was thought worthwhile to investigate the radical scavenging activity.

Materials and Methods

***Plant Materials*:** The fresh leaves of *Rhinacanthus nasutus* were collected from Tirumala Hills, Tirupati, Chittoor district of Andhra Pradesh in the month of July – October

and identified by Dr. K. Madhava chetty, Assistant Professor, Department of Botany, S.V.University, Tirupati.

***Preparation of Extract*:** Fresh leaves of *Rhinacanthus nasutus* (L) were shade dried and milled to fine powder using a mechanical grinder. The powdered plant material was macerated and shaken in methanol for 48 h using a bath shaker. The extract was then filtered with filter paper (Whatman No.1) and concentrated to dryness under vacuum and reduced pressure using Rota evaporator at 40 °C. The concentrate was then layered on aluminum foil and freeze dried for further use.

***Antioxidant Assay*:** The antioxidant activity of Plant extract was determined by different in vitro methods such as the DPPH free radical scavenging assay and reducing power methods. All the assays were carried out in triplicate and average values were considered.

DPPH Radical Scavenging Activity [8]: The free radical scavenging capacity of the methanolic extract of *Rhinacanthus nasutus* was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanol extract of *Rhinacanthus nasutus* was mixed with 95% methanol to prepare the stock solution (10mg/100mL). The concentration of this *Rhinacanthus nasutus* Methanolic extract solution was 10 mg /100 ml or 100ì g/ml. From stock solution 2ml, 4ml, 6ml, 8ml & 10ml of dilution with same solvent was made the final volume of each test tube up to 10 ml whose concentration was then 25ìg/ml, 50ìg/ml 75ìg/ml & 100ì g/ml respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing *Rhinacanthus nasutus* methanolic extract (25ìg/ml, 50ìg/ml, 75ìg/ml, and 100ìg/ml) and after 10 min, the absorbance was taken at 517 nm using a spectrophotometer. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (100ì g/ml) of Methanolic extract of *Rhinacanthus nasutus*. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank.

DPPH free radical scavenging activity was measured using the following equation-

$$\% \text{ DPPH radical-scavenging} = \frac{[(\text{Absorbance of control} - \text{Absorbance of test Sample})]}{(\text{Absorbance Of control}) \times 100}$$

***Assay of Reducing Power*:** 1 ml of plant extract solution (final concentration 100- 500 mg/l) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [K_3Fe (CN6)] (10g/l), then mixture was incubated at 50º C for 20 minutes[9]. 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

Results and Discussion

DPPH radical scavenging activity and reducing power activities of methanolic extract of *Rhinacanthus nasutus* were compared with ascorbic acid were presented in figure1 and 2. Methanolic extract of *Rhinacanthus nasutus* leaves has got profound antioxidant activity in both the methods when compared with the standard antioxidant ascorbic acid. The DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The methanol extract of *Rhinacanthus nasutus* leaves exhibited a significant dose dependent inhibition of DPPH activity. The IC_{50} value of the methanol leaves extract of *Rhinacanthus nasutus* and ascorbic acid were found to be at 34.4 ìg/mL and 40.8 ìg/mL respectively. The reducing ability of a compound generally depends on the presence of reductants which have been exhibited anti oxidative potential by breaking the free radical chain, donating a hydrogen atom. The presence of reductants (i.e. antioxidants) in *Rhinacanthus nasutus* leaves extract causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. It has been reported that reactive oxygen species contribute to various patho physiological conditions and endogenous defense mechanisms have evolved to offer protection in these conditions. An increase in the antioxidant reserves of the organism can reduce oxidative stress and some of the plant derived agents may help to reduce it. Determination of the natural antioxidant compounds of plant extract will help to develop new drug candidates for antioxidant therapy [10, 11].

Conclusion

The plants may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms. The present study concludes that the methanolic extract of Rhinacanthus nasutus leaves exhibit potential antioxidant activity in both the methods. Further investigation on the isolation and identification of antioxidant component(s) in the plant may lead to chemical entities with potential for clinical use.

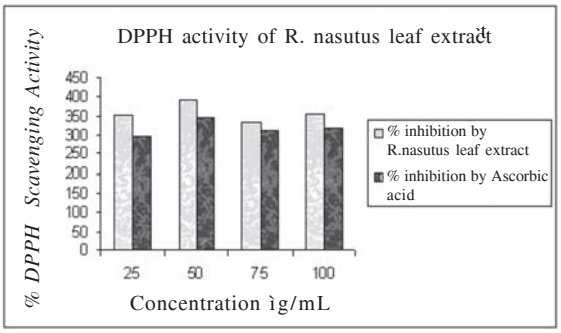

Fig.1. DPPH activity of *Rhinacanthus nasutus* leaf extract

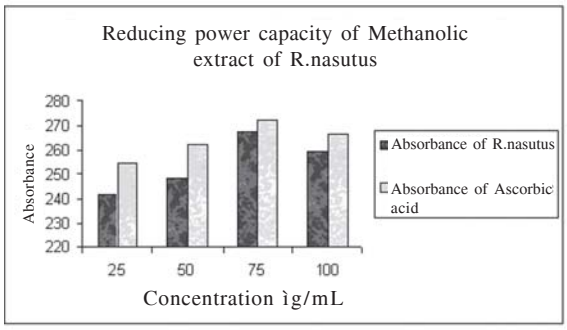

Fig 2. Reducing power capacity of Mehanolic extract of *Rhinacanthus nasutus*

Radical Scavenging capacity

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Optimization and Production of extracellular amylase from newly isolated *Aspergillus* species by Submerged Fermentation

Murali Krishna Chimata[1], Sasidhar Pasumarthi[1] and Suresh Challa[2*]
[1]Department of Biotechnology, Acharya Nagarjuna University, Guntur, India
[2]Department of Biochemistry, National Institute of Nutrition, Tarnaka, Hyderabad - 500 007, India
*For Correspondence - sureshnin2000@gmail.com

Abstract

The production of extracellular amylases by SmF was investigated employing our laboratory isolate *Aspergillus* sp.MK07. Various process variables namely Incubation Period, Temperature, pH, Inoculum level were tested. Varying concentrations of CSL from 1 to 4% were supplemented to the synthetic media used in the study to evaluate the nitrogen source effect on the media with our laboratory isolate *Aspergillus* sp.MK07. Corn steep liquor played an important role in amylase production and an additional supplementation of 2% CSL to the media increased amylase production compared to control without CSL in the media. Among the different parameters tested pH, Temperature and substrates played a very important role. Highest amylase production was obtained with a temperature of 30°C, pH 5.0, Inoculum level 5%, incubation period of 72 h and among the carbon substrates tested highest amylase production was obtained when Sucrose was used as a substrate. Highest amylase production obtained under all optimized parameters was 91 U/ml.

Key words: Submerged, Optimization, *Aspergillus*, Fermentation, Amylases

Abbreviations: SSF (Solid State Fermentation), SmF (Sub merged Fermentation), CSL (Corn Steep Liquor).

Introduction

Amylases are a group of important enzymes which are mainly employed in the starch processing industries for the hydrolysis of polysaccharides like starch into simple sugars (12). Due to wide range of application of amylases in various sectors like Confectionary, Baking, Paper, Textile, Detergent, Pharmaceutical and many more are gaining the attention of researchers (6). Amylases are produced on a large scale normally by submerged fermentation (SmF) because of the ease of handling the cultures and physical parameters like pH and temperature could be controlled easily (5). Mostly synthetic media is used for the production of amylases by SmF (6). Optimization of various physical parameters along with manipulations in media constituents plays an important role in determining the cost of the produced enzymes (2). Many researchers have done enormous amount of work with various bacterial strains using SmF (8). Production of amylases in fungi is known to depend on both morphological and metabolic state of the culture (4). Various individual parameters like pH, temperature, incubation period, carbon sources, inducers, surfactants and agitation with respect to SmF are widely discussed in literature (23) .Many researchers have studied the amylases production with a wide variety of substrates and microorganisms ranging from bacteria, yeast and

fungus (6). Amylases accounts for about 30% of the world's enzyme production (14). Due to the ever increasing demand of this enzyme people are still trying to increase the productivity of amylases by a variety of approaches like selection of a high enzyme producing strain, process optimization, usage of cheaper substrates and effective downstream processing etc (20). Researchers are mainly working on process optimization, media optimization and some researchers have done good improvement in isolating various strains which can use a wide variety of carbon substrates like banana peel also (20).Among the various types of amylases produced commercially thermostable amylases are gaining much more attention in comparison to other types (19). Many researchers have done good amount of work on isolation of some of the thermo tolerant strains (2). Several reports have been published by many researchers showing Amylase production with *Aspergillus sp* (5). However it would be economically competitive if the isolated strain shows some advantages over existing products and every strain has its own unique microbial and Biochemical properties (16). The present investigation is aimed to isolate an *Aspergillus sp* locally which can produce a fairly good amount of amylase which on further process and media optimization could result in enhanced levels of amylase at an affordable cost when scaled up.

Material and Methods

Fungal Strain: Isolation of the fungal strains was carried out with various soil samples collected from local starch plant near Hyderabad. *Aspergillus* species were identified by standard blotter method (12).They were identified on the basis of morphological characteristics and certain standard confirmatory tests.

Isolation of the Strain: The soil suspension was diluted upto 10^{-3} to 10^{-6} times and 0.5 ml of each diluted suspension was then transferred by spread plate method with a sterile glass spreader on petri plates containing Czapex Dox Starch agar medium .The petri plates were incubated at 30°C for 4-5 days. Based on the zone of clearance on the starch agar plates, 13 colonies were picked up and individual amylase activity of selected colonies were carried out and strain MK 07 was used for further studies. The young colonies of fungal cultures were aseptically picked up and transferred to Czapex Dox Starch agar slants with 1% starch. These slants were then incubated at 30°C for 4 days and after sufficient growth they were stored at 4°C in the refrigerator.

Inoculum Preparation: Actively growing and heavily speculating ten days old Czapex Dox Starch agar slant culture was added to 10 ml sterile 0.85% sodium chloride salt solution. The spores were gently scraped off with the help of a sterile needle and contents were passed through glass wool so as to obtain spore inoculums free from mycelial bits. A volume of one ml of spore suspension contained more than 2.6 x 10^6 spores.

Chemicals: All chemicals used in the study were purchased from Hi-Media (Mumbai, India) and Sigma Chemicals (USA).

Production Media Used: The composition of the production media used was sucrose 30 g/l, magnesium sulphate 0.5 g/l, potassium chloride 0.5 g/l, ferrous sulphate 0.01 g/l, peptone 5 g/l, potassium phosphate 1 g/l, corn steep liquor 10 g/l and to the media added 1000 ml of distilled water and pH was adjusted to 5.5 and the media was sterilized in an autoclave for 15 min at 121°C. The media was inoculated with a loop-full of spore suspension of *Aspergillus* sp isolated and then incubated in 30°C at 200 RPM for 96 h. The media was centrifuged at 5000 RPM for 15 min and the crude enzyme isolate was used for further studies.

***Measurement of Amylase activity*:** Amylase activity was determined by the spectrophotometric method described by Bernfield (3). In an assay mixture containing enzyme extract, starch as substrate and DNS as coupling reagent, one unit of amylase activity is defined as the amount of enzyme that releases 1 micromole of reducing sugar as glucose per minute under the assay conditions. Enzyme activity is expressed as units per milliliters (U/ml).

Results and Discussion

***Role of Incubation Time*:** Maximum enzyme production could be obtained only after a certain incubation time which allows the culture to grow at a study state (16).Enzyme production of each strain is based on the specific growth rate of the strain. Growth rate and enzyme synthesis of the culture are the two main characteristics which are mainly influenced by incubation time (6).The results of the present study showed that amylase production increased with increase in incubation time as shown in Fig.1 linearly till 72 h and on further incubation there was a decrease in the amylase production. Maximum amylase production was obtained at 72 h (55 U/ml).

***Influence of Temperature*:** Temperature is one of the most important parameter to be optimized for maximum enzyme production. Optimum temperature for maximum enzyme production depends on the characteristics of the strain (5) .In submerged and SSF fermentation temperature plays a very important role in enzymatic synthesis (12).The isolated *Aspergillus* strain was tested in a wide range of temperatures ranging from 20°C to 40°C. Maximum amylase production was obtained at a temperature of 30°C (59 U/ml) as shown in Figure 2. In the present experiment with increase in temperature enzyme production increased up to a certain level and upon further increase of temperature, production decreased. Mukherjee (13) also reported 30°C as optimum temperature for *Aspergillus flavus.*

Influence of pH: Amylase production by microbial strains strongly depends on the extracellular pH as culture pH strongly influences many enzymatic reactions and also for the transport of various components across the cell membrane (14, 4). Growth and metabolism along with enzyme production is governed by an important factor called pH (5).Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity (16). The results in the present experiment as shown in Fig 3 revealed that the strain isolated had an optimum pH of 5.0 with a maximum enzyme activity of 63 U/ml. With increase in pH value from strong acidic phase to a neutral phase enzyme activity increased up to a pH of 5.0 and upon further increase in pH,

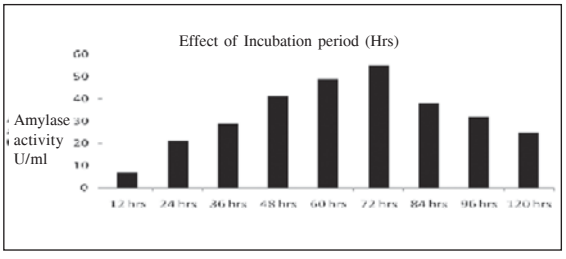

Fig. 1. Effect of amylase activity at different incubation periods

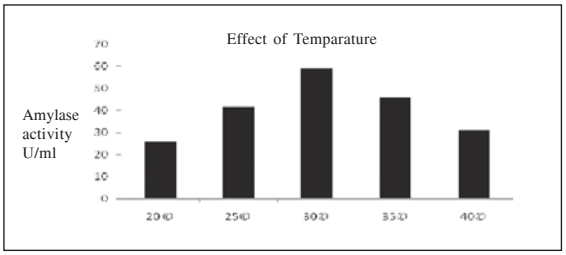

Fig. 2. Effect of amylase activity at different temperatures

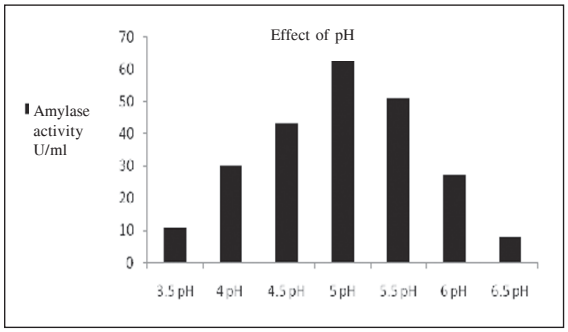

Fig. 3. Effect of amylase activity at varying pH

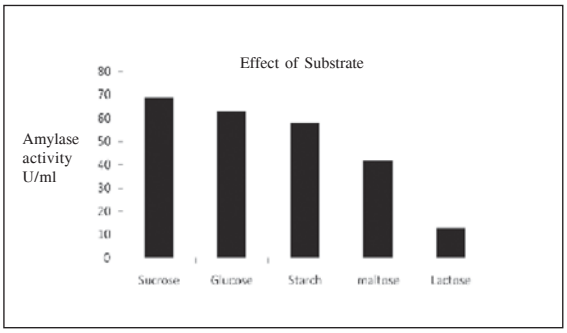

Fig. 5. Effect of amylase activity with different carbon substrates

enzymatic activity decreased. Other researchers (15) stated that pH 7.0 was optima for *Aspergillus flavus*.

Influence of varying concentrations of inoculum: Varying concentrations of Inoculum levels were studied in this experiment and the various range of inoculum (ml) selected in this experiment ranged from 3% to 15%. Enzyme production varied with percentage of inoculum and the maximum enzyme production was 61 U/ml (as shown in Fig 4) with 5% Inoculum. Increase of inoculum level from 5% to 10% or 15% showed a marginal decrease in amylase production.

***Evaluation of different carbon substrates in the production medium*:** Several carbon substrates like Glucose, Starch, Maltose, Lactose, and Sucrose were tested to evaluate the enzyme production by SmF. On supplementation of various carbon substrates maximum enzyme production was exhibited by sucrose (3% w/v). The results as shown in fig 5 revealed a different impact on enzyme production with different substrates. The maximum enzyme production obtained was 69 U/ml with 3% w/v Sucrose. Glucose also enhances enzyme production.

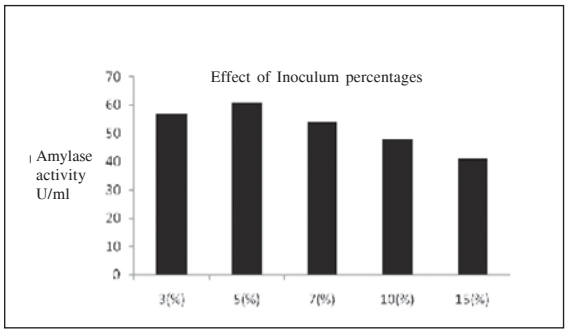

Fig. 4. Effect of amylase activity at varying concentrations of Inoculum levels

***Evaluation of varying concentrations of CSL to the production medium*:** The effect of varying concentrations of CSL as nitrogen sources was studied with supplementation to the production medium and the results were further analyzed. A control flask was maintained without additional CSL supplementation. Among the various ranges of CSL supplementation tested to the media, 2% supplementation of CSL has showed an enhanced enzyme production. Maximum enzyme production was obtained with CSL (2% v/v) and it was 84 U/ml. 1% CSL supplementation also showed a considerable amount of increase in amylase production compared to the control.

Conclusion

The results obtained in the present study indicated that *Aspergillus sp*.could be a potential strain for amylase production by SmF with sucrose

as the substrate. The enzyme production was influenced by various physiological and chemical nature of the substrate as well as the media ingredients during SmF. Among the various parameters screened for submerged fermentation; pH, temperature, incubation period and CSL as a nitrogen source played an important role for higher amount of amylase production. After optimization of individual parameters separately when further experiment was carried out with all optimized parameters, amylase production increased to a maximum of 91 U/ml.

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Angiotensin-Converting Enzyme inhibition by certain fruits: an *in vitro* study

Narasimhacharya A. V. R. L.* , Rupal A. Vasant and Pradeep C. Prajapati
Department of Biosciences, Sardar Patel University,
Vallabh Vidyanagar - 388120, Gujarat, India.
* For Correspondence - narasimhacharya@yahoo.com

Abstract

Angiotensin-converting enzyme (ACE) (EC 3.4.15.1) is a nonspecific dipeptidyl-carboxypeptidase that converts angiotensin I into a potent vasoconstricting, hypertensive angiotensin II. Inhibition of ACE results in an overall antihypertensive effect and several drugs are currently in use to inhibit the ACE activity such as captopril, enalapril etc. Purpose of the study was to determine the efficacy of Indian Gooseberry, Guava, Wood Apple and Star fruit for angiotensin converting enzyme (ACE) inhibitory activity using Hippuryl-L-histidyl-L-leucine (HHL) as a substrate. An *in vitro* study was carried out using sheep lung as an enzyme source and both dialyzed and undialyzed fruit extracts were used for testing their ability to reduce the ACE activity. The study revealed that both undialyzed and dialyzed extracts of all four fruits exhibited ACE inhibitory activity which could be due to the varied amounts of phytoconstituents present in the extracts (i. e., phenols, flavanoids, ascorbic acid and protein contents).

Key words: Angiotensin converting enzyme inhibition; Fruit extracts; Sheep lung

Introduction

Angiotensin-converting enzyme (ACE) (EC 3.4.15.1) is a zinc metallo- peptidase associated with the blood pressure regulating renin-angiotensin system. This enzyme increases blood pressure by converting the decapeptide angiotensin I (A-I) into the potent vasoconstricting octapeptide angiotensin II (A-II) and it also inactivates the vasodilator, bradykinin through its cleavage. ACE is widely present in vascular endothelium, particularly in high concentrations in lungs. This is significant because of the entire venous flow is through the lungs which provide a mechanism for rapid conversion of A-I to A-II. ACE also plays an important role in renin-angiotensin-aldosterone system (1-4).

Currently, more than 10 ACE inhibitors, such as-captopril, enalapril, lisinopril and temocapril are available in various countries as monotherapeutic agents (5). Although the ACE inhibitors are well established in therapy of hypertension and heart failure, they have also been shown to exert organ protective effects (6). These drugs however have several side-effects causing hypotension, reduced renal function, cough and fetal abnormalities (7). This has stimulated the search for natural ACE inhibitors and consequently several peptides have been identified in a range of food proteins, justifying the search for natural ACE inhibitors for safe and economical use (8). Naturally occurring ACE inhibitors were first discovered in snake venom (9,10). Several traditionally used medicinal plants and those with antihypertensive and diuretic properties have been tested for their

ACE inhibiting capacities (11, 12, 8, 13). Fruits such as banana and pomegranate have been shown to possess ACE inhibitory activity and possibly reduce blood pressure (14, 15).

In the present study, Indian Gooseberry (Amla), Wood apple (Kothu), Star fruit (Kamrakh) and Guava (Jamphal) were screened for ACE inhibitory activity as they are consumed commonly and inexpensive and also used widely in traditional medicinal preparations. Gooseberry (*Emblica officinalis*; F. Euphorbiaceae) is reported to have antioxidant, hypolipidemic, and hypoglycemic activities. It possesses expectorant, cardiotonic, antipyretic, anti viral and anti-emetic properties. The fruit is also used in the treatment of leucorrhea and atherosclerosis, pulmonary tuberculosis and scurvy (16-21). Guava (*Psidium guajava*; F. Myrtaceae) is a widely distributed plant, which has several medicinal uses such as-antifilarial, antidiarrheal, CNS depressant, antiamebic, antispasmodic, and antimicrobial activity (22-26). The unripened fruits of guava have been used throughout the tropics to halt gastroenteritis, diarrhea and dysentery (27). Fruits of Wood apple (*Limonia acidissima*; F. Rutaceae) are much used in India as liver and cardio-tonic and when unripe in halting diarrhea, dysentery and in the treatment for hiccough, sore throat, in diseases of the gums and in scurvy (27, 28). Star fruit (*Averrhoa carambola*; F. Oxalidaceae) has been used in counteracting fever, in kidney and bladder complaints, in the treatment of eczema, administered to halt hemorrhages and in biliousness and diarrhea (27). Despite the availability of literature on the medicinal properties of these selected fruits, no reports are available on angiotensin converting enzyme inhibitory activities of these fruits. Therefore, we have attempted to investigate the potential of these fruits as ACE inhibitors.

Materials and Methods

***Reagents and Chemicals*:** Hippuryl-l-histidyl-l-leucine was obtained from Sigma Aldrich (St. Louis, MO); Cyanuric chloride was purchased from Alfa Aesar (Lancaster). Standard drug, Captopril 25 (Tridoss Laboratories Pvt. Ltd., India) was bought from a local medical shop. All other reagents and chemicals used were of analytical grade (Loba chemie, Mumbai, India).

***Collection of Tissue*:** Fresh lung tissue of sheep was obtained from the slaughter house, situated in GIDC, Vitthal Udyognagar and brought to the laboratory immediately, under frozen conditions and stored at low temperatures until further use.

***Extraction of ACE (Angiotensin Converting Enzyme) from lung homogenate*:** Angiotensin converting enzyme was prepared by method described by Mallikarjuna Rao *et al*. (14). Briefly, the method is as followed: One gram of lung tissue was diced and homogenized in 10 ml of the ice-cold 100 mM borate buffer (pH-8.3) containing 50 mM KCl using a homogenizer at 4° C. The homogenate was centrifuged at 20,000g for 20 min at 4 ° C; the supernatant was collected and dialyzed for 12 h against 20 volumes of the same buffer at 4 °C, to remove the endogenous low molecular weight inhibitors. The dialyzed supernatant was used as an enzyme source for angiotensin converting enzyme.

***Collection and Extraction of fruits*:** Starfruit was collected from the Botanical Gardens of Sardar Patel University, Vallabh Vidyanagar; Indian goose berry (amla), wood apple and guava were procured from the local market. Fruit extracts were prepared by homogenizing 5 gm of unripened fruit pulp in 5 ml of 50 mM borate buffer (pH-8.3). The homogenate was centrifuged at 10,000 g and the supernatant was collected and dialyzed for 12 hours. Both dialyzed and

undialyzed extracts were used for the experimentation.

***Assay of ACE inhibitory activity*:** ACE activity was measured by the method described by Mallikarjuna Rao *et al.* (14) using Hippuryl-L-histidyl-L-leucine (HHL) as a substrate. The reaction mixture contained 0.2 ml of 5 mM HHL prepared in 200 mM borate buffer (pH-8.3) with 1000 mM KCl. The reaction was initiated by adding 50µl lung extract, incubated for 30 minutes at 37 °C. The reaction was stopped by adding 2 ml of HEPES after which, 1 ml of 136 mM cyanuric chloride was added to the reaction mixture and mixed vigorously for 15 sec. The absorbance of the yellow color developed was measured at 405 nm. The specificity of the reaction for ACE was tested by adding 10 µl of 10 µM captopril to the incubation mixture.

ACE inhibitory activity was measured by including the suitable aliquots (100, 200, 300 µl) of both dialyzed and undialyzed fruit extracts in the assay system. Captopril was used as a standard drug with assay system for comparison. Various concentrations of hippuric acid (250-2500 µmol/L) were used for preparation of a standard graph. ACE Activity of the lung extracts incubated with captopril and fruit extracts (both dialyzed and undialyzed) was read against the standard curve. One unit of ACE is defined as the amount of an enzyme required to release 1 µmol of hippuric acid over incubation time.

***Phytochemical Analyses*:** Ascorbic acid was estimated using DNPH reagent (29). Polyphenol and flavonoid contents were measured using Folin-Ciocalteu reagent and vanillin reagent respectively (30). Total Protein content was determined by the method of Lowry *et al* (31).

Statistical Analysis: Results are expressed as mean ± SD. The data was statistically analyzed using student's t-test (Graph Pad Prism Version 3.0), P values <0.05 were considered significant.

Results and Discussion

The percent inhibition of ACE activity by various undialyzed fruit extracts ranged between 82.96 % (for Wood Apple, 300 µl dose) to as low as 0.61 % (for Gooseberry at 200 µl dose) where as the percent inhibition of ACE activity in dialyzed fruit extracts was in the range of 12.84 % (in Gooseberry, 200 µl dose) to 49.47% (Guava, 300µl dose). However, Wood Apple extract's (undialyzed, 300 µl dose) ability to inhibit the ACE activity was comparable to that of Captopril (94.27%). On the other hand, Gooseberry (200 and 300 µl), Guava, Wood Apple and Star fruits' dialyzed extracts with all three doses exhibited ACE inhibitory activity (Table 1). When these inhibitory actions were compared to those of native (undialyzed) extracts the following observations emerged: While the dialyzed Gooseberry extracts (200, 300 µl) showed stronger ACE inhibitory activity compared to undialyzed, the dialyzed Guava and wood-apple extracts were found to have (100, 200, 300 µl) have weaker ACE inhibitory activity compared the undialyzed counterparts. The dialyzed Star fruit extracts at all doses were found to have marginally higher ACE inhibitory activity compared to the undialyzed extracts.

The native undialyzed extracts of these fruits at all concentrations tested (100, 200 and 300 µl) with an exception of Gooseberry at 100 µl, indicated that all extracts have inhibited ACE in *in vitro* conditions although at different levels. For instance, the undialyzed wood apple fruit extract showed an ACE inhibitory activity up to 82.96 % where as the dialyzed extract exhibited 37.08% ACE inhibitory activity with 300 µl dose. Similarly the native extracts of Guava also exhibited higher ACE inhibitory activity compared to that of dialyzed extracts (63.50% and 49.47 %

Table 1. ACE inhibitory activity by dialyzed and undialyzed (Native) fruit extracts

Sr. No.	Test Extract	µl of the extract	Enzyme Activity (Units/ 25µl)*		% Inhibition	
	Captopril (10 µM)	10	3.53		94.27	
			Dialyzed Extracts		Undialyzed Extracts	
1	Gooseberry	100	73.17±10.27	---	74.81±5.63	----
		200	53.71±2.34	12.84±3.84	61.80±1.01	0.61±0.26*
		300	49.03±2.10	20.4±3.41	51.92±8.17	15.72±13.27
2	Guava	100	37.50±6.26	37.84±12.13	26.22 ±0.26	57.43±0.42*
		200	38.29±7.47	39.12±10.17	25.75± 0.58	58.19±0.95*
		300	31.12±11.27	49.47±18.30	22.48±0.71	63.50±1.15
3	Wood aple	100	38.76±4.61	17.67±8.84	41.48±1.63	32.66± 2.65*
		200	48.42±2.93	21.39±4.76	19.91±0.35	67.67±0.57*
		300	54.72±7.94	37.08±7.49	10.49±1.07	82.96±1.75*
4	Starfruit	100	50.28±5.44	18.37±8.84	52.54±1.35	14.71±2.19
		200	33.93±4.86	44.92±7.89	44.52±4.66	27.73±7.57
		300	33.62±7.03	45.42±11.41	39.61±7.89	43.91±5.48

Data presented are the mean of triplicates ± SD; — No inhibition detected under assay conditions; * P value < 0.05 were considered statistically significant.

respectively at 300 µl dose). However, the percent inhibition of ACE is more or less similar when both dialyzed and undialyzed star fruit extracts (300 µl dose) were employed. When the ACE inhibitory activities are compared with the phytochemical quantities of the extracts, in general the ACE inhibitory activity was higher where the phytochemical concentration was higher (in guava and wood apple). The phytochemical analyses of undialyzed and dialyzed fruit extracts revealed that flavonoid, phenol, ascorbic acid and protein contents were significantly higher in undialyzed fruit extracts compared to the dialyzed extracts (Table 2). For instance, both flavanoid and polyphenol contents were highest in dialyzed as well as undialyzed extracts of wood apple. While the lowest concentration of flavanoids were noted in gooseberry dialyzed extract, the polyphenols were present in lesser quantity in star fruit's dialyzed extract. Total ascorbic acid and protein contents were found maximum in the undialyzed and dialyzed extracts of gooseberry and minimum was registered in star fruit's dialyzed and undialyzed extracts.

A number of plants which are known to be effective in reducing the cardiovascular diseases for instance, Hawthorne has been shown to exert a mild blood pressure lowering effects as it contains a number of active constituents including flavonoids, catechins, triterpene, saponins, amines and oligomeric proanthocyanidins (OPCs) (32, 33). Besides, various other herbs also have been reported for their hypotensive effects including European mistletoe (34), olive leaf (35, 36), Indian snakeroot (37), and garlic (38). The association between the regular intake of flavonoid-rich foods and a decreased risk of cardiovascular diseases is well documented by epidemiological studies (39).

Table 2. Phytochemical analyses of dialyzed (D) and undialyzed (UD) fruit extracts (mg %)

Fruits	Gooseberry		Guava		Wood apple		Star fruit	
	D	UD	D	UD	D	UD	D	UD
Flavanoids	3.13	16.20	6.35	80.07	94.96	102.04	5.54	62.33
Phenols	75.74	107.82	26.73	100.69	87.02	133.96	10.99	26.13
Ascorbic acid	7.64	44.76	5.14	40.00	2.25	15.67	3.38	13.54
Proteins	343.30	698.25	53.20	434.17	59.02	365.82	45.04	121.16

Evidences that ACE inhibition can explain the health effect of flavonoids in general are given by the ability of ACE inhibitors to modulate anti-oxidant defenses and regulate mitochondrial NO (Nitric Oxide) production (40). In this context, it has been postulated that flavonoids may regulate anti-oxidant defenses through mechanisms that involve ACE, highlighting the participation of flavan-3-ols and procyanidins in modulation of oxidative stress, vascular function and cardiovascular diseases (41). A number of compounds such as tannins, phenylpropanes, proanthocyanidins, anthocyanins, flavonoids, xanthones, fatty acids, terpenoids, alkaloids, oligosaccharides and peptide amino acid from plants have been reported to possess *in vitro* ACE inhibitory activity (42,43,44). Supplements such as omega-3-fatty acids (45), amino acids L-arginine (46), and taurine (47), and vitamins C and E (48) have been effectively used in the treatment of cardiovascular disease, including hypertension.

Thus in the present study % inhibition exhibited by different fruits was not uniform with respect to dialyzed and undialyzed extracts. As can be gleaned from Table 1 both guava and wood apple native (undialyzed) extracts exhibited a high % of ACE inhibition compared to dialyzed extracts. These observations are in agreement with the findings of Mallikarjuna Rao et al (14). On the other hand, no significant differences were found in ACE inhibitory activities of dialyzed and undialyzed extracts of gooseberry (300 µl) and star fruit (100, 200 and 300 µl). While the ACE inhibition by guava and wood apple correlates well with the phytochemical contents of both the native (undialyzed) and dialyzed extracts, such a correlation is not found with respect to gooseberry and star fruit extracts as the ACE inhibitory activities are not significantly different among the dialyzed and undialyzed extracts. The marginal differences found in ACE inhibitory activities of both types of extracts of these fruits in spite of the large differences in phytochemical contents indicate that yet unexplored inhibitory components could be responsible for a marginally higher ACE activity in the dialyzed extracts of gooseberry and star fruit. Further work is in progress in this direction.

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Antibacterial properties of *Spirulina platensis, Haematococcus pluvialis, Botryococcus braunii* micro algal extracts

A. Ranga Rao[1], A. Harshavardhan Reddy[2], S. M. Aradhya[2]*

[1]Plant Cell Biotechnology Department, [2]Fruit and Vegetable Technology Department
Central Food Technological Research Institute (Council of Scientific & Industrial Research),
Mysore-570 020, India
*For Correspondence - smaradhya@yahoo.co.in

Abstract

Micro algae *Spirulina platensis, Haematococcus pluvialis* and *Botryococcus braunii* are cultured commercially and their productions are established in different parts of the world. In the present investigation the antibacterial properties of different solvent extracts of these three micro algae were evaluated. The maximum phenolic contents (128, 131, 110 μg/mg) was recorded in chloroform extracts of *S. platensis, H. pluvialis* and ethyl acetate extract of *B. braunii*. Hexane, chloroform, ethylacetate, acetone and methanol extracts of *S. platensis, B. braunii* and *H. pluvialis* were tested against important clinical bacterial isolates such as *Bacillus subtilus, Bacillus cereus, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus fecalis* and *Yersinia enterocolitica.* The antibacterial activity was determined by agar-well diffusion assay and minimum inhibitory concentration (MIC). Chloroform and ethlyacetate extracts of *S. platensis* showed highest inhibition against *B. subtilus* (18.12 mm and MIC at 200 ppm), while chloroform extract of *H. pluvialis* recorded highest inhibition against *B. subtilus* (17.32 mm and MIC at 150 ppm). In *B. braunii,* ethlyacetate extract exhibited maximum inhibition against *E. aerogenes* (15.11 mm and MIC at 300 ppm). We conclude that, *S. platensis, H. pluvialis* and *B. braunii* extracts can be used as bacteriostatic agents for suitable applications.

Key words*:* Micro algae**,** *S. platensis*, *H. pluvilais*, *B. braunii*, phenolics, antibacterial properties

Introduction

Micro algae are known to produce natural pigments such as carotenoids, chlorophylls, phenolics, phycocyanin, phycoerythrin and phycobiliproteins. They are used in nutraceutical, pharmaceutical and in food applications (1). Various strains of cyanobacteria are known to produce intracellular and extraceulluar metabolites with diverse biological activities such as antialgal, antibacterial, antifungal, and antiviral activity (2)

Spirulina is a photosynthetic, filamentous, spiral-shaped, multicellular form. *Spirulina platensis* has been largely studied due to its commercial importance as a source of proteins, vitamins, essential amino acids, and fatty

acids (3). More recently, special attention has been given to *S. platensis* as a potential source of nutraceuticals (4-5).

Astaxanthin (3, 3'-dihydroxy-â, â'-carotene – 4, 4' dione) is a ketocarotenoid produced by the green alga *Haematococcus pluvialis* which accumulates astaxanthin upto 2-3% on dry weight basis. Astaxanthin is the major carotenoid in *Haematococcus* and it exists mainly as astaxanthin esters (monoester 70%, di ester 10-15% and free form 4-5%) of various fatty acids (6). Astaxanthin is extensively used as pigmentation source in farmed salmon and trout (7). Astaxanthin as a nutraceutical and a medicinal ingredient against degenerative disease such as cancer, for prevention of age-related macular degeneration, inflammation, *Helicobacter pylori* infection (7). Miki et al. (8) reported an antioxidant effect of astaxanthin on serum low density lipoprotein (LDL) in human subjects. It also has important metabolic functions in animals and man, enhancement of the immune response, and protection against degenerative diseases by scavenging oxygen radicals (9). Astaxanthin has shown superior antioxidant properties to β-carotene in a number of *in vitro* studies (10-11).

B. braunii is a green colonial micro alga belonging to the family chlorophyceae. . This alga is mainly known for the production of hydrocarbons, exopolysaccharides and carotenoids (12). The presence of carotenoids is more pronounced in races B and L (13). In the linear stage of growth, both the races produce almost equal amounts of β-carotene, echinenone, canthaxanthin, lutein, violaxanthin, loroxanthin, and neoxanthin. However, lutein is the major carotenoid (22-29%) in the linear phase of these races. The carotenoid composition and antioxidant properties of *B. braunii* (Race 'A') were reported by Ranga Rao et al. (14).

There is growing awareness of microalgae as a source of food and for several therapeutic purposes. In view of this has placed great demand on the present study was undertaken to evaluate the antibacterial properties of micro algal extracts of *S. platensis, H. pluvialis*, and *B. braunii* against important pathogenic bacteria.

Material and Methods

Chemicals: All the chemicals and analytical grade solvents hexane, chloroform, ethylacetate, acetone, methanol, ethanol, dimethyl sulphoxide (DMSO), hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), folin ciocalteu reagent from Ranbaxy fine chemicals Ltd, (Mumbai, India). Caffeic acid standard was obtained from Sigma Chemicals, Co (St. Louis, MO).

Micro algal culture: *S. platensis: S. platensis* (CFTRI) It is a local isolate and maintained in modified Zarrouks medium (15). The *S. platensis* culture was grown indoors at 28 ± 2 °C with an illumination of 4 klux light intensity.

H. pluvialis: *H. pluvialis* (19-1a) It was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany and was maintained on autotrophic medium agar slants. *Haematococcus* culture was grown in modified autotrophic Bold basal medium (16) and carotenoid formation was obtained under salinity stress as reported earlier by Sarada et al. (17). The encysted red cells rich in carotenoids were harvested, freeze dried and stored at low temperature.

B. braunii: The sample was collected from Indian fresh water bodies of Kodaikanal (latitude 10.31N and longitude 77.32E), India, and identified as Race 'A' (18) and maintained in modified Chu13 medium (19). Stock cultures of *B. braunii* were maintained routinely on agar slants of Chu13 medium as well as in liquid medium. The cultures

were incubated at 25 ± 1°C under 1.5 ± 0.2 klux light intensity with 16 h light: 8 h dark photoperiod.

Biomass estimation: The cultures were harvested by centrifugation at 5,000 rpm and the cells were washed with distilled water. Then the pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight (g/l).

Bacterial strains and culture conditions: The antibacterial activity was tested against B*acillus subtilus, Bacillus cereus Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus fecalis* and *Yersinia enterocolitica.* The above bacterial strains were obtained from the stock cultures of Microbiology laboratory, Department of Microbiology, Mysore Medical College, Mysore, India. The bacterial stock cultures were maintained on nutrient agar (HiMedia lab. Pvt., Ltd, Bombay, India) slants at 37 ± 1°C.

Preparation of micro algal extracts: Known quantity of freeze dried algal biomass (2 g) was ground well in a mortar and pestle and extracted with different solvents (each 10 mL) acetone, methanol, chloroform, and ethylacetate (20). The extraction was repeated until the sample became colourless (total volume 50 mL). The pooled extract (60 mL) were taken, concentrated to dryness by flash evaporation (Buchi rota vapor R205, Germany) at 30-35 °C and redissolved in a known volume of ethanol (20 mL) and tested for antibacterial activity.

Determination of total phenolic compounds in micro algal extracts: The concentration of total phenolic compounds in the extracts was determined according to the method of Taga et al. (21) and expressed as caffeic acid equivalents. In brief, samples and standards were prepared in acidified (3 g/l HCl) methanol/water (60:40 v/v) and 100 µL of each were added separately to 2 mL of 2% Na_2CO_3. After 5 min, 100 µL of 50% Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. Absorbance was measured at 750 nm using spectrophotometer (Shimadzu 160A). The blank consisted of all reagents and solvents without sample or standard. The standard caffeic acid was prepared at concentrations of 10-100 µg/mL. The phenolic concentration in the algal extract was determined by comparison with the standards.

In vitro screening for antibacterial activity

Agar-well diffusion assay: *In vitro* antibacterial activity of five extracts was determined by agar well diffusion method (22). Extracts dissolved in DMSO (5 mg/mL) was used for the assay. About 50 µl of the sample was placed in the wells and allowed to diffuse for 2 hrs. Plates were incubated at 37 °C for 48 hrs and the activity was determined by measuring the diameter of inhibition zones. DMSO alone was used as a control and amoxycillin as a positive control. The assay was carried out in triplicate.

Minimum inhibitory concentration (MIC): The MIC was determined by the modified method developed by Dufour et al. (23) and Gary et al. (24). Different concentrations of test sample and 100 µL of the bacterial suspension (10^5 CFU/mL) was placed aseptically in 10 mL of nutrient broth and incubated for 24 hrs at 37 °C. The growth was observed both visually and by measuring O.D. at 600 nm (Shimadzu 160A) at regular intervals followed by plating with nutrient agar. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. The sample tubes were maintained

for each concentration of test sample and the readings were plotted against O.D. at 600 nm as growth curves.

***Statistical analysis*:** All experiments were done in triplicates and the data presented are the averages of mean of three independent experiments with standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and the *post-hoc* mean separations were performed by Duncan's multiple-range test at $p < 0.05$ (25).

Results and Discussion

Growth of micro algal biomass: The biomass yield of three micro algae was given in Figure 1. The biomass yield of *S. platensis*, *H. pluvialis*, *B. braunii* that were grown in three different media such as Zarrouk's, Bold basal and Chu13 medium yielded 2.2, 2.4 and 2.5 g/l(dry weight) respectively.

Total phenolic content in micro algal extracts: The total phenolic content was estimated in hexane, chloroform, ethylacetate, acetone and methanol extracts from *S. platensis*, *H. pluvialis* and *B. braunii* and the results are presented in Table 1. *S. platensis* and *H. pluvialis* exhibited highest phenolic content of 128 and 131 (µg/mg) respectively in chloroform extract, while *B. braunii* showed highest phenolics 110 (µg/mg) in ethylacetate extract (Table 1).

Antibacterial properties of micro algal extracts: Antibacterial activity of *S. platensis* against selected bacteria is presented in Table 2. Among the different solvent extracts, chloroform extract showed antibacterial effect on wide range of bacteria and recorded highest zone of inhibition against *B. subtilus* (18.12 mm) followed by *P. mirabilis* (17.45 mm). While methanol extract exhibited growth inhibition against *P. mirabilis* (15.21 mm), *S. aureus* (15.20 mm) and *B. cereus* (15.77 mm). Similarly, chloroform extract of *H. pluvialis,* exhibited highest antibacterial effect against *B. subtilus* (17.32 mm) followed by *E. coli* (15.87 mm). Interstingly, ethylacetate and methanol extracts showed inhibition against *L. monocytogenes*, *P. mirabilis* and *S. aureus* respectively (Table 3). In case of *B. braunii* ethylacetate extract recorded highest inhibition against *E. aerogenes* (15.11 mm) followed by *P. mirabilis* in comparison with other extracts (Table 4).

MIC values of micro algal extracts: The MIC values of *S. platensis* extracts ranged from 200 to 500 ppm against varied type of bacteria tested (Table 5). However chloroform extract recorded least MIC values against *B. subtilis* and *M. luteus.* While, complete inhibition of *P. mirabilis*, *S. aureus*, *S. fecalis*, and *L. monocytogenes* were observed at 300 ppm (Table 5). Similarly, chloroform extract inhibited effectively *B. subtilus* at 150 ppm. While, *M. luteus* and *E. coli* were inhibited effectively at 250 ppm (Table 6). Unlike others ethyl acetate extract of *B. braunii* showed highest inhibitory effect against *E. aerogenes* at MIC of 300 ppm. Further, it also showed inhibition against the growth of *S. aureus* and *P. mirabilis* at slightly higher concentration (Table 7).

The present study evaluated the antibacterial properties of micro algal crude extracts of *S. platensis, H. pluvialis*, and *B. braunii* against important pathogenic bacteria. The chloroform, ethlyacetate, acetone and methanol extracts of *S. platensis, B. braunii* and *H. pluvialis* extracts showed higher activity compared to hexane extracts. Chloroform and ethyl acetate extracts of *S. platensis* showed higher inhibitory activity against *B. subtilus, P. mirabilis* and M. *luteus*, whereas methanol extract showed inhibitory effect against *B. cereus*, *P. mirabilis S. aureus*. The acetone and

hexane extracts of *S. platensis* showed lower inhibitory activity against *B. subtilus* and *P. mirabilis*, when compared to chloroform, ethylacetate and methanol extracts. The *H. pluvailis* extracts of chloroform and ethylacetate extracts showed higher inhibitory activity against *B. subtilus, E. coli* and *E. aerogenes*, when compared with other extracts such as methanol, ethylacetate, acetone and hexane. However, ethlyacetate extract of *B. braunii* showed higher inhibitory activity against *P. mirabilis* and *S. aureus*, while methanol and chloroform extracts showed inhibitory activity against *E. coli,* and *S. aureus.* The acetone and hexane exhibited lower antibacterial activity, when compared to other extracts. Thus establishing a positive correlation between the inhibitory activity with highest phenolic content in the respective extracts.

Taken together the results indicated that the antimicrobial activity was related to the amounts of phenolic compounds contained in *S. platensis, H. pluvialis* and *B. braunii* organic extracts. Mundt et al. (26) and Ozdemir et al. (27) reported that antimicrobial activity of micro algae against some pathogenic organisms could be due to its fatty acids and hydroxyl unsaturated fatty acids, glycolipid and phenolic compounds. In the present investigation also the non-polar extracts of some algae exhibited greater antimicrobial activity than polar ones and their activity may also be attributed to the presence of fatty acids and lipid-soluble phenolic. At low concentration, phenols are reported to affect enzyme activity, especially of those enzymes associated with energy production while at higher concentrations, they cause protein denaturation. In addition, effect of phenolic and fatty acids on microbial growth could be the result of the ability of these compounds to alter microbial cell permeability, permitting the loss of macro-molecules from the interior and could be also interact with membrane proteins causing a deformation in their structure and functionality as well as affecting cellular activity as reported by Mundt et al. (26). In the present investigation linear inhibition of bacteria with increased concentration of extracts was noticed. Earlier report also indicated that antibacterial effects of phenolic compounds are concentration dependent (28). Patterson et al. (29) reported that the blue green alga extracts contained different antibacterial substances and reflect the variety of secondary metabolites. Kreitlow et al. (30) reported 12 cyanobacterial strains for their antibiotic activities against 7 microorganisms, whereas antibacterial activity of fresh water micro algae was reported by Jaya prakash Goud et al. (31). The algal species in the present study were individually positive to one or more phycochemicals, which might have contributed to their antibacterial efficacy. The significant antibacterial activity of S. *platensis, B. braunii* and *H. pluvialis* extracts against bacteria may be attributed to their chemical nature.

Conclusion

The chloroform, methanol and ethyl acetate extracts of *S. platensis, B. braunii,* and *H. pluvialis* respectively were found to have potential antibacterial activity against *B. subtilus, B. cereus, E. aerogenes, E. coli, K. pneumoniae, L. monocytogenes, M. luteus, P. mirabilis, P. aeruginosa, S. typhi, S. aureus, S. fecalis* and *Y. enterocolitica* pathogens of clinical importance. Chloroform extract of *S. platensis, H. pluvialis* and ethylacetate extract of *B. braunii* were found to have higher antibacterial activity when compared to other forms of extracts. The antibacterial effect of *S. platensis* and *H. pluvialis* against clinically important pathogenic bacteria can be a preferred supplement to their known health benefits as antibacterial agents and usage in food system. Further investigations are in progress to study the biological activity of these algae.

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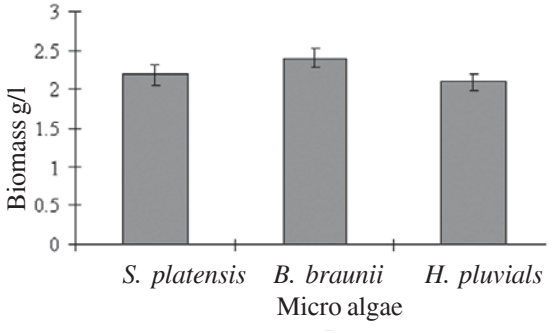

Fig. 1. Biomass yield of *S. platensis, B. braunii* and *H. pluvials.* Values are mean ± SD, Data recorded on 3 weeks old culture.

Table 1. Total phenolic content in different extracts of three species of micro algae.

Solvent system	Phenolic content (ìg/mg)		
	S. platensis	***H. pluvialis***	***B. bruanii***
Hexane	41.42 ± 2.56	47.52 ± 1.25	41.68 ± 0.42
Chloroform	128.38 ± 4.86	131.98 ± 5.23	85.74 ± 0.82
Ethylacetate	108.74 ± 3.14	95.02 ± 0.89	110.45 ± 2.14
Acetone	65.98 ± 5.87	68.03 ± 0.25	65.23 ± 2.60
Methanol	98.24 ± 2.65	85.23 ± 0.18	70.23 ± 0.23

Each value represents mean of three different observations ± SD.

Table 2. Antibacterial activity of *S. platensis* extracts[a].

Bacteria	Diameter of zone of inhibition (mm)				
	Hexane	**Chloroform**	**Ethylacetate**	**Acetone**	**Methanol**
B. subtilus,	—	18.12 ± 0.67^{a}	15.52 ± 0.27^{b}	12.05 ± 0.25^{a}	12.63 ± 0.28^{d}
E. aerogenes	—	14.23 ± 0.61^{d}	13.34 ± 0.18^{d}	10.31 ± 0.46^{c}	14.45 ± 0.23^{b}
P. mirabilis	—	17.45 ± 0.75^{b}	16.67 ± 0.36^{a}	12.22 ± 0.24^{a}	15.21 ± 0.31^{a}
M. *luteus*	9.12 ± 0.11^{c}	15.09 ± 0.37^{e}	13.05 ± 0.28^{d}	11.08 ± 0.18^{b}	14.85 ± 0.54^{b}
E. coli	—	11.87 ± 0.2^{i}	14.89 ± 0.53^{c}	—	—
S. aureus	11.24 ± 0.18^{a}	12.43 ± 0.19^{g}	13.67 ± 0.17^{d}	12.34 ± 0.17^{a}	15.20 ± 0.16^{a}
S. typhi	—	—	—	—	11.28 ± 0.24^{e}
P. aeruginosa	—	14.26 ± 0.34^{d}	12.31 ± 0.34^{e}	—	—
K. pneumoniae	—	11.73 ± 0.25^{i}	12.35 ± 0.19^{e}	11.98 ± 0.32^{b}	12.65 ± 0.52^{d}
B. cereus	—	10.02 ± 0.28^{j}	—	11.37 ± 0.21^{b}	15.77 ± 0.23^{a}
L. monocytogenes	—	12.85 ± 0.12^{f}	11.24 ± 0.18^{f}	—	12.98 ± 0.37^{d}
S. fecalis	10.09 ± 0.14^{b}	13.98 ± 0.18^{e}	12.93 ± 0.31^{e}	10.51 ± 0.16^{c}	—
Y. enterocolitica.	—	12.09 ± 0.34^{h}	—	—	13.23 ± 0.11^{c}

Values are expressed as mean ± SD. Values are not sharing a similar superscript with in the same column are significantly different (P<0.05) as determined by ANOVA.

Table 3. Antibacterial activity of *H. pluvialis* extracts[a].

Bacteria	Diameter of zone of inhibition (mm)				
	Hexane	Chloroform	Ethyl acetate	Acetone	Methanol
B. subtilus,	11.01 ± 0.12[a]	17.32 ± 0.18[a]	11.23 ± 0.14[d]	—	10.43 ± 0.12[d]
E. aerogenes	—	14.54 ± 0.18[c]	12.34 ± 0.26[c]	10.05 ± 0.26[e]	—
P. mirabilis	—	—	13.82 ± 0.18[c]	—	—
M. *luteus*	—	12.09 ± 0.10[e]	—	12.78 ± 0.24[b]	12.81 ± 0.20[b]
E. coli	—	15.87 ± 0.20[b]	—	—	12.34 ± 0.13[b]
S. aureus	10.23 ± 0.17[b]	13.75 ± 0.31[d]	12.61± 0.23[c]	11.45 ± 0.17[c]	13.52 ± 0.34[a]
S. typhi	—	—	12.02 ± 0.11[c]	—	—
P. aeruginosa	—	12.02 ± 0.21[e]	—	—	—
K. pneumoniae	—	—	—	—	—
B. cereus	—	11.93 ± 0.14[f]	—	—	12.27 ± 0.18[b]
L. monocytogenes	10.66 ± 0.20[b]	—	11.34 ± 0.18[d]	13.96 ± 0.25[a]	13.18 ± 0.31[a]
S. fecalis	—	13.21 ± 0.28[d]	12.98 ± 0.21[d]	11.72 ± 0.10[c]	—
Y. enterocolitica.	—	10.34 ± 0.17[e]	10.54 ± 0.17[g]	10.65 ±0 .21[d]	11.32 ± 0.19[c]

[a]Experimental details are the same as in Table 2.

Table 4. Antibacterial activity of *B. bruanii* extracts[a].

Bacteria	Diameter of zone of inhibition (mm)				
	Hexane	Chloroform	Ethylacetate	Acetone	Methanol
B. subtilus,	—	11.56 ± 0.18[c]	12.08 ± 0.18[f]	—	11.32 ± 0.34[d]
E. aerogenes	—	—	15.11± 0.56[a]	12.41 ± 0.19[a]	—
P. mirabilis	—	—	14.33 ± 0.38[c]	11.05 ± 0.14[d]	—
M. *luteus*	11.34 ± 0.21[a]	11.78 ± 0.25[c]	—	—	12.71 ± 0.34[b]
E. coli		—	13.08 ± 0.26[d]	11.56 ± 0.3[c]	13.14 ± 0.28[a]
S. aureus	10.21 ± 0.15[b]	13.71 ± 0.31[a]	14.09 ± 0.41[b]	—	12.05 ± 0.27[c]
S. typhi	—	12.05 ± 0.23[b]	—	—	—
P. aeruginosa	—	—	12.76 ± 0.17[e]	11.89 ± 0.12[b]	—
K. pneumoniae	—	—	13.19 ± 0.33[d]	12.34 ± 0.30[a]	11.15 ± 0.12[d]
B. cereus	—	10.75 ± 0.18[d]	—	—	—
L. monocytogenes	10.53± 0.14[b]	12.62 ± 0.17[b]	11.18 ± 0.21[g]	—	12.18 ± 0.27[c]
S. fecalis	10.46± 0.27[b]	—	12.23 ± 0.14[f]	11.25 ± 0.23[cd]	—
Y. enterocolitica.	—	11.76 ± 0.26[c]	10.18 ± 0.17[h]	10.51 ± 0.18[e]	

[a]Experimental details are the same as in Table 2.

Table 5. MIC values for *S. platensis* extracts against food borne pathogens.

Bacteria	MIC (ppm) Hexane	Chloroform	Ethylacetate	Acetone	Methanol
B. subtilus,	—	200	250	400	400
E. aerogenes	—	400	450	450	300
P. mirabilis	—	300	200	400	250
M. luteus	500	250	400	350	300
E. coli	—	400	300	—	—
S. aureus	300	300	400	450	300
S. typhi	—	—	—	—	400
P. aeruginosa	—	400	400	—	—
K. pneumoniae	—	400	400	400	400
B. cereus	—	450	—	400	300
L. monocytogenes	—	300	500	—	400
S. fecalis	450	300	400	450	—
Y. enterocolitica.	—	400	—	—	300

Table 6. MIC values for *H. pluvialis* extracts against food borne pathogens.

Bacteria	MIC (ppm) Hexane	Chloroform	Ethylacetate	Acetone	Methanol
B. subtilus,	400	150	350	—	350
E. aerogenes	—	250	300	350	—
P. mirabilis	—	—	250	—	—
M. *luteus*	—	200	—	350	300
E. coli	—	200	—	—	300
S. aureus	350	250	300	400	250
S. typhi	—	—	300	—	—
P. aeruginosa	—	350	—	—	—
K. pneumoniae	—	—	—	—	—
B. cereus	—	350	300	—	300
L. monocytogenes	300	—	300	300	250
S. fecalis	—	250	200	350	—
Y. enterocolitica.	—	350	350	300	350

Table 7. MIC values for *B. braunii* extracts against food borne pathogens.

Bacteria	MIC (ppm) Hexane	Chloroform	Ethylacetate	Acetone	Methanol
B. subtilus,	—	450	450	—	500
E. aerogenes	—	—	300	450	—
P. mirabilis	—	—	350	500	—
M. *luteus*	450	400	—	—	450
E. coli	—	—	400	400	400
S. aureus	550	350	350	—	450
S. typhi	—	450	—	—	—
P. aeruginosa	—		450	450	—
K. pneumoniae	—	—	400	400	450
B. cereus	—	500	—	_–	—
L. monocytogenes	400	400	450	—	400
S. fecalis	500	—	400	450	—-
Y. enterocolitica.	—	450	400	500	—

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Expression of a bacterial chitinase (ChiB) gene enhances antifungal potential in transgenic *Litchi chinensis* Sonn. (cv. Bedana)

Das, D.K.* and Rahman, A.
Post Graduate Department of Biotechnology, T.M.Bhagalpur University,
Bhagalpur-812007, India
*For Correspondence - dilipdas1@live.com

Abstract

Transgenic litchi harboring chitinase and gus genes were generated via Agrobacterium mediated transformation of zygotic embryos. Confirmation of the transgenic was done by PCR, RT-PCR, Southern blotting and by Western blot. Functional analysis of the transgenic was also attempted

Key words: *Agrobacterium tumefaciens*, Somatic embryogenesis, die back, leaf spots and blight, Transformation, *Phomopsis sp.*

Abbreviations HPT: Hygromycin phospotransferase gene, Bacterial chitinase gene, WPM: woody plant media (Lloyd and McCown 1981), MS: Murashige and Skoog (1962), IBA: Indole3-butyric acid, 2,4-D: 2,4-dichlorophenoxyacetic acid, GA : Gibberellic acid, B5: Gamborg et al.(1968)

Introduction

Litchi is an important fruit tree in China, India, Pakistan, Bangladesh, Burma, former Indochina, Taiwan, Japan, the Philippines, Queensland, Madagascar, Brazil and South Africa. Major varieties produced in India are Purbi, Early and Late Bedana, Kashba, Shahi, China, Deshi, Dehra Rose, Longia etc. However, these varieties are susceptible to an array of diseases. Fungal diseases, which cause extensive losses in yield and quality, have been the most serious problems for cultivation. The most important among them are die back, leaf spots and leaf blight caused by *Phomopsis sp*. And leaf blight by *Gleosporium sp*. respectively. Indian litchi cultivars are mostly threatened by these two pathogens. Though the diseases are controllable by fungicides, considering the cost and safety of the application resistant varieties offer a viable alternative. Some attempts to produce transgenic litchi using transformation of zygotic embryos have been attempted. In this paper, we report on the use of *Agrobacterium* method to overexpress bacterial chitinase gene (ChiB) in *Litchi chinensis* cv. Bedana. The transgenic plants that exhibited higher chitinase activity than non-transgenic plants showed increased resistance to die back, leaf spot and blight.

Materials and Methods

***Plant materials*:** Immature litchi fruits cv. Bedana were obtained from Horticulture Division, Bihar Agricultural College, Sabour, Bhagalpur, India after four weeks of anthesis, were washed in running tap water to free them from dust. Followed by these immature fruits were dipped in water mixed with 2-4 drops of tween 20 and

remained there for 5 min. After that these fruits were taken out and again dipped into 0.1 % $HgCl_2$ and remained there for 10min. Followed by these fruits were rinsed thrice in sterile distilled water under aseptic conditions. These fruit's walls were incised and zygotic embryos were isolated. These zygotic embryos were the materials for transformation experiments.

Determination of antibiotic sensitivity: Prior to genetic transformation experiments, the amount of kanamycin required to inhibit germination and regeneration of embryogenic calli was determined using untransformed cultures. The fresh proembrygenic calli were placed on (1) or (2) medium supplemented with various concentrations of kanamycin (0, 10, 20, 50 and 100 mgl^{-1}), to study the effects of the antibiotic. A completely randomized design was used for these experiments. Each experiment was repeated three times. For each treatment 10 calli of size ranging from 0.3 to 0.4 cm were used. Cultures were observed after 5 weeks of incubation.

***Transformation of litchi*:** Zygotic embryos of litchi (*Litchi chinensis* Sonn. cv. Bedana) were transformed as described by (3) by infection with *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pBI121-ChiB, which contains the *chitinase* coding region (GenBank Acc. No. X54367). Following 3 days of co-cultivation, the zygotic embryos were sub-cultured in 2-weeks interval following the protocol of (4). Subsequently, the calli were collected and transferred to cell suspension culture and remain there for 4-9 months. Followed by protoplasts were isolated from harvested cells and formed Calcium alginate beads. These beads were cultured on P11 liquid medium consisted of major salts of (5) medium (NH_4NO_3 600, KNO_3 1900, $CaCl_2.2H_2O$ 600, MgSO4.7H2O 300, KH_2PO_4 170,KCl 300 mgl^{-1}), minor salts, iron and vitamins of (1) basal medium 2,4-D (1 mgl^{-1}),NAA (0.2 mgl^{-1}), glutamine (250 mgl^{-1}), Zeatin (0.5 mgl^{-1}), coconut water (200 ml l^{-1}), glucose (0.45 M) and Sucrose (0.1M) for embryogenic calli development. These calli develop a number of putatively transformed small somatic embryos (2 mm) on (1) medium consisted of (1) salts and (6) vitamin with Kinetin 1mgl^{-1}, NAA 0.1mgl^{-1}, glutamine 500 mgl^{-1}, 8% sucrose and 15gl^{-1} agar which later developed into large and matured somatic embryos (5-10 mm) (1) medium consisted of (1) salts and (6) vitamins with glutamine 500 mgl^{-1}, coconut water 50 mll^{-1}, 5% sucrose and 9 gl^{-1} with appropriate concentration of antibiotics for selection and 400 mgl^{-1} cefotaxime (0.7% agar, pH 5.7). The cultures were maintained at 25°C under a 16-h photoperiod. Somatic embryos were germinated on (1) medium consisted of (1) salts and (6) vitamins with kinetin 1mgl^{-1}, GA 5 mgl^{-1}, coconut water 50 mll^{-1}, 3% sucrose and 7 gl^{-1} agar. Shoot elongation, proliferation, rooting and subsequent plantlet development were induced on half concentration of (2) (BAP 0.25 µM + NAA 0.1 µM) supplemented with antibiotics. Rooted plantlets were further acclimatized in vermiculite and finally transferred into garden soil and grown in a green house.

Polymerase chain reaction (PCR): To confirm the presence of the *Bacterial chitinase* gene in transgenic plants, genomic DNA was isolated from 0.5 g of fresh young litchi leaves as described by (7). For the PCR analysis, 200 ng of plant DNA or 4 ng of plasmid DNA was used per 25-µl reaction mixture. The primers were designed to amplify 465 bp fragments of chitinase at 63.6°C (F 5'GCTAC TGCTTC AA G GAGGAG AAACA3'; R 5'C TGGTTGT AGCAATCCAGGTTATCG-3') and 508-bp fragments of *npt* gene at 52°C (F-5'AGCTG CGCCGATGGTTTCTACAA3'; R-5'ATCGC CTCGCTCCAGTCAATG 3'). The PCR program profile for both the genes was as follows; initial denaturation at 94°C for 4 min, followed by

30 cycles of 94°C for 1 min, 1.5 min at the annealing temperature of each gene and 1 min at 72°C, with a final extension at 72°C for 10 min. The amplified products were run on 1 % agarose gels and visualized by ethidium bromide staining.

***Southern blot analysis*:** In order to confirm the transgene integration and to determine the number of copies of transgene *(ChiB)* integrated, Southern blotting (8) experiments were performed. Genomic DNA (10 µg) and plasmid as positive control were digested with *XbaI or BamHI* (New England Biolab), fragments were separated on 1% (w/v) agarose gels at 25 V for 16 h. The fractionated DNA was denatured with 0.5 M NaOH for 30 min, neutralized with 25 mM sodium phosphate buffer, pH 6.5 and transferred onto positively-charged nylon membranes (Hybond N+, Amersham Biosciences, Hongkong). The transferred DNA was fixed to the membrane by baking at 80° C for 30 min followed by UV irradiation (12×10^4 µJ cm-2) using a UV cross-linker (UV-Stratalinker 1800, USA).

***Total RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis*:** Total RNA was prepared from leaf tissues using Trizol Reagent as per the manufacturer's instructions (Trizol Reagent, Invitrogen life technologies, San Diego, California, USA). The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry. To detect the presence of bacterial chitinase mRNA transcripts in transformants, RT-PCR was carried out. To 5 µg of RNA in a total volume of 12 µl of RNAse free H_2O, 1 µl of 18-mer oligo dT primer was added and the mixture was incubated for 10 min at 70°C. Then the RNA was reverse transcribed by addition of 4 µl of RT, 1x of buffer, 1 µl of RNAse inhibitor (40 U/µl) and 2 µl of 5mM dNTPs. The mixture was incubated for 5 min at 37°C, and then 1 µl M-MuLV (Moloney Murine Leukemia Virus) Reverse Transcriptase polymerase (200 U/ µl) was added and incubated for 1 hour at 42°C followed by 10 min. inactivation at 70°C. PCR was carried out on 5 µl of this RT product using *ChiB* gene specific primers at gene specific primers at 52°C (F 5'TCTCCT TCAAGACGGC GTTCTGGTTC-3'; R5'CT GGTTGTAGCA ATC CAGGTTATC G-3') and cycle parameter was kept as mentioned in the section earlier to amplify 290-bp of bacterial chitinase. Untransformed plants cDNA was used as experimental control.

***Chitinase assay*:** The chitinase levels in transgenic litchi were determined by colorimetric enzyme assay, and western blot assay. Total soluble proteins were extracted from frozen zygotic embryos of the transformed and non-transformed samples. Zygotic embryos were homogenized with a mortar and pestle in liquid nitrogen and the frozen powder was suspended in 5 volumes of 0.1 M sodium citrate buffer (pH 6.0) containing 20 mM sodium ascorbate and polyclar AT. After two rounds of centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant was recovered (9). The protein concentration in the extracts was estimated by the method (10). Equal amount (25 µg) of soluble proteins were loaded in 12% SDS-gels and stained with Coomassie brilliant blue dye to ensure the equal loading of protein and proceeded for Western blotting by transferring proteins on to the nitrocellulose membrane and probed with antiserum to bean chitinase using horseradish peroxidase (HRP) conjugated anti-rabbit IgG as the secondary antibody chitinase (Generously provided by Dr.M.V.Razam, Deptt. Of Genetics, South Campus, University of Delhi, India) at 1:5000 dilution. The specific position of antigen-antibody complex on the membrane was visualized

n the exposed to Kodak X-ray film by using super signal west pico chemiluminescent substrate (Thermo scientific).

Quantitative assay of Chitinase enzyme activity - A solubilized, ethylene glycol-chitin (Sigma-Aldrich) was used as a substrate for chitinase activity assay. The colorimetric analysis of chitinase enzyme activity of PCR, Southern and RT-PCR positive transgenic plants was done following the protocol of (11) with slight modifications in triplicate, where aliquots of 300 µl of ethylene glycol-chitin (stock 2 mg/ml) were mixed with 100 µl of 200 mM sodium acetate buffer, pH 5.0 and 0.5 ml enzyme solution, then incubated for 60 min. at 37°C in circulating water bath. The reaction was terminated by the addition of 100 µl HCl (1.0 N) on ice and incubated for 10 min. to facilitate precipitation of the non-degraded substrate (chitin), was centrifuged at 14000g for 5 min. The resulting N-acetyl glucosamine (GlcNAc) residues were colorimetrically measured by the dinitrosalicylic acid (DNSA) method (12). To 1 ml of the reaction mixture, 1 ml of DNSA was added and boiled for 10 min. and then 0.4 ml of Potassium-Sodium tartarate was added. The mixture was cooled at room temperature and OD was taken at 540 nm. As an appropriate controls enzyme and substrate blanks were included in the experiment. One unit was defined as the amount of enzyme that produced 1 µmol of reducing sugars corresponding to *N*-acety*l*-D-glucosamine in 1 min.

***Pathogenicity test against die back, leaf spots and blight*:** We evaluated the potential of tolerance of transgenic litchi transformed with *Bacterial chitinase* gene to die back, leaf spots and blight caused by *Phomopsis sp.* newly developing secondary or tertiary leaves was detached from the in vitro grown transgenic plants. Two leaves from each transgenic plant were placed adaxial side up onto 0.6% (w/v) agar containing 40 mgl^{-1} benzodiazoloe in a petri dish. As a control leaves from non-transformed plants were taken. *Phomopsis sp.* is an obligate biotrophe so, can not be cultured on medium. The spores were collected by washing die back, leaf spots and blight infected leaves from Horticulture department, Bihar Agricultural College, Sabour, Bhagalpur in distilled water containing 0.01% (v/v) Tween 20. The spore suspension (0.5 ml) was sprayed on to each petri dish containing leaf and kept at saturated humidity at 25°C. The degree of disease severity was scored using a visual assessment scale based on the size and characteristics of necrotic lesions. A 5-point disease rating scale based on the approximate percentage of leaf necrotic area after 15-28 days of inoculation (1 = 0%; 2 = 1-20%; 2 = 20-30%; 3 = 30-40%; 4 = 40-50%; 5 = >50%) similar to grape (9) and (13) was employed. We also recorded the number of days required for the onset and complete chlorosis in each leaf in comparison to control. The diseased leaves were digitally photographed. A portion of leaves of both non transgenic and transgenic plants were electron micro graphed which showed that pathogenic fungal spores could easily germinate, ramify mycelium and also invade on leaf surface cells of non transgenic litchi plants so that they developed die back, leaf spots and blight disease but pathogenic spores were unable to germinate, ramify and invade the transgenic plants so that there was no development of disease (Fig 6 a & b). Each disease value was taken on averages of three experiments and all data were analyzed for significance differences by one way analysis of variance (ANOVA) for detached leaf assay infection scores followed by Turkey's multiple comparison tests using the Graph Pad software (Graph pad In Stat. Software Inc. San Digeo 92130, USA).

Results

***Kanamycin sensitivity assay*:** After 4 weeks of culturing we observed that, as the concentrations of kanamycin was increased from 0 to 100mgl^{-1}, the percentage of survival decreased and the browning area increased and the calli failed to proliferate. Without kanamycin, about 78% survived and 17% were dead calli. In the plate containing 100 mgl^{-1} kanamycin, almost all the calli died and completely turned brown.

***Transformation of litchi with the Bacterial chitinase gene*:** The globular embryos were formed after transfer of proembryogenic callus ,developed from zygotic embryos which were transformed by infecting with the Agrobacterium tumefaciens strain LBA4404 containing the binary vector pBI121 ChiB (Fig 1.1), to different developmental stages i.e. suspension culture, protoplast culture, calcium alginate bead culture, liberation of embryogenic calli from calcium alginate bead on different MS modified medium supplemented with different hormones i.e. 2,4-D (1mgl^{-1}), Zeatin (0.5 mgl^{-1}), Kinetin (1mgl^{-1}), NAA (0.1mgl^{-1}), STS (29.4 µM), glutamine (250 mgl^{-1}), coconut water (20 ml l^{-1}) in different stages of embryo development (Fig. 1.2 A). Under these conditions we were able to block necrogenesis following of *Agrobacterium* co-cultivation. The stable transformed embryos were selected by periodic increase in kanamycin concentration. Initially after 20 days on medium containing 50mgl^{-1} kanamycin, a high number of germinated somatic embryos were obtained. When transferred to medium containing higher concentrations of the antibiotic (75, 100 mgl^{-1}), many of the regenerants became yellowish, and were discarded after 50 days. Stable matured somatic embryos were selected after additional 20 days of incubation on (1) medium consisted of (1) salts and (6) vitamins with kinetin 1mgl^{-1}, NAA 0.1 mgl^{-1}, glutamine 500 mgl^{-1} containing 50 mgl^{-1} kanamycin and 400 mgl^{-1} cefotaxime. During in vitro selection, only the transformed cells maintained a high regenerative capacity, while the other parts became progressively necrotic.

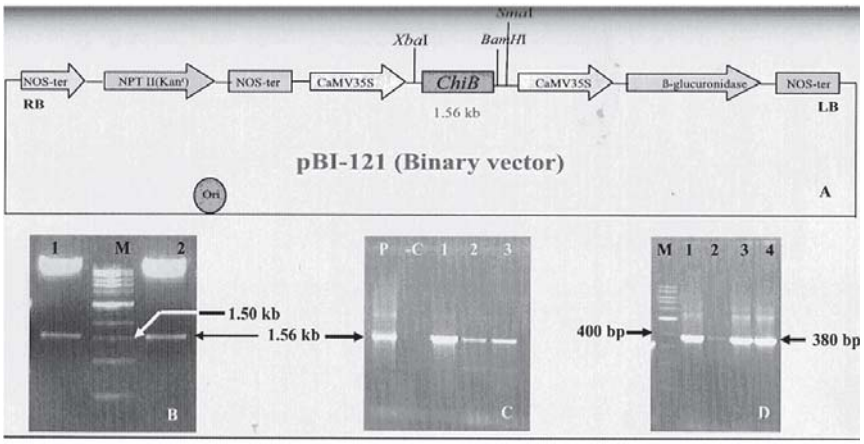

Fig. 1. ChiB genes construct in plasmid pBI 121 and its analysis. A. Map of *ChiB* gene constructs. Expression of gene is under the control of CaMV 35 S constitutive promoter. **B.** Enzymatic digestion pattern of recombinant clone by Xba I and SmaI; LaneM: 1 kb DNA ladder, Lanes1 and 2: showing 1.56 kb *ChiB* fragment. **C.** Colony PCR of recombinant clone using gene specified primers. Lane P: recombinant plasmid as a positive control, Lane C: negative control without the recombinant plasmid, Lanes 1, 2 and 3 showing amplicons of 1.56 kb ChiB gene. **D.** Colony PCR of recombinant clone using GUS gene specific primers, Lane M: 100bp DNA ladder, Lanes 1, 2, 3 and 4 showing GUS specific amplicon of 380 bp.

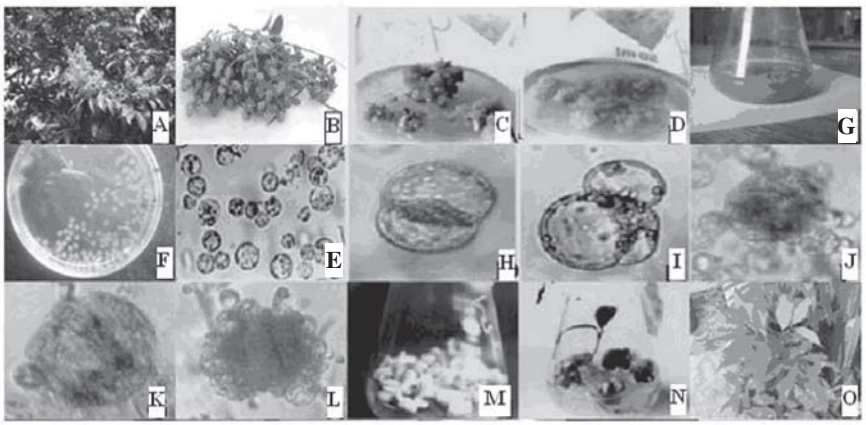

Fig. 1.2. production of transgenic litchi plants harboring the bacterial chitinase gene (*ChiB*) by the *Agrobacterium tumefaciens* infection method.

A-Embryogenic calli of Bedana obtained on MS medium containing IBA. **B, C** - Transformed embryos selected on woody plant medium (WPM) containing 50 mgl^{-1} kanamycin after co-cultivation with *A. tumefaciens*. **D, E**-Organization of many shoots on calli of somatic embryos on the same medium containing BAP and NAA. **F, G**-Secondary somatic embryos with its germling and germinated plantlets on same medium **H, I, J**- Full-grown in-vitro plantlet. **K**- Plantlet in vermiculite

About twenty putatively transformed embryogenic clusters and somatic embryos were developed from initial proembryogenic calli (3 g) developed from zygotic embryos which were used for co-cultivation and selection on kanamycin containing medium (Fig. 1.2 C-M). The putatively transformed somatic embryos were germinated on (1) medium consisted of (1) salts and (6) vitamins with kinetin $1mgl^{-1}$, GA 5 $mg\ l^{-1}$, coconut water 50 ml l^{-1} , sucrose 3 % (w/v) agar 7 gl^{-1} (pH 5.8). The overall frequency of plantlet regeneration through the steps of embryo germination on the same medium was 20.9% (18 plantlets/86 embryos) (Fig. 1.2 N, P). Of the germinating kanamycin resistant transgenic embryos, about 20% showed normal shoot and root development compared to a conversion frequency of 67% in the control plantlets. Nine regenerated lines (B-Chi-1, 2, 4, 5, 9, 10, 14, 15 and 18) were used for further analyses. Each transgenic line was derived from a single somatic embryo and grew on kanamycin-containing media. The putative transgenic plants had no phenotypic abnormalities in comparison to the untransformed control plants.

***Molecular and Biochemical characterization of transgenic plants*:** Existence of the pBI121-ChiB gene in the regenerated litchi plantlets was confirmed by PCR analysis using gene-specific primers to amplify 465 bp internal fragments. The npt (neomycin phosphotransferase) gene was also detected by PCR with gene specific primers to amplify 508 bp regions. All nine transformants (B-Chi1, 2, 4, 5, 9, 10, 14, 15, and 18) were positive for the 508-bp npt band (Fig 2 A) but only five regenerants (B-Chi 1, 4, 9,15 and 18) were confirmed to possess 465-bp (Fig 2 B) *Bacterial chitinase* gene. There was no amplification observed in the untransformed plant. For further work only these five plants were tested.

PCR analysis: Existence of the Bacterial chitinase gene in the regenerated litchi plantlets was confirmed by PCR analysis using *Bacterial chitinase* gene-specific primers to amplify 465 bp internal fragments. The *npt* gene was also detected by PCR with *npt* gene specific primers to amplify 508 bp of the structural gene. All nine transformants (B-Chi1, 2, 4, 5, 9, 10, 14, 15, and 18) were positive for the 508-bp npt band (Fig 2

Fig. 2

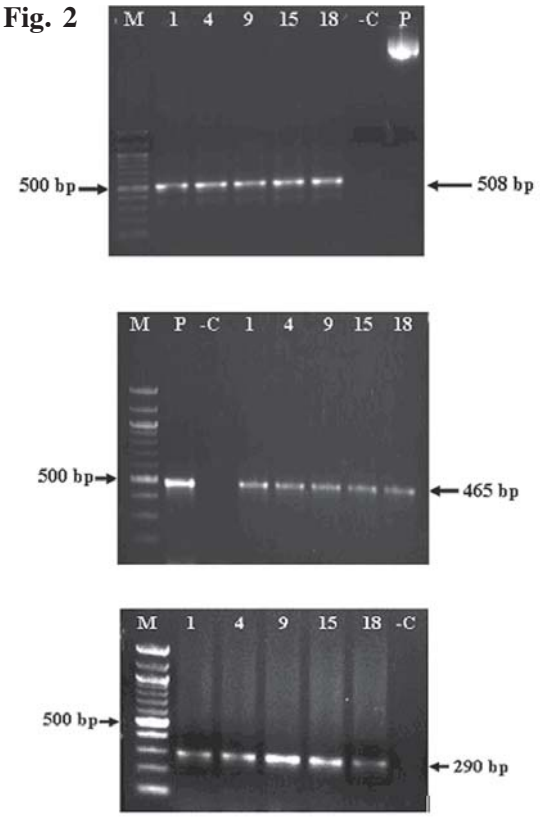

Fig. 2. PCR analysis of plant lines for transgenes (A-*npt* gene), (ChiB gene). 200 ng of plant genomic DNA was used for each reaction. *Numbers* indicate plant lines (1, 4, 9, 15 and 18), *P*- Plasmid positive control; -C- untransformed plant negative control; *M*- 1 Kb marker; *Arrow* indicates amplicon size.**C.** RT-PCR analysis of plant lines for transgenes. 400 ng of total RNA was used for each reaction. *Numbers* indicate plant lines (1, 4, 9, 15 and 18); -C- untransformed plant negative control; *M*- 100 bp marker; *Arrow* indicates amplicon size.

A) but only five regenerants (B-Chi 1, 4, 9,15 and 18) were confirmed to possess 465-bp (Fig 2 B) *Bacterial chitinase* gene. There was no amplification observed in the untransformed plant. For further work only these five plants were tested.

The introgression of foreign genes and copy number pattern of integrated transgene in the nuclear genome of the PCR positive transgenic lines was confirmed by Southern hybridization. The genomic DNA (5 µg) was digested with *XbaI and BamHI* restriction endonuclease enzymes in order to release the *ChiB* cDNA-terminator cassette (~1.56 kb). The blot was probed with ^{32}P-dCTP labeled *ChiB* cDNA. In all the five transgenic lines analyzed, the expected ~1.56 kb band lighted up at the position corresponding to the chitinase gene within their genome while it was absent in the untransformed control plant. Genomic DNA was also digested with *Sac*II, which has single recognition site on the T-DNA region to determine the number of integration events and probed with ^{32}P-dCTP labeled *npt* gene fragment. Single bands were seen in all the transgenic lines confirming the single copy integration in all five lines (Fig. 3 A and B).

We used RT-PCR analysis to check the expression of *ChiB* gene. In order to study the expression of mRNA for *ChiB* gene, total RNA was taken and RT-PCR analysis was performed from all five PCR positive lines using primers

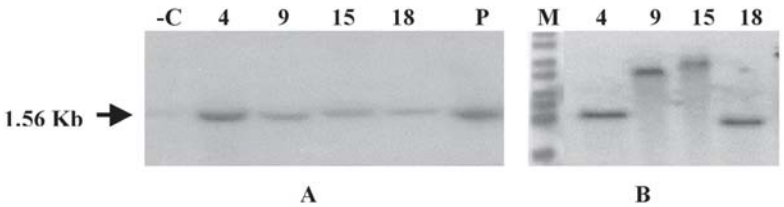

Fig. 3. Southern Blot analysis of transgenic litchi cv Bedana. Genomic DNA (5 µg) and plasmid digested with *Xba*I (A) or *BamH*I (B) were probed with PCR-generated fragments of chitinase (*ChiB*) and *npt* gene. (*Lanes – C*- untransformed plant, *P*- plasmid- pBI121-ChiB, *4, 9, 15, 18*- different transgenic lines, *M*- 1 Kb marker).

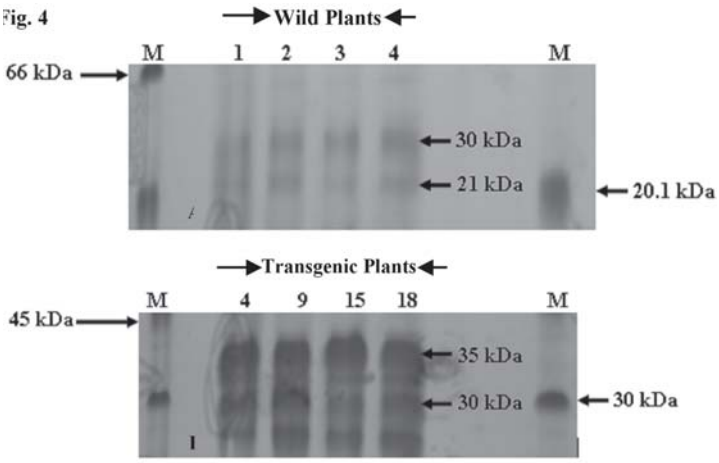

Fig. 4. Biochemical analysis of transformed and untransformed litchi plants (cv Bedana). A and B: Detection of chitinolytic activity of chitinase after glycol chitin SDS-PAGE electrophoresis of untrans-formed and transformed litchi plants cv Bedana. (Lanes- 1, 2, 3 and 4 is untrans-formed plants showing two chitinase isoforms of 21 and 30 kDa. Lanes- 4, 9, 15, and 18 are individual transformed lines showing three isoforms of chitinsae i e. 21 kDa and 30 kDa like normal untransformed plants and 35 kDa of unique size translated from bacterial chitinase (*ChiB*) gene. M-Rainbow marker was used as protein molecular weight standard.

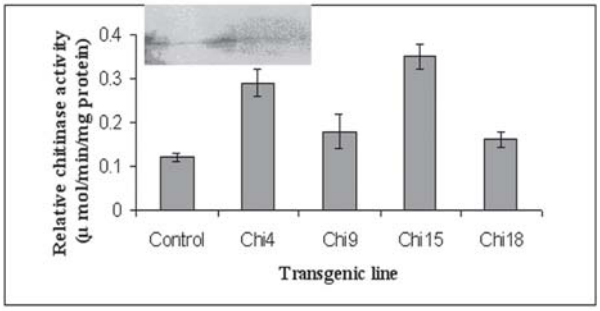

Fig. 5. Chitinase enzyme assay of different transgenic lines of litchi cv. Bedana. Average values of three different experiments each in duplicates were plotted in the graphs. Inset shows Western blots analysis showing 35 kDa bands.

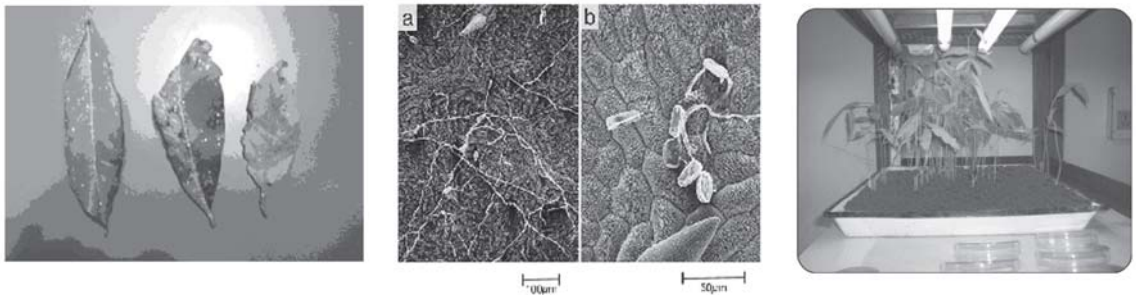

Fig. 6. Fewer symptoms of leaf die back disease in a transgenic plant 18 days after inoculation on detached leaves from a transgenic plant chi-15. **A** Transgenic litchi cv Bedana leaf showing spores of *Phomopsis sp*. couldn't grow and consequently no development of disease. **B**. Untransformed litchi plant's leaf showing growth of spores of pathogen *Phomopsis sp*. on leaf surface and development of die back, leaf spots and blight disease. **C**. Untransformed litchi plant' leaf turned death. **D.** Transformed litchi plant (matured) cv Bedana. **E**.-Electron micrographs of untransformed and transformed litchi leave showing the growth of mycelium of pathogen (Phomopsis sp.) a. Electron micrograph of untransformed litchi plant's leaf showing germination of fungal spores and growth of mycelium and entering inside leaf cells b. Electron micrograph of transformed litchi plant's leaf showing very small germination of fungal spores and no growth of mycelium **F.** Transformed litchi plants in field soil.

specific for the mRNA sequence of *ChiB*. A 290-bp amplified fragment of mRNA corresponding to the *ChiB* transcript confirmed the expression of the Bacterial chitinase gene. No amplification was observed in RNA samples isolated from un-transformed plant. RT-PCR results of all five independent transgenic lines (B-Chi 1, 4, 9, 15 and 18) are shown in Fig. 2C.

Western blot analysis of representative lines employing polyclonal antibodies raised against bacterial chitinase showed presence of a single prominent band corresponding to the size of 35 kDa i.e. the expected size, indicating that the transgene is being expressed constitutively at a higher rate (Fig. 5C). Higher chitinase activity was detected in all transgenic plants expressing the *ChiB* gene investigated (chi4, chi9, chi15 and chi18) than in the nontransgenic plant (Table 2). Lines 4 and 15 showed approximately two and three fold increase in the enzyme activity than nontransgenic plants, while lines 9 and 18 showed approximately one and half fold increase in the activity (Fig. 5).

Table 1. Kanamycin sensitivity of zygotic embryos of litchi

Conc. of Kanamycin (mgl^{-1})	% of survival calli[a]
0	81.67 ±2.33
10	52.66 ±2.72
20	28.63 ±2.90
50	25.34 ±1.20
100	0.00 ±0.00

[a]-Freshly cultured 3-4 mm calli (n = 10) were used in triplicate in each experiment.

Table 2. Assay of chitinase activity in crude extracts of litchi cv Bedana

Control	0.12	±0.01
Chi1	0.22	±0.019
Chi4	0.29	±0.031
Chi9	0.18	±0.04
Chi15	0.35	±0.03
Chi18	0.16	±0.017

Table 3. Functional assay of transgenic litchi to die back. leaf spots and blight

Line	Disease rating
B-Chi-1	4.6±0.52[b]
B -Chi-4	2.0±0.55
B-Chi-9	4.5±0.46
B -Chi-15	1.3±0.58
B-Chi-18	4.0±0.49
Non-transformed	4.7±0.51

a: 0, No symptoms; 5, very severe symptoms
b: mean SD (±) of disease rating scales

***Evaluation of die back, leaf spots and blight resistance in transgenic plants*:** Detached leaves of transgenic plants were tested for resistance to the phytopathogenic fungus, *Phomopsis sp.* Both Chi-4 and Chi-15 lines showed disease rating scores of 3.0 and 3.3 as an average score of three experiments, respectively, versus a score of 4.7 for the non-transformant (Table 3). These results indicated that the two transformants exhibited partial resistance to *Phomopsis sp.*, which was sufficient to delay the spread of lesion areas from the disease. The degree of disease symptoms correlated well with the level of chitinase enzyme accumulation. That is, the transgenic plants with a higher level of chitinase activity tended to have a smaller total lesion area.

We also recorded the number of days required for the onset of necrosis. Since the leaves used for the experiment were similar in leaf position and size, we also recorded the number of days required for the complete necrosis in each leaf. The results are shown in Table 4. Similar to the result of disease index, both Chi-4 and Chi-15 lines took longer period for the necrosis to develop and completely cover the whole leaf area. However, with longer durations, all leaves succumbed to the disease (Fig. 6).

Table 4. Disease development in complete leaf necrosis caused by die back, leaf spots and blight on detached leaves from *Bacterial chitinase* transgenic lines.

Lines	First symptom (days)	Fully covered
B -Chi-1	6	19
B -Chi-4	9	24
B -Chi-9	6	18
B -Chi-15	18	35
B-Chi-18	7	20
Non-transformed	3	14

Discussion

It has previously been reported that tobacco species expressing the *Bacterial chitinase* gene (*ChiB*) conferred an enhanced resistance to fungal disease (14) but no report on other plant species. It has been confirmed with rice chitinae to show resistance to fungal disease in many species i.e., resistance of rice against the *Sheath Blight* (15), transgenic indica rice variety Pusa Basmati1 exhibits enhanced resistance to *Rhizoctonia solani* (16) and genetically transformed Pigeon pea for enhanced resistance to *Fusarium spp*. causing the wilt disease (17). Here, we present another illustration of enhanced resistance to die back, leaf spots and blight in transgenic litchi. The present study shows that zygotic embryos derived embryogenic calli could be transformed by *Agrobacterium*-mediated transformation harboring the binary vectors pBI121-*ChiB* (Fig 1.1). These results demonstrated that zygotic embryos are well suited as target material for *Agrobacterium tumefaciens*-mediated transformation in litchi. The transformation process via *Agrobacterium* inoculation can cause necrosis of the tissue; however this could be reduced by special treatments as has been done in grape (18); (19). Stress response may involve release of polyphenol from the vacuole (20) as well as de novo phenol synthesis (21) causing browning. However, browning phenomenon is also commonly observed in non-transformed calli cultures and seems to be necessary for the induction of large amounts of somatic embryos (22). Using the procedure of (4), the overall transformants frequency were 20.9% (18 plantlets/86 embryos) of kanamycin resistant plantlets regenerated through the steps of embryo germination, shoot formation and rooting could be achieved. It is known that the response of explants to regeneration is affected by genotype and developmental stage as well as endogenous growth regulators.

Kanamycin was used seldom in selection (18). Generally, the faster the effect of the antibiotic on cell death the more effective is the selection at high concentration (23). These results showed that kanamycin was toxic to non-transformed calli as it was able to severely inhibit growth at 100 mgl^{-1} or higher concentrations. We found that LC_{50} of kanamycin was 50mgl^{-1}. In general, hygromycin B is more toxic than kanamycin at lower concentration (20 mgl^{-1}) but at higher concentration (50-100 mgl^{-1}) the later kills non-transformed cells more quickly. This sensitivity varies depending on the type of tissues exposed to the antibiotic. We have observed that more advanced developmental stages such as embryos or germinating embryos could be selected on higher concentrations of 50-100 mgl^{-1} kanamycins. It is apparently difficult to maintain a good balance between selection using a necessarily high concentration of kanamycin and the inhibitory effect on shoot and root induction.

Molecular analyses indicated successful integration of T-DNA into genomic DNA as confirmed by PCR and Southern blot analysis. A positive PCR signal depended upon complete insertion of the entire fragment into the genomic DNA. Any loss in nucleotides at either primer-

annealing site would prevent annealing and amplification would fail (24). This could explain that all *npt* positive transformants did not show the insertion of *ChiB* gene. Five transformants (Chi 1, 4, 9, 15 and 18) exhibited clear band when probed with the *ChiB* gene. When *Xba*I was used and probed with *npt* gene, the integration patterns were detected with single copy number. The difference detected using the two different restriction enzymes could be ascribed according to the digestion site, where *XbaI and BamHI* removes 1.56 kb gene cassette while *Sma*I would cut once in the T-DNA region. Band sizes differed among the transgenic lines, indicating independent transformation events. The presence of *ChiB* gene transcripts in transgenic plants was demonstrated by RT-PCR analysis. Densitometry assessment gave the integrated density value (IDV) of each band. This showed that line Chi15 has high IDV then the rest. The high expression of transgene translated into more chitinase.

Expression of the ChiB by Western blotting clearly detected a specific signal for the bacterial chitinase in the transgenic plants. Based on in-gel assay, we observed the presence of two chitinase isoforms in the leaves of untransformed *Litchi chinensis* cv. Bedana with an additional 35 kDa chitinase band, indicating that the foreign gene was translated into the protein of expected molecular weight. The estimated molecular weights of chitinase in this study ranged from 25 to 35 kDa as compared to litchi chitinase molecular weights of 31 to 34 kDa (25), (26). Earlier (27) found four isoforms of chitinase activity in soybean nodules. It is possible that different chitinase isoforms are effective against particular fungi species and ineffective against others (28) and many have different roles in the regulation of plant development.

Transgenic line Chi-15 showed approximately two fold increase in the enzyme activity while the transgenic lines Chi-1, 4, 9 and 18 showed approximately one and half fold increase in the enzyme activity and this could be correlated well with the degree of resistance to the pathogens. Previous reports have also shown that elevated chitins' activity in transgenic canola (29), strawberry (30), rice (31), tobacco (29) and cotton (32). Similar delays in the development of symptoms of fungal disease were also observed in these transgenic plants expressing an exogenous chitinase gene (33); (30); (9). (9) produced transgenic grape plants expressing the rice chitinase gene and indicated that longer inoculation with *Elisinoe ampelina* resulted in severe symptoms in both the transgenic and non-transgenic plants; nevertheless, after inoculation with *Uncinula necator*, such further development of the disease was not observed even after prolonged culturing. Similarly, very high inoculation densities of leaf rust spores led to the same severity of symptoms in transgenic wheat plants expressing an integrated chitinase gene as in control plants (34). We also observed, the symptoms of die back, leaf spots and blight gradually progressed on leaves from nontransgenic plants that exhibited very low chitinase activity, so that the transgenic and non transgenic plants were distinguishable when culture of the infected leaves was prolonged (>18 days). The present study indicates that high expression of the chitinase gene surely confers resistance to fungal disease on plants; the enhanced resistance is partial and quantitative. In the present study, we used the *in vitro* inoculation method with detached leaves to evaluate the increased resistance against die back, leaf spots and blight in transgenic plants. This method showed high accuracy for the detection of slight differences such as a delay in the development of disease symptoms, in fully controlled conditions, between the original nontransgenic plant and primary transformants

having the same genetic background.

In conclusion, we demonstrate the successful transformation of zygotic embryos derived somatic embryos of *Litchi chinensis* cv. Bedana and regenerated into rooted plants. Following the successful integration of the transferred bacterial chitinase gene into the litchi genome, the expression of transferred gene and their activity, showed an increased resistance to leaf die back by in vitro inoculation on detached leaves. Our next step will be focused on the evaluation and further improvement of the resistance against various fungal pathogens under greenhouse and field conditions. Together, these findings suggest that the Bacterial chitinase *ChiB* gene could be utilized as a genetic source of disease resistance for breeding and improving crop species. This is our first report of expression of bacterial chitinase in litchi cv Bedana for making fungal tolerant which is agronomically very important cash crop.

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Perspectives on Basidiomycete Genomes for Biotechnology and Pharmacy

Ursula Kües*
Division of Molecular Wood Biotechnology and Technical Mycology,
Büsgen-Institute, University of Göttingen, Göttingen, Germany
*For Correspondence - ukuees@gwdg.de

Dr. Igor Grigoriev from the American Department of Energy Joint Genome Institute (JGI) organised at March 22nd - 23rd 2010 in Walnut Creek (CA) a Jamboree on Basidiomycete genomes. The progress and current status on understanding genomes of distinct basidiomycetes were summarized by the principal investigators of genome consortia and future perspectives in basidiomycete genome research were discussed.

At this point of time, complete genomes from 16 species are publically available for basidiomycetes (http://genome.jgi-psf.org/pages/fungi/home.jsf), representing saprotrophs growing on organic waste materials in composts and soils (*Agaricus bisporus*, *Coprinopsis cinerea, Sporobolomyces roseus*), white-rotting species living preferentially on dead and decaying wood (*Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Schizophyllum commune*, *Tremella mesenterica*) and brown-rotting wood decay fungi (*Postia placenta*, *Serpula lacrymans*), plant symbionts (*Laccaria bicolor*), biotrophic (*Melampsora laricis-populina*, *Puccinia graminis*, *Ustilago maydis*) and necrotrophic plant pathogens (*Heterobasidion annosum*), as well as human pathogens infecting lungs (*Cryptococcus neoformans*) and skins (*Malassezia globosa*). Currently, summarising views on the completed genome sequences are published for six of these fungi (1-6) and reports for others are in the pipeline. Following first computational genome analysis, gene predictions and annotations, consortia of scientists are actively continuing annotating genes in the genomes and sorting them into new large groups and families. Most of the sequenced species have around 10,000 to 12,000 predicted genes, however with *S. roseus* (http://genome.jgi-psf.org/Sporo1/Sporo1.home.html) and *U. maydis* (2) with around 5,500 and 6,900 genes, respectively, being at a lower scale and *L. bicolor* with about 20,000 genes (5) at an upper scale of gene content.

Why is it so interesting to focus on these specific fungal genomes? Pathogenic species attacking our crops or valuable tree species destroy valuable food and renewable energy resources which results in huge economical losses with also apparent socio-political and environmental consequences. For example, alone in Europe wood losses by the conifer pathogen *H. annosum* are estimated 800 million Euro annually (7). The maize pathogen *U. maydis* is of less economic importance but a range of close relatives of this experimentally easy-to-access smut model-organism causes significant losses to crops worldwide, including to wheat, barley and sugarcane (8). Wheat stem rust caused by *P. graminis* has been in control for 50 years by using resistant wheat cultivars but, in fact, new highly virulent races appeared recently in Africa that rapidly spread into other continents as existent

thread of food security (9,10). Studying the genomes of plant pathogenic fungi and encoded protein functions is done in order to find new efficient ways in pathogen management and in the constant fight against dreadful plant diseases.

Wanting to understand the mode of infection of the human opportunistic pathogen *C. neoformans* in both immuno-compromised and immuno-competent individuals for human health protection is evident (11) – genome studies can help to identify efficient targets for drugs and methods of defeat. The biochemical pathway of melanization for example has been recognized has a concept to target disease (12). The US detergent and cosmetics company Proctor & Gamble financed the sequencing of the dandruff fungus *M. globosa* in order to improve their shampoos for better dandruff beat and prevention (4). Whole genome analysis aims at defining new treatments by changing the level or activities of *Malassezia* genes that act for example in skin colonization (13).

The mission of JGI relates to clean energy generation and environmental characterization and cleanup in systems-based scientific approaches, addressed by integrated high-throughput sequencing and computational analysis (http://www.jgi.doe.gov/whoweare/index.html). Particularly the basidiomycetes are in the focus of the JGI bioenergy program along the line biomass production – protection – conversion.

Symbionts such as *L. bicolor* stand at the beginning of a production chain of biomass as renewable energy. The symbiotic species helps in tree growth and thus ultimately in the fixation of CO_2 in the form of wood (5) - a reason for JGI to initiate studies on the genomes of such organisms. In combination with the genomes of their tree hosts and studies on genome-wide resistance and defence responses host genes, (14,15), a logic choice in the progression of wood production and protection is to establish genomes of pathogenic fungi like *M. laricis-populina* and *H. annosum* harassing the health and biomass output of trees by infecting either their leaves or their roots and stems.

Once harvested, wood made of energy-rich lignocellulose might be transferred by into sugars through the action of wood-decay fungi or, at least, through fungal lignin degradation the celluloses and hemicelluloses in the wood might become more accessible to microbial hydrolases. Freed cellulose and hemicellulose and released sugars might serve as substrates for environmentally friendly bioethanol production, for example with the help of natural or engineered yeasts (16). Lignocellulose is the most abundant carbohydrate source in nature and represents an ideal renewable energy source to which we will have to turn for replacing the expiring fossil energy by bioethanol and biofuels that are produced without stressing any foodstuff. However, due to lignin incorporation lignocellulose is extremely recalcitrant to depolymerisation into its simple chemical units. Plant waste degrading saprotrophic and particularly wood-decay basidiomycetes are especially rich in genes for enzymes expected to act at degrading plant cell wall components including the difficult lignin. Quite often, large families of genes for such enzymes have been generated in the individual species by repeated gene duplications (1,6,17).

Surprisingly, the cannon of genes potentially functioning in degradation of lignocellulose very much differs from basidiomycete to basidiomycete, not only between species of different life style but also between species of seemingly a same life style (1,5,6,18 and reports on basidiomycete genomes expected to come up in the nearer future). Whilst in one fungus a gene family can be fully missing, in other species genes of the same family are multiple duplicated. For

example, *P. chrysosporium* is lacking any laccase genes (1), whereas the brown-rot *P. placenta* surprisingly has two (6), the saprotrophic *C. cinerea* in total seventeen (17) and the symbiont *L. bicolor* 9 different laccase genes (18). On the contrary, *C. cinerea* has only one gene for a basal lineage class II fungal peroxidase (i.e. the well known Cip; 19), similarly as *P. placenta* and *L. bicolor* (5,6). In contrast, *P. chrysosporium* has 10 gene copies for lignin peroxidases (LiPs), five for manganese-dependent peroxidases (MnP) as well as a gene for a basal peroxidase (Nop) (20,21). Only a minority of the encoded redox-enzymes have so far been biochemically characterized (22-27) as a step forward for their usage in biotechnological applications. Recombinant expression and production of the enzymes can be a requisite to both, biochemical enzyme characterization and biotechnological application (23-25,28,29).

The wealth of the multiple gene families in the genomes of basidiomycetes offers plentiful new opportunities in biotechnology. Only a few other very interesting families with enlarged numbers of genes were so far analyzed with genes for enzymes such as for P450 oxygenases in *P. chrysosporium* (30) and genes for metallopeptidases of the fungalysin family (31), for glycoside hydrolases (32-34), and for P450 oxygenases and sesquiterpene synthases in *C. cinerea* (35). Particularly the currently known largest fungal P450ome found in *P. chrysosporium* accelerated numerous innovative studies on biochemical conversion of a diversity of organic compounds with often a harmful character for the environment (36-39). Many of the P450 oxygenases have thus potential in detoxification of hazardous and toxic organic substances and may be applied in remediation or bioremediation of polluted water and soils and other substrates but there might be also enzymes useful in biotechnological production of desperately wanted chemicals such as drugs for medical purposes and other fine chemicals (40).

For all of the above mentioned species being now fully in the post-genomics-era – at this point applying on them the versatile fast developing and newly emerging methods of transcriptomics, proteomics, metabolomics and other omics and the computational options of networking of data obtained from an omics study and between omics studies (41-43) – we likely will be provided with many more yet unseen discoveries with high value in practical applications. As clearly seen from the so far available genomes, various different ways in degradation of lignocellulose in general and in attack of lignin by different sets enzymes in special have been verified by the basidiomycetes. The JGI launched new calls in their bioenergy program for sequencing genomes of further fungi, with good potential in biomass production and/or degradation, global carbon cycling and biogeochemistry. In addition, there are ongoing projects and calls to sequence metagenomes of microbial communities e.g. in soils (http://www.jgi.doe.gov/CSP/user_guide/index.html). Of these, we can also expect to obtain more exciting data on basidiomycetes. Since amongst the so far sequenced basidiomycetes no enzyme fitting for decay of plant cell wall material has been found twice, we anticipate to discover new cocktails of interesting enzymes from sequencing more species. The rapid advances in basidiomycete genomics promise that more impact on biotechnology and pharmacy has to be expected by this work in the near future.

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In Vitro Organogenesis and Plant Regeneration in Eggplants (*Solanum melongena* L.)

Shivaraj G. and Srinath Rao*
Department of Botany, Gulbarga University,
Gulbarga-585 106, Karnataka India.
*For Correspondence - srinathraom@rediffmail.com

Abstract

In vitro organogenesis was achieved from cotyledonary leaf derived callus in eggplant (*Solanum melongena* L.) cultivars, Pusa Purple Long (PPL) and Black Beauty (BB) on MS medium. Organogenic callus was induced from cotyledonary leaf excised from 20 day old seedlings on MS medium containing NAA, 2, 4-D and PAA either alone or in combination with KN. Maximum amount of callus was induced on MS medium supplemented with 3 mg/l NAA + 0.5mg/l Kn. Induction of multiple shoots from cotyledonary leaf derived callus was achieved when pieces of callus were cultured on MS medium supplemented with either 6-benzyl amino purine (BAP) or thidiazuron (TDZ) alone or in combination with Kn. High frequency (100%) and maximum number of shoots (19.6 ± 0.71) were obtained on MS medium containing 2mg/ BAP +1mg/l Kn. The *in vitro* regenerated dwarf shoots were further elongated on MS medium supplemented with 1.5mg/l gibberellic acid (GA_3). Elongated shoots were then excised from shoot clumps and transferred to rooting medium containing indole butyric acid (IBA) at 3mg/l. The rooted plantlets were hardened on MS basal liquid medium and subsequently transferred to polycups containing vermiculate: soil: sand in a ratio of 1:2:2. Plantlets, thus developed were successfully established and finally transferred to greenhouse. The plantlets showed high survival rate (80%) in the soil.

Key words: BAP, Callus, Cotyledonary leaf, Eggplant, Regeneration

Introduction

Eggplant (*Solanum melongena* L.) is a common vegetable crop of India. It has high nutritive value and can well be compared with tomato (1). Eggplant is a vegetable crop of the family Solanaceae grown in the sub-tropics and tropics. The crop is cultivated on small family farms and considered to be an important source of nutrition and cash income for many resource poor farmers. A number of cultivars are grown through out the country depending upon the yield, consumer's preference about colour, size and shape of various cultivars (2). Eggplants can be cultivated and grown round the year but the productivity and quality of this crop suffer due to its susceptibility to a number of diseases and insect pests (3). In India, it is also used for the treatment of diabetes, bronchitis, asthma, dysuria, dysentery etc (4). The progress towards the improvement of this crop for insect pest and disease resistance and introduction of new varieties is hampered mainly due to the wide prevalence of sterility in the progeny and occurrence of genetic incompatibility following intergeneric and

interspecific crosses, respectively (5) and (6). Tissue culture techniques are widely used for the improvements of various crops. *In vitro* shoot induction from callus culture can induce genetic and epigenetic changes in the regenerated plants. Plant regeneration through callus culture is one of the most important steps for any kind of genetic transformation studies (7). An efficient and reproducible *in vitro* regeneration system through callus is an important pre-requisite for selection of stress tolerant cell lines and genetic transformation studies.

In this investigation, we report reproducible protocol for efficient and high frequency plant regeneration from callus of eggplant of two Indian popular varieties Pusa Purple Long and Black Beauty.

Materials and Methods

Plant Materials : Seeds of egg plant (*Solanum melongena* L.) cultivar Pusa Purple Long (PPL) and Black Beauty (BB) were obtained from the Pocha Agro Science Pvt. Ltd., Delhi. Seeds were germinated in the pots containing sterilized soil. Cotyledonary leaf (20) hypocotyl (12), and matured leaf of 30 days old seedlings were used as explants for callus induction. These explants were washed with 1% bavistin for 3-4 min, followed by successive four washing with distilled water to make explants free from bavistin. The explants were then surface sterilized with 0.1% (w/v) mercuric chloride for 1-2 min. and were washed with sterile distilled water 4-5 times to remove traces of mercuric chloride. All the explants were inoculated onto MS medium (8) supplemented with different concentrations of 2,4-D, NAA and PAA alone or in combination with Kn for induction of callus and regeneration of shoots through callus., BAP and TDZ alone or in combination with KN were also used. The medium contained 3% sucrose (w/v) and pH of the medium was adjusted to 5.8 and solidified with 0.8% agar before autoclaving at a pressure of 1.06kg cm^2 for 20 min. The cultures were maintained at a temperature of 25 ± 2^0C under 16/8 h (light/ dark) under 3000 lux intensity provided by cool white fluorescent lamps.

The *in vitro* initiated individual shoots were separated and transferred to MS basal medium containing different concentrations of GA_3 for elongation of shoots. These elongated shoots bearing at least 4-5 internodes were excised from the mass of proliferated shoots and were transferred to rooting media supplemented with different concentrations of NAA and IBA. The rooted plants were taken out from the culture tubes, washed to remove adhered agar with distilled water and transferred to MS liquid medium for two weeks for hardening and subsequently transferred to plastic pots with sterile vermiculate, sand and soil 1: 2: 2. The plantlets were kept in a greenhouse at 80% relative humidity, 32 ± 2^0C under a 16h photoperiod for acclimatization. The plants were given fertilizer with $1/8^{th}$ MS macronutrients biweekly. Established plants were transplanted to earthen pots under natural conditions and the survival rate was recorded.

Statistical analysis: The experimental design was random and factorial with auxins and cytokinins as independent variables. The data pertaining to amount of callus induction, number of shoots, shoot length and roots were subjected to analysis of variance (ANOVA) and mean were determined by Duncan's New Multiple Range Test (DNMRT). Twenty five cultures were raised for each treatment and all the experiments were repeated thrice.

Results and Discussion

Callus was initiated by using different explants such as cotyledonary leaf, hypocotyl, and matured leaf. Callus initiated within 6-11 days of

culture and mass of callus formation was observed within four weeks of culture. Among these explants cotyledonary leaf explants showed early initiation of callus and the highest frequency of callus induction when compared to other explants (Table-1). Different sources of explants were used for the induction of callus in eggplants including cotyledon and midrib (9) leaf (10) root, Franklin et al. (11) and anther, (12). Three different auxins (2, 4-D, NAA and PAA) were used singly or in combination with Kn for callus induction. Different concentrations and combinations of growth regulators had marked influence on callus induction and the results are presented in Table-2. Among the three auxins, 2,4-D, NAA and PAA, NAA was found to be the most suitable for the induction of callus in this species. (13) also reported that NAA gave better response for callus induction than 2,4-D. Further, it was noticed that callus derived from 2,4-D supplemented medium did not show organogenesis. High frequency of callus induction and maximum growth of callus were observed on medium supplemented with an auxin and a cytokinin when compared to medium supplemented with an auxin alone. The growth of the callus was maximum (3.54 g) and with a frequency of 100% on MS medium supplemented with 3mg/l NAA + 0.5mg/l Kn followed by 3mg/l NAA + 1mg/l Kn (Table-2; Fig-a). (9) reported that the combination of NAA and BAP supported best growth of callus.

Regeneration through callus : Shoot induction was not noticed on MS basal medium devoid of growth regulators after four weeks of callus culture. Callus when transferred to regeneration medium supplemented with different concentrations of BAP or TDZ alone or in combination with KN, induction of multiple shoots was observed. There was a gradual increase in the number of shoots with an increases in the concentration of BAP from 1-2 mg/l .However BAP at 3mg/l proved inhibitory for induction of multiple shoots. BAP induced multiple shoot formation was reported earlier by Prakasha et al. (14) in this species.

Cytokinins, especially BAP were reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation (15). Maximum number of shoots were noticed on MS medium fortified with 2mg/l BAP (16.3 ± 0.34) followed by 1mg/l BAP (12.2 ± 0.50) in the cultivar PPL. At a given concentration and combination of medium containg BAP, PPL veritiy showed more callus and multiple shoot induction than the cv. BB. Varietal differences

Table-1 Number of days taken for initiation of callus and frequency of callus induction from different explants of eggplant (*Solanum melongena* L.*)* on MS medium

Explants	Initiation of callus (Days)		Frequency of callus induction (%)	
	PPL	BB	PPL	BB
Cotyledonary leaf	6-7[a]	7-8[a]	100[a]	100[a]
Hypocotyl	9-10[c]	11-12[c]	80[b]	80[b]
Matured Leaf	8-9[b]	8-9[b]	80[b]	80[b]

Data represents average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error. Mean followed by the same superscript in a columns is not significantly different at P = 0.05 levels.

Table-2. Effect of different auxins or in combination with Kn on callus induction derived from cotyledonary leaf explants of eggplant (*Solanum melongena* L.)

Concentrations of growth regulators (mg/l)	PPL			BB		
	Frequency (%)	Fresh weight of callus (g)	Nature of callus	Frequency (%)	Fresh weight of callus (g)	Nature of callus
2,4-D						
1.0	100	0.95 ± 0.34^{g}	SW	100	0.83 ± 0.78^{g}	SW
2.0	100	1.60 ± 0.85^{e}	SW	100	1.50 ± 0.93^{e}	SW
3.0	100	0.70 ± 0.23^{g}	SW	100	0.65 ± 0.46^{g}	SW
4.0	100	0.62 ± 0.54^{h}	SW	100	0.55 ± 0.38^{h}	SW
NAA						
1.0	100	1.24 ± 0.75^{f}	SW	100	1.23 ± 0.34^{f}	SW
2.0	100	1.80 ± 0.98^{e}	SW	100	1.54 ± 0.72^{e}	SW
3.0	100	2.60 ± 0.73^{c}	SW	100	2.06 ± 0.49^{e}	SW
4.0	100	2.14 ± 0.24^{d}	SW	100	1.84 ± 0.25^{d}	SW
PAA						
1.0	100	1.14 ± 0.45^{f}	HW	100	0.75 ± 0.64^{g}	HW
2.0	100	1.41 ± 0.35^{f}	HW	100	1.23 ± 0.42^{f}	HW
3.0	100	0.50 ± 0.56^{h}	HW	100	0.67 ± 0.76^{g}	HW
4.0	100	0.43 ± 0.66^{h}	HW	100	0.32 ± 0.52^{h}	HW
2,4-D + Kn						
2.0 + 0.5	100	1.78 ± 0.68^{e}	SW	100	1.43 ± 0.34^{e}	SW
2.0 + 1.0	100	2.75 ± 0.78^{b}	SW	100	2.28 ± 0.76^{b}	SW
2.0 + 1.5	100	1.64 ± 0.45^{e}	SW	100	1.52 ± 0.33^{e}	SW
2.0 + 2.0	100	1.74 ± 0.19^{e}	SW	100	1.15 ± 0.65^{f}	SW
NAA + Kn						
3.0 + 0.5	100	3.54 ± 0.26^{a}	SG	100	3.16 ± 0.64^{a}	SG
3.0 + 1.0	100	3.45 ± 0.33^{a}	SG	100	3.24 ± 0.66^{a}	SG
3.0 + 1.5	100	2.87 ± 0.78^{b}	SG	100	2.37 ± 0.99^{b}	SG
3.0 + 2.0	100	2.56 ± 0.19^{b}	SG	100	2.15 ± 0.20^{b}	SG

SW: Spongy white; SG: Spongy green; HW: Hard white; Data represents average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error. Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

Table-3 Frequency, number of shoot and shoot length obtained from cotyledonary leaf derived callus of eggplant (Solanum melongena L.) supplemented with different concentrations of cytokinins on MS medium

Concentrations of growth regulators (mg/l)	PPL			BB		
	Frequency (%)	No. Shoots/ Culture	Shoot length/ culture (cm)	Frequency (%)	No. Shoots/ culture	Shoot length/ culture (cm)
BAP						
1.0	100	12.2 ± 0.50^{b}	4.6 ± 0.27^{b}	90	6.8 ± 0.34^{b}	3.5 ± 0.39^{b}
2.0	100	16.3 ± 0.34^{a}	4.6 ± 0.33^{b}	90	7.9 ± 0.45^{ab}	3.9 ± 0.34^{a}
3.0	70	09.8 ± 0.86^{c}	2.1 ± 0.23^{d}	60	5.0 ± 0.25^{c}	3.1 ± 0.77^{b}
TDZ						
0.25	100	10.5 ± 0.12^{c}	3.3 ± 0.21^{c}	80	7.2 ± 0.33^{b}	3.1 ± 0.50^{b}
0.50	80	11.6 ± 0.95^{b}	4.5 ± 0.98^{b}	70	8.8 ± 0.66^{a}	4.3 ± 0.56^{a}
0.75	60	8.8 ± 0.34^{d}	4.9 ± 0.45^{b}	60	5.6 ± 0.89^{c}	3.1 ± 0.23^{b}
1.0	60	6.7 ± 0.45^{e}	5.3 ± 0.87^{a}	60	3.4 ± 0.56^{d}	2.4 ± 0.22^{c}

Data represents average of three replicates; each replicate consists of 25 cultures. Mean ± Standard error. Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

with respect to callus induction and regeneration potential were earlier reported by (16, 14) in this species. The lower concentration of TDZ (0.25 and 0.5mg/l) induced maximum shoots/explants when compared to higher concentrations. (17, 11) reported induction of shoots lower concentration of TDZ and their inhibition at higher concentrations. BAP proved to be better cytokinin than TDZ in terms of induction of multiple shoots (Table-3).

Interaction of kinetin on multiple shoot induction : It is well known that a proper concentration and combination of different cytokinins is necessary for morphogenesis, leading to the formation of complete plantlets (18). In the present investigations it was noticed that supplementing Kn along with BAP or TDZ further enhanced, the frequency of shoot induction and number of shoots formed per explants (Table-4). Maximum number of multiple shoots (19.6 ±0.71) were noticed when cotyledonary leaf derived callus was cultured on MS medium supplemented with 2mg/l BAP + 1mg/l KN (Fig-b, c). (16) also reported that BAP along with KN proved superior for the induction of multiple shoots in this species. The shoots were healthy thick and dark green in colour when compared to shoots obtained on MS medium containing combination of TDZ and Kn. When left on shoot induction medium, no further growth of the shoots was noticed; hence they were transferred to the shoot elongation medium.

Separated individual shoots from shoot clumps were transferred onto MS medium supplemented with different concentration of GA_3 (0.5-1.5mg/l) for elongation of shoots.

Table-4 Frequency, number of shoot and shoot length obtained from cotyledonary leaf derived callus of eggplant (Solanum melongena L.) supplemented with different concentrations of cytokinins along with KN on MS medium

Concentrations of growth regulators (mg/l)	PPL			BB		
	Frequency (%)	No. Shoots/ Culture	Shoot length/ culture (cm)	Frequency (%)	No. Shoots/ culture	Shoot length/ culture (cm)
BAP + Kn						
2.0+0.5	100	18.8 ± 0.92^{a}	8.0 ± 0.99^{b}	100	12.0 ± 0.26^{a}	4.3 ± 0.25^{d}
2.0+1.0	100	19.6 ± 0.71^{a}	6.3 ± 0.93^{d}	100	10.8 ± 0.50^{b}	6.9 ± 0.90^{a}
2.0+1.5	100	15.6 ± 0.23^{b}	9.4 ± 0.36^{a}	100	7.9 ± 0.85^{d}	5.7 ± 0.75^{b}
TDZ + Kn						
0.5+0.5	100	12.4 ± 0.66^{c}	6.3 ± 0.23^{d}	100	9.5 ± 0.29^{b}	4.7 ± 0.25^{c}
0.5+1.0	100	10.5 ± 0.48^{a}	7.4 ± 0.94^{c}	100	8.6 ± 0.35^{c}	4.6 ± 0.16^{c}
0.5+1.5	100	8.6 ± 0.46^{e}	7.2 ± 0.57^{c}	100	7.7 ± 0.56^{d}	6.7 ± 0.47^{a}

Data represents average of three replicates; each replicate consists of 25 cultures.Mean ± Standard error. Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

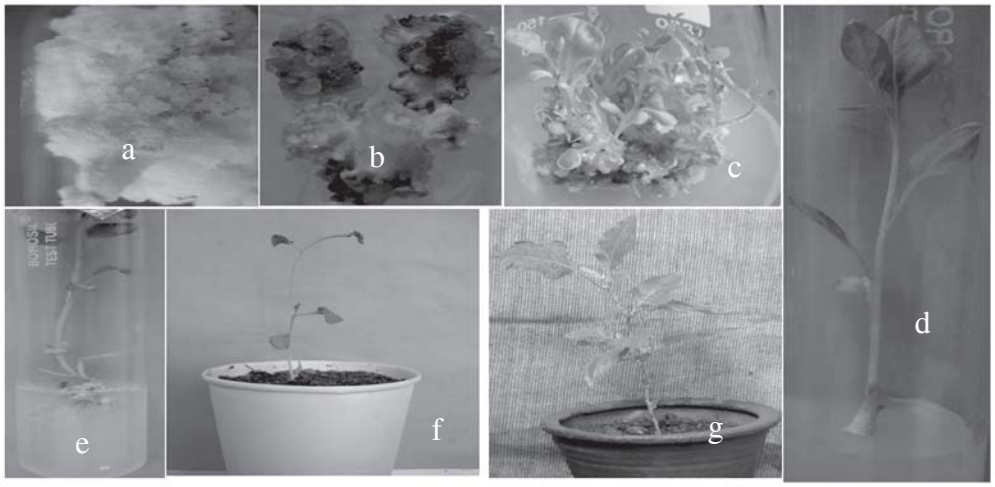

Fig. 1. *In vitro* organogenesis of eggplant

a) Cotyledonary leaf derived callus on MS medium supplemented with 3mg/l NAA + 0.5mg/l Kn after 30 days of culture. b) Initiation of multiple shoots from cotyledonary leaf derived callus on MS medium containing 2mg/l BAP + 1mg/l Kn after 12 days of culture. c) Proliferation of shoots on from cotyledonary leaf derived callus on MS medium containing 2mg/l BAP + 1mg/l Kn after one month of culture. d) Elongation of *in-vitro* shoots on MS medium supplemented with 1.5mg/l GA_3. e) Direct rooting from regenerated shoots on MS medium containing 3mg/l IBA after 30 days of culture. f) Hardened plants in polycups containing vermiculate, sand and soil (1:2:2). *g) In vitro* raised plants in green house.

Maximum shoot length (13.3 ± 0.50) was recorded on medium containing 1.5mg/l GA_3 (Fig-d) followed by 1mg/l GA_3. Our results are in agreement with the results of (9) and (13) who reported shoot bud elongation using GA_3. However, (11) reported elongation of shoots on hormone free medium in this species.

Rhizogenesis

Roots were not induced during shoot multiplication in the presence of cytokinins. Individual shoots when implanted on half or full strength MS medium free from growth regulators resulted in few weak roots with very low frequency. Supplementing the medium with auxins like NAA or IBA enhanced the frequency of root induction as well as number of roots formed per plantlet. Among the two auxins tested IBA at 3mg/l induced 89.3 ± 0.75 roots per shoot (Fig-e) as compared to other concentrations of IBA or NAA. Similar results have also been reported by Rahman *et al*. (9) Hossain et al. (13) and Chakravarthi Dhavala *et al*. (19) in this species. In contrast Hossain et al. (13) reported that, there was no root formation on MS medium supplemented with IBA. The roots formed on IBA supplemented medium were thick, long and dark coloured as compared to roots induced on NAA. After four to five weeks of culture in the rooting medium the rooted shoots were transferred to MS basal liquid medium for two weeks for hardening and then transferred to pots. About 60% of the transplanted plantlets survived. It was noticed that if the plants in the culture tubes were kept at normal room temperature for seven to eight days prior to transplantation in pots the survival rate of plantlets in the pots increased to 80%. The plants were grown under semi-controlled temperature (32 ± 2^0C) and light (2000lux) in green house. During this period of acclimatized, shoots elongated, leaves expanded and turned deep green and looked healthier (Fig-f).

After three weeks, plants were transferred to an open field and gradually acclimatized to outdoor condition, where 80% of plants survived and produced normal flowers and fruits. The technique described here appears to be readily adaptable for large scale clonal propagation and the genetic improvement of egg plant.

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NEWS ITEM

India needs more quality research in science: Dr.Manmohan Singh, Prime Minister of India

Prime Minister Manmohan Singh in his recent address at Kanpur has emphasized the country's urgent need to increase quality research in science and technology to meet newer challenges like climate change. He also said that the government has set in a motion an ambitious programme to completely restructure the legal and regulatory environment of higher education. Observing that science and technology played a dominant role in determining the power and progress of a nation, Dr.Singh said, "This role has become even more critical in the wake of newer challenges like climate change." He also mentioned that the country needed more innovation in areas like sustainable agriculture, affordable health care and energy security. Dr Singh also emphasized for greater collaboration between institutions like IITs and the corporate sector, he said, "This would be of mutual benefit to both- to the corporate sector it would mean cost effective solutions and newer technology while for the IITs it would bring much needed funds and enhance their research capabilities."

Economic, Diplomatic, Educational, Scientific and Cultural ties between India and Canada

The Prime Minister of India, Dr. Manmohan Singh, visited Canada on June 26 at the invitation of the Prime Minister of Canada, Mr. Stephen Harper. During his visit, Prime Minister Singh participated in the G-20 Toronto Summit and held bilateral discussions with Prime Minister Harper. The two Prime Ministers, in reviewing the state of bilateral relations, welcomed the enhanced interaction in a broad range of areas, visits and other exchanges between the two countries. They expressed their desire to broaden and deepen economic, diplomatic, educational, scientific and cultural ties between India and Canada. To further enhance the considerable momentum achieved in exchanges and collaboration amongst a broad range of higher education institutions in both countries, the Prime Ministers welcomed the signing of a Memorandum of Understanding on Higher Education Cooperation. This framework will facilitate academic exchanges, partnerships and mobility between higher educational institutions in the two countries. The Prime Ministers also noted initiatives to link Canadian and Indian universities and colleges, including in curriculum development and the creation of Chairs and Centres for Indian studies at a number of Canadian universities, such as, for example, those in the process of being established at Carleton University and McGill University.

Indian prime minister backs IPCC boss Rajendra Pachauri

The Inter-governmental Panel on Climate Change (IPCC) and its Indian chairman, Rajendra Pachauri, have come under intense criticism for a false claim in its 2007 report that Himalayan glaciers would disappear as early as 2035. Pachauri has refused to apologize for the claim, though the IPCC has issued a statement expressing regret for the mistake. India's Prime Minister defended a beleaguered UN climate change body today, saying India has full confidence in the panel, despite an error in its report on global warming. The Prime Minister, Manmohan Singh, said the error did not change the facts regarding the harmful impact of greenhouse gases on the planet. "This debate does not challenge the core projections of the IPCC about the impact of greenhouse gas accumulations on temperature, rainfall and sea-level rise. India has full confidence in the IPCC process and its leadership and will support it in every way that we can," Singh said. He asserted that India backed both the panel and its chairman. He urged developed countries to extend greater financial and technical assistance to poor countries to help them cope with the consequences of rising carbon dioxide emissions. Singh also said that Industrialised countries should recognise their role in the accumulation of greenhouse gases in the atmosphere and respond with bolder initiatives to cut back on future emissions

Statement by the Prime Minister of India Dr. Manmohan Singh at the Nuclear Security Summit

Prime Minister Dr. Manmohan Singh visited Washington, DC for Nuclear Security Summit on April 10-14, 2010. A summit-eve statement prepared for Prime Minister Manmohan Singh, initially said the Nuclear Security Summit (NSS) would focus on nuclear terrorism and the "proliferation of sensitive nuclear materials and technologies". Nuclear security is one of the foremost challenges faced today. He wished to commend President Barack Obama for his initiative in convening this Summit on Nuclear Security. Singh said "We would like the Summit to lead to concrete outcomes which help make our world a safer place. The developmental applications of nuclear science in areas such as medicine, agriculture, food preservation and availability of fresh water are by now well established. Today, nuclear energy has emerged as a viable source of energy to meet the growing needs of the world in a manner that is environmentally sustainable. India is deeply concerned about the danger it faces, as do other States, from this threat.

He also told that since 2002, we have piloted a resolution at the United Nations General Assembly on measures to deny terrorist's access to Weapons of Mass Destruction. We fully support the implementation of United Nations Security Council Resolution 1540 and the United Nations Global Counter Terrorism Strategy." Finally he announced that they have decided to set up a "Global Centre for Nuclear Energy Partnership" in India.

SCIENTIFIC NEWS

Animals That Live Without Oxygen - True!

Multi-cellular animals live entirely without oxygen has been discovered recently. The creatures that reside deep in one of the harshest environments on earth: the Mediterranean Ocean's L'Atalante basin, which contains salt brine so dense that it doesn't mix with the oxygen-containing waters above. The previous reports showed that single-celled life was present, but the new study published recently in *BMC Biology* has identified multi-cellular animals that apparently live and reproduce in the sediments under the salt brine. Italian and Danish researchers describe three new species of tiny animals called Loricifera. The animals took up radioactively tagged leucine (an amino acid), and a fluorescent probe that labels living cells, evidence that they were alive when they were collected. Their cells apparently lack mitochondria, the organelles that use oxygen to power a cell. Instead they are rich in what seem to be hydrogenosomes, organelles that can do a similar job in anaerobic environments. The findings could help scientists to understand what life might have looked like in the earth's early oceans, which also had very little oxygen.

Dr.T.Leelanath

Vitamin B3 as a Novel Approach to Treat Fungal Infections

Vitamin B3 was identified as potential and novel antifungal drug. Infections by the yeast *Candida albicans* represent a significant public health problem and a common complication in immunodeficient individuals such as AIDS patients, cancer patients undergoing chemotherapy and recipients of organ transplants. While some treatments are available, their efficacy can be compromised by the emergence of drug-resistant strains. The recent study published in Nature Medicine by a team of scientists from the Institute for Research in Immunology and Cancer (IRIC) of the University of Montreal have identified that a *C. albicans* enzyme, known as Hst3, is essential to the growth and survival of the yeast. Researchers found that genetic or pharmacological inhibition of Hst3 with nicotinamide, a form of vitamin B3, strongly reduced *C. albicans* virulence in a mouse model. Both normal and drug-resistant strains of *C. albicans* were susceptible to nicotinamide. In addition, nicotinamide prevented the growth of other pathogenic *Candida* species and *Aspergillus fumigatus* (another human pathogen), thus demonstrating the broad antifungal properties of nicotinamide. The results of the study are very exciting and they constitute an important first step in the development of new therapeutic agents to treat fungal infections without major side effects for patients.

N.Vijaya Sree

Way to Lower Tumor Risk in Stem Cell Therapies

One of the characteristics of embryonic stem cells is their ability to form unusual tumors called teratomas. These tumors, which contain a mixture of cells from a variety of tissues and organs of the body, are typically benign. But they present a major obstacle to the development of human embryonic stem cell therapies that seek to treat a variety of human ailments such as Parkinson's, diabetes, genetic blood disorders and spinal cord injuries. Now a team of biologists at UC San Diego funded by a grant from the California Institute for Regenerative Medicine, the state's stem-cell funding agency, has discovered a way to limit the formation of teratomas. In the Proceedings of the National Academy of Sciences, the researchers report that they have identified a new signaling pathway critical for unlimited self propagation of embryonic stem cells. Using small molecule compounds that inhibit this pathway, the scientists were able to dramatically reduce the potential of embryonic stem cells to form teratomas. If researchers could halt the propagation of human embryonic stem cells during lineage-specific differentiation before they are transplanted, they could avoid the risk of producing teratomas. Once we identify more pathways required for teratoma formation by embryonic stem cells, we might be able to completely suppress the formation of teratomas by targeting multiple pathways simultaneously.

P.Udaya Sri

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KNOWLEDGE IS POWER

National Conference

&

Fourth Annual Convention of Association of Biotechnology and Pharmacy

Fourth Annual Convention of Association of Biotechnology and Pharmacy and the National Conference on "Emerging Trends in Biopharmaceuticals – Relevance to Environment and Health" is being organized jointly by Department of Biotechnology and Association of Biotechnology and Pharmacy at Thapar University, Patiala during 11-13 November, 2010.

For further details contact

Prof. M. Sudhakara Reddy

Director of the Symposium &Professor, Department of Biotechnology

Thapar University, Patiala – 147 004, Punjab, India

Office : 0175- 2393743; Fax: 0175-2393738, Email: pharmacy@thapar.edu

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