ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

Volume 6 Issue 1 January 2012

www.abap.co.in

Current Trends in Biotechnology and Pharmacy
ISSN 0973-8916 (Print), 2230-7303 (Online)

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ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

(An International Scientific Journal)

Volume 6 Issue 1 January 2012

www.abap.co.in

Indexed in Chemical Abstracts, EMBASE, ProQuest, Academic SearchTM, DOAJ, CAB Abstracts, Index Copernicus, Ulrich's Periodicals Directory, Open J-Gate Pharmoinfonet.in Indianjournals.com and Indian Science Abstracts.

Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

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Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Current Trends in Biotechnology and Pharmacy

ISSN 0973-8916

Volume 6 (1) **January- 2012**

CONTENTS

Review papers

Probiotics: Recent Understandings and Biomedical Applications 1-14
Jamila K. Adam, Bharti Odhav and K. Suresh Babu Naidu

Food, Health and Agricultural Importance of Truffles: A Review of Current Scientific Literature 15-27
Seema Patel

Plagiarism in Scientific Research: Needs Lock-up to Unlock the Ethical Publications 28-34
Chandrakantsing V. Pardeshi, Pravin V. Rajput, Kapil S. Chaudhary and Ganesh B. Patil

Research Papers

Development and Validation of Reversed Phase HPLC Method for Analysis of Immunotherapeutic Peptide PADRE and its Applications 35-43
Srinivas Poondru, Venugopal Marasanapalle, Poonam Saraf and Bhaskara Jasti

Successful Transportation and *in vitro* Expansion of Human Retinal Pigment Epithelium and its Characterization; a step towards Cell-based Therapy for Age related Macular Degeneration 44-54
Rajappa Senthilkumar, Sadananda Rao Manjunath, Subramani Baskar, Vidyasagar Devaprasad Dedeepiya, Sundaram Natarajan, Sudhakar John, Periyasamy Parikumar, Insaan Aditya Yuichi Mori, Hiroshi Yoshioka David Green, Madasamy Balamurugan, Shigeo Tsukahara and Samuel Abraham

Proteolytic Enzyme Production by Isolated *Serratia* sp RSPB11: Role of Environmental Parameters 55-65
P. Lakshmi Bhargavi and R. S. Prakasham

In vitro regeneration of *Capsicum chinense* Jacq. 66-72
K. Sanatombi and G. J. Sharma

Molecular Characterization of Antibiotic Producing Bacteria *Pseudomonas sp*.BP-1 from Nagavali river basin of Srikakulam, Andhra Pradesh, India 73-77
Praveen B. and Bisht S.P.S.

RAPD Based Genetic Diversity Analysis within the Genus *Solanum* (Solanaceae) 78-83
Srinath Rao, G. Shivaraj, C. Kaviraj, P. Surender Reddy and P. B. Kavi Kishor

Formulation and Evaluation of Fast Disintegrating Zolmitriptan Sublingual Tablets 84-98
Haarika Balusu and Prabhakar Reddy Veerareddy

Influence of Cultural Conditions for Improved Production of Bioactive Metabolites by *Streptomyces cheonanensis* VUK-A Isolated from Coringa Mangrove Ecosystem 99-111
Usha Kiranmayi Mangamuri, Sudhakar Poda, Krishna Naragani and Vijayalakshmi Muvva

Short Communication

Differential Expression of ADH and ALDH2 can be a Diagnostic Marker in Gastroesophageal Cancer 112-117
Srikanth L., Ramaiah J., Venkatesh K., Sriram P., Tejachandra G., Kannan T. and Sarma P.V.G.K.

News Item i - vi

Information to Authors

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Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Vol. 6 (1) 1-14 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Probiotics: Recent Understandings and Biomedical Applications

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Abstract

Probiotics are defined as "microbial food supplements" with beneficial effects on the consumers. Several aspects including safety, functional and technological characteristics, have to be taken into consideration in the selection process of probiotic microorganisms. Our Knowledge about probiotics and their interactions with the host has grown ever since Metchnikoff's theory of longevity and proven mechanism of action on probiotics published elsewhere in medical literature. Certainly, now there is enough clinical evidence to support claimed health attributes to selected strains of *Lactobacillus* and *Bifidobacterium* spp. The aim of this review article is to summarise selection criteria of probiotics, technological challenges for probiotic formulations, safety assessment and potential applications of probiotics for health care professionals and common man.

Keywords: Probiotics, *Lactobacillus, Bifidobacterium, Bacillus*, Biomedical Applications

1. Introduction

Probiotics have been defined as living microorganisms which upon ingestion in adequate numbers exert positive health effects beyond inherent basic nutrition (22). There health benefits include improvement of the normal microflora, prevention of infectious diseases (39), food allergies (14), reduction of serum cholesterol (42), anticarcinogenic activity (15, 27), stabilization of gut mucosal barrier (21), immune adjuvant properties (17), alleviation of intestinal bowel disease symptoms and improvement in the digestion of lactose intolerance hosts (25). For decades probiotics have been used in fermented dairy products such as yogurts and fermented milks and the technologies to incorporate these organisms into fresh, refrigerated dairy products is now a mature science. The continuing emergence of clinical evidence for benefits to consumers and the subsequent marketing power of these ingredients have now seen probiotics become the fastest growing category of functional food (FF) ingredients (63). Presently, probiotics play a vital role in promoting health for humans and are also used as therapeutic, prophylactic and growth supplements in animal production (1, 53, 54, 58, 74). From birth till death, all animals are exposed and colonized by a vast, complex and vibrant group of microorganisms that have important effects on immune functions, nutrient process and a broad range of other host activities (36). These microorganisms can mediate the critical balance between health and disease; provide therapeutics for animal and human inflammatory

Vol. 6 (1) 1-14 January 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

disorders on the basis of novel biological principles (26).

The most widely researched and used species belong to *Lactobacilli* and *bifidobacteria* (60, 75). *Pediococcus acidilactici* has a wide range of potential benefits of probiotic properties which are still being studied. Though it is being used as probiotic supplements in treating constipation, diarrhea, relieving stress, enhancing immune response among birds and small animals, human trials are still limited. *Pediococcus acidilactici* is also known to prevent colonization of the small intestine by pathogens like *Shigella*, *Salmonella*, *Clostridium difficile* and *Escherichia coli* among small animals. *Pediococcus acidilactici* in conjunction with *Saccharomyces boulardii*is found to stimulate humoral immune response to produce higher Eimeria-specific antibodies while also reducing the number of oocysts shed by possible competitive inhibition and pediocin production which inhibit pathogenic bacteria and other gram-positive spoilage. Other commonly studied probiotics include the spore forming *Bacillus* spp. Cutting (10) have given a critical review of *Bacillus* probiotics and products for human use.

Probiotics have created considerable interest in the scientific community, and this has resulted in a very substantial increase in published research. The preponderance of the publications reported positive results for probiotic therapy as an important adjunct in the treatment of a host of pathologic processes (9, 71). The aim of the present paper was to review the recent understandings, biotechnological applications and various parameters in considering a microorganism for potential probiotic applications.

2. Selecting Probiotic Microorganism: Fundamental aspects : According to FAO/WHO guidelines it is necessary to identify the microorganism to species/strain level given that the evidence suggests that the probiotic effects are strain specific (45). It has been recommended to employ a combination of phenotypic and genetic techniques to accomplish the identification, classification, and typing of microorganism. For the nomenclature of bacteria, scientifically recognized names must be employed and it is recommended to deposit the strains in an internationally recognized culture collection. Further characterization of strains must be undertaken taking into account the "functional" or probiotic aspects and safety assessment. In addition, even if these genera have a long history of safe consumption in traditionally fermented products and several species have been awarded as "General Recognised As Safe" (GRAS) status by the American Food and Drug Association or a qualified presumption of safety (QPS) consideration by the European Food Safety Authority (EFSA), some characteristics (Fig. 1) must be studied to ensure the safety of the novel *Llactobacilli* and *Bifidobacteria* strains. Several of the *in vitro* tests can be correlated with *in vivo* studies with animal models, but probiotics for human use must be validated with human studies covering both safety (Phase 1 trials) and efficacy (Phase 2 trials) aspects. Phase 2 studies should be designed as double-blind, randomized, and placebo-controlled to measure the efficacy of the probiotic strain compared with a placebo and also to determine possible adverse effects (22). Briefly, the critical parameters to be considered for probiotic candidate are discussed.

2.1 Isolation and Selection criteria : Currently, probiotics are being used successfully to improve the quality of feed provided to domestic animals. The resulting benefits of effective administration of probiotics in feed to cattle, pigs, and chickens include enhanced general health, faster growth

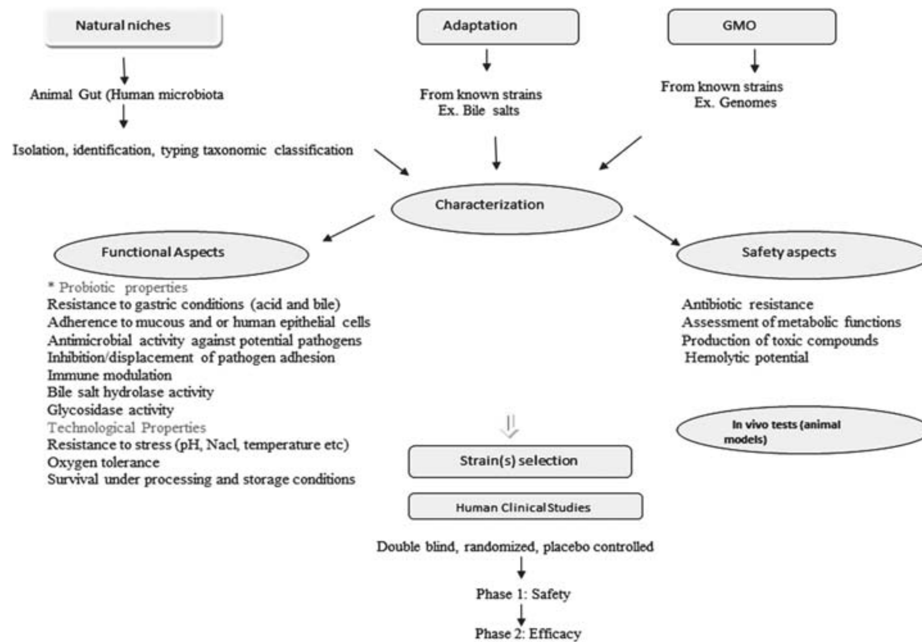

Fig. 1. Procedure for the isolation and characterization of novel strains with putative probiotic Status (43)

rates as a result of improved nutrition, enhanced immunity and increased production of milk and eggs. Some of the microorganisms most commonly used to promote animal health and nutrition include strains of the *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus* genera and yeast of the *Saccharomyces*, *Aspergillus*, and *Torulopsis* genera (71). In the development of probiotic foods intended for human consumption,strains of lactic acid bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, have been used most commonly, primarily because of the perception that they are desirable members of the intestinal microflora (28) (Table 1). In addition, these bacteria have traditionally been used in the manufacture of fermented dairy products and have GRAS status. However, some of the probiotic isolates currently used in the dairy food industry are not of human origin and therefore do not meet the criteria outlined for the selection of acceptable probiotic microorganisms, hence thorough characterization of strains must be undertaken taking into account the functional or probiotic aspects and safety assessment. *In vitro* tests, some of them summarized in Fig. 1. are useful to gain knowledge of both strains and mechanisms of the probiotic effect. The selection criteria for a microorganism to be used as 'probiotic' include the following

1. Safety
2. Detailed definition and typing
3. Absence of pathogenic characteristics (including production of enterotoxins and cytotoxins, enteroinvasivity, pathogenic adhesion, hemolysis, serum resistance, serum pathogenicity, presence of genes of antibiotic resistance)
4. Resistance to gastric acid and to bile
5. Ability to adhere to intestinal epithelium
6. Ability to colonize the colon
7. Clinically proven beneficial health effects
8. Ability to withstand into food stuff at high cell counts and remain viable through the shelf-life of the product (23)

Vol. 6 (1) 1-14 January 2011, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 1. The most commonly used species in probiotic preparations

Lactobacillus Sp.	*Bifidobacterium* Sp.	*Enterococcus* Sp.	*Streptococcus* Sp.	*Bacillus* Sp.	*Pediococcus* Sp.
L. acidophilus	*B. bifidum*	*Ent. faecalis*	*S. cremoris*	*B. subtilis*	*Pediococcus acidilactici*
L. casei	*B. adolescentis*	*Ent. faecium*	*S. salivarius*	*B. licheniformis*	
L. delbrueckii ssp. (bulgaricus)	*B. animalis*		*S. diacetylactis*	*B.polyferminticus*	
L. cellobiosus	*B. infantis*		*S. intermedius*	*B.coagulans*	
L. curvatus	*B. thermophilum*			*B. laterosporus*	
L. fermentum	*B. longum*			*B.polymyxa*	
L. lactis				*B.pumilus*	
L. plantarum				*B.clausii*	
L. reuteri				*B. cereus var toyoi*	
L. brevis					

The use of human origin probiotic and its administration in living form are only relative criteria. *Saccharomyces boulardii*, the probiotic character of which has been sufficiently proven has no human origin and hence it may probably more suitable to name it as a "biotherapeutic agent" simulataneously. Anti-inflammatory effects of probiotics in experimental colitis may be mediated by microbial components such as peptidoglucan, lipopolysaccharide, and non methylated DNA (57, 53). Much attention is paid to detail typing of probiotics. DNA-DNA hybridization or sequencing DNA regions encoding species-specific areas of 16S rRNA are routinely used molecular techniques to test species classification. These techniques are combined with specific cultivation methods for verifying the microbial phenotype.

Genomic analysis is of principal importance for the detailed knowledge of individual probiotics. The first probiotic to be sequenced of whole genome was *Lactococcus lactis* subsp. IL1403 (3). This was followed by the complete genomic analysis of *Lactobacillus plantarum* (35), *Bifidobacterium longum* (68), and *Escherichia coli* (26). Nevertheless, genomically non- defined microorganisms (e.g. *L. casei Shirota, L. reuteri, Streptococcus salivarius* subsp. thermophilus, and non pathogenic *E. coli* O83: K24: H1) or even mixtures of microbes are also used as probiotics. Genomic analysis is indispensable for predicting the effects of individual probiotics as well as for studying the relationship between probiotics and prebiotics and life conditions of the intestinal microflora. The final goal of these studies is to create a global genome bank of intestinal prokaryotes (30).

2.2 Molecular tools for assessment of Probiotic microorganism : Interest of the research community in the study of microbial ecosystem at molecular level is expanding. The Human Microbiome Project (HMP) has recently been launched by the National Institutes of Health (NIH) with the mission of generating resources enabling comprehensive characterization of the human microbiota and analysis of its role in human health and disease. In Europe, the MetaHIT (Metagenomics of the Human Intestinal Tract) project funded by the European Commission is investigating the role of the gut microbiota in obesity and inflammatory bowel diseases (48). Topics surrounding the gut microbiota and its intrinsic relations with health and disease also interest the food and pharmaceutical industries (10).

Broadly, several different methodologies are in use to assess intestinal microbiota which fall in two distinct categories: culture- dependent and culture independent methods (Fig. 2). Culture-dependent methods aim both qualitatively and quantitatively and has been traditionally carried out by cultivation of feces and reviewed in detail by Lee and Salminen (44). One of the most widely applied approaches deals with the use of 16S rRNA and its encoding genes as target molecules. The 16S rDNA gene contains highly conserved regions, present in all bacteria, and highly variable ones that are specific for certain microbes. Specific polymerase chain reaction (PCR) primers and probes can thus be designed based on these variable regions to detectcertain species or groups of bacteria. These culture-independent approaches include16S rRNA measurements, PCR amplification with specific primers of 16S rDNA extracted from faecal or mucosal samples, universal or group 16S rDNA PCR amplification followed by cloning and sequencing, Temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) analysis, fluorescence *in situ* hybridization (FISH), real-time quantitative PCR (RT-PCR), and oligonucleotide-microarrays. In more recent years metagenomic and metaproteomic approaches have also been applied to the intestinal microbiota assessment.

2.3 Technological Challenges for Probiotic formulations: The development of new novel food products and their delivery in acceptable form turns out to be challenging, as it has to fulfill the consumer's expectancy for products that are simultaneously relish and healthy (15). Probiotic

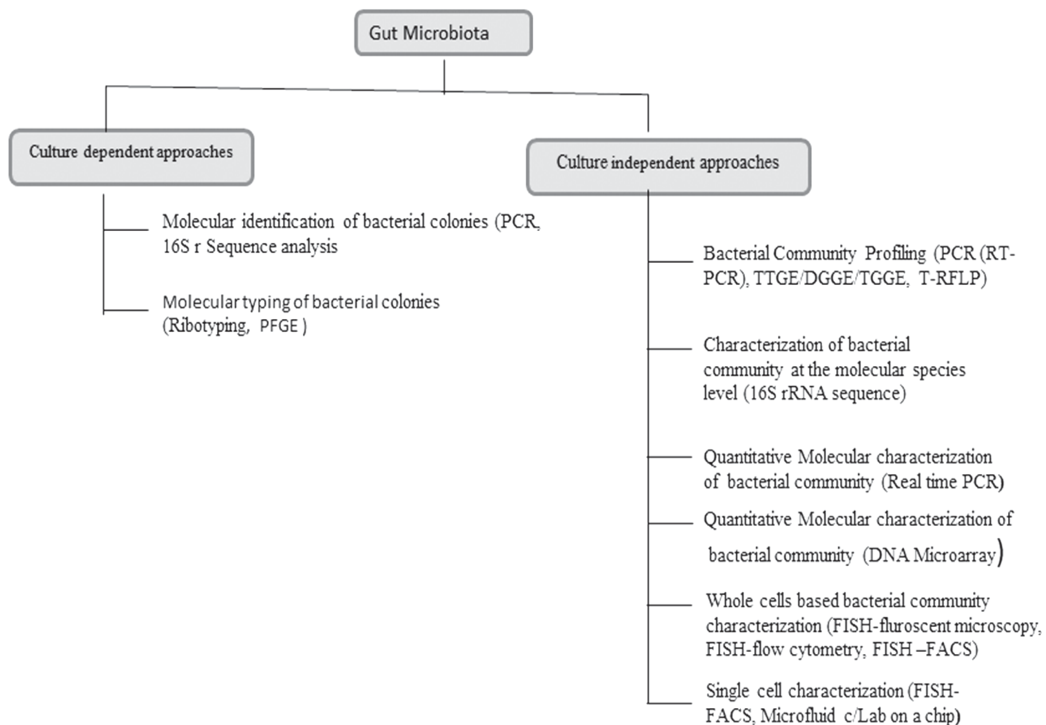

Fig. 2. Molecular tools to investigate the gut Microbiota for probiotic properties (10)

food is defined as a food product that contains viable probiotic microorganisms in sufficient populations incorporated in a suitable matrix (29, 61). This means that their viability and metabolic activity must be maintained in all the steps of the food processing operation, from their production stage to ingestion by the consumer, and also that they must be able to survive in the gastrointestinal tract (63). The information available concerning the concentration of probiotic microorganisms needed for biological effects leads to the conclusion that it will vary as a function of the strain and the health effect desired (12). Nevertheless, populations of 10^6–10^7 CFU/g in the final product are established as therapeutic quantities of probiotic cultures in processed foods (72).

From a technological stand point, there are many challenges in the development of probiotic containing food product such as selection of strain(s), inoculum preparation, survival during processing, viability and functionality during storage, access the viable counts of the probiotic strain(s) (particularly when multiple probiotic strains are added along with starter cultures) and management of effects on sensory properties (11). Processing of microbial systems for functional food (FF) is also dependent on the composition and processing history of the raw material used as substrate, the viability and productivity of the starter cultures applied, processing and storage conditions of the final food products. The viability and activity of probiotic cultures are affected during all steps involved in a delivery process through the exposure to different stress factors (74). In general, probiotics are extremely susceptible to environmental conditions such as water activity, redox potential (presence of oxygen), temperature, and acidity (70). Different stress factors associated with development of probiotic formula during processing are summarized in table 2. The reader is suggested to consult Girgis *et al*. (28) for in-depth review of this subject. Most conventional food processing efforts are aimed towards the reduction or inactivation of unwanted microbial populations. This is achieved by thermal processing (i.e. blanching, pasteurization, sterilization) using water, steam, electrical, light or microwave energy as well as ultrasound or hot air as a means for heat transfer

Table 2. Different stress factors affecting viability of probiotic during processing (74)

Processing step	Stress factor
Production of probiotic preparations	Presence of organic acids during cultivation Concentration-high osmotic pressure, low water activity, higher concentration of particular ion Temperature freezing, vacuum and spray drying Prolonged storage Oxygen exposure
Production of probiotic containing product	Nutrient depletion, strain antagonism, increased acidity, positive redox potential, presence of antimicrobial compounds (Hydrogen peroxide and bacteriocin), storage temperature.
Gastrointestinal transit	Gastric acid, bile salt, microbial antagonism.

(31). Ambient temperature processes include the use of anti-microbials, irradiation, high hydrostatic pressure treatment, high intensity electric fields or light pulses as processing tools, as well as combination processes such as the use of the hurdle technology concept (30). In addition, modified atmosphere packaging and cold or frozen storage is designed to reduce or control microbial growth. Another way of achieving this is to use competitive microbial flora (including probiotic organisms) thus mimicking the situation in the human gut where the competitive microflora also needs to be constantly maintained, improved or re-established. On the other hand, fermentation which is a process to improve the digestibility, quality, safety and physico-chemical properties of the raw material and primarily aimed to produce probiotics and FF or food ingredients can be counterproductive to the viability of microorganisms because it requires maximum productivity of microorganisms which can lead to poor microbial viabilities in the fermented product. Consequently, challenges to retain and optimize microbial viability and at the same time for improving productivity are reviewed by Knorr (41).

Safety of Probiotics : There is abundant experimental evidence to support the health benefits of probiotics, including improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergies, enhancement of immune potency, antitumorigenicactivities, antioxidative and antiatherogenic effects, and a hypo-cholesterolemic property (49, 52) (Table 3).

However, in recent years, many species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* have been isolated from infected lesionsin patients with such conditions as bacterial endocarditis and blood stream infections (26, 46, 47). This is a clear indication of probiotic translocation from gastrointestinal tract to extra intestinal sites which can be measured by the recovery of viable bacteria from lymph nodes, spleen, liver, blood stream and other tissues. This phenomenon is observed due to defective intestinal barrier, immuno-suppression or prematurity. Translocation may result in the transfer of bacteria to other organs, thereby potentially causing bacteremia, septicemia, and multiple organ failure. The ability of microorganisms to translocate, survive, and proliferate in extra intestinal tissues involves complex interactions between the host defense mechanisms and the bacteria's ability to invade host tissues; however, the precise mechanisms involved remain unknown (56).

Bacteria of the indigenous microflora are not normally found in the mesenteric lymph nodes, spleen, liver, or blood of healthy animals. The host's immune defenses normally eliminate indigenous bacteria that would translocate across the mucosal epithelium. Thus, most of the studies in which probiotics were administered at high dosages to healthy subjects found an absence of probiotic translocation. Indeed, probiotics rarely cause severe disease in healthy subjects, even when probiotic bacteria translocate from the gastrointestinal tract. Despite these reports of an absence of translocation of probiotics in healthy subjects, *Lactobacilli* or *Enterococci* have been identified as the strains translocating most commonly into the mesenteric lymph nodes of healthy pathogen-free mice (4) and in immunocompromised patients (64). Although *Lactobacilli* are usually considered contaminants in blood cultures, they have been identified in some clinical reports as causal agents of dentalcaries, infectious endocarditis, urinary

Table 3. Beneficial health effects of specific probiotics (43)

Microflora	Mode of action	Reference
Bifidobacteria species	Reduced incidence of neonatal necrotizing enterocolitis	(52)
	Treatment of rotavirus diarrhea, balancing of intestinal microflora, treatment of viral diarrhea	(37)
Enterococcus faecium	Decreased duration of acute diarrhoea from gastroenteritis	(38)
	Probiotic assessment of *Enterococcus faecalis* CP58 isolated during an *in vitro* screening of lactic acid bacteria	(49)
Lactobacillus strains	Administration of multiple organisms, predominantly *Lactobacillus* strains shown to be effective in ameliorating pouchitis	(74)
	Lactose digestion improved, decreased diarrhoea and symptoms of intolerance in lactose intolerant individuals, children with diarrhoea, and individuals with short-bowel syndrome	(38)
	Microbial interference therapy – the use of nonpathogenic bacteria to eliminate pathogens and as an adjunct to antibiotics	(6)
	Improved mucosal immune function, mucin secretion and prevention of disease	(47)
	Vaccine adjuvant, adherence to human intestinal cells, balancing of intestinal microflora	(5)
Saccharomyces boulardii	Prevention of traveler's diarrhea, prevention and treatment of *C. difficile* diarrhea Shortened the duration of acute gastroenteritis	(7, 38)
Bacillus Sp.	Heat stable oral vaccine delivery, prophylactics and prevention of gastrointestinal infections	(58)
Pediococcus	Enhanced immune responses against infectious coccidioidal diseases	(44)

tract infections, corioamniotitis, endometritis, meningitis and intra-abdominal, liver and spleen abscesses (34). Commonly, these infections can be correlated with previous illnesses (recent surgery, transplants, valvulopathy, diabetes mellitus, AIDS and cancer) with either immunosuppressive therapy or antibiotic treatment, which could promote the development or the selection of the microorganism.

Despite these findings, most studies conducted with healthy subjects have not reported severe disease caused by probiotics even when they do translocate from the gastrointestinal tract. The reasons for this remain unclear and several theories have been proposed. One possibility may be that probiotics are more susceptible to intracellular killing by macrophages upon translocation, since phagocytes are known to exhibit a protective effect during the induction of infective endocarditis caused by gram-positive bacteria (17). The long history of consumption, available epidemiological data, clinical trials, acute toxicity studies that have been conducted all suggest that the *Lactobacillus* sp. commonly occurring in fermented foods and used in current probiotics are safe. It is likely, however, that expansion in this area and the introduction of new probiotic strains will have to take safety aspects into more detailed consideration, particularly should those strains be genetically modified or derived from animals.

Applications

Fermented dairy products enriched with probiotic bacteria have developed into one of the most successful categories of functional foods. They gave rise to the creation of a completely new category of probiotic products like the daily-dose drinks in small bottles, yoghurt, ice creams, milk based desserts, powdered milk for infants, butter, may onnaise, cheese, products in the form of capsules or fermented food of vegetable origin. It has been estimated that there were approximately 70 probiotic-containing products marketed in the world (65), the list is continuously expanding. Moreover, probiotic products containing *Bacillus* species have been in market for at least 50 years with the Italian product known as Enterogermina®registered 1958 in Italy as an OTC medicinal supplement (9). Of the species that have been most extensively examined are *B. subtilis, B. clausii, B. cereus, B. coagulans* and *B. licheniformis*. Spores being heat-stable have a number of advantages over other non-spore formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier (2) which is a limitation for all species of *Lactobacillus* (72).

Conclusion

The global market for functional foods is growing at a very fast rate and probiotic products represent a potential growth area. Intense research efforts are under way to develop products into which probiotic organisms such as *Lactobacillus* and *Bifidobacterium* species are incorporated. The long term exploitation of probiotics would depend on scientifically proven clinical evidence of health benefit, of consumer expectation and of effective marketing strategies (66).Today, probiotics are used to prevent and treat a wide variety of conditions. The evidence is strongest in support of their use for gastrointestinal disorders including diarrhea, pouchitis, inflammatory bowel disease, traveller's diarrhea, allergy, antibiotic associated diarrhea and *Clostridium difficle* infection (32). Many consider probiotics to be Complementary or alternative medicine (CAM). The use of CAM is increasing rapidly in United States and recent survey found that 36% of all adults used

some form of CAM and US $ 37- $ 47 billion is spent annually Interestingly, probiotic therapy and CAM are widely accepted and used frequently by children and adolescents attending gastroenterology clinics (16).

One approach that should be encouraged for health effects is the concept of Synbiotic (use of prebiotic and probiotics), use of genetically engineered recombinant micro-organisms and in combination with food enzymes and antibiotics. There are many probiotic products in the market place and most have supporting evidence behind the advertized health claims (9). However, there is increasing demand for technology for probiotic formulation which can be prepared in viable manner on large scale at low operational cost. Probiotics should not be treated as "elixir of life" but can be incorporated into a balanced diet to exercise good health. Here we hope to have provided an important insight into the recent developments of probiotics and its potential in future to serve in food industry.

Acknowledgements

We extend our sincere acknowledgement to Prof. S. Mayo, Research Management Division, Durban University of Technology, South Africa for awarding Post Doctoral fellowship to Dr. K. Suresh Babu Naidu.

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Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) 15-27 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Food, Health and Agricultural Importance of Truffles: A Review of Current Scientific Literature

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Abstract

Truffles, the subterranean mycelial fruit bodies are wonderful gustatory delights across the globe. Of late, several therapeutic compounds with antioxidant, immunosuppressor, antimicrobial, and anticarcinogenic properties have been discovered from truffles. The so far unexploited fungal resource truffles have been recently discovered to possess great economic potential. However, their cultivation methods have not proved much successful. Submerged fermentation for higher production yield, use of response surface methods for optimum polysaccharide extraction, innovative storage techniques to eradicate spoilage, adulterant determination in canned truffles by molecular tools are strategies for their optimal utilization. In the present paper, the issues hindering the popularity of truffles, the current scenario and the future potentials are reviewed.

Key words: Truffles, antioxidant, antimicrobial, anticancer, mycorrhiza, fermentation

Introduction

The etymological origin of truffle is from *tuber*, meaning "lump". The Latins refer to it as Tuber, derived from the word tumere (to swell) to indicate its globoid form. In scientific jargon, truffles are microaerobic, hypogeous, ascomycetous fungi that form ectomycorrhizae (ECM) with the roots of both angiosperms and gymnosperms. Generally, truffles form long-living symbioses with oak, elm, poplar, chestnut, willow, hazel, beech, birch, hemlock, fir and pine (1). Since antiquity truffles are regarded as the ultimate gastronomic delight or "the diamond of the kitchen"(2). This group of fungi generally belong to the family Tuberaceae or Pezizaceae (3).

Despite their multiple nutritional and therapeutic importance, truffles are shrouded with mystery since antiquity. The scripts on papyrus document that, truffles have been relished by the rich and famous since the Pharaoh age. The truffles are thought to be a "miracle of nature" since ancient Greek civilization. Theophrastus, a pupil of Aristotle's, referred to truffles in 500 BC as "a natural phenomenon of great complexity, one of the strangest plants, without root, stem, fiber, branch, bud, leaf or flower." The precious desert truffle of the Middle East is believed to spawn when lightning and thunder strikes. Since, 18th-century, edible truffles are held in high esteem in French, Spanish, northern Italian, Croatian and many other international cuisines. Truffles are considered to be one of the oldest food-stuff used by Arabs. The Bedouins used truffles as a substitute for meat in their diet (4).

Desert truffles are edible, seasonal and socio-economically important fungi, growing wild in the central-southern part of Bahrain. The truffles usually appear in the deserts of Gulf States following the rainy season (5). Demand of truffle exceeds supply because only ~20 tons are produced worldwide, so price even reaches to an exorbitant US$3000 per kg for white truffles. To cater to the overwhelming consumer demand, large plantations have been established in southern European countries, New Zealand, Australia and the USA, for harvesting truffles.

***Morphology, types and distribution*:** Freshly dug up truffles are aromatic, wrinkled and have bruised, lobed potato-like appearance. Tuber species can generally be distinguished on the basis of their fruit bodies and mycorrhizae (6). The white species are *viz. Tuber magnatum*, *T. maculatum*, *T. borchii*, *T. dryophilum*, *T. puberulum* and the black are *T. brumale*, *T. melanosporum*, *T. indicum*, *T. himalayense*. *T. magnatum* is the most hunted and prized truffles species (7) (Fig 1). The flavour of black truffles is far less pungent and reminiscent of fresh earth and mushrooms.

It is generally assumed that truffles species diversity is favoured by warm, fairly dry climates and calcareous soils. There are about 100 different kinds of truffles around the world, most of which grow in various parts of Europe, particularly in France, Italy, Australia, China, deserts (3). In Piedmont, Tuscany, Umbria, and Le Marche regions of Italy, truffles are found in plentiful during October and November. Arid desert areas harbour truffles, notably *Terfezia* and *Tirmania* species belonging to Pezizaceae family (8). *Tirmania nivea* or "Zubaidi" is the most preferred and expensive truffles in Baharin due to its musky smell, delicacy and soft white tissues. This is followed by *Terfezia claveryi* or "Ikhlasi". *Terfezia boudieri* chatin is widely distributed in arid and semi-arid regions of Tunisia. In the Pacific coast of North America, includng Oregon and California, white truffles *(Tuber oregonense)* and *Tuber gibbosum* are harvested.

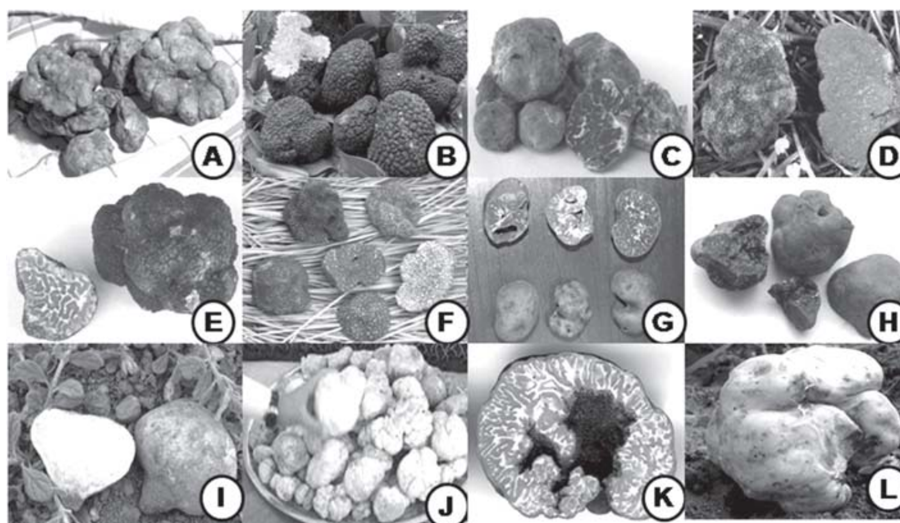

Fig. 1. **A.** *Tuber magnatum*, **B.** *Tuber aestivum*, **C.** *Tuber borchii*, **D**. *Tuber melanosporum*, **E.** *Tuber brumale,* **F.** *Tuber indicum*, **G.** *Tuber excavatum*, **H.** *Melanogaster intermedius,* **I.** *Terfezia claveryi*, **J.** *Tirmania nivea,* **K.** *Tuber pseudoexcavatum,* **L.** *Choiromyces meandriformis Vitt.* (Collected from www.wikipedia.org/)

***Biochemical composition*:** The chemical composition and nutritional quality of *T. claveryi* and *T. nivea* have been studied, which show the presence of protein, fat, dietary fibre, ash, ascorbic acid and essential amino acids, K, P, Fe, Cu, Zn and Mn (9). Recent studies have proven that some truffles contain ergosteroids, ergosterol, the most widespread fungal sterol and brassicasterol having the characteristic sulphurous aroma (3, 10). The typical taste of truffles is the result of a unique combination of several volatile organic compounds (VOCs) *viz.* aldehydes, alcohols, ketones, organic acids and sulphurous compounds (11). The aroma of truffles are diverse, ranging from sulphur, onion, meaty, fruity green apple, anise, cheese, phenolic, metallic, mushroom, roses, earthy, dust, gasoline, leather to animal, butter, creamy, fruity, fatty, waxy, deep-fried, rotten food, cotton candy and cooked potatoes (12). The most important aroma compounds of black truffles are 2, 3-butanedione, dimethyl disulphide (DMDS), ethyl butyrate, dimethyl sulphide (DMS), 3-methyl-1-butanol and 3-ethyl-5-methylphenol (12) (Fig 2). The polyhydroxylated ergosterol glycoside, the dominant unsaturated fatty acids as well as a minor polyhydroxylated C18 fatty acid have been discovered from the fruiting bodies of *T. indicum* (13). Quinonoid and polyphenolic compounds are the most important constituents of truffles pigments. The black pigments are found to be allomelanins of polyketide origin (14). Truffles VOCs derived from various metabolic pathways *viz.* fatty acid catabolism, polyketidic and isoprenoid pathways, are small hydrocarbons containing alcohol, ester, ketone, aromatic groups and sulfur atoms (15). The factors characterizing the mature truffles are a relatively high level of carbohydrates and melanin (30% and 15% by dry weight, respectively) and the presence of rhamnose, calcium and iron. These biochemical markers could be used as indicators of the degree of ascocarp development and the attainment of maturity (16).

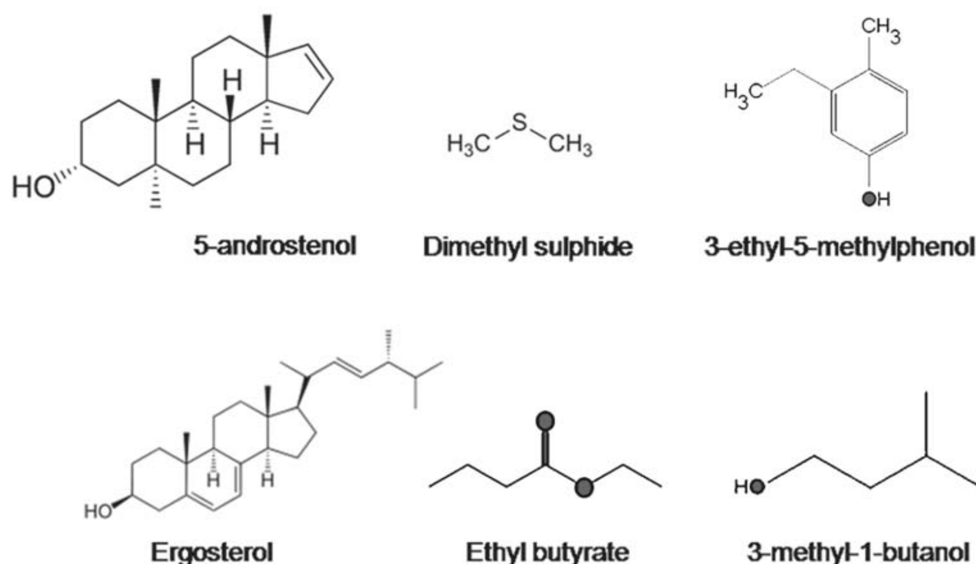

Fig. 2. Various active compounds of truffles *viz.* 5-androstenol, dimethul sulphide, 3-ethyl 5-methylphenol, ergosterol, ethyl butyrate and 3-methyl 1-butanol.

***Structure elucidation of bioactive compounds*:** Bioactive compounds are extracted from dried and powdered fruiting bodies using $CHCl_3$ and methanol, followed by silica gel column chromatography (17). In order to determine the alkyl chain length in fatty acids and sphingosine components, the acid hydrolysis is carried out (3). A headspace solid-phase micro-extraction combined to gas chromatography mass-spectrometry (GC/MS) has permitted the aroma identification of diverse truffles species. (18). A novel method using solid-phase extraction coupled with gas chromatography (GC) and flame ionization detector for the quantitative determination of 5-androstenol in truffles fermentation broth is developed (19). The methods of head-space and vapour analysis at high mass resolution have permitted the identification of 36 compounds in the volatile fractions from 6 species of truffles collected from France. The stir bar sorptive extraction in head space mode coupled with GC/MS has been successfully applied to compare the aroma profile of three truffles species (15). The aromatic composition is evaluated by gas chromatography-olfactometry, complemented by an aroma extract dilution analysis (12).

***Multiple uses of truffles*:** Apart from their immense demand in gastronomic platter, the truffles have also therapeutic potential. The role of truffles in ethno-medicine of Bedouins is documented in Islamic literature (20). These fungal wonders have also antioxidant, antimicrobial, antimutagenic and aphrodisiac properties. Truffles are also good source of sterols. Truffles also can supplement the meagre income of the tribals. Truffles play pivotal role as mycorrhiza too, suggesting their obvious contribution to agriculture.

***As food*:** The arresting aroma and delectable taste makes truffles prized in the culinary world. Connoisseurs relish truffles products like truffles oil, jams or biscuits (20). Truffles, generally the white varieties can be served uncooked as pasta, pizza, omelette and salads (2).Truffles can also be cooked in a myriad ways. The most favoured way of cooking truffles include boiling the cleaned, sliced truffles in salts and spices followed by deep frying in local lamb oil and spicing. Bahrainis prepare sliced clean truffles with rice or other vegetables. Kuwaiti truffles prefer to boil truffles in salty, fresh cow or camel yoghurt or roast them in melted butter, while other Bedouins eat them roasted or as soup. Roasting in campfore ashes is another method of cooking. Sometimes the boiled trufflesare added to a sauteed onion, garlic, and tomato sauce flavoured with spices. White or black paper-thin truffle slices may be inserted into meats or sprinkled as garnish. Some speciality cheeses contain truffles as well. A savoury Tuscan cheese, Boschetto al Tartufo is made with cow and sheep milk infused with thin slices of local truffles. Sottocenere, Caciotta and Pecorino are some other popular Italian truffle speckled-cheeses. Bedouins, usually use truffles as a substitute for meat. Truffles are also used to prepare truffle vodka. Truffles can be kept for future use by drying. The 'desert truffles' are of high dietary value. The most valued truffles in Iraq cookery is the *Terfizia* species for its delicious taste. *T. nivea* can convert a bland recipe into a delicacy (5). Truffles are one of the most expensive food articles in Europe, especially in Italy and France which are mainly relished uncooked (21, 12). *Tartufo Bianco,* a risotto made with white truffle is a delicious Italian special dish. The nutritional value of truffles are higher than that of the cultivated mushrooms, largely owing to lower water contents. The sweet taste is because of the high carbohydrate content. Peeling of truffles before eating has been reported to considerably decrease the content of protein, fat, ash, ascorbic acid and

mineral elements especially calcium and iron. While in the past, chefs used to peel truffles, in modern times most restaurants brush the truffle carefully and shave it or dice it with the skin on so as to retain the nutrients as well as to use most of this expensive stuff. Supplies can be found commercially as fresh produce or preserved, typically in brine, as canned truffles.

As antioxidants**:** Truffles have high content of antioxidants such as vitamin A, C, β -carotene and phenolic compounds, which can scavenge peroxy radicals and chelate ferric ions, thus reducing lipid peroxidation (5). The effect of industrial processing as canning and freezing on antioxidant activity of edible truffles *T. claveryi* and *Picoa juniper* were studied (15). The antioxidants in dried desert truffles from Bahraini, Iranian, Moroccan and Saudi origins have been examined for their antiradical activities by ferric reducing ability of plasma (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), deoxyribose, and nitric oxide (NO) methods (5). Chemical constituents responsible for the antioxidant activities have been identified as ascorbic acid, anthocyanins, total esterified phenolics, total free phenolics and total flavonoids and total carotenoids. The antioxidant rich aqueous extract of *T. claveryi* have demonstrated a very powerful hepatoprotective activity, when evaluated in rats using a potent hepatotoxin carbon tetrachloride (CCl_4) (20). Freezing and canning decreased the antioxidant activity of truffles (22).

Antimicrobial property**:** Recently reported antibiotics extracted from the desert truffles *T. nivea* and *T. claveryi* have proved effective against a broad range of Gram (-) and Gram (+) bacteria (20, 23). *T.claveryi* was reported to be useful in the treatment of opthalmic ailments. Boiled water extract of truffle was claimed effective by the Bedouins for trachoma remedy. The extract showed inhibition towards the aetiological agent of trachoma, *Chlamydia trachomatis* (20). The antimicrobial activity of aqueous and methanolic extracts, as well as partially purified protein extracts from *T.claveryi* was investigated against *Staphylococcus aureus (*24*)* and *P. aeruginosa* (25) *in vitro*. Aqueous extract of *T.claveryi* was reported to contain a potent proteinaceous antimicrobial agent for treatment of eye infections caused by *P. aeruginosa*.

Immunomodulating, antitumor and antimutagenic property**:** Possible mutagenic and antimutagenic properties of aqueous and ethanolic extracts from *T. aestivum* have been studied (11). Intracellular polysaccharides (IPS) isolated from the fruiting-body of *T. sinense* have demonstrated immunomodulatory and antitumor activities (26). Neurotrophic, antitumor, immunostimulatory, anti-phospholipase A2 and cholesteryl ester transfer protein inhibitory activity have also been reported from some truffles (3).

As aphrodisiac, anti-depressant and sterol source**:** For centuries, truffles have been believed to possess mystical aphrodisiac powers. Food connoisseurs describe the scent of truffles as sensual and seductive. A survey has reported that, about 95% of the non-Bahraini respondents and 72% of the Bahraini respondents eat truffles for sexual reasons (20). 5α-Androst-16-en-3-α-ol (androstenol), a steroidal compound belonging to the group of musk odorous 16-androstenes, recognized as a pheromone that could increase the sexual arousal of human female, adjust moods as submissive rather than aggressive in female menstrual cycle and antagonize anxiety and convulsion by positively modulating the GABA receptors. Due to its pleasant odour and pharmaceutical function, androstenol has been developed to perfume with high value in market.

Also, it has potential in drug development for anti-depression (19). From the fruiting bodies of *T. indicum*, a new polyhydroxy sterol glycoside named tuberoside has been isolated along with four known ergostanes. These compounds are assumed as structural constituents of cellular biomembranes and precursors of steroid hormones (17).

***As model for investigation of enzymatic adaptation*:** Truffles show both morphological, physiological and biochemical adaptations to poorly oxygenated area and are good models for investigation of enzymatic adaptation to microaerobic conditions. The antioxidant and glutathione dependent enzymes of truffles *viz.* superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glyoxalase-1 and glyoxalase-2 are expressed and correlated with the microaerobic metabolism, growth rate and mycorrhizal symbiosis of truffles (1).

***As source of income*:** Many indigenous tribal communities are dependent upon truffles not only as diet but as earning source. Bedouin families in the deserts are motivated to supplement their income by collecting truffles (20). The Karuk, Yurok and Hupa people of Pacific Northwest region practise truffle harvesting since ancient times. For the Igbo and Esan people of Nigeria and Cameroon, mushroom hunting is dominated by the women, who gather and sell truffles for seasonal income. While *T. melanosporum* is generally sold at euro 30-40/100g in France, *T. magnatum* reached euro 300–400/100g in fall 2003. A productive truffle-ground represents therefore a significant economic source.

***As mycorrhiza and nitrogen fixing agent*:** Ectomycorrhizal fungi have received a great deal of attention in recent years and have been used world-wide in reforestation, since they can ramify their hyphae in soil and enhance water and mineral absorption of host trees and provide protection against root diseases. In this regard, truffles are important. Morphological characterization of mycorrhiza formed by *Helianthemum almeriense* with *T. claveryi* and *Picoa lefebvrei* have been studied (27). Ectomycorrhizal fungi are also evidenced to confer their host plants with increased tolerance toward toxic metals. Metal homeostasis-related genes in *T. melanosporum* have been recently identified. Genes associated with metal transport exhibited preferentially highest expression level in mycorrhiza, suggesting extensive trafficking of metals as Cu and Zn to host root cells (28). Diversity of nitrogen-fixing bacteria and their activity was investigated in *T. magnatum*, the most well-known prized species of Italy. Degenerate PCR primers are applied to amplify the nitrogenase gene *nifH* from *T. magnatum* ascomata which revealed the presence of *Alphaproteobacteria* belonging to *Bradyrhizobium* spp. (29).

***Searching and detection*:** The high cost of truffles, arise from their difficult and labour-intensive treasure-hunting dedication. Pigs can detect truffles underground owing to aromatic pheromones emanating from both black and white truffles (3). The female pig's instinct to comb the forest floor for truffle is due to a compound within the truffle similar to androstenol, the sex pheromone. In Italy and France, small groups of truffle hunters scour the woods with dogs and pigs looking for truffles in secret spots. Trained pigs and dogs through their ability to detect and recognize the odorant VOCs, determine the underground locations of truffles. While pigs have the keener nose for truffles, they tend to eat the truffles, so dogs are preferred as they have little appetite for mushrooms. Dimethyl sulfide appears to be the key-odour compound for truffle location. Two other

sulfurous and three C8 compounds are reported to be attractant for truffle flies (2). The locations of truffles can be detected by observing the hovering of *Suillia* flies as they lay eggs on the ground above truffles, to provide food for the larvae. Knowledge on the microbial environment where truffles develop is surely a pre-requisite for a better exploitation of the natural truffle-grounds (30). Truffle hunters are a secretive breed who rarely part with the tricks of their trade, do not encourage outsiders into their fraternity and fight all attempts to regulate or organise the truffle business and this craft is usually handed down from generation to generation. Truffles are hunted in the Kalahari Desert by men and women who look for cracks or humps in the soil, which are then extracted with hands or digging sticks.

Dispersal and cultivation**:** Squirrels, wallabies, mice and voles browse on truffles and play an essential role in spore distribution. Mycologists have tried to cultivate truffles by injecting the spores into the roots of trees. This process has met with some success in the case of the black truffle; however, it has proved futile with the most expensive and prized white truffle. Irrigation enhanced the production of truffles due to the abundance of the *Helianthemum* herbs in the Iraqi, Syrian, Jordanian and Saudi desert (21). Several statistical studies have indicated that a high concentration of active carbonate in the soil favours *T. melanosporum* fruit body production. It is explained as the acidification of the immediate soil environment and solubilisation of carbonated fractions by *T. melanosporum* mycelia favouring their mycelia growth (31). *T. melanosporum* has ability to create vegetation-free area because its mycelium and fruit bodies produce multiple substances that adversely affect young plants and seed germination, such as 2-methylpropanal, 2-methylbutanal and 2-methyl-1-butanol (31).

Hurdles in popularity**:** Because of their elusiveness and high price, truffles are used sparingly. Only the hunters or the rich have access to the taste the delectable truffle dishes, common people are often deprived. Limited shelf life of truffle as fresh product is another deterrent, as storage robs off its taste, aroma and hence marketability. This problem is particularly prevalent in Italy, where production has high potentialities, but low possibilities for its wide marketing, due to its limited shelf life as a fresh product. The mating system and the mode of spore dispersal are still under shroud, although some mammals or insects are suspected to act as potential vectors. This general lack of ecological knowledge account for the difficulty in artificial inoculation of host trees with truffle spores (32).

Spoilage**:** Stress factors cause release of enzymes phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) in truffles. PAL is involved in polyphenol synthesis, whereas PPO is responsible for catalyzing the biochemical conversion of the phenolic compounds. PPO oxidizes polyphenols to quinones, which form brown melanin pigments (23). Enzymatic browning affects the flavour and taste of truffles. Perishability of truffles is accelerated when stored in humid places. Fresh truffles are strongly contaminated from different groups of microorganism *viz. Pseudomonas*, *Listeria*, *Salmonella*, mold and yeast species (33).

Strategies for enhanced production**:** Because of the great demand for truffle in market and the shortage in wild resources, an innovative strategy for truffle production is urgent. Comparing to the natural fleld collection and semi-artificial simulation cultivation, submerged fermentation is a promising alternative for the efficient production of truffle and its metabolites. A fed-batch mode of submerged fermentation process is developed for the efficient production of bioactive mycelia and polysaccharides from *T.*

melanosporum. The production of exopolysaccharides (EPS) and intrapolysaccharides (IPS) are markedly improved by carbon and nitrogen sources mixing feeding strategy (34). Another novel method using Plackett-Burman design (PBD) coupled with Draper-Lin small composite design (SCD) and desirability function (DF) was developed to optimize *Tuber melanosporum* fermentation medium. Compared with the original medium, the biomass, production of EPS and IPS were increased upto 54%, 72% and 124%, respectively (35). Effects of nitrogen source and its concentration were studied during the submerged fermentation of *T. sinense*. A mathematical model constructed by Box-Behnken design and response surface methodology was applied to study the synergic effect of carbon and nitrogen sources which identiûed yeast extract and peptone as the most favourable ingredients for mycelial growth (36). For large-scale fermentation of truffles for simultaneous production of biomass and tuber polysaccharides, fermentation technique proved promising.

***Storage and sterilization*:** As already mentioned above, the truffles are highly perishable. These are incapable to retain their sensory and biochemical peculiarities for a long time. The common methods used to store truffles include chilling, drying and freezing. Some Bedouins preserve clean truffles by pickling in 3-6% vinegar and salt (20). Use of gamma rays, are potentially attractive to improve the shelf life and safeguard sensory characteristics of truffles. Effect of 1.5 kGy gamma-ray dose on some biochemical and microbiological profiles of black truffles were monitored, immediately after treatment and after 30 days of storage at 4°C and found to be suitable for preservation (23). However, if applied in an inappropriate mode, irradiation could trigger unwanted sensory and chemical changes, i.e. resulting in free radicals, whose reaction with proteins, lipids and polyphenols, could give rise to detrimental effects. The effectiveness of radiation, heat and fungicides separately or synergistically to inactivate the fungal flora present on truffles were investigated. A trial of triple combination of 2000 ppm propionic acid at 56°C for 5 min and 150 krad of ionizing radiation brought complete sterilization against microbial spoilage (21). Electrophoretic and chromatographic analyses of proteins and peptides allowed a better understanding of the mechanisms responsible for biochemical alterations and bacterial pattern in black truffles during their storage. The γ–irradiation at 1.5 kGy appeared as threshold dose for preserving the characteristics of the fresh product beyond which polyphenol degradation is observed (37). Recent progress in food technology as well as a better knowledge of food physics and chemistry, microbiology and engineering, has allowed the introduction of more innovative food storage systems (23). Storage at 4°C is the treatment that best preserves the biochemical and microbiological characteristics of fresh truffles (38). Subjecting truffles to high CO_2 and low O_2 atmospheres reduce the polyphenol metabolism, anerobic pathways and polyamine biosynthesis slowing senescence (39). Falsconi *et al.* (2005) studied the relative change of the white truffle's aroma (*Tuber magnatum* Pico) in the days following the harvesting, in order to determine the maximum preservation time for the white truffles (Alba's truffle). The flavour of the white truffle is mainly characterized by four parameters: the type, the origin, the ripening and the freshness (aging). The truffle freshness is extremely important for both consumer's safety and commercial points of view, i.e. determining the quality and price of the product. It would be therefore interesting to have a reliable system for truffle freshness evaluation. The change in aromatic compounds in the headspace of white

truffles using SPME-GC-MS technique and the Pico 2-electronic nose (EN) were observed (40). A significant change in truffle's aroma has been observed after 5 days from harvesting. This variation has been mainly attributed to an increase in the headspace of four compounds: acetic acid, ethanol, 2-methyl-1-butanol, 2-methyl-1-propanol. All these compounds are originated by truffle fermentation and can be considered as markers of the product degradation.

***Adulterant determination*:** Purveyors often cheat the consumers by selling minor league truffles at major league prices. To protect consumers from fraud, to identify less tasty and cheaper truffle species, PCR DNA-based method is helpful. This technique unequivocally identifies the nature of the product. A DNA extraction kit in association with a mitochondrial PCR marker is useful to analyze canned truffles (22).

***Molecular studies* :** Integration of molecular and geographic diversity patterns can allow the selection of sites for *Tuber* biodiversity conservation (41). Phylogenetic relationships among truffle species from Europe and China were investigated through parsimony analysis of the ITS sequences. Three major clades were obtained (42). Nuclear and mitochondrial ribosomal DNA (rDNA) polymorphisms were used to analyse the genetic variability in natural populations at different geographical scales (32). Genetic diversification of *Tuber magnatum* depending on the different environmental conditions were analysed with multilocus horizontal starch gel electrophoresis (7). Speciûc primers, based on the *T. magnatum* internal transcribed spacer (ITS) of the ribosomal DNA sequence, were used for a molecular characterization of mycorrhizal seedlings raised under controlled conditions. Morphotyping and ITS sequencing of these mycorrhizal samples provided novel information on the ectomycorrhizal and endophytic species living in a *T. magnatum* truffle-ground (30). Degenerate PCR primers were used to amplify a conserved gene portion coding chitin synthase from genomic DNA of six species of ectomycorrhizal truffles, *T. magnatum* and *T. ferrugineum*, while TubCHSZ was derived from the in vitro growing mycelium of *T. borchii* (43). The cloning, expression and characterization of the hxk-1 gene of white truffle *Tuber borchii* Vittad was studied to understand sugar metabolism (44). Degenerated oligonucleotides were used as primers for polymerase chain reactions to amplify PKC-related sequences from the white truffle species *Tuber magnatum* and *Tuber borchii*. The deduced amino acid sequences of cloned sequences reveal domains homologous to the regulatory and kinase domains of PKC-related proteins, but lack typical Ca^{+2} binding domain and therefore should be classified as nPKCs. Both contain a large extended N-terminus which is found exclusively in fungi PKCs (45). Three black truffle species were studied and found that they can unambiguously be differentiated by performing a single amplification reaction and comparing the length of amplicons obtained (46). Truffles grow in arid reasons. Owing to disturbance of the sandy soils around the Kalahari villages by cattle and goats, the truffle harvest is steadily getting endangered. Taylor *et al* 1995 worked on restoration of production and devising ways for enabling the rural poor community to cultivate truffles for food (47).

Conclusions

Truffles despite their wonderful organoleptic, pharmaceutical and agricultural possibilities have not got its due recognition, which needs to be unravelled for their maximum

exploitation. To raise public awareness, truffle fairs are being held in the regions where these are harvested *viz.* Italy and Oregon. It is a matter of concern that, natural truffle production has declined dramatically over the past century, the main obstacle being the inadequate knowledge of their cultivation. However, the advent of molecular biology is expected to give a boost to truffle research. The rapid strides in food processing techniques have accorded truffles a reputed status in international food platter as gastronomic delight and potential nutraceuticals. However, further scientific studies are warranted in order to develop value-added products, functional foods and pharmaceuticals from bioactive compounds from truffles. As the popularity of truffles is growing, the developing countries are attempting to cultivate truffles for exporting, to boost their economy. Mycologists are engaged in active research identifying new truffle species, testing new hosts, inventing innovative cultivation practices for mass farming of truffles. For all the information available on truffles, it is still in infancy, still an untapped source with multi-pronged prospects. This updated review is strongly believed to provide future direction in truffle research.

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Vol. 6 (1) 28-34 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Plagiarism in Scientific Research: Needs Lock-up to Unlock the Ethical Publications

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Abstract

Plagiarism, a concern of copying the text and claiming to be one's own, is posing a never-ceasing challenge for those engaged in scientific research and ethical publications. Committing plagiarism intentionally is a punishable act, violating the moral and intellectual property of original author. Researchers must be aware of this sensitive issue and it may be amenable to further exploit this prominent aspect. Plagiarism needs to be potentially explored and exposed to all those contributing to the scientific research so as to lock it up universally. The present discussion is designed with a prime objective to provide an insight on how plagiarism is corrupting the scientific research and the keys to troubleshoot the declining reputation of the profession with special emphasis on pharmaceutical field. Herein, authors have tried to focus over the basics pertaining to plagiarism, a questionable fact in ethical publications. The manuscript could be beneficial to one who is at the entrance of research gate in scientific fields including pharmaceutical sector. The review embodies the fact that the plagiarism must be looked up by the researchers around the globe and awareness needs to be cultivated amongst them to foster the culture tied with honesty, and professionalism.

Keywords: Plagiarism, Academic dishonesty, Research misconduct, Professional malpractices, Publication ethics

Introduction

Royal College of Physicians of Edinburg defined Research Misconduct as "any behavior of researcher, whether intentional or not, that fails to scrupulously respect high scientific and ethics standards". The research misconduct may include fabrication or falsification of data, problematic data presentation or analysis, failure to obtain ethical approval by a research ethics committee or to obtain the subject's informed consent, inappropriate claims of authorship, duplicated publications, undisclosed conflicts of interest, and plagiarism (1). An ethical research is all about giving credit to the owner of any intellect wherever it is desired.

Plagiarism is one of the most ignitable issues in the scientific research and the scientific journals around the globe are looking forward about the evaluation of research investigations or manuscripts for plagiarism. Plagiarism is a

serious breach of research ethics and if it is committed intentionally, it is considered as a punishable offence. It is unethical practice and it means failure to give appropriate credit, either accidently or intentionally, for ideas or words or intellect that came from someone else's mind. It is not only to presenting the work of others as owns but also presenting the identical words or identical portions of manuscripts without giving appropriate citations or acknowledgement constitute plagiarism (2).

Plagiarism is very common in educational sector or academia, often to minimum extent, where authors do plagiarism by editing the original text and replacing the original words with the synonymous words or terms (1). Outside educational institutions, those who committed plagiarism are most likely to be prosecuted for breach of copyright. Copyright, in actual, is designed to protect the rights of an author. Plagiarism, while certainly breaching such rights, has a greater impact on reader's mind, by misleading origin for the material what the reader is reading (3). In brief, plagiarism is violation of the publication ethics.

Victoria university defines plagiarism as "plagiarism is presenting someone else's work as if it were your own, whether you mean to or not". Even if it is presented in one's own style or one's own words, still one must acknowledge the source fully and appropriately (4).

The Compact Oxford English Dictionary (2009) defines plagiarism as the act of "taking the work or idea of someone else and pass it off as one's own".

The term plagiarism was came from the English word "Plagiary" meaning 'one who wrongfully takes another's words or ideas' and derived from the Latin word "Plagarius" meaning 'kidnapper' (5).

There are many synonymous words reflecting the plagiarism practice like stealing, cheating, theft, misconduct, and dishonest which are used alternatively in the scientific field to address the plagiarism, not with correct terminology but with somewhat similar meaning.

In case, where there is a direct adoption of statement from another source and if we give the citation for this statement from the source from where it was adopted, it would not constitute any kind of plagiarism. Thus, it would be even more better, if we put the information in our own words, in a more informative and updated words alongwith the proper and full citation then, there would not be any issue of plagiarism.

***Difference between Plagiarism and Paraphrasing*:** Plagiarism is if one uses someone else's findings or results or writing without giving the proper quote and without adding the source or citation in bibliography at the end of the manuscript. Sometimes giving only a part of the quotation and showing the rest of the quotation is one's own or giving the full quotation but without citation also contribute to plagiarism.

It is a common practice that someone may include a statement from a particular source and same statement has copied from an original articled reported published previously a very long time ago. But, it does not constitute plagiarism if authors tend to cite the source in which that statement has been reported or the original article from where that statement has taken many times. Copying of the statement and putting it into manuscript without giving the citation of the source is plagiarism, in actual sense. Cross-referencing of the citations would always be better to avoid any chances of occurrence of plagiarism.

Paraphrasing is stating someone else's ideas or rewriting someone else's words in your own words. Quotation marks should be used to indicate the exact words of another author. If one uses the same words and grammatical structure as the original source (6).

***Need and objectives*:** With the raising need of preventing or minimizing research misconduct in scientific and other allied research areas including the pharmaceutical-research sector, the editorials of various journals, research scientists, academicians, and students pursuing higher education must understand the concepts relevant to plagiarism and shoulder their responsibilities in avoiding the scientific or professional malpractices as a part of the scientific community.

The manuscript was designed herewith the objectives to provide primary and basic information related to the plagiarism, its types, detection, consequences to victim and plagiarist, strategies to avoid or minimize the plagiarism, and future challenges.

***Reasons behind plagiarism (7)*:** The prime reasons behind the occurrence of or prevalence of plagiarism, whether intentionally or unintentionally, includes;

- Lack of author's knowledge about ethical writing
- Improper or poor functioning of ethical committee
- Availability of very few journals on MEDLINE
- The rarity of authors commenting on the research integrity and maintenance of the same
- Some academicians need to increase the number of their publications
- Some students need to dropdown the heavy work load or the assignments given to them
- One may not understand how to use and appropriately cite or acknowledge the sources or references
- One may not have awareness regarding the citation of online material and unintentionally may commit plagiarism
- Author's ignorance about proper citation or referencing style
- Failure to keep notes or sources

***Types of Plagiarism*:** The major types of plagiarism are described below.

***Intentional plagiarism*:** The deliberate or deceptive act of copying is called as intentional plagiarism (5). The term intentional plagiarism could be attributed to the fact that the one who misuse the work of another author without giving proper credit or citation and reflect others as it was of his own, intentionally for his benefit. Intentional plagiarism is a worst offence in scientific research.

The major remarks of intentional plagiarism include:

- Buying readymade material from the internet vendors
- Downloading free access articles from the commercial websites or internet paper mills and utilizing the same as own
- Copying articles from the internet databases and presenting it as own
- Copying words or sentences or even paragraph from previously published articles
- Quoting only a part of the quote and pertaining that the remaining is own
- Making cut-paste of material from various sources without giving whole citation or quotation or acknowledging the attribution

***Unintentional Plagiarism*:** The act of accidental copying and presenting contribute unintentional plagiarism. The unintentional plagiarism may be committed by the research students, research scientists or by academicians.

The major remarks of unintentional plagiarism include:

- Improper citation of a source
- Copying whole sentence and replacing or substituting few words
- Paraphrasing without giving citation
- Summarizing without citation
- Missing out punctuation in citation
- Giving an inappropriate or improper or incorrect information about the source of a quotation

The basic types of plagiarism are mentioned below (8, 9)

***Plagiarism of Source*:** Plagiarism of source is committed if an author uses the citation of one source without acknowledging that the citation came from that source.

***Plagiarism of Authorship*:** The term plagiarism of authorship occurs when one person claims to be an author of an entire bunch of work, fully or substantially authored by another person. The best example to understand this type is when one scientist submits a paper for publication that has already been published by another scientist.

***Self-plagiarism*:** Self-plagiarism means duplication of publication. When one author replace few words or substitute few words in new manuscript taken from his own previously published manuscript, then such an act is referred to as self-plagiarism. Authors generally commit this type of plagiarism to increase their number of publications.

Plagiarism of Online Material

Many of the research scientists and writers hesitate to publish their research investigations or findings or intellect over the internet as online because they may be afraid of the fact that their work will be plagiarized and used without acknowledgement or attribution elsewhere. A junior researcher may have a fear in their mind that if they publish their research work online, their findings will be copied and published under the name of senior researchers and then it will become difficult for them to render readers to realize that the idea had originated elsewhere.

However, if such occurrence happens then the plagiarism may be detected by a service called Internet Wayback Archive Machine (archive.org). This service utilizes the dates of publication for detection of whether the work has been published previously or not.

Internet Wayback Archive Machine is a freely available and potentially very useful service for protecting the intellectual property rights or author's intellect (10).

Detection of Plagiarism

Following are the ways of detection of plagiarism:

1. Evaluation of manuscripts by expertise readers and reviewers those have an ability to judge the difference between the common knowledge and novelty in the manuscripts.
2. Checking for the references cited or listed in the bibliography section of the manuscript.
3. Checking the availability of the similar words or phrases simply by submitting 4-5 keywords from quotation marks in Google search box.
4. Use of detection software.

Use of plagiarism software now-a-days, is a common practice since one's research work needs to be guaranteed to be free from plagiarism

of any kind. This is better to use plagiarism detection software because it utilizes the advanced technological tool to find out whether the research work is plagiarized. These software are advantageous over other plagiarism detection tools because they allow the detection of plagiarism within the manuscript text by scanning it within few minutes to ensure that the manuscript is absolutely free of plagiarism and safe and one can go for its submission to any scientific journal. Also, if author is sure about his manuscript to be plagiarism free, then it would be beneficial to keep the research standard high, whether it is scientific or professional (industrial) or academic (institutional) (11, 12).

Following are few of the freely available plagiarism detection software:

1. VIPER
2. WRITE-CHECK (TURNITIN)
3. EVE (Essay Verification Engine)

   Also, following listed are few of the links of websites where one can put their manuscripts so as to detect plagiarism:

a) http://www.articlechecker.com/ (Free)
b) http://www.dustball.com/cs/plagiarism.checker/ (Free)
c) http://www.scanmyessay.com/index.php (Free)
d) http://www.duplichecker.com (Free)
e) http://www.plagiarismchecker.com
f) http://www.plagiarismdetect.com
g) http://www.ithenticate.com

However, the major limitation associated with some plagiarism detection software is that they does not search the books whilst they compare only the submitted work with the material that is already exist in an electronic format. Also, these software only detects the plagiarized words or phrases but not the plagiarized thoughts or ideas (11).

Consequences of plagiarism

***Consequences to Plagiarist*:** The possible consequences that plagiarist may suffer after finding that he/she has been suspected as plagiarized include;

- Loss of reputation or professional stature among the scientific community
- Loss of research fundings granted
- Rejection of manuscript if the suspect is a research scientist
- Withdrawal of manuscript, if published
- Author may be restricted or suspended from writing or may be black-listed for manuscript submission to the scientific journals
- Dissertation may not be accepted, in case if the plagiarist is a research student
- Grades may not be given or may be declined for research projects
- Degree may not be awarded to the student suspected as plagiarist
- Expulsion from the institution or university of the suspect
- Loss of job in case if the plagiarist is an employee of any professional organization

***Consequences to Victim (14)*:** The possible consequences that a plagiarized victim may suffer include;

- Violation of the collegial trust among the researchers
- Loss of research interest of the plagiarized author
- Theft of intellectual property of the plagiarized author

Strategies to Prevent or Avoid or Minimize Plagiarism (13, 15, 16)

Following are the strategies which one must follow to prevent or avoid or minimize plagiarism:

- Successful implementation of anti-plagiarism policies in institutions or universities
- Create an environment where high standard of academic and research integrity is valued and maintained
- Design of novel research projects that deter research students from plagiarizing
- Promotion of novel and original ideas by encouraging students
- Let everybody know, doing research or writing research manuscripts, about research writings, academic honesty, professional conduct, publication ethics, and plagiarism
- Keep portfolios of the research writings
- Appropriate note taking and citing each note in a sequential manner at appropriate places in the text and numbering properly in the bibliographic section
- Cite everything where one has doubt and if so, talk to research supervisor
- Review of manuscript by the expertise who can differentiate common knowledge from the novel ideas
- Foster a culture of honesty and integrity amongst the scientific community

***Plagiarism Controversy (17)*:** Here, we are making you all to know one issue of plagiarism suspected in India, In 2007, appeared a controversy in Anna University (India). Authors from Anna University and Indira Gandhi Centre for Atomic Research (IGCAR) published an article in the Journal of Materials Science (Springer Link). The article written by K. Muthukumar, T. Mathews, S. Selladurai and R. Bokalawela was reported to be a reproduction of an article published earlier in Proceedings of the National Academy of Sciences (PNAS) by David Andersson and others at the Royal Institute of Technology, Sweden. Later on, the journal reported that the article 'does not just plagiarize the results presented in the PNAS paper but actually copies most of it word for word'. The three authors other than the first author had distanced themselves from the paper and the first author has accepted his mistake. In the meanwhile, the Anna University had barred Dr. Selladurai from guiding any more doctoral students. Also added each and everyone suspected Dr. Selladurai was putting blame on his student, because the professor was not capable of scientific activities. And also the Editorial of the journal found Prof. Selladurai was the corresponding author because the paper uploaded from his (University) computer IP address and the copyright form signed by him, the university found the student as a scapegoat for Prof. Selladurai.

***Future perspectives and Challenges*:** Plagiarism is a problem of global concern and everybody, contributing to the scientific community, must be aware about the plagiarism. There is a stringent need of future to educate the students, researchers, academicians and industrial professionals about the relevant ethical issues of scientific publications and the plagiarism.

With the ever-increasing incidences of research misconduct, academic dishonesty, and professional malpractices, plagiarism is posing a big challenge in front of scientific community, converting professionalism into unprofessionalism and disturbing the reputation of scientific profession.

Conclusion

First and frontline key aspect which serves as a foundation to build a culture of honesty and professionalism in the vistas of scientific research is the ethical writing of research findings. A genuine researcher needs to adapt this culture to harvest the truthful attributes in the research so as to keep the researcher's intellect safe but unlocked.

The high standard of academic and research integrity could only be underpinned when the researchers successfully tackle the universal barrier of plagiarism. Once the sensitive issues of plagiarism have been thoroughly communicated among the scientific community, we may not be too far off seeing a culture bloomed with honesty, trustworthiness and ethical publications, which are of paramount importance in scientific pharmaceutical research.

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Development and Validation of Reversed Phase HPLC Method for Analysis of Immunotherapeutic Peptide PADRE and its Applications

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Abstract

A reversed phase high performance chromatography (RP-HPLC)method was developed and validated for the quantification of PADRE, a peptide with proven immunogenic activity *in vitro*.The developed method was used to determine PADRE stability within biomatrices like plasma, intact tumor and tumor homogenate *in vitro* and to determine PADRE release from its PLGA base dmicroparticle formulation.A reversed phase C8 column was used and the elution was performed using a concave gradient flow. Mobile phase consisted of acetonitirile and 0.01M phosphate buffer at 10:90 ratio containing 0.01% trifluoroacetic acid, adjusted to pH 3, was increased using a concave gradient to 40:60 v/v in 15 minutes. The retention time of PADRE was found to be 10.4 minutes. The method was linear in the range of 0.5-100 µg/ml and the regression coefficient was obtained as 0.9997. The method was found to be accurate with recovery in the range of 97.80 and 104.64%. Precision ofthe proposed method was established by determination of intra day and inter day variability using standard solutions. The percentage relative standard deviation values were all within acceptable range. The detection and quantitation limit for the method were found to be 0.156µg/ml and 0.5 µg/ml. Utilizing the proposed method, degradation profiles of PADRE within biomatrices were obtained. PADRE exhibited rapid and maximum degradation within plasma incomparison to intact tumor or tumor homogenates. PADRE was encapsulated within PLGA microparticles as a means to improve the stability *in vivo*. The proposed HPLC method was also used to characterize PADRE encapsulation within its PLGA based microparticle dosage form. The encapsulation efficiency was found to be 27.2±6.3% and the surface associated peptide was quantified as 21.6±2.2%. The release of PADRE from the dosage form was found to be biphasic. The method may further be utilized to quantify PADRE at tumor site and in plasma upon systemic injection and also to study improvement in PADRE stability using enzyme inhibitors and various drug delivery systems.

Keywords: PADRE, Reversed phase HPLC, Stability, Release

Introduction

The utility of peptide-based vaccines has been exploited for treatment of various types of cancers. Certain tumor associated antigens (TAAs) expressed by cancer cells may be utilized as a trigger to generate a potent systemic immune

response to specifically destroy proliferating and metastasizing cancer cells (1). The TAAs bind to the peptide sequences present onmajor histocompatibility complex (MHC), also called HLA, which essentially are proteins expressed on most cell surfaces.This binding is effective only when certain amino acidsare preserved in thepeptide sequence at fixed positions. The TAA-MHC complex is then recognized byT lymphocytes to generate an immune response. The ability of a peptide to generate an immune response therefore depends on how efficiently it binds to MHC and also on the efficiency of recognition of the TAA-MHC complex by the T cells (2).

Several peptides have shown activity when tested on *in vitro* culture systems and also produce effective immunogenic responses *in vivo* when coated onto surfaces of tumor cells before inoculation in animals. However potent immunogenic responses are not observed when the peptide is injected systemically. The possible reasons for this could be the physical, physiological and metabolic barriers presented by tumor tissue. It is important to determine if the peptide is susceptible to enzymatic degradation byexo and endo peptidases present within biological matrices like plasma, extracellular matrix and tumor resulting into loss of immunogenic activity (3).

Pan DR Reactive Epitope (PADRE) is a syntheticnon-naturallinear peptidebelonging to the MHC II family. The peptide PADRE having a 13 amino acid sequence, aK(X) VAAWTLKAAa, has a high binding affinity towards a number of MHC allelic variants which is advantageous in imparting immunogenicity. Also, when tested on *in vitro* culture systems using T cell proliferation assay, PADRE has shown to be a powerful antigen with 100 fold higher potency than a control tetanus toxoid (4,5). With its proven immunogenicity *in vitro*, the peptide has a strong potential as a vaccine for cancer therapy.However when injected systemically, the peptide failed to produce delayed type hypersensitivity or tumor rejection, possibly due to physical and metabolic barriers (6), which may be overcome by suitable delivery strategies.

The development of a peptide as a therapeutic demands a reliable and rapid analytical method. Previously RP-HPLC has been used for the analysis of immunogenic peptides such as a 34 amino acid peptide called HEL (Hen egg white lysozyme) has been characterized by HPLC and separated from degradation products upon trypsin and chymotrypsin treatment.The method however was not validated to test the sensitivity, accuracy and precision and had along run time of 60 minutes (7). The present work aimed at development and validation of a reversed phase HPLC method for determination of PADRE with good sensitivity and short analysis time. The method will be particularly useful to determine the levels of PADRE at the tumor site. It will alsobe useful to determine stability of PADRE within biological matrices like plasma, solid tumor and tumor homogenates and to characterize the encapsulation of PADRE in drug delivery systems like microparticles (8,9) and to study subsequent release kinetics of PADRE from these systems.

Materials and Methods

The peptide PADRE was synthesized by Genemed Synthesis Inc., San Francisco, USA. All reagents and solvents, namely,trifluoroacteic acid (reagent grade), monobasic potassium phosphate (reagent grade) and acetonitrile (HPLC grade) were obtained from Sigma Aldrich, St.Louis, USA. Buffers were prepared in deionized water.

***Instrumentation*:** The high performance liquid chromatography was performed using LC- HP 1100 series (Hewlett Packard Corporation, USA) system, equipped with G1311A pump, G1313A auto sampler and G1315A diode array detector. The system was equipped with a NovapakC8 reversed phase column (Waters corporation, USA), 5 µm particle size, 4.6 mm internal diameter and 150 mm in length.The data analysis and acquisition was executed using Chem Station software (Agilent, Santa Clara, CA, USA).

***Optimization of Chromatographic conditions*:** The separation was performed using a concave gradient flow. At the start of the separation, the mobile phase consisted of acetonitrile (10%) and 0.01 M phosphate buffer containing 0.01% trifluoroacetic acid, adjusted to pH 3, (90%) at a flow rate of 1 ml/min. The mobile phase ratio was increased from 10:90% v/v to 40:60%v/v from 0 to 15 minutes using a concave gradient.A wavelength of detection of 210 nm was used. For each study, mobile phase was prepared by filtration through 0.45 µm nylon filter and was degassed prior to use.

***Preparation of standard solutions*:** PADRE was solubilized in phosphate buffered saline (PBS). Standard solutions were prepared at the concentrations 0.5, 1, 2.5, 5, 20, 25, 50, 75 and 100 µg/ml. Freshly prepared stock solutions were used to perform all analysis and final dilutions of standard solutions were prepared in mobile phase.

***Validation of the method*:** The developed HPLC method was validated using certain analysis parameters like linearity, accuracy, precision, range, limit of detection and limit of quantification (9,10). Various concentrations of the standard solution of PADRE were run and the peak area was determined by integration. A standard curve of concentration (µg/ml) v/s peak area (mAU) was plotted and regression coefficient of the plot was determined as a measure of linearity.Accuracy of the method was assessed by testing different concentrations of standard solution of PADRE and determining the percentage recoveryat each concentration (n=6). The intra-day and inter-day variability was studied to determine precision of the method. To obtain the intra-day and inter-day variation, each standard solution of PADRE was analysed six times on five different days. The percentage of relative standard deviation (% RSD), also known as % CV was determined as a measure of precision. The limit of quantification (LOQ) and limit of detection (LOD) were determined as per the method specified in ICH guidelines using empirical method (11, 12).

Application of the method

a) The developed HPLC method was utilized for determining the stability of PADRE in biomatrices like mouse plasma, intacttumor and tumor homogenate.Fresh plasma and tumor were extracted from tumor bearing mice after CO_2 euthanasia. For preparing tumor homogenate, the tumor is homogenized in 2 ml of phosphate buffer and centrifuged to obtain a peptidase rich supernatant, which is used for further studies.The peptide was incubated in PBS, mouse plasma, solid tumor and homogenized tumor (supernatant)at 37°C and aliquots were withdrawn at 0, 1, 2, 3, 18 hours. The plasma samples were de-proteinated with equal volume of acetonitrile and centrifuged at 10,000 rpm for 10 minutes and the supernatant was subjected to further analysis. All the samples were analyzed by the proposed HPLC method.

b) The proposed method was also used to determine the encapsulation efficiency of the peptide PADRE within poly lactic co glycolic acid (PLGA, M.W. 60,000) microparticles. A w/o/o emulsion method was used for preparation of the microparticles. Briefly, the PADRE was dissolved in 0.5% sodium lauryl sulfate and then

dispered in acetonitrile:dichloromethane (50:50) as the oil phase. The primary emulsion so formed was homogenized in mineral oil and double emulsion so formed was evaporated under vaccum to remove the organic phase. The resultant particles were hardened using hexane. For determination of encapsulation efficiency (%EE), the microparticles were digested in acetonitrile and peptide was extracted in water and analyzed using the proposed HPLC method.The amount of surface associated PADRE was determined by simply washing the microparticles with water. The release of PADRE from microparticleswas studied at 0, 2,4, 6, 8 and 24 hours in PBS using the HPLC method.

Results and Discussion

***Optimization of method*:** A method for determination of a novel peptide PADRE by reversed phase high performance liquid chromatography was proposed. The development of the HPLC method for PADRE involved optimization of the mobile phase to obtain a relatively sharp and symmetrical peak with a suitable retention time. Using the proposed HPLC method, PADRE showed a retention time of 10.4 minutes. Fig. 1 is a representative chromatogram for the proposed method for determination of PADRE. Pure synthetic grade PADRE was used in the development of this method. No additional peaks due to any possible degradation of the peptide were observed in the chromatogram, indicating that the method conditions and solvents were suitable for the analysis.

***Linearity*:** The standard curve was obtained by plotting the peak area (mAU) and concentration (µg/ml) using nine standard solutions of PADRE (Fig. 2).The linear equation of the standard curve and regression coefficientobtained was as follows:

$$\text{Area} = 29.12\ (\pm 0.17)^* \text{ Concentration} - 2.49\ (\pm 8.01)$$
$$(\text{Correlation coefficient } [R]^2) = 0.99$$

The calibration curve of PADRE showed a good linearity within the tested concentration range with a regression coefficient of 0.99 (13).

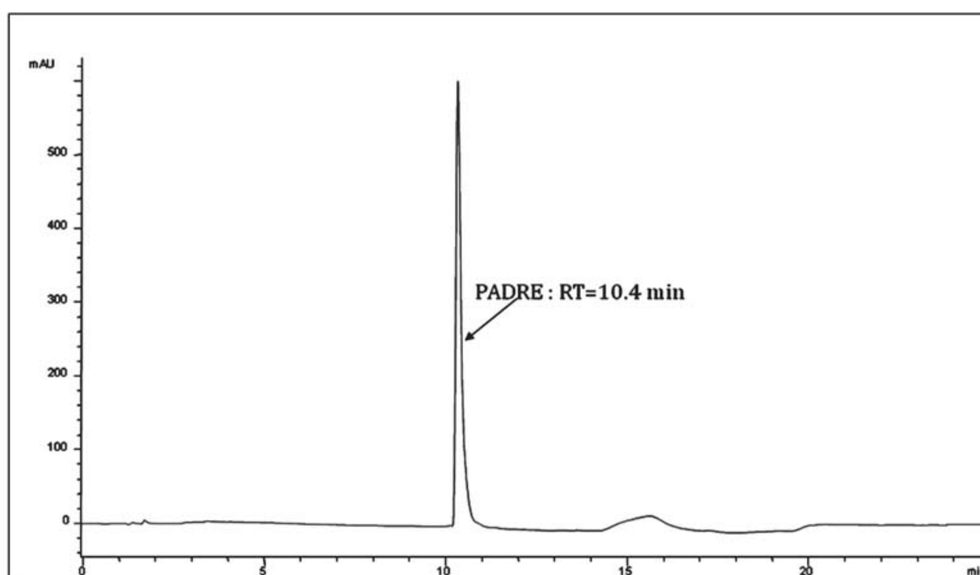

Fig. 1. Representative chromatogram for determination of immunogenic peptide PADRE

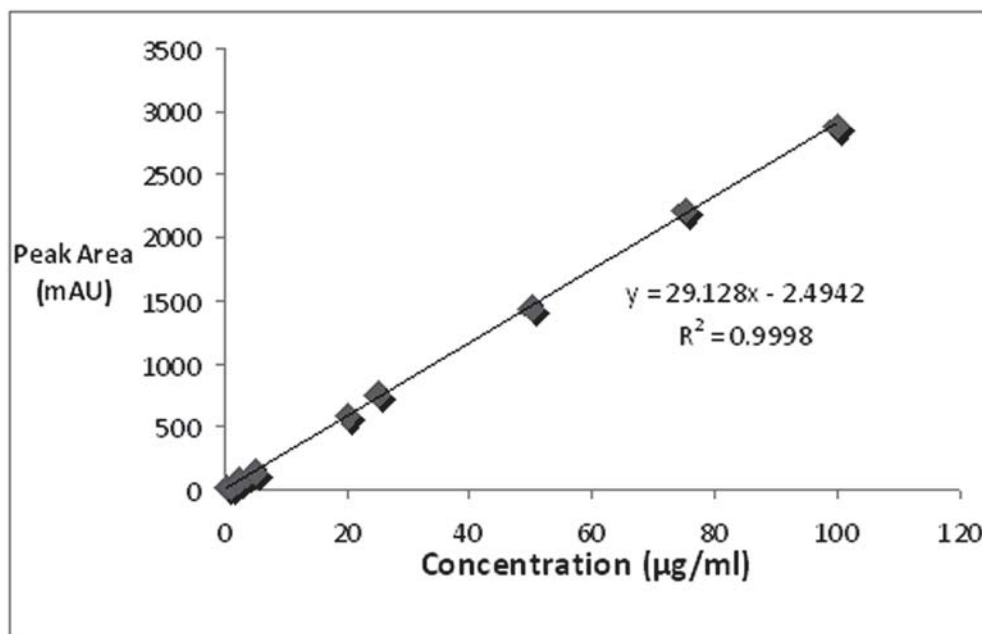

Fig. 2. Calibration curve obtained from standard solutions of PADRE by HPLC analysis

***Accuracyand Precision*:** The method was tested for accuracy, which is the closeness of the observed results to the actual results. The accuracy of the method is expressed as percent recovery calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Observed Concentration}}{\text{Actual Concentration}} \times 100$$

From the results of the analysis (Table 1), the average recovery was observed to be 102, 97.80, 97.90, 104.64 and 99.50% at the standard concentrations of 1, 5, 10, 50 and 100 respectively. An overall average recovery of 100.36% was obtained. An average percentage recovery value between 95 to 105% is considered to be acceptable. Also no individual measurement of percentage recovery was less than 80% or greater than 120%. Thus the method was found to be accurate. Precision, which is a measure of repeatability of the data was also assessed for the method. The relative standard deviation (%RSD), also known as % coefficient of variation was determined by analyzing standard solutions of various concentrations on 5 consecutive days. The following formula was used to calculate the relative standard deviation:

$$\% \text{ RSD} = \frac{\text{Standard deviation of measurements}}{\text{Average of measurements}} \times 100$$

The variability improved at higher concentrations of the standard solutions.The %RSD was found to be reasonable for both intra-day and inter-day variability (Table 2). The proposed method for PADRE determination was found to be precise.

***Limit of detection (LOD) and limit of quantification (LOQ)*:** The limit of detection and

limit of quantification are measure of sensitivity of the method.The limit of quantification for the method at the tested concentrations of standards was found to be 0.5 μg/ml. At this concentration, both precision and accuracy were found to be less than 20%.The limit of detection, which is the lowest concentration of the analytethat can be detected by the proposed method was found to be 0.156 μg/ml.

RP-HPLC is a good technique to analyze peptides qualitatively and quantitatively due to the effective separation of peptides with nearly identical sequences. Enzymatic digestion products of peptides and proteins have previouslybeen effectively separated by RP-HPLC. For example,phosphorylation was utilized as an approach to improve the stability of an immunogenic peptide in serum.The stability of the unphosphorylated parent analogs; T 207-222, T 224-240, and T 390-408 and itsmonophosphorylated and diphosphorylated derivatives in serum was determined. The improvement in serum stability of phosphopeptides due to resistance of the serine and threonine phosphate ester bonds to serum proteases was linked to the improved immunogenicity of these peptides (14). The only disadvantage of using RP-HPLCfor peptide analysis is that, the peptide undergoes denaturation during the chromatographic run and therefore the method is not useful for preparative separations (15).

Application of the method

a) The analysispeptide PADRE after incubation in various biomatrices showed that the peptide was susceptible to degradation. Other than the peptide peak, three degradation peaks were observed in the chromatogram. PADRE showed extremely rapid degradation in plasma and therefore rate calculations could not be performed. The peptide degraded much more slowly if injected into the intact tumor mass and samples were taken from the medium but was much rapid in the presence of homogenized tumor. The peptide half-life was 30 minutes and 21 minutes in intact tumor and homogenized tumor, respectively (Fig.3). Thus the peptide

Table 1. Determination of accuracy for validation of the HPLC method for determination of PADRE (n=6)

Actual/ Prepared concentration (μg/ml)	Measured concentration (μg/ml)	Percentage Recovery (%)
1	1.02	102
5	4.89	97.80
10	9.79	97.90
50	52.32	104.64
100	99.50	99.50

Average percentage recovery (%) 100.368

Table 2. Determination of inter-day and intra-day variability to assessprecision of the HPLC method for determination of PADRE (n=6)

Actual concentration (μg/ml)	Relative standard % deviation (RSD)	
	Intra-day variability[1]	Inter-day varability[2]
1	2.6	10
5	3.8	8
10	1.6	3.7
50	1.2	2.2
100	2.1	1.9

[1]Intraday variability is determined by analyzing each concentration of standard solution 6 times within a day (n=6)

[2]Inter-day variability is determined by analyzing each concentration of standard solution on 5 consecutive days (n=5)

PADRE was relatively unstable in biomatrices. Several strategies have been proposed to improve plasma half-life of peptides (16) and these may be beneficial in improving the in vivo immnunogenic potential of PADRE.

b) PADRE was successfully entrapped in microparticles of PLGA. By HPLC analysis using the proposed method, the amount of PADRE at the surface of the particles was found to be 21.6± 2.2%. Similarly the amount of drug encapsulated within the PLGA matrix was found to be 27.2± 6.3%. Thus total peptide associated with the microparticles was found to be 54.3± 3.7%. Also, the release of PADRE was studied from the microparticles at two different levels of drug loading (1% and 5%w/w of peptide to polymer). The drug release (Fig. 4) was found to be biphasic, with an initial rapid phase and a

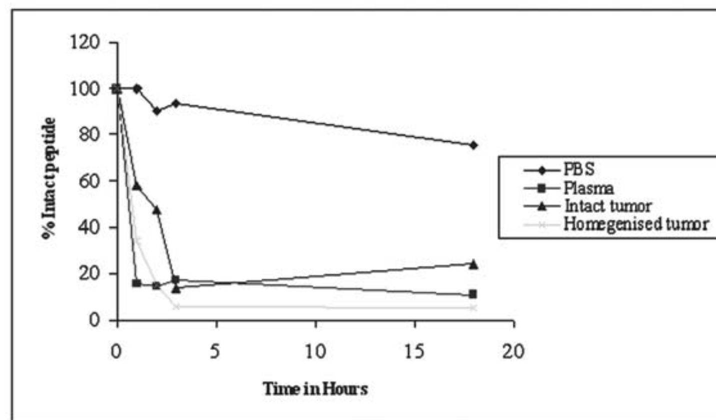

Fig. 3. Stability of the peptide PADRE in various biomatrices determined using the proposed HPLC method.

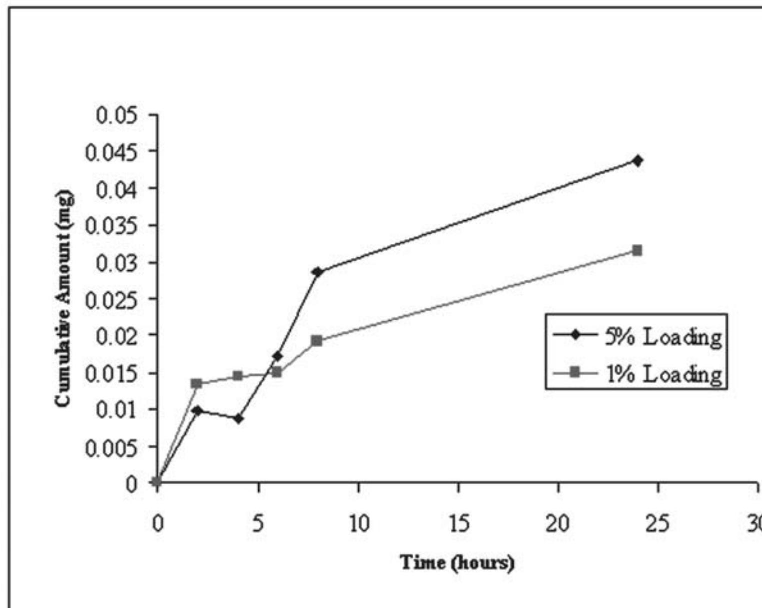

Fig. 4. Drug release of PADRE from PLGA microparticles

slow phase corresponding to the release of the encapsulated peptide.This pattern should mimic the profile of antigen concentrations that are seen in the course of a natural infection; i.e., a high dose of antigen within a few days of the injection followed by a period of decreasing amounts of antigen. The initial high load of antigen is expected to influence the extent of memory T-cell formation, whereas the subsequent steady decrease in antigen load will aid the development of antibody affinity maturation (17,18). PADRE release rate from the particles with 5% loading was higher when compared to the particles with 1% loading.

Conclusion

A reversed phase HPLC method was developed for the analysis of PADRE. The method was validated and found to be linear, precise, accurate and sensitive for detection of relatively low concentrations of analyte upto 0.5 µg/ml.The method was rapid with elution occurring at 10.4 minutes and runtime for each sample being 17 minutes. The proposed method was used to study stability of the peptide within biomatrices like plasma, tumor and tumor homogenates*in vitro*. Also, the method was used to study the encapsulation of PADRE within PLGA microparticles as drug delivery system and also used to characterize the subsequent release of PADRE from the microparticles. The method may further be applied to quantify PADRE at tumor site and in plasma upon systemic injection and also to study improvement in PADRE stability using enzyme inhibitors and various drug delivery systems.

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Vol. 6 (1) 44-54 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Successful Transportation and *in vitro* Expansion of Human Retinal Pigment Epithelium and its Characterization; A step towards Cell-based Therapy for Age related Macular Degeneration

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Abstract

Age related Macular Degeneration (AMD) is a disease of the retina that leads to deterioration in vision and eventually permanent blindness. As yet there are no definitive ways of repairing the damage caused by AMD. Recently evidence is mounting that cell-based therapy using Retinal Pigment Epithelium (RPE) could be a feasible option for treating this disease. For example, autologous RPE transplantation has been successful at providing a functioning replacement for the diseased retina in animal models and humans. However, degeneration can re-occur requiring more RPE cells from the patient. Therefore, considering the option of one-time harvested RPE tissue from the periphery of the patient's eye, safe transportation between clinics/hospitals, efficient *in vitro* RPE expansion at the destination and long-term cryopreservation for future applications, we have developed a biodegradable RPE carrying medium in 3D, made from a growth factor-free Thermo-reversible gelation polymer (TGP - Mebiol gel). RPE cell layers harvested from cadaver eyes were embedded in the TGP hydrogel and divided into three groups: Group 1, were processed immediately, Group 2 after 18-24 hours and Group 3 after 40-48 hrs of harvesting. Each group had one control sub-group grown in conventional media and one TGP sub-group grown embedded in TGP scaffold. No growth factors were used in the culture, when grown for three weeks. RPE cell counts were done at regular intervals during the expansion phase, and were then characterized by RT-PCR to confirm their RPE phenotype. The cells in all the three TGP preserved groups and the controls were equally viable after different periods of preservation, with a maximum

duration of 48 Hrs. In cultivation, TGP preserved RPE cells formed a monolayer with a typical honeycomb/cobblestone appearance characteristic of native RPE. The degree of pigmentation is increased in the TGP group compared to the control group indicating that the RPE possesses a native RPE phenotype. The proliferative capacity of RPE was also increased when embedded in TGP. Cells from both the groups expressed Cellular Retinaldehyde-Binding Protein (CRALBP) and RPE65, which are abundantly expressed in the RPE cells and Mueller cells of the retina. We have established a simple and efficient transportation module for RPE at varying climatic conditions without the need for cool preservation using a polymer hydrogel cocktail and a culture method without using any growth factors. These cells can be a potential source for transplantation in treating retinal disorders upon further confirmation of their functional characteristics.

Key words: Retinal pigment epithelium, macular degeneration, hydrogel scaffold.

Introduction

The Retinal Pigment Epithelium (RPE) is a neuroepithelium-derived, cellular monolayer that lies on Bruch's membrane between the photoreceptor outer-segments and the choriocapillaris. It serves to provide the transducing interface for visual perception (1) and hence is critical to providing vision (2). The RPE is also important for local homeostasis and maintenance of the extraphotoreceptor matrix.

Age related Macular Degeneration (AMD) is the third leading cause of worldwide blindness in the elderly that results in a loss of vision in the center of the visual field either due to atrophy of the retinal pigment epithelial layer, Dry Age related Macular Degeneration (Dry AMD) or due to abnormal blood vessel growth, Wet Age related Macular Degeneration (Wet AMD) (3). The current treatments for wet AMD include: photodynamic therapy (PDT), laser therapy, anti-vascular endothelial growth factor (anti-VEGF) therapy etc., all of which have limited success and are non-permanent solutions. For dry AMD there are not many options available. The ideal treatment will be to replace the dysfunctional RPE cells by cell-based therapies which can repair the damaged retina and restore normal vision. RPE transplantation in humans was first performed in 1991 by Peyman *et al.* but with little success (4). Later allogenic fetal RPE cell transplantation was tried in which, immune rejection of the graft was a major issue. It was observed that rejection rates were lower in dry AMD than wet AMD (5). In many cases transplantation without Immunosuppression therapy led to leakage on fluorescein angiography and eventual fibrosis (6). All these lead to the era of autologous RPE transplantation which is conventionally done employing two techniques namely retinal pigment epithelial suspension and autologous full-thickness retinal pigment epithelial-choroid transplantation (7-11). Encouraging clinical outcomes has already been reported with the transplantation of the autologous RPE- Choroid from the periphery of the eye to a disease affected portion (10, 12, 13). Hurdles in autologous RPE transplantation include, the RPE cells being fragile when hit with surgical material, it is impossible to transplant a layer uniformly and in case of full-thickness transplantations including retinal pigment epithelial- Bruch's membrane- choroid transplantation, formation of multilayered folds and contraction of the graft pose as major hurdles (14). Also the option of autologous graft is constrained by the size of the full thickness RPE graft that can be taken from the periphery of the same eye to patch the defect

and this is usually insufficient. As a result, an alternative source of cells to replace the diseased RPE is needed (15). For this, we believe that a safe, reproducible and efficient *in vitro* expansion of the human RPE could be developed for the clinic. Hence, there arises the need for a scaffold material that will support RPE cells while culture similar to a Bruch's membrane, helping in fluid transport with porosity and helps in transplantation of RPE in the sub- retinal space and is bio degradable. Recent approaches employ the use of substrates like amniotic membrane (16 - 18). Singhal *et al*., has grown RPE cells obtained from human cadaver eyes on human Amniotic Membrane *(hAM)* as culture substrate and reported that the Primary adult human RPE cell cultures retain epithelial morphology *in vitro* when cultured on human amniotic membranes. However after the expansion, separation of the RPE cell from amniotic membrane and transferring of the expanded RPE cells without contamination of amniotic membrane is difficult. The use of feeder layers and growth factors are another disadvantage, as a source of contamination and immunoreactivity in the host.

Herein, we report the transportation of RPE tissue layers in a novel polymer based transportation cocktail from hospitals to a central processing facility without cool preservation under varying climatic conditions and subsequent isolation, expansion and characterization of the RPE cells in a synthetic biodegradable polymer scaffold without use of growth factors.

Materials and Methods

***Isolation of Retinal Pigment Epithelium*:** Twelve RPE samples obtained from eyes of human cadavers after proper informed consent from the guardians were used in the study. These RPE samples were obtained from three different hospitals located at varying distances of 300 to 3000 kms from the central processing facility taking a maximum time of 48 hrs. All the procedures were in accordance with the declaration of Helsinki and approved by the Institutional ethical committees of the hospitals involved in the study.

The RPE tissues were dissected and embedded in Thermo Reversible Polymer based cocktail by an experienced ophthalmologist.

***Preparation of Transportation cocktail*:** The transportation cocktail consisted of TGP (Commercial name: Mebiol Gel) which is a copolymer composed of thermo-responsive polymer blocks [poly(N-isopropylacrylamide-co-n-butyl methacrylate) poly(NIPAAm-co-BMA)] and hydrophilic polymer blocks [polyethylene glycol (PEG)]. The TGP is in liquid state at lower than the sol-gel transition temperature but turns to gel immediately upon heating and returns to a liquid state again when cooled. This sol-gel transition temperature can be altered (19). The lyophilized TGP (1gm) vial was provided for this study by Nichi-In Biosciences (P) Ltd, Chennai, India. The TGP was reconstituted with 10 ml of DMEM/F12 (Gibco BRL, Gaitherburg, MD, USA) and incubated at 4°C overnight. 200 µl of reconstituted TGP was added in each sterile vial and the basal culture medium which need to be overlaid contained 300µl of DMEM/F12 culture medium without growth factors.

***Transportation of RPE in TGP based cocktail* :** The TGP vial was taken from the refrigerator and the RPE tissue was embedded in the TGP in the vial over which additional TGP kept under cold condition was added and left undisturbed for five minutes for the cold TGP to solidify. After solidification of TGP, basal culture medium described above was overlaid and the tissue containing vials were transported to the central

processing facility without cool preservation. One RPE tissue was transported in one TGP vial. Thus the RPE tissues embedded in TGP vials were divided into three groups, Group 1, Group 2 and Group 3. RPE tissues in Group 1 were those tissues which were processed within 18 hrs of transport to the central processing facility, Group 2 contained RPE tissues which were processed after 18-24 hours and Group 3 contained those which were processed after 40 – 48 hours.

***Tissue Processing*:** For tissue processing, the RPE tissue transported in TGP vials were kept in cold condition for the TGP to liquefy. All the RPE tissues were subjected to washing by adding cold Calcium Magnesium free CM-HBSS with antibiotics (penicillin 50 U/ml, Streptomycin 0.1 mg/ml and Amphotericin B 0.0025 mg/ml) and centrifugation at 1200 rpm for 10 minutes at 4 p C using an ultra low temperature centrifuge. After centrifugation, the supernatant was discarded and the pellet was washed twice. The washed RPE tissue pieces were subjected to enzymatic digestion with 0.25% of Trypsin and 0.02% of EDTA for 10 minutes. After 10 minutes of incubation, equivalent amount of Dulbecco's Modified Eagle's medium (DMEM)/Ham F12 medium with 10% FBS was added to neutralize the Trypsin activity. The cell suspension was collected and centrifuged at 1200 rpm for 5 minutes. Cell count was done using Trypan Blue Dye exclusion method. The cells obtained were seeded equally in two groups for *in vitro* expansion.

***Control Group*:** The cell pellet was mixed in Dulbecco's Modified Eagle's medium/Ham F12 medium supplemented with 10% Fetal Bovine Serum and antibiotics (penicillin 50 U/ml, Streptomycin 0.1 mg/ml and Amphotericin B 0.0025 mg/ml) in 24 well culture plates.

***TGP Group*:** The cell pellet was plated in 24 well culture plate containing TGP. Additional TGP kept under cold conditions was added on top of the cells and they were left undisturbed for 5 minutes for the cold TGP to solidify. DMEM/Ham F12 medium supplemented with 10% Fetal Bovine Serum and antibiotics (penicillin 50 U/ml, Streptomycin 0.1 mg/ml and Amphotericin B 0.0025 mg/ml) was overlaid on TGP after solidification.

Both the groups were cultured for 21 Days in 5% CO_2 at 37°C and documented. Periodical adding of culture medium and observation were done in the same manner for both groups of specimens.

***Cell count and characterization of cultured Retinal Pigment Epithelium*:** On the 21st day the cells were harvested and subjected to cell counting using tryphan blue exclusion method and documented. The cultured RPE cells from treated and un-treated groups (Control Group and TGP Group) were subjected to H&E staining and RT-PCR analysis. The total RNA content was isolated from cultured Retinal Pigment Epithelial Cells. Gene expression for major proteins specific to the native RPE was confirmed using RT-PCR specific primers such as RPE 65 which is a RPE specific 65 kDa protein abundantly expressed in the RPE cells and Mueller cells of the retina (20) and Cellular Retinaldehyde-Binding Protein (CRALBP) which is a 36-kD water soluble protein and carries 11 cis-retinaldehyde or 11 cis-retinal as physiological ligands (21). GAPDH was used as control.

Results

The viability of the RPE tissues were well maintained after transportation in the TGP based preservation cocktail, and we could obtain an average of 0.91×10^6 cells after preservation for a duration of 12- 48 hrs and the duration of preservation did not have any statistically significant effect on the viability of the cells. The

Cells counts obtained after processing and culture for 21 days are depicted in Table-1. The RPE cell proliferation was higher in the TGP group in majority (83.3%) of the specimens (Graph 1). The TGP group had a 2.38 fold increase in cells on an average whereas the control group had a 2.17 fold increase (Graph 2). RPE Cells in TGP Group formed a monolayer, showing a typical honeycomb appearance (Fig.1) whereas in the Control Group these characteristics were not observed. The cells in the Control Group exhibited Glial cell like morphology (Fig. 2), whereas in TGP Group the cells resembled neuronal like spheres (Fig. 3). The expression of pigments was better in TGP Group compared to Control Group (Figs. 4, 5). Histologically (H & E staining), dark pigmented

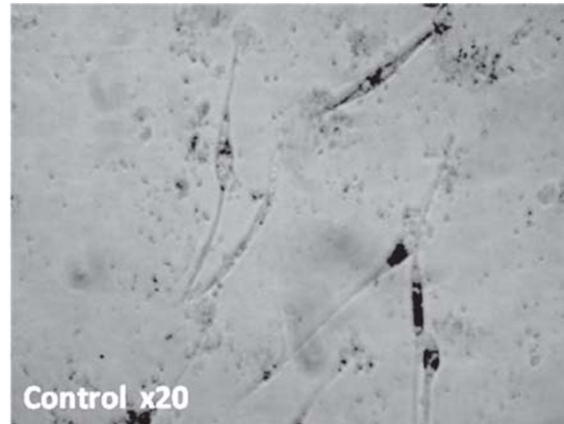


Fig. 2. RPE cells in control group shows Glial Like cells *in vitro*

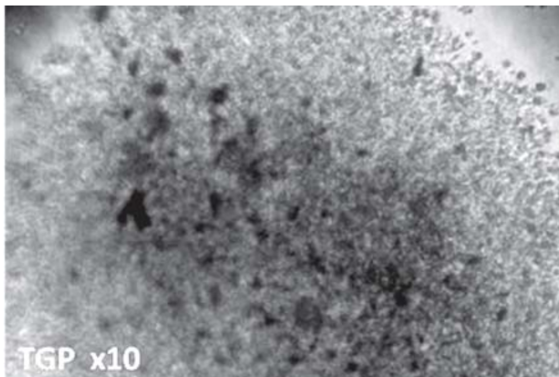


Fig. 1. *In vitro* expanded RPE cells in TGP group showing honey comb morphology

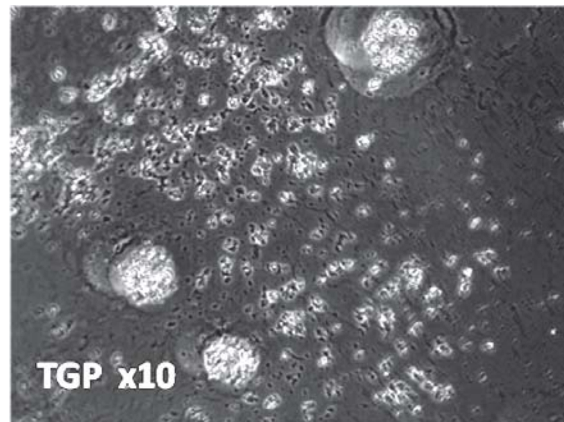
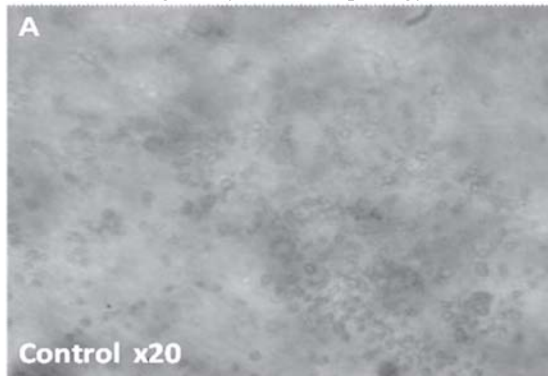


Fig.3. *In vitro* expanded RPE cells in TGP group exhibiting neuronal like spheres



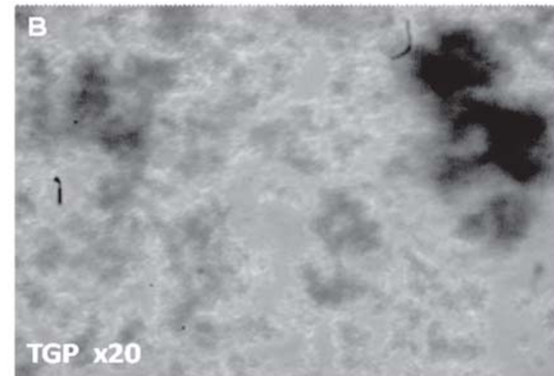


Fig. 4. RPE cells showing relatively more pigmentation in TGP group (4B) when compared with control group (4A) after 21 Days

epithelial cells were observed in both the groups, confirming the *in vitro* expanded cells to be RPE cells. Large cells with large nuclei suggestive of immature neuronal cells were observed in the TGP group (Fig. 6). RT-PCR showed the expression of the retinal pigment epithelial (RPE) cells specific markers, Cellular Retinaldehyde-Binding Protein (CRALBP) and retinal pigment epithelial 65 (RPE65) in both the groups (Fig.7).

Discussion

The World Health organization (WHO) states AMD to be the third leading cause of visual impairment with nearly 9% of the world population affected by this disorder (22) and the prevalence is expected to double by 2020 (23). Current therapeutic approaches to AMD include thermal laser photocoagulation, surgical approaches like excision or displacement, photo-dynamic therapy and more recently anti-vascular endothelial growth factor (VEGF) therapies. However all these therapies aim to minimize visual loss and optimize vision-related quality of life (14). The search of an ideal therapeutic approach that would help in regaining complete vision, still continues in which, the approach of RPE transplantation holds great promise. Autologous RPE transplantation faces hurdles such as (i) insufficient quantity of cells in the graft and (ii) risk of biological contamination with use of feeder layers, animal derived growth factors for cell expansion and biological scaffolds like amniotic membrane which have an inherent threat of contamination after

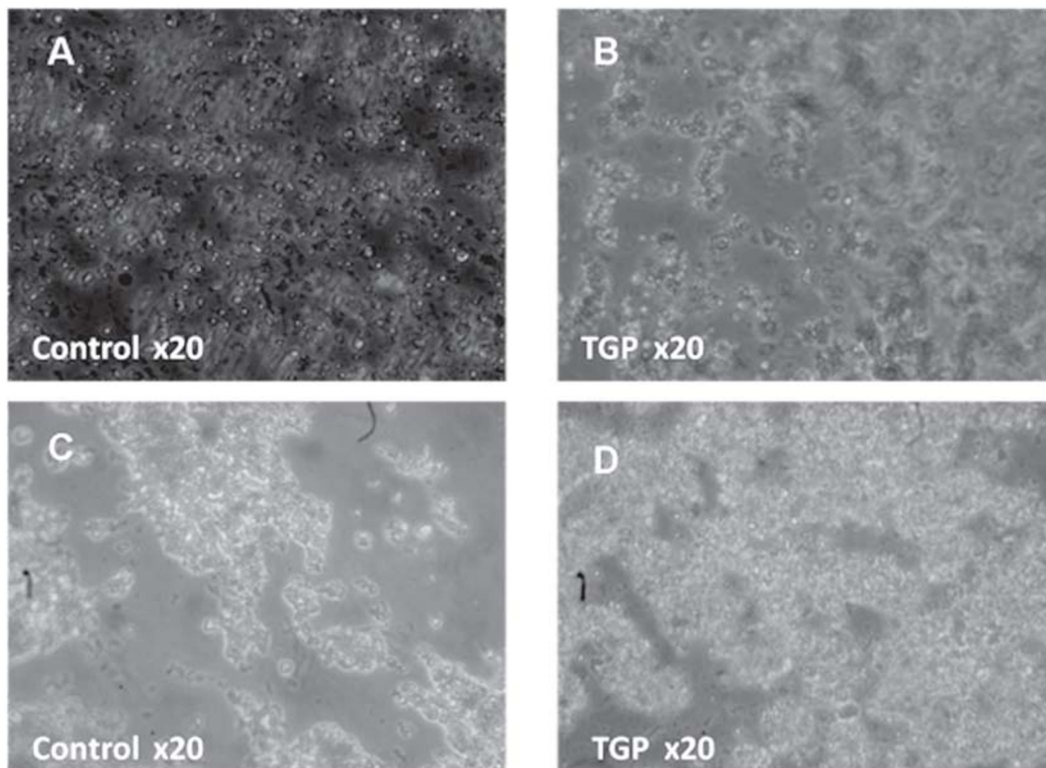


Fig. 5. Inverted microscope images of the RPE cells during *in vitro* expansion: A, B (Day 7) and C, D (Day 14)

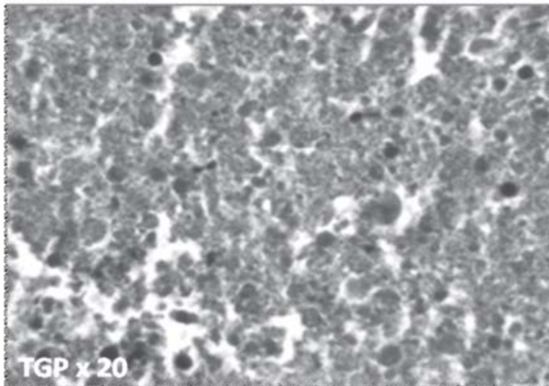


Fig. 6. H&E staining of the *in vitro* expanded cells show scattered dark pigmented epithelial cells. Large cells with large nuclei suggestive of immature neuronal cells are also seen.

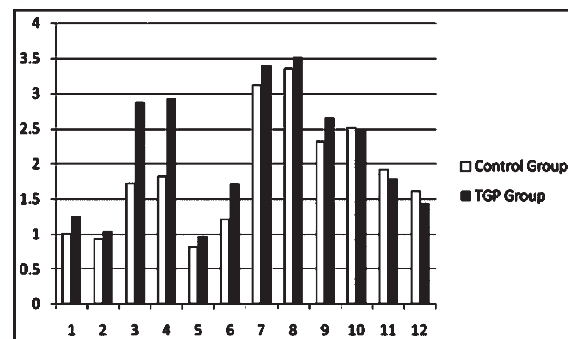
Fig. 7: RT-PCR characteristics of the *in vitro* expanded RPE Cells. Lane 1: 100 bp Ladder; Lane 2(control) : GAPDH and CRALBP ; Lane 3 (TGP) : GAPDH and CRALBP; Lane 4(control) : RPE65; Lane 5(TGP): RPE65

transplantation. To our knowledge, an ideal scaffold to support RPE cells for *in vitro* expansion and to act as a carrier for transplantation has not yet been reported. We have accomplished the successful transportation of RPE tissues in a novel Thermo-reversible Gelation polymer (TGP) based transportation cocktail from remote hospitals to a central processing facility without cool preservation under varying climatic conditions and using the same polymer as a scaffold, subsequent isolation, expansion and characterization of the human retinal pigment epithelial cells without use of growth factors.

We performed this study using TGP as a 3D scaffold for transportation and cell expansion *in vitro* because TGP has shown to support the culture of several cell types including corneal limbal stem cells (24), Hepatocytes (25),

Table 1. Cell count after *in vitro* expansion for 21 Days

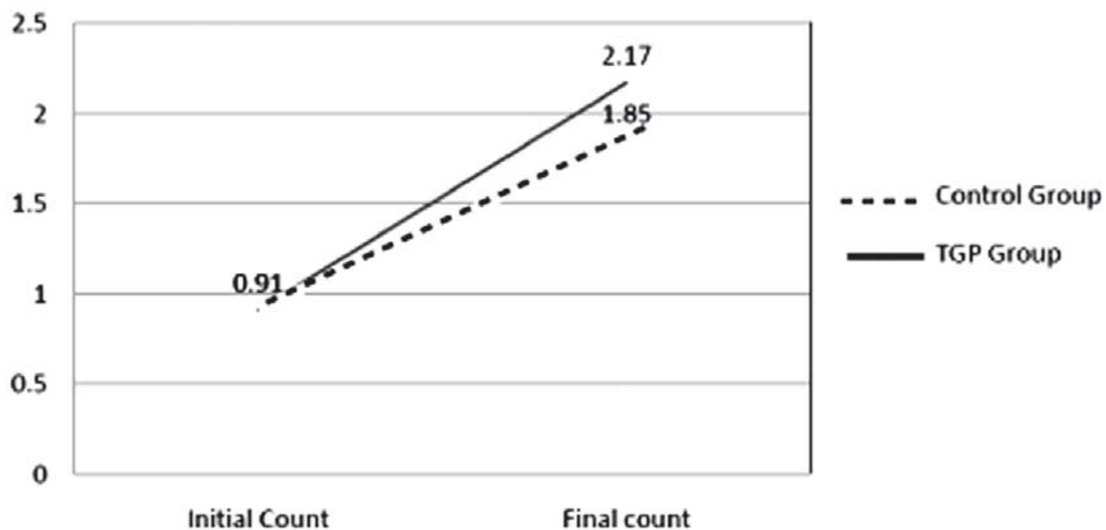
S. No	Initial cell count	Cell Count after *in vitro* expansion for 21 Days	
		Control Group	TGP Group
1	0.71	0.99	1.25
2	0.85	0.93	1.04
3	0.96	1.71	2.88
4	1.05	1.82	2.93
5	0.61	0.81	0.97
6	0.86	1.2	1.72
7	1.15	3.12	3.4
8	1.09	3.35	3.52
9	1.44	2.32	2.65
10	1.06	2.51	2.48
11	0.64	1.92	1.78
12	0.57	1.6	1.44



Graph 1. Comparison of RPE cell count after *in vitro* expansion in Control Group vs TGP Group after 21 days

Continuous Cell lines (26), chondrocytes (27), neural cells (28) etc. TGP has already been proven to preserve the cell viability of corneal endothelial cells after transportation in varying climatic conditions without cool preservation (29). In this study, this result has been replicated for RPE transportation. In addition cell viability of the RPE tissues was maintained after transportation in TGP and these cells could be expanded substantially *in vitro* which is evident by the higher fold increase of cells in the TGP group compared to control group (Graph 2). The transportation without cool preservation would prove valuable in developing nations like India where cold chain preservation in remote areas is difficult. Another major obstacle faced in the culture of RPE is the loss of phenotype or de-differentiation of the pigmented cells resulting in loss of pigmentation. This loss of pigmentation has been observed after sub-retinal transplantation of the cultured RPE too (30). In the present study, the ability of TGP to maintain the phenotype of cultured RPE was confirmed by the presence of neuronal spheres in TGP Group which were not observed in Control Group. Also the presence of intracellular pigments was greater in TGP Group cultures compared to Control Group. TGP provides a three-dimensional tissue culture environment that helps the cells to grow for longer period of time without losing their viability (31). In many studies on RPE culture, 3T3 feeder layers are employed. Johnen *et al.* reported that when RPE was cultured on 3T3 fibroblasts, RPE cells exhibited stable expression of pigment epithelial genes, but expression of mouse collagen type 1 was also observed (18) which is a disadvantage for clinical translation.

The methodology employed in the present study eliminates the need for growth factors or feeder layers. When TGP is used as a substrate for RPE cell culture and transplantation into the sub retinal space, it could be advantageous because the separation of cells from the substrate is easy. There is no need for enzymatic digestion to separate the cells due to the unique sol-gel transition property of the TGP, which reverts into



Graph 2. Comparison of Percentage of increase in RPE cell count between Control Group and TGP Group after 21 Days

liquid state when it is cooled. TGP has been previously used by Sitalakshmi *et al.* in the transplantation of corneal limbal stem cells into the eyes of rabbits (24). TGP is biologically inert (32) and biodegradable (33). Thus, TGP satisfies most of the properties that is required for successful RPE transplantation. This study has established the utility of TGP for transport of RPE tissues in a viable manner and its efficacy to support RPE tissues in culture. Further studies are needed to compare the functional characteristics and post-transplantation efficacy of allogenic RPE Vs autologous RPE transplantation cultured in TGP scaffolds in animal models before a clinical transplantation. Sub-retinal space being an immunoprivileged site, the allogenic RPE transplantation if proven to be successful without any adverse reactions would provide a simple and attractive new solution. Thereby, the RPE tissues, which are discarded once the corneal button is harvested from cadaver eye, could be an indispensable source of RPE cells for treating the AMD of many patients.

Conclusion

We have established a simple and efficient transportation modality of human cadaver donor derived RPE tissues at varying climatic conditions without cool preservation embedded in a hydrogel cocktail and a methodology for *in vitro* expansion of the RPE cells without using any growth factors. RPE cells showed better growth characteristics in TGP even after transportation at varying climatic conditions for 48 hours suggesting that TGP can be used as a suitable substrate for both transportation and *in vitro* expansion. The *in vitro* expanded RPE cells using TGP could be a potential source of RPE transplantation procedures for treating Age related macular degeneration after confirmation of their functional efficacy in animal models.

Acknowledgement

The authors thank M/S Hope Foundation (Trust), India for funding the study, M/S. Chennai Cell Cluster for technical advice Ms. Eiko Amemiya for her secretarial assistance

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Proteolytic Enzyme Production by Isolated *Serratia* sp RSPB11: Role of Environmental Parameters

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Abstract

An effective proteolytic enzyme producing microbial strain has been isolated from marine habitats and evaluated its extracellular protease production properties with respect to different fermentative physiological parameters. The strain has been identified based on biochemical tests according to Bergey's Manual of Systematic Bacteriology as *Serratia* sp and designated as RSPB11. This strain has potential to hydrolyze chitin, gelatin and casein revealing its industrial potential for production of multi-enzyme complex. Since the isolated strain belongs to *Serratia* genus, the protease produced by this strain is considered as serralysin and which is not inhibited by PMSF suggesting the enzyme belongs to other than serine type of protease. Further analysis denoted that this enzyme belongs to metalloprotease which is confirmed based on negatively regulation of caseinolytic (proteolytic) activity by EDTA. The maximized enzyme production occurred at medium initially adjusted to pH 7.0 incubated at 33°C under aerated environment. Analysis of the pH profile before and after fermentation depicted that irrespective of initial medium pH, it is shifted to pH 9.0 after fermentation suggesting the enzyme produced is alkaline in nature.

Keywords: Enzyme, Fermentation, Isolation, Protease, *Serratia* sp.

Introduction

Proteases/ proteinases are a group of hydrolytic enzymes (catalyse the reaction of hydrolysis of bonds with the participation of a water molecule) which are specific for digestion of proteins in to peptides and amino acids. Proteases differ in their ability to hydrolyze various peptide bonds hence, specificity associated with each enzyme differs based on catalytic site. Several classification systems currently available, provides rich and vast information about each and every identified protease. These schemes can be categorized under 4 major categories based on the characteristic features like: pH (acidic/neutral/ alkaline), peptide bond specificity (endo/exo peptidases), functional group present at active site (serine/ cysteine/ aspartic/ metalloproteases) of proteolytic activity with respect to functional group present at the active site. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases.

Most commercially available proteases belong to the class serine proteases, produced

by organisms belonging to the genus *Bacillus, Apergillus* and *Streptomyces*, Alkaline proteases are more preferable at industrial scale compared other acidic proteases. Metalloproteases are those enzymes whose catalytic mechanism involves a metal which plays an important role in pathogenesis hence have advantage in health care sector. Most of pharma related metalloproteases are dependent on zinc (1) and a few uses cobalt, iron, manganese, etc (2).

Serratiapeptidase is a type of metallo-protease (EC 3.4.24.40) originally isolated from digestive system of silkworm (3). Subsequently, this enzyme also reported from different bacterial strains including *Pseudomonas* auri *Serratia marcescens*, *Proteus mirabilis* and *Escherichia freundii* (4). Functionally this enzyme breaks peptide bond of non-terminal amino acids under alkaline environment hence also referred as alkaline-endopeptidase. Serrapeptidase differs from other metalloend-opeptidases in the catalytic reaction, where the enzyme preferentially cleaves the peptide bonds associated with hydrophobic residues (5).

Initially this peptidase production was noticed during infection by *Serratia* sp. as well as other bacterial strains such as *Pseudomonas aeruginosa* or *Erwinia chrysanthemi*. The physiological function of serratia peptidase in these microbial strains is yet to be identified, however the enzyme seems to play a significant role in nutrient digestion/uptake in these bacterial strains (6). Maeda and coworkers reported that this protease plays a critical role in pathogenesis of *Serratia marcescens* (4). Interestingly, this enzyme production is mostly reported from clinical isolates (4, 7) however, reports from marine microbial strain YS-80-122, *Pseudomonas* sp., also noticed in the literature (8, 9).

Much attention has been focused on alkaline metalloprotease production by species of *Serratia* especially *Serratia marcescens* due to its potential for higher enzyme yields compared to literature reports and has been used as an anti-inflammatory agent all over the world (10). Alkaline metalloprotease production from different microbes has been evaluated by various researchers with respect to enzyme regulation and excretion mechanisms (11, 12), characterization and purification (10, 13), and genetic analysis of these enzymes (14, 15). Efforts also have been made to improve the economics of the bioprocess by using non conventional media components such as whey powder or squid pen powder (16-18). At present, Serratiapeptidase, a major alkaline metalloprotease is commercially produced with a SMP-overproducing mutant of *S. marcescens* ATCC-21074 (19).

Analysis of biochemical and biocatalytic properties of alkaline metalloprotease produced by different microbial strains revealed variation in specificity of action, metal component, optimal pH, temperature, etc (10) which influences biotechnological application specificity. This, in addition to the high price for this enzyme in the market are some of the powerful appeals that lead to search for new protease producing sources and subsequent bioprocess development. Microbial enzyme production is highly influenced by media components like carbon and nitrogen sources besides several other factors such as aeration/agitation, pH and temperature, salinity and incubation time (20, 21, 22). Hence, the present study focused on isolation of a bacterial strain with high protease production property, growth characterization, and enzyme production properties in addition to identification. The data indicated that the isolate belongs to *Serratia* sp and has higher enzyme titers compared to literature reports therefore this

isolate could be effective strain for industrial production. This strain's protease production is highly regulated by chelating agents, incubation temperature, hydrogen ion concentration in the medium, and incubation temperature.

Materials and Methods

***Screening of chitinase and protease producing microbes*:** Soil samples collected from marine environment (contaminated with sewage from fish processing plants) located near Bapatla, Andhra Pradesh, India were used in this study. A serial dilution method has been followed after enrichment technique for isolation of microbial strains. In brief, one gm of soil sample was added to sterile 100 ml media containing mineral salts ($(NH_4)_2SO_4$ 0.1 g, KH_2PO_4 0.02 g, NaCl 0.5g, $MgSO_4$ 0.05g) and 5gm dried shrimp as well as crab shell powder followed by incubation for 48h at 30°C. After serial dilution, the obtained samples were spread over chitin agar plate (Colloidal Chitin 1.5 g, mineral salts, agar 20 g, distilled water 50 ml, sea water 50 ml, pH 7.0) and incubated for 5 days at 30°C. A clear- zone forming bacteria were selected and inoculated on 2% casein and 1% gelatin agar plates at pH 7.0 for identification of protease production. After incubation for 2 days at room temperature, clear hydrolytic zone forming bacterial strains were selected for further studies and maintained on agar slants.

***Biochemical and phenotypic characterization*:** Selective isolate was identified through its biochemical and physiological properties according to Bergey's Manual of Systematic Bacteriology (23).

***Scanning Electron Microscope analysis (SEM)*:** SEM was used to investigate the morphology of isolated strain. The sample for SEM was prepared by transferring the microbial strain to a clean eppendorff tube containing approximately 1.5 ml of 3.5% glutaraldehyde solution. Then, culture was incubated for 4 h at room temperature followed by wash with phosphate buffer (100 mM, pH 7.2). The culture is then dehydrated using alcohol gradient from 10 to 100%. The dehydrated samples were then air dried and fixed on the stubs using double adhesive tape. A thin layer of gold was coated over the sample using HUS-5GB Hitachi vacuum evaporator for 90 sec. These samples were then observed under scanning electron microscopy (Hitachi S- 3000N, Japan) at various magnifications at acceleration voltage of 10.0 KV.

Production media and culture conditions : One of the bacterial isolate designated as RSPB11 was maintained regularly on nutrient agar slants and used in this study. For enzyme production, yeast extract – peptone media consisting (%, g/100ml) of yeast extract – 1.0, Peptone – 1.0, Dextrose - 0.2, $MgSO_4$ - 0.02, KH_2PO_4 - 0.02, NaCl -0.02 at pH 7.0 was used. Inoculum (OD_{600nm}~1.5) was developed by growing the isolate in nutrient broth for 18h. For production of enzyme, 1.0% inoculum was added to 50ml production medium in 250ml conical flasks and then incubated at 30°C for 3-4 days. Samples withdrawn at specific time intervals were centrifuged at 10,000 rpm for 10 min and the supernatant has been used as enzyme source for assay. All the culture conditions were same unless otherwise mentioned.

***Protease assay*:** Protease activity was assayed according to Anson method and was slightly modified (24). The reaction mixture contained 2.5 ml of 0.65% Hammerstein casein and 0.5 ml of appropriately diluted enzyme in the presence of 50 mM Glycine NaOH buffer pH 9.0. The reactants were incubated at 37°C for 10 min and the reaction was stopped by adding 2.5 ml of 110 mM trichloroacetic acid (TCA). A suitable blank was run simultaneously, in which TCA was added to the enzyme solution, followed by

Vol. 6 (1) 55-65 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

substrate addition. After incubating at room temperature for 30min both test and blank solutions were centrifuged at 10,000g for 10min. To the 0.4ml supernatant, 1.0ml 50mM Na_2CO_3 and 0.2ml Folin-ciocalteau reagent was added, the reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 660nm. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions.

***Optimization of culture conditions for the bacterial growth and the protease production by isolated strain*:** To select the optimum pH, temperature, aeration and agitation, enzyme production was investigated at different pH environments (pH 5.0-9.0), at different temperatures (27°C-40°C), aeration conditions with respect to volume of media in 250ml conical flasks (25ml-125ml) and speed of agitation from static to 200 rpm, respectively in separate flasks. The samples were collected every 24 h for 72h to measure the enzyme activity.

Results and Discussion

***Isolation and screening of protease producing microbes*:** Various soil samples collected from marine environment were used for isolation of effective protease producing microbe. Chitin in the exoskeleton of shrimp and crab shells is associated intimately with proteins therefore for the isolation of chitionolytic and proteolytic bacteria, chitin utilization in the screening media offers a great advantage (18, 25). Therefore, enriched microbial population from the shrimp and crab shell media has been spread over chitin agar plate and incubated for 5 days until the visibility of hydrolytic zones. Selected microbes from the chitin agar plates were again spot inoculated on casein agar plate containing 1mM PMSF (serine protease inhibitor) and gelatin agar plate. After 2 days colonies showing maximum proteolytic zones were picked and purified over nutrient agar plates. Among the 10 potent chitinase producers only 3 microbes showed high casein hydrolytic zones, revealing that these isolated strains were not serine protease producers. This is concluded based on the fact that these colonies are showing the proteolytic activity in presence of PMSF which binds specifically to the active site serine residue in a serine protease leading to inactivation of serine proteases (26). As our protease of interest is metalloprotease due to their pharmaceutical importance, addition of PMSF in the casein agar plate during the screening of microbes helped in differentiating serine protease producers from other class of protease producers. All three

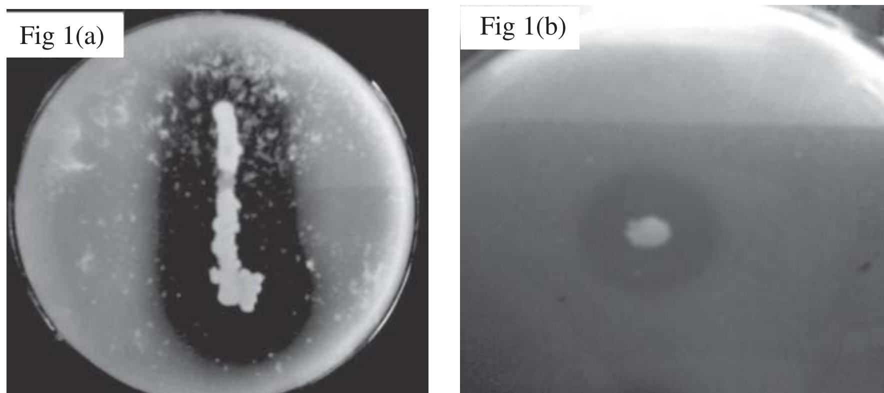

Fig 1. Petri plates showing the marine isolate with a potential to produce extracellular protease (a) and chitinase (b)

isolates were named and sub cultured in nutrient broth. Based on the larger hydrolytic zones on chitin (Fig 1a), casein (Fig 1b) agar plates and protease assay, one isolate designated as RSPB11 was selected for further studies.

***Biochemical, phenotypic and morphological characteristics of the RSPB11*:** Biochemical and physiological properties of RSPB11 was identified according to Bergey's Manual of Systematic Bacteriology (2). Table 1 indicates the isolated strain physical properties and Table 2 denote biochemical characterization tests and their results. Colonies of RSPB11 on nutrient agar plates were white, round with smooth and glossy surface as well as slightly opaque in nature. The isolate is characterized as aerobic, gram negative and rod shaped bacteria. Fig 2 shows the scanning electron microscope picture of the isolate and cells were visulaized as single, short rod shaped bacteria. Physiological tests show that the cells could survive and grow in the medium pH ranging from 5.0 to 11.0 and under saline conditions of 3.0% NaCl. From the biochemical tests, it was concluded that this isolate RSPB11 belong to Enterobacteriace family, and it is a member of *Serratia* genus. This genus is characterized with ten species (strains) distributed in two sub species (27).

***Optimization of process parameters for protease production*:** Protease production by the isolate, *Serratia* sp RSPB11, was noticed at 12 hours of growth and enzyme production reached to the maximum level after 48h of cultivation. Growth of the isolate, monitored by taking the absorbance of media against blank at 600nm at specific time intervals shows a gradual increase in biomass production. Protease production was also noticed in correlation with biomass production (Fig 3) but a notable protease activity has been observed after 24h. This data suggested that the protease production by this strain is growth associated. This is further supported by the fact that a constant protease activity was observed during stationary phase. The results further suggested that improved level of protease production could be possible with high active biomass production.

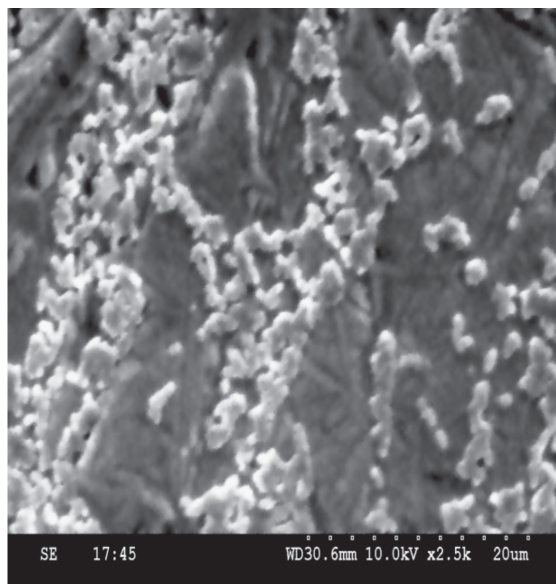

Fig 2. Scanning electron micrograph of marine isolate *Serratia* sp. RSPB11

The impact of incubation time as well as other physical parameters like initial pH, temperature, and agitation on protease production by isolated *Serratia* sp RSPB11 was investigated. Initial pH of the media was adjusted with either 1M HCl or 1M NaOH for attaining desired pH conditions. From the results it is clear that initial pH of 7.0 supported the growth of bacterium, where a maximum biomass and enzyme activity (5230U/ml) was noted (Fig 4). Analysis of the pH of the fermentation medium at specific time intervals indicated a gradual rise in pH and reached alkaline condition (pH ~ 9.0) after 48h (data not shown) indicating that this enzyme is an alkaline protease. Media adjusted with different pH conditions (pH 5.0, 6.0, 8.0,

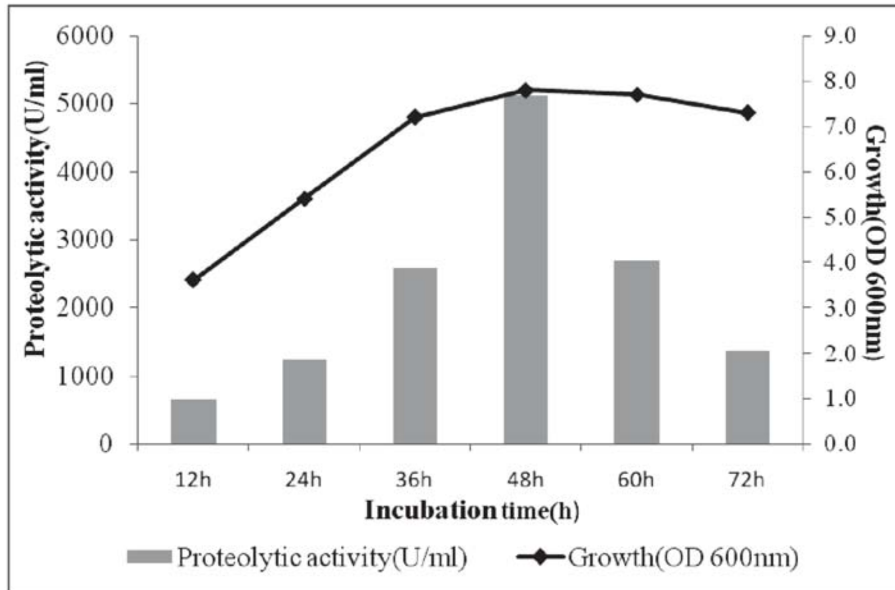

Fig 3. Effect of incubation time on growth and protease production by the marine isolate *Serratia* sp. RSPB11

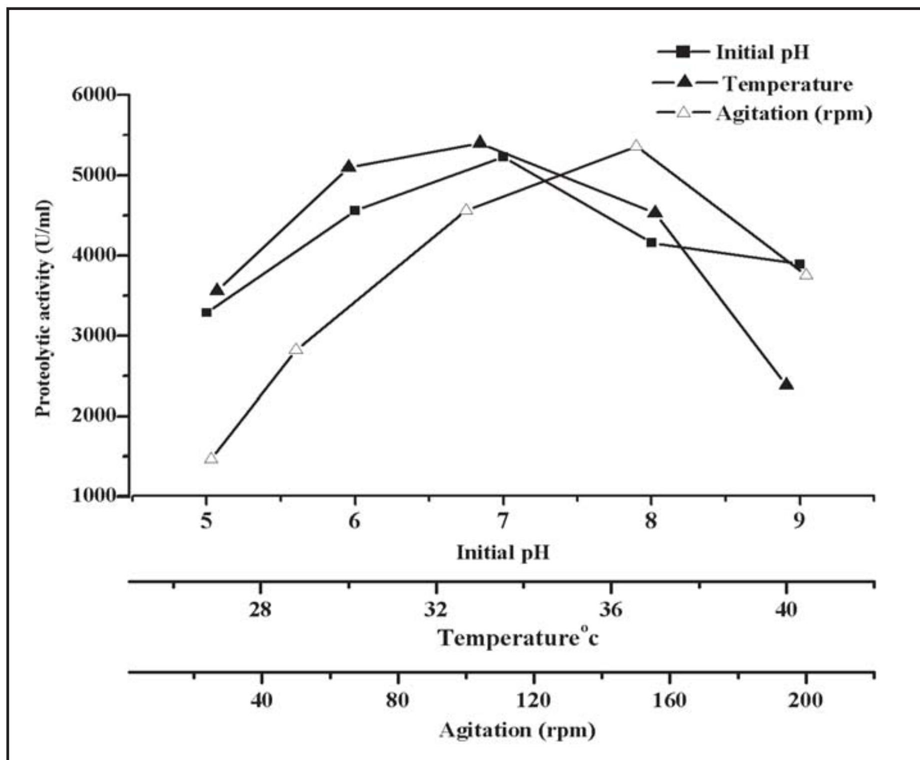

Fig 4. The effect of pH, temperature, and speed of agitation on production of protease by the marine isolate *Serratia* sp. RSPB11

9.0) also reached a final pH ~ 9.0 at the end of the fermentation, but the enzyme production was low at other than pH 7.0 conditions. This increase in pH value along with fermentation process hence this protease production bioprocess may be attributed to ammonia produced as a consequence of the aminoacids catabolism released by protein hydrolysis by produced protease enzyme as reported (28). pH dependent protease production by different microbial strains were also observed by several investigators, where the initial pH 6.0 (29) to 8.0 (30) could support the increase in biomass as well as production of protease. The above data also depict that higher yields may be effectively obtained by constant maintenance of pH of the medium during fermentation process as noticed by Venil, (31) and Panasuriya (32). Efforts are being made in this direction.

Among the physical factors, temperature is one of the most critical parameters that could affect the bioprocessing. To evaluate the same, media inoculated with *Serratia* sp RSPB11 has been incubated at different temperatures ranging from 27°C to 40°C. The enzyme production has been noticed in all tested temperature conditions suggesting at this temperature range the isolated *Serratia* sp. RSPB11 survives and produces metabolism linked protease production. Though protease production noticed all most all studied environments higher production was noticed at 33°C (5400U/ml). A very low enzyme activity of 2385U/ml observed at 40°C (Fig 4). Several studies revealed that temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane (33). The optimum temperature of *Serratia* sp RSPB11 was slightly higher than *Serratia marcescens* NRRL B-23112 at 25°C (32), *Serratia* sp DT3 at 28°C (29), and lower than *Serratia marcescens* sp7 at 40°C (30), *Pseudomonas aeruginosa* MTCC 7926 metalloprotease at 40°C (13). A temperature range of 30-37°C has been employed in several works (17, 34).

In general, it is well known that all organisms and microbes vary in their aeration requirement. In particular, during aerated environment, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks and also affect the nutrient availability (35). In view of the above, speed of agitation was studied by incubating the flasks on orbital shakers at various rpm (25-200 rpm) conditions. The data depicted that fermentation processed at 25 rpm, a very low production (1460U/ml) of protease was noted and a maximum production of 5363U/ml observed at 150rpm. This data denote that this isolate needs a particular speed of agitation for achieving the maximum biomass along with the production of enzyme.

Partial characterization of protease produced by isolated Serratia sp. RSPB11 : To evaluate the nature of proteolytic enzyme produced by isolated *Serratia* sp. RSPB11, the enzyme activity studied in the presence of 1 mM EDTA to know whether this enzyme belongs to metalloprotease family. Use of 1mM EDTA in the reaction medium inhibited the proteolytic activity of the produced enzyme, indicting negative regulation of protease activity by this chelating agent. The result suggested that the enzyme produced by isolated *Serratia* sp. RSPB11 belongs to metalloprotease. This was further confirmed from the literature reports where protease activity was observed to be inhibited by a metalloprotease inhibitor like EDTA (36). To evaluate further the nature of enzyme, the enzyme activity was measured in terms of zone of inhibition in the presence of

PMSF (results not shown). This further confirmed that this enzyme is not belonging to serine type of protease as reported in other proteases (37).

The protease produced by isolated microbial strain is considered as one of the serralysin type of protein. This is confirmed based on the following observations- a) The isolated strain belongs to genus *Serratia*, b) The produced proteolytic enzyme is negatively regulated by EDTA, c) all serralysin type proteases are metalloproteases and not belongs to serine type of proteases, d) The proteolytic enzyme production is observed in presence of serine protease inhibitor, PMSF and e) The enzyme production is maximum at alkaline environment, hence this protease does not belongs to aspartate type.

Conclusion

In the present investigation, a serralysin type of protease producing microbial strain has been isolated using marine soil samples. The strain has been purified and characterized in terms of its biochemical and physiological growth properties. Based on biochemical tests, the isolate has been identified up to genus level and observed that this strain belongs to *Serratia* sp. Extracellular enzyme production properties were studied and observed that this strain produces more than one extracellular enzymes; such as chitinase and gelatinase depending up on the nutritional conditions. This strains ability towards protease production and since the strain belongs to *Serratia* sp. hence, the produced protease is considered as serralysin type of protease which is known for its commercial importance. In this context, the protease production was investigated further in terms of optimal requirements for physiological growth factors. The optimized protease production has been noticed at a physiological pH of 7.0 and at temperature 33°C as well as at 150 rpm therefore, these parameters are crucial for effective production yields.

Acknowledgement

One of the authors, P.Lakshmi Bhargavi is greatly indebted to Council of Scientific and Industrial Research, New Delhi for financial support in the form of Senior Research Fellowship.

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Vol. 6 (1) 66-72 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

In vitro regeneration of *Capsicum chinense* Jacq.

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Abstract

An efficient *in vitro* regeneration protocol was developed for *Capsicum chinense* Jacq. cv. 'Chiengpi' using shoot-tip and axillary shoot explants. Shoot-tip explants proliferated shoot buds in Murashige and Skoog (MS) medium supplemented with 5 or 10 mg/l 6-benzylaminopurine (BAP). The regenerated shoot buds showed rooting on medium containing 0.5 mg/l IAA. Axillary shoots were induced in the rooted plantlets by decapitating them and multiple shoots were further induced from the axillary shoot explants in medium containing BAP alone or in combination with IAA. Maximum shoot bud proliferation from the axillary shoot explants occurred in medium supplemented with 5 or 10 mg/l BAP. The proliferated shoot buds also showed rooting and elongation on medium containing 0.5 mg/l IAA. Rooted plantlets were successfully established in the soil.

Keywords: axillary shoot culture, *Capsicum chinense*, decapitation, shoot-tip culture.

Introduction

Chillies are the fruits of the genus *Capsicum* belonging to the Nightshade family, Solanaceae. The genus *Capsicum* consists of about 25 wild and 5 domesticated species. The five domesticated species are *Capsicum annuum* L., *Capsicum frutescens* L., *Capsicum chinense* Jacq., *Capsicum baccatum* L., and *Capsicum pubescens* R & P.(1). Chillies have been forming a part of the human diet since the beginning of civilization in the western hemisphere from about 7500 B.C. (2). Chillies contain numerous chemicals including steam-volatile oil, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fibre and mineral elements (3) and are used in a wide assortment of foods, drugs, and cosmetics. Besides the above-mentioned uses, chillies also have medicinal uses. The medicinal use of chillies has a long history, dating back to the Maya and Aztec tribes of ancient America who used them to treat asthma, coughs and sore throats (4). The medicinal value of *Capsicum* is well-recognized today and it is included in the American Illustrated Medical Dictionary, the Merck Manual and Materia Medica, where it is referred to as a rubefacient, local stimulant and diaphoretic (5). Recently, several studies have also demonstrated anti-cancer or anti-mutagenic effect of chilli extracts (6-8).

Capsicum chinense Jacq. cv. 'Chiengpi' is an indigenous chilli cultivar cultivated in different parts of Manipur. The fruits of the cultivar are pungent with a characteristic aroma and flavour and both fresh and dried fruits of

this cultivar are used as hot spice. Although the plants of this cultivar are perennial and persist for 2-3 years, the production decreases successively from the first year and requires new planting material every year for optimum production. The production potential of this cultivar does not often show the achievable targets due to various reasons, and to meet the increasing demand for the crops, faster propagation techniques for mass multiplication has become necessary. Since the plants also lack natural vegetative propagation tissue culture methods provide a novel way for the asexual multiplication of these chilli pepper plants.

In *Capsicum*, several procedures are available for inducing *in vitro* plant regeneration using different explants (9-27). However, several of these reports suggest a strong influence of genotype on the regeneration process (13,15,16,19). Moreover, *Capsicum* tissue culture is mostly confined to the more common species, *Capsicum annuum* L. and there has been no report for the *in vitro* plant regeneration of *Capsicum chinense* Jacq. Therefore, the present study was undertaken to develop efficient *in vitro* plant regeneration protocol for the economically important chilli cultivar.

Materials and Methods

Seeds extracted from fresh and healthy ripe fruits collected from local cultivation fields were used for initiation of *in vitro* cultures. The seeds were first washed with tap water and treated with 0.1% *Dhanustin* (carbendazim 50% w/w) for 10-15 min followed by washing with distilled water. The seeds were then surface sterilized under aseptic conditions with 0.1% $HgCl_2$ solution for 5 min followed by several washes with sterile distilled water. The surface-sterilized seeds were inoculated in 250 ml flasks containing sterile filter papers soaked in sterile distilled water and incubated in the dark for 7-10 days at 25±2 °C. After germination, the seeds were transferred to culture tubes containing Murashige and Skoog (MS) (28) basal medium. Shoot-tip explants (1-1.5 cm long shoot apices) were derived from four week-old *in vitro* germinated seedlings and inoculated on shoot bud multiplication medium consisting of MS basal medium supplemented with different concentrations of cytokinins, 6-benzylamino-purine (BAP) or kinetin (Kin) alone or combinations of BAP with indole-3-acetic acid (IAA). The number of shoot buds was counted after four weeks. The elongated shoot buds (about 2 cm long) proliferating from the shoot-tip explants were excised and cultured in 250 ml flasks containing 70 ml of MS medium supplemented with concentrations of 0.5 or 1 mg/l of IAA, indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) for rooting of the shoot buds. The percentage of rooting, number of roots (including the main roots and laterals), shoot length and the length of the roots were recorded after six-weeks of culture.

For induction of enhanced axillary shoot development, the axillary shoots were induced on four week-old rooted plantlets. These plantlets having 5-9 leaves were decapitated for inducing axillary shoot development by cutting the tips with a sterile blade. Axillary shoots developing in the axils of leaves of the decapitated plantlets were used for further multiple shoot bud induction by culturing on medium containing BAP alone or in combination with IAA and the number of shoot buds were counted after six weeks. The shoot buds elongating from axillary shoot-tip explants were excised and cultured on rooting medium consisting of MS medium supplemented with 0.5 mg/l IAA or IBA. All cultures were maintained in a growth chamber at a temperature of 25±2 °C and 16-h photoperiod provided by white fluorescent tubes (30 µmol $m^{-2}s^{-1}$).

The rooted plantlets were gently removed from the flasks and the roots were washed in tap water to remove traces of agar. The plantlets were then transplanted in perforated paper cups containing sand: soil (1:1) and kept covered with clear polythene bags having a few holes on it for the initial 10 days. The plantlets were kept in a 50% shaded net-house and watered daily with tap water to maintain high humidity. After 10 days, humidity was gradually decreased by increasing the size of holes in the polythene bags and the polythene bags were finally removed. Four week-old hardened plants were then transplanted to bigger earthen pots or to the field.

All the experiments were repeated thrice and each treatment for shoot bud induction and rooting of the shoot buds consisted of ten replicates. Data on multiple bud induction and rooting were analyzed by Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test.

Results

After 2-3 weeks of culture on the shoot bud multiplication medium, about 2-8 multiple shoot buds developed from the shoot-tip explants derived from *in vitro* germinated seedlings. The effect of growth regulators in shoot bud multiplication from shoot-tip explants is shown in Table 1. Maximum proliferation of shoot buds occurred on medium containing 5 or 10 mg/l BAP (Fig. 1a). The frequency of shoot buds obtained on MS medium containing Kin alone was low (1 to 3) with explants showing little or no growth followed by browning and therefore were not used for further experiments. When the regenerated shoot buds (about 1 cm long) were separated and transferred to MS medium supplemented with IAA, IBA or NAA, rhizogenesis occurred followed by elongation of the shoots (Fig. 1b). IAA and IBA were found superior to NAA with respect to the induction of roots and 42-100% rooting efficiency was recorded for the cultivar (Table- 2). On NAA containing medium, the roots produced were thick and short with fine root hairs while on medium containing IAA or IBA, the roots were thin and long with branches and root hairs. Best rooting occurred on medium containing 0.5 mg/l IAA or IBA and maximum shoot elongation occurred on medium supplemented with 0.5 mg/l IAA or 1 mg/l IBA (Table- 2).

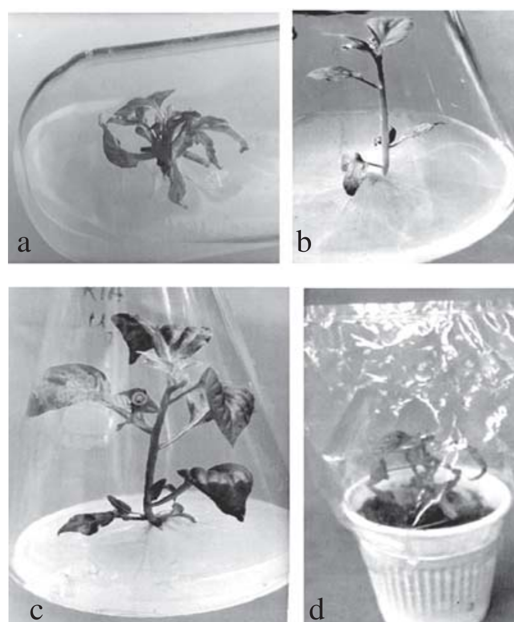

Fig. 1. *In vitro* plant regeneration of *Capsicum chinense* Jacq. cv. 'Chiengpi': a) shoot bud multiplication from shoot-tip explant; b) rooting of shoot buds; c) axillary shoot bud induction by decapitation of rooted plantlet; d) hardening of plantlet in plastic pots.

In the next set of experiments, the effect of decapitation on enhanced axillary branching in the rooted plantlets and the effect of growth regulators on the multiplication of shoot buds from the axillary shoot-tip explants were studied. The decapitated plantlets showed the development of axillary shoots/branches within

Vol. 6 (1) 66-72 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 1. Effect of growth regulators on multiple shoot bud multiplication from shoot-tip explants of *Capsicum chinense* Jacq. cv. 'Chiengpi' after four-weeks of culture

Growth regulators (mg/l) BAP	IAA	Mean number of shoots per explant (mean ± S.E.) 'Chiengpi'
2	-	1.2 ± 0.13^{c}
2	1	1.3 ± 0.21^{c}
2	2	1.1 ± 0.10^{c}
5	-	3.3 ± 0.5^{4b}
5	1	1.4 ± 0.22^{c}
5	5	1.2 ± 0.13^{c}
10	-	4.5 ± 0.43^{a}
10	1	2.6 ± 0.45^{b}
10	5	1.3 ± 0.21^{c}

Means followed by the same letters are not significantly different at $P < 0.01$

Table 2. Effect of auxins on rooting and elongation of *in vitro* induced shoot buds from shoot-tip explants of *Capsicum chinense* Jacq. cv. 'Chiengpi' after six-weeks of culture

Auxins (mg/l)			Rooting	Shoot length (cm)	No. of roots	Root length (cm)
IAA	IBA	NAA	(%)	(mean ± S.E.)	(mean ± S.E.)	(mean ± S.E.)
0.5	-	-	100	$2.5^{a} \pm 0.28$	$21.2^{b} \pm 1.29$	$6.3^{a} \pm 0.31$
1	-	-	50	$1.4^{b} \pm 0.25$	$5.7^{cd} \pm 1.93$	$2.1^{b} \pm 0.72$
-	0.5	-	100	$1.2^{b} \pm 0.16$	$28.4^{a} \pm 1.17$	$5.3^{a} \pm 0.49$
-	1	-	60	$2.7^{a} \pm 0.46$	$7.7^{cd} \pm 2.16$	$2.2^{b} \pm 0.63$
-	-	0.5	100	$1.3^{b} \pm 0.17$	$10.8^{c} \pm 0.81$	$1.0^{bc} \pm 0.14$
-	-	1	40	$0.6^{b} \pm 0.08$	$2.4^{d} \pm 1.06$	$0.2^{c} \pm 0.07$

Means followed by the same letters are not significantly different at $P < 0.01$

Table 3. Effect of growth regulators on multiple shoot bud multiplication from axillary shoot explants of *Capsicum chinense* Jacq. cv. 'Chiengpi' after six-weeks of culture

Growth regulators (mg/l) BAP	IAA	Number of shoot buds/explant (mean ± S.E.)
2	-	$1.1^{d} \pm 0.10$
2	1	$1.2^{d} \pm 0.13$
5	-	$3.1^{b} \pm 0.18$
5	1	$1.7^{cd} \pm 0.21$
10	-	$3.9^{a} \pm 0.31$
10	1	$2.1^{c} \pm 0.23$

Means followed by the same letters are not significantly different at $P < 0.01$

two weeks of culture. About 3-6 young shoots per plantlet were formed within two weeks of culture (Fig. 1c). On culturing the axillary shoots with a few leaf primordia in bud induction media, these proliferated to produce multiple shoot buds. The maximum proliferation of shoot buds from the axillary shoot-tip explants occurred on medium containing 5 or 10 mg/l BAP (Table-3). The proliferated shoot buds also showed rooting and elongation on medium containing 0.5 mg/l IAA. The regenerated plants showed 80-90% survival during hardening (Fig. 1d) and acclimatization and there were no morphological variations between the parent plants and *in vitro* raised plants. The transplanted plantlets were established well in pots and later in the field.

Discussion

The effectiveness of different combinations of BAP and IAA in inducing shoot buds from different explants of *Capsicum* was reported in earlier studies (9,10,12,14,16,19,20). Therefore, *in vitro* culture response of shoot-tip and axillary shoot explants of the cultivar cultured on MS medium supplemented with various concentrations of cytokinins (BAP or Kin) and combinations of BAP with IAA have been investigated. Proliferation of multiple shoot buds from shoot-tip explants of *Capsicum* were reported in limited cases (15,24-26) and since *in vitro* clonal propagation via meristem culture is one of the ways for producing large number of disease-free plantlets, protocol for *in vitro* clonal propagation of the cultivar was developed using shoot-tip explants. The maximum proliferation of shoot buds occurred on medium containing 5 or 10 mg/l BAP. Similar effectiveness of MS medium containing BAP alone in inducing multiple shoot buds in chilli tissue culture was reported earlier (12,15,17,18). MS medium containing Kin alone was found to be the least effective between the two cytokinins (BAP and Kin) tested. Such ineffectiveness of Kin in shoot bud induction from chilli tissue culture has also been reported earlier (9,10,12). The shoot buds derived from the shoot-tip explants rooted efficiently on MS medium containing 0.5 or 1 mg/l IAA or IBA. Similar effectiveness of IBA and IAA on rooting of *in vitro* regenerated chilli plantlets was reported by several workers (9,12,15-17,22,23,26). Studies also reported higher effectiveness of NAA in inducing rhizogenesis of the regenerated shoots in *Capsicum* (21,29,30). However, in the present study, NAA was found to be less effective for root induction.

Earlier, we reported axillary shoot proliferation (up to 5) from *in vitro* raised chilli seedlings by decapitation (27). The response of axillary shoo-tip explants to bud multiplication media containing different concentrations and combinations of growth regulators were identical to the shoot-tip explants. Since the shoot buds derived from the shoot-tip explants rooted efficiently on MS medium containing 0.5 or 1 mg/l IAA or IBA, the buds derived from the axillary shoot-tip explants were also rooted on medium containing 0.5 or 1 mg/l IAA or IBA.

Thus, by inducing multiple shoots from the shoot-tip explant of a seedling followed by *in vitro* induction of axillary shoots from the regenerated plantlets and further induction of multiple shoot buds from the axillary shoot explants, it is possible to produce large number of plantlets from a single seedling. This technique, therefore, presents an efficient system of *in vitro* clonal propagation for conserving and mass multiplication of the chilli cultivar.

Acknowledgement

One of the authors (K.S.) is grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi for the award of a Senior Research Fellowship (*vide* No. 9/476(26)/2K3-EMR-1).

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Vol. 6 (1) 66-72 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

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Vol. 6 (1) 73-77 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Molecular Characterization of Antibiotic Producing Bacteria *Pseudomonas sp*.BP-1 from Nagavali river basin of Srikakulam, Andhra Pradesh, India

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Abstract

The *Pseudomonas* sp.BP-1 strain (NCBI gene accession number HM359121) was isolated from Nagavali river basin of Srikakulam, Andhra Pradesh India. The strain is capable of inhibiting the growth of a wide variety of Gram +ve and Gram –ve bacteria. Partial 16S rRNA gene sequence of the isolate was sequenced and compared with the sequences of representative *Pseudomonas sp.* The 16S rRNA data supported the phylogenetic position of the strain within the genus *Pseudomonas* and was closely related to *Pseudomonas sp*. 19-28; (GenBank entry: EU167964) and *Pseudomonas pseudoalcaligenes;* RW31; (EU419918) with a sequence similarity of 99%.

Key Words: *Pseudomonas sp*.BP-1 strain, Nagavali, antibiotic, 16S rRNA sequencing.

Introduction

All organisms need to compete in order to survive in their respective habitats. This biological task can be achieved by the development of competitive mechanisms such as the production of toxins, enzymes and antimicrobial agents like antibiotics. One of the areas in soil where one can find abundance in microbial populations is the rhizosphere. The high nutritional content of Nagavali river basin area promotes a high microbial colonization which includes bacteria, fungi and nematodes. Even though this area has a high nutritional content, the organisms that colonize it have to compete for space, water availability, and other physical factors (1). Therefore, these communities exhibit and maintain their competition and survival mechanisms which includes symbiotic relations, parasitism and the production of antagonistic substances such as antimicrobial agents and hydrolytic enzymes. A compilation of the microbial sources of antibiotics in the soil discovered in the United States and Japan between 1953 and 1970 revealed that approximately 85% are produced by actinomycetes, 11% by fungi and 4% by bacteria (2). Bacteria of the genus *Pseudomonas* are able to survive and prosper in a wide range of environmental conditions. This genus not only contains plant, animal and human pathogens but also accommodates species of environmental interest such as plant growth promoters, xenobiotic degraders and bio-control agents (3, 4). Among the bio-control agents, antibiotic producing strains have received considerable attention.

Antibiotics encompass a chemically heterogeneous group of organic low molecular weight compounds which are deleterious to the growth or metabolic activities of other

microorganisms at low concentrations (5, 6). The fact that there is an abundant amount of studies on antibiotics produced by *Pseudomonas* sp. has several reasons: *Pseudomonads* are common inhabitants of rhizosphere and phyllosphere are easily isolated from natural environments and utilize a wide range of substrates easy culturing and their genetic manipulations makes them more amenable to experimentation (7, 8).

Material and Methods

***Conventional identification tests*:** The strain BP-1 was isolated from Nagavali river basin (18° 10'to 19° 44' 0N lat and 82° 53' to 84° 05' 0E long) of Srikakulam District, Andhra Pradesh, India, maintained on nutrient agar and stored at 4°C. The isolate was initially evaluated by conventional tests i.e. Gram stain, growth and morphometric characteristics on nutrient agar, growth at 37°C, catalase, oxidase, motility, indole production, gelatin liquefaction, oxidative fermentative carbohydrate utilization, decarboxylation of lysine, urease activity etc. Additional tests included phenylalanine deamination, nitrate reduction, citrate utilization and H_2S production.

***Screening for antibiotic producing bacteria*:** The soil suspensions were homogenized by shaking at 200 rpm for 15 minutes at 30°C and serial dilutions were carried up to 10^{-3}. Two repetitions of each of the dilutions were inoculated on nutrient agar and the cultures (or master plates) were incubated at 37°C for 24 hours. After the incubation period, colonies that exhibited antagonism were designated as antimicrobial agent producing microbes (AAPM) and were sub cultured and purified by streaking them on nutrient agar plates. After purification the isolated AAPM's were preserved and stored at -20°C for further tests.

***Antibiograms*:** Susceptibility test with whole cell extract: Cultures were centrifuged at 3000g for 15 min, then the cell pellets were resuspended in 15% glycerol, 1% SDS, 0.1M Tris-HCl pH 6.8 and denatured by treatment at 100°C for 20 min. Non-solubilized material was removed by centrifugation at 3000g for 15 min and the resulting supernatant was used as crude whole cell extract. The antimicrobial agent producing capability of the isolate was tested by a modification of the Kirby-Bauer method

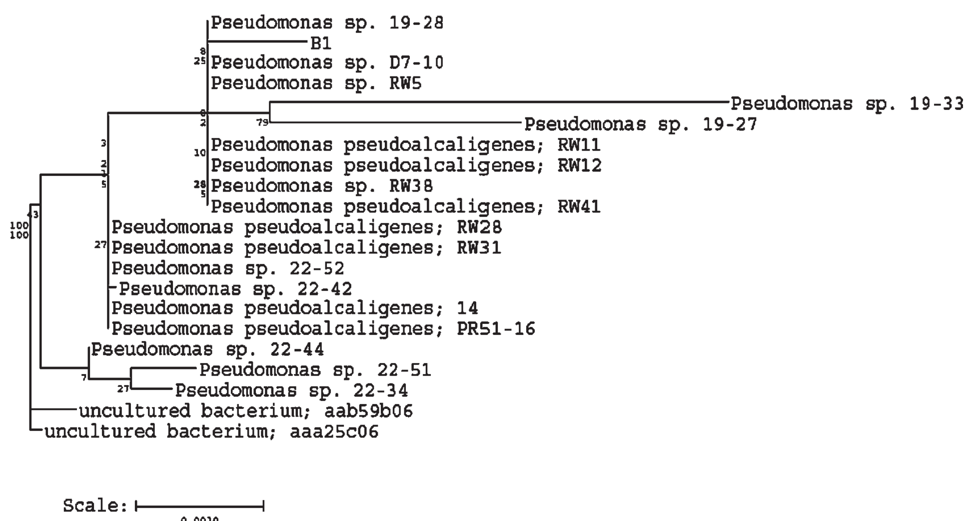

Fig. 1. Phylogenetic position based on 16S rRNA gene sequence analysis of strain BP-1

described by Boyle et al.(9). The target microorganisms used were *Escherichia coli* MTCC 40, *Pseudomonas aeruginosa* MTCC 424, *Proteus vulgaris* MTCC 426 and *Staphylococcus aureus* MTCC 87. AAPM's were incubated 24 hours at 37°C and the targets were also incubated for 24 hours at 37°C. In order to create a bacterial lawn of the targets on nutrient agar, the spread plate technique was employed by using 200 µl of each target. Wells were prepared by a sterile borer and loaded with 20 µl of the AAPM's supernatant and placed over the bacterial lawns. A positive control was included by using a sterile tetracycline antibiotic disc. Antibiograms were incubated 24-48 hours at 37°C. After this period inhibition zones were measured with a ruler using a cm scale.

***DNA preparation and PCR amplification*:** Genomic DNA was extracted from the isolates using Chromous Genomic DNA isolation kit (RKT09). Each genomic DNA used as template was amplified by PCR with the aid of 16S rDNA primers (16S Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3' 16S Reverse Primer: 5'-CGYTAMC TTWTTACGRCT-3' and the programme consisted of denaturation at 94°C for 5 min and subsequent 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in 1% agarose gel.

***16S rRNA sequencing and data analysis*:** Sequencing analysis was performed on a 1500 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The three 16S rRNA sequences were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI Basic Local Alignment Search tool (BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16S rRNA gene sequences have been deposited to Genbank using BankIt submission tool and has been assigned with NCBI accession number (HM359121).

Results and Discussion

The antimicrobial activity of the genus *Pseudomonas* has been reported by many workers [10, 11] from various sources and ecological regions. The bacterial strain was isolated and identified as *Pseudomonas* sp. based on the results of 16S rRNA sequencing. The 16S rDNA amplification by forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3', Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3' followed by sequencing reveals that the strain BP-1 had highest homology (99%) with *Pseudomonas* sp. 19-28; (GenBank entry: EU167964) and *Pseudomonas pseudoalcaligenes*; RW31; (EU419918). More than 1400bp of the 16S rRNA genes of the strain BP-1 was sequenced. Analysis of the 16S rRNA sequences confirmed the strain BP-1 was found to be most similar to *Pseudomonas* sp. 19-28 (GenBank entry: EU167964) (Fig 1). The 16S rRNA gene sequence has been deposited to Genbank using BankIt submission tool and has been assigned with NCBI (National Centre for Biotechnology information) accession number HM359121.The results of inhibition zones against the target microorganisms are shown in Table 1. The strain BP-1 is a Gram negative, rod shaped bacterium with a circular, flat, translucent colony morphology with entire margin. Table 2 shows the results of the biochemical tests carried out for identification.

Table 1: Susceptibility test using filtered supernatants

Microbial targets				
Specimen	***E.coli***	***P.aeruginosa***	***S.aureus***	***P.Vulgaris***
BP-1	1.6	1.0	1.8	1.0
Tetracycline(+ve control)	1.2	1.0	1.0	1.1

Table 2: Results of biochemical tests

Biochemical tests	BP-1 (Remark)
Carbohydrate fermentation	
Glucose	-ve
Adonitol	-ve
Lactose	-ve
Arabinose	-ve
Sorbitol	-ve
Citrate utilization	+ve
Nitrate reduction	-ve
Lysine utilization	+ve
Ornithine utilization	-ve
Phenylalanine deamination	-ve
Urease production	-ve
H_2S production	-ve
Indole Test	-ve
MR-VP Test	-ve

Conclusion

The authors are of the opinion that metagenomic studies are required to exploit the less known and virgin habitats for the possible new antibiotics from nature as most of the synthetic antibiotics has one or other problems including the base problem of developing multiple drug resistant microbes.

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Vol. 6 (1) 78-83 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

RAPD Based Genetic Diversity Analysis within the Genus *Solanum* (Solanaceae)

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Abstract

The advances in molecular biology techniques have led to identification of large number of highly informative DNA markers that are useful for the identification of genetic polymorphism. In the present study, Random Amplified Polymorphic DNA (RAPD) technique was used as a tool for assessing genetic diversity and species relationship among the thirteen accessions of eggplants. Thirteen seed samples of eggplants were collected from different parts of the country. A total of 116 polymorphic amplified products were obtained from seven decamer primers, which discriminated all the accessions. The similarity results indicate presence of high levels of genetic diversity in eggplants and a dendrogram constructed by TFPGA methods shows 86.5% polymorphism. Genetically distinct genotypes identified using RAPD markers could be a potential source of germplasm for eggplant's improvement.

Keywords: Brinjal, Genetic diversity, RAPD markers, Solanaceae.

Introduction

Eggplant *(Solanum melongena* L.) is a vegetable crop of the family Solanaceae, grown in the sub-tropics and tropics. It is one of the most popular vegetables in many parts of the world including India. 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 (1). Eggplants can be cultivated and grown round the year but the productivity and quality of this crop suffers due to its susceptibility to a number of diseases and insect pests (2). In India, it is also used for the treatment of diabetes, bronchitis, dysuria and dysentery (3). Many other *Solanum* species are also used for medicinal purposes (4). For an effective breeding programme, information concerning the extent and nature of genetic diversity within a crop species is essential. It is particularly useful for characterizing individual accessions and cultivars and as a general guide in the selection of the parents for hybridization. Several workers have contributed to the characterization of the largest genus, *Solanum,* of Solanaceae family (5-9). Great degree of taxonomic confusion exists as regards to genus *Solanum* (10). India or Indochina is the center of eggplants diversity (11), but the affinities of S. melongena to related species are uncertain (9).
Genetic fingerprinting has been accomplished traditionally through the use of isozymes, total seed protein and more recently through various types of molecular markers. However, DNA based markers provide powerful tool for discerning variations within crop germplasm and

for studying evolutionary relationships (9). Among molecular markers, random amplified polymorphic DNAs (RAPDs) have been extensively used in genetic research owing to their speed and simplicity (12, 13). The use of molecular techniques in genetic diversity studies is supported by the finding that evolutionary forces such as natural selection and genetic drift produce divergent phylogenetic branchings which can be recognized because the molecular sequences on which they are based share a common ancestor. The present study was aimed at analyzing the eggplant germplasm with RAPD markers and classifying the relationship and variability using RAPD data among the eggplant taxa with numerical taxonomic techniques.

Materials and Methods

***Plant material*:** The plant material for the study comprised 13 accessions (morphologically and geographically distinct genotypes), representing twelve species of cultivated and one species of wild eggplant (Table-1). The materials were collected from different parts of India, grown and maintained at our green house. Young leaves were collected form thirteen samples grown in the green house and immediately stored at -80^{0}C.

***Genomic DNA isolation*:** Genomic DNA from thirteen cultivars of eggplants was isolated by CTAB method (14) with certain modifications. Five grams of young leaves frozen in liquid nitrogen were ground to fine powder and transferred to 15 ml pre-warmed DNA extraction buffer [10mM, Tris-HCl (pH-8.0), 20mM, EDTA, 1.4M, NaCl, 2%, CTAB (cetyl trimethyl ammonium bromide)] and 0.2%, ß-mercaptoethanol. The samples were incubated at 60^{0}C for 1 h with occasional mixing by gentle shaking. To this, 15 ml of chloroform isoamyl alcohol (24:1 v/v) was added, mixed gently by inversion and kept for 20 min, followed by centrifugation at 15,000 rpm for 10 min at room temperature. The aqueous phase was recovered and mixed with 2/3 volume of isopropanol to precipitate DNA, which was recovered with a glass rod, washed with 70% ethanol and dried overnight. DNA was finally suspended in 2 ml of TE buffer {10mM, Tris-HCl (pH-8.0), 0.1mM, EDTA}. DNA was extracted from all the samples and ten samples of each cultivar from different vines were pooled together for further steps.

***DNA purification*:** The isolated DNA was further purified to remove RNA, proteins, polysaccharides and phenols using RNase, proteinase, phenol: chloroform: isoamyl alcohol (25:24:1), phenol: chloroform (24:1), chloroform: isoamyl alcohol and sodium acetate treatments.

***DNA quantification*:** The purified DNA was quantified following the protocol given by Pharmacia Biotech DyNA Quant TM 200 flurometer instruction manual. Finally the isolated DNA was diluted to 25ng/µl.

Polymerase chain reaction (PCR) conditions: PCR amplification reactions were carried out as described by Williams (12) with minor modifications. Reaction mixture (25µl) contained genomic DNA (50ng), Tris-HCl (10mM), $MgCl_2$ (1.9mM) and 100µM DNTPS, primer (0.4mM) and 0.5 units of AmpliTaq DNA polymerase. The tubes were centrifuged for few seconds and placed in a thermocycler for cyclic amplification using the following parameters: 1 cycle of 5 min at 94^{0}C followed by 35 cycles of 1 min each at 94^{0}C for denaturation, 1 min at 38^{0}C for primer annealing and a 2 min extension at 72^{0}C followed by a 5 min cycle at 72^{0}C and finally the machine was held at 4^{0}C till analysis. Amplification products were analyzed by gel electrophoresis on 1% agarose gel incorporated with 1.0 µl/ml of ethidium bromide in 0.5 X TBE buffer. Gels were visualized on a UV

transilluminator and photographs were taken with the help of a Polaroid camera.

Data analysis**:** For RAPD analysis, the bands with same molecular weight and mobility were treated as identical fragments. The data matrices were analyzed by TFPGA program of dendrogram (Version 0.3) and similarities between cultivars were estimated using Nei's co-efficient method to develop a dendrogram by UPGMA.

Results and Discussion

A total of 134 bands were amplified with 7 primers and 13 cultivars, and these were in the size ranges of 0.1 to 5.0 kbp. The individual primers produced between 7 (OPF-01) and 14 (OPF-4, OPF-8) bands. Out of 134 bands, 116 (86.5%) were found polymorphic for one or more accessions. Polymorphism was observed with four primers OPF-3, 4, 8 and 9 which are shown in Plate-1. The primer of OPF-01 amplified minimum number of unique accession-specific bands, whereas OPF-4 amplified maximum number of polymorphic bands. The results show an average of 86.5% polymorphic bands per primer. The remaining 18 of the 134 bands (13.4%) were monomorphic i.e. they were observed in all the 13 cultivars. Fourteen bands were identified as unique to a particular accession which made it distinct from all the other accessions. Out of all the primers, OPF-04 gave the maximum number of 6 unique bands with four different accessions (Table-2; Plate-1).

The pair wise Nei's co-efficient (1972) for the genetic similarities among the 13 accessions are presented in Table-2. The cluster analysis of the distribution of 134 RAPD bands is shown as a dendrogram (Fig-1). This analysis clearly distinguished all the 13 accessions from each other, the accessions Debgiri and wild brinjal were grouped together as they were separated from the remaining accessions with only 25% similarity. Mangiri Gutta and Covai Vari Kathiri were separated from the other members of its cluster with only 40% similarity. The accessions Surachi selection-10 and Padma were grouped together with a maximum similarity of 90% followed by Purple round cluster and Kranthi which showed a similarity of 85%. No duplicates were found among the 13 accessions.

In the present study, a dendrogram was constructed based on the PCR (RAPD) markers

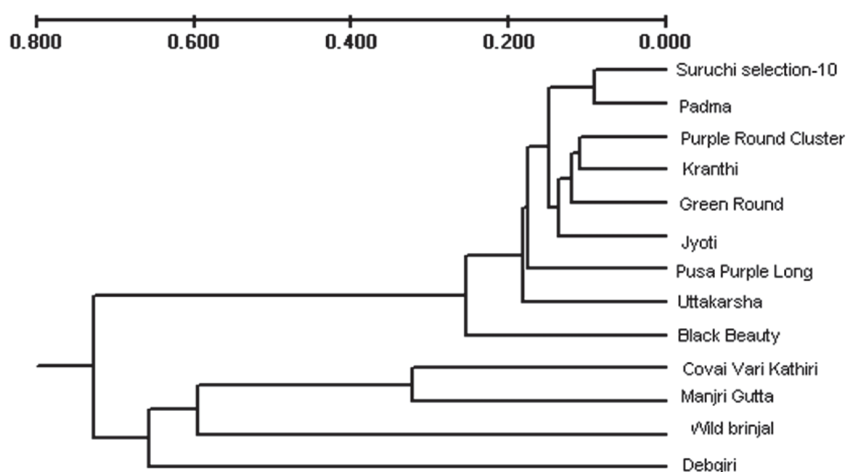

Fig. 1. Genetic relatedness among thirteen cultivars of *Solanum* Sps. based on all seven primers.

Table 1. Accessions of eggplant and its related species analyzed for RAPDs

Sl. No	Materials	Taxon	Source
1	Covai Vari Kathiri	*Solanum melongena*	Bangalore
2	Suruchi selection-10	*Solanum melongena*	Jalna
3	Padma	*Solanum melongena*	Secunderabad
4	Debgiri	*Solanum melongena*	Kolkata
5	Manjri Gutta	*Solanum melongena*	Jalna
6	Pusa Purple Long	*Solanum melongena*	Delhi
7	Black Beauty	*Solanum melongena*	Delhi
8	Green Round	*Solanum melongena*	Hyderabad
9	Purple Round Cluster	*Solanum melongena*	Bangalore
10	Jyoti	*Solanum melongena*	Hyderabad
11	Kranthi	*Solanum melongena*	Secunderabad
12	Uttakarsha	*Solanum melongena*	Hyderabad
13	Wild Brinjal	*Solanum violaceum*	Gulbarga

Table 2. Selected primers along with their sequence and some characteristics of amplification products in accessions analyzed

Sl. No.	Primers	Sequence 5' to 3'	Maximum Number of bands	Number of polymorphic bands	% of polymorphic bands	Mol. Wt. Range (kb)
1	OPF-01	ACGGATCCTG	14	12	85.71	0.2-2.0
2	OPF-02	GAGGATCCCT	16	15	93.75	0.1-1.0
3	OPF-03	CCTGATCACC	19	17	89.47	0.2-1.0
4	OPF-04	GGTGATCAGG	24	22	91.66	0.1-0.5
5	OPF-06	GGGAATTCGG	18	14	77.77	0.075-0.7
6	OPF-08	GGGATATCGG	21	18	85.71	0.1-0.5
7	OPF-09	CCAAGCTTCC	22	18	81.81	0.1-1.0

which showed that 86.5% of the bands observed were polymorphic between the 13 brinjal accessions. This seems to be relatively high when compared to the reports of other RAPD studies, as in the case of sweet potato (15, 16), tomato (17, 18), chilli (19) and other species of Solanaceae. One of the reasons for this high level of polymorphism could be that the interspecific variation in brinjal is extensive. Even though the number of genotypes tested here has come from similar locations, duplicates were not observed among the 13 brinjal accessions. This indicates the level of genetic variability among the local accessions which would be useful for selection of parents in the development of scented brinjal varieties. It will be worth to investigate specific traits in the wild species and they may be introgressed by sexual crossing or somatic

Vol. 6 (1) 78-83 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

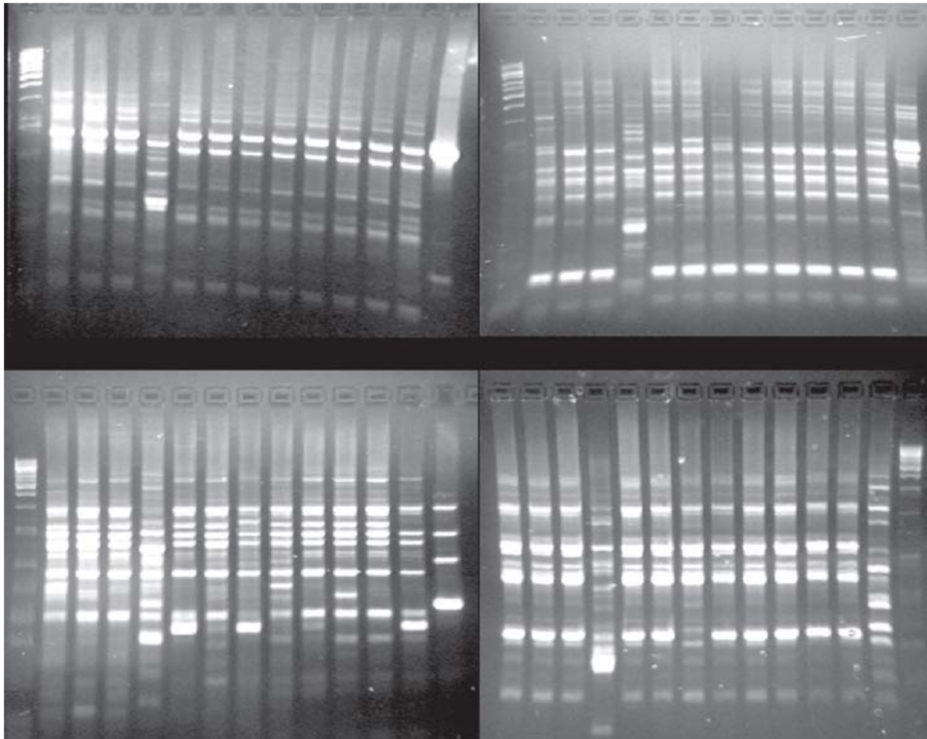

Plate-1. Amplification products from genomic DNAs of 13 accession of Indian Brinjal using a) primer OPF-3, b) OPF-4, c) OPF-8 and d) OPF-9. The lanes represents M- molecular weight 1) Covai vari kathiri, 2) Suruchi selection-10, 3) Padma, 4) Debgiri, 5) Manjri, 6) Pusa Purple Long, 7) Black beauty, 8) Green round, 9) Purple round cluster, 10) Jyothi, 11) Kranthi, 12) Uttakarsha and 13) Wild Brinjal

hybridization into commercial varieties of S. melongena. It is strongly believed that RAPD markers can be applied to other *Solanum* species to assess the genetic relationship among them and assist in the introgression of genes.

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Vol. 6 (1) 84-98 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Formulation and Evaluation of Fast Disintegrating Zolmitriptan Sublingual Tablets

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Abstract

The drug delivery via sublingual drug route is considered to be a one of promising alternative to oral route and quick entry of drug into the systemic circulation can be possible. A diseases state such as migraine, considering pharmacological response was fast which is an important criteria. In the present study sublingual tablets of a potent anti-migraine drug of zolmitriptan 5mg per tablet were prepared. The powdered materials are compressed by direct compression method incorporating various pharmaceutical excipients. The super disintegrates were used are sodium starch glycolate, cross carmellose sodium, cross povidone. The flow properties of powder are important in handling and processing operations. The blend was examined for angle of repose, Carr's compressibility index and Hausner's ratio. The Angle of repose was determined by using conventional fixed funnel method. The Carr's compressibility index and Hausner's ratio were calculated from Bulk and tapped density of the zolmitriptan sublingual powder.The tablets were studied for physiochemical properties and dissolution efficiency. The optimised formulation was used for *in vivo* studies on rabbit as an animal model. The optimised formulation was disintegrated rapidly and from the dissolution studies, it was within the limits of compendia. This specially reveals that the concentration range of mannitol, cross povidone and avicel 102 are in the appropriate ratios and are formulated in good proportions. From *in vivo* studies it showed that optimised formulation containing cross povidone has the minimum T_{max} and $T_{½}$ values (P<0.05), and show effective therapeutic Cmax when compared to clinical dose and it's a promising alternate to oral administration route in acute management of migraine.

Keywords: Fast disintegrating sublingual tablets, Pharmacokinetics, LC-MS/MS.

Introduction

Cluster headache is rare but extremely debilitating disorder that is characterised by the rapid onset of unilateral , per orbital headache that quickly escalates to maximum intensity, patients routinely report the pain of an attack as being the most severe they have ever experienced. Based on the definition of the International Headache Society attacks headache typically last for 15 to 180 minutes when it is untreated and subjected by one more cranial autonomic features such as ipsiletaral, conjunctival injections, lacrimation and rhinorrhoea or nasal congestion (1,2). In this case rapid onset of pharmacological effect is an often desired from drugs. This can be possible through parenteral route of

administration, but this method, sometime may give some inconvenience to the patient. Therefore there is a need to develop such new, non parenteral, and convenient dosage forms using other administration routes where the drug rapidly dissolved and immediately available for the systemic circulation (3).

Sublingual administration can offer an attractive alternative route of oral administration. The advantage of the sublingual drug delivery is that the drug can be directly absorbed into systemic circulation, bypassing the enzyme degradation in the gut and liver. In addition to the thin sublingual mucosa (about 190 µm compared to 500-800 µm of the buccal mucosa) and the abundance of blood supply at the sublingual reason allow excellent drug penetration (absorption) to achieve high plasma drug concentration with a rapid onset of action. A well established example is nitroglycerin which is used for the treatment of acute angina (4). Zolmitriptan is a second- generation triptan prescribed for patients with migraine attacks, with and without an aura, and cluster headaches. It has a selective action on serotonin receptors and is very effective in reducing migraine symptoms, including pain, nausea, and photo or phono phobia. It is currently available as a oral tablet, an orally disintegrating tablet and a nasal spray (2.5 mg and 5 mg per dose) (5,6). In present study, we have developed zolmitriptan fast disintegrating sublingual tablets using pharmaceutical excipients in appropriate proportions (7), and manufactured with optimal techniques. For this reason, the developed sublingual tablet formulations were evaluated with basic tablet physicochemical tests, *in vitro* release studies and *in vivo* studies using rabbit as the animal model.

Materials and Methods

Zolmitriptan was kindly supplied by Suven Pharma Limited Hyderabad, India. Sodium starch glycollate, cross carmellose sodium, cross povidone, avicel pH102, aspartame, mannitol and magnesium stearate were supplied as gift sample by Cheminova Remedies, Hyderabad, India. All analytical grade chemicals and HPLC grade solvents were used. Double distilled water was used throughout the experiment.

***Formulation of fast disintegrating sublingual tablets*:** Sublingual tablets of zolmitriptan were prepared using the method of direct compression. The excipients used were mannital (8), avicel pH102 (diluent), sodium Starch glycollate (9, 10), cros carmellose sodium, cros povidone (super disintegrant) (11-13), aspartame (sweetening agent), magnesium stearate (lubricant). Accurate amount of the active ingredient and all additives were homogenously blended using geometric dilution after passing through sieve number 60 (standard test sieves) and finally magnesium stearate was added for lubrication and triturated well. Different concentrations of excipients were used to prepare different formulations of sublingual tablets. The blended material was compressed on 8mm standard concave punch using a minipress (RIMEK, India). The total weight of tablet was 150 mg.

Evaluation of sublingual tablets

***Micromeritic properties of zolmitriptan powder formulations*:** The flow properties of powder are important in handling and processing operations. The blend was examined for angle of repose, Carr's compressibility index and Hausner's ratio. The Angle of repose was determined by using conventional fixed funnel method. The Carr's index or % compressibility can be expressed using the following formula

$$I = \frac{D_t - D_b}{D_t} \times 100$$

Where D_t is tapped density of the powder and D_b is bulk density of the powder.

Vol. 6 (1) 84-98 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Hausner's ratio were calculated from bulk and tapped density of the zolmitriptan sublingual powder formulation and it is expressed as

$$\text{Hausner's ratio} = \frac{D_t}{D_b}$$

Where D_t is tapped density and D_b is bulk density.

***Determination of physiochemical parameters*:** Drug content uniformity (14) was determined by dissolving the crushed tablets in mobile phase (90:10 %(v/v)) mix buffer (pH 4.0) and acetonitrile respectively and filtered through 0.45 µm membrane filter and degas. It was made necessary dilutions and analysed using High Performance Liquid Chromatography (HPLC - Agilent 1100 series, USA)at the wavelength of 210nm .The liquid chromatography equipped with UV detector and column YMC-pack ODS-AQ (150x4.6mm, 5µm) was used. Isocratic elution was carried out at a flow rate 1.0 ml/min. The injection volume was 10µl and the column temperature was 30° C. Weight variation test was done by weighing 20 tablets, and individual tablet weights were compared with calculated average weights.

The thickness and diameter of the tablets was measured with a vernier calliper (Mututoyo, Japan). The strength of tablet is expressed as tensile strength (N: Newton). The tablet crushing load was the force required to break a tablet into two halves by applying compression. It was measured using a tablet Hardness tester (Tab machines, India).The Friability test is performed to assess the effects of friction and shocks, which may often cause tablet to chip, cap or break. Friabilator (Electrolab, India) was used for this purpose. Pre-weighed twenty tablets was placed in the Friabilator and operated for 100 revolutions (15). The tablets was dusted and reweighed. Compressed tablets should not lose more than 1% of their weight.

Wetting time of the tablets was performed by placing the tablet on tissue paper which was placed in a petri dish of 6.5cm in diameter containing 10ml of water at room temperature, and the time for complete wetting was recorded. *In vitro* disintegration time was carried out using a modified disintegration method (n=6) using disintegration tester (Lab India, DS 1400, India) at 37± 0.5°C in distilled water. The tablet was kept in the basket and noted the time taken for the tablet to disintegrate completely into smaller particles (16).

In vivo disintegration time was carried out by placing a tablet in the floor of the mouth of the volunteer (n=6) and the time required for complete disintegration in the mouth was noted. The taste and mouth feel was also observed (17). *In vitro* dissolution for all the formulations were studied (13), employing a USP Dissolution test apparatus type II (Paddle method (Lab India, DS 14000, India)) at a rotating speed of 50 rpm according to US FDA guidelines. The medium used for these dissolution tests was 500 ml of 0. 1N hydrochloric acid maintained at 37±0.5°C. The solution was filtered though a 0.45µ pore size (PVDF filter) .The samples were collected at predetermined time intervals (5,10,15,20,30,45 and60 min) and analysed for drug content with a UV-Visible spectrophotometer (Schimadzu, model UV1601, Japan) set at 223nm. All the dissolution studies were made six replicate (n=6) to ensure a high sample power and confidence in the results. The calibration curve for zolmitriptan in 0.1N hydrochloric acid was linear from 1-8 µg per ml (r^2>0.99).

***Scanning Electron Microscopy (SEM)*:** The surface characteristics of the zolmitriptan sublingual tablets and standard zolmitriptan were examined using Scanning Electron Microscope (SEM)(Scanning Electron Microscopy, JEOL 5400, Japan) . Samples were fixed on a brass stub

using double sided adhesive tape and were made electrically conductive by coating with five to six times and formed a thin layer of gold, then SEM images were recorded at acceleration voltage of 5 kv.

***Differrential Scanning Calorimetry (DSC)*:** The molecular state of the drug was evaluated by performing DSC analysis of placebo (tablet), standard zolmitriptan, zolmitriptan physical mixture without drug, physical mixture with drug and sublingual zolmitriptan formulations. Using differential scanning calorimeter (DSC 6, Perkin Elmer, USA) curves of the samples were obtained. The samples were heated in hermetically sealed aluminium fans over a temperature range of 35°C - 350°C at a constant rate of 10.0°C per min under nitrogen purge at 20ml /min.

***Powder X-Ray Diffractometry (PXRD)*:** Physical mixture with drug, standard zolmitriptan and sublingual zolmitriptan formulation were measured using X-Ray powder diffract meter (XRD x' pert PRO MPD PAN Analytical, USA). The diffraction pattern was measured using Ni filtred Cu Ka (45kV/40mA) radiation. The samples were measured between the angular range of 2°-50°(2θ) using 0.017° steps and a 10 s counting time per step.

***Fourier Transform infrared spectroscopy (FT-IR)*:** Infrared spectrum peaks of placebo(tablets), physical mixture without drug, physical mixture with drug, zolmitriptan sublingual formulation was compared with zolmitriptan reference standard using FT-IR spectrophotometer (Perkin Elmer Spectrum one series, USA) by KBr pellet method. The scanning range was in between 400 to 4000 cm^{-1} and 1 cm^{-1} resolution.

***Pharmacokinetic analysis*:** Physicochemical properties of formulation nine (F9) was selected and pharmacokinetic studies of sublingual tablets were compared with intravenous administration (18-20).The experimental protocol approved by institutional animal ethics committee (Vimta Labs, Pre clinical Division, Hyderabad, India, Study number: VLL/0611/NG/D007). Six male New Zealand rabbits (1.4 -2 kg) were purchased from Sainath agencies, Hyderabad, India. All rabbits were housed in stainless steel cages (size approximately width 45X length 60X height 35cm). Rabbits were housed separately; the cases were equipped with facilities for holding pellet food and drinking water in bottle with stainless steel sipper tube. All rabbits were free access to reverse osmosis (RO) generated potable water, standard animal diet (provimi animal nutrition). During the study, the room temperature and relative humidity was maintained at 22°C ± 3°C and 30% - 70% RH respectively. Prior to treatment, initiation rabbits subjected to randomization based on their body weights and distributed equally into two groups (21, 22).

Each animal (n=3) in first group was administrated single sublingual tablet (5mg) irrespective of the body weight under mild anaesthesia (isoflurane). The rabbit mouth was opened, tongue was elevated by using a flat forceps, tablet and small amount of water was added to surface of the tablet before administering. It was placed underneath of the mouth by using forceps (30). The mouth was closed for few minutes, to avoid chewing or swallowing of the tablet (23-25) Group-II rabbits were received intravenous preparation of zolmitriptan (standard, 5mg per kg body weight) according to body weight. Animals were bled at pre-determined time points through marginal ear vein (0, 0.83, 0.25, 0.5, 1, 2, 4, 6 and 8 hours). The samples were centrifuged (Cetrifuse, thermoscientific X3R, USA) and serum was separated, stored at -20°C.

***Analysis of blood samples*:** Study sample, calibration curve samples and quality control

samples were transferred to a pre labelled ria vials and added 20µ of internal standard (rizatriptan 2 µg /ml) was added and vortexed, 2.5 ml of diethyl ether: dichlomethane (70:30) was added and shaken for 15 minutes, then centrifuged for 10 minutes at 20°C at 400 rpm. The supernatant was transferred into pre labelled ria vial, evaporated under a stream of nitrogen at 35°C until it completely dried, reconstituted the dried residue with 0.2 ml of mobile phase and vortexed. Samples were loaded into pre-labelled auto-injector vials and 10 µl of samples were injected onto LC-MS/MS system containing HPLC (Perkin Elmer PE 200 series and mass spectrophotometer, API 2000, USA). The Devilosil ODS-3 column (4.6x150mm, 3.5 µm) and the oven temperature was maintained at 40°C and mobile phase was 0.1% formic acid: acetonitrile (25 :75 V/V) with a flow rate of 0.45ml/min and an injection volume with 10µl. The total run time was about 4 minutes and the electron spray ionization was performed in the selected ion monitoring mode. The detection ions were at mass-to-charge ratios m/z of 288.2amu (parent) to 182amu (product) and 270.2amu (parent) to 201amu (product) for zolmitriptan tablets and internal standard rizatriptan respectively. The chromatograms were analysed by using 1.4-2 version software and the concentration of zolmitriptan was calculated. Pharmacokinetic parameters were calculated by non-compartmental analysis using WinNonlin (R) 5.2 software.

Pharmacokinetic parameters**:** The following parameters were primarily calculated from *in vivo* study. The Peak serum concentration attained by the drug (Cmax) and its time required to attain peak serum concentration (Tmax) (26-29) where obtained directly from the plasma concentration time profile. The area under the curve (AUC0-t) was calculated by using trapezoidal rule method. The AUC0-8 , time taken for a test items undergoing decay to decrease by half ($T_{1/2}$) was calculated (31) and the volume of distribution (Vd), clearance of the drug is calculated (Cl) . The bioavailability (%F) was estimated (32-34).

Statistical analysis**:** Statistical analysis was expressed as mean± standard deviation (SD) and performed with (repeated measures) which controls the experimental wise error at rate α=0.05, was used to determine significance among all possible pairs of formulations and interactions. The level of statistical significance was chosen as $p \leq 0.05$ (ANOVA).

Results and Discussion

Preparation of zolmitriptan sublingual tablets**:** Total nine formulations of zolmitriptan sublingual tablets were prepared using three super disintigrents (sodium starch glycolllate, cross carmellose sodium and cross povidone) with variation in there percentage (2-6%) and variation in excipients. The diluents used were mannitol and avicel pH 102. The composition of zolmitriptan sublingual formulation was shown in Table 1.

The micromeritic properties of the zolmitriptan powder formulations are vital in handling processing operations because the dose uniformity and ease of filling into container is detected by the powder flow properties. The powder flow properties can be accessed from Angle of repose, Carr's index and Hausner's ratio. The results for powder formulations were represented in Table 2. Our results indicate small angle of repose (<33°) assuring good flow properties for formulations. In addition to this Carr's index and Hausner's ratio were also less than 14 and 1.17 respectively ensuring all nine formulations resulted in good mixing, flow ability and compressible characteristics. Table 3 and 4 shows the physiochemical characterization of sublingual formulations. Drug uniformity results

were found to be good among different batches of tablets and the percentage of the drug content was more than 97.5% ($P<0.05$). The results also showed acceptable and homogeneous distribution of drug in all tablets. The average weight of the tablet in all formulations ranged from 150.07 ± 0.06 mg to 151.37 ± 0.23mg. All tablets prepared in this study meet the USP requirements for weight variation <2% (USP 31).The diameter and thickness of the formulations ranged from 8.03± 0.06mm to 8.17± 0.06mm and from 3.1±0.00mm to 3.2±0.00 mm respectively. All the formulations of tablets indicated good mechanical strength (4-5kg/cm 2), where as friability is less than 0.5%, indicating the friability was within the compendia limits (USP 31), which showed that the tablets possess good mechanical resistance.

***In vitro disintegration study*:** The disintegration time of the tablets was one of the most important parameter which is supposed to be optimised in the development of sublingual tablets. In this present study, all the tablets were disintegrated in the range varied from 84.3±0.58s to 7.7±0.58s. As per USP, the disintegration test for sublingual tablets, the disintegration apparatus for oral tablets is used without covering plastic disks and 2 minutes is the time limit specified as an acceptable limit for tablet disintegration fulfilling the official requirements (<2minutes) for sublingual tablets (USP31).

All of our formulations meet the requirements for disintegration. Out of all, the formulation 9 (F9) was quickly disintegrated compared to other formulations and showed disintegration time of 7.7±0.58s.The *in vivo* disintegration time was in the range of 115.33± 0.58s to 12.33±0.58s and the time was found to be 12.33±0.58s for formulation nine (F9). The wetting time and water absorption was 66.0±1.0s to 5.0±1.0s and 154.32±0.01% to 90.75±0.01% respectively. It was observed in formulation 9 (F9), the tablet wetted and disintegrated completely within 5s.

***In vitro dissolution study*:** Table 5 showed the dissolution profile of zolmitriptan from all formulations. After starting the experiment, more than 85% of drug was dissolved within 15 minutes. According to the literature, amount of drug dissolved from sublingual tablets must exceed 80% in 15 min. Therefore the resulted dissolution profile met the above-mentioned requirement. The formulation nine (F9) showed 100.34± 1.19 dissolution efficiency in 15 min than other formulations, Which contains 6% cross.povidone, shown less disintegration time and more dissolution efficiency. Hence formulation 9(F9) was selected as optimized formula and is characterised.

***Scanning Electron Microscopy (SEM)*:** The surface morphology of sublingual zolmitriptan formulation and standard zolmitriptan and were examined by scanning electron microscopy (Fig.1). The SEM micrographs reveal that there is no segregation or deposition of particles on the surface of sublingual tablets.

***Differential scanning calorimetry (DSC)*:** The thermo tropic behaviour, the physical states of the drug in sublingual tablets were ascertained from the DSC thermo grams of placebo (tablet), physical mixture without drug, standard zolmitriptan, physical mixture with drug and zolmitriptan sublingual formulation (F9). It is shown from Fig. 2 that the onset of peak for standard zolmitriptan was found to at 136°C; a sharp intensive peak at 140°C was observed. In the zolmitriptan sublingual tablets and physical mixture with drug, there is a small peak was noticed at 138°C (135°C-140°C). The small size of peak is attributed to the fact that the amount of zolmitriptan in physical mixture with drug and tablets was less than 10% by weight. The peak at 169°C in all the formulations except in standard

Vol. 6 (1) 84-98 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

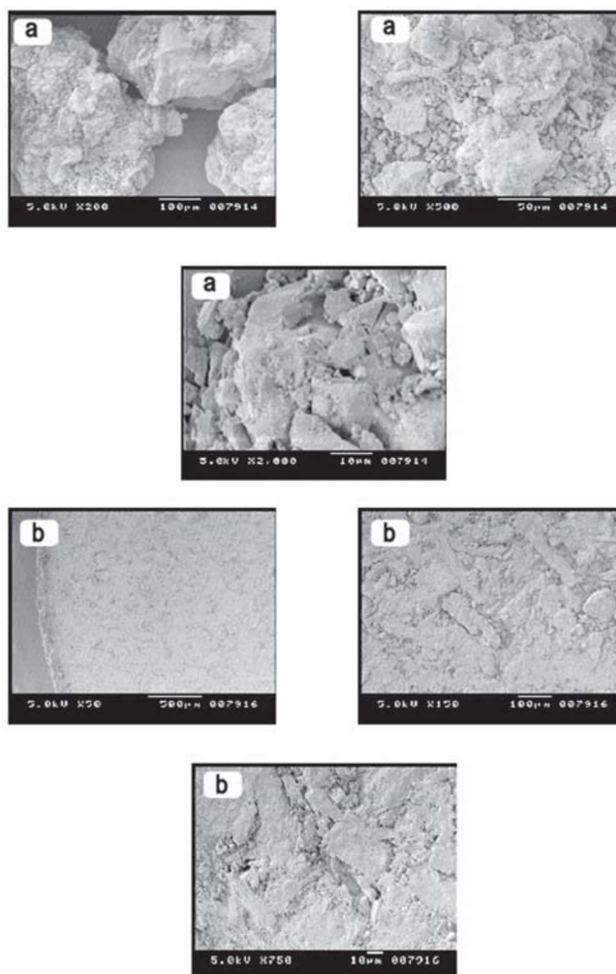

Fig. 1. Scanning electron microscope images of a) Zolmitriptan standard b) Zolmitriptan sublingual tablets

zolmitriptan was due the presence of excipients. This shows that there was no polymorphic change occurring in these formulations.

Powder X-Ray diffraction study (PXRD)**:** The pure drug showed numerous characteristic high intensity diffraction peaks demonstrating the crystalline nature of the drug (Fig. 3). The peak at about 19.32(2θ) corresponds to main peak in standard zolmitriptan. The same peak was also found in physical mixture with drug and in zolmitriptan sublingual tablets at 19.32(2θ). This indicates that the crystallinity of zolmitriptan was not changed in physical mixture with drug and in zolmitriptan sublingual formulation.

Fourier transform infrared spectroscopy (FTIR)**:** FTIR spectra are of placebo(tablet), physical mixture without drug, physical mixture with drug, standard zolmitriptan and zolmitriptan sublingual formulation (Fig. 4). The pure zolmitriptan exhibits characteristic peaks at 3350cm^{-1}(aromatic secondary amine N-H stretching), 2974cm^{-1}(aromatic C-H stretching),

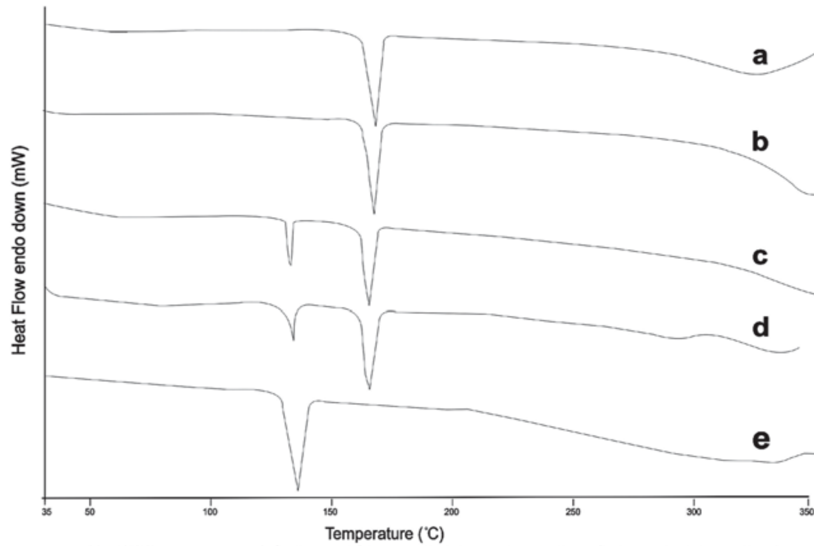

Fig.2. DSC thermograms of a) Placebo b) Physical mixture without drug c) Physical mixture with drug d) Sublingual Zolmitriptan Tablet e) Zolmitriptan Standard

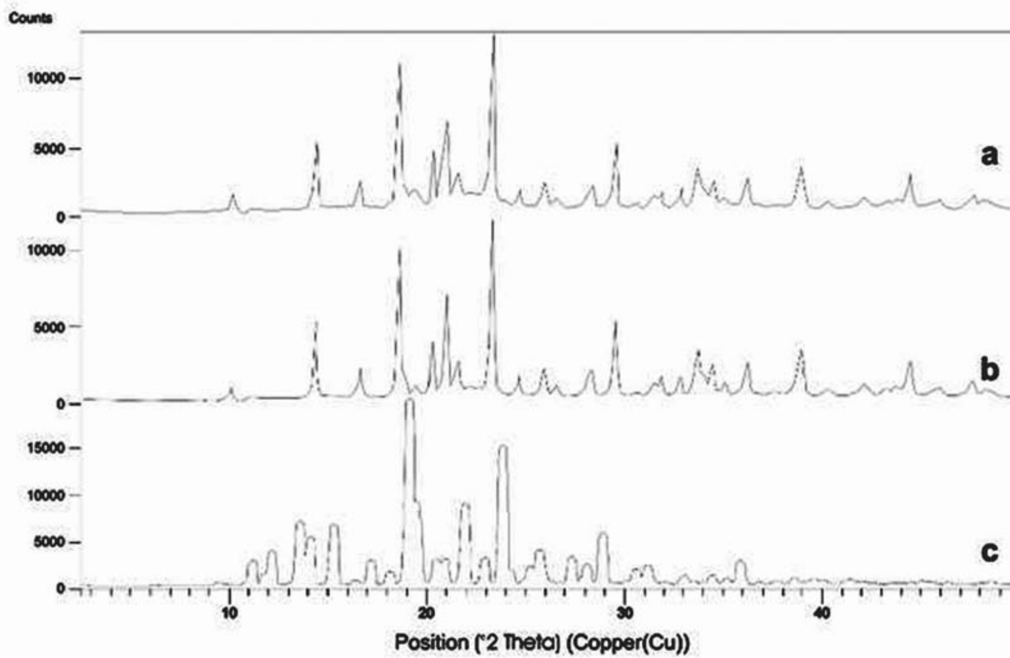

Fig. 3. Powder X-ray diffraction patterns of a) Zolmitriptan sublingual tablets b) Zolmitriptan physical mixture with drug c) Zolmitriptan standard.

Vol. 6 (1) 84-98 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

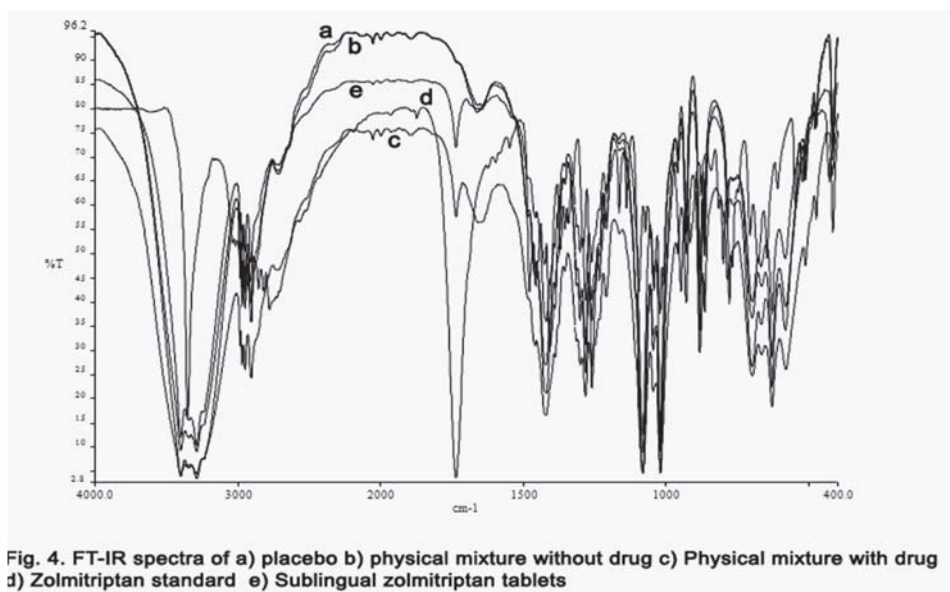

Fig. 4. FT-IR spectra of a) placebo b) physical mixture without drug c) Physical mixture with drug d) Zolmitriptan standard e) Sublingual zolmitriptan tablets

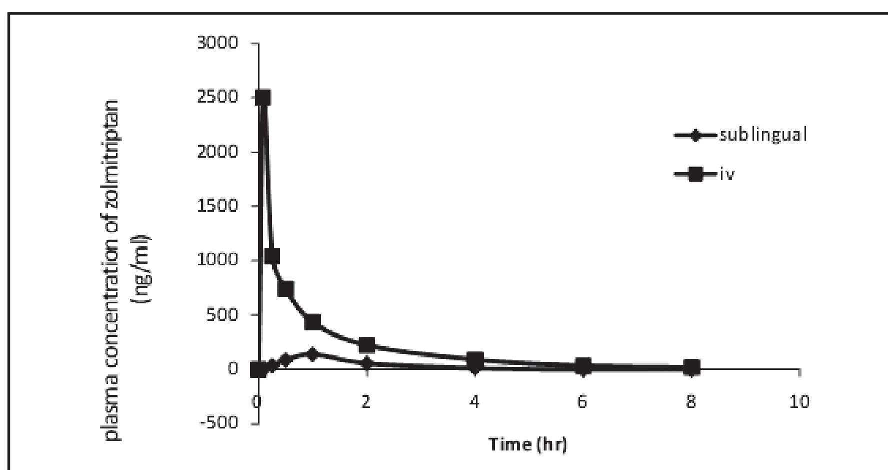

Figure 5. The plasma concentration time curve of zolmitriptan sublingual tablets and intravenous (Zolmitriptan standard) administration. Results are expressed as the mean± SD (n=3)

1736 cm^{-1} (C=O five member cyclic stretching), 1259 cm^{-1} (C-N aliphatic amine stretching) (Fig. 4). All these peaks have appeared in zolmitriptan formulation(F9) at 3292 cm-1 (aromatic secondary amine N-H stretching), 2970cm-1(aromatic C-H stretching), 1736 cm^{-1} (C=O five member cyclic stretching), 1260 cm^{-1}(C-N aliphatic amine stretching), indicate no chemical interaction during formulation of zolmitriptan tablets.

Pharmacokinetic studies :The optimised formulation was chosen according to *in vitro* results by means of exhibiting fast disintegration and dissolution profile. Formulation nine (F9) was directly included for *in vivo* experiments.

Table 1. Composition of the Zolmitriptan formulations

Ingredients(mg)	F1	F2	F3	F4	F5	F6	F7	F8	F9
Zolmitriptan	5	5	5	5	5	5	5	5	5
Sodium Starch Glycollate	3	6	9	-	-	-	-	-	-
Cross Carmellose Sodium	-	-	-	3	6	9	-	-	-
Cross Povidone	-	-	-	-	-	-	3	6	9
Avicel 102	39.75	36.75	33.75	39.75	36.75	33.75	39.75	36.75	33.75
Aspartame	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Mannitol	100	100	100	100	100	100	100	100	100
Magnesium stearate	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Total weight(mg)	150	150	150	150	150	150	150	150	150

Each tablet contains 5mg of Zolmitriptan.

Table 2. Powder flow properties of the Zolmitriptan formulations Data are expressed as mean ± SD. (n=3).

Code	Angle of repose (θ)	Bulk density (gm/cm^3)	Tapped density (gm/cm^3)	Compressibility index (I)	Hausner's ratio
F1	32.4±0.1	0.23±0.01	0.26±0.01	11.39±0.25	1.15±0.03
F2	32.00±0.11	0.31±0.01	0.35±0.01	11.54±0.19	1.13±0.00
F3	31.6±0.18	0.22±0.01	0.25±0.01	12.01±0.48	1.13±0.01
F4	31.1±0.17	0.25±0.01	0.29±0.01	13.64±0.27	1.16±0.01
F5	31.3±0.04	0.25±0.01	0.28±0.01	10.86±0.61	1.12±0.00
F6	30.4±0.13	0.25±0.00	0.28±0.01	11.74±1.78	1.13±0.02
F7	30.0±0.13	0.27±0.01	0.30±0.01	10.11±0.20	1.11±0.01
F8	30.4±0.13	0.25±0.00	0.28±0.01	10.84±0.23	1.11±0.02
F9	29.8±0.14	0.26±0.01	0.29±0.01	10.46±0.21	1.12±0.00

Sterile solution of zolmitriptan at a concentration of 5mg/kg body weight was used to calculate relative bioavailability. The mean serum concentration-time data of zolmitriptan following the administration of the sterile solution formula via intravenous and sublingual tablet formulations is shown in Figure 5. Table 6 showed pharmacokinetic parameters for both the formulations. Peak serum concentration attained by drug was 140.62 ± 18.39ng/ml and 2500.85±1004.02 ng/ml following sublingual and intravenous administration respectively. Time required for attaining peak serum concentration by drug, following sublingual and intravenous administration was 1 hr and 0.083 hr respectively .Area under the curve AUC0-24 was found to be 231.77±81.50 ng.hr/ml and 1712.74±606.65 ng.hr/ml for sublingual and intravenous administration respectively.AUC0-8 was calculated and was found to be 295.131±15.40 and 1750.45±619.57 ng.hr/ml respectively for sublingual and intravenous administration. Time

Table 3. Physicochemical properties of sublingual tablets of Zolmitriptan Data are expressed as mean ± SD. (n=3).

Code	Content uniformity (%)	Thickness(mm)	Hardness (Kg/cm²)	Friability (%)
F1	98.06±0.89	3.17±0.06	4.33±0.58	0.28±0.02
F2	98.76±1.30	3.13±0.06	4.33±0.58	0.18±0.01
F3	99.09±0.19	3.17±0.06	4.33±0.58	0.13±0.01
F4	102.9±0.62	3.10±0.06	4.66±0.58	0.24±0.01
F5	103.31±0.56	3.20±0.00	4.33±0.29	0.30±0.01
F6	97.66±0.41	3.17±0.06	4.16±0.29	0.33±0.01
F7	101.82±0.33	3.20±0.00	4.16±0.29	0.29±0.01
F8	101.23±0.41	3.10±0.00	4.5±0.50	0.33±0.01
F9	102.23±0.42	3.17±0.06	4.33±0.29	0.30±0.02

Table 4. Physicochemical properties of sublingual tablets of Zolmitriptan Data are expressed as mean ± SD. (n=3).

Code	Average tablet weight(mg)	Diameter (mm)	DT (seconds)	*In vivo* DT (seconds)	Wetting Time (seconds)	Water Absorption Ratio (%)
F1	151.3 ±0.04	8.03±0.06	84.3 ±0.58	115.33±0.58	66±1	154.32±0.01
F2	151.37±0.23	8.07±0.06	57.7±0.58	104.67±0.58	55.67±0.58	147.22±0.01
F3	151.33±0.58	8.13±0.06	53.7±0.58	82.33±0.58	43.67±0.58	141.06±0.00
F4	151.23±0.12	8.13±0.06	16.0±1.0	33.0±0.00	15.3±0.58	129.06±0.00
F5	151.13±0.12	8.13±0.06	15.67±0.58	31.67±0.58	14.3±0.58	121.32±0.00
F6	151.5±0.10	8.17±0.06	15.0±1	27.0±0.00	12.3±0.58	117.68±0.00
F7	150.07±0.06	8.13±0.06	11.3±0.58	25.33±0.58	10.67±0.58	109.45±0.00
F8	151.17±0.06	8.10±0.00	9.3±0.58	20.0±0.0	6.67±0.58	106.18±0.01
F9	151.03±0.06	8.17±0.06	7.7±0.58	12.33±0.58	5±1	90.75±0.01

required for a drug to decrease by half (ie T1/2) was found to be 0.86hr and 1.66 hr following sublingual and intravenous administration. Volume of distribution for a drug (vd) was 20914.02±657.47 ml and 0.458±0.16 ml following sublingual and intravenous administration. Clearence of the drug was found to be 16964.75±885.29 ml/hr and 3079.49±654.44 ml/ hr following sublingual and intravenous administration. Percentage availability (% F) was found to be 20.

The therapeutic dose of zolmitriptan oral dose in rabbits is 0.083 mg/kg. In current study, 5 mg/kg was administered to rabbits which correspond to 1.25 mg/kg in humans. In conclusion, the mean plasma concentration time profile for zolmitriptan 5 mg tablet by sublingual

Table 5. *In Vitro* Release profile of the Zolmitriptan formulations Data are expressed as mean ± SD. (n=3).

Time (Min)/ Code(%)	F1	F2	F3	F4	F5	F6	F7	F8	F9
5	71.16 ± 2.93	78.83 ± 2.52	83.52 ± 1.01	77.72± 0.73	81.04± 0.81	84.13± 0.46	79.15± 0.78	80.89± 0.81	85.49± 0.46
10	78.17± 3.60	83.29 ± 3. 70	93.75± 1.74	84.93± 0.80	84.87± 0.54	95.17± 0.90	86.95± 0.49	88.94± 0.65	97.10± 0.54
15	85.15 ± 2.49	93.32 ± 3.04	97.37 ± 0.69	91.92± 0.82	88.70± 0.27	96.08± 0.54	97.28± 0.59	97.21± 0.40	100.34± 1.19
20	91.90 ± 1.62	98.67 ± 0.64	97.60± 0.20	97.25± 0.81	95.42± 0.75	98.38± 0.29	97.05± 0.95	97.67± 0.40	102.41± 0.49
30	97.47 ± 1.25	101.71 ± 1.41	99.41± 0.64	99.13± 0.72	99.74± 0.92	99.52± 0.66	101.64± 0.99	101.58± 0.38	102.86± 0.40
45	98.68 ± 2.56	103.10 ± 0.40	102.81 ± 0.80	99.13± 0.72	99.74± 0.92	100.91± 0.62	102.09± 0.69	102.73± 0.68	103.10± 0.41
60	102.62 ± 1.75	103.57 ± 0.68	103.50 ± 0.12	101.21± 0.85	102.35± 0.87	102.97± 0.08	103.01± 0.77	102.50± 0.39	103.33± 0.09

Table 6. Pharmacokinetic Parameters of Zolmitriptan sublingual tablets in rabbits following administration of Optimised Zolmitriptan sublingual tablets (F9) and Intravenous administration.

PK parameters	Sublingual tablets (5 mg/tablet)	Intravenous administration (5 mg/kg)
C_{max}(ng/ml)	140.62±18.39	2500.85±1004.02*
T_{max}(hr)	1±0	0.083±0*
AUC(0-t) (ng.hr/ml)	231.77±81.50	1712.74±606.65*
AUC(0-8) (ng.hr/ml)	295.13±15.40	1750.45±619.57*
T1/2 (hr)	0.86±0.02	1.67±0.645*
CL (ml)	16964.75±885.29	3079.49±654.44*
VD (ml)	20914.02±657.47	0.458±0.16*
%F		20

Data are expressed as mean ± SD. (n=3). *Significant difference at P≤0.05 vs control.

route show initially it is very rapid absorption of drug and reached an average 80% of eventual Cmax within 1 hour and 5 mg/kg intravenous route show time to peak plasma concentration within 1 hour. zolmitriptan 5 mg tablet by sublingual route in rabbits show effective therapeutic Cmax (140.62 ng/ml) when compared to clinical dose by oral route (5.6 ng/ml)(26).

Conclusions

All tablets met the compendia limits in terms of physicochemical parameters, disintegration and dissolution studies. When given sublingually, zolmitriptan is well absorbed, and its bioavailability by this route is significantly. From this study, the optimised sublingual tablets of zolmitriptan appeared to be

a promising alternative to oral drug administration route in acute management of migraine.

Acknowledgement

The authors are thankful to Suven pharma, Hyderabad, India for providing zolmitriptan as a gift sample and acknowledge the Sipra Labs, Hyderabad, India for *in vitro* studies and Vimta Labs, Hyderabad, India for *In vivo* Studies.

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Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) 99-111 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Influence of Cultural Conditions for Improved Production of Bioactive Metabolites by *Streptomyces cheonanensis* VUK-A Isolated from Coringa Mangrove Ecosystem

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Abstract

The influence of culture conditions and the effect of environmental factors on the growth and production of bioactive metabolites by *Streptomyces cheonanensis* VUK-A was the focus of this study. The strain exhibited broad spectrum antimicrobial activity against Gram-positive, Gram-negative, unicellular and multicellular fungi. The optimum pH and temperature for bioactive metabolite production were 7 and 30°C respectively. Production of bioactive metabolites by the strain was high in asparagine glucose broth as compared to other media tested. Studies on nutritional factors revealed that highest antimicrobial metabolite production was obtained when lactose and peptone at 1% and 0.25% were used as carbon and nitrogen sources respectively. Ninty six hours of incubation was found to be the optimum for bioactive metabolite production by the strain. As the strain exhibited potent antimicrobial activity, it may be explored for biotechnological purposes.

Keywords: Optimization, Bioactive metabolites, Nutritional factors, Culture conditions, Environmental Parameters.

Introduction

There is an immediate need to discover and develop new antibiotics as spread and prevalence of infectious diseases resistant to chemotherapy is on the rise (1). Special attention was focused on the microbes that have been proved as the natural dumps for the bioactive metabolites since decades for resolving the problem of the antibiotic resistance (2). These microbes are capable of producing a wide array of the potent antimicrobial compounds that have broad application in the field of medicine (3). Many microbes that thrive in extreme conditions have the potentiality to produce unusual bioactive metabolites that acts as a chemical defense against the pathogenic microbes (4). The most promising source of the future antibiotics that the society expects is the natural microbial products (5). The exploration for the new antimicrobial compounds and the new strains continue to be the most important research programme around the world (6). The microbial natural products remain the most potent and important source for the novel antibiotics, although new methodologies are needed to improve the efficiency of the discovery of compounds. Numerous antibiotics have been

obtained from various microbes isolated from the marine environment played a significant role in the discovery of anti-metabolites (7). Production of the secondary metabolites by the microbes differs in quality and quantity based on the type of strains and also species used (8). It is also a known fact that appropriate fermentation medium is critical and crucial for the production of the secondary bioactive metabolites (9) and prior experience and knowledge is required in developing a suitable basal medium since it plays an important role in further media optimization (10). Additionally, biosynthesis of the secondary metabolites is influenced by numerous environmental factors including nutrients (nitrogen, phosphorous and carbon sources), growth rate, feedback control, and other physical conditions (oxygen supply, temperature and pH) (11, 12, 13, 14). Therefore, influence of the growth conditions and environmental conditions are important to improve the production of the secondary metabolites.

Streptomyces species has been widely reported for the production of a number of anti-microbial metabolites that have therapeutic applications (15). These species have gained a special prominence for their characteristic ability to produce potent antibiotics and other secondary metabolites including anti-tumor agents (7). Each of the strain has a genetic set up that influences the production of 10-20 different kinds of secondary compounds. The objective of the present study is to design an appropriate culture medium and also optimize the cultural conditions of the *Streptomyces cheonanensis* VUK-A strain in order to reduce the cost of fermentation process and improve the formation of antimicrobial compounds.

Materials and Methods

***Isolation*:** The *Streptomyces cheonanensis* VUK-A was used in the investigation. The strain was isolated from soils of "Coringa Mangrove Ecosystem" of south coastal Andhra Pradesh, India by using soil dilution plate technique on starch-casein agar medium (16) and further maintained on yeast extract malt extract dextrose (ISP-2) agar medium at 4°C (17). The 16s rRNA sequence of the strain *Streptomyces cheonanensis* VUK-A was submitted to the Genbank (accession number JN087502) (48).

***Incubation Period on Bioactive Metabolite Production*:** The growth pattern and bioactive metabolite production of the strain was studied at regular intervals up to 7 days. One week old culture of the strain was cultivated in seed medium (starch casein broth) at room temperature for 48 h. Seed culture at a rate of 10% was inoculated into the production medium of the same composition. The fermentation process was carried out for one week under agitation at 120 rpm. At every 24 h interval, the flasks were harvested and the biomass was separated from the culture filtrate. Biomass was determined in terms of total cell dry weight. Antimicrobial metabolite production determined in terms of their antimicrobial spectrum (18). The culture filtrates were extracted with ethyl acetate and evaporated to dryness in a water-bath at 80°C. The solvent extracts were concentrated and 50µl of crude extract was tested for antimicrobial activity by employing agar well–diffusion method (19) against test organisms like *Bacillus subtilis* (ATCC 6633), *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231).

***Culture Conditions for the Optimum Production of Bioactive Metabolites*:** Antimicrobial metabolite production of the strain was optimized by using different parameters such as pH,

temperature, culture media, carbon and nitrogen sources and minerals.

***pH and Incubation Temperature on Biomass and Bioactive Metabolite Production*:** To determine the influence of initial pH on growth and bioactive metabolite production, *Streptomyces cheonanensis* VUK-A was cultured in the medium with different initial pH, ranging from 4-10 and at different starting temperatures, from 20 - 60°C. The biomass and bioactive metabolite production were estimated to determine optimal pH and temperature conditions which were used in this study (20, 21).

***Culture Media on Biomass and Production of Bioactive Metabolite*:** In order to determine ideal conditions for the maximum production of antimicrobial metabolite from the *Streptomyces cheonanensis* VUK-A, the strain was cultivated in 10 different media such as asparagine-glucose broth, tyrosine broth (ISP-7), starch inorganic salts broth (ISP-4), glycerol-asparagine broth (ISP-5), yeast-starch broth, malt extract broth, tryptone yeast extract broth (ISP-1), czapek-dox broth, maltose-tryptone broth and soya-bean meal broth. The biomass accumulation and bioactive metabolite production in each medium was evaluated. The medium in which the strain exhibits optimum levels of bio-active metabolite production was used for subsequent study.

***Carbon and Nitrogen Sources on Biomass and Bioactive Metabolite Production*:** To determine the effect of carbon sources on biomass and bioactive metabolite production of the strain, different carbon sources like maltose, lactose, fructose, sucrose, dextrose, starch, mannitol, arabinose, xylose, glycerol and inositol (each at a concentration of 1%) were added separately to the optimized medium. Furthermore, the effect of varying concentrations of the best carbon source (0.5 - 5%) on bioactive metabolite production was also determined. Similarly, influence of various nitrogen sources on bioactive metabolite production was evaluated by supplementing different nitrogen sources like sodium nitrate, ammonium sulfate, ammonium oxalate, peptone, yeast-extract, tryptone, casein, tyrosine, phenyl alanine, glycine and glutamine (each at a concentration of 0.5%) to the optimized medium containing an optimum amount of the superior carbon source as determined above (22). Furthermore, the impact of varying concentrations of optimized nitrogen source (0.1 - 2%) was studied to standardize the maximum antimicrobial metabolite production.

***Minerals on Biomass and Bioactive Metabolite Production*:** Impact of minerals on the production of biomass and bioactive metabolites was studied by supplementing different minerals like K_2HPO_4, $MgSO_4$, $FeSO_4$, K_2HPO_4 and $ZnSO_4$ each at a concentration of 0.05% (w/v) to the optimized medium (23).

***Antimicrobial Activity Against Test Organisms*:** The antimicrobial metabolites of the strain produced under optimized conditions were tested against various strains of bacteria viz., *Streptococcus mutans* (MTCC 497), *Lactobacillus casei* (MTCC 1423), *Lactobacillus acidophilus* (MTCC 495), *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (MTCC 7299), *Shigella flexneri* (MTCC 1457) and *Xanthomonas campestris* (MTCC 2286) and fungi such as *Candida albicans* (ATCC 10231), *Aspergillus niger*, *Aspergillus flavus, Fusarium oxysporum* (MTCC 3075) and *Penicillum citrinum* by agar-diffusion assay (19).

***Statistical Analysis*:** Data obtained on the bioactive metabolite production under different

microbial culture conditions were statistically analyzed and expressed as mean ± standard error with one-way analysis of variance (ANOVA).

Results

***Incubation Period on Biomass and Bioactive Metabolite Production*:** The growth pattern of *Streptomyces cheonanensis* VUK-A was studied on starch casein broth. The stationary phase of *Streptomyces cheonanensis* VUK-A extended from 72 h to 120 h of incubation (Fig.1). The secondary metabolites obtained from four day old culture exhibited high antimicrobial activity against the test microorganisms, which is in agreement with the earlier reports (24, 25, 26, 27, 28).

***pH and Incubation Temperature on Biomass and Bioactive Metabolite Production*:** The maximum growth and antimicrobial activity of the strain was obtained at pH 7 (Fig. 2) suggesting its inclusion in the neutrophilic actinomycetes group. Bhattacharya *et al.* (29) reported that 7 is the optimum pH for antibiotic production by *Streptomyces hygroscopicus* D1.5. Atta *et al.* (30) stated that the optimum initial pH value capable of promoting biosynthesis of anti-microbial agents by *Streptomyces torulosus* KH-4 was found to be 7. Similarly, bioactive metabolites obtained from the isolate *Streptomyces* sp. VITSVK 9 at pH 7 exhibited good antimicrobial activity (21). There was an increase in the growth of the cell as well as the production of bioactivie metabolic production with the increase of the incubation temperature from 20°C -30°C (Fig 3). However, further increase in temperature (above 30°C) resulted in the decreased growth rate and decline in the production of bioactive metabolite (Fig.3). In terms of its optimum temperature for growth, the organism appeared to be mesophilic in nature. This is in agreement with earlier reports for several of the *Streptomyces* species (31, 32, 33, 34, 35).

***Culture Media on Biomass and Bioactive Metabolite Production*:** Biomass and bioactive metabolite production of the strain was studied in different culture media (Fig. 4). Among the media tested, asparagine-glucose broth produced higher levels of bioactive metabolites followed by tryptone broth (ISP 1) and tyrosine broth (ISP 7). Similarly production of biomas was higher in czapek–dox broth followed by soya-bean meal

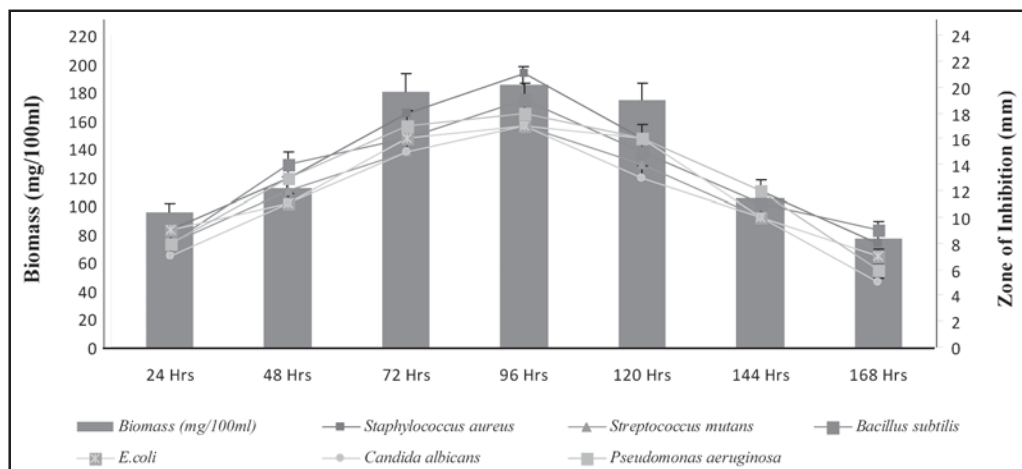

Fig 1. Growth pattern of *Streptomyces cheonanensis* VUK-A. Data are statistically analyzed and found to be significant at 5%.

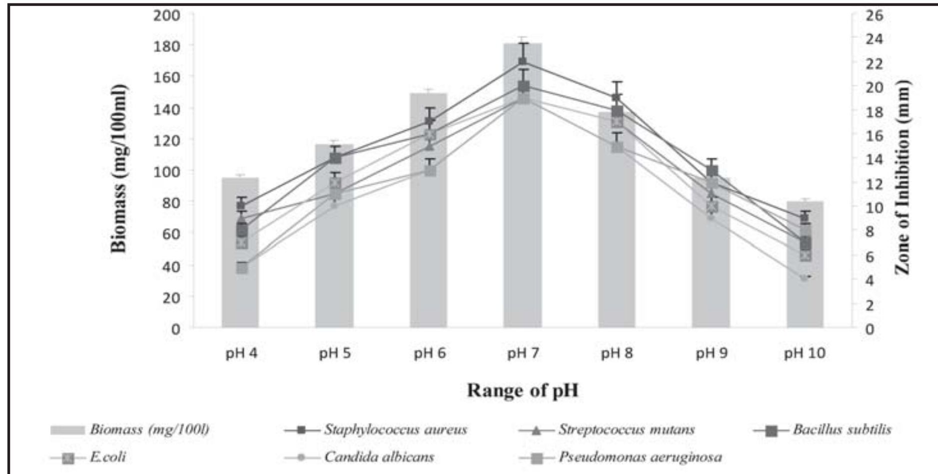

Fig. 2. Effect of pH on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK –A. Data are statistically analyzed and found to be significant at 5%.

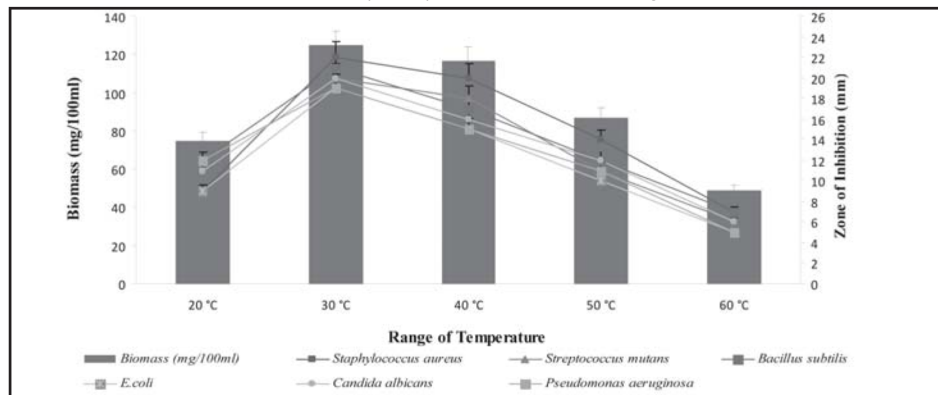

Fig. 3. Effect of temperature on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK-A. Data are statistically analyzed and found to be significant at 5%.

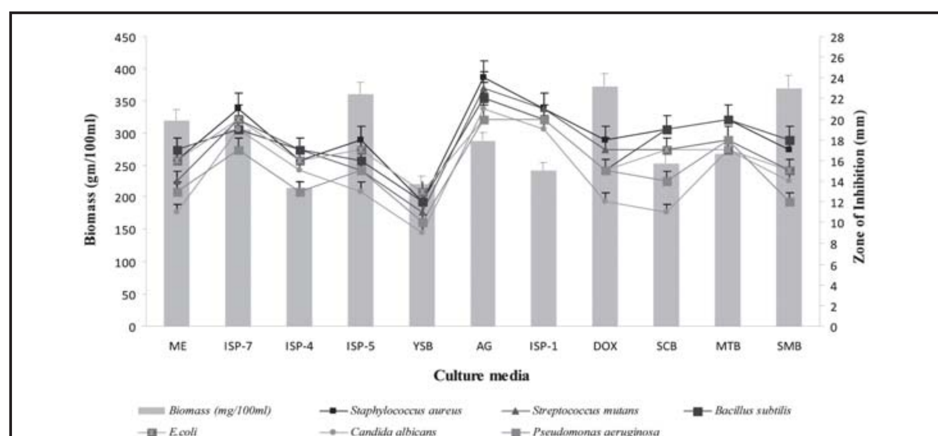

Fig. 4. Effect of different culture media on cell growth and bioactive metabolite production by *Streptomy ces cheonanensis* VUK-10. Data are statistically analyzed and found to be significant at 5%.

broth and asparagine-glycerol broth (ISP 5). There is a significant increase in the bioactive metabolite production cultured in asparagine-glucose broth. Saha *et al.* (7) reported that czapek- dox broth favored high rates of antibiotic production by *Streptomyces* sp. MNK-7 isolated from soil samples from Bangladesh. Kavitha and Vijayalakshmi (28) showed that maltose-tryptone broth favored maximum production of bioactive metabolite production by *Nocardia levis* MK-VL-113.

Carbon and Nitrogen Sources on Biomass and Bioactive Metabolite Production: The details of the effect of carbon and nitrogen sources on production of biomass and bioactive metabolites by *Streptomyces cheonanensis* VUK-A were showed in figs. 5 and 6. Significant production of bioactive metabolite was obtained in lactose amended media followed by fructose and sucrose. Similarly, the production of biomass was high with fructose followed by sucrose and lactose. These results are comparable with Streptomyces hygroscopicus strains AK-111-81, CH-7, which utilized lactose as carbon source for antibiotic production (36, 37). As lactose emerged as the most preferred carbon source for bioactive metabolite production by the strain, varying concentrations of lactose (0.5-5%) was tested to determine its optimal concentration. As shown in Fig 7, lactose at levels of 2% and 1% showed optimal yields of biomass and bioactive metabolites, respectively. A few reports suggested that maximum growth and bioactive metabolite production occurred with glucose (1%) as sole carbon source (32, 38, 39). In order to develop effective composition of growth medium, the roles of different nitrogen sources were evaluated for their influence on growth and antimicrobial agent production by the strain. Of all examined nitrogen sources, bacteriological peptone was found to be the best nitrogen source for growth as well as bioactive metabolite production. It should be noted that tryptone and tyrosine, as nitrogen sources, also favored good growth but the antimicrobial compound yield was less in comparison to peptone. Inorganic nitrogen sources like ammonium sulfate, ammonium oxalate, sodium nitrate and some organic nitrogen sources like yeast extract and casein did not show significant effect on antibiotic production by the strain. Peptone enhanced the biomass and

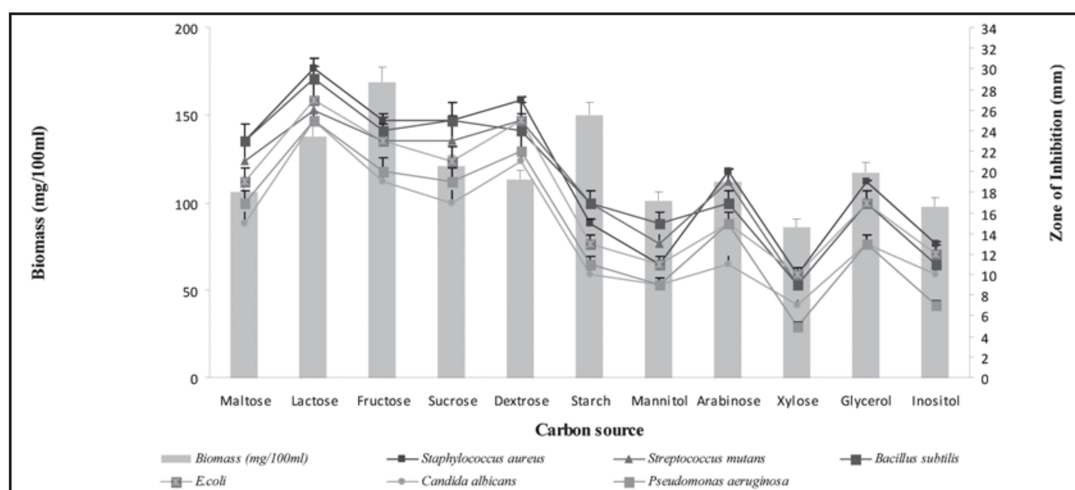

Fig. 5. Effect of different carbon sources on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK –A. Data are statistically analyzed and found to be significant at 5%

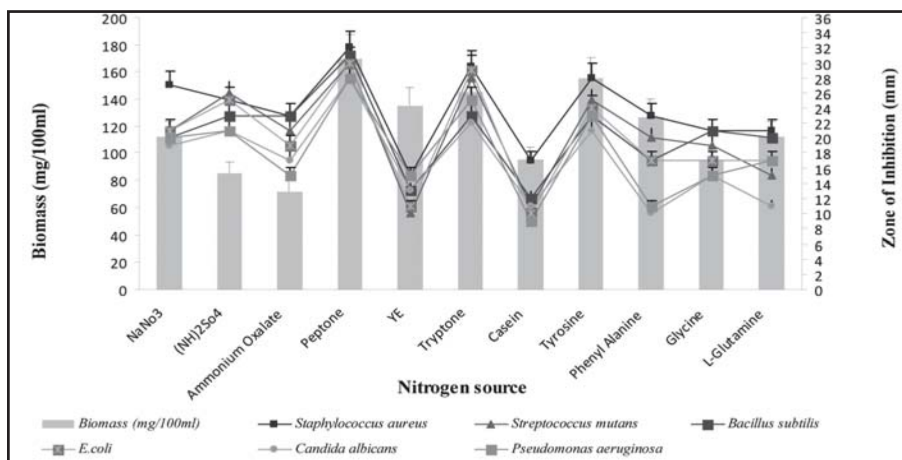

Fig. 6. Effect of different nitrogen sources on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK –A. Data are statistically analyzed and found to be significant with 5%.

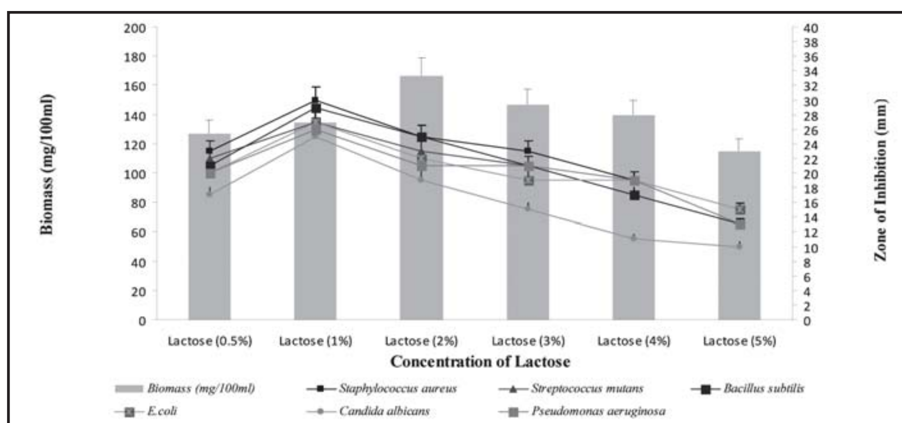

Fig. 7. Effect of different concentrations of Lactose on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK –A. Data are statistically analyzed and found to be significant at 5%.

bioactive metabolite production by *Streptomyces cheonanensis* VUK-A which is in conformity with earlier reports (21, 40, 41, 32). Effect of different concentrations of peptone on the production of bioactive metabolite production has been shown in Fig.8. It should be noted that peptone at a concentration of 1% and 0.25% exhibited optimal production of biomass and bioactive metabolite, respectively. Hassan *et al.* (42) recorded that $NaNO_3$ followed by peptone and alanine at a concentration of 0.25% increased antibiotic production by Streptomyces violates. Ismet *et al.* (43) also reported that corn steep powder at a concentration of 0.25% enhanced cell growth and bioactive metabolite production by *Micromonospora* sp. M 39.

Minerals on Biomass and Bioactive Metabolite Production: Effect of minerals on biomass and bioactive metabolite production by the strain has been shown in Fig 9. Among the minerals tested, K_2HPO_4 supported biomass and bioactive metabolite production whereas lower antimicrobial metabolite production was obtained with $FeSO_4$ and $ZnSO_4$. Ripa et al. (44) also reported that K_2HPO_4 showed positive influence on antibiotic production by the strain. Narayana

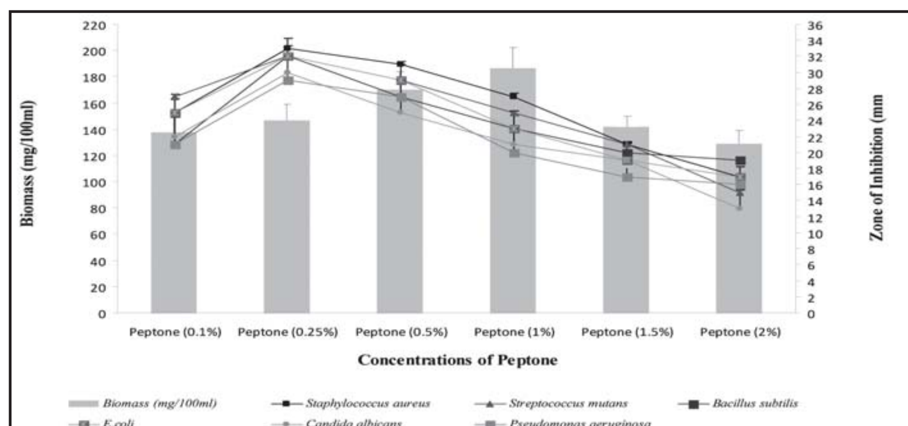

Fig. 8. Effect of different concentrations of peptone on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK –A. Data are statistically analyzed and found to be significant at 5%.

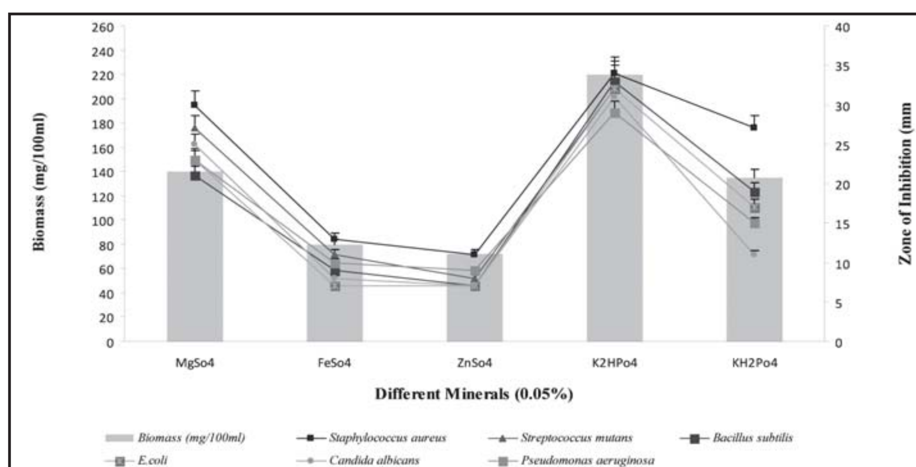

Fig. 9. Effect of different minerals on biomass and bioactive metabolite production by *Streptomyces cheonanensis* VUK –10. Data are statistically analyzed and found to be significant at 5%.

and Vijayalakshmi, (18) also noted that K_2HPO_4 slightly enhanced the production of cell mass and bioactive metabolites of *Streptomyces albidoflavus.*

Discussion

A broad spectrum bioactive metabolite producing actinomycetes isolate VUK-A from Coringa mangrove ecosystem, Andhra Pradesh, India has been identified as *Streptomyces cheonanensis* VUK-A. Ninety six hours of incubation at 30ºC and pH 7 was found to be optimum for bioactive metabolite production. It has been reported that the environmental factors like temperature, pH and incubation time have profound influence on antibiotic production (45). It was found that the bioactive metabolite production by *Streptomyces cheonanensis* VUK-A was positively influenced by the carbohydrates, nitrogen sources and minerals. The results suggest that antibiotic production was higher in medium having 1% lactose as carbon source. The

Vol. 6 (1) 99-111 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

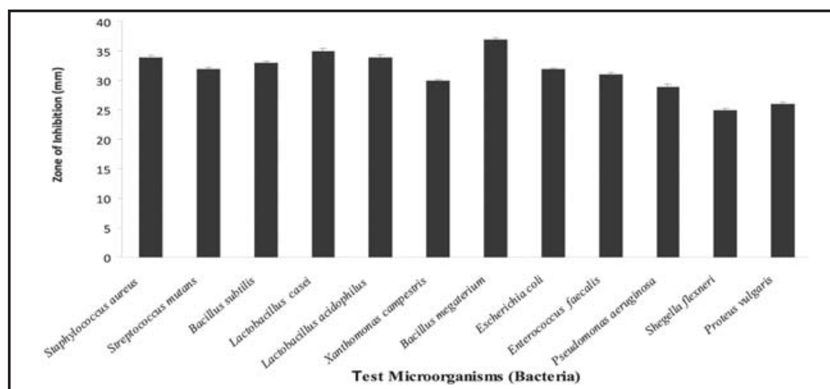

Fig. 10. Figure representing the anti-microbial metabolite produced by *Streptomyces cheonanensis* VUK-A (presented in terms of zone of inhibition) under optimized conditions tested against various bacteria. Data are statistically analyzed and found to be significant at 5%.

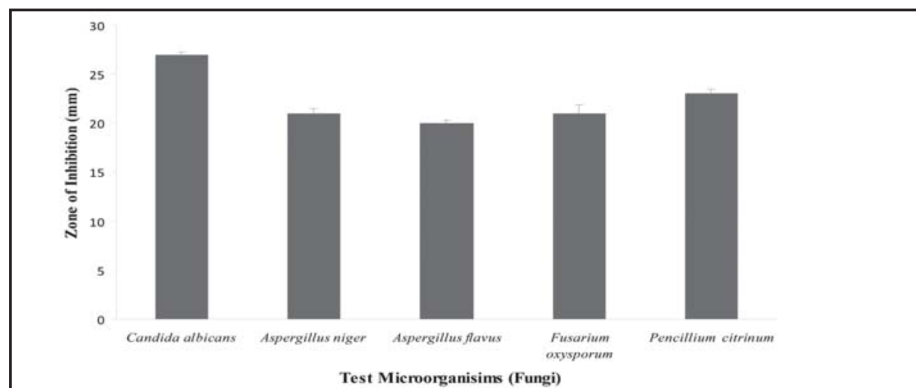

Fig. 11. Figure representing the anti-microbial metabolite produced by *Streptomyces cheonanensis* VUK-A (presented in zone of inhibition) under optimized conditions tested against various fungi. Data are statistically analyzed and found to be significant at 5%.

bioactive metabolite production got reduced with increase or decrease of lactose concentration. Among the nitrogen sources tested maximum antibiotic production was obtained with 0.25% peptone. In comparison with inorganic nitrogen sources, organic nitrogen sources gave relatively higher antimicrobial agent production by *Streptomyces cheonanensis* VUK-A. This is in conformity with the findings of Vahidi *et al.,* (46) which showed that the organic nitrogen sources are better for the production of antifungal agents. Among minerals, K_2HPO_4 favored slight enhancement of antibiotic production. The results indicated that antibiotic production is greatly influenced by medium constituents. Vilches *et al.*, (47) stated that the nature of carbon and nitrogen sources strongly effect antibiotic production in different organisms. In the present study, the metabolites produced by *Streptomyces cheonanensis* VUK-A grown under optimized conditions exhibited good antimicrobial activity against gram positive, gram negative bacteria and fungi (Figs. 10, 11 and 12). Hence, further studies regarding the purification, characterization and identification of bioactive compounds produced by *Streptomyces cheonanensis* VUK-A are in progress.

Acknowledgements:

The authors are thankful to CSIR, India for providing the financial support.

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Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) 112-118 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Differential Expression of ADH and ALDH2 can be a Diagnostic Marker in Gastroesophageal Cancer

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Abstract

Acetaldehyde interacts covalently with DNA to form major stable acetaldehyde–DNA adduct N2- ethyl-2'- de-oxyguanosine, which may be critical initiating event in the multistage process of chemical carcinogenesis primarily observed in gastroesophageal cancers. In the present study, RT-PCR experiment results showed increased expression of ADH and decreased expression of ALDH2 in GE cancerous conditions when compared with the normal GE tissue indicating the variation in the catabolism of acetaldehyde to acetate in the cancerous tissue. These results were further corroborated by enzyme assay where increased ADH enzyme activity was observed (0.445$\pm$0.02μM/ml/minute) in cancerous tissue compared to normal tissue (0.335$\pm$0.01 μM/ml/minute) whereas; ALDH2 enzyme activity (0.15$\pm$0.01 μM/ml/minute) decreased appreciably in cancerous tissue and compared to normal tissue (0.356$\pm$0.04 μM/ml/minute). Further, SDS-PAGE results too indicated differential expression of 40KD and 55KD proteins in normal and in GE cancerous tissue. Therefore, detection of ADH and ALDH2 expression levels in alcoholics will assist in the early diagnosis of gastro esophageal cancers.

Key Words: Acetaldehyde, DNA adducts, Gastroesophageal cancer, N2-ethyl-2'-deoxyguanosine

Introduction

Alcohol consumed by individuals is catabolised in the body with the help of Alcohol dehydrogenase (ADH) and Aldehyde dehydrogenases (ALDH). ADH located in the cytosol, promotes the oxidation of ethanol into acetaldehyde, where they release H^+ ions along with reduction of NAD to NADH. Multisubunit superfamily enzyme ALDH catalyzes the oxidation of acetaldehyde to acetate during ethanol metabolism Fig 1. In humans, there are multiple forms of ALDH that consist of nine major families, ALDH1 to ALDH9 (1-3). The best studied isoenzymes are the cytosolic and mitochondrial forms, designated as ALDH1 and ALDH2, respectively (4). ALDH1 and ALDH2 are both tetrameric with individual subunits comprising 499–500 amino acids and they share 68% sequence identity with each other (4).

Among them, mitochondrial ALDH2 plays a major role in human acetaldehyde metabolism because of its very low Km (< 5 μM) comparing with the other isoforms (5) Even though, aldehyde formation is majorly through by ADH, cytochrome P4502E1 (CYP2E1) and catalase also contributes its formation (6).

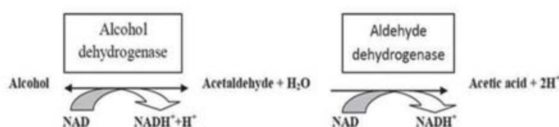

Fig 1. Mechanism of alcohol consumption in human beings

All human are not alcoholics but alcohol may derive from fermented foods and metabolites of microbial flora. More than 90% of ingested alcohol is eliminated via metabolic degradation mainly in the liver. The International Agency for Research on Cancer (IARC/WHO) has recently concluded that acetaldehyde derived from the alcoholic beverage itself or formed endogenously is carcinogenic to humans (7-10), which is a highly reactive intermediate that may cause cellular and DNA damage. Several possible mechanistic pathways through which drinking alcohol may cause cancer are: alcohol's contact-related local effects on the upper gastrointestinal tract, the induction of microsomal enzymes involved in carcinogen metabolism, the generation of oxygen radicals and lipid peroxidation products, nutritional deficiency, especially vitamin and mineral deficiencies and suppressed immune function (11). The inhalation of acetaldehyde produced tumors of the respiratory tract, specifically adenocarcinomas and squamous cell carcinomas of the nasal mucosa in rats (12) and laryngeal carcinomas in hamsters (13), in which this metabolite also served as a promoter in carcinogenesis attributable to benzo(a)pyrene (14). Acetaldehyde interacts covalently with DNA to form a major stable acetaldehyde–DNA adduct, N2-ethyl-2'- deoxyguanosine, which is a critical event in the multistage process of chemical carcinogenesis (15, 16). N2-ethyl-2'-deoxyguanosine can be used efficiently by eukaryotic DNA polymerase (17). Alcoholic patients' levels of acetaldehyde adduct in lymphocyte and granulocyte DNA was much higher than the corresponding levels in healthy control individuals (18, 19). In view of importance of alcohol metabolism; in the present study ADH and ALDH2 expressions were evaluated in human gastro esophageal cancer for the probable use as prognostic marker.

Materials and Methods

Biopsy collection**:** For this study, the cancerous and normal human gastro esophageal tissues of 6 patients were obtained from the Department of gastroenterology, Sri Venkateswara Institute of Medical Sciences (SVIMS).

Reverse Transcription -Polymerase Chain Reaction (RT-PCR)**:** The total RNA was extracted from both normal and cancerous tissues and from them the mRNA was isolated by oligo dT cellulose column chromatography. Thus, 1ìg of total mRNA from both normal and diseased tissues were isolated, the first strand of cDNA synthesis was carried by using 1 unit of AMV-reverse transcriptase (as per manufacturer's protocol -Promega) for 1hr, the above reaction mixture was used as template for RT-PCR which was performed in a Thermal cycler (Eppendorf master cycler gradient) by using specific primers and conditions mentioned in Table 1. The results were recorded in Vilber Lourmat gel documentation system (20-22).

Tissue Homogenization**:** 20 mg of diseased and normal tissues were homogenized by adding 1ml homogenizing buffer (0.1M Tris-HCl PH = 7.5; 15 mM EDTA; 0.25N sucrose) each.

Table 1. The details of Forward and Reverse Primers and PCR reaction conditions

	Forward primer 5' '! 3'	Reverse primer 5' '! 3'	PCR Product size
ADH	GTGCTTGTGCCAAGGTTTC	CAGGCCACGTGCTTGGCAG	0.762Kb
ALDH2	CAGCAGCAATGCCCCCA	CCAGCTGGGCTCACCTT	0.917Kb

PCR conditions	Temperature	Time
Initial denaturation	94^0C	5 min
Denaturation	94^0C	1 min
Annealing	52^0C	45 sec
Amplification X 40 cycles	72^0C	1 min
Extension temperature	72^0C	30sec

Homogenized solutions were collected, centrifuged for 2min at 1,500 g at 4^oC cell debris were removed, and supernatant was again centrifuged at 33,000 g for 10 min at 4^oC. The supernatant was again centrifuged at 1,00,000 g for 90 min at 4^oC and the supernatant thus, obtained was used for enzyme assay.

Enzyme Assay for ADH and ALDH2**:** Enzymes ADH and ALDH2 were assayed by comparing their respective normal and diseased tissues to estimate their enzyme activities, Km towards substrates. 3ml assay mixture contained 100mM Tris HCl pH 8.0, 1mM NAD, 50 ìl of ADH / 50ìl of ALDH2 enzyme (source tissue extract), 0.5M ethanol (for ADH assay) / 1mM acetaldehyde (for ALDH2 assay) were taken and incubated for 10 min and O.D. values read at 340nm. Enzyme activities, specific activities, Vmax and Km were determined through Hanes-Woolf plot. On X-axis (So) substrate concentration and on Y-axis [(So)/ Vo] were plotted.

Proteins Profile Analysis**:** The extracts of both normal and diseased tissue were electrophoresed in 10% SDS-PAGE and analysed by silver staining (23, 24).

Result

In the present study human Gastro esophageal cancerous tissue was obtained from Department of Gastroenterology, Sri Venkateswara Institute of Medical Sciences; Tirupati, which was evaluated and ascertained clinically. From the cancerous and normal tissues total RNA was extracted and from that, total mRNA was fractionated using Oligo-dT cellulose column chromatography. Thus obtained mRNA

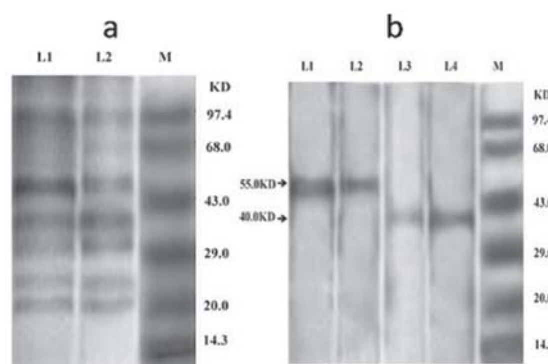

Fig. 2. Detection of ADH and ALDH2 mRNA expression in gastroesophageal cancer tissue compared with normal tissue. a. Lanes L1 and L2 RT-PCR products of ADH from normal tissue and lanes L3 and L4 ADH RT-PCR products from cancerous tissue. b. Lanes L1 and L2 RT-PCR product of ALDH2 from normal tissue and lanes L3 and L4 ALDH2 RT-PCR product from cancerous tissue.

from the column was about 0.1% of the total RNA and this total mRNA was used as template in RT-PCR experiment. The results indicated an increased expression of ADH gene in cancerous condition compared to normal (Fig 2a). Similarly, another important enzyme of alcohol metabolism ALDH2 was also evaluated using RT-PCR which showed low expression level compared to normal (Fig 2b).

These results were further evaluated using enzyme kinetics (Table 2) .The enzyme activity of ADH was found to be EA = 0.335±0.01µM/ml/min; Km = 0.193±0.01 µM and ALDH2 was found to be 0.356±0.04µM/ml/min; Km = 0.0299±0.003 µM in the normal tissue However, in the Gastro esophageal cancerous tissue the enzyme kinetics for ADH and ALDH2 were EA = 0.445±0.02 µM/ml/min and 0.150±0.01 µM/ml/min; Km = 0.082±0.01 µM and 0.2916±0.003 µM respectively. The SDS PAGE analysis of normal and cancerous tissue showed high expression at 40KD. This study was performed in order to ensure increased protein content due to higher expression of ADH since, the molecular weight of human ADH is about 40KD. These results clearly explain probable accumulation of acetaldehyde in the cancerous tissue.

Discussion

Acetaldehyde, the major ethanol metabolite that is extreme toxic and reactive than ethanol, has been postulated to be responsible for alcohol-induced tissue and cell injury. The problem arises when there is any malfunction of ALDH2 i.e., prevention of oxidizing the acetaldehyde (toxic) to acetate (non toxic) thus, the accumulation of acetaldehyde, a Carcinogen in the body leads to cancer. Many researchers have shown that accumulation of acetaldehyde in the body induces chromosomal aberrations, micronuclei and sister chromatid exchanges in cultured mammalian cells (25). It can also interact covalently with DNA to form DNA adducts, which may be involved in cancerous conditions. The experiments in mice explaining the formation of N2-ethyl-2'- deoxyguanosine, one major stable acetaldehyde–DNA adduct (26), was detected in the liver of ethanol-treated mice (27) and it has been observed that N2- ethyl-2'-de-oxyguanosine is used more efficiently by eukaryotic DNA polymerase to incorporate in

Table 2. The kinetics of ADH and ALDH2

ADH			ALDH2		
	Normal tissue	**Cancerous tissue**		**Normal tissue**	**Cancerous tissue**
Enzyme activity	0.335±0.01 µM/ml/min	0.445±0.02 µM/ml/min	**Enzyme activity**	0.356±0.04 µM/ml/min	0.150±0.01 µM/ml/min
Specific activity, V_o	134±4 µM/mg/min	30.90±2 µM/mg/min	**Specific activity, V_o**	24.72±1 µM/mg/min	60±3 µM/mg/min
V_{max}	4.84±0.04 µM/mg/min	1.36±0.06 µM/mg/min	**V_{max}**	0.598±0.05 µM/mg/min	8.333±0.09 µM/mg/min
Km	0.193±0.01	0.082 ±0.01 µM	**Km** µM	0.0299±0.003	0.2916±0.03 µM µM

SD ± for four determinations

DNA (28). In Alcoholic patients, levels of acetaldehyde adduct in lymphocyte and granulocyte DNA was much higher than the corresponding levels in healthy control individuals (29). *In vitro* experiments have shown that lymphocytes from habitual drinkers with the inactive form of ALDH2, which cannot detoxify acetaldehyde efficiently, have higher frequencies of sister chromatid exchanges than lymphocytes from individuals with normal, active ALDH2 (30). In the present study corroborated results shown increased ADH activity with very low Km and decreased ALDH2 activity with very high Km in the cancerous tissue compared to normal tissue (Table 2). Very low Km also indicates very high Kcat (31, 32) leading to high amount of ADH expression and thus, increased formation of acetaldehyde (Fig. 1). These results were further substantiated in RT-PCR experiment where higher and lower expression levels of ADH and ALDH2 respectively were observed in the cancerous tissue (Fig. 3). Hence, the ADH and ALDH2 activity and expression in the system could be handy in the early diagnosis of GE cancer.

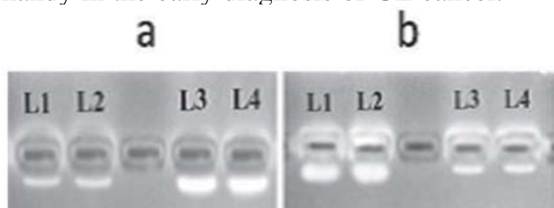

Fig. 3. SDS PAGE analysis of total protein extracted from cancerous tissue and normal tissue. Lane L1 cancerous tissue total protein and L2 normal tissue total protein.

Conclusion

The present study clearly suggests that the increased consumption of alcohol has profound effect resulting in Gastro esophageal cancer thus; it is imperative to diagnose GE cancers at an early stage. In this respect the differential levels of ADH and ALDH2 can be diagnostic markers.

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Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) i-iv January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

NEWS ITEM

Science need to be more relevant for poor

Prime Minister, Sri.Manmohan Singh called for greater alignment of the science and technology sector with the inclusive development needs of the country. Inaugurating the 99th Indian Science Congress at KIIT University, Bhubaneswar, he emphasized that science is often pre-occupied with problems of the rich, ignoring the enormous and in many ways more challenging problems of the poor and the under-privileged and so we must make scientific output much relevant to our stage of development. The Prime Minister stressed up on ensuing a major increase in investment in research and development including by industry and strategic sectors along with creation of a new innovation ecosystem. He explained the need of expanding basic science infrastructure, while enlarging the reach of international collaboration. Expressing the need to do much more to change the face of Indian science to over 20,000 delegates, including nobel laureates and renowned scientists from India and abroad at 99th Indian Science Congress.

Need to use Satellite Technology for progress of Nation

Noting that Satellite platforms can help our economic growth, President Smt.Pratibha Devisingh Patil emphasized that the tele-education and tele-medicine are among the readily identifiable areas for this. The president emphasized that the space-based applications had revolutionized the way that government machinery reaches out to its citizens, even in the far flung remote areas of the country. Utilizing the application of its research and work for the benefit of society need to be the main objectives of science and technology. The president lauded the way ISRI had reached out to other countries in science and technology, and she said that, India does enjoy a niche amongst the space faring nations of the world.

More autonomy to IIMs, IITs

The Minister of State for Human Resource Development, Smt.D. Purandeswari stated that the Union Government is willing to give greater autonomy to premier professional educational institutes such as IIMs and IITs. Addressing an international conference on Human Values in Higher Education, she said that that the government believe in autonomy because that will bring in greater flexibility within the system and encourage the institutes to do much better than what they are doing now. She further stated that the HRD Ministry in principle agreed that older IIMs like Ahmadabad, Bangalore and Calcutta, which do not claim non-plan grants, should have independence to create posts within approved norms and can establish open centres in India and abroad so that the further growth can be expected from these organizations.

PM urges Youth to take up career in Mathematics

Prime Minister, Sri.Manmohan Singh expressed concern that for a population size of India, the number of competent mathematicians we have is badly inadequate declaring the year 2012 as “The National Mathematical Year” as a tribute to the great mathematician Srinivasa Ramanujan while inaugurating the year long celebrations at University of Madras on the eve of 125th birth anniversary of Ramanujan and announced that the birthday of Ramanujan, December 22 as “National Mathematics Day”. The Primer Minister expressed concern over a slide in the number of students pursuing higher studies in mathematics. He rebuffed perceptions that pursuing mathematics does not end up in a

rewarding career. The mathematicians now have a wide array of attractive careers to choose from, even “the teaching profession itself becoming much more attractive.”

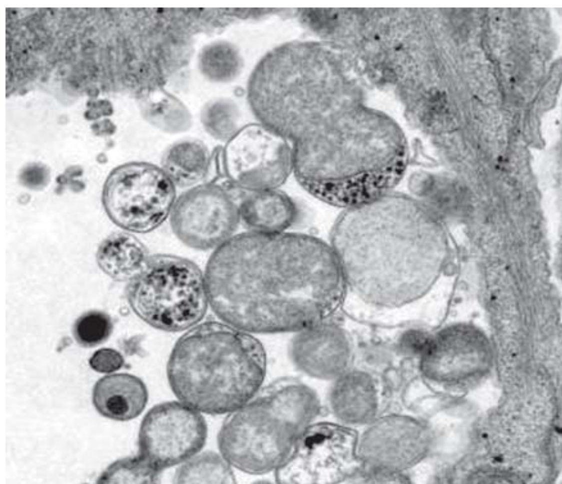
Spearheading of Aakash-II project

The HRD Minister, Sri.Kapil Sibal addressing a gathering stated that our country is aimed at developing enhanced version of low cost tablet Aakash, indicating that the improved version would be launched by April. The Aakash was launched last year with much fanfare, developed as part of the National Mission on Education through Information and Communication Technologies of the Ministry of Human Resource Development.

SCIENTIFIC NEWS

Species of Horse Fly named after Beyonce

A previously un-named species of horse fly whose appearance is dominated by its glamorous golden lower abdomen has been named in honour of American pop diva, Beyonce. According to the Australian National Insect Collection researcher responsible for officially describing the fly as Scaptia (Plinthina) beyonceae, CSIRO’s Bryan Lessard, the fly’s spectacular gold colour makes it the “all time diva of flies”. According to Mr Lessard’s paper, published in the Australian Journal of Entomology, this discovery has doubled the known size of the Scaptia (Plinthina) sub-genus and extended the known distribution of Scaptia into the Northern Territory and north-western Australia where they were previously thought not to exist. Mr Lessard said that it was the unique dense golden hairs on the fly’s abdomen that led me to name this fly in honour of the performer Beyoncé as well as giving me the chance to demonstrate the fun side of taxonomy – the naming of species.



S. Vinod

New way to Identify and Block — Traits in Harmful Pathogens

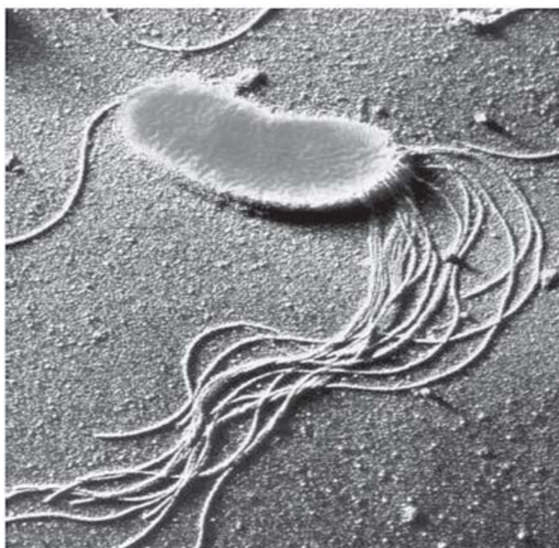
Researchers at Duke University Medical Center have developed a new way to identify the genes of harmful microbes, particularly those that have been difficult to study in the laboratory. This new method uses chemicals to create mutant bacteria, followed by genomic sequencing to identify all mutations. By looking for common genes that were mutated in Chlamydia sharing a particular trait, the investigators were able to rapidly “zero in” on the genes responsible for that trait. Raphael Valdivia, an Associate Professor of Molecular Genetics and Microbiology at Duke said that the approach is versatile and inexpensive enough that it could be applied to study a range of microorganisms. One of the goals in studying microbial pathogens that harm humans and animals is to locate and disrupt the genes required for infection. The microbe in this study, Chlamydia, is usually sexually transmitted, hides in human cells, and is a type of bacteria that must cause disease to be transmitted from one host to another. Valdivia suggested that even microbes associated with our normal intestinal flora, which are notoriously difficult to manipulate, are now open to

exploration so that we can learn how their genes influence human health, including dietary disorders and inflammatory bowel disease. Valdivia also said that the new technique could help to create Chlamydia vaccines that have a combination of mutations that affect the pathogen's virulence. "That way we can cripple some aspects of the bacterium's ability to thrive intact in a host, while still allowing the bacterium to replicate enough to prime the immune system against it", Valdivia said.

R. Sudhir Kumar

Novel Antibacterial Protein Blocks *E. coli* O157:H7 Infection

The use of a novel antibacterial protein to selectively destroy diarrhea-causing *Escherichia coli* O157:H7 both prior to and after infection was reported in a rabbit model. The biotech company AvidBiotics, Inc., USA has been developing new antibacterial agents based on bioengineered pyocins. Pyocins are polypeptide toxins produced by some *Pseudomonas aeruginosa* strains that kill other types of bacteria by piercing their cell envelopes. AvidBiotics' proprietary "Avidocin" pyocin efficiently kills antibiotic-resistant bacteria and does not promote the spread of multidrug resistance. Avidocin proteins are nontoxic to animals or nontargeted bacteria, and are biodegradable. Results of studies in which Avidocin was used to treat rabbits exposed to *E. coli* O157:H7 reported that Avidocin remained active within the treated animals' intestinal tract for at least 24 hours after administration. Avidocin protein targeted against *E. coli* O157:H7 offers promise for both the prevention and treatment of infection by this important enteric pathogen. Moreover, this agent provides several significant advantages over conventional antibiotics, including a lack of drug-induced shiga toxin production and unintended collateral damage to normal intestinal bacterial populations. "Antibiotics are contraindicated for patients infected with enterohemorrhagic *E. coli* (EHEC) strains like O157:H7, because many of those drugs induce the bacteria to produce and release harmful toxins. Antidiarrheal medications also do not benefit infected patients, as they cause the bacteria to be retained in the intestines, leading to greater toxin exposure. Thus the successful development of treatments that can prevent infection or limit symptoms and disease duration and the possible further spread of harmful bacteria without increasing toxin release could benefit both individual patients and affected communities."



K. Greeshma

Electricity Production by *Geobacter sulfurreducens*

It was reported that the members of the Geobacteraceae can use electrodes as electron acceptors for anaerobic respiration. In order to understand this electron transfer process for energy production, *Geobacter sulfurreducens* was inoculated into chambers in which a graphite electrode served as the sole electron acceptor and acetate or hydrogen was the electron donor. The electron-accepting electrodes were maintained at oxidizing potentials by connecting them to

similar electrodes in oxygenated medium (fuel cells) or to potentiostats that poised electrodes at +0.2 V versus an Ag/AgCl reference electrode (poised potential). When a small inoculum of *G. sulfurreducens* was introduced into electrode-containing chambers, electrical current production was dependent upon oxidation of acetate to carbon dioxide and increased exponentially, indicating for the first time that electrode reduction supported the growth of this organism. When the medium was replaced with an anaerobic buffer lacking nutrients required for growth, acetate-dependent electrical current production was unaffected and cells attached to these electrodes continued to generate electrical current for weeks. This represents the first report of microbial electricity production solely by cells attached to an electrode. Electrode-attached cells completely oxidized acetate to levels below detection (<10 μM), and hydrogen was metabolized to a threshold of 3 Pa. The rates of electron transfer to electrodes (0.21 to 1.2 μmol of electrons/mg of protein/min) were similar to those observed for respiration with Fe(III) citrate as the electron acceptor (Eo' =+0.37 V). The production of current in microbial fuel cell (65 mA/m2 of electrode surface) or poised-potential (163 to 1,143 mA/m2) mode was greater than what has been reported for other microbial systems, even those that employed higher cell densities and electron-shuttling compounds. Since acetate was completely oxidized, the efficiency of conversion of organic electron donor to electricity was significantly higher than in previously described microbial fuel cells. These results suggest that the effectiveness of microbial fuel cells can be increased with organisms such as *G. sulfurreducens* that can attach to electrodes and remain viable for long periods of time while completely oxidizing organic substrates with quantitative transfer of electrons to an electrode.

P.Ananth Swaroop

Coffee May Protect Against Endometrial Cancer

Long-term coffee consumption may be linked with a decreased risk for endometrial cancer, according to new findings. Edward Giovannucci, Professor of Nutrition and epidemiology at the Harvard School of Public Health, reported that coffee is emerging as a protective agent in cancers that are linked to obesity, estrogen, and insulin. Drinking more than four cups of coffee per day was associated with a 25% reduced risk for endometrial cancer. Drinking between two and three cups per day was linked with a 7% reduced risk. A comparable link was seen in decaffeinated coffee, where drinking more than two cups per day was associated with a 22% reduced risk for endometrial cancer. Dr. Giovannucci states that he hopes this study leads to additional inquiries about the effect of coffee on cancer because in this and similar studies, coffee intake is self-selected and not randomized. "Coffee has long been linked with smoking, and if you drink coffee and smoke, the positive effects of coffee are going to be more than outweighed by the negative effects of smoking," said Dr. Giovannucci. "However, laboratory testing has found that coffee has much more antioxidants than most vegetables and fruits."

V.V.V.R.P. Kumar

EDUCATION

PhD/Post Doctoral Programs

Admission to Ph.D. Programme: Applications are invited for admission to Ph.D programmes in Pompeu Fabra University, Barcelona, Spain in the relevant areas of research in the Institute like bioinformatics, molecular biology and evolution. The PhD project will be directed by Dr. Tomás Marquès-Bonet. The project consists in the detailed analysis and characterization of changes of gene expression in primates due to structural variation such as duplications or

inversions. Candidates with Bachelor's degree (or equivalent) in Biology, Computer science or similar and with strong programming, knowledge on next-generation sequence methods and experimental background are eligible. Applicants should send, before February 2012 and preferably via e-mail, a CV and a short letter of interest including the names of two persons able to provide references to: Tomás Marquès-Bonet (tomas.marques@upf.edu).

Admission to Post Doctoral Programme:

Applications are invited for admission to Post Doctoral programme at Genomic and Structural BioInformatics Unit, BioSystems, BioModeling & BioProcesses Department, Universite Libre de Bruxelles, Belgium in the Development of computational tools for the rational design of modified proteins for a period of 2 years. The candidate should have experience in structural bioinformatics, in particular in the design and development of knowledge-based approaches for predicting the structure, stability, interactions or function of a protein from its sequence, preferentially in C programming language, or in the applications of such approaches to systems of biological or medical interest. Eligible candidates can send applications with cover letter, full CV and the names and email addresses of two contact persons for reference letters to Marianne Rooman (mrooman@ulb.ac.be).

OPPORTUNITIES

Department of Genetics & Plant Breeding, G.B.Pant University of Agriculture & Technology, Pantnagar – 263145, Uttarakhand, India. Applications are invited from eligible candidates for One position of SRF in research projects entitled "Mapping of Mungbean Yellow Mosaic virus resistance loci in soybean" funded by Department of Biotechnology, Ministry of Science & Technology, Govt. of India on consolidated fellowship of Rs.14000/- per month. Candidates with M.Sc in Genetics & Plant breeding/ Biotechnology/Plant Breeding/Genetics with working knowledge of molecular markers. Eligible candidates may apply on plain paper to Dr. Pushpendra, Principal Investigator, DBT Project, Dept. of Genetics & Plant Breeding, College of Agriculture, G.B.Pant University of Agriculture & Technology, Pantnagar – 263145, Udham Singh Nagar, Uttarakhand by 04/02/2012. and appear for Interview on 06/02/2012along with the duly filled in application form supported by Bio-data and one set of attested photo copies of Certificates of educational qualification, age, experience, caste (in case of SC/ST/OBC candidates), latest passport size photograph. Date of Walk-in-Interview: 6th February, 2012 at Dept. of Genetics & Plant Breeding, College of Agriculture, G.B.Pant University of Agriculture & Technology, Pantnagar–263145, Udham Singh Nagar, Uttarakhand.

Centre for DNA FingerPrinting & Diagnostics, Nampally, Hyderabad – 500001, India. Applications are invited from eligible candidates for the post of Project Associate/ Research Associate and Project - Junior Research Fellow (JRF)/Project Assistant in research projects funded by National/ International agencies at CDFD like DST/DBT/ICMR/CSIR etc. For the post of Project Associate/ Research Associate candidates with Ph.D. in Physics / Chemistry / Life Science / Biotechnology / Genetics/ Biochemistry / Microbiology / Bioinformatics from recognized university or M.Sc. in Physics / Chemistry / Life Science / Biotechnology / Genetics/ Biochemistry / Microbiology / Bioinformatics from recognized university with three years of research experience are eligible. For the post of Project - Junior Research Fellow (JRF)/Project Assistant

candidates with M.Sc. in Physics / Chemistry / Life Science / Biotechnology / Genetics/ Biochemistry / Microbiology / Bioinformatics / Medicinal Chemistry from recognized university with research experience are eligible. Candidates may register their names by applying in the prescribed online application form at www.cdfd.org.in. and send the print out of the application to The Head, Administration, Centre for DNA FingerPrinting & Diagnostics (CDFD), 2nd Floor, Bldg. 7, Gruhakalpa, 5-4-399/B, M.J.Road, Nampally, Hyderabad – 500001, India by **17/02/2012**.

SEMINARS/WORKSHOPS/ CONFERENCES

World Congress on Biotechnology 2012: World Congress on Biotechnology 2012 was going to held on 4th - 6th May, 2012 at Leonia Holistic Destination, Hyderabad, India organized by Bright International Conferences & Events, Hyderabad, India. Abstract can be submitted online through Email: biotechnology2012@brightice.org/ info.brightice@gmail.com / info@brightice.org on or before April 6th, 2012. For further details contact: Organizing Comittee-Biotechnology2012, Bright Technologies, Plot No.109, Chanikya Puri, IDA Mallapur, Hyderabad-500076, India. Website: www.brightice.org.

Biotech 2012, Annual Conference on "Current Advances in Biotechnology & Medicine (YSC-2011): A Biotech 2012, Annual Conference on "Current Advances in Biotechnology & Medicine was going to held on February 24-25, 2012 at Institute of Liver & Biliary Sciences, New Delhi organized by Department of Research, Institute of Liver & Biliary Sciences, D – 1, Vasant Kunj, New Delhi, India. Abstract can be submitted online through E-mail: biotech2012.ilbs@gmail.com on or before February 1st, 2012. Registration Fee: For BSI Member Delegate- Rs.1000/- and for Non Member Delegate - Rs.1500/- and for BSI Member Students - Rs.500/ -and for Non Member Students - Rs.750/- and for Industry Individual – Rs. 4000/- (DD drawn in favor of Institute of Liver & Biliary Sciences payable at New Delhi). For further details contact: Dr. NirupmaTrehan Pati, Department of Research, ILBS, D – 1, Vasant Kunj, New Delhi, India.

4th National Symposium cum Workshop on Recent Trends in Structural Bioinformatics and Computer Aided Drug Design (SBCADD'2012): A 4th National Symposium cum Workshop on "Recent Trends in Structural Bioinformatics and Computer Aided Drug Design" (SBCADD'2012) was going to held on February 20-23, 2012 at L.Ct.L. Palaniappa Chettiar Memorial Auditorium, Alagappa University, Karaikudi, Tamilnadu, India organized by Department of Bioinformatics, Science Block, 4th Floor, Alagappa University, Karaikudi - 630004, Tamilnadu, India. Abstract can be submitted online through E-mail: bioinfoau@gmail.com on or before February 10, 2012. Registration Fee: For Faculty - Rs.1000/- and for Non Academic/Industry – Rs.1500/- and for Research Scholars - Rs.750/- and for Students - Rs.500/- (DD drawn in favor of Organizing Secretary, payable at Karaikudi). For further details contact: Dr. M. Karthikeyan, Organizing Secretary, SBCADD'2012, Department of Bioinformatics, Science Block, 4th Floor Alagappa University, Karaikudi - 630 004, Tamil Nadu, India. Phone – + 91-4565-230725.

6th Annual Convention of Association of Biotechnology and Pharmacy -

International Conference on Environmental Impact on Human Health and Therapeutic Challenges

December 20-22, 2012

6th Annual Convention of ABAP and International Conference on Environmental Impact of Human Health and Therapeutic Challenges is being organized at Sri Venkateswara University, Tirupathi, India during 20-22 December, 2012-01-15

Broad Areas of Focus

- Biodiversity and its Conservation
- Conservation Techniques (*in situ* and *ex situ*)
- Biotechnology in Biodiversity & Bar-coding
- Ecotoxicology impact on Biodiversity
- Water, Soil and Air Pollution & diseases
- Environmental Pollution and Control
- Environmental Impact Assessment
- Toxicology and Human Health
- Bioremediation and Biodegradation
- GM Crops & Ecofriendly Technologies
- Integrated Pest Management & Organic farming
- Nanotechnology & Drug Discovery
- Novel Therapeutics & other related areas

For further details contact

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Printed, Published and owned by Association of Bio-Technology and Pharmacy # 6-69-64 : 6/19, Brodipet, Guntur - 522 002, Andhra pradesh, India. **Printed at :** Don Bosco Tech. School Press, Ring Road, Guntur - 522 007. A.P., India **Published at :** Association of Bio-Technology and Pharmacy # 6-69-64 : 6/19, Brodipet, Guntur - 522 002, Andhra pradesh, India.
Editors : Prof. K.R.S. Sambasiva Rao, Prof. Karnam S. Murthy