ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

Volume 7 Issue 3 July 2013

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

www.abap.co.in

ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

(An International Scientific Journal)

Volume 7 Issue 3 July 2013

www.abap.co.in

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Current Trends in Biotechnology and Pharmacy

ISSN 0973-8916

Volume 7 (3) **CONTENTS** **July - 2013**

Research Papers

DNA Methylation at the *RARβ* Promoter: A Potential Biomarker for Cervical Cancer 708-715
Lao Duc Thuan, Truong Kim Phuong, Quynh-Anh NguyenThai, Neelesh Sharma and Le Huyen Ai Thuy

Design of Polymeric Nanoparticles of *Emblica officinalis* extracts and study of *in vitro* therapeutic effects 716-724
Renuka R., Sandhya P., Vedha Hari B.N. and Ramya Devi D.

Authentication of Herbal Medicinal Plant- *Boerhavia diffusa* L. Using PCR-RFLP 725-731
Kakoli Biswas, Ashita Kapoor and Rajesh Biswas

Purification, Partial Characterization and Applications of Alkaline Protease from *Bacillus subtilis* NCIM No. 2724 732-742
Sujatha P. and Radhika P.

Liquefied Sorghum Starch Medium as Novel Substrate for Production of Lactic Acid by Immobilized Acid Resistant *Rhizopus oryzae* in Natural and Synthetic Sponges 743-754
N.Sailaja, M.Sreedharamurthy, R.S. Prakasham and O.V.S.Reddy

Elicitation of Forskolin in Suspension Cultures of *Coleus forskohlii* (Willd.) Briq. Using Elicitors of Fungal Origin 755-762
G. Swaroopa, M. Anuradha and T. Pullaiah

Optimization and Production of Fibrinolytic Protease (GD kinase) from Different Agro Industrial Wastes in Solid State Fermentation 763-772
E. Venkata Naga Raju and G. Divakar

Insecticidal properties of water diffusible prodigiosin produced by *Serratia nematodiphila* 213C 773-781
Patil N.G., Kadam M.S. , Patil V.R. and Chincholkar S.B.

Antimicrobial property of *Datura* leaf extract against Methicillin-resistant *Staphylococcus aureus* isolated from Urethral and Skin Suppurative Infections 782-792
B. Venkanna, A Uma, Ch. Suvarnalaxmi, N. Chandrasekharnath, R. S. Prakasham and L. Jayalaxmi

Optimization of fermentation culture conditions for Alkaline Lipase Production by *Bacillus flexus* XJU-1 793-803
Francois N. Niyonzima,Sunil S. More and Uday Muddapur

Short Communication

Evaluation of Hematological and Biochemical Parameters of Magnetic Field Exposed Albino Rats 804-807
Purushothaman G., Gajalakshmi G., Qairunnisa, S., Madhuri B.A. and Chandrasekhar M.

Review

Psoriasis: History, Present and Future Prospects 808-824
Ajay Singh and Neena Bedi

News Item i - vi

Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

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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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 708-715 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

DNA Methylation at the *RARβ* Promoter: A Potential Biomarker for Cervical Cancer

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Abstract

Cervical cancer is the leading cause of cancer death for women worldwide. Besides human papilloma virus (HPV) infections which are main cause of cervical cancer global, the disruptions of the genetic material such as the epigenetics including DNA methylation are also demonstrated to be in related to cervical cancer. Recent studies indicated that DNA methylation at promoter region in *RARβ* gene related to the formation and progression in human cervical cancer. The *RARβ* gene is a member of the tumor repressor gene family, located at 3p24 region. The function is thought that RARβ protein limited the proliferation of cervical tumor. In an attempt to establish early molecular diagnostic biomarker for cervical cancer, at first, we performed a systematic evaluation about the methylation frequencies of *RARβ*. Subsequently, 36 studies were evaluated for the methylation status for *RARβ* by Methylation Specific PCR (MSP) assay. As the results, we correctly confirmed that the methylation frequencies as 77% on CpG of *RARβ* promoter. Experimentally, we concluded that methylation levels associated with HPV genotype infections. Therefore, methylated *RARβ* could be a potential biomarker for cervical cancer.

Keywords: Biomarker, Cervical cancer, DNA methylation, *RARβ*, MSP.

Introduction

Cervical cancer is the leading cause of cancer death for women worldwide. In Vietnam, the death ratio of cervical cancer patients was up to with 52% (1). Besides human papilloma virus (HPV) infections which caused 70% of cervical cancer global, the disruptions of the genetic material such as the epigenetics including DNA methylation, are also demonstrated to be related to cervical cancer (2).

DNA methylation, as a covalent chemical modification, is resulting in the addition of a methyl group (CH3-) at the carbon 5 position of the cytosine ring. The establishment and maintenance of DNA methylation is achieved by specific enzymes known as DNA methyl transferases. DNA methylation with the regulation of gene expression leds to human cancers has become one of the most important criteria in cancer investigation. Recent studies showed that the disruptions in DNA's methylation in malignant cells are the abnormal methylation which promote CpG islands of tumor suppressor genes or another cancer related genes lead to tumorgenesis (3, 4). The alteration in DNA methylation of promoter CpG islands of human genes which alternative mechanism of gene activation leads to several human cancers as cervical cancer, lung cancer.

The *RARβ* gene is a member of tumor repressor gene family, located at 3p24 region. This gene encodes *RARβ* receptor for retinoic acid, a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators (5,6,7). It bind with retinoic acid, the biologically active form of vitamin A which mediates cellular signaling in embryonic morphogenesis, cell growth and differentiation (6,

8). It is thought that this protein limits the growth of many different tumor cells by regulating gene expression. Recently, it was found that hypermethylation in promoter CpG islands of *RARβ* occur to the loss of expression of *RARβ*, mostly in the cervical cancer. Loss of retinoic acid receptor for beta gene expression is linked to aberrant histone H3 acetylation in lung cancer cell lines. According to the research conducted by Ivanova *et al.*,(2008) the loss of expression of *RARβ* in 40% of squamous cell carcinoma is caused by hypermethylation (8). The methylation of the 5' region of *RARβ* gene contribute in gene silencing which may be an important point to generate the early event in cervical carcinogenesis (9). As compared with the normal cervical cells, the *RARβ* is normally expressed without methylation, but the hypermethylation in *RARβ* in the invasive cervical cancer rounds 33 to 66% (8,10).

In this study, we used the MSP (Methylation Specific PCR), a rather simple rapid and specific method to determine the methylation status of promoter CpG islands of *RARβ* (Retinoic Acid Receptor Beta) described by Herman *et al* (11).

Materials and Methods

Data mining : More than 36 studies (update to January, 2013) were evaluated to examine the methylation of *RARβ* in across all stages of cervical cancer and assays in *RARβ* gene. The target sequences were collected from the Genbank using the identification number (Gene ID) 1029. The online bioinformatics tools, such as Methprimer (http://www.urogen.org), Tf Search (www.cbcr.jp/research/db/TFSEARCH.html) to determine CpG islands in promoter and transcription factor binding sites in the target gene were used, respectively. The designing primers for MSP as well as evaluation of primers' parameter were examined by Methprimer (http://www.urogene.org/methprimer/) and IDT analyzer (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/), respectively. The specificity of primers was evaluated then by using Meth Blast database (http://medgen.ugent.be/methBLAST/).

DNA extraction, bisulfite and methylated specific PCR : Biological samples consisted in 32 clinical samples including 27 samples of Pap'smear, provided by R and D Department, Viet-A Corporation, Vietnam. These samples were positive with high-rick HPV genotyped using PCR-Reverse Dot Blot Kit (Viet-A Corporation, Vietnam). The fresh five frozen tissues were collected from cervical cancer patients, from Hung Vuong Hospital (Ho Chi Minh city, Vietnam).

Approximately 2 mg genomic DNA of each sample was bisulfite- modified and purified by using the DNA modification kit (Epitect Kit, Qiagen) and the final precipitate was eluted in a volume of 20 ml. Methylation- specific PCR was done in a total volume of 15ml, containing 3ml bisulfite- modified template DNA, 0.75 unit iTaq DNA polymerase (Biorad). MSP reaction was subjected to initial incubation at 95^{o}C for 5 min, followed by 40 cycles at 95^{o}C for 30s, 51^{o}C for 30s, 72^{o}C for 30s and 72^{o}C for 6 min for final incubation. *RARβ* -MF/MR primers: Forward TCGAGAACGCGAGCGATTC and Reverse GACCAATCCAACCGAAACGA, *RARβ* -UF/UR primers: Forward TTGAGAA TGTGAG TGATT TGA and Reverse AACCAATCCAAC CAAAA CAA. MSP products were resolved on 2% agarose gel and visualized with Ethidium Bromide (Biorad).

MSP sequencing : MSP products were directly performed sequencing in Macrogen Co. (Korea) to confirm the specificity of primers, to examine the efficiency of bisulfite modification reaction and the methylation status of the tested region as well.

Results and Disscussion

Data mining : We systematically evaluated 36 studies in total to examine the methylation of *RARβ* in cervical cancer cells from the various sources such as Pap'smear, fresh frozen tissue, paraffin embedded tissue, cervical scrapings and exfoliative cells. The results showed that the majority of studies used MSP counted for 50% (18 of 36) and Quantitative MSP (QMSP) counted for 30% (11 of 36) in examining methylation status in cervical cancer. Alternatively, fresh frozen tissue

was commonly, counted for 52.8% (19 of 36), used in studying.

The fact that the aberrant modulation frequencies of *RARβ* surveyed in total of 491 samples showed the variation, that might be caused due to kind of sample are used, population, method followed or other unidentified factors given effect on the result. The results are shown in table 2.

In this current research, among four studies about the methylation status, there were two reports indicated that the methylation of *RARâ* with over 40 percent and the two others were approximately 0 percent. As the result, we based on the mean of percentage weighted to evaluate the methylation frequencies as 35.6% which were fairly high. It was thought to be related to its function which mediates cellular signaling in embryonic morphogenesis, cell growth and differentiation (6, 8). However, the methylation frequencies were dramatically high in the adenoma carcinoma (75%). According to this report, it is considered that hypermethylation in *RARβ* was a significant criterion biomarker for cervical cancer. We relied on the information about the methylation in *RARβ* gene, MSP method is chosen for evaluating the methylation status in fresh frozen cells which are isolated directly from patients.

Table 1. Overview of 36 studies across various sources and methods

Methods Sources	**MSP**	**QMSP**	**Nested MSP**	**Bisulfite sequencing**	**Other**	**Total**
Pap' smear	1	1	-	-	-	2
Fresh frozen tissue	9	5	1	2	2	19
Paraffin embedded tissue	5	1	-	1	-	7
Cervical scrapings	1	2	-	-	-	3
Exfoliative cells	2	2	1	-	-	5
Total	18	11	2	3	2	36

Table 2. Comparison of methylation frequencies and methods in various stages of cervical cancer. The percentages (%) indicated methylation frequencies based on the number of samples which are put in parentheses.

Stages	MSP			QMSP			References
	(a)	(b)		(c)	(d)	(a)	
LSIL	-	11%(37)	5%(181)	-	37.5%(8)	65%(20)	(13,14,15)
HSIL	-	29%(17)	56,6%(23)	-	55.6%(18)	75%(20)	
SCC	-	40%(20)	75%(8)	-	93.8%(16)	85%(40)	(8,15,16)
AC	-	-	-	-	100%(4)	85%(20)	(15)
Normal	-	0%(22)	0%(8)	-	44.4%(9)	85%(20)	(13,15)

(a) Paraffin embedded tissues; (b) Fresh frozen cells; (c) Pap smear; (d) Cervical scrapings. LSIL – Low-grade Squamous Intraepithelial Lesions, HSIL – High-grade Squamous Intraepithelial; SCC – Squamous cell carcinoma; AC – Adenoma carcinoma.

Vol. 7 (3) 708-715 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

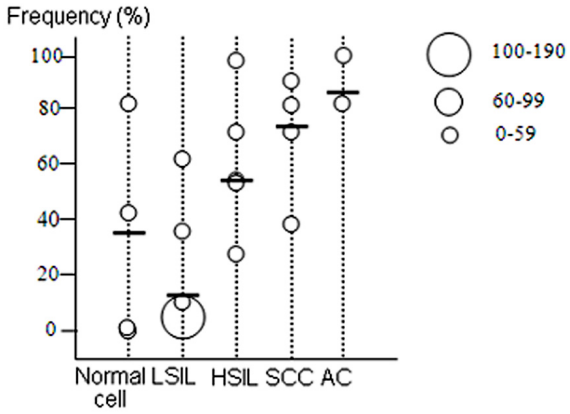

Fig. 1. Methylation frequencies were reported in cervical cancer for RARβ. The center of the circle indicated the reported frequencies. The size of circle indicated the size of studies. Horizontal lines indicated the mean of percentage weighted methylation frequencies for RARβ based on various sources of cervical cancer.

Determination of CpG islands and MSP primer design : The promoter of CpG islands was identified by using MethPrimer program. CpG island reached length of 150 bp situating in the region from +65 to +215. Several different transcription factors including Sp1, MZF, CP2, ANML1a, p300, C/EBP, GATA were identified by TF RESEARCH. These transcription factors presented in promoter are evidently demonstrated associated with gene expression in human. Therefore, we designed MSP primers to specifically evaluate methylation status in recognized positions of these transcription factors, mean that we had able to evaluate efficiently and exactly methylation status of promoter CpG of *RARâ* gene.

To evaluate the methylation status of bisulfite-treated DNAs, we designed primer pairs which have to contain at least three CpG in between annealing sites, one CpG preferably at the most 3'-end. All forward primers must to include much Thymine bases and all reverse primers must also contain Adenine bases that possible to discriminate completely methylated or completely unmethylated bisulfite converted DNAs. In fact, all cytosines were converted to uracil in bisulfite-treated DNAs, but 5-methylcytosines remained as cytosines.

Methylation-specific primers were chosen to cover eight CpGs numbered 43–61 (forward primer) and 169–188 (reverse primer), whereas unmethylation-specific primers covered eight CpGs numbered 43–63 (forward primer)) and 169–188 (reverse primer). Sequence differences between methylated primers and unmethylated are boldface and underline types at CpG sites. Moreover, those primers sequences cover seven important transcription factors were shown on Fig. 2.

Parameters of two primer pairs were shown in table 3 such as melting temperature, length, GC-base pair ratios, Gibbs Free Energy (ΔG) for secondary structures (hairpin, self-dimer, and heterodimer. Those parameters are good except the Gibbs free energy of the homodimer structure of MF primer and %GC of two unmethylated primers.

The sample tested : In this study, we examined methylation frequencies of *RARβ* of 31 clinic samples including 4 tissues and 27 Pap'smear by MSP. The results showed in Fig. 3, in which the methylated and unmethylated products in

Table 3. Primer sequence and parameter. Gibbs free energy (kcal/mole) for hairpin loop (1); homodimer (2) and heterodimer (3) structure formations.

Gene	T_m (°C)	Length	%GC	(1)	(2)	(3)	Products
RARβ-MF	57,6	19	57,9	-1,84	-10,36	-5,19	146 bp
RARβ-MR	55,4	20	50,0	-0,11	-3,61		
RARβ-UF	49,6	21	33,3	2,24	-3,42	-5,84	146 bp
RARβ-UR	50,8	20	35	-	-1,74		

Table 4. Summarized report of HPV type infection, characteristics clinic samples and results of MSP of *RARβ* gene. PM: Pap' smear; TS: Tissue; (+) Positive; (-) Negative for MSP reaction; ND: not detected

Sample	HPV Genotype	Sample Type	M	U
H8	18	PM	+	+
H9	ND	TS	+	+
H10	ND	TS	+	+
H11	ND	TS	-	+
H12	ND	TS	-	+
31	18, 33	PM	+	+
51	18	PM	+	+
77	11, 16, 18	PM	+	+
151	11, 18, 33	PM	+	+
260	16	PM	-	+
285	16	PM	+	+
424	16, 18	PM	+	+
428	16, 18	PM	+	+
434	18	PM	+	+
438	18	PM	+	+
452	18	PM	+	+
461	16	PM	+	+
473	16, 18	PM	-	+
477	18	PM	+	+
520	16	PM	-	+
545	18	PM	+	+
629	18	PM	+	+
633	18	PM	+	+
858	18	PM	-	+
1209	16	PM	+	+
1210	16, 18	PM	+	+
1211	16	PM	+	+
1212	16	PM	+	+
1213	16, 18	PM	+	+
1498	16	PM	+	+
1499	18	PM	-	+

Table 5. Methylation frequencies of *RARβ* on cervical cancer tissues and vaginal Pap' smear.

Sample	Numbers of methylated sample n(%)
Cervical cancer tissues (n=5)	2 (50)
Vaginal Pap' smear (n= 27)	22 (81)
Total samples (n=31)	24 (77)

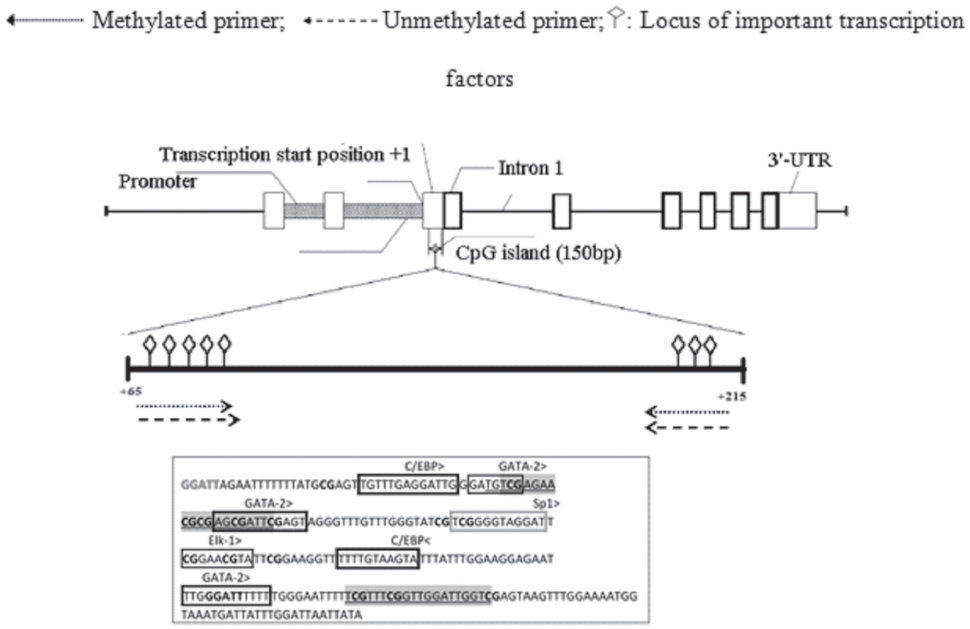

Fig. 2. CpG structure of RARß

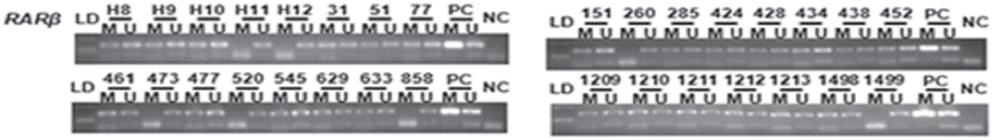

Fig. 3. Methylated promoter of *RARβ* analysis on clinic samples by MSP. M: MSP products with methylated primers; U: MSP products with un-methylated primers; NC: Negative control, MSP product methylated primer set with water; PC: positive control, control DNAs; LD: DNA ladder.

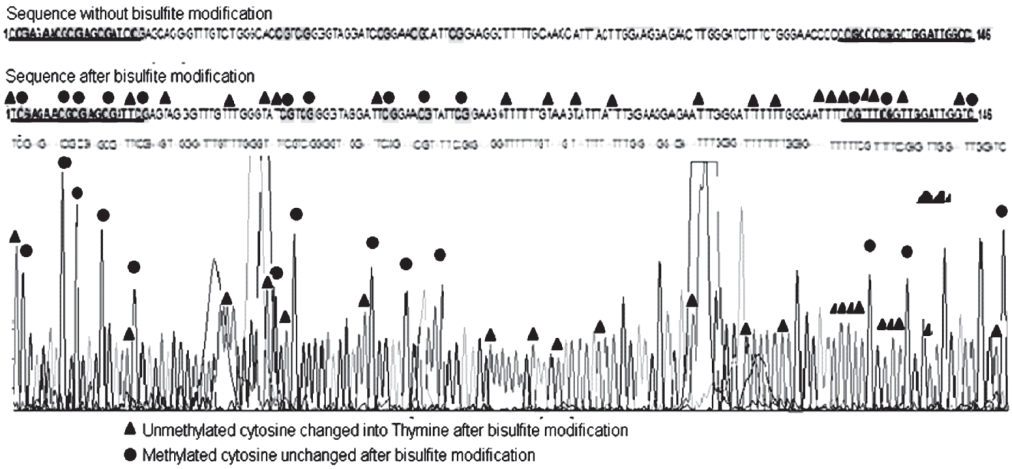

Fig. 4. Sequence profile of methylated MSP fragment of *RARβ* gene. Underlined, bold sequences were sites forming duplex with MSP primer pair. Yellow and triangle labeled nucleotides were non CpG 'C'; Green and circle labeled nucleotides were CpG 'C'.

Table 6. Correlation between methylation frequencies of RARβ promoter and HPV genotype Infections

Sample	Numbers of methylated sample n(%)
Pap'smear (n=27)	22 (81)
HPV type 18 (n=11)	9 (82)
HPV type 16 (n=8)	6 (75)
Co-infection with HPV 18 and 16	4 (80)
Co-infection with other HPV types	3 (100)
*p**	< 0.05

accordance. Moreover, MSP sequencing confirmed the results of the MSP assay as well as indicated the specificity of the MSP primer sets. The MSP fragment of *RARβ* gene sequencing result showed that after using the bisulfite treatment, all unmethylated Cytosine changed into Thymine, and the other methylated Cytosine unchanged after bisulfite modification (Fig. 4). Therefore, we could assure that genomic DNA had completely modified through bisulfite modification reaction. The methylated status and characteristics of clinical samples were summarized in table 4.

As the result, the proportion of methylations status of *RARβ* has shown 77.4 % (24/31) (Table 5). There were differences between our study and previous studies such as, compare with Narayan *et. al.,* (16), Reesink-Peters *et al.* (17), and Yang *at el.* (13) as 29.3%, 38.2% and 53.6%, respectively. As the mean of percentage weighted methylation frequencies in our study were higher than the methylation frequencies described in Fig.1 (75%). However, we did not examine non-HPV-infectious tissues and Pap'smear, so that we have to make more examinations to compare the methylated and unmethylated between the infectious and non-infectious tissues or Pap' smear. Besides, we did not consider the differences in methylation frequencies of *RARβ* between cervical cancer tissue and Pap'smear (Table 5). Those results indicated abnormal methylation at CpG islands on promoter may occur early on tumorgenesis. Therefore, methylated *RARβ* could be potential biomarker for cervical cancer prognosis. However, the limitation of this study was that we did not examine non-HPV-infectious tissues and Pap'smear to compare the methylated and unmethylated between the infectious and non-infectious tissues or Pap' smear. Thus, in future, we will try to examine the methylation status of healing samples (non-HPV infectious and low-risk HPV genotypes) in order to predict the relative risk of methylated or unmethylated of *RARβ* gene which may lead to development of cervical cancer.

Correlation between methylation frequencies of RARβ promoter and HPV genotype infections : According to our study, the results of comparison of methylation frequencies of *RARβ* promoter with HPV types showed that HPV genotype infections related to the methylation level ($p<0.05$) (Table 6). Actually, all HPV genotypes 16, 18 which presented in our study's samples were high-risk types or oncogenic virus, acted as carcinogens in the development of cervical cancer by integrating the viral DNA into human genome and expressing the E6 and E7 oncoproteins. It could be deduced that the methylation of *RARβ* gene as the potential biomarker for cervical cancer prognosis.

Conclusion

From data mining, DNA methylation in the *RARβ* gene could be considered as promising biomarkers for cervical cancer. We had also successfully designed primers to evaluate the methylation status of *RARβ* gene by MSP. Experimentally, we determined the methylation frequencies as 77% on CpGs located at *RARβ* gene and this methylation level associated with HPV genotype infections.

In this study, the sample size is not immense and tumors whether from non-cervical cancer patients or non-HPV infectious Pap'smear samples have not been analyzed. However, outcome could still allow us to have a vision for this methylation characteristic of cervical malignancy in Vietnam. These data would contribute to demonstrate the role of methylation information as a biomarker for early prognosis and therapeutic drug application.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 716-724 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Design of Polymeric Nanoparticles of *Emblica officinalis* extracts and study of *in vitro* therapeutic effects

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Abstract

The therapeutic values of commonly available plants are of more interest due to its beneficial pharmacological activities. *Emblica officinalis* (EO) belongs to Euphorbiaceae family, a most commonly available fruit plant in the tropical and subtropical regions of India. This plant is observed to show several medicinal properties and especially, its fresh fruit juice is used for ophthalmic, carminative, digestive, stomachic, laxative, aphrodisiac, rejuvenative, diuretic, antipyretic purposes and to enhance the memory. The objective of present work is to extract the active ingredients present in the dried fruits of *Emblica officinalis* (EO) by cold maceration using ethanol and hot decoction process using water. The crude extracts are developed into polymer-herbal nanoparticle by solvent evaporation process using polyvinyl pyrrolidine (PVP) as the polymer and characterized for its size and stability. The entrapment efficiency and release study is performed for the nanoparticle formulations. The crude herbal extracts and their nanoparticle formulations are evaluated for anti-oxidant and anti-inflammatory activity by *in vitro* methods. Also the stability and skin irritation test are performed to understand the effect of the formulation.

Keywords: *Emblica officinalis,* Maceration, Decoction, Herbal Nanoparticles, Anti-oxidant, Anti-inflammatory

Introduction

Emblica officinalis (EO), commonly called as the Indian gooseberry has a very reputed place in the field of Ayurveda as is believed that the plant EO was the first plant on earth. This is a native Indian plant commonly seen in tropical and subtropical regions. There are several therapeutic properties believed and proved for the parts of the plant. The fruit exhibits several activities like antioxidant, immune modulatory, antipyretic, analgesic, cytoprotective, anti tussive and gastro protective. They are also found to be beneficial in the treatment against cancer, diabetes, liver diseases, heart problems etc. From past many decades *Emblica Officinalis* was used in home as a memory enhancer and to lower cholesterol levels. It is also helpful in neutralizing snake venom and as an antimicrobial agent (1, 2).

There are several compounds primarily identified in EO like tannins, carbohydrates, amino acids and alkaloids. It is also observed that the fruits juice contained a high amount of vitamin-C about 478.56 mg/100 mL. Also when the EO fruit juice is blended with other fruit juices, there is boost up effect on the nutritional content and quality, in terms of vitamin-C.

Low molecular weight (<1000) of hydrolysable gallotannins (EOT) isolated from the fresh juice of EO proved to contain the emblicanin A, emblicanin B, punigluconin and pedunculagin, specifically showed a significant antioxidant effect

by the *in vitro* tests performed (3, 4). Even the *in-vivo* test showed to exert a significant antioxidant effect against iron-overload hepatotoxicity and it elevated the rat fronatal cortical and striatal concentrations of superoxide dismutase, catalase and glutathione peroxide and reduce lipid peroxidation, especially in the brain region of rats. EO usually protects the cells against free radical damage by giving an antioxidant protection. There were several studies that proved the enhancement of the activity of immune system as there was a constant increase in the white blood count and the strengthening of the immune system. This fruit showed a reduced UV-induced erythema and was observed to be an excellent free-radical quencher. Even the chelating ability of the fruit to compounds like iron and copper, helped in the inhibitory activity of MMP-1 and MMP-3 was proved (5, 6, 7).

With all these efficient properties of EO, the present work concentrate on designing herbal extract nanoparticle formulation which can improve the potential activities. The entrapment efficiency and release studies of the formulations are tested and compared to the crude extracts. The formulation is exposed to test its improvement of Anti-oxidant and Anti-inflammatory properties.

Materials and Methods

Plant material collection: The fruits of *Emblica officinalis* (Euphorbiaceae family) was collected, identified and authenticated by Dr. Ravichandran, Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur, India.

Extraction by cold maceration: The fruits of EO was first cut into small pieces, seed removed and dried completely without any amount of moisture present in it. This dried fruit was then pulverised and made into fine powder. About 75g of the powder was taken in a beaker and a required amount of petroleum ether was poured to the content and allowed to macerate overnight. The next day the content of primary extract filtrate was discarded and the residue was air dried for 30 min. This dry sample was again treated with 100mL of ethanol and kept overnight for extraction. The next day the filtrate was collected and evaporated in a water bath for 24 hours at 70°C. The dry extract was obtained after the evaporation process was complete (8).

Extraction by hot decoction: Another 75g of EO powder was taken in a beaker with 100mL of distilled water. This was boiled to 100°C for 15 min and stirred continuously. Then the solution was filtered and the filtrate was taken for solvent evaporation process. The process was done for 24 hours at 70°C till a dry powder extract of the fruits was obtained (9, 10).

Preparation of Polymeric Nano-particles: The nanoparticles of herbal extract with polymer were formulated by solvent evaporation process. In this process an aqueous solution of the extract was prepared by dissolving 0.02 g of dry extract with 10ml of water and 0.01g of Pluronic F68 was added as a surfactant. The organic phase containing the polymer was prepared with 0.05g, 0.10g and 0.15g of PVP with 2ml of dichloro methane (DCM) separately. The aqueous solution was sonicated for 20 min at 100 Kv using a probe sonicator (P250 Vibronics, India), into which the organic solvent was poured in drops. Then the mixture was placed for constant stirring in a magnetic stirrer for 20 min, until the organic solvent is completely evaporated (11, 12). This process was performed for the extracts obtained by cold maceration and hot decoction process to formulate the nanoparticles in different composition of polymer. A blank nanoparticle containing the polymer and surfactant (without extract) was also formulated for comparative studies.

Calibration of the EO extract by UV-Visible Spectrophotometer: The aqueous extract was dissolved in water, whereas the ethanol extract was dissolved in small amount of ethanol, followed by addition of water, and then suitably diluted. The solutions were scanned for its

specific UV-Visible range of absorbance maxima. Then the absorbance of the different serial diluted samples was measured at the λmax, and a standard calibration curve was plotted with concentration against absorbance.

Entrapment efficiency of the polymeric herbal nanoparticles: The nanoparticle suspension formulated with the extract and polymer was ultra centrifuged at 18,000 rpm for 30 minutes in a cooling centrifuge apparatus (Sigma 3K30, Germany) at -10ºC and then the supernatant solution was diluted suitably to measure the absorbance, from which the concentration of drug in supernatant was calculated using the standard calibration data. The entrapment efficiency of the extract in the polymeric nanoparticles was calculated using the formula (13),

$$\%\ Entrapment\ Efficiency = \frac{Total\ drug\ content - Drug\ content\ in\ supernatant}{Total\ drug\ content}\ x\ 100$$

Particle size and surface charge analysis of the nanoparticles: The nanoparticle formulations of both the extracts were analysed by a zeta analyser (Malverm Nano Series ZS, UK) to verify for the particle size based on dynamic light scattering technique and the zeta potential based on charge conductivity principle, to ensure the uniformity of size distribution and the stability of the formulation, respectively (14).

In-vitro anti-Inflammatory bioassay: The *in-vitro* anti inflammatory bioassay was performed based on the protein denaturation principle method. A mixture containing a total of 5ml was prepared that consisted of 0.2ml of egg albumin with 2ml of varying concentrations of the sample (aqueous crude extract prepared by cold and hot maceration and their respective nanoparticles in separate test tubes) so that final concentrations become 30, 60, 120, 240, 500 µg/ml with 2.8ml of phosphate buffered saline (PBS, pH 6.4) in each solution. Distilled water of similar volume was used as control. Then these samples were incubated at 37 ± 2ºC for 15 minutes and then heated for 5 minutes at 70ºC. After cooling, the absorbance of the solutions was measured at 660 nm by using the respective blank. Diclofenac sodium was used as a reference drug, which was treated similarly with egg albumin and the final concentration (78.125, 156.25, 312.5, 625, 1250 µg/mL) were measured for its absorbance (15). The percentage inhibition of protein denaturation was calculated for all the formulations and crude extract using the formula,

$$\%\ Inhibition = \frac{Vt}{Vc - 1}\ x\ 100$$

Where, Vt = absorbance of test sample, Vc = absorbance of control.

Anti-oxidant activity by ferric reducing assay: Different concentrations of the crude extracts obtained by cold and hot maceration at their respective nanoparticles at various concentration (100, 200, 300, 400 and 500µg/ml) was dissolved in 1 ml of methanol separately. To each test tube 2.5 ml of phosphate buffer of pH 6.6 and 2.5 ml of 1% potassium ferri cyanide was added. These tubes were kept in water bath at 50°C for 20 minutes and then cooled rapidly. The cooled samples were mixed with 2.5 ml and 0.5 ml of 10% trichloroacetic acid and 0.1% ferric chloride respectively and this mixture was incubated for 10 min. A Perl's Prussian blue colour was formed due to the presence of iron (II)-ferricyanide complex formation. This was determined by measuring the absorbance at 700 nm (16, 17, 18).

In vitro release studies: The extracts obtained by cold maceration and hot decoction, and their nanoparticles prepared with different ratios of polymers was evaluated for the percentage of release of the extract constituents, for 6 hours with first one hour samples estimated for every 15 min followed by sampling at every one hour to estimate the immediate or sustained release effect of extract from the polymeric nanoencapsulation complex (19). 2ml of the nanoparticle formulation was placed in a plastic tube with one side sealed with dialysis membrane and this tube is placed into the basket of USP type I apparatus (DS 8000 Labindia, India) and

dipped into the dissolution chamber containing 100 mL of distilled water as media with the bath temperature of 37 ± 0.5ºC. The study was carried at 100 rpm and the samples of 10 mL were withdrawn using a syringe at the end specified predetermined time intervals. The collected samples were analyzed using UV-Vis Spectrophotometer at the λmax.

In- vitro drug release kinetics: Kinetic modeling was performed to understand the kinetics and mechanism of extract release from the nanoparticles. In these analysis results of *in vitro* dissolution studies were fitted with various kinetic equations like zero order (cumulative % release vs. time), first order (log % drug remaining vs time), Higuchi's model (cumulative % drug release vs. square root of time), Hixon model (Cube root of drug % remaining in matrix vs. Time) and Korsmeyer-Peppas (Log cumulative % drug release vs. log time). The R^2 and n values obtained from the linear plots were used to analyze the mode of extract reelase from the nanoparticulate system (20).

Results and Discussion

Calibration of the crude extract: The crude ethanolic and aqueous extract of EO dissolved in suitable solvent, diluted in distilled water and scanned in a UV-spectrophotometer have shown the maximum absorbance wavelength at 273.7 nm. The serially diluted samples of both the extracts exhibited the absorbance values for the calibration as shown in Table 1 and their regression was found to be 0.999 and 0.993 for ethanolic and aqueous extract respectively (Fig. 1).

Entrapment efficiency: Amount of extract entrapped in the polymeric nanoparticle determined using UV-VIS spectrophotometer showed that both the extracts of different nanoparticle formulation had higher entrapment efficiency in the range of 58 – 70%. There was no significant difference observed in the formulations prepared with different concentrations of the polymer, since the surfactant level, amount of extract used and all other process variables were kept constant during the nanoparticles development.

Particle size and zeta potential of nanoparticles: The average size of the synthesised nanoparticles of ethanolic and aqueous extract was found to lie within the range of 550-825 nm. Size range of polymeric nanoparticles as 100-1000 nm could provide stable colloidal dispersion (14). Surface charge measured in terms of zeta potential plays a major role for nanoparticles stability as the standard criteria. The zeta potential of the synthesized herbal nanoparticles was found to be -7.49 mV to -13.0 mV. Zeta potential greater than +25mV and less than -25mV correlate to higher stability of the nanoparticles(12). Thus the below

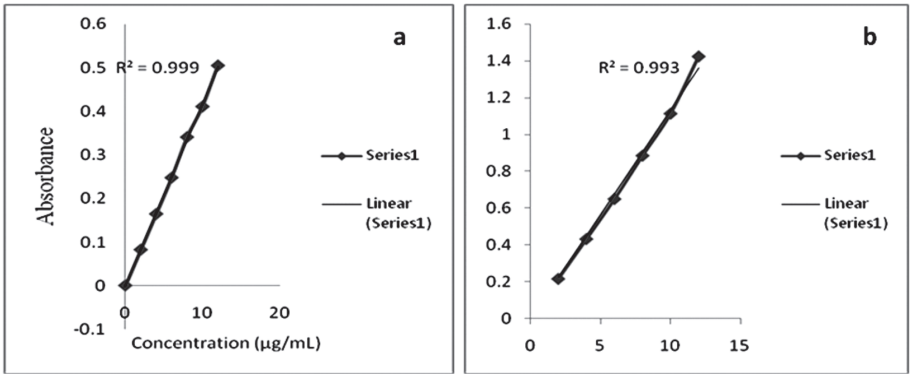

Fig. 1. Calibration curve of *Emblica officinalis* extract (a) Ethanolic (b) Aqueous

tabulated values illustrated that the synthesised nanoparticles exhibited average stability.

The results of entrapment, size and charge of the nanoparticles are shown in table 2, where E1, E2, E3 – Nanoparticles of *EO* ethanolic extract with 50 mg, 100 mg and 150 mg PVP, respectively, A1, A2, A3 – Nanoparticles of *EO* aqueous extract with 50 mg, 100mg, 150 mg PVP, respectively.

Anti-oxidant activity of the nanoparticles: The results of anti-oxidant assay performed for 5 different concentrations (100, 200, 300, 400, 500 µg/mL) for the ethanolic extract nanoparticles (E1, E2, E3) and aqueous extract nanoparticles (A1, A2, A3) was compared with the standard values taken from Ascorbic acid as shown in Table 3. As the data of nanoparticles were compared with standard Ascorbic acid the Fig. 3 showed the higher reducing power of the EO nanoparticles converting ferrous to ferric which depict the scavenging of the free radicals. The increase in absorbance of the reaction mixtures indicated increased reducing power. The ethanolic extract nanoparticles showed that E1 formulation with 50mg of PVP showed a higher activity than the formulations E2 and E3, due to the faster release of the EO extract from the polymeric coating with low amount of polymer (50 mg), than the formulations E2 and E3 with higher polymer concentration (100 and 150 mg respectively). In case of the aqueous extracts A1, A2 and A3, even with increasing amount of polymeric coating in all the three formulations, they showed an increased consumption of the free radicals than the standard Ascorbic acid or E2 and E3 formulation confirming the highest potential activity of the aqueous extract of EO. The antioxidant activity found in all the EO formulations showed increased activity and this could be due to the presence of high Flavonoids in the extracts that was confirmed from the phytochemical analysis done previously (18).

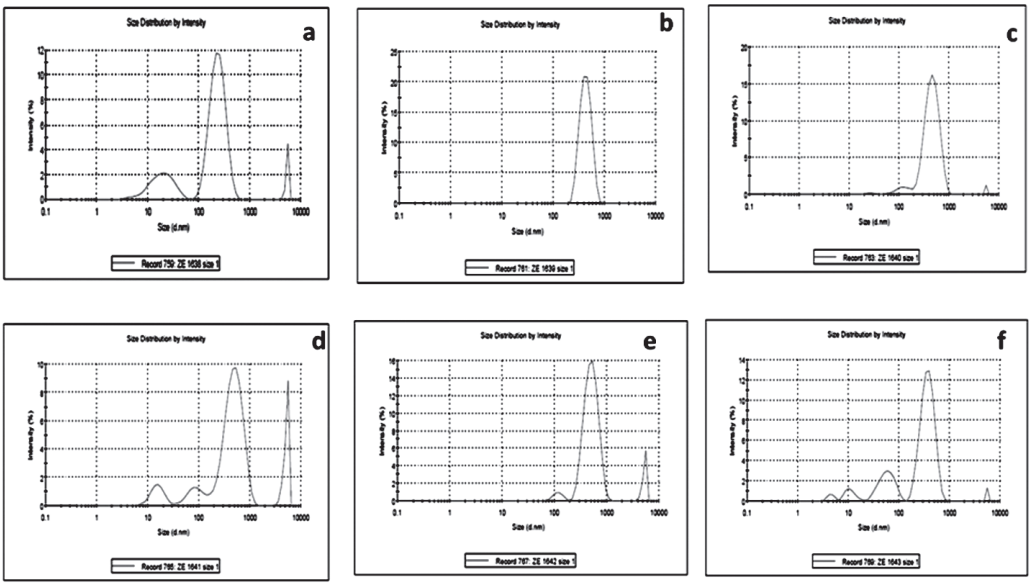

Fig. 2. Size and Zeta potential of *Emblica Officinalis* Nanoparticles (a) Ethanolic extract with 50mg PVP (b) Ethanolic extract with 100mg PVP (c) Ethanolic extract 150 mg PVP (d) Aqueous extract with 50 mg PVP (e) Aqueous extract with 100 mg PVP (f) Aqueous extract 150 mg PVP

Anti-inflammatory activity: EO showed a very high protein denaturation effect *in vitro* when egg albumin was used as the protein (15, 21). EO extract polymeric nanoparticles of different concentrations showed a higher anti-inflammatory activity when compared to Diclofenac sodium as a standard. The comparative evaluation shown in Fig. 4 depicted a cumulative evaluation of the standard and the nanoparticles formulated from both extracts, wherein the higher activity in the nanoparticle formulations was observed, especially with decreasing concentration of the polymeric coat in ethanolic extract formulation as E1 showed higher activity than E2 and E3. But all the aqueous extracts A1, A2 and A3 showed higher activity than the ethanolic and the standard Diclofenac sodium, inspite of the changes in concentration of polymer used. This proved that the percentage inhibition effect was higher in the nanoparticle formulation of aqueous extract prepared when compared to the ethanolic extract samples, due to the presence of active anti inflammatory constituents in the aqueous extract of *Emblica Officinalis*.

Release studies from nanoparticles: The release of the extract from the nanoparticles prepared with different concentrations of polymer was compared for both the extracts (Fig. 5). Nanoparticle formulation A1 containing aqueous extract with 50 mg of polymer, have shown the highest release of 56% at the end of 6 hours. Whereas, when the polymer composition was increased to 100 mg and 150 mg in A2 and A3 formulation respectively, the drug release was found to decrease to 30% and 26% due to the higher polymer coating or encapsulation. A similar profile was observed in ethanolic extract nanoparticles as 33%, 30% and 23% release of the extract at the end of 6 hours from E1, E2 and E3 respectively. Also in case of the ethanolic extract nanoparticles, there was a lag time observed in the release profile, as the extract was released form the nanoencapsulated system only after 1.5 hours. Whereas in case of aqueous extract sample, the extract release was observed

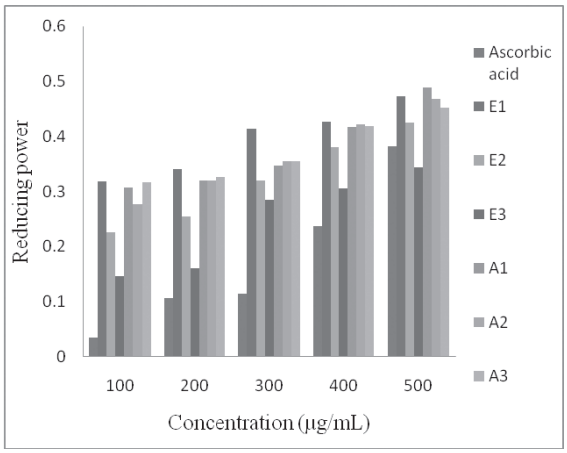

Fig. 3. Comparitive analysis of Anti- oxidant activity of nanoparticle formulations with standard sample by FRAP method

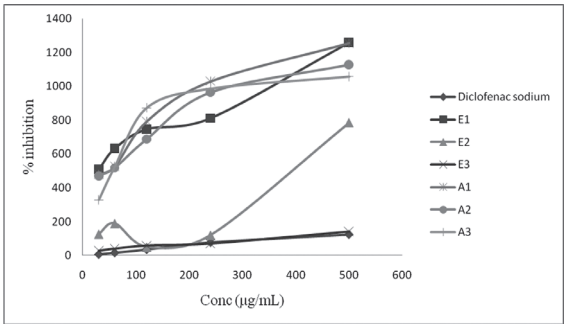

Fig. 4. Anti-Inflammatory activity of *Emblica officinalis* Nanoparticles

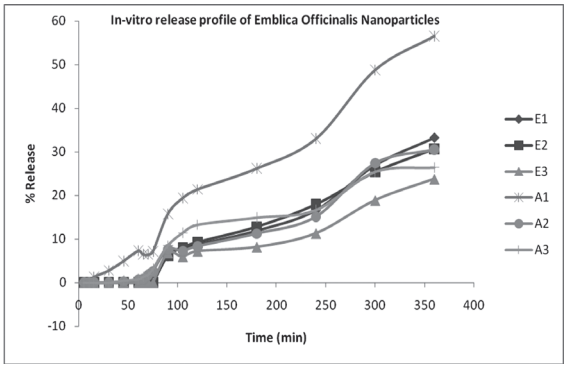

Fig. 5. *In-vitro* drug release profile of *Emblica officinalis* Nanoparticles

Table 1. Calibration data of *Emblica officinalis* extract at 273 nm

Concentration (ig/mL)	Absorbance of ethanolic extract	Absorbance of Aqueous extract
2	0.082	0.218
4	0.164	0.434
6	0.247	0.651
8	0.34	0.886
10	0.41	1.114

from initial 15 min, providing linear release profile. When the aqueous and ethanolic extract nanoparticles were compared, we could observe that the percentage release was higher in aqueous extract due to its exposure to aqueous dissolution media. The significant reason for the faster disssolution of the aqueous extract may be due to the presence of aqueous soluble active constitutents in the sample exposed to the aqueous dissolution media. Also the nanosize of the sample and its solubilization played a special role in the dissolution rate of the formulationss.

Table 2. Physico chemical characterization of *Emblica officinalis* nanoparticles

Formulation Code	E1	E2	E3	A1	A2	A3
Entrapment efficiency (%)	68.5	66	67.5	66.05	70.65	58.94
Size (nm)	548.1	793.3	629.7	693.9	825.8	770.9
Zeta potential (mV)	-9.91	-13.0	-12.3	-11.4	-11.3	-7.49

Table 3. Anti- oxidant activity of *Emblica officinalis* Nanoparticles by FRAP method

Conc. (µg/mL)	Ascorbic acid	E1	E2	E3	A1	A2	A3
100	0.0343±0.01	0.318±0.003	0.226±0.002	0.146±0.05	0.307±0.012	0.276±0.034	0.317±0.002
200	0.106±0.003	0.34±0.004	0.255±0.026	0.161±0.06	0.32±0.007	0.32±0.004	0.325±0.004
300	0.115±0.001	0.413±0.009	0.319±0.018	0.285±0.089	0.346±0.001	0.354±0.009	0.358±0.009
400	0.237±0.007	0.426±0.004	0.38±0.013	0.306±0.072	0.417±0.012	0.421±0.023	0.418±0.041
500	0.382±0.016	0.472±0.006	0.424±0.006	0.343±0.039	0.488±0.005	0.468±0.03	0.452±0.039

Drug release kinetics: The release study data fitted to various kinetic models revealed the mechanism of extract release from the polymeric nanoparticle formulations. As shown by the results in table 4, the R^2 value observed for all the nanoparticles of both aqueous extract and ethanolic extract showed the mechanism of release of the active constituents from the nanosystem could be attributed to Korsmeye-Peppas model, which explain the diffusion of the extract from the polymeric matrix. Also the mode of diffusion was found to be super case II transport phenomenon based on the n-value > 0.89.

Conclusion

The development of herbal nanoparticles using polymeric susbtance was found to be very successful in providing linear release of the encapsulated extract containing active ingredinets in it. The entrapment efficiciency and required size could be achieved by optimizing the composition of the variables used and the

Table 4. Release kinetics of the *Emblica officinalis* Nanoparticles

Formulation Code	Zero Order	First order	Higuchi R^2 values	Korsemeyer-Peppas	Hixon - Crowell	n-value
A1	0.9718	0.9406	0.7192	0.9761	0.9538	1.090
A2	0.9017	0.8743	0.5803	0.9606	0.8838	1.421
A3	0.8974	0.8913	0.6514	0.9005	0.8942	1.074
E1	0.9391	0.9041	0.6439	0.9787	0.9163	1.317
E2	0.9800	0.9562	0.7055	0.9931	0.9650	1.158
E3	0.9155	0.8959	0.6564	0.9310	0.9028	1.184

process parameters. Also the *in vitro* therapeutic effects of the formulated nanoparticles showed significant improvement in the anti oxidant and anti inflammatory activity of both the aqueous and alcohol extracted contents.

Acknowledgement

The authors are thankful to SASTRA University for providing the necessary infrastructure, online resources and lab facilities to carry out the study.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 716-724 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Authentication of Herbal Medicinal Plant- *Boerhavia diffusa* L. Using PCR-RFLP

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Abstract

Herbal drugs are integrated part of both modern and traditional systems of medicine and are frequently found to be adulterated with other drugs of same morphological features, thereby requires scientific methods for their authentication and standardization. Various methods like chemo-profiling using chromatographic and spectroscopic techniques are used for the purpose but have their limitations because of the variations in the chemistry of the metabolites due to the age of the plants and their varied geographical distribution. DNA-based techniques have been widely used as a method of authentication for such types of herbal drugs. In the present study, using a DNA based technique known as PCR-RFLP, an attempt has been made to authenticate one such herbal drug *Boerhavia diffusa* which gets adulterated with *Trianthema portulacastrum* and *Trianthema monogyna*. The PCR amplified product of ribosomal ITS gene when subjected to restriction digestion with five different restriction enzymes viz. *MspI, HinfI, MboI, EcoRI and EcoRV* showed varied banding patterns for *Boerhavia diffusa* and adulterated plants. Of the five enzymes *MspI*, *HinfI* and *MboI* could be used for authentication of *B. diffusa* as they gave unique patterns for this plant and were different from that of adulterants. Rest of the enzymes either could not digest the ITS product or was not showing sufficient polymorphism to assign it to a particular genotype. Our study has established ITS-RFLP based markers for the authentication of *B. diffusa*, *T. portulacastrum* and *T. monogyna* with enzymes *MspI*, *HinfI* and *MboI*.

Keywords: *Boerhavia diffusa, Trianthema portulacastrum, T. monogyna,* Adulteration, Authentication, Herbal drugs, ITS-RFLP, Molecular markers and Polymorphism.

Introduction

Plants play a major role in the drug industry – be it synthetic or traditional. As for the synthetic drugs, there exists a standardized authenticated process for its quality control, but no such mechanism exists for raw materials or finished products in the traditional drugs. Hence, it is not possible to check the adulteration in the finished drug in traditional medicine. Adulteration in herbal drugs may be intentional for monetary gains or unintentional due to lack of awareness and knowledge about the authentic plants, confusion in vernacular names between indigenous systems of medicine and local dialect, similarity in morphological and aromatic features of the different plant sources, non-availability of the authentic plants, careless collection and other unknown reasons (1). Due to adulteration, faith in herbal drugs has declined (2). Adulteration in market samples is one of the greatest drawbacks in promotion of herbal products (3). It is invariably found that the adverse event reports are not due to the intended herb, but rather due to the presence of an unintended herb (4). This kind of adulteration is found among three plants,

Boerhavia diffusa (family Nyctaginaceae), *Trianthema portulacastrum* and *Trianthema monogyna* (family Aizoaceae) belonging to different families and having similar medicinal properties. These plants differ in their morphology and availability to a certain extent, but since they grow in the same vicinity, it becomes undistinguishable for the pluckers, who are interested in the roots having similar appearance and hence are wrongly supplied to the drug companies. *Trianthema portulacastrum* has dark green leaves like *Boerhavia diffusa* but its leaves have a maroon outline, however, not significantly distinguishable by people who are unaware of these differences and tend to confuse between the two causing unintentional adulteration. *Trianthema monogyna* has light colored leaves, thorns in the axils of branches and different flowers.

Boerhavia is also known by the names of *Boerhavia adscendens, B. caribaea, B. coccinea, B. erecta, B. paniculata, B. repens,* and *B.viscosa*. Though considered as a weed, its leaves, seeds and roots are edible and are rich in carbohydrates and proteins. The roots are diuretic, emetic, expectorant, laxative and stomachic. They are used in the treatment of asthma, oedema, anaemia, diabetes (5), jaundice (6), ascites, scanty urine and internal inflammation (7). *Trianthema portulacastrum* also called as *Desert horsepurslane* is shown to have anti-carcinogenic potential (8, 9), used in the treatment of oedema in the liver and the spleen (10). It has good hepato-protective activity against paracetamol and thio-acetamide intoxication in mice (11). This plant is shown to be diuretic, locative, analgesic and anti-inflammatory and is used in the treatment of asthma, jaundice, abdominal diseases and fever. These plants although have similar medicinal properties, vary in their active secondary metabolites which may produce different effects and hence the prevention of adulteration is important.

Therefore, to get the most beneficial effect, adulteration has to be checked for which a precise method should be applied to authenticate these plants apart from morphological and histological methods. One method, which can be relied on is the use of molecular markers which are generally referred to biochemical constituents, including primary and secondary metabolites (phenotype) and other macromolecules such as nucleic acids (genotype). In medicinal plants, majority of active chemicals acting as drugs are secondary metabolites which vary with the age and different geographic locations thereby not reliable as molecular markers. Also there is a potential for adulteration of plants with extracts from plants that have lower drug content. DNA based molecular markers have several advantages over typical phenotypic markers and are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental factors (12). DNA can be extracted from fresh or dried organic tissue (13) of the botanical material hence the physical form of the sample for assessment does not restrict detection. Based on the specificity of the genotype of a system, a particular DNA profile being unique can be ascribed to a particular organism. Hence, various DNA marker based methods can be used for species characterization and adulteration detection in medicinal plants.

Various types of DNA based molecular techniques utilized to evaluate DNA polymorphism include PCR based methods which include random amplified polymorphic DNA (RAPD) (14, 15) and amplified fragment length polymorphism (AFLP) (16), minisatellites and microsatellites (17), restriction fragment length polymorphism (RFLP), PCR-RFLP and sequencing based markers. Due to their high level of polymorphism, they have been extensively used for DNA fingerprinting as well as for genetic markers. RFLP of the internal transcribed spacer (ITS) gene is one of the methods to detect polymorphism to establish species specific patterns. The internal

Vol. 7 (3) 725-731 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

transcribed spacer is a sequence of RNA in a primary transcript that lies between precursor ribosomal subunits. These sequences are coded by ribosomal DNA. Eukaryotic organisms have two internal transcribed spacers; ITS-1 located between the 18S gene and the 5.8S gene, while ITS-2 is located between the 5.8S and the 28S gene. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races) because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA). Variation among individual rDNA repeats can sometimes be observed within the ITS. In the ITS region, restriction digestion shows specific patterns for a species and a variation in the pattern within a species, which can be detected as polymorphism. Ribosomal DNA – ITS-RFLP of *Vigna mungo* var *silvestris*, *V. trilobata, V. glabrescens* and diverse cultivars of *V. mungo*, have been used for species identification (18). We have made an attempt to establish fingerprinting pattern for *Boerhavia diffusa, Trianthema portulacastrum,* and *T.monogyna* using ITS-RFLP and thus authenticate these plants and prevent the adulteration between the three.

Material and Methods

Collection of plant material: Leaves of *Boerhavia diffusa*, *Trianthema portulacastrum* and *Trianthema monogyna* were collected from various areas of Chandigarh, India; Panchkula, Ambala, and Yamunanagar, Haryana, India; Mohali, Amritsar and Patiala, Punjab, India; Delhi, India; Mumbai, Maharashtra, India; and Baroda, Gujarat, India. Five samples from each region were collected for the experiments.

Reagents for Restriction Fragment Length Polymorphism:Enzymes *MspI, Eco RI, Hinf I, Eco RV, MboI*, Tango buffer (Fermentas), Buffer R (Fermentas), Buffer *Eco RI* (Fermentas) were used in the experiments.

Plant DNA extraction: A modified method of Biswas and Biswas (13) was used to isolate genomic DNA from plants without liquid nitrogen.

Amplification of the Internal Transcribed Spacer region by Polymerase Chain Reaction: PCR was set up for 50 µl reaction mixture containing 5.0 µl 10X Taq buffer with 15 mM magnesium chloride, 1.0 µl magnesium chloride (25 mM), 5.0 µl dNTPs (2 mM), forward primer (ITS F) (5' TCC TCC GCT TAT TGA TAT GC 3') 1.0 µl, reverse primer (ITS 1F) (5' AAG TCG TAA CAA GGT TTC CGT AG 3') 1.0 µl, milli Q autoclaved water 33.5 µl, 0.5 µl *Taq polymerase* (1.25 U), and template DNA 3.0 µl. The PCR mixtures were exposed to denaturation at 95º C for 5 min followed by 30 cycles of denaturation at 95º C for 30 sec, annealing at 52º C for 30 sec, extension at 72º C for 1 min, final extension at 72º C for 7 min in the Eppendorf Mastercycler Personal Thermal cycler. After the completion of the cycles, the sample was tested for amplification by electrophoresing on 1% agarose gel using 3 µl ITS amplified product and 1 µl loading dye at a voltage of about 70 V for 60 to 90 min. The marker used for size determination was 100 bp ladder.

Restriction Fragment Length Polymorphism (RFLP) of the amplified ITS regions of the plant DNA:The amplified ITS products, enzyme buffers and autoclaved distilled water were thawed from -20º C to 4º C by placing on ice for 1 to 2 h. Twenty µl mixtures were made for the enzymes to act on the templates with the respective buffers. Amplified ITS (template) 10 µl (1 µg), buffer (10X) -2 µl (1X), enzyme (10 U/µl) – 1 µl (0.5 U/µl), and 7 µl of autoclaved distilled water were added. Following mixtures were made: enzyme *Eco RI,* amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.75 µl (0.375 U/µl) and autoclaved distilled water – 7.25 µl; enzyme *Mbo I:* amplified ITS (template) 10 µl (1µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.5 µl (0.25 U/µl) and autoclaved distilled water - 7.5 µl; enzyme *HinfI:* amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.3 µl (0.15 U/µl) and autoclaved distilled water - 7.7 µl; enzyme *Eco RV:* amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.3 µl

(0.15 U/µl) and autoclaved distilled water - 7.7 µl. The mixtures were placed in the water bath (Tanco India) at 37º C for 16 – 20 h according to the time required by the enzymes for digestion. The mixtures were then placed in water bath at 65º C for 20 min to inactivate the enzymes. These were then electrophoresed in 2% agarose gel with 5 µl sample and 1 µl loading dye (as in DNA isolation procedure) and 100 bp ladder was used as marker at 66V for 60-90 min to check the RFLP patterns obtained by restriction digestion of the enzymes on the ITS regions of the plant genome. The bands were analyzed for their size variation by comparing them with the standard marker.

Results and Discussion

DNA of high quality was obtained from all the samples. All the samples of each plant collected from different regions showed same results, therefore representative data is presented in the article. A product of 700 bp of ITS region was obtained after PCR amplification for *Boerhavia diffusa* samples. The same molecular weight products (700 bp) of ITS were also observed for *Trianthema portulacastrum* and *Trianthema monogyna* samples (Fig. 1). All the obtained ITS products of *B. diffusa* plants from different regions, when subjected to restriction digestion with five enzymes *MspI*, *Eco RI*, *Hinf I*, *Eco RV*, *MboI* separately to study the polymorphism, each individual enzyme showed unique pattern. The digested products of ITS of both *T. portulacastrum* and *T. monogyna* showed exactly same banding pattern with the five enzymes but were different from that of *B. diffusa.* With *MspI*, ITS of *B. diffusa, T. portulacastrum* and *T. monogyna* gave four fragments. However, the four fragments obtained in *B. diffusa* were different from that of *T. portulacastrum* and *T. monogyna* which showed similar patterns (Fig. 2, Table 1). When digested with the enzymes *Eco RI* and *MboI*, a single band of ITS was obtained for all the samples of *B. diffusa* which shows that there was no restriction site for these enzymes. In *T. portulacastrum* and *T. monogyna*, similar results were obtained with *Eco RI* but with *MboI* two fragments of 470 bp and 220 bp were obtained. On digestion of the ITS with the enzyme *HinfI*, five fragments were observed with four restriction sites in *B. diffusa,* while *T. portulacastrum* and *T. monogyna* displayed three restriction sites each for same enzyme (Fig. 3, Table 1). *Eco RV* digestion of the ITS produced two fragments of 300 bp and 400 bp for all the

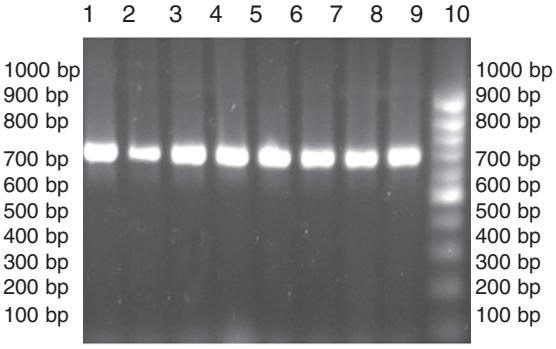

Fig. 1. ITS products after PCR amplification Lane 1-100 bp ladder, lane 2- *B. diffusa* from Chandigarh, lane 3- *B. diffusa* from Patiala, lane 4- Delhi, lane 5- *B. diffusa* from Baroda, lane 6- *T. portulacastrum* from Chandigarh, lane 7- *T. portulacastrum* from Patiala, lane 8- *T. monogyna* from Chandigarh, lane 9- T. monogyna from Patiala, lane 10- 100 bp ladder

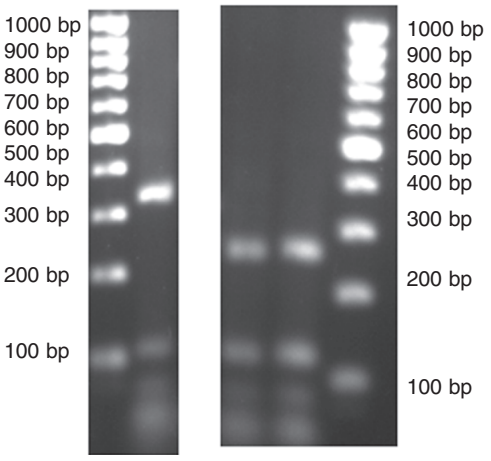

Fig. 2. Restriction digestion of the ITS with Msp I; Lane 1-100 bp ladder, lane 2- *B.diffusa* from Chandigarh, lane 3- *T. portulacastrum.* from Chandigarh, lane 4- *T. monogyna* from Chandigarh, lane 5- 100 bp ladder

Vol. 7 (3) 725-731 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

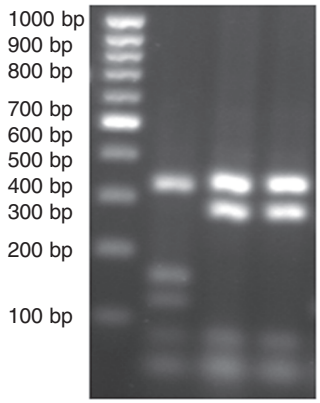

Fig. 3. Restriction digestion of the ITS with HinfI; Lane 1- 100 bp ladder, lane 2- *B. diffusa* from Chandigarh, lane 3- *T. portulacastrum.* from Chandigarh, lane 4- *T. monogyna* from Chandigarh

samples of three plants. Polymorphisms among plant varieties are widely found and they have been studied based on biochemical markers (19) as well as DNA markers (18, 20) mostly with respect to geographical distribution and to establish their phylogenetic relationships (18, 20, 21). DNA based techniques have also been widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable (22).

The use of biochemical markers like isozymes showed limited polymorphism which was found to be insufficient to distinguish closely related cultivars in *Musa* species (19). DNA based polymorphisms provide a broader arena to establish markers for different species and/or varieties of plants (23, 24). ITS-RFLP is among some of the reliable methods to establish markers for the authenticated identification of plants (23) as ITS has both conserved (5.8S) and variable (ITS 1 and ITS 2) regions. The ITS obtained in all the three plants of *B. diffusa, T. portulacastrum* and *T. monogyna* were of 700 bp, and hence their RFLP was necessary to establish variation. The restriction enzymes used for RFLP were selected on the basis of previous reports showing their use on plant genomic DNA (18, 19). In the present study, it has been observed that the enzyme *Eco RI* could not restrict the ITS of all the three plants and *MboI* could not cut the ITS of only *B. diffusa*. The inability of these two enzymes to cut the rRNA unit of all these plants can be attributed either to lack of a restriction site or the restriction site in the DNA may be methylated. While restriction sites were observed with the enzymes *MspI, HinfI* and *Eco RV* in all the three plants, restriction sites were seen only in *T. portulacastrum* and *T. monogyna* with the enzyme *MboI*. The RFLP pattern revealed that only *MspI, HinfI* and *MboI* could be used to establish markers for *B. diffusa* and *T. portulacastrum* as they gave totally different patterns. No intra-species variation was found among the *B. diffusa* plants. As reported earlier, *HinfI* and *MspI* generally have sites in plant ITS (18, 19). *T. portulacastrum* and *T. monogyna* did not show variation among them with any enzyme, but the RFLP patterns were exactly similar with the enzymes used in this study. Therefore, it will be necessary to use a number of other enzymes to establish a variation as it is unexpected to have no variation among the two different species. Our

Table 1. ITS-RFLP of the plants and various restriction enzymes used in the study.

Plant Samples	Fragment Size (bp)				
	MspI	**EcoRI**	**HinfI**	**Eco RV**	**MboI**
Boerhavia diffusa	350+120+80+50	700	310+160+120+80+30	300+400	700
Trianthema portulacastrum	290+130+80+50	700	320+280+80+30	300+400	470+220
Trianthema monogyna	290+130+80+50	700	320+280+80+30	300+400	470+220

study has established ITS-RFLP based markers for the authentication of *B. diffusa* and *T. portulacastrum* using the enzymes *MspI*, *HinfI* and *MboI*. These restriction enzymes can be applied to find out authenticity of plants used in drug preparations and avoid adulteration. Similar works on the authentication of medicinal plants of *Dendrobium* species by the internally transcribed spacer of rDNA has been reported earlier (23). RAPD technique has been used to identify eight types of dried *Coptis* rhizomes and one type of *Picrorrhiza* rhizome, a substitute for the former in the Chinese herbal market (24). AP-PCR, RAPD and RFLP have been successfully applied for differentiation of *Panax ginseng* and *Panax quinquefolius* (20) and to detect substitution by other closely related species (25). The present techniques have allowed an effective and reliable differentiation of *B. diffusa* from its adulterants. Proper combination of molecular techniques and analytical tools will lead to the development of comprehensive systems for the characterization of herbal medicinal plants and can be conveniently applied at the industrial level for quality control of these drugs. The authentication of plants based on genetic/molecular markers is important for quick identification in crude, semi-processed and processed herbal formulations wherever they are used.

Conclusion

Among the three plants *Boerhavia diffusa*, *T. portulacastrum* and *T. monogyna*, no variation was observed in the size of the ITS region. However, ITS-RFLP with experimental restriction enzymes showed absolutely similar patterns for *T. portulacastrum* and *T. monogyna* which varied from that of *B. diffusa*. Thus, restriction enzymes *MspI*, *HinfI* and *MboI* can be used to develop molecular markers that can differentiate these plants, authenticate them and prevent adulteration.

Acknowledgement

The authors duly acknowledge the support rendered by the Principal, DAV College for providing the infrastructure and other facilities.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 732-742 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Purification, Partial Characterization and Applications of Alkaline Protease from *Bacillus subtilis* NCIM No. 2724

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Abstract

Thermostable alkaline protease production from *Bacillus subtilis* NCIM No. 2724, its purification and characterization was investigated. The purified enzyme revealed MW of 26 KDa. This enzyme is stable at a pH and temperature range of 7-11 with optimum pH at 10.0 and 30 to 70°C with optimum activity at 60°C, respectively. The enzyme is strongly activated by metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} while the activity is negatively regulated by EDTA, urea, guanidine hydrochloride and SDS. Whereas >50% activity of the purified enzyme was observed in the presence of commercial detergents tested, at 60°C. Blood stain removal was effective with this enzyme when supplemented with $CaCl_2$ and glycine.

Keywords: Alkaline protease, *Bacillus subtilis*, characterization, detergent compatibility, purification, thermostability.

Introduction

The industrial enzyme market represents a major part of the global enzyme business ($ 1.4 US billion annually). Among these enzymes, protease is the most important enzyme and accounts for about 60% of the total worldwide enzyme sales (1). In general alkaline proteases molecular masses range from 18-90 KDa and are characterized by their high activity at alkaline pH range of 8-12 optimum being at pH 10 with an isoelectric point around pH 9.0. They are stable at temperatures of 50 and 70°C with broad substrate specificity and require divalent cations like Ca^{2+} and Mn^{2+} or combination for their activity. Their application potential is seen in different industries viz. detergents, food, brewing (2), meat tenderization, baking, dairy, leather (3, 4) and primarily as cleansing additives. Mostly, all industrial processes are carried out at higher temperature where normal enzymes become unstable. Ideally, proteases used in a detergent formulation should have a high level of activity over a broad range of pH and temperatures (5).

Bacterial proteases are the most significant, when compared with plant, animal and fungal proteases (6). Of all proteases, alkaline proteases produced by *Bacillus* species are of great importance in detergent industry due to their high thermo and pH stabilities. The optimum temperature for protease from *Bacillus* HSO8 was 65°C (7), and from *Bacillus subtilis* PE-11 and *Bacillus mojavensis* was 60°C (8, 9). The optimum incubation period for the protease production was 96 h for *Bacillus licheniformis* and *B.coagulans* (10). The other alkaline proteases reported with good thermostability are those of *Bacillus* sp. B18, which was retaining 60% of the original activity after incubation at 70°C at pH 10.0 for 60 min (11). Alkaline proteases from high yielding strains have been studied extensively (12). The literature reports revealed that the maximum alkaline protease production of 4,98,123 PU/l from isolated *Bacillus* sp. pE-11 (8) and protease production by thermophilic

Vol. 7 (3) 732-742 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Bacillus sp. strain SMIA-2 cultivated in liquid cultures reached a maximum in 9 h, with levels of 1.93 U/mg protein (13). However, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process (10).

The aim of the present work was to carry out purification, partial characterization, and determination of the properties of a protease enzyme produced by *Bacillus subtilis* NCIM No. 2724 and to evaluate the pH optima and thermostability of the enzyme. An attempt has been made to assess the compatibility of the enzyme with commercial detergents.

Materials and Methods

***Microorganism and Inoculum development*:** *Bacillus subtilis* NCIM No. 2724 was obtained from NCIM, Pune and grown on nutrient agar slants at 37°C for 24 h and preserved at 4°C for one month. The preserved culture was revived on fresh agar slants after every week. The inoculum was prepared by transferring a loopful of the culture into 50 ml of nutrient broth and kept for incubation at 37°C for 24 h. The diluted sample of culture (1 in 5) was plated onto skimmed milk agar plates containing peptone (0.1%), NaCl (0.5%), agar (2%), and skimmed milk (10%). Plates were incubated at 37°C for 24 h to measure the zone of hydrolysis (14).

***Protease Production*:** Six milliliter of the inoculum was transferred into 200 ml of the production medium. The production medium (glucose-1%, casein-0.5%, yeast extract-0.55%, potassium dihydrogenphosphate-0.2%, sodium carbonate-1%, magnesium sulphate-0.2%, pH 8) was incubated in a temperature controlled orbital shaker at 37°C and 140 rpm for 72 h. The biomass was separated by centrifugation at 4°C and 2,000 rpm. The supernatant was stored at 4°C for further analysis.

Protease Enzyme Assay: The protease enzyme activity was determined by the method of Tsuchida *et al.* (15). One protease unit is defined as the amount of enzyme that releases 1 µg of tyrosine/ml/min (0.18 µg of tyrosine = 1 µ mole of tyrosine) under standard assay conditions.

Protein determination: Total protein concentrations were determined by the method of Lowry *et al.* (16) using bovine serum albumin (BSA) as a standard.

Enzyme Purification and Partial Characterization

***Ammonium Sulphate Precipitation*:** The supernatant was used for purification of the enzyme. Initially ammonium sulphate precipitation was carried out at concentrations ranging from 50 to 80% using 3.135 g, 3.90 g, 4.723 g and 5.611 g of ammonium sulphate/10 ml of supernatant. All subsequent steps were carried out at 4°C. The ammonium sulphate fraction (70%) pellet was suspended in 0.2 M carbonate-bicarbonate buffer (pH 10) and dialyzed against the same buffer (diluted to 1:5).

Ion Exchange Chromatography: A column of length (30 × 2.5 cm) filled with activated DEAE-cellulose (20 g) was equilibrated with 250 ml of 0.1M Tris-HCl buffer (pH 7.8). The dialyzed sample (180.1 mg) was loaded onto ion exchange column contain DEAE-cellulose. Unbound fractions were collected at a flow rate of 5 ml/10 min by washing with 0.1M Tris-HCl buffer, pH 7.8. The bound enzyme was eluted from the column by increasing the ionic strength of the buffer with NaCl from 0.1-0.3 M.

***Gel Filtration Chromatography*:** The DEAE-cellulose fraction (32 mg) was then loaded onto Sephadex G-100 (2 g) gel filtration column (1.5 x 24 cm) which was previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.8). Fractions of 2 ml were collected at a flow rate of 15 ml/h. The absorbance was measured at 660 nm for all the fractions. The fractions with maximum activity were pooled, dialyzed, concentrated by lyophilization and used for further characterization.

Polyacrylamide Gel Electrophoresis: Polyacrylamide gel electrophoresis, at pH 8.3, was performed in 8% slab gels according to the method of Reisfeld *et al.* (17). About 50 µg (30 µl) of protein samples were loaded into the wells.

Proteins were visualized using Coomassie brilliant blue R 250 staining. The specific staining of protease was done in 8% slab gels by incorporating casein (0.5%) in the gel and visualizing the transparent band.

***Determination of Molecular Weight by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*:** Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis was carried out by the method of Laemmli (18) using 10% acrylamide. The marker proteins used were, bovine serum albumin (67 KDa), ovalbumin (44 KDa), chymotrypsinsogen A (25 KDa), and lysozyme (14 KDa). From the distance migrated by marker proteins, the molecular weight of the enzyme was determined.

***Determination of Optimum pH and Temperature for Enzyme Activity and Stability*:** For determining the optimum pH of the enzyme, 2% casein (substrate) was prepared in different buffers viz., 0.1 M sodium acetate buffer (pH 3-4), 0.1 M citrate buffer (pH 5-6), 0.1 M phosphate buffer (pH 7-8), 0.1 M Tris-HCl (pH 9), 0.1 M carbonate-bicarbonate buffer (pH 10-11), glycine-NaOH buffer (pH 12). Reaction mixtures were incubated at 37°C for 30 minutes and the activity of the enzyme was determined. To determine the pH stability of the protease, 1 ml of the purified enzyme (0.1 M buffer) was kept at 4°C for 12 h, aliquots of 1.5 ml were used for enzyme assay with the buffers mentioned earlier. For temperature studies, optimum temperature was determined by measuring the enzyme activity in the temperature range 30-100°C. The stability of the enzyme at different temperatures was determined by incubating the enzyme for 30 min over a temperature range of 30°C to 100°C.

***Effect of Metal Ions, Inhibitors/Chelators, Denaturants and Different Natural Substrates on Purified Protease Activity*:** In order to determine the effect of metal ions on enzyme activity, enzyme solutions were pre incubated for 30 min with metal chlorides ($ZnCl_2$, $CuCl_2$, $MgCl_2$, $HgCl_2$, $CoCl_2$, $CaCl_2$, NaCl, $CdCl_2$, $AlCl_3$, and $MnCl_2$ at a final concentration of 5 mM) at 5°C and assayed for protease activity and residual activity. The effect of various inhibitors (5 mM) such as β-mercaptoethanol, iodoacetate, and EDTA were determined by pre incubation with the enzyme solution for 30 min at 37°C before the addition of substrate. The effect of various denaturants such as Urea (3 M, 6 M), Guanidine-HCl (6 M), and SDS (0.1%) were determined by pre incubation with the enzyme solution for 30 min at 37°C before the addition of the substrate. The effect of various natural substrates such as Casein, Egg albumin, Bovine Serum Albumin and Gelatin were determined after incubation with the enzyme solution for 30 min at 60°C. The specific protease activity towards casein was taken as a control. The relative protease activity was determined.

***Compatibility of Protease with Various Detergents*:** The compatibility of the protease with commercial detergents in the presence of 10 mM $CaCl_2$ and glycine at 60°C was studied. Seven commercial detergents were used in this study. The detergents were diluted with distilled water and incubated with protease for 3 h at 60°C, and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%.

***Blood Stain Removal Capacity of Protease*:** Application of protease (4000 U/ml) as a detergent additive was studied on white cotton cloth pieces (5 × 5 cm) stained with blood. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied. 1. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood). 2. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood) + 1 ml of brand 2 (10 mg/ml) and 3. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood) + 1 ml of brand 2 (10 mg/ml) + 2 ml of enzyme solution. The above flasks were incubated at 60°C for 15 min. After incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of various pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with blood were taken as control.

Vol. 7 (3) 732-742 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

***Determination of Dehairing and Gelatin Removal Property of Purified Protease*:** The dehairing property was studied by using goat skin (10 cm^2). Purified enzyme solution was applied at 1% concentration on goat skin pieces and kept at ambient temperature (35°C), pH 8.0 and incubation period of 18-24 h. For gelatin removal, the X-ray film (1 g) pieces were incubated with enzyme (100 U/ml) at pH 9 (Tris-HCl, 50 mM) and the reaction was carried out at 45-55°C for 30 min to 1 h and the time taken for complete gelatin removal was observed (19).

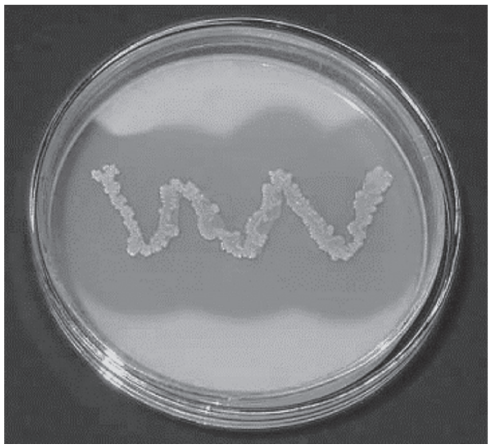

Fig. 1. The zone of hydrolysis of *Bacillus subtilis* NCIM No. 2724 on skimmed milk agar

Results and Discussion

The protease producing capability of the organism was initially determined by measuring the zone of hydrolysis obtained after inoculating the diluted sample of culture onto plates containing skimmed milk agar. Plates produced a significant clear zone of hydrolysis with a diameter of 15 cm and a growth zone was 1.5 cm (Fig. 1). The production of protease enzyme was carried out by submerged fermentation in a medium described earlier in two 1l flasks, each containing 200 ml of production medium. The protease activity was determined after 18 h during the log phase of the growth and was found to be 102.2 U/ml.

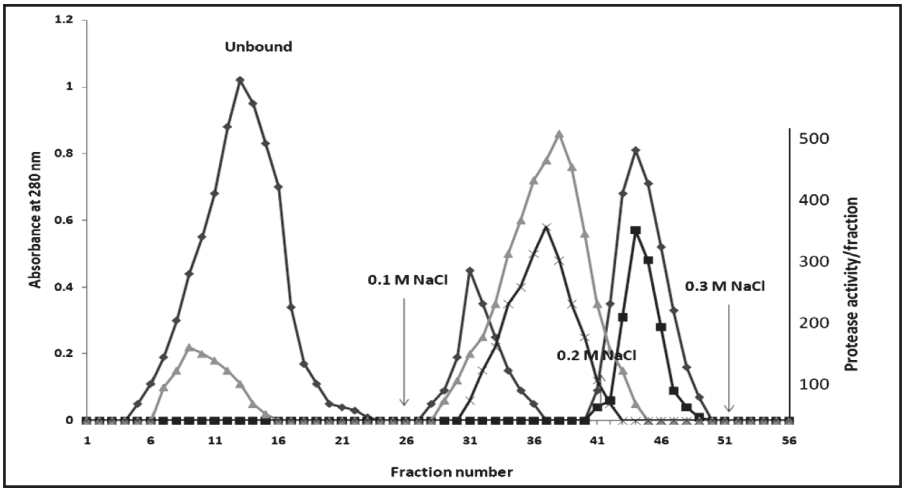

Fig. 2. Elution profile of purified protease by DEAE-cellulose Ion-exchange Chromatography and Sephadex G-100 Gel filtration

(▲ ——— ▲) Absorbance at 280 nm of eluted fractions from DEAE-cellulose column
(■ ——— ■) Purified protease activity of eluted fractions from DEAE-cellulose column
(▲ ——— ▲) Absorbance at 280 nm of eluted fractions from Sephadex G-100 column
(x ——— x) Purified protease activity of eluted fractions from Sephadex G-100 column

After ammonium sulphate precipitation and dialysis 180.1 mg of total protein with a specific activity of 395.2 U/mg was obtained. After purification by Ion exchange chromatography and gel filtration, the total protein was 32 mg and 25.2 mg with a specific activity of 872.2 U/mg and 935.8 U/mg respectively which represents a purification fold of 2.62. The elution profiles of Ion exchange chromatography and gel filtration chromatography were shown in Fig. 2. The final recovery of purified protease obtained by these purification methods was 18.13%. The summary of purification steps of protease enzyme from *Bacillus subtilis* NCIM No. 2724 is presented in Table 1. The protease enzyme isolated from *Bacillus subtilis* (20) with specific activity of 6381.75 (units/mg prot/ml^{-1}) with purification of 7.87 folds. Gerze *et al*. (21) reported that the specific activity of partially purified enzyme from *Bacillus subtilis megaterium* was 13.5 U/mg after 80% ammonium sulphate fractionation followed by dialysis. Ahmed *et al*. (22) reported that an alkaline protease produced from *Bacillus subtilis* was purified 1.49-fold with specific activity of 74.66 U/mg and a yield of 3.11%. With our present strain, 6 fold increase in yield and 12 fold increase in activity was observed when compared with the values of Ahmed *et al*. (22).

The homogeneity of purified protease was determined by native PAGE. Depending on the relative mobility, the molecular weight of the protein band was calculated to be 26 KDa. The band obtained was slightly above the protein marker Chymotrypsinogen A (25 KDa) indicating that the molecular weight of the enzyme is about 26 KDa. In specific staining a single transparent band corresponding to the band obtained in the native page (Fig. 3) was observed. The molecular weight of the obtained protease is similar to that of the protease isolated from *Bacillus subtilis* reported by Ahmed *et al.* (22). The alkaline protease from *Bacillus* sp. is reported as a single band with molecular weight from 16-32 KDa (23, 24), where as other reported halophilic and alkaline proteases have molecular weights in the range of 40 to 130 KDa (25).

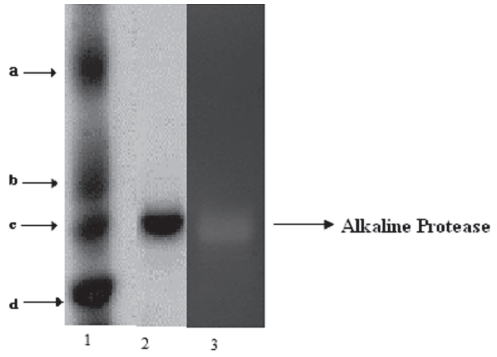

Fig. 3. SDS-PAGE and specific staining of purified protease of *Bacillus subtilis* NCIM No. 2724 Lane 1. Standard protein markers; a) Bovine serum albumin (67 KDa) b) Ovalbumin (44 KDa) c) Chymotrypsinogen A (25 KDa) d) Lysozyme (14 KDa) Lane 2. Purified protease enzyme; Lane 3. Specific staining

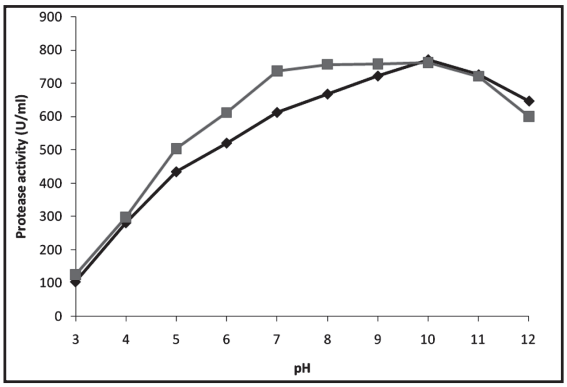

Fig. 4. pH optimum and stability of purified protease of *Bacillus subtilis* NCIM No. 2724 (♦————-♦ Optimum p^{H} and ■————-■ pH stability)

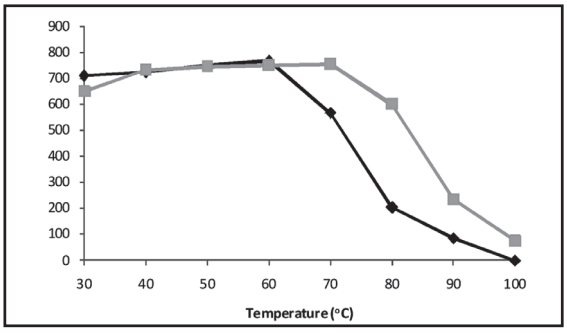

Fig. 5. Temperature optimum and stability of purified protease of *Bacillus subtilis* NCIM No. 2724 (♦ ——♦ Optimum temperature and ■————■ Temperature stability)

The maximum protease activity was found to be at pH 10. The pH optimum for the purified alkaline protease was found to be at pH 10 and stable over a pH range of 7-11 (Fig. 4). The pH optima for alkaline protease of *Bacillus* sp. has been reported to be vary from 8-11 and species specific (26). Our results are correlated with Adinarayana *et al*. (8), Patel *et al*. (24) and Deng *et al*. (27). The optimum temperature determined for the purified enzyme was found to be 60°C. The enzyme activity gradually declined at temperatures beyond 60°C. The enzyme was stable up to 70°C for an incubation period of 30 min and 1 h (Fig. 5). Thermostability of industrial enzymes is a desirable property (28). Our enzyme exhibited thermostability and the optimum temperature was 60°C and was stable for one hour. A similar type of result was obtained by other investigators where a maximum temperature of 55°C was recorded for an alkaline protease from *B. stearothermophilus* AP-4 (29) and 60°C for a protease derived from *Bacillus* sp. B21-2 (30). The kinetic parameters K_M and V_{max} of the protease enzyme produced by *B. subtilis* NCIM No. 2724 were estimated by Lineweaver-Burk plot employing various concentrations of casein as substrate (Fig. 6). The Lineweaver- Burk plot for the proteolytic reaction of the casein revealed that the K_M and V_{max} values of the reaction were 5 mg/ml and 1428 U/ml respectively. Patel *et al*. (24) reported that the K_M and V_{max} of Ve1 protease were

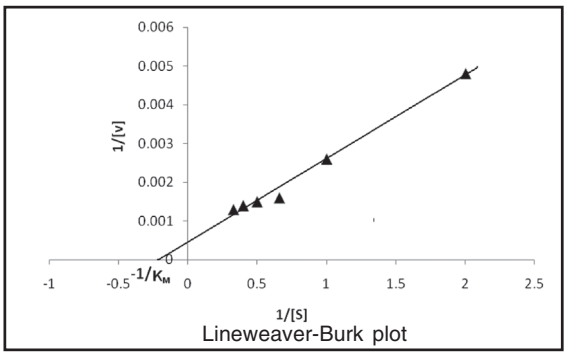

Fig. 6. Effect of substrate concentration on purified protease of *Bacillus subtilis* NCIM No. 2724

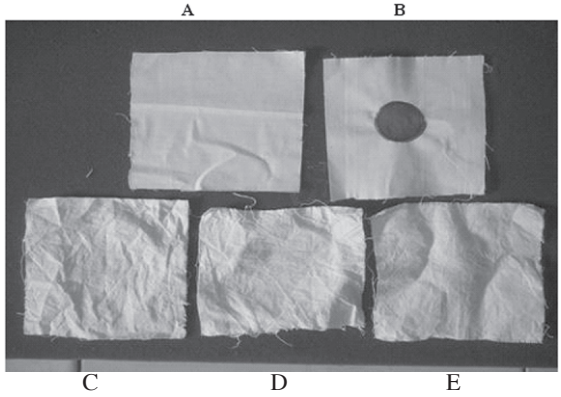

Fig. 7. Blood stain removal capacity of purified protease from *Bacillus subtilis* NCIM 2724 in the presence of detergent (brand 2) A. Unstained cloth; B. Blood stained cloth; C. Blood stained cloth washed with enzyme; D. Blood stained cloth washed with detergent and enzyme; E. Blood stained cloth washed with detergent

Table 1. Summary of purification steps of protease from *Bacillus subtilis* NCIM No. 2724

Purification step	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% Recovery
Crude enzyme (150 ml)	1,30,083.3	364.2	357.2	1.00	100.00
Ammonium Sulphate precipitation (70%) Dialyzed (30 ml)	71,190.4	180.1	395.2	1.10	54.72
DEAE-cellulose (30 ml)	27,911.1	32.0	872.2	2.44	21.45
Sephadex G-100 (30 ml)	23,584.4	25.2	935.8	2.62	18.13

Yield and fold purification were calculated on the basis of total activity units and specific activity units respectively

Table 2. Effect of various metal ions, inhibitors/chelators, denaturants and natural substrates on purified protease activity

Metal ion (5 mM)	Residual Protease Activity (%)	Inhibitor/ Chelator (5 mM)	Relative Enzyme Activity (%)	Denaturant	Relative Enzyme Activity (%)	Substrate (2 mg/ml)	Relative Enzyme Activity (%)
Control	100	Control	100	Control	100	Casein	100
$Zn^{2+}(ZnCl_2)$	94	β-mercaptoethanol	95	Urea (3 M)	98	BSA	44
$Cu^{2+}(CuCl_2)$	96	Iodoacetate	92	(6 M)	96	Egg albumin	20
$Mg^{2+}(MgCl_2)$	115	EDTA	98	Guanidine –HCl (6 M)	94	Gelatin	10
$Hg^{2+}(HgCl_2)$	94			SDS (0.1%)	92		
$Co^{2+}(CoCl_2)$	92						
$Ca^{2+}(CaCl_2)$	133						
$Na^{+}(NaCl)$	96						
$Cd^{2+}(CdCl_2)$	92						
$Al^{3+}(AlCl_3)$	94						
$Mn^{2+}(MnCl_2)$	109						

0.153 g/100 ml and 454 U/ml respectively. Ahmed *et al*. (22) reported that using casein as substrate, the enzyme showed maximum activity (V_{max}) of 148 U/ml with its corresponding K_M value of 58 μM. The V_{max} value of our enzyme is found to be 3 times and 10 times more than that of values of Patel *et al.* and Ahmed *et al.* respectively.

Most of the metal ions tested had a stimulatory effect (Ca^{2+}, Mg^{2+} and Mn^{2+}) or a slight inhibitory effect (Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Na^{+}, Cd^{2+}, Al^{3+}) on enzyme activity (Table 2). Ca^{2+}, Mg^{2+} and Mn^{2+} increased and stabilized the protease activity of the enzyme. These cations also have been reported to increase the thermalstability of other *Bacillus* alkaline proteases (31). These results suggest that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at higher temperatures (32). Other metal ions such as Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Na^{+}, Cd^{2+}, Al^{3+} and EDTA did not show any appreciable effect on the enzyme activity. These results were found to be similar to those reported by Adinarayana *et al.* (8). The effect of different inhibitors on the enzyme activity of the purified protease was determined (Table 2). The protease enzyme activity was unaffected when it was exposed to various inhibitors viz., β-mercaptoethanol, iodoacetate, and EDTA of 5 mM concentration. The protease was not inhibited by EDTA, while a slight inhibition was observed with iodoacetate and β-mercaptoethanol. These results were found to be similar to those reported by Adinarayana *et al.* (8). Ramakrishna *et al*. (33) reported that EDTA slightly activated the protease activity whereas, according to the report of Arulmani *et al*. (34), EDTA showed mild inhibitory effect on serine protease from thermostable alkalophilic *Bacillus laterosporus*-AK1. Alkaline protease activity was unaffected when it was exposed to various denaturants such as Urea (3 M, 6 M), Guanidine-HCl (6 M), SDS (0.1%) (Table 2). Gupta *et al.* (2) reported that as the concentration of SDS increased, the activity of protease decreased. The purified protease showed a high

Table 3. Compatibility of purified protease activity from *Bacillus subtilis* NCIM No. 2724 with commercial detergents in the presence of 10 mM $CaCl_2$ and Glycine at 60°C

Relative Residual Protease Activity (%)								
Time (h)	**Control**	***1**	**2**	**3**	**4**	**5**	**6**	**7**
0.0	100	100	100	100	100	100	100	100
0.5	97	90	94	92	93	92	88	87
1.0	94	87	90	89	88	90	85	84
1.5	90	84	88	86	83	80	81	80
2.0	86	79	81	78	75	80	74	70
2.5	80	69	71	67	61	71	63	59
3.0	73	56	63	54	51	52	53	50

* *Commercial detergent brands 1, 2, 3, 4, 5, 6 and 7*

level of hydrolytic activity against casein and poor to moderate hydrolysis of BSA and egg albumin, although the hydrolysis was hardly observed with gelatin (Table 2). An alkaline protease is highly substrate specific and exhibit maximum activity towards casein as substrate (34). Adinarayana *et al*. (8) reported that protease have a high level of hydrolytic activity against the substrate casein and poor to moderate hydrolysis of BSA and egg albumin respectively.

The protease from *B. subtilis* NCIM No. 2724 showed excellent stability and compatibility in the presence of commercial detergents (1, 2, 3, 4, 5, 6 and 7) at 60°C in the presence of $CaCl_2$ and glycine as stabilizers. Our protease enzyme showed good stability and compatibility in the presence of 2 followed by 5 when compared to other detergents (1, 3, 4, 6 and 7) (Table 3). The enzyme retained more than 50% activity with most of the detergents tested even after 3 hours of incubation at 60°C after the supplementation of $CaCl_2$ and glycine. Bhosale *et al.* (35) reported that high activity of alkaline protease from *C. coronatus* showing compatibility at 50°C, in the presence of 25 mM $CaCl_2$ with a variety of commercial detergents and also reported 16 % activity in Revel, 11.4% activity in Arial, and 6.6% activity in Wheel. Comparing the results of Bhosale *et al.,* the isolated enzyme from *B. subtilis* NCIM No. 2724 was significantly more stable in the presence of commercial detergents. The supplementation of the enzyme preparation in detergent (brand 2) significantly improved the removal of the blood stains (Fig. 7). This result is similar to that reported by Adinarayana *et al*. (8), where detergent Wheel was used in their experiments. The dehairing capacity of the protease was poor when compared to sodium sulphide solution. Studies carried out by different workers have demonstrated the successful use of alkaline proteases in leather tanning from *B. amyloliquefaciens* (36) and *B. subtilis* (37). The protease did not remove gelatin from the X-ray film. In an earlier report, an alkaline protease from *B. subtilis* decomposed the gelatin layer within 30 min at 50-60°C and released the silver (38). Prakash *et al.* (19) have been reported that the complete removal of gelatin layer from X-ray films was achieved in 25 min at 45°C with *Bacillus subtilis* MTCC 9226 protease. The poor dehairing and gelatin removal from X-ray films of our enzyme may be due to the low concentration of the enzyme.

In conclusion, the protease enzyme purified from *Bacillus subtilis* NCIM No. 2724 selected for this study has an optimum temperature of 60°C, possessing good thermostability for 1 h at 70°C, with a pH optimum of 10 indicating that it is a thermostable alkaline protease with potential industrial applications especially in the detergent industry. This enzyme can be commercially exploited.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 743-754 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Liquefied Sorghum Starch Medium as Novel Substrate for Production of Lactic Acid by Immobilized Acid Resistant *Rhizopus oryzae* in Natural and Synthetic Sponges

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Abstract

Lactic acid production by acid resistance UV mutated *Rhizopus oryzae* UV-40 strain immobilized on Natural-Vegetable Loofa Sponge (NVLS) and Synthetic Sponge (SS) was evaluated. Shape and texture of these matrices remained invariable with pH and temperature. Four pieces of NVLS and/or SS inocula per 150 ml of liquefied sorghum medium inoculated with spore suspension of 3×10^6 and 2×10^6 spores/ml resulted in 2.69 and 2.56 g/l/h lactic acid up to 4th and 3rd cycles of repeated batch fermentations, respectively. Scanning electron microscopic studies revealed NVLS is better adsorbing and immobilizing agent than SS. The mycelia in case of NVLS, inocula was strongly bound and entangled in between the matrices of the loofa sponge while in case of SS, inoculum was found to be better than the free cell and NVLS as well as production of maximum lactic acid.

Keywords: Immobilization, *Rhizopus oryzae* UV-40, Liquefied sorghum medium, Lactic acid

Introduction

Lactic acid has received attention in the global market due to its utilitarian values in the industrial applications. Polymer of lactic acid, a plant and crop based renewable plastic produced by fermentation process is considered as alternative to petroleum based plastics (1, 2). Fermentation based lactic acid production is unique in its nature and product profile i.e., bioproduction using microbial strain is specific in synthesizing either D or L-lactic acid or both. In view of colossal disadvantages of bacteria and yeast in lactic acid production, an increased research interest has been observed by fungal fermentation in recent decades (3, 4). The best known fungal source for production of L(+)-lactic acid using starch as carbon source and with a low nutritional requirement is *Rhizopus oryzae* (5, 6, 7). The selection of this strain is considered as advantageous as this strain has potential to carry out simultaneous saccharification and fermentation (8, 9).

Crop residues are annually renewable sources of energy; and approximately 3.5 billion tons of these are produced per annum globally. Finding new and efficient uses for agricultural crops and their residues is imperative for competing in today's global market. Among the renewable sources, various starchy substances appear to be attractive as raw materials for production of industrial important products through fermentation process. However, most of these starchy substrates such as wheat, rice, potato, corn, etc are commonly used as food materials by human beings hence use of these

substrate materials for production of biotech products results in food-fodder issue. Therefore, a non-conventional starchy substrates and non-edible spoiled food grains, etc which is being utilized to a less extent for human consumption could be exploited as substrate material for lactic acid production at industrial sectors (10, 11). Sorghum *(Sorghum bicolor* (L.) Moench) is the 5th most important cereal crop after wheat, rice, maize, and barley in terms of production. Sorghum provides one of the most efficient sources of starches owing to its high carbohydrate content (12). Substantial amounts of sorghum are utilized for food, feed and other products. However, some of the by-products of sorghum, such as broken or damaged grains are produced as uneconomical products. Therefore, these can be economically converted into lactic acid by simultaneous saccharification and fermentation of liquefied sorghum starch media. Moreover owing to its high carbohydrate content, sorghum provides one of the most efficient sources of starches (12). Zhan et al. (13) reported an average lactic acid yield of 32.5 g/l from 150 g/l of direct ground grain sorghum flour (without any treatment). The authors report that the low productivity may be attributed to variation in viscosity of the medium and also due to the presence of considerable amounts of anti-nutritional factors such as tannin, phytic acid, polyphenol and trypsin inhibitors in the crude flour that are undesirable for fermentation (14). However, it was also reported that the anti nutritional factors like tannins rich sorghum flour may cause reduction of viscosity which in turn results in change lactic acid production as well as regulation of amylase activity during saccharification process (15).

Fermentation process efficiently could be enhanced by immobilization strategy (16, 17, 18). There has been a considerable appreciation on the potential of immobilized fungal cells for the production of metabolites like various organic acids in view of repeated batch fermentation and continuous processes which economizes the production yields (19, 20, 21). In addition, fermentation by immobilization cells offer several advantages such as higher productivity, operational stability, and easy mass transfer and decreased contamination of the product as compared to free cell fermentation. Since many studies have revealed that the adsorption method of immobilizing microorganisms has edge over the entrapment method using gel beads (22, 23), the same has been adopted in the present study. One of the important desired objective of the present work is development of an economically viable process for lactic acid production from liquefied sorghum starch with higher productivity using *Rhizopus oryzae* UV-40 mutant strain immobilized with cheaper adsorbing materials like loofa sponge and synthetic sponge.

Materials and Methods

Microorganism: *Rhizopus oryzae* UV-40 strain attained by UV-induced mutagenesis of the wild strain of *Rhizopus oryzae* that was isolated from vermi-compost soil was used in the present study. The mutant strain was grown on PDA slants at room temperature for 7 days and preserved as stock culture in refrigerator at 4°C temperature for maintenance and periodical use.

Immobilization materials: Naturally available loofa vegetable sponge obtained from the ripened dried fruit of *Luffa cylindrica* (24) and synthetic sponge pieces, which were acquired from the local market, are used as immobilizing matrices.

Preparation of loofa sponge and synthetic sponge pieces for immobilization: From the dried fruit of *Luffa cylindrica*, the reticulated sponge was separated from the soft tissues of the dried fruit and prepared for immobilization. Luffa sponge and synthetic sponge were cut into small pieces, soaked in boiling water for 30 min, washed and left for 24 h in distilled water for 3-4 times. The obtained discs were then dried at 70°C, weighed, and stored in a dessicator until use.

Preparation of liquefied sorghum starch medium: The crude sorghum flour was gelatinized and liquefied using commercial

thermostable α-amylase, traded as Biotempase by Biocon India Pvt. Ltd., Bangalore. As the crude sorghum flour from the damaged grains contain only 65% starch, a concentration of 20 µl of the enzyme/100 g of flour was added and autoclaved at 110°C for 5 min with a pH 6.0. After cooling, the solution was filtered and stored till use. The concentration of total sugars of the liquefied sorghum starch solution was estimated and the concentration was adjusted according to the requirement for fermentation.

Fermentation with immobilized Rhizopus oryzae UV-40: The production of lactic acid was carried out in two phases. In the first phase, *Rhizopus oryzae* UV-40 strain was inoculated into pre-culture medium (g/l): Liquefied sorghum-50, $(NH_4)_2SO_4$-1.35, KH_2PO_4-0.15, $MgSO_4$-0.25 and $ZnSO_4$-0.04. After immobilization of enough cells, the medium was replaced with production medium consisting (g/l): 150-Liquefied sorghum, $(NH_4)_2SO_4$-3.0, KH_2PO_4-0.15, $MgSO_4$-0.25, $ZnSO_4$-0.25 with 1×10^6 spores/ml concentration of inocula and without the addition of neutralizing agents. Fermented samples were withdrawn at required time intervals, filtered through Whattman No.1 filter paper, centrifuged at 6000 rpm for 10 min and the supernatant was further filtered through sterile 0.22 ìm filter (Millipore, USA) and used for determination of product. The immobilized biocatalyst was confirmed using Scanning Electron Microscope (SEM).

Preparation of immobilized fungi for SEM studies: For SEM, the samples were processed in four steps namely washing, fixation, dehydration and drying (25).

.***Washing:*** Loofa sponge and cellulose sponge pieces after sieving from the fermentation broth were rinsed with deionized water till the pH of the washings were neutral. These were then sliced with a sharp and sterile lancet.

Fixation: Briefly the immobilized fungal mat was taken and primary fixation was done by 2.5 % gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C and post-fixed 2% in aqueous osmium tetroxide for 4 h, in the same buffer.

Dehydration: The samples were then washed and dehydrated by treating through successive alcohol gradients (30, 50 and 70% ethyl alcohol for 15 min at each step; 80, 90 and 100% ethyl alcohol for 30 min at each step) in a stepwise manner.

Drying: After complete dehydration, samples were dried to Critical Point Drying with Electron Microscopy CPD unit. The dried samples were mounted over the stubs with double-sided carbon tape. A thin layer of gold coat is applied over the samples using an automated sputter coater (JEOL JFC-1600) by gold shadowing technique for 3 min and then the samples were scanned under Scanning Electron Microscope (Model: JOEL-JSM 5600) at desired magnifications.

Storage: The immobilized biomass as washed with sterile distilled water immersed in 0.9% NaCl and stored in a refrigerator at 4°C until use.

Immobilized biomass measurement: The immobilized biomass was separated from the production media after fermentation by filteration and washed twice thoroughly with distilled water before freeze-drying. Weight of the immobilized biomass was measured by substracting the weight of the sponge pieces (uninoculated) from the weight of the immobilized sponge pieces, which was freeze-dried for at least three consecutive days till the achievement of constant weight.

Statistical analysis: Statistical analysis was done using INSTAT SOFT software. Design of selection was done automatically based on the data provided. Analysis of Variance (ANOVA) was selected and the comparison of means was done using Tukey-Kramer Multiple Comparison Test (p value less than 0.05 were highly significant).

Results

Recombinant or mutant strains when immobilized alleviate strain genetic stability problems (26). Hence, whole cell immobilization was applied to *Rhizopus oryzae* UV-40 mutant strain using cheaper adsorbing materials like

loofa sponge and synthetic sponge, as the selection of immobilization matrix was one of the important parameters among different fermentation conditions to get sustained/ improved microbial products without cell loss (27).

pH and temperature stability of loofa and synthetic sponge pieces: The pH stability of the sponges was determined by soaking the sponge in the buffer solutions of varying pH (1.1–14) for two weeks and then observing the change in shape, structure and texture of the sponge. The temperature stability of the sponges was also observed by repeatedly autoclaving the sponges at 121°C for various lengths of time (20–40 min). It was noticed that after these repeated temperature and pH treatments, there was no influence on the shape and texture of these immobilization matrices. These results indicated that both the sponge discs can be repeatedly used in adverse conditions because neither pH nor autoclaving resulted in any change in the shape and structure of the sponge.

Effect of sponge size: Sponge size of 1.6×1.8 mm led to the maximum levels (120.9 g/l (F: 3.168, P: 0.08) and 117.02 g/l (F: 11.319, P: 0.003) of lactic acid production in both the sponge varieties with the use of 150 g/l of substrate concentration with loofa sponge and synthetic sponge, respectively. With respect to increase in size of the sponge, the biomass continued to increase to a level of 5.3 g/l in case of loofa sponge, whereas the biomass concentration was only 4.06 g/l in case of synthetic sponge (Fig. 1 and 2). In the case of NVLS the increase of sponge size at 2.0X2.2mm level produced 102.62 g/L of lactic acid and 4.04 gm of biomass whereas in case of SS it was 105.04 g/L of lactic acid and 3.01 g of biomass.

Effect of spore suspension: Both the sponges of 1.6×1.8 mm size was added with inocula containing varying levels of spores from 1×10^6 to 4×10^6 spores/ml concentration. The increase in spore concentration up to 3×10^6 spores/ml led

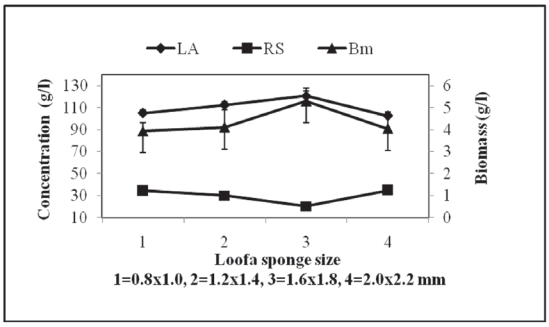

Fig. 1. Effect of loofa sponge (NVLS) size

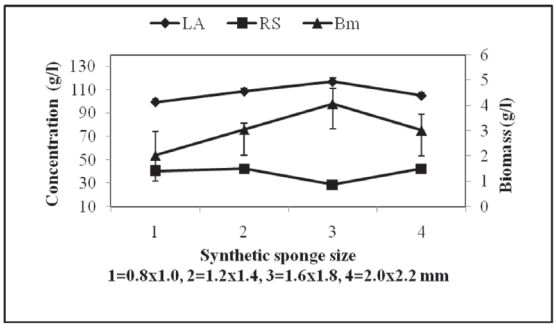

Fig. 2. Effect of synthetic sponge (SS) size

to significant increase (F: 5.761, P: 0.021) of 120.6 g/l lactic acid production with higher levels of biomass accumulated (5.05 g/l) under these conditions in case of loofa sponge. On the other hand, 2×10^6 spores/ml concentration led to significant increase (F: 11.604, P: 0.0028) of 114.64 g/l lactic acid and 5.1 g/l of biomass in the case of synthetic sponge. Further increase in spore concentration above the given optimal conditions was detrimental for the acid production ability of the organism (Fig. 3 and 4).

Effect of inocula level pieces*:* Varying levels (2–8%) of fungus immobilized loofa sponge (NVLS) and fungus immobilized synthetic sponge (SS) inocula with spore suspension of 3×10^6 and 2×10^6 spores/ml was used, respectively for understanding the impact of inoculum concentration and subsequent variations in lactic acid productivity yields. An inocula level of 4% (4 pieces of loofa sponge of 1.6×1.8 mm) had led

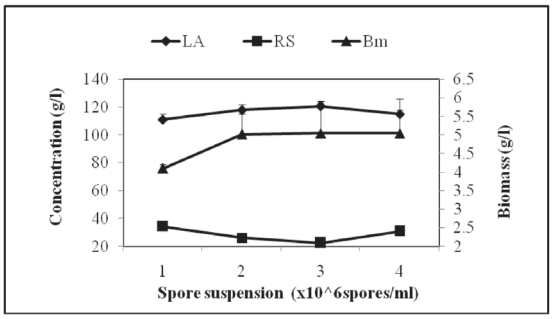

Fig. 3. Effect of spore suspension on NVLS

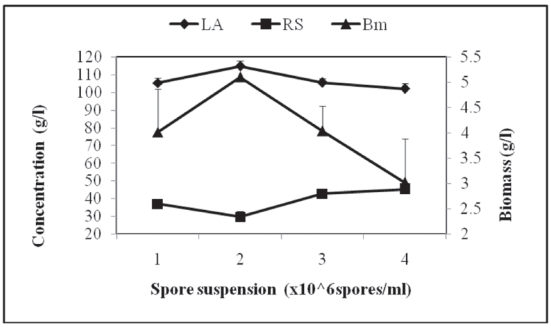

Fig. 4. Effect of spore suspension on SS

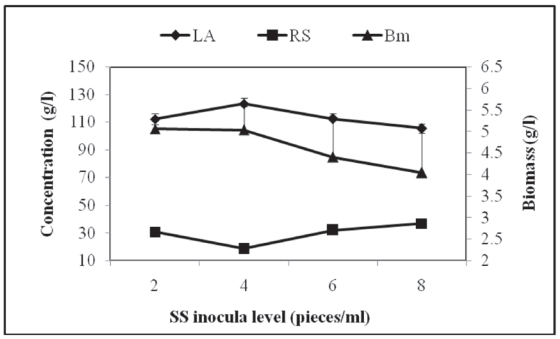

Fig. 5. Effect of inocula level on NVLS

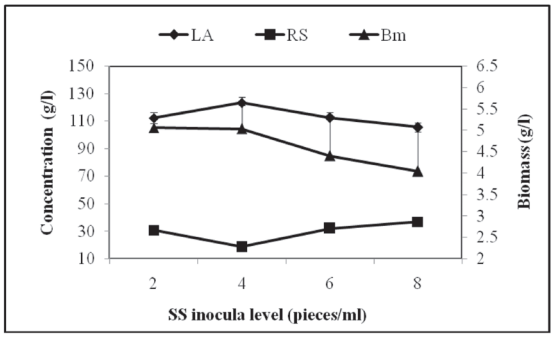

Fig. 6. Effect of inocula level on SS

to maximum significant levels (F: 10.886, P: 0.0034 and F: 11.319, P: 0.003) of acid production and a similar pattern was observed for the growth of the fungus (Fig. 5 and 6).

Fungi show many morphological variations in submerged fermentation. Since, fungi forms mat like biomass during fermentation process, it was difficult to control morphology of the fungi to obtain best lactic acid productivity in the case of free cell fermentation. Whereas, during immobilization, the fungal cells distributed more or less equally in the entire immobilization matrix and do not undergo a morphological change. However, the free fungal cells in the submerged fermentation resulted in production of mat formation causing unequal distribution of gas mass balance where higher availability to outer mass compared inner fungal mass in the mat. The immobilized cells were grown superficially on to the support and only their extracellular secretions were leaked out into the medium providing space for improved mass balance as any fermentation process improves with effective mass balance of gas and other nutrients (28).

SEM studies: It was used to confirm the immobilization of fungal cells. The immobilization of the fungus in the loofa sponge pieces was found to be strongly bound and entangled in between the matrices of the loofa sponge, whereas the fungus immobilized in case of synthetic sponge was found to be superficial with less contact with the support, and it was observed that fungal mycelia were not properly entangled in between the matrices of synthetic sponge (Fig. 7).

Effect on repeated batches: Immobilization effect on repeated batches was observed up to 7 batch cycles. The immobilized clumpy inocula was spliced into pieces with the help of a sterile lancet at the end of each batch and was further used as inocula for the next batch by storing in 0.9% saline.

Discussion

In the present study the impact of natural and synthetic sponge material as novel

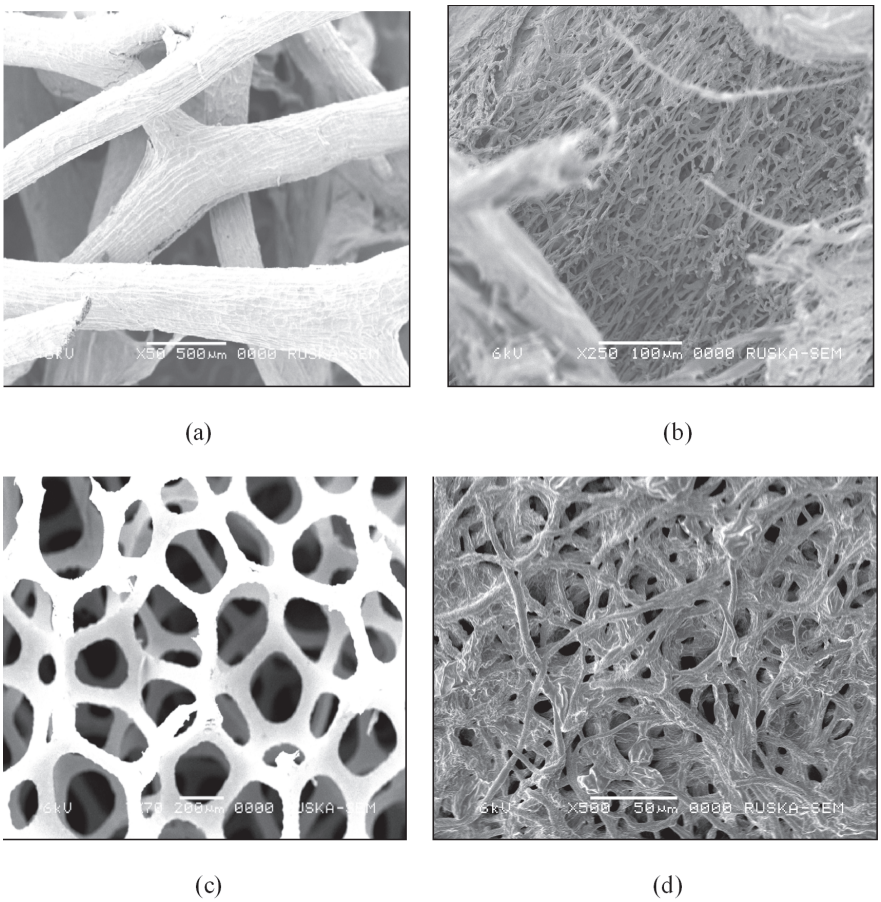

Fig. 7. Scanning electron micrographs of NVLS and SS (a. NVLS control; b. Fungal immobilized NVLS; c. SS control; d. Fungal immobilized SS).

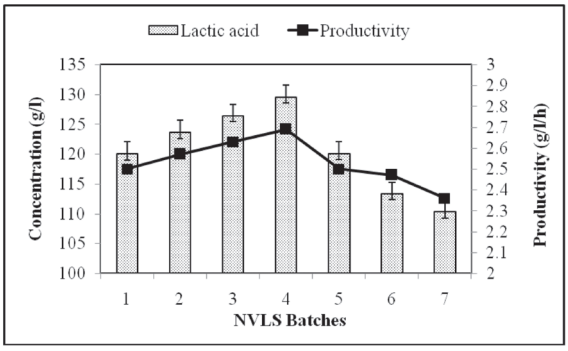

Fig. 8. Lactic acid production and productivity levels using NVLS

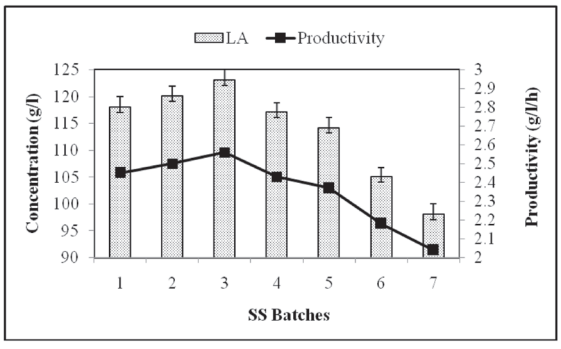

Fig. 9. Lactic acid production and productivity levels using SS

Vol. 7 (3) 743-754 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

immobilization matrices was investigated for industrial product, lactic acid, production using mutated *Rhizopus oryzae.* Considerable variations in productivity levels and reusability of immobilized cells have been noticed. For example, with the use of natural immobilization matrix i.e., loofa sponge (NVLS), the productivity yield was maximum (129.45 g/l) during 4th recycle (Fig. 8) while in case of synthetic sponge (SS) optimized lactic acid production was observed during third cycle (Fig. 9). This data is in contrast with the reported results on reusability of the lactic acid production by free cells for at least seven cycles (29). Hong-Wei Yen and Yi-Chih Lee used alginate as immobilization matrix for lactic acid production. Hence the observed variation in the present study and reported data (30) may be attributed to matrix structure and nature mediated variation in entrapment and subsequent metabolism of immobilized cells associated with mass transfer of nutrients, products and other fermentation physiological parameters such as pH, temperature, etc fluctuations during fermentation process. In addition, the observed differences may also attributed to mutation mediated changes in metabolic process as in the present study the lactic acid production was monitored using mutated strain of *Rizophus oryzae* while Hong-Wei Yen and Yi-Chih Lee (30) used natural strain of same sp.

The observed lactic acid production range also differed with respect to natural and synthetic sponge materials where cells immobilized in loofa sponge revealed high productivity compared to synthetic sponge material as immobilization matrix. This could be exemplified based on observations that lactic acid concentration ranged from 120.02 to 129.45 g/l with a productivity range of 2.5 to 2.69 g/l/h during 1-5 batches of repeated fermentation with NVLS as immobilization matrix while the productivity values observed with SS were found to be slightly less (Fig. 8 and 9). The data presented here is in comparison with literature reports of Hong-Wei Yen and Yi-Chih Lee (30) with alginate immobilized cells.

Analysis of lactic acid production data with respect to repeated cycles and variation of immobilization matrices further suggested that more number of reusability cycles could be possible with the use of naturally synthesized material immobilization matrix for *Rhizopus oryzae* filaments compared to chemically synthesized material. The evidence for this may be noticed with the reusability data, where cells immobilized in NVLS, the lactic acid production was noticed more than seven repeated cycles whereas with the use of SS (chemically synthesized sponge as immobilization matrix), the reusability immobilized cells was observed for less cycles but less productivity yields (Fig 8 and 9).

The nature of immobilization matrix also influenced the fermentation process mediated product production. This could be confirmed based on the observation that, the lactic acid production was increased with increase in repeated use till fourth batch of fermentation where the observed lactic acid production was noticed to be 129.45 g/l and subsequent use of same immobilized cells resulted in decreased production (Fig. 8). These results are in accordance with literature reports were authors reported the repeated use of immobilized cells for lactic acid production however the present study differs in constant increase of production of lactic acid up to seventh batch and further increase in repeated batch resulted in deceased yield which was attributed loss of microbial population in immobilized matrix (29, 30).

Immobilization matrice dependent lactic acid production was noticed in the present study indicating the impact of matrix nature on microbial metabolism related carbon metabolic process and subsequent product yield variation. This could be exemplified based on the observation that lactic acid production levels altered with type of immobilization sponges used. When NVLS used as immobilization matrix, the maximum level of lactic acid concentration noticed to be 129 g/l with a productivity yield of 2.69 g/l/h at fourth batch of fermentation while in case of SS

the optimum lactic acid production level observed to be 123 g/l with a productivity yield of 2.56 g/l/h at third batch of fermentation (Fig. 8 and 9). The observed reduced production of product beyond fourth and third batch with immobilized cells of *Rhizopus oryzae* UV-40 in NVLS and SS matrices may be attributed to leakage of fungal cells. Visual observation of fermentation broth did confirm the appearance of fungal cell biomass in the medium. Similar trend has been observed by researchers working on lactic acid production with immobilized cells where the authors reported that long-term repeated use of immobilized cells resulted in a decrease in the lactic acid production (31) and suggested that occurrence of the morphological and physiological changes due to stress conditions during the prolonged use of biocatalyst which may be due to stress responsive elements that are induced during such conditions. In addition, the observed variation in the productivity values may be associated with the produced lactic acid mediated influence on the metabolism as the increased product accumulation in the fermentation broth causes reduction of pH levels compared to initial similar to that noticed in the literature (32).

Substrate availability and its depletion pattern are most important aspects for optimized product production during fermentation process which influences the overall productivity yields. In fact under immobilization environment, substrate mass transfer play significant role on overall process. Analysis of substrate depletion and lactic acid production pattern during various cycles of immobilization based fermentation conditions indicated that higher utilization of sugars in case of NVLS compared to SS which may be one of the factors for observed changes in lactic acid production levels in the present study as noticed by immobilized *R. oryzae* RBU2-10 using 12% of hydrolyzed rice starch (33).

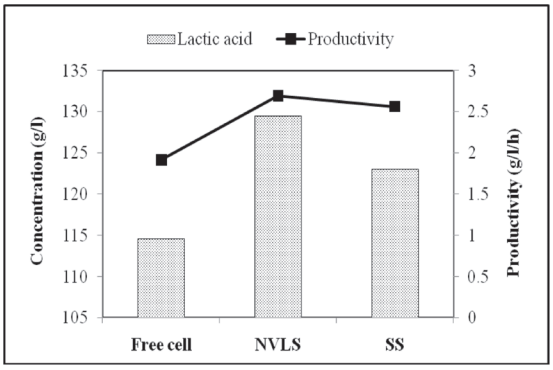

Fig. 10. Comparative study between free and immobilized cells (NVLS and SS)

Table 1. List of loofa sponge immobilized organisms used in the production of various industrially important products

Organism immobilised	Product	Reference
Thermomucor indicae-seudaticae	Glucoamylase	(18)
Aspergillus niger	Glucoamylase	(44)
Trichoderma reesi	Cellulose	(45)
Saccharomyces cerevesiae	Ethanol	(46)
Zymomonas mobilis	Sorbitol	(47)
Aspergillus niger	Glucose oxidase	(48)
Saccharomyces cerevisiae IR2 and *A. awamori IAM 2389*	Ethanol	(49)
Aspergillus niger	Polymethylgalacturonase	(50)

Loofa sponge as immobilization matrix was also evaluated after coating with sodium alginate in case of biosorption studies (36, 37) for blue green microalga like *Synechococcus sp.,* (38), bacteria like *Escherichia coli* and *Nocardia mediterranei* (39), basidiomycete like *Phanerochaete chrysosporium* (40), *Schizophyllum commune* (39), ascomycete like *Aspergillus niger,* phycomycete like *Rhizopus nigricans* and also used for plant cells (41, 42) and animal cells (43) due to its specific properties like stability at whole range of pH and resistance to any change in shape, texture and structure as a result of repeated autoclaving at high temperatures. In the present study too loofa sponge has been found to be a better immobilizing matrix than synthetic sponge, though cellulose-based sponges are used for beverage fermentations and bacterial immobilization methods (34,35). The immobilization capacity of loofa sponge was found to be comparable to that of polyurethane foam regardless of the cultivation time (33). Loofa sponge was also used as carrier for various microorganisms in the production of industrially important products as listed in the Table 1. Comparative analysis of process parameters obtained for free suspended cells and immobilized cells (NVLS and SS) is necessary to confirm the advantage of the latter and the prospects of immobilized biocatalyst application in practice. Lactic acid yield against substrate concentration in free cells, NVLS and SS was observed to be 76.4, 86.3 and 82.02 % with productivities 1.91, 2.69 and 2.56 g/l/h, respectively (Fig. 10) suggesting more than 40% improvement in productivity yields with immobilized cells. Therefore, adsorption or entrapment of immobilization process by low cost natural loofa sponge might be applied in large scale production.

Acknowledgements

We are thankful to UGC, New Delhi, for the financial assistance. We acknowledge Dr.M.Lakshman, RUSKA Labs, Rajendra Nagar, Hyderabad for genially executing SEM studies.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 755-762 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Elicitation of Forskolin in Suspension Cultures of *Coleus forskohlii* (Willd.) Briq. Using Elicitors of Fungal Origin

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Abstract

MS liquid medium supplemented with 3 mg/l NAA and 1 mg/l BAP with pH 5.7 was used for suspension cultures. Suspension cultures subjected to biotic elicitors *Aspergillus niger*, *Fusarium oxysporium*, *Rhizopus oryzae* and *Penicillium notatum* induced increase in forskolin content. Among all the elicitors tried *Aspergillus niger* proved to be best elicitor at 5% CF which was 6 times higher than the control.

Keywords: Callus cultures, Secondary metabolites, *Coleus forskohlii*, forskolin

Introduction

Elicitor induced accumulation of secondary metabolites received wide acceptance because of its ability to improve the productivity of the plant cell systems significantly. The effects of fungal elicitors on the growth and forskolin content were studied in the suspension cultures of *Coleus forskohlii*. *In vitro* production of bioactive forskolin with increased focus towards yield enhancement can provide new means of obtaining large quantity of forskolin in shorter period of time. In the present study an attempt to enhance the yield of forskolin in *in vitro* using fungal elicitors is carried out.

Materials and Methods

Different explants from *in vitro* grown plantlets such as root, leaf, petiole, internode and node were excised, surface sterilized and inoculated on MS medium fortified with 2 mg/l 2,4-D. pH of the media was adjusted to 5.7-5.8 prior to the addition of agar. The media was autoclaved at 15 psi, 121°C for 20 minutes. The cultures were incubated at 25±2°C temperature under dark for callus proliferation.

Initiation and maintenance of suspension cultures: MS liquid medium supplemented with 3 mg/l NAA and 1 mg/l BAP with pH 5.7 was used for suspension cultures. About 50 ml of liquid medium was dispensed into 250 ml Erlenmeyer flasks and friable callus weighing approximately 5 gms was transferred to the flasks under aseptic conditions. Cultures were incubated on a rotary shaker at 120 rpm in darkness and temperature was maintained at 25 ± 2°C. After 15 days of incubation cell suspensions were filtered through appropriated sterile sieves to obtain single cells and a few celled aggregates which were used as inoculums for subculture. Fine cell suspensions were obtained by repeated subculturing of callus and removing small clumps at every stage. The cell suspension cultures were maintained on the same medium by subculturing at 2-week interval. *Aspergillus niger, Rhizopus oryzae, Fusarium oxysporum* and *Penicillium notatum* cultures were procured from Padmashree Institute of Management Studies, Bangalore.

Elicitor preparation: Potatoes (200g) were cut into slices and boiled with 200 ml of distilled water and then filtered through muslin cloth. Twenty grams of dextrose was added to the filtrate and the volume was made up to 1000 ml. The pH of the medium was adjusted to 6.5. Into each

250 ml conical flask 50 ml of the media was dispensed and autoclaved. Loop full of fungal inoculum grown on solid medium was inoculated into the medium. The flasks were incubated and allowed to grow. After 20 days, during sporulation flasks with the cultures were autoclaved. The fungal mat was separated by filtration and then washed several times with distilled water. Aqueous extract was made by homogenizing the mat in a mortar and pestle using acid washed neutralized sand. The homogenized mat was filtered through muslin cloth and the volume was made equal to that of spent medium. The mat extracts and filtrates were used as elicitors.

Four concentrations of cell filtrate (at 5, 10, 15 and 20 per cent v /v) and four concentrations of cell extract (5, 10, 15 and 20 per cent v /v) of all fungi added to the established cultures to study their effect on active principle production. Control cultures were supplemented with volumes and concentrations of the microbial culture medium equivalent to that of elicitor.

Results and Discussion

Influence of Aspergillus niger: Most of the strategies employing fungal elicitors utilize fairly undefined mixtures such as autoclaved fungal homogenate (1) or fungal culture filtrates (2, 3). It can be seen that each elicitor had an optimum at a particular concentration and the extent of elicitation also varied. Different concentrations of *Aspergillus niger* elicitor were added to suspension cultures of *Coleus forskohlii* at day 6 of the cultivation period, which was determined to be the optimal time for elicitation of forskolin accumulation. Elicitor concentrations were varied between 5 and 20%. After addition of elicitor to cell cultures the cells were harvested and assayed for dry weight and forskolin content after 24 and 48 hours. The addition of 5% MF was found to result in forskolin levels 1178 ± 6.1 mg/kg DW in the cells which were comparable to the control 220 ± 5.1 mg/kg DW at 24 hours after addition of elicitor (Table 1). ME at 5% also resulted in forskolin content 315.3 ± 5.2 mg/kg DW which was comparable to the control. The other levels of the elicitor tried had resulted in low levels of forskolin. Addition of ME and MF of *A. niger* to the cell suspension cultures of *Coleus forskohlii* resulted in a reduction in the biomass accumulation which was dependent on the concentration of the elicitor added with higher concentrations resulting in lower biomass accumulation and forskolin production. The data subjected for statistical F-test indicate a significant difference at 5% level in response to different concentrations of mat extract and filtrate of *Aspergillus niger* in terms of forskolin content at 24 hours (F=1302*) and 48 hours (F=66*) The elicitor concentration plays a very important role in elicitation process and this was reported in several plant cell cultures. Namdeo *et al*. (4) reported higher accumulation of ajmalicine in *Catharanthus roseus* when treated with different concentrations of elicitor extracts of *Trichoderma viride*, *Aspergillus niger* and *Fusarium moniliforme*. Ajmalicine accumulation was higher in cells elicited with higher concentration (5.0%) of mat extracts as compared to lower concentration (0.5%). However, increasing the concentration further up to 10% adversely affected the accumulation of ajmalicine. These results are also supported by the findings of Nef-Campa et al. (5) and Rijhwani and Shanks (6). High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction (7, 8. 9). About 3-fold increase in ajmalicine production by *Catharanthus roseus* cells elicited with extracts of *Trichoderma viride* for 48 hours, whereas, about 2-fold increase was observed in cells elicited with *Aspergillus niger* and *Fusarium moniliforme* (4, 10). However further increasing exposure time resulted in decrease in ajmalicine content. Similar results were reported by Rijhwani and Shanks (6), Moreno et al. (11) and Negeral and Javelle (12). When the excretion of forskolin to the medium was evaluated, no response could be observed with any of the strains employed in this work. This fact contrasts markedly with the observations of Nef *et al.,* (13) reported that low concentrations of the fungal homogenates induced the liberation

of 90% of the total ajmalicine in to the medium, while serpentine accumulated preferentially in the tissue. The comparative decrease in the levels of forskolin treated with a relatively high concentration of the elicitor could be due to the toxicity of the elicitor to the cells, which was observed as necrotic browning.

Influence of Fusarium oxysporium: The effect of *Fusarium oxysporium* as an elicitor was tested by adding the ME and MF at concentrations of 5, 10, 5 and 20% to the suspension culture (Table 2). Forskolin contents were analysed after 24 and 48 hours of incubation and compared with control. The data subjected to statistical analysis indicate a significant difference at 5% level with response to different concentrations of elicitor in terms of forskolin content at 24 hours (F=915*) and 48 hours (F=3373*). This shows that forskolin levels increased for several hours after addition of elicitor as also monitored for cell cultures of *Lithospermum erythrorhizon* (14). The incubation time of the elicitor can be an important factor. A minimal time is required to induce the process of elevated secondary metabolite production. An exposure that lasts too long could have harmful effects on growth and production of secondary metabolites. The accumulation of secondary metabolites depends on the dose of elicitor employed and on the type of this strain has been reported in the other systems (8, 15, 16, 17, 18). The response of plant cells and tissues to elicitation with fungal homogenates is in direct relation with the composition of the cell wall of the fungi, where their different components are the true elicitors. Some types of cellulose–glucans are better elicitors than chitin-glucan (15). For the present work, the oligosacchrides liberated from the cell wall of *Aspergillus* were the best elicitors, even though their identity is still unknown. Six

Table 1. Effect of *Aspergillus niger* on elicitation of forskolin production in suspension cultures of *Coleus forskohlii*

Elicitor	Concentration (%)	Forskolin content (mg/kg DCW)	
		24 hours Mean ± SD	48 hours Mean ± SD
Control	5	220.1 ± 5.1	92.2 ± 2.1
	10	150.2 ± 4.1	72.4 ± 2.1
	15	120.3 ± 2.2	66.2 ± 3.9
	20	96.1 ± 3.1	45.2 ± 3.0
Cell Extract	5	315.3 ± 5.2	50.3 ± 2.2
	10	79.3 ± 3.1	53.3 ± 2.2
	15	59.3 ± 2.3	55.6 ± 4.2
	20	40.1 ± 1.0	58.3 ± 2.3
Cell Filtrate	5	1178.5 ± 6.1	50.2 ± 2.1
	10	138.1 ± 4.0	61.1 ± 4.1
	15	95.1 ± 2.1	73.2 ± 2.1
	20	60.2 ± 3.2	80.2 ± 4.2
	F-Value	1302*	66*
	SE	2.183	1.732
	CD at 5 %	6.372	5.056

* Significant at 5 % Level, Number of Replication=3

Table 2. Effect of *Fusarium oxysporium* on elicitation of forskolin production in suspension cultures of *Coleus forskohlii*

Elicitor	Concentration (%)	Forskolin content (mg/kg DCW)	
		24 hours Mean ± SD	48 hours Mean ± SD
Control	5	220.1 ± 5.1	92.2 ± 2.1
	10	150.2 ± 4.1	72.4 ± 2.1
	15	120.3 ± 2.2	66.2 ± 3.9
	20	96.1 ± 3.1	45.2 ± 3.0
Cell Extract	5	107.4 ± 3.3	177.3 4.1
	10	48.2 ± 2.1	272.3 ± 4.3
	15	46.2 ± 2.1	350.3 ± 5.4
	20	44.4 ± 2.0	500.3 ± 4.9
Cell Filtrate	5	208.3 ± 4.4	117.3 ± 3.1
	10	185.3 ± 5.0	122.3 ± 4.3
	15	150.2 ± 5.1	144.3 ± 4.2
	20	70.4 ± 2.4	170.3 ± 5.2
	F-Value	915*	3373*
	SE	2.082	2.317
	CD at 5 %	6.077	6.673

* Significant at 5 % Level, Number of Replication=3

Fusarium oxysporium strains were tested for their influence on the production of thiophenes by hairy roots of *Tagetes patula*, the optimum concentration was 1% extract and the yield was 55% more compared to the control (19). Mycelia of *Fusarium oxysporium* added on the ninth day of growth of *Dioscorea deltoidea* cell cultures decreased dry weight and increased total amount of diosgenin from 10 to 72% compared to the controls (20). Combining the use of fungal elicitor like *Fusarium* with immobilized cell suspension cultures of *Solanum surattense* showed stimulation in solasodine production, of about seven times as compared to cell suspensions (21). Elicitation of banana plantlets with *Fusarium oxysporium* elicitor led to increase in phenolic acid synthesis by 7-fold, which probably suppressed the pathogen invasion (22).

Influence of Rhizopus oryzae: Different concentrations of ME and MF were tested to study the effect of *Rhizopus oryzae* on elicitation of forskolin content in suspension cultures of *Coleus forskohlii* (Table 3). The ME elicitor (w/v) extracted from *Rhizopus oryzae* at a level of 15% exhibited 72% increase in the forskolin production after 24 hours of elicitation over control cultures of *Coleus forskohlii*. The data subjected to statistical F-test indicated a significant difference at 5% level in cultures treated with various concentrations of *Rhizopus oryzae* in terms of forskolin content at 24 hours (F=1298*) and 48 hours (F=272*). Treatment with *Rhizopus oryzae* derived elicitor at a level of 1.0% (v/v), exhibited more than 3-fold increase in the plumbagin production (15.18 mg/gm DCW) in suspension cultures of *Plumbago rosea* (23). Rokem *et al*. (20) reported 72% increased diosgenin production when the cell

Table 3. Effect of *Rhizopus oryzae* on elicitation of forskolin production in suspension cultures of *Coleus forskohlii*

Elicitor	Concentration (%)	Forskolin content (mg/kg DCW)	
		24 hours Mean ± SD	48 hours Mean ± SD
Cell Extract	5	259.5 ± 5.6	114.7 4.6
	10	228.3 ± 3.3	84.3 ± 3.1
	15	180.2 ± 4.2	70.3 ± 3.2
	20	170.2 ± 4.2	60.4 ± 3.2
Cell Filtrate	5	58.3 ± 3.3	159.4 4.3
	10	39.2 ± 2.3	130.2 ± 5.1
	15	40.1 ± 3.0	80.3 ± 2.2
	20	49.2 ± 4.3	63.3 ± 3.1
Control	5	220.1 ± 5.1	92.2 ± 2.1
	10	150.2 ± 4.1	72.4 ± 2.1
	15	120.3 ± 2.2	66.2 ± 3.9
	20	96.1 ± 3.1	45.2 ± 3.0
	F-Value	1298*	272*
	SE	2.183	1.992
	CD at 5 %	6.372	5.815

* Significant at 5 % Level, Number of Replication=3

suspension cultures of *Dioscorea deltoidea* were elicited with *Rhizopus* sp. in comparison with control cultures without elicitor. Lee and West (24) have shown that homogeneous α-1, 4-endopolygalacturonase from culture filtrates of the fungus *Rhizopus stolonifer* elicits casbene synthetase activity in castor bean (*Ricinus communis* L.). There was no effect on biomass production when treated with *Rhizopus oryzae*. *Rhizopus* sp. affected the levels of taxol to 5.8 times when compared to control cultures however there is decrease in cell growth in cell cultures of *Taxus yunnanensis* (25).

Influence of Penicillium notatum: The elicitor extracted from *Penicillium* sp. brought about a 4-fold increase in salidroside yield over that in the control condition in cell suspension cultures of *Rhodiola sachalinensis* (26). The effects of *Penicillium notatum* as elicitor at concentrations, 5, 10, 15 and 20% and exposure times on forskolin production were studied. A higher concentration of elicitor filtrate (15%) responded positively in terms of forskolin accumulation which was increased by 3-fold after 48 hours of incubation. The concentrations higher than this resulted in decreased levels of forskolin and also biomass production. The data subjected for statistical F-Test indicate a significant difference at 5% level in cultures treated with various concentrations of *Penicillium notatum* in terms of forskolin content at 24 hours (F=3395*) and 48 hours (F=8214*) (Table 4.) In a similar study crude extract, obtained by autoclaving the fragment suspensions of mycelia from *Penicillium* sp. dramatically increased the taxol accumulation in the cell culture of *Taxus yunnanensis* (25). In contrast to this result *Penicillium notatum* was not effective on enhancement of anthocyanin

Table 4. Effect of *Penicillium notatum* on elicitation of forskolin production in suspension cultures of *Coleus forskohlii*

Elicitor	Concentration (%)	Forskolin content (mg/kg DCW)	
		24 hours Mean ± SD	48 hours Mean ± SD
Cell Extract	5	47.3 ± 2.1	35.10 2.0
	10	74.4 ± 2.2	81.3 ± 3.2
	15	200.3 ± 5.3	150.3 ± 5.3
	20	450.5 ± 5.2	300.3 ± 4.2
Cell Filtrade	5	64.3 ± 2.4	42.3 ± 2.2
	10	60.3 ± 2.2	412.4 ± 4.3
	15	58.3 ± 2.3	620.3 ± 5.3
	20	49.1 ± 2.4	500.4 ± 6.3
Control	5	220.1 ± 5.1	92.2 ± 2.1
	10	150.2 ± 4.1	72.4 ± 2.1
	15	120.3 ± 2.2	66.2 ± 3.9
	20	96.1 ± 3.1	45.2 ± 3.0
	F-Value	3395*	8214*
	SE	1.992	2.236
	CD at 5 %	5.815	6.527

* Significant at 5 % Level, Number of Replication=3

production in callus cultures of *Daucus carota* (27). This clearly indicates that each species exhibits specific response to the elicitor type and concentration.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 763-772 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Optimization and Production of Fibrinolytic Protease (GD kinase) from Different Agro Industrial Wastes in Solid State Fermentation

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Abstract

The cost of fibrinolytic protease (GD kinase) production may be significantly decreased by using inexpensive carbon substrates like agricultural residues. The optimization of different physical parameters such as various substrates, initial moisture level, pH, substrate particle size, incubation temperature, inoculum size, incubation period and chemical parameters such as additional carbon and nitrogen sources were studied for the production of fibrinolytic protease (GD kinase) in solid-state fermentation using mutant *Bacillus cereus* GD55. The maximum fibrinolytic protease (GD kinase) production was observed with apple pomace (52.20 ± 0.21 U/g), peptone (50.87 ± 0.21 U/g), NH_4NO_3 (50.19 ± 0.65 U/g), incubation period 4 Days (48.32 ± 0.24 U/g), inoculum size 2.0% (46.24 ± 0.98 U/g), incubation temperature 35°C (40.85 ± 0.19 U/g), substrate particle size 1.2 mm (40.15 ± 0.17 U/g), initial pH 8 (38.39 ± 0.36 U/g), initial moisture level 70% (34.28 ± 0.10U/g), carbon source fructose (46.64 ± 0.13 U/g) and least fibrinolytic protease (GD kinase) production was observed with rice chaff (12.8± 0.12 U/g) in the production medium.

Keywords: Solid-state fermentation, *Bacillus cereus* GD55, Optimization, fibrinolytic protease (GD kinase).

Introduction

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation (1). The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (2). Of these, strains of *Bacillus sp*. dominate the industrial sector (3). Fibrinolytic enzyme is well known as a sub class of protease, which has an ability to degrade fibrin (4-8). Blood clots (fibrin) are formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator (9). Deposition of fibrin in blood vessels normally increases thrombosis, resulting in myocardial infarction and other cardiovascular diseases (10-11).

Many microorganisms *viz., Pseudomonas, Staphylococcus, Alteromonas, Coryneform bacteria, Penicillium, Asperigillus, Fusarium, Trichotecium, Actinomyces, Streptomyces, Escherichia coli* and *Bacillus* (12) have been evaluated for the production of fibrinolytic enzymes. However, high cost and low yields of fibrinolytic enzymes have been the main problems for its industrial production. Therefore, there is a

great need to develop a new fermentation medium with inexpensive substrates that provides a high fibrinolytic enzymes yield. It is well known that 30-40% of the production cost of fibrinolytic enzymes is taken up by the cost of growth medium (13). Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture. Among existing technologies in the fermentation industry, solid-state fermentation (SSF) shows many advantages over fermentation with submerged culture, such as lower cost and much higher reactor volume. The application of SSF process has a considerable economical potential in the food, feed, pharmaceutical and agricultural industries (13-14). There are a great number of literatures reported to use the SSF process for producing fibrinolytic enzymes with industrial importance such as nattokinase, streptokinase and urokinase (15). However, it has not been reported using the SSF for production of fibrinolytic protease (GD kinase) using apple pomace and cotton seed meal. India has the largest production of apple, cotton, soybean and rice in the world. Millions of tons of apple pomace, cotton seed meal, rice chaff and soybean meal are also produced each year. In 2011, the yields of apple pomace and cotton seed meal were about 1 and 6 million tons, respectively (16-17). These relatively low-priced agro industrial residues, containing abundant nutrients (hemicellulose, cellulose, proteins and starch), have a great potential to be utilized as alternative fermentation substrates. Therefore, in this article, apple pomace, cotton seed meal, soy bean meal and rice chaff were selected and used as basic carbon and nitrogen sources for production of fibrinolytic protease (GD kinase). In the present study, the productivity of fibrinolytic protease (GD kinase) by mutant *Bacillus* cereus GD55 using solid agro-industrial residues such as apple pomace, cotton seed meal, soy bean meal and rice chaff was evaluated. In addition, the culture conditions as initial moisture content, initial pH, inoculum size, incubation temperature, and substrate particle size as well as the extra supplementation of carbon and nitrogen sources were optimized to maximize the fibrinolytic protease (GD kinase) yield.

Materials and Methods

Microorganism and inoculum preparation: The bacterial strain of *Bacillus cereus* GD55 isolated from soils of various regions in Bangalore, India was mutated by UV irradiation (18), Ethyl methyl sulphonate (EMS) and Ethedium bromide (EtBr) (19) and was identified at Institute of Microbial Technology (IMTECH) Chandigarh, India. Stock cultures were maintained in nutrient broth medium with 70% glycerol; cultures were preserved at -20^{o}C (20). The inoculum was prepared by transferring a loopful of stock culture (*Bacillus cereus* GD55) to a certain volume of sterile nutrient broth stock medium, and then incubated it overnight at 37^{o}C in a rotary shaker with 200 rpm, before being used for inoculation (21). A stock suspension was prepared and adjusted to 7×10^3 cells ml^{-1}.

Substrates: Apple pomace was obtained from a local apple juice concentrate company in Bangalore, India. It was dried in an oven at 60^{o}C and ground in a hammer mill. The ground material was passed through 30- and 50-mesh sieves. The fraction which passed through the 30-mesh sieve but retained by the 50-mesh sieve was collected and used as basic fermentation media. The cottonseed meal was obtained from a market at Bangalore, India. The meal was made after cotton seed oil extraction using a compression method and was pre-treated in the same way as the apple pomace. Soy bean meal and rice chaff obtained from local market Bangalore, India was pre treated as same as for the apple pomace.

Solid state fermentation: Ten grams of solid substrate, in a 250 ml Erlenmeyer flask, were moistened with mineral salt solution (g l^{-1}: Ferrous sulfate 1; magnesium chloride 1; manganese sulfate 1; pH 7.0), thoroughly mixed and autoclaved at 121^{o}C for 30 min. The cooled medium was inoculated with 48 h old inoculum (2.0×10^6CFU/g initial dry substrate) and incubated at 30^{o}C for 4 days. The moisture content of the medium after inoculation was 50%.

Unless otherwise specified, these fermentation conditions were maintained throughout the experiment.

Measurement of pH and Moisture Content: The pH was determined using 1.0 g of fermented material in 10 ml of distilled water, and then the mixture was agitated. After 10 min, the pH was measured in the supernatant using a pH meter. The moisture content of the medium was estimated by drying 5 g of the wet sample to a constant weight at 105°C and the dry weight was recorded (22).

Fibrinolytic Protease (GD kinase) Extraction: At the end of fermentation, the harvested biomass was soaked in 50 ml distilled water and agitated thoroughly in a rotary shaker at 130 rpm at 30^{o}C for 1 h. The whole contents were filtered through sterile muslin cloth, and residues were again treated with another aliquot of 50 ml of distilled water as previously and subsequently filtered. The filtrates were pooled then centrifuged, and the final clear supernatant was used as the fibrinolytic protease (GD kinase) source (23)

Fibrinolytic protease (GD kinase) assay: Fibrinolytic protease (GD kinase) activity was carried out according to the method described by Greenberg 1957(24). The reaction mixture contained 8 mg bovine fibrin, 500ìl enzyme in Phosphate buffer (0.05mM, pH 7.8) in a total volume of 1ml. This mixture was incubated for 30 min at 37^{o}C in a water bath. The reaction was stopped by adding 0.5ml of 15% cold tri chloroacetic acid (TCA). The mixture was centrifuged at 3,000 rpm for 10 min to remove precipitated fibrin. To 0.5ml of acid soluble filtrate 2.5ml of 0.3 N sodium hydroxide and 2.9% (w/v) sodium carbonate was added, followed by 0.75ml of Folin's phenol reagent. The mixture was incubated for 25 min at room temperature and the color developed was read at 650 nm (24). The above said procedure was followed with heat killed enzyme kept as blank. One unit of enzyme activity was calculated as the amount of enzyme which releases 1ìg of tyrosine per minute under the specified reaction conditions.

Optimization of the culture condition for Fibrinolytic Protease (GD kinase) Production: The different physicochemical parameters to maximize the yield of fibrinolytic protease (GD kinase) by *Bacillus cereus* GD55 under solid state fermentation were investigated. The optimized parameter was incorporated at its optimized level in the subsequent optimization experiments. The impact of initial moisture content (20-90%), initial pH (3-11, adjusted with 1N HCl or 1N NaOH), incubation temperature (20- 40°C), incubation period (2-12 days), particle size and size of inoculum on fibrinolytic protease (GD kinase) production using solid state fermentation of *Bacillus cereus* GD55 was evaluated. Moreover, the effect of incorporation of additional carbon sources (glucose, sucrose, fructose, maltose, arabinose, starch and mannose at 1%w/v), additional nitrogenous compounds (KNO_3, NH_4Cl, $(NH_4)_2HPO_4$, NH_4NO_3, $(NH_4)_2SO_4$, casein, yeast extract, peptone, serine, histidine and aspartate at 1% w/v), to the production medium were studied. All the experiments were conducted in triplicate and the mean values are considered. After incubation of each fermentation sample, the crude extract was prepared.

Results and Discussion

Evaluation of different agro-industrial material for fibrinolytic protease production: The fermentation profile of fibrinolytic protease (GD kinase) production in SSF varied with the type of agro material used. Highest fibrinolytic protease (GD kinase) production (52.20 ± 0.21U/g substrate) was observed with apple pomace and the least fibrinolytic protease (GD kinase) production (12.8± 0.12 U/g substrate) was observed with rice chaff (Table 1 and Fig. 1). A 4-fold variation was noticed with these materials. This could be attributed to solid materials dual role-supply of nutrients and anchorage to the growing microbial culture which influence the microbial growth and subsequent metabolite production. Such substrate dependent microbial product yield variations were also reported in literature. These results depict that the selection of an ideal agro biotech source for fibrinolytic

protease (GD kinase) production depends primarily on the availability of carbon and nitrogen source and thus screening of several agro-industrial residues is essential. Gopinath et al 2011 (45) working with *Penicillium chrysogenum* SGAD12, reported that rice chaff is better solid support material for the production of fibrinolytic enzyme under SSF. However, in the present study, among all studied materials, rice chaff supported least production of fibrinolytic protease (GD kinase). This may be attributed to the fact that the strains used by them may vary in their metabolic pattern compared to *Bacillus cereus* GD55 used in the present study or carbon source material associated with rice chaff may not be utilized by the *Bacillus cereus* GD55. To evaluate the same, the hemicellulose and cellulose hydrolysis ability of the strain was investigated.

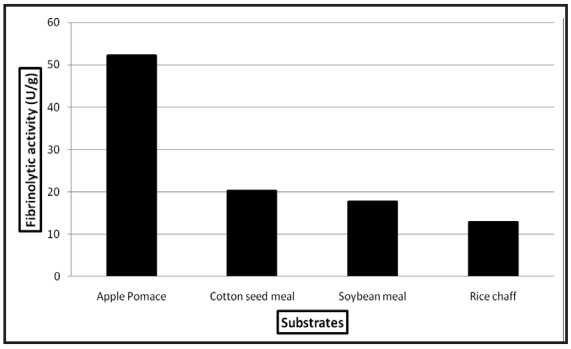


Fig.1. Effect of various substrates on fibrinolytic protease (GD kinase) production by Bacillus cereus GD55 under SSF

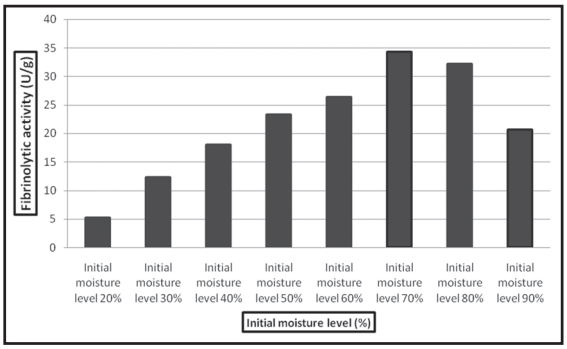


Fig.2. Effect of various moisture level (%) on fibrinolytic protease (GD kinase) production by Bacillus cereus GD55 under SSF

This data further confirmed that high fibrinolytic protease (GD kinase) associated with apple pomace were due to the maximum production of hemicellulose and cellulose hydrolyzing enzyme by the strain and as strain *Bacillus cereus* GD55 is hemicellulase positive hence could utilize hemicellulose as carbon source. Hence, it could be concluded that the selected strain requires substrates that provide hemicellulose as its enzymatic machinery that hydrolyzes the polysaccharides present in substrates.

Effect of moisture level: The moisture level in the solid-state fermentation critically affects the process due to its interference in the physical properties of the solid particle. Increased moisture is believed to reduce the porosity of substrate, thus limiting the oxygen transfer. The decreased moisture content cause lower availability of media nutrients to the *Bacillus cereus* GD55 resulting into lower extent of production. The effect of total moisture content on fibrinolytic protease (GD kinase) production for 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90% moisture was investigated. The result indicated that 70% moisture gave the higher fibrinolytic protease (GD kinase) production during fermentation compared to other treatments. The maximum yield of fibrinolytic protease (GD kinase) production (34.28 ± 0.10 U/g substrate) was obtained from 70% moisture (Table 2 and Fig. 2). The results from the previous study stated that the ideal moisture content was 70% and the reduction in enzyme yield could occur with low and to higher moisture level.

Effect of initial pH of the medium on fibrinolytic protease Production: The initial pH of the fermentation media may change during fermentation because the substrates employed in SSF usually have the least buffering. Some samples from the fermented mass were aseptically withdrawn, homogenized and pH was checked. The pH of the medium during fermentation was found to be between 3.0 and 11.0, i.e. around acidic to alkaline condition. The initial pH is another important factor which affects the growth and enzyme production during solid-

state fermentation. Substrate was adjusted to different initial pH using 1N HCl and 1N NaOH prior to inoculation. The maximum yield of fibrinolytic protease (GD kinase) production

Table 1. Effect of various substrates on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

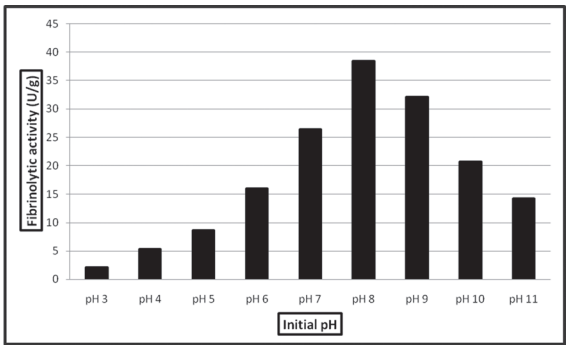
Substrate	Fibrinolytic activity(U/g)
Apple Pomace	**52.20 ± 0.21**
Cotton seed meal	20.2 ± 0.15
Soybean meal	17.69 ± 0.62
Rice chaff	12.8± 0.12

Table 2. Effect of various moisture level (%) on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Initial moisture level (%)	Fibrinolytic activity (U/g)
Initial moisture level 20%	5.20 ± 0.10
Initial moisture level 30%	12.3 ± 0.13
Initial moisture level 40%	17.98 ± 0.12
Initial moisture level 50%	23.26 ± 0.15
Initial moisture level 60%	26.36 ± 0.29
Initial moisture level 70%	**34.28 ± 0.10**
Initial moisture level 80%	32.16 ± 0.62
Initial moisture level 90%	20.68 ± 0.18

Table 3. Effect of various initial pH on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Initial pH	Fibrinolytic activity (U/g)
pH 3	2.10 ± 0.22
pH 4	5.30 ± 0.20
pH 5	8.68 ± 0.27
pH 6	15.98 ± 0.20
pH 7	26.36 ± 0.17
pH 8	**38.39 ± 0.36**
pH 9	32.10 ± 0.62
pH 10	20.68 ± 0.12
pH 11	14.22 ± 0.42



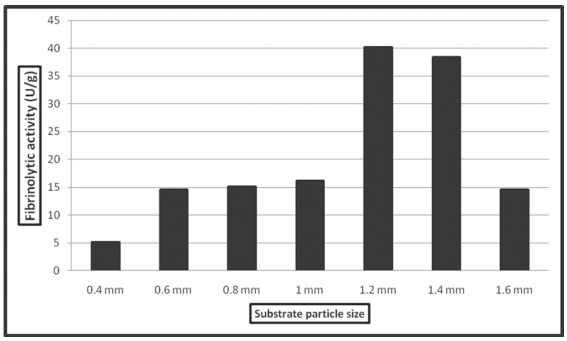


Fig.4. Effect of various substrate particle size on fibrinolytic protease production by Bacillus cereus GD55 under SSF

(38.39 ± 0.36 U/g substrate) was observed at pH 8.0 (Table 3 and Fig. 3).

Effect of substrate particle size: In solid-state fermentation process, the availability of surface area play a vital role for microbial attachment, mass transfer of various nutrients and substrates and subsequent growth of microbial strain and product production. The availability of surface area in turn depends on the particle size of the substrate or support matrix. The experimental data revealed that fibrinolytic protease (GD kinase) production was affected by the particle size. Maximum fibrinolytic protease (GD kinase) production (40.15 ± 0.17 U/g substrate) was noticed with 1.2 mm substrate particle size (Table 4 and Fig. 4). Altering the substrate particle size in either side of this resulted in reduction of

fibrinolytic protease (GD kinase) production. The observed reduction of fibrinolytic protease (GD kinase) production with altered particle size could be attributed to intra-particulate associated aeration, available surface area for microbial attachment and substrate mass transfer and subsequent growth and enzyme production.

Effect of temperature: The maintenance of an optimal process temperature is one of the major factors in the economics of a process. Temperature affects microbial cellular growth, spore formation, germination and microbial physiology, thus affecting product formation in turn. 35°C was found to be the optimum temperature in this case. The maximum yield of fibrinolytic protease (GD kinase) production (40.85 ± 0.19 U/g substrate) was observed at 35°C (Table 5 and Fig. 5).

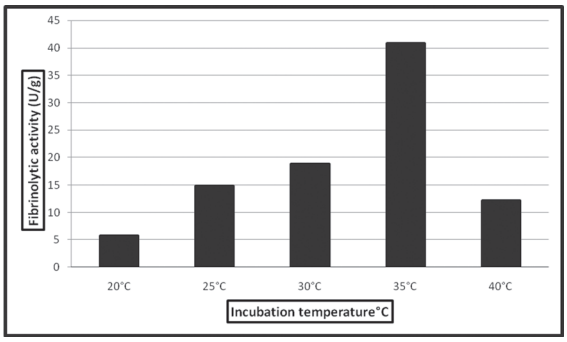


Fig.5. Effect of various incubation temperatures on fibrinolytic protease production by Bacillus cereus GD55 under SSF

Effect of inoculum size: The optimum inoculum size for fibrinolytic protease (GD kinase) production (46.24 ± 0.98 U/g substrate) by *Bacillus cereus* GD55 was 2×10^6 CFU/g initial dry substrate (Table 6 and Fig. 6). Adequate inoculum can initiate fast growth and product formation, thereby reducing the growth of contaminants. A decrease in enzyme production was observed when the inoculums size was increased beyond the optimum level. Enzyme production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased fibrinolytic protease (GD kinase) synthesis.

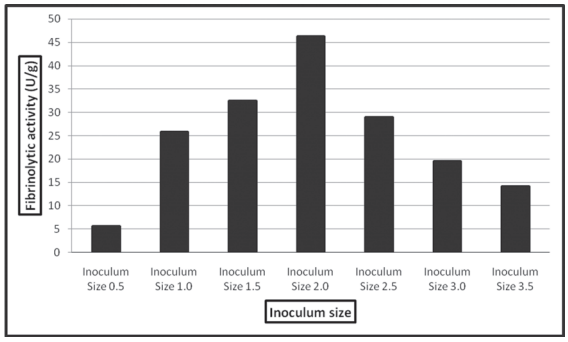


Fig.6. Effect of various inoculum size (×106) on fibrinolytic protease production by Bacillus cereus GD55 under SSF

Effect of incubation period: Solid-state process was performed for various incubation periods. Remarkably higher levels of fibrinolytic protease (GD kinase) production were observed following 2–4 days of the process and maximal levels (48.32 ± 0.24U/g substrate) was achieved at the 4^{th} day of fermentation (Table 7 and Fig. 7). Important ascend in fibrinolytic protease (GD kinase) yield with increased biomass was observed during 2^{nd}–6^{th} days of fermentation cycle. Significant variation in fibrinolytic protease (GD kinase) production was observed during different fermentation periods.

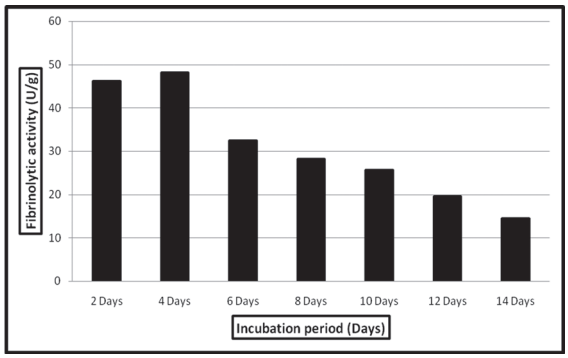


Fig.7. Effect of various incubation periods on fibrinolytic protease production by Bacillus cereus GD55 under SSF.

Table 4. Effect of various substrate particle size on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Substrate particle size	Fibrinolytic activity (U/g)
0.4 mm	05.18 ± 0.29
0.6 mm	14.62 ± 0.24
0.8 mm	15.18 ± 0.21
1.0 mm	16.24 ± 0.23
1.2 mm	40.15 ± 0.17
1.4 mm	38.39 ± 0.41
1.6 mm	14.60 ± 0.23

Table 5. Effect of various incubation temperatures on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Incubation temperature (^{o}C)	Fibrinolytic activity (U/g)
20°C	05.82 ± 0.26
25°C	14.91 ± 0.13
30°C	18.84 ± 0.18
35°C	40.85 ± 0.19

Table 6. Effect of various inoculum size ($\times 10^6$) on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Inoculum Size (%)	Fibrinolytic activity (U/g)
Inoculum Size 0.5	05.64 ± 0.12
Inoculum Size 1.0	25.80 ± 0.56
Inoculum Size 1.5	32.40 ± 0.11
Inoculum Size 2.0	46.24 ± 0.98
Inoculum Size 2.5	28.96 ± 0.12
Inoculum Size 3.0	19.58 ± 0.16
Inoculum Size 3.5	14.18 ± 0.52

Effect of nitrogen source on fibrinolytic protease (GD kinase) production: Generally, the high concentration of nitrogen sources in media is effective in enhancing the production of fibrinolytic protease (GD kinase) by *Bacillus cereus* GD55. The protein content in apple pomace is very low so that the nitrogen levels as well as the commercial value all decrease greatly.

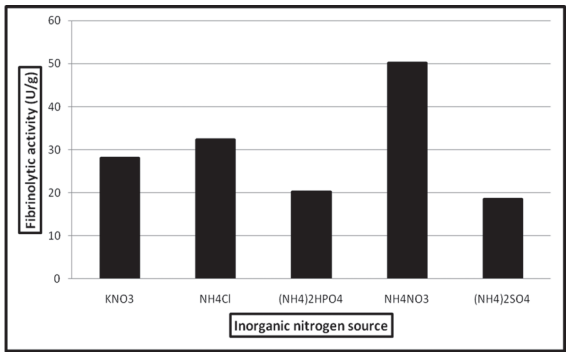


Fig.8. Effect of various inorganic nitrogen sources (1%w/v) on fibrinolytic protease production by Bacillus cereus GD55 under SSF

Table 7. Effect of various incubation periods on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Incubation period (Days)	Fibrinolytic activity (U/g)
2 Days	46.20 ± 0.29
4 Days	48.32 ± 0.24
6 Days	32.40 ± 0.41
8 Days	28.16 ± 0.85
10 Days	25.68 ± 0.26
12 Days	19.58 ± 0.28
14 Days	14.42 ± 0.24

Table 8. Effect of various inorganic nitrogen sources (1%w/v) on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Inorganic nitrogen source (1%)	Fibrinolytic activity (U/g)
KNO3	28.10 ± 0.22
NH4Cl	32.36 ± 0.14
(NH4)2HPO4	20.17 ± 0.41
NH4NO3	50.19 ± 0.65
(NH4)2SO4	18.58 ± 0.26

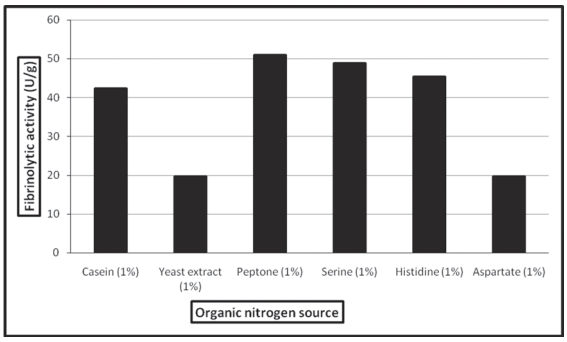


Fig.9. Effect of various organic sources (1%w/v) on fibrinolytic protease production by Bacillus cereus GD55 under SSF

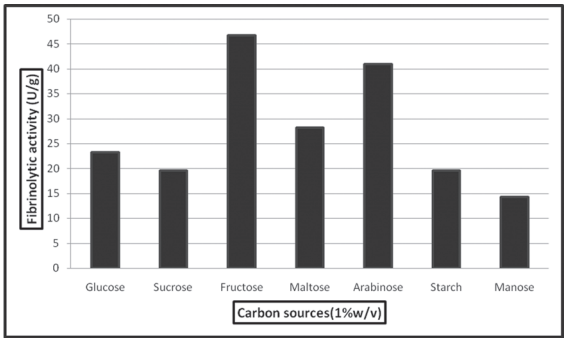


Fig.10. Effect of various carbon sources (1%w/v) on fibrinolytic protease production by Bacillus cereus GD55 under SSF

Table 9. Effect of various organic sources (1%w/v) on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Organic nitrogen source (1%)	Fibrinolytic activity (U/g)
Casein (1%)	42.32 ± 0.16
Yeast extract (1%)	19.58 ± 0.41
Peptone (1%)	50.87 ± 0.21
Serine (1%)	48.82 ± 0.45
Histidine (1%)	45.38 ± 0.28
Aspartate (1%)	19.58 ± 0.31

Table 10. Effect of various carbon sources (1%w/v) on fibrinolytic protease production by *Bacillus cereus* GD55 under SSF

Carbon sources (1%w/v)	Fibrinolytic activity (U/g)
Glucose	23.26 ± 0.10
Sucrose	19.58 ± 0.56
Fructose	46.64 ± 0.13
Maltose	28.14 ± 0.78
Arabinose	40.96 ± 0.16
Starch	19.58 ± 0.36
Manose	14.28 ± 0.62

Hence, the exogenous addition of various nitrogen levels to the solid medium was studied. KNO_3, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4NO_3 as inorganic and casein, yeast extract, peptone, serine, histidine and aspartate was used as complex organic nitrogen source. Based on the results it was found that peptone was the best organic nitrogen source (50.87 ± 0.21 U/g substrate) and NH_4NO_3 was the best inorganic nitrogen source (50.19 ± 0.65 U/g substrate) its supplementation led to further increase in fibrinolytic protease (GD kinase) production (Table. 8,9 and Fig. 8, 9).

Effect of carbon source on fibrinolytic protease (GD kinase) production: Although apple pomace can support the growth of *Bacillus cereus* GD55 and fibrinolytic protease (GD kinase) production, it may not provide enough carbon sources needed by the organism for maximum enzyme production. Hence, the exogenous addition of various carbon sources to the medium may improve cell growth and enzyme production. Generally, the high concentration of carbon sources in media is effective in enhancing the production of enzymes by microorganisms. The impact of supplementation of external carbon sources on fibrinolytic protease (GD kinase) was studied. Addition of carbon sources with 1% w/v concentration to the medium showed different effects on fibrinolytic protease (GD kinase) production. The *Bacillus cereus* GD55 was grown on the medium with carbon source more rapidly at the first 2-4 days, but it turned to autolysis soon,

resulted in less growth and then less fibrinolytic protease (GD kinase) at day 14. So among all the compounds tested, fructose yielded the highest fibrinolytic protease (GD kinase) production (46.64 ± 0.13U/g substrate) (Table. 10 and Fig. 10), followed by arabinose (40.96 ± 0.16 U/g substrate), maltose (28.14 ± 0.78 U/g substrate) and glucose (23.26 ± 0.10 U/g substrate). So fructose and arabinose can be added as supplementation of carbon sources in basal substrate to prolong cell growth or to improve fibrinolytic protease (GD kinase) secretion. Results obtained in this study indicated that among the various agro-industrial residues studied, apple pomace was the suitable substrate for fibrinolytic protease (GD kinase) synthesis by *Bacillus cereus* GD55 in SSF. SSF showed its superiority for fibrinolytic protease (GD kinase) production and also revealed the possibilities of effective utilization of apple pomace (and possibly other agro industrial residues) for value addition through biotechnological means.

Conclusion

The role of agro wastes in fibrinolytic protease (GD kinase) production was identified and production parameters were determined. The apple pomace and cotton seed meal can be the less expensive alternative active substrates in the production of fibrinolytic protease (GD kinase). The optimal conditions for fibrinolytic protease (GD kinase) production using SSF for apple pomace were initial pH (8), initial moisture level (70%), substrate particle size (1.2 mm), inoculums size (2 x 10^6 CFU/g), incubation temperature(35°C), incubation period (day 4), fructose (1% w/v), NH_4NO_3 (1% w/v), peptone (1%w/v) respectively. Such processes would not only help in reducing the cost of production but also pave the way in effective solid waste management. With the above encouraging leads, it will be interesting to study the apple pomace as substrate for the production of other enzymes from different microbes.

Acknowledgments

The authors would like to thank the chairman Acharya institutes Sri B.Premnath Reddy for providing Laboratory Facilities and supporting this research work.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 773-781 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Insecticidal properties of water diffusible prodigiosin produced by *Serratia nematodiphila* 213C

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Abstract

Water diffusible pigment producing rhizospheric bacteria was isolated and identified as *Serratia nematodiphila* 213C through phenotypic characters, FAME and 16S rDNA analysis. In glycerol - peptone media at 28 ^{O}C, 72 h and 180 rpm; dark red colored pigment was biosynthesized, which showed UV – Visible absorbance at λ_{535} and Rf of 0.9 on TLC (10 M ethyl acetate: methanol- 6:4), suggesting close similarity toward prodigiosin type of pigment. Insecticidal performance of isolated pigment on *Helicoverpa armigera* and *Spodoptera litura* showed 70 and 100 % larval mortality at 20 and 30 mgml^{-1} concentrations.

Keywords: *Serratia*, insecticide, prodigiosin, *Helicoverpa armigera*

Introduction

The extensive use of chemical pesticides has resulted in (i) environmental degradation, (ii) adverse effects on human health, (iii) effect on other organisms, (iv) eradication of beneficial insects and (v) resistance developed by insect pest with broad host range e.g. *Helicoverpa. armigera* (4, 7, 40) and *Spodoptera litura*. (26). Thus, failures of chemical pesticide and demand of organic food has led to search for new avenue for insect pest control (11). In this regard, bio-pesticide demonstrated a sustainable approach over chemical pesticide because of its (i) target specific, (ii) easy biodegradable, (iii) less shelf-life and (iv) user friendly for sustainable agriculture (6, 30). Nature had maintained host parasite relations; hence entamopathogenic bacterium and their nematode association can be considering trail for this challenges.

Serratia genus is omnipresent in the environment and found in different geoclimatic conditions as well as in close association with other microbes, insects (nematode), mammals and plants (12). Root association of *Serratia* sp. and corn root worm (Coleoptera: Chrysomelidae) was explained earlier (24). Similarly biocontrol potential of *Serratia* sp., like *S. marcescens*, S*. ficaria*, *S. rubidaea* etc. has been explained previously (18, 23, 27, 28, 32). In view of the broad action of *Serratia* sp. the biochemical mechanism shows production of potentially active molecules (secondary metabolite as well as proteins) like pyrrolnitrin, oocydin A, carbapenem, prodigiosin, surfactant serrawettin as well cell-wall/membrane degrading enzymes like chitinase (1, 15, 19, 21, 35).

Serratia produces both water insoluble as well as water soluble pigments, like in *Serratia marcescens* red water insoluble pigment has antibiotic activity (16, 34, 38, 39) whereas water soluble reddish-violet pigment with superoxidase dismutase mimetic activity (14, 36). Amongst all the pigments, prodigiosin is well studied because its abundance in *Serratia* sp.; consisting of alkaloid with a tripyrrole group i.e. 5[(3-methoxy-5-pyrrol-2-yildene-pyrrol-2-yildene)-methyl]-2-methyl-3-pentyl-1H pyrrole ($C_{20}H_{25}N_3O$) covering three rings and forming a pyrrolylpyrromethane

skeleton with a C-4 methoxy group of average molecular weight of 323.44 D (33, 41). It showed its bioactive potential as antibacterial, antimycotic, immunomodulating, antitumor and anti-malarial agent (2, 8).

In order to elucidate natural biocontrol mechanism in insect pest infested sorghum rhizosphere, current study relates the pigment producing bacteria from rhizosphere and insecticidal activity of pigment with the objectives (i) isolation of pigment producing bacteria and its identification by phenotypic, FAME and 16S rDNA analysis, (ii) isolation and identification of pigment and (iii) determination of activity of pigment against the common insect pest i. e. *H. armigera* and *S. litura*.

Materials and Methods

Isolation and identification of organism: Sorghum rhizospheric soil sample was serially diluted up to 10^{-7} dilution on suspending in sterile phosphate buffer saline (pH – 7.0) in proportion of 1% and mixed it carefully. A 0.1 ml sample was spread on nutrient agar and incubated at room temperature for 24 hrs. Colonies with diffusible pigment was observed and isolated colony was sub cultured and stored at 4°C. The isolated bacteria was further characterized for identification by phenotypic characters i.e. morphological (staining and motility), cultural (nutrient agar, glycerol-peptone medium), biochemical tests (IMVIC test, catalase test, oxidase tests) (3).

Further confirmation was done with (i) whole-cell fatty acids derivatized to methyl esters (FAME) were analyzed using Gas Chromatography (Agilent 6850 Series II) with the help of MIDI Sherlock software for FAME (MIDI, Newark, DE, USA) as per Rajan *et al*. (2011) at Royal Life Sciences Pvt. Ltd, Secunderabad and (ii) nucleotide sequence of 16 S rRNA gene.

The genomic DNA of strain 213C was isolated as per Rajan *et al*. (2011) at Royal Life Sciences Pvt. Ltd, Secunderabad where , 16S rDNA subjected to amplification (1.5 kb) using universal primer 16S F (5' AGA GTT TGA TCC TGG CTC AG 3') and 16S R (5' ACG GCT ACC TTG TTA CGA CTT 3'). The PCR amplification conditions were (i) 25 repeat cycles of 95 °C denaturation for 1 min, (ii) annealing at 55 °C for 1 min and extension of annealing at 72 °C for 1.30 min (Gene AMP PCR System 9700) in 0.2 mL PCR tubes. The PCR products were purified using Qiagen PCR product purification kit. Purified PCR products were taken for cycle sequencing with ABI Big Dye Terminator chemistry using either forward primer or reverse primer in Gene AMP PCR System 9700. The cycle sequencing products were purified according to Applied Biosystems protocol. Purified products were sequenced in ABI 3730XL Sequencer. The 16S rRNA nucleotide sequences were aligned using BLAST analysis at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). For comparison currently available sequences at NCBI were used and multiple sequence alignment performed by using Bioedit 7.0. The evolutionary dendrogram was inferred using the Neighbor-Joining method (29). The bootstrap consensus tree inferred from 1000 replicates (5) was taken to represent the evolutionary history of the taxa analyzed (5). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (5). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (17) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (37). The nucleotide sequence of 16 S rRNA was deposited to Gene Bank database under accession number JN 166084.

Fermentation condition: A pre-culture was developed in 50 ml of nutrient broth in Erlenmeyer flask by inoculating a single colony of selected bacterial strain (213 C) and incubating at room temperature, 180 rpm for 24 h. A 50 ml of freshly prepared fermentation medium (gL^{-1}): glycerol,

0.2; peptone, 0.5 (10) of pH 7.0 in 250 ml Erlenmeyer flasks was inoculated with the pre-culture of 1.0 absorbance at 600 nm at 1% (v/v) concentration and incubated at 28 ^{O}C, 180 rpm for 72 h.

Isolation of Pigment: The pigment naturally concealed into the glycerol - peptone broth medium after 72 h incubation was separated by either filtration in glass wool tied funnel or by centrifugation at 10,000 rpm for 15 min and subjected to concentrated in rotary vacuum evaporator (BÜCHI Rotavapor, R -124) at 50 ^{O}C under vacuum (10^{-5} torr).

Determination of chromatographic and spectral properties: Thin layer chromatography - The concentrated pigment was checked for its electrophoretic mobility as well separation using TLC. The silica gel TLC plate (20 x 20 cm) were prepared and developed as per standard protocol (22, 31). A mobile phase (10 M ethyl acetate: methanol – 6: 4) was run to separate the pigment components as well as to determine its retardation factor (Rf) (20).

UV-Visible spectral trait: The concentrated pigment (10 mgL^{-1}) was dissolved in de-ionized Millipore water and scanned for its absorbance on UV – Visible spectrophotometer (UV-1800, Shimadzu, Japan) from 700 – 400 nm.

Insect bioassay: The insect bioassay was carried out on second instar larvae of *H. armigera* (pod borer) and *S. litura* (cotton leaf eating caterpillar) in 12 or 24 well flat bottom plates using pigment as a diet feed in triplicate plate was sealed and placed in a humidified growth chamber at 28 ^{O}C. 24 - 30 larvae were placed in each of the petri plates containing different concentrations of pigment (10, 20, 30, 40, 50 $mgmL^{-1}$) sprayed on okra pods as diet, with a control kept without pigment spray. The larvicidal effect of pigment was determined by counting the number of dead larvae after 72 h on observing failed motility after probing with needle.

Statistical analysis*:* The dose–response data was subjected to probit regression analysis to determine the statistical significance of performed experiment.

Results and Discussion

Different *Serratia* sp. has been studied for their physiological and pigment production (9, 13). Sorghum is one of the contamination sites for different insects like *H. armigera*, *S. litura.* Very few reports are available for isolation of *Serratia* sp. from Sorghum rhizospere. Present study is related with nematode contaminate sorghum field and its natural control by pigment, producing microbes as biocontrol agent. In view of this, nine different pigment producing bacteria were isolated on nutrient agar after incubation for 24 h at room temperature. Among 09 isolates, one red pigmented mucoid bacterial colony (designated as 213 C) showing water diffusible pigment production was selected for further study. Colonies were small, low convex, smooth, bright red colored, non spore forming, Gram negative, motile, rods. The biochemical performance showed -, -, +, + reaction trait for IMViC test and catalase (+) and oxidase (-) test suggest relevance of *Serratia* genus. The FAME analysis showed similarity index of 0.654 with *Serratia marcescens*-GC-subgroup with 14 reference peaks (data not shown). The partial 16S rRNA gene sequence was compared with sequences available from databases. Phylogenetic tree was constructed from available 16S rRNA of *Serratia* sp. and selected *B. subtilis* using molecular evolutionary analysis tool i. e. Clustual X and MEGA software (Fig. 1). Based

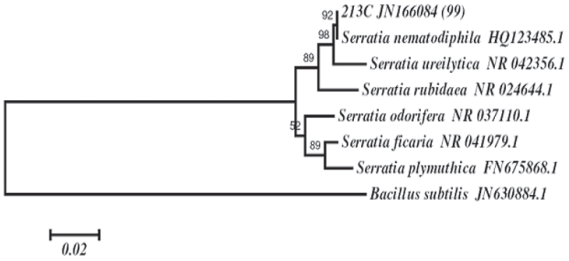

Fig. 1. Phylogenetic position of strain 213C among genus Serratia in a neighbour-joining tree based on analysis of 16S rRNA gene sequence.

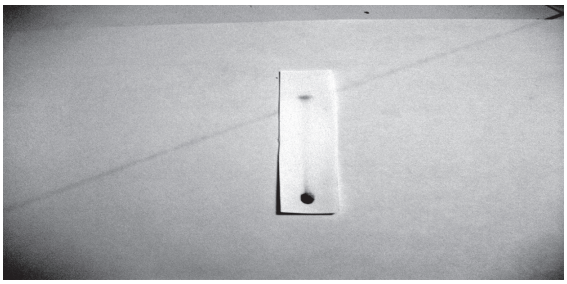

Fig. 2. Thin Layer Chromatography (TLC) of crude concentrate pigment in 10 M Ethyl acetate: Methanol – 6: 4 mobile phase separated as single red spot of Rf - 0.90.

on 16 S rRNA homology, the isolate 213 C suggest (99%) with a *Serratia nematodiphila* HQ123425. Thus the phenotypic, FAME and 16S r DNA reveals the similarity of isolate 213 is a *Serratia nematodiphila* (Gene bank accession no. JN166084). The results obtained are similar to Zhang *et al*. (2009).

Pigment isolation and its preliminary characterization: After 72 h of incubation at 28 ^{O}C, yellow white colored glycerol peptone media of pH 7.0 turns in lust red. Pigment isolated after centrifugation at 10,000 rpm for 10 min and concentrated in rotary vacuum evaporator and finally showed dark red colored powder.

The TLC scan (Fig. 2) showed single spot after evaporation of solvent at room temperature. The Rf value at 0.9 is similar to prodigiosin type of pigments i.e. 0.9 – 0.95 as reported earlier (22, 31). Similarly the UV-Visible spectrum (Fig. 3) from 700 – 400 nm gives absorption maxima at 536 nm which is analogous to prodigiosin type of pigment as reported by Patil *et al*. (2012). The electrophoretic and spectral evidence reveals the biosynthesis of extracellular water diffusible sole prodigiosin type of pigment by an isolate *Serratia nematodiphila* 213C in glycerol peptone media. Prodigiosin is high value pigment as described earlier and hence sole synthesis of pigment in fermentation media will be helpful in down streaming of large scale pigment production.

Insecticidal performance of pigment: In earlier reports (23, 24) *Serratia marcescens* and other *Serratia* sp. have been shown its biocontrol potential. Although, Patil and coworker have shown role of prodigiosin in mosquito control, till

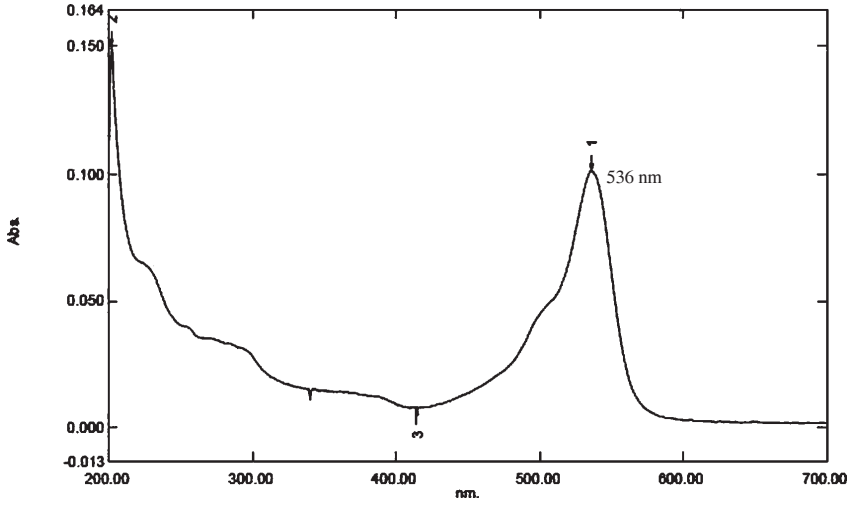

Fig. 3. UV- Visible (400 - 700) spectrum of crude pigment isolated from S. nematodiphila 213C showing ?max at 536 nm

Vol. 7 (3) 773-781 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

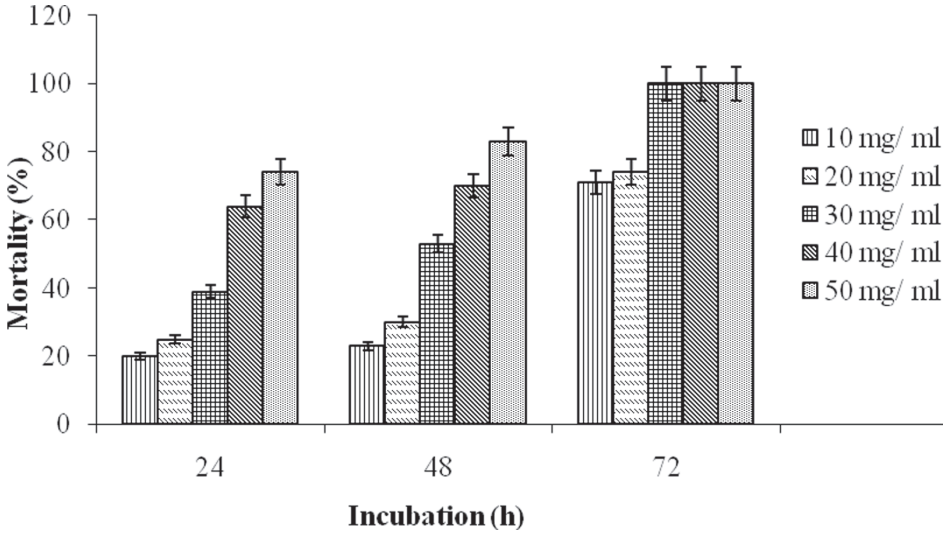

Fig. 4. Effect of crude prodigiosin on second instar *H. armigera* larvae with increase in dose (10 – 50 $mgml^{-1}$) and incubation time (24 – 72 h) with 5 % SD

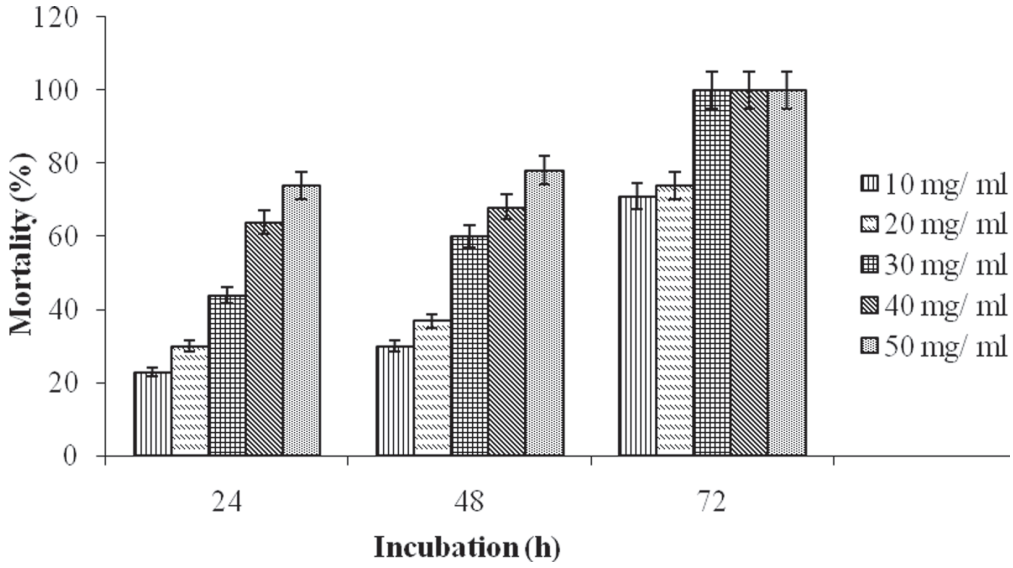

Fig. 5. Effect of crude pigment on second instar *S. litura* larvae with increase in dose (10 – 50 $mgml^{-1}$) and incubation time (24 – 72 h) with 5 % SD

Supportive information - Preliminary phenotypic and biochemical characteristics of bacterial isolate 213C

Test	Result
Colony on nutrient agar	Mucoid, sticky, convex , entire surrounded with red pigment
Gram character	Gram negative, rod, single,
Microscopy	motile, non
spore forming	-
Indole production	-
Methyl red	-
Voges-proskauer	+
Citrate utilization	+
Catalase	+
Oxidase	-
Urease	-
Gelatinase	+
Amylase	+
H_2S production	- or weak +

role of prodigiosin in insect pest is missing. Okra pods i.e. diet adsorbs the pigment after spraying on it. Hence the pigment acts on insect through its digestive track. Different concentration of pigment (10 – 50 $mgmL^{-1}$) in diet feed of *H. armigera* and *S. litura* showed dose dependent mortality where 10 – 20 and 30 - 50 $mgmL^{-1}$ prodigiosin resulted in 70 and 100 % mortality after 72 h incubation of both insect in humidified chamber (70 % humidity). Also the mortality seems to be time dependent, where at 30 $mgml^{-1}$ concentration 39, 53 and 100 % mortality were seen after 24, 48 and 72 h incubation of *H. armigera* (Fig. 4). Similarly, at 30 $mgmL^{-1}$ concentration 44, 60 and 100 % mortality were seen after 24, 48 and 72 h incubation of *S. litura* (Fig. 5). Prodigiosin has different target site which includes induction of single and double-strand DNA breaks, modulation of pH, regulation of nitrogen-activated protein kinase, and inhibition of cell cycle progression leading to apoptosis. Mentioned factor might lead to insect mortality. The prodigiosin biosynthesized by *S.*

Statistical analysis for insecticidal performance

1. *H. armigera*

Parameters for correlation	24h	46h	72h
Number of XY Pairs	5	5	5
Pearson r	0.9776	0.9902	0.8796
95% confidence interval	0.6928 to 0.9986	0.8543 to 0.9994	-0.01228 to 0.9920
P value (two-tailed)	0.004	0.0012	0.0492
P value summary	**	**	*
Is the correlation significant? (alpha=0.05)	Yes	Yes	Yes
R square	0.9556	0.9805	0.7737

2. *S. litura*

Parameters for correlation	24h	46h	72h
Number of XY Pairs	5	5	5
Pearson r	0.9887	0.9824	0.8796
95% confidence interval	0.8338 to 0.9993	0.7513 to 0.9989	-0.01228 to 0.9920
P value (two-tailed)	0.0014	0.0028	0.0492
P value summary	**	**	*
Is the correlation significant? (alpha=0.05)	Yes	Yes	Yes
R square	0.9776	0.9651	0.7737

nematodiphila 213C, proved to have insecticidal effect on Lepidopteran pests i.e. *H. armigera* and *S. litura*. The statistical analysis recommends the data in correlation is significant with $p \leq 0.05$.

Conclusion

Present investigations showed insecticidal capability of prodigiosin at laboratory conditions on *H. armigera* and *S. litura*. To our knowledge, this is the first report of *S. nematodiphila* isolated from sorghum rhizosphere as well as having water diffusible pigment production capability. Sole prodigiosin synthesis, suggest efficacy of bacterium for biocolor production. At crude concentrate level, prodigiosin showed insecticidal activity implies its econo - value. However, further purification, cytotoxicity testing and field trial will be carried out in future for its commercial use.

Acknowledgement

Authors are thankful to Royal Life Sciences, Secunderabad, India for FAME and 16S rRNA analysis, as well as to Dr. J. C. Rajput, Nirmal seeds Laboratories, Pachora, India for carrying out of insecticidal activity.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 782-792 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Antimicrobial property of *Datura* leaf extract against Methicillin-resistant *Staphylococcus aureus* isolated from Urethral and Skin Suppurative Infections

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Abstract

Datura stramonium is commonly known as Jimson weed and is widely used in phyto-medicine to treat epilepsy, skin ulcers, etc as well as to kill the microbes associated with wound lesions. Antimicrobial activity of ethyl acetate and methanol extracts of leaves and flowers of *Datura stramonium* was investigated against antibiotic resistant pathogenic bacteria isolated from pus samples of skin and urethral infections. The crude ethyl acetate extracts of leaves showed good inhibitory effects against Methicillin-resistant *Staphylococcus aureus* (MRSA) pathogens. The extract was separated by TLC and silica gel column chromatography to fractionate the bioactive constituents. Evaluation of chemical nature of ethyl acetate fractions revealed that the presence of alkaloids, steroids, phenolic compounds, tannins, tripenoids and saponins. The isolated compounds were further screened for antimicrobial activity against MRSA. Bioactive alkaloid fraction showed 86.11% purity by HPTLC was subjected to FTIR analysis. Further chemical characterization of the active fraction was carried out by LC-MS/ESI.

Keywords: *Datura stramonium;* Bioactive; Alkaloid; Skin infection; Urethral infection, *Staphylococcus aureus*.

Introduction

Staphylococcus aureus causes numerous infectious diseases in humans including minor skin infections, such as pimples, impetigo and scalded skin syndrome as well as life-threatening diseases such as pneumonia, meningitis and toxic shock syndrome. It is majorly involved in nosocomial infection of surgical wounds and infections associated with indwelling medical devices (1). *Staphylococcus aureus* is also responsible pathogen for superficial skin and urethral pus forming lesions (2) resulting the symptoms like formation of styes, boils with pus and furunculosis. The choice of drugs to be used against skin and urinary tract infection causative strains is declining day by day since the vulnerability of these strains to drugs is failing due to molecular level changes adopted by these microbes (3).

Methicillin-resistant *staphylococcus aureus* (MRSA) or oxacillin-resistent *Staphylococcus aureus (ORSA)* associated infections in human are difficult-to-treat mainly due to its acquired multi-drug resistance. A genetic element that can be transferred from one bacterium to another causes *Staphylococcus aureus* to develop resistance to antibiotics. At least five genes namely SSCmec genes I-V have been identified. Hospital-acquired MRSA (HA-MRSA) usually have genes I-III while Community-acquired MRSA (CA-MRSA) have genes IV-V (4). HA-MRSA is resistant to more antibiotics than CA-MRSA. MRSA can be transmitted by direct (through skin and body fluids) and indirect contact (from towels, diapers and toys) to uninfected people.

The skin and urinary tract supportive infection causative MRSA strains exhibit resistance to drugs by the secretion of a huge array of cell-associated proteins (5). Commonly, phagocytosis is the major defense mechanism for combating any sort of pathogen but the proteinaceous toxins secreted by skin and urethral infection causing resistant strains impervious to opsonization which dictates the host immune system resulting fail in defence (5). All these factors lead to the skin and UTI pathogens to develop immense resistance to numerous drugs. Pathogenic bacterial strains develop several defense mechanisms against antimicrobial agents for their evolutionary survival process. However, these phenomenons may differ from microbe to microbe as well as nature of antibiotic environment (6). Another major factor is indiscriminate use of various antibiotics which leads the emergence of multi drug-resistant pathogens (7). Although several novel antibiotics are being developed, the efforts become futile once the microbe develops the resistance to the drug. Hence it becomes important to sort after and adopt novel therapeutic methodologies to override these disease causing agents. Exploiting the synergy of natural plant extracts with antibiotics can pave a new strategy to combat the ever increasing magnitude of antibiotic resistance (8). The crude preparations of medicinal plant extracts have exhibited a great potential in clinical pathology. Several studies show that the magnitude of synthetic antibiotic can be enhanced by combining it with plant extracts (9).

Datura stramonium commonly known as *Datura* or Jimson weed or Thornappleis, is well known for its medicinal properties (10) due to the presence of various phytochemicals that exhibit extraordinary array of antimicrobial activity against drug resistant pathogens. Throughout the world there are approximately 25 different species of *Datura* exists. The whole plant, particularly the leaves and seeds are used as anesthetic, anodyne, anti-asthmatic, anti-spasmodic, anti-septic, narcotic, sedative, bronchodilator, hallucinogenic and is useful for asthma (11-15). In view of the above, present study is carried out phytochemical analysis, composition determination, standardization and chemical characterization of *Datura stramonium* leaf ethyl extract to provide scientific evidence for its anti-microbial potential against MRSA strains isolated from pus samples.

Materials and Methods

***Chemicals*:** Muller Hinton Broth, Muller Hinton Agar, methanol, ethyl acetate, n-hexane, etc., were obtained from Hi Media, Mumbai, India. Silica gel (60-80) used for column chromatography was obtained from Finar Chemicals India Pvt. Ltd. The standard reference antibiotic, Cefalotin and TLC plates were purchased from Sigma-Aldrich (St. Louis, MO, USA).

***Microorganisms*:** Two different strains of MRSA were isolated from skin and urethral pus samples of MRSA infected patients, at Chest Hospital, Hyderabad. For comparison, the standard MRSA and standard MSSA were used (procured from same place). All the four cultures were maintained in Muller Hinton broth as well as on agar slants at 4°C. The Clinical studies were carried out at Department of Pathology, Chest Hospital, Hyderabad. All the screening experiments were carried out in triplicates and the mean values were expressed along with their standard deviations.

Preparation of solvent extracts from different parts of plant material: Fresh leaves and flowers of *Datura stramonium* were procured from local gardens at Hyderabad, India. The collected material was washed thoroughly with distilled water and freeze dried. The dried samples were ground to powder and stored in tight container at -20°C until further use. Then the processed plant materials were subjected to fractionation using n-hexane, ethyl acetate, methanol and water in the order of increasing in polarity. The extraction procedure was carried out at room temperature for 72h using mass to volume ratio of 1:10. By using a Rotary Evaporator each solvent present in the extracted material was evaporated at its

respective boiling point. The solvent free extracts were dissolved in DMSO and were screened for their activity against the selected multi drug resistant pathogens.

***The primary screening for evaluation of antibacterial activity of solvent extracts*:** All the extracts were evaluated for *in vitro* antibacterial activity against three strains of multi drug resistant *S. aureus* (MRSA) and one multidrug susceptible *S. aureus* (MSSA). A modified Kirby Bauer method (an agar well-diffusion) (16) was performed for screening the antibacterial activities of extracts of leaves and flowers of *Datura stramonium*. The bacterial strains were sub culturing into fresh medium at 37°C for 18 h in an incubator. An inoculum size of $1x10^6$ CFU/ml was inoculated aseptically to Muller Hinton Agar medium by pour plate method. 50 µl of each test extract from the stock concentration (1mg/ml) was added to the wells (8mm) punched on agar surface. Reference standard was maintained with Cefalotin (1mg/ml). Plates were incubated overnight at 37°C. The activities were evaluated by measuring the diameter of inhibition zone (DIZ in mm). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts were determined by broth dilution methods (17). A serial two-fold dilution ranging from 4.06-0.008 mg/ml was used for determining the minimum inhibitory concentrations. The minimum inhibitory concentration is the lowest concentration that demonstrates no visible growth by macroscopic evaluation. The tubes showing no turbidity were diluted to 100-fold with drug-free media and incubated at 37°C for 48 h. The lowest concentration of tube that showed no visible growth in drug-free cultivation is considered as minimum bactericidal concentration.

***Phytochemical screening and separation, purification of bioactive components*:** The ethyl acetate extracts of *D. stramonium* were subjected to phytochemical screening for major constituents including alkaloids, tannins, flavonoids, phenols, saponins, tripenoid, steroids, triterpenes and glycosides using standard qualitative method as described earlier (18-20). The column chromatography separated and purified bioactive compounds from crude extracts were analyzed by TLC using mobile phase comprising of n-Hexane: Ethyl acetate in a ratio of 4:1. The developed plates were exposed to iodine vaporousin order to detect the spots. In brief, 1 gm of dried bioactive plant extract was dissolved in suitable solvent (Ethyl acetate) and ground with 3 gm of silica gel to eliminate the formation of air bubbles. Then the trodden material was loaded on the top of silica gel packed in a glass column. Initially, 100 ml of a non-polar solvent such as n-Hexane was passed in order to elute residual non-polar compounds. Later, the polarity of eluting solvent was increased with ethyl acetate. Each fraction was collected and stored in separate screw cap container. Thin layer chromatography was performed for each fraction and then the fractions were evaluated for antibacterial activities.

***Analysis of purity of bioactive fractions*:** The samples were spotted on pre-coated silica gel plates 60 F 254 (2cm x 2cm with 0.2mm thickness, E. Merck, KGaA 64271 Darmstadt, Germany) using a CAMAG automatic sample spotter (CAMAG TLC Scanner 3). Initially the TLC plates were washed with ethyl acetate and activated at 60°C for 5 minutes prior to performing. The sample was spotted just 1cm above from the bottom and 1cm from the left edge of the TLC plate. The loaded plates were placed in a solvent system containing hexane-ethyl acetate 4:1 ratio in glass TLC chamber (20±10cm) previously saturated with the solvent for 30min (temperature 25±2°C) till the solvent front reached to 5.9 cm for separation of components. TLC plates were air dried and scanning was performed on a CAMAG TLC scanner 3 at absorbance mode of 254nm fitted with win CATS planar chromatography manager software V1.4.4 version.

***Chemical characterization of bioactive compound*:** The seventh fraction obtained from column chromatography of ethyl acetate

extraction of leaf was taken as sample for Fourier-transform infrared spectroscopy (FTIR). The bioactive fraction of the ethyl acetate extract of leaf was further analyzed further using Fourier-Transform Infrared Spectroscopy (FT-IR). One drop of sample was placed between two cells of sodium chloride to form a thin film. The spectrum was recorded and the unknown compound was identified from the library of known compounds. LC-MS was carried out for further analysis of the same fraction. For the chromatographic separation, Acquity-BEH-C18-(50×2.1mm) 1.7-µm column was used. A combination of solvent A (0.1% HCOOH in water) and Solvent B (0.1% HCOOH in acetonitrile) were considered for mobile phase solvents. The flow rate was adjusted to 0.5ml/min and Acetonitrile was taken as diluents.

Results

***Evaluation of antibacterial activity of solvent extracts*:** Ethyl acetate and Methanol extracts of leaf and flower of *Datura strmonium* showed varying degrees of antibacterial activities against the clinical isolates from pus samples of skin and urethral lesions (Table 2 and 3) as well as standard strains of MRSA and MSSA of *Staphylococcus aureus* (Gram positive) as control organisms. The Antibacterial activity of *Datura stramonium* of ethyl acetate and methanol extracts were compared with that of standard antibiotic (Cefalotin). The ethyl acetate extract of dried leaves exhibited promising inhibitory effect on standard strains and clinical isolates compared to flowers (Tables 2 and 3). The inhibition zones for MRSA strains of urethral infection were found to be 67.85% more (28±1mm) in comparison to MSSA strain (inhibition zone 19±0.5mm). The methanol extract showed low antibacterial activity with inhibition zones ranging between 18±1 to 19±0.5 mm for MRSA and MSSA strains (Table 3). The methanol extract of dried leaves exhibited comparatively moderate activity on four strains whereas the flower extract did not exhibit any inhibitory effects (Table 3). The Solvent extracts of leaves and flowers of *Datura stramonium* showed minimal inhibitory concentrations against all the selected resistant bacterial strains. From tables 4 and 5, it is evident that among all the extracts, the ethyl acetate extract of dried leaves was found to be more effective in inhibiting the growth of MRSA strains of skin and urethral infections. The observed minimum inhibitory concentration (MIC) for this extract was 0.039±0.01mg/ml, the positive reference standard Cefalotin, a powerful β-lactam antibiotic (21) was 0.019±0.02mg/ml. The MRSA of urethral sample on Cefalotin antibiotic has the maximum MBC effect of 0.0625±0.02mg/ml.

The purified fractions of ethyl acetate extract (leaves) were also screened for determination of minimum bactericidal concentrations against all the MRSA strains and effective bactericidal concentration was shown by the active bio-fraction (7^{th} fraction). MSSA standard strain showed a good zone of inhibition of 33±1mm followed by MRSA standard, MRSA skin sample, MRSA urethral sample showed zone of inhibition of 30±0.2mm, 29±0.3mm and 29±0.2mm (Table 6) respectively. The reference antibiotic cefalotin showed its maximum MIC of 0.156±0.02 mg/ml and MBC of 0.625±0.03mg/ml on MRSA of urethral sample while a minimum MIC of 0.078±0.01 and MBC of 0.312±0.01mg/ml on MRSA standard skin sample (Table 7).

The minimum inhibitory concentration and minimum bactericidal concentration were checked for the 7^{th} fraction which is having highest zone of inhibition as noted in table 7. The MIC for MRSA skin strain found to be highest minimum inhibitory concentration (0.039 ±0.01 mg/ml), while all other strain showed MIC of 0.019±0.02 mg/ml. Whereas MBC for MRSA standard indicated the maximum (0.625±0.02 mg/ml) while the lowest value (0.156±0.02 mg/ml) has been observed for MRSA strain isolated from pus samples of the skin.

***The Preliminary Screening of Phytochemical*:** The preliminary phytochemical investigation was done for the *Datura stramonium* leaf extracts to identify various phytochemical constituents present in the extracts and evaluated for

antimicrobial activity against MRSA by agar well diffusion method. The preliminary phytochemical studies revealed the presence of alkaloids, flavonoids, steroids, tripenoids, phenolic compounds, glycosides, teriterpens, tannins, and saponins in the leaf extract (Table 1).

Table 1. Phytochemical components present in *Datura stramoinum* ethyl acetate dried leaf extract.

S. No.	Bioactive compounds	Result
1.	Alkaloids	+
2.	Steroids	+
3.	Tripenoids	±
4.	Phenolic Compounds	+
5.	Tannins	+
6.	Saponins	±
7.	Glycosides	"
8.	Triterpenes	"

+= Present; **"** = absent; **±** = Trace.

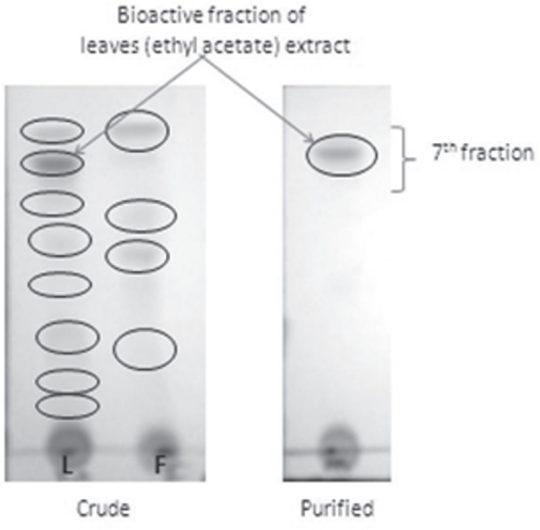

Fig. 1. Thin layer chromatography analysis of dried leaves ethyl acetate extract of Datura stramonium.

***Purification of bioactive compound*:** Since the ethyl acetate extract of dried leaves showed good microbial inhibitory activity, the whole extract was subjected to silica gel column chromatography to separate the active fraction. The purification was performed with solvent gradient elution with a mixture of n-hexane and ethyl acetate in the ratio of 98:2 (n-Hexane: Ethyl acetate). The column was eluted slowly by increasing the polarity of eluting solvent and the extract was fractionated in to eight individual fractions (spots were detected by TLC) (Fig.1.). The 7th fraction of extract showed the highest activity against all the resistant strains (Table 6). Hence, this fraction was further subjected to HPTLC, FTIR and LC-MS/ESI for determining the purity, functional group and nature of active compound (Fig.3, 4, 5, 6 and 7).

HPTLC analysis: The 7th fraction obtained from ethyl acetate extraction of leaf was subjected to HPTLC analysis for generation of HPTLC finger printing profile which is represented as a chromatogram. The solvent system used in the investigation was found to give compact spots for extracts at different Rf values and there was no overlap with any other component in the analyzed sample at 254 nm. The solvent system used in the investigation was found to give a

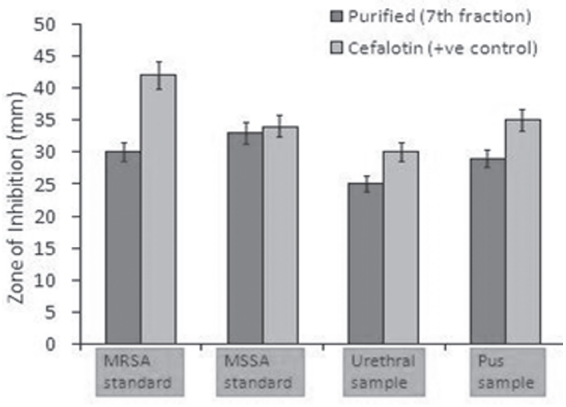

Fig.2. Antibacterial effect of purified bioactive compound of (7th fraction) ethyl acetate leaf extract in comparison with Cefalotin (a positive control antibiotic).

strong uphill peak with purity of about 86.53% for the seventh fraction with Rf values between 0.94-1.06. No overlap with any other peaks was observed in the analyzed sample; this shows the purity of a bioactive fraction. The results and observations are presented in table 8 and figs. 3 and 4.

***FTIR analysis*:** Spectroscopic analysis was performed for the TLC fractions that showed antibacterial activity. The FTIR spectrum obtained with that of the reference chart revealed the presence of functional groups such as alcohols, alkyls, alkynes, esters, alkanes and amines in the leaf extract. The seventh fraction of ethyl acetate extract of leaf was analysed by FTIR for determining the functional groups of the bioactive component based on its peak ratio and electron transition of compounds. The peak values observed within the IR range (4000-400cm^{-1}) include 3631.75 and 3465.07 which depictsalcohol and 2985.43 as alkyl, 2085.83 as alkynes, 1742.14 as ester,1241.51 and 1046.94 as amines, 1373.98, 937.93, 847.10, 786.38 as alkanes and 608.24 as alkynes. More alkane functional groups in the active fraction (22) are revealed in Table 9 and Fig. 5.

LC-MS analysis: The LC-MS chromatogram of ethyl acetate extract of *Datura stromanium* at 254 nm showed the retention times, the mode (+/-), λ max, and molecular weight of the respective compounds. In the LC-MS analysis of *Datura stromanium* a negative molecular ion of molecular weight with retention time 310.5 (2.32) corresponding to 3-tygloyloxy-6-isobutyrloxy-tropane, 422.3 (1.94) corresponding to Quinine sulphate, 521.5 (2.05) corresponding to hysicyamine, 437.4 (1.89) corresponding to hyoscine, 255.2 (1.50) corresponding to 3-tygloyloxy-6, 7-dihydroy tropane were observed respectively (23) (Fig. 6).

Discussion

Plants comprise of numerous bioactive phyto-compounds which are imperative for the development of antimicrobial agents. The plants belonging to Solanaceae family have emperical

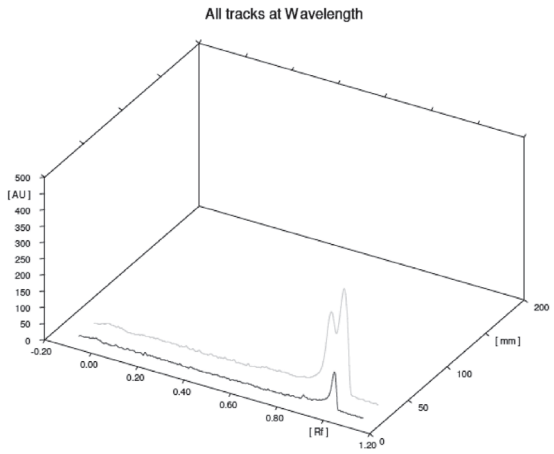

Fig.3. HPTLC analysis showing finger printing of the bioactive compound (7 th fraction) of ethyl acetate leaf extract of Datura stramonium.

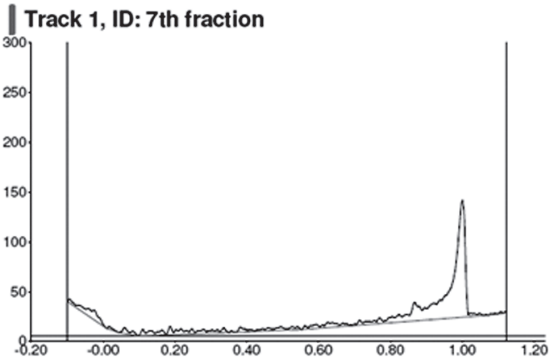

Fig.4 . Graphical representation of HPTLC analysis of the bioactive compound (7th fraction) of the ethyl acetate leaf extract of Datura stramonium.

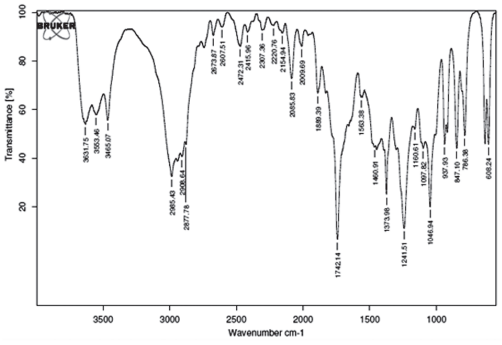

Fig. 5. FTIR spectra peak values of the 7th fraction (ethyl acetate leaf extracts) of Datura stramonium.

Table 2. Antibacterial effect of Datura *stramonium* leaf extract against MRSA and MSSA strains.

Test organism	Diameter of zone inhibition (mm)				
Extract concentration (1mg/ml)	Solvent controls			Cefalotin	
	Ethyl acetate	Methanol	E. A[a]	MeOH[b]	(100µg/ml)
MRSA of urethral infection	28±1	NA	NA	NA	33±1
MRSA of skin infection	NA	NA	NA	NA	34±1
MRSA standard strain	22±0.5	19±0.5	NA	NA	30±1
MSSA standard strain	24±1	18±1	NA	NA	38±2

[a]E.A- Ethyl acetate solvent; [b]MeOH-Methanol solvent; NA-No inhibition activity.

Table 3. Antibacterial effect of Datura *stramonium* flower extract against MRSA and MSSA strains.

Test organism	Diameter of zone inhibition (mm)				
Extract concentration (1mg/ml)	Solvent controls				Cefalotin
	Ethyl acetate	Methanol	E. A[a]	MeOH[b]	(100µg/ml)
MRSA of urethral infection	12±1	NA	NA	NA	33±1
MRSA of skin infection	14±0.5	NA	NA	NA	34±1
MRSA standard strain	13±0.5	NA	NA	NA	30±1
MSSA standard strain	15±1	NA	NA	NA	38±2

[a]E.A- Ethyl acetate solvent; [b]MeOH-Methanol solvent; NA-No inhibition activity.

Table 4. Determination of Minimum inhibitory and bactericidal concentrations of *Datura stramonium* leaf extracts &Cefalotin effect on pathogenic MRSA and MSSA strains.

Test Organism	Ethyl acetate (mg/ml)		Methanol (mg/ml)		Cefalotin (mg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
MRSA of Urethral infection	0.156±0.02	0.625±0.06	0.078±0.02	1.25±0.04	0.156±0.02	0.625±0.03
MRSA of skin infection	0.078±0.02	0.312±0.03	0.312±0.03	0.439±0.01	0.078±0.01	0.312±0.01
MRSA standard strain	0.078±0.01	0.312±0.02	0.312±0.02	0.439±0.01	0.078±0.01	0.312±0.01
MSSA standard strain	0.625±0.06	1.25±0.1	0.156±0.02	0.625±0.01	0.078±0.01	0.156±0.02

Table 5. Determination of Minimum inhibitory and bactericidal concentrations of *Datura stramonium* flower extracts &Cefalotin effect on pathogenic MRSA and MSSA strains.

Test Organism	Ethyl acetate (mg/ml)		Methanol (mg/ml)		Cefalotin (mg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
MRSA of Urethral infection	0.078±0.01	0.312±0.02	0.039±0.01	1.25±0.02	0.156±0.02	0.625±0.03
MRSA of skin infection	0.039±0.01	0.078±0.01	0.078±0.01	0.156±0.01	0.078±0.01	0.312±0.01
MRSA standard strain	0.156±0.01	0.312±0.01	0.312±0.01	0.625±0.01	0.078±0.01	0.312±0.01
MSSA standard strain	0.312±0.01	0.625±0.02	0.156±0.01	1.25±0.02	0.078±0.01	0.156±0.02

applications in the treatment of skin diseases, epilepsy and wounds (24). The phytochemical components of *Datura stramonium* have been established in previous studies and these include majorly tropane alkaloids such as hyoscyamine and scopolamine which could be responsible for its herbal gram applications (25). Successive isolation of a bioactive compound from any herbal plant is mainly based on the type of solvent employed in the extraction procedures (26). In the present investigation, primarily two different polar solvents such as ethyl acetate and methanol were employed for extracting the bioactive constituents from leaves and flowers of *Datura stramonium*. The ethyl acetate extracts of leaves showed good inhibitory effect against antibiotic resistant strains isolated from pus samples of skin and urethral infections (Table 4 and 5). Even the aqueous leaf extract of four species of *Datura* screened against human pathogenic bacteria showed similar type of results as reported by Jamdhade and his coworkers (27). While as shown in the present study, differential activities were observed between a less polar (ethyl acetate) solvent and a high (methanol) polar solvent extracts of leaves and flowers. Methanol extract of leaves have shown comparatively lower activity than ethyl acetate extract, whereas flowers exhibited moderate activities in case of both solvents.

Table 6. Antibacterial effect of column chromatography purified fraction of dried leaves ethyl acetate extract of *Datura stramonium.*

Test organism	Diameter of zone inhibition (mm) Dried leaves Ethyl acetate Fractions (1mg/ml)								Cefalotin
	F 1	F 2	F 3	F 4	F 5	F 6	F 7	F 8	(100µg/ml)
MRSA of Urethral infection	NI	NI	NI	NI	NI	NI	29±0.2	7±0.1	33±1
MRSA of skin infection	NI	NI	NI	NI	NI	NI	29±0.3	9±0.2	34±1
MRSA standard strain	NI	NI	NI	NI	NI	NI	30±0.2	4±0.1	30±1
MSSA standard strain	NI	NI	NI	NI	NI	NI	33±1	5±0.1	38±2

F- Fractions (1-8), NI- No zone of Inhibition.

Table 7. Minimum inhibitory and bactericidal concentrations of the 7th fraction (purified) of dried leaves ethyl acetate extract of *Datura stramonium.*

Bacterial strains	7th fraction (mg/ml)		Cefalotin (mg/ml)	
	MIC	MBC	MIC	MBC
MRSA of Urethral infection	0.019±0.02	0.312±0.01	0.156±0.02	0.625±0.03
MRSA of skin infection	0.039±0.01	0.156±0.02	0.078±0.01	0.312±0.01
MRSA standard strain	0.019±0.02	0.625±0.02	0.078±0.01	0.312±0.01
MSSA standard strain	0.019±0.02	0.312±0.01	0.078±0.01	0.156±0.02

Table 8. HPTLC analysis for purity of bioactive compound of the 7 th fraction (dried) leaves ethyl acetate extract *Datura stramonium.*

Peak	StartRf	Start Height	MaxRf	MaxHeight	Max%	EndRf	EndHeight	Area	Area%
1	0.94	18.5	1.00	117.7	86.11	1.06	0.0	1920.1	86.53

Though several compounds are known to present in various Datura species and only some will show the bioactivity. This is further evidenced in the present study too where only one fraction of the ethyl acetate extract of leaves revealed bioactivity. Further analysis of active fraction by FTIR and LC-MS/ESI (Fig.5 and 6) depicted ahyscocyamine alkaloid nature. However, observations reported in literature suggest that the leaves of *Datura* species comprise majorly tropane alkaloids which are bioactive in nature (25). Some reports revealed that atropine, hyoscyamine and scopolamine (hyoscine) were the tropane alkaloids found in all species of the genus *Datura* however their concentrations varied depending on species and on the part of the plant (28-30). In fact, data on concentration of total alkaloids in leaves and flowers of *D. metel* (Hindu *Datura*), *D. stramonium* (Jimson weed) and *D. innoxia* (Thorn apple) were presented by Duke (31). This emphasizes on tropane alkaloids is responsible for pharmaceutical importance of this plant. This is further confirmed based on the fact that testing of crude ethyl acetate extract fraction and purified fraction of leaves with an employed panel of resistant bacterial strains showed that the pure fraction was more effective against standard MRSA strain and clinical isolates of skin and urethral infections (Fig. 2).

Table 9. FTIR peak values of the 7th fractions of ethyl acetate leaf extracts of *Datura stramoinum.*

S. No.	Peak values Absorption (cm^{-1})	Functional groups Names
1.	3631.75	Alcohol
2.	3465.07	Alcohol
3.	2985.43	Alkyls
4.	2085.83	Alkynes
5.	1742.14	Esters
6.	1373.98	Alkanes
7.	1241.51	Amines
8.	1046.94	Amines
9.	937.93	Alkanes
10.	847.10	Alkanes
11.	786.38	Alkanes
12.	608.24	Alkynes

Studies revealed the importance of *D. stramonium* in traditional medicine for treating

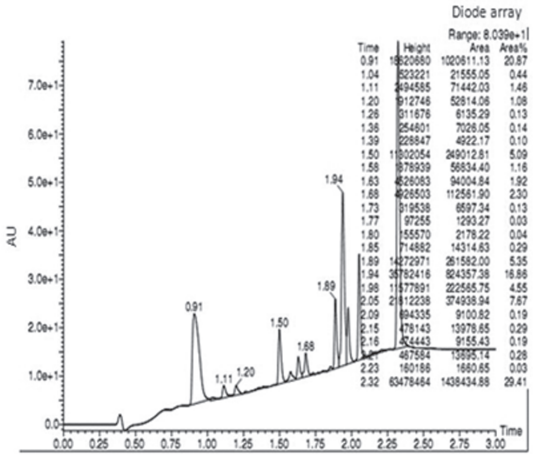

Fig.6. LC-MS analysis of all the bioactive fractions from ethyl acetate leaf extract of Datura stramonium.

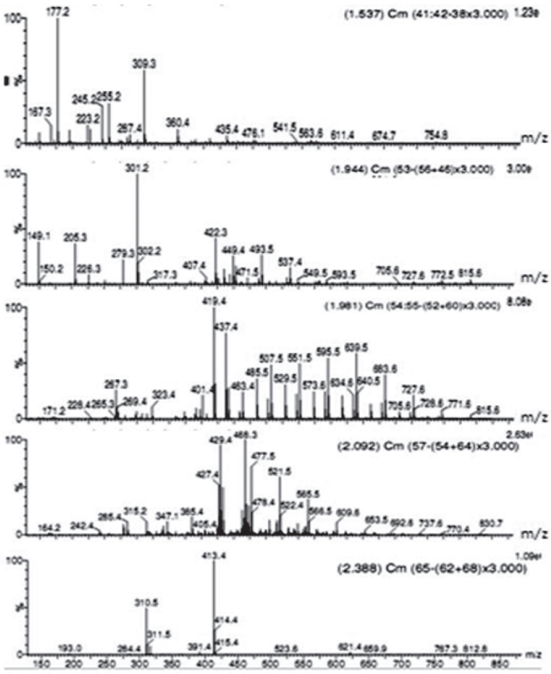

Fig. 7. Mass Spectroscopic analysis of all bioactive fractions from ethyl acetate dried leaves extract.

Asthma& Intestinal problems (32). However, shows toxicity when taken in high concentration or through oral route of administration and no toxic effects when used in low concentrations. In addition, it displays non-toxic effects when applied on upper layers of skin (33). Data related to acute toxicity of the compound hyoscyamine indicated that LD50 in mouse following intravenous administration is 95 mg/kg and in human 1.471 mg/kg. In human, probable LD oral dose is of 5 mg/Kg. In view of the above and present data on the demonstration of antibacterial activity of *Datura stramonium* against both standard and clinical isolates of resistant *Staphylococcus aureus* strains involved in skin and urethral supportive infections provide an immense scope for developing topical agents to cure bacterial skin and urethral infections. However, the actual structure involved in the purified bioactive fraction, its mode of action as well as any allergic and toxic effects on human skins need to be elucidated before going for clinical trials.

Conclusion

In view of the continuous rise of antibiotic resistant bacterial strains, the present study was undertaken to analyze antibacterial activity of the extracts of *Datura stromanium* and the findings clearly indicate that *D. stramonium* has profound antimicrobial activity against the Methicillin-resistant *staphylococcus aureus* strains. These results are supportive to the traditional use of *Datura metel* in phyto-medicine against multidrug resistant parasites of MRSA. The present investigation has opened up the possibilities for the use of purified bioactive fraction hyoscyamine from *D. stramonium* in the development of topical drugs for the treatment of skin lesions, wound infections and urinary tract infections caused by MRSA strains. Further research is being carried out in our laboratory for the determination of mechanism of action, toxicological studies and formulation of the compound.

Acknowledgements

The authors are grateful to University Grants Commission (UGC), New Delhi, India for the Research grants.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 793-803 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Optimization of fermentation culture conditions for Alkaline Lipase Production by *Bacillus flexus* XJU-1

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Abstract

Studies were carried out for optimizing extracellular alkaline lipase production by *Bacillus flexus* XJU-1 under submerged fermentation. Maximum alkaline lipase activity was observed at 36 h when 2% (v/v) refined cotton seed oil was used as a sole carbon source at an initial pH 11.0 at 37 °C with 2% inoculum (v/v), 1.5% (w/v) yeast extract as nitrogen source and 0.5% (v/v) Tween 80 as a surfactant. An overall 6.47-fold increase in alkaline lipase activity (from 2.83 U/ml to 18.31 U/ml) was achieved after optimizing the various nutritional and physicochemical parameters. The extracellular alkaline lipase was purified to homogeneity in a two-step procedure involving acetone precipitation and anion exchange chromatography. A specific activity of 312.4 U/mg with 40.1% recovery in enzyme activity in comparison to crude enzyme extract was recorded. The purified alkaline lipase was homogeneous as shown by a single band on SDS-PAGE with an apparent molecular weight of 16.1 kDa. The production of higher amount of lipase under strong alkaline conditions using a cheap oil substrate makes this strain a good candidate for detergent and leather industries.

Keywords: Optimization, cotton-seed oil, alkaline lipase, submerged fermentation, *Bacillus flexus* XJU-1

Introduction

Lipases (EC 3.1.1.3) hydrolyse lipids via the addition of water across ester bonds and constitute the third largest group of enzymes used in industries after proteases and carbohydrases. They have many potential applications in various industries and are chosen for each application based on the substrate specificity, position and stereospecificity as well as temperature and pH stability (1). However, lipases stable under higher alkaline conditions remain a major challenge till date. Animals, plants and microorganisms are source of lipases. The microorganisms are important source of lipases used in commerce owing to better properties (1,2). The bacterial lipases are often used in biotechnological applications due to good thermostability and improved storage conditions (2). The species of the genera *Bacillus*, *Pseudomonas* and *Burkholderia* are most important lipase producers (3). Gram-positive lipase producers include *Bacillus*, *Staphylococcus* and *Streptomyces* species. Among the different Gram-positive bacteria used in industry, the chief lipolytic producers belong to the genus *Bacillus* (2,4).

The lipase production by the bacteria is influenced by nutritional factors such as inorganic salts, carbon and nitrogen sources and physicochemical factors such as incubation period, inoculum concentration, incubation temperature, initial pH and dissolved oxygen concentration (2,3). Submerged fermentation is frequently used to produce bacterial lipases since most of these enzymes are extracellular as they are excreted through the cell membrane into the fermentation medium. In addition it allows a proper control of culture conditions (3). Optimization of nutritional and physicochemical parameters for maximum lipase production is vital

as these conditions help to improve the production levels and to develop an efficient cost effective fermentation process (5,6).

Although most commercial applications of lipases such as detergent formulation do not necessarily require pure enzyme, enzyme purification in some cases is essential as it allows studying the enzyme properties and understanding the three dimensional structure and the functioning of the enzyme. Pharmaceutical and medical industries also required purified enzymes (1). The precipitation, gel filtration chromatography, affinity chromatography and ion exchange chromatography are the common chromatographic techniques used to purify lipases and all have been led to better results. A combination of these methods is usually performed for the same purpose (1).

Reports on the alkaline lipases production by *Bacillus* species are scarce and the optimum pH reported in the alkaline region for various *Bacillus* species varies from 7.5 to 10.6 (4-13). There are no reports available so far with *Bacillus* species capable of producing alkaline lipase enzymes at an optimum initial pH of 11.0. We have recently reported the screening and identification of a novel alkaline lipase producing bacterium (14). In this paper, we report the optimization of fermentation conditions for alkaline lipase production by *Bacillus flexus* XJU-1 under shake flask fermentation using low cost substrate. Although many lipases of *Bacillus* species have been purified to homogeneity (4,6,15,16), the alkaline lipase of *Bacillus flexus* has never been purified. The paper also reports the purification of the enzyme to homogeneity.

Materials and Methods

***Microorganism*:** The microorganism screened earlier from a potato grown field of Bangalore (India) was used in the present work. It was identified as *Bacillus flexus* XJU-1 based on morphological and physiological properties as well as nucleotides homology and phylogenetic analysis (14).

Preparation of bacterial inoculum: A loopful of bacterial culture was taken from a 48 h agar slant and was used to inoculate 25 ml of nutrient broth contained in 50-ml Erlenmeyer flask. The flask was incubated overnight at 37 °C with shaking (S150 orbital incubator, Stuart, India) at 100 rpm. The broth obtained served as inoculum (10^8 CFU/ml) for submerged fermentation. The nutrient broth prepared and sterilized in the same conditions was used to standardize the inoculum.

Submerged fermentation and crude alkaline lipase preparation: The fermentation medium proposed by Bora and Bora (6) was used with slight modification and was composed of groundnut oil (1%, v/v), peptone (1%, w/v), $MgSO_4.7H_2O$ (0.04% w/v), KH_2PO4 (0.3%, w/v) and NaCl 0.01%. The pH of the medium was set to 10.0 with sodium carbonate of 2 M strength. 100 ml of cultivation medium in 250-ml conical flask was inoculated with 1 ml of inoculum and incubated at 37 °C for 48 h with shaking at 100 rpm. After incubation period, the fermentation broth was centrifuged at 10000 rpm in a C-30BL cooling centrifuge (Remi instruments, India) at 4 °C for 10 min. The supernatant served as enzyme source and was used to determine alkaline lipase activity.

Determination of crude alkaline lipase activity: The alkaline lipase activity was assayed spectrophotometrically as per the modified method of Sumathi and Meerabai (17) using 4-nitrophenylpalmitate (pNPP) in the substrate solution. 1 mg of pNPP was dissolved in 1 ml of isopropanol with the help of a CM 101 cyclomixer (Remi equipments, India) for 5 min at 30 ± 2 °C (solution A). 0.01 g of gum Arabic, 0.04 ml of triton X-100 and 9 ml of 50 mM Tris HCl buffer (pH 8.5) were mixed together with the help of a 2MLH magnetic stirrer (Remi equipments, India) for 10 min (solution B). The solution A was added in drops to the solution B on a magnetic stirrer with intense stirring to get a substrate solution. 0.05 ml of crude enzyme was added to 0.95 ml of substrate solution and incubated for 20 min at 37°C. After incubation period, the reaction was arrested by incubating in boiling water at 95 °C

for 5 min. The volume was made up to 4 ml with 50 mM Tris HCl buffer (pH 8.5). The blank used contained 0.05 ml of 50 mM Tris HCl buffer (pH 8.5) instead of enzyme. The released p-nitrophenol was assayed at 410 nm with a SL-159 UV VIS spectrophotometer (Elico, India). The reference curve constructed with p-nitrophenol in the 0.01 to 0.06 mM range was used to find out the unknown concentration of p-nitrophenol liberated. The amount of enzyme necessary to liberate 1 ìmole of p-nitrophenol per min per ml under the assay conditions was the unity (U) of the alkaline lipase activity.

Protein estimation: The amount of protein present in the samples was quantified using the method of Lowry et al. (18). A standard curve was constructed with bovine serum albumin (BSA).

Optimization of nutritional and physicochemical parameters: One factor was optimized at a time and the optimized factor was incorporated into the next experiment. The following culture conditions were optimized to obtain maximum alkaline lipase yield.

Effect of carbon source on alkaline lipase production: The influence of carbon source on enzyme production was investigated by replacing the ground nut oil in the cultivation medium with various oils. The coconut, sunflower, cottonseed, mustard, gingelly, ricebran and soybean oil were used. The inoculated flasks were incubated at 37 °C on an orbital shaker at 100 rpm for 48 h. The fermentation broth was then centrifuged at 10000 rpm in a cooling centrifuge at 4 °C for 10 min. The supernatant was used for alkaline lipase assay as described earlier. The effect of concentration of best carbon source was investigated by varying the concentration of the oil in the 0.5 to 3.5% (v/v) range. The lipase activity was assayed as described earlier.

Effect of inoculum concentration on alkaline lipase production: The effect of inoculum size on alkaline lipase production was studied by inoculating the fermentation media with different levels of inoculum ranging from 1 to 5% (v/v). The lipase activity was quantified as described earlier.

Effect of pH on alkaline lipase production: Effect of different initial pHs of the cultivation medium ranging from 7.0 to13.0 (with a gradual increase of one pH unit) on the enzyme production was investigated. Various buffers were used. The incubation was done at 37 °C for 48 h in a shaker incubator at 100 rpm. The enzyme activity was measured as earlier.

Effect of incubation temperature on alkaline lipase production: The effect of incubation temperature on alkaline lipase production was analyzed by incubating at 30, 37, 45 and 55 °C. The lipase activity was determined as described earlier.

Effect of nitrogen source on alkaline lipase production: The effect of nitrogen source at 1% (w/v) on lipase synthesis was studied by incorporating different organic and inorganic nitrogen compounds individually in the production medium in the place of peptone with the same amount. The organic nitrogen sources used were tryptone, yeast extract, asparagine, soybean meal, beef extract and urea, whereas potassium nitrate, ammonium chloride and ammonium nitrate were used as inorganic nitrogen sources. The effect of concentration of best nitrogen source was analysed in the 0.5 to 3% (w/v) range.

Effect of surfactants on alkaline lipase production: The effect of surfactant on alkaline lipase production was studied by adding individually Tween-80, Triton X-100, Dimethyl sulfoxide (DMSO) and sodium dodecyl sulfate (SDS) to the fermentation medium.

Time course of an alkaline lipase production: The time course studies on lipase production were carried out under optimized fermentation conditions namely (2% (v/v) refined cotton seed oil, initial pH 11.0, incubation temperature of 37 °C, 2% inoculum (v/v), 1.5% (w/v) yeast extract and 0.5% (v/v) Tween 80). The enzyme activities were recorded after every 12 h for a period of 72 h.

Purification of alkaline lipase of Bacillus flexus-XJU-1:

Partial purification by acetone precipitation: The crude enzyme was partially purified by acetone precipitation as per the modified method of Obi et al. (19) method with minor changes. 3 volumes of chilled acetone were slowly added to 1 volume of enzyme. The mixture was vortexed and incubated for 2 h at -20 °C. It was then centrifuged at 10000 rpm for 15 min at 4 ^{O}C. The supernatant was discarded and the pellet obtained resuspended in a minimum volume of 0.1 M Tris-HCl buffer pH 9.0. Resuspended partially purified enzyme was further concentrated by lyophilisation (Freeze dryer, Model LY3TTE, Snijders Scientific, Tilburg Holland) and subjected to the total purification.

Total purification by anion exchange chromatography: An anion exchange chromatography was carried out using DEAE-cellulose column (2.0 X 25 cm) as reported by Annamalai et al. (15). The enzyme solution obtained in the above step was applied to the column pre-equilibrated with 0.1 M Tris-HCl buffer pH 9.0. Fractions of 3 ml each were collected in different test tubes by a linear gradient of 0.1 - 1 M NaCl (prepared using Tris-HCl buffer instead of distilled water) at a flow rate of 0.4 ml/min. The lipolytic activity was determined in each fraction. Fractions having higher activities were pooled together and concentrated by lyophilization. The lipase activity, protein content and specific activity of the crude, partially and completely purified enzyme were determined. The purified enzyme was used in sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and molecular weight determination: The molecular weight was determined by SDS-PAGE as per Laemmli (20). It was carried out on 15% polyacrylamide gel with 6% stacking gel. SDS-PAGE was also used to check the purity of the alkaline lipase. The protein bands were visualized by coomassie brilliant blue R-250 stain after the electrophoresis. The molecular mass of the alkaline protease was estimated by comparing its mobility with that of the proteins in the marker (kDa): phosphorylase b (97.4), bovine serum albumin (66.0), ovalbumin (43.0), carbonic anhydrase (29.0), soyabean trypsin inhibitor (20.1) and lysozyme (14.3).

Statistical analysis: Three independent experiments for each treatment were carried out to determine enzyme activities. Means of variable and standard deviation were calculated. The significant differences among the enzyme activities were tested by one way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) by running SPSS software at the 5% significance level.

Results and Discussion

The *Bacillus flexus* XJU-1 used in the present study was screened from potato field soil and identified based on cultural, microscopic, biochemical and molecular techniques (14). Submerged fermentation was chosen because most of bacterial lipases are extracellular as they are excreted through the cell membrane into the fermentation medium (3). Since lipase production by the bacteria is influenced by nutritional and physicochemical factors, they were optimized in order to maximally produce the enzyme. As the p-nitrophenylpalmitate led to a low background color and formed a suspension in the buffer similar to that of fat compounds, it was selected for lipolytic activity determination (16).

Effect of carbon source on alkaline lipase production by Bacillus flexus XJU-1: Lipid substrates like vegetable oils in combination with nitrogen sources such as peptone and yeast extract are generally used by microorganisms to manufacture lipases (3). Among the different carbon sources used, a significant lipolytic activity was recorded with cotton seed oil although statistically at par with sunflower oil (Fig. 1). This may be attributed to its higher unsaturated fatty acids content. Similarly, Chakraborty and Raj (4) have tested various vegetable oils for lipase production and the maximum yield was found with sunflower oil and olive oil because of their higher amounts of unsaturated fatty acids. Likewise, the lipase isolated from *Bacillus* sp. LBN2 was able

to rapidly hydrolyze oleic acid-containing oils (cottonseed oil, groundnut oil, olive oil) (6). The cottonseed oil was also adequate carbon source for lipase production by *Aspergillus terreus* (17).

Effect of varying carbon source concentration on alkaline lipase production by Bacillus flexus XJU-1: The influence of varying cotton seed oil concentration was studied and the maximum alkaline lipase production was noticed with 2% oil while a decrease was observed for higher concentrations (Fig. 2). When a higher oil concentration is used, the bacterial culture aeration is affected leading to a poor growth resulting in less enzyme yield. Likewise, a little yield was noted due to a poor growth resulted from an improper culture aeration caused by a high oil concentration (21). The decrease in lipase production was also ascribed to proteases in the culture medium (6,11). In Bacillus sp. RSJ1, maximum lipase production was obtained with cottonseed oil at concentration of 0.75% (in combination with 0.5% Tween-80) (9).

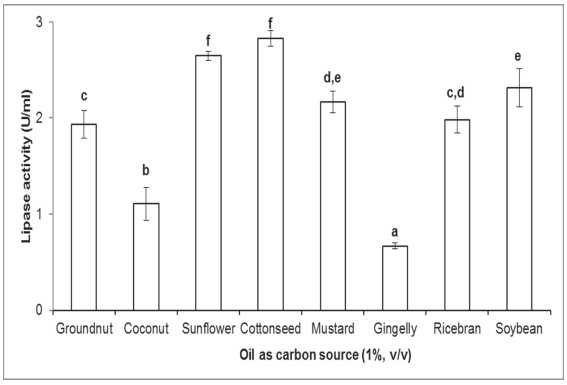

Fig. 1. Effect of different vegetable oils as carbon sources on alkaline lipase production by Bacillus flexus XJU-1. The cultivation media of pH 10.0 were inoculated with 1% inoculum size and incubated at 37 °C with shaking at 100 rpm for 48 h. The groundnut was used as control. The different letters on the error bars show significant difference at $P_{0.05}$.

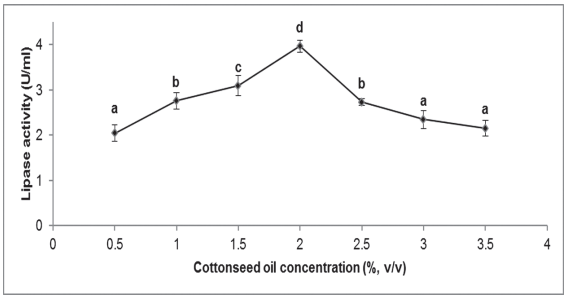

Fig. 2. Influence of different concentrations of cottonseed oil on alkaline lipase production by *Bacillus flexus* XJU-1. The cultivation media of pH 10.0 were inoculated with 1% inoculum and incubated at 37 °C with shaking at 100 rpm for 48 h. 1% cotton seed oil served as control. The different letters on the error bars show significant difference at $P_{0.05}$.

Effect of inoculum concentration on alkaline lipase production by Bacillus flexus XJU-1: The inoculum concentration plays a crucial role in bacterial growth and thus in lipase production. Different inoculum concentrations ranging from 1 to 5% were investigated for alkaline lipase production by *Bacillus flexus* XJU-1. A marked increase in lipase production (2.2-fold) was observed with 2% inoculum when compared to control (1%) (Table 1). Baharum et al. (22) reported that the nutrient and oxygen levels support sufficient bacterial growth at appropriate inoculum level leading to maximal lipase production. At low or high inoculum concentration, the lipase activity was low. Indeed, if the inoculum level is too low, a little growth is observed and less alkaline lipase yield is obtained whereas at higher inoculum, a decrease in enzyme yield is seen owing to insufficiency of total dissolved oxygen and nutrient supply in the fermentation media. Supportively, a decrease in lipase activity was seen when a higher inoculum concentration was used (23). 1% (v/v) inoculum gave the highest lipase production in *Bacillus licheniformis* MTCC 6824 (4) and in *Bacillus tequilensis* NRRL B-41771 (24). Contrastingly, the maximum lipase production by *Bacillus pumilus* was noted when a higher inoculum size of 10% (v/v) was used in the fermentation on medium (10). Therefore, the influence of inoculum on lipase production may depend on the type of the bacterium and on the size of the bacterium which will determine the number of bacterial colony in an inoculum.

Table 1. Effect of inoculum concentration, incubation temperature, yeast extract concentration and surfactant on alkaline lipase production by *Bacillus flexus* XJU-1. Means and standard deviation are shown for each treatment. In rows, the values with different letters are significantly different at $P_{0.05}$.

Inoculum concentration (%, v/v)	1	2	3	4	5	6
Lipase activity (U/ml)	3.95±0.19^{b}	8.78±0.30^{d}	6.15±0.34^{c}	4.15±0.17^{b}	1.95±0.14^{a}	
Incubation temperature (°C) Lipase activity (U/ml)	30 9.08±0.18^{b}	37 10.54±0.14^{c}	45 10.16±0.23^{b,c}	55 6.34±1.11^{a}		
Yeast extract concentration (%, w/v) Lipase activity (U/ml)	0.5 12.06±0.32^{c}	1.0 13.43±0.40^{d}	1.5 14.55±0.44^{e}	2.0 14.31±0.50^{e}	2.5 10.56±0.41^{b}	3.0 8.49±0.71^{a}
Surfactant (0.5%, v/v) Lipase activity (U/ml)	Control 14.95±0.70^{a}	Tween-80 16.53±0.58^{b}	Triton X-100 15.45±0.21^{a}	DMSO 14.60±0.68^{a}	SDS 14.70±0.15^{a}	

Effect of initial pH on alkaline lipase production by Bacillus flexus XJU-1: The pH of the fermentation medium plays a vital role in nutrients absorption, bacterial growth, enzyme production and enzyme release (6). The effect of different initial pHs on lipase production was analyzed in the alkaline region. The bacterium was able to grow on all the initial pHs tested and maximum enzyme production occurred in the range of 9.0 to 12.0 with optimal production at pH 11.0 (Fig. 3). Hasan and Hameed (8) reported that the variation in the enzyme yield at different initial pHs may be attributed to the strain specificity. At optimum pH, the enzyme yield is maximum; however any deviation lead to a decrease in enzyme production. This may be attributable to the disruption of transport mechanisms across the microbial membrane that may prevent enzyme release (25). The maximum alkaline lipase production by different Bacillus species in the alkaline region from 7.5 to 10.6 has been reported (4-13).

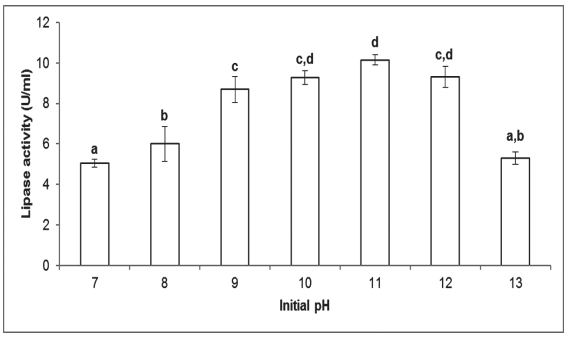

Fig. 3. Influence of different initial pHs on an enzyme production by *Bacillus flexus* XJU-1. The cultivation media were inoculated with 2% inoculum and incubated at 37 °C with shaking at 100 rpm for 48 h. The pH 10.0 was control. The different letters on the error bars show significant difference at $P_{0.05}$.

Effect of incubation temperature on alkaline lipase production by Bacillus flexus XJU-1: The physical factors like incubation temperature, initial pH and aeration play a vital role in lipase production as they modulate bacterial growth (3). The influence of lipase production by *Bacillus flexus* XJU-1 was studied in the temperature range 30-55 °C. The maximum incubation temperature was observed at 37 °C although statistically at par with 45 °C (Table 1). Similarly, the optimum incubation temperature for lipase production by Bacillus species in the range of 30 to 50 °C was reported by various researchers (6,8,10,11,13,24). The low activity value was recorded at 55 °C (Table 1). Lipase production in Bacillus mycoides was stopped when the incubation temperature was above 50 °C (5). The low value at 55 °C may be due to thermoliability of the alkaline lipase.

Effect of nitrogen source on alkaline lipase production by Bacillus flexus XJU-1: A nitrogen

source in the cultivation medium is required for the synthesis of proteins (including enzymes) and nucleotides. The influence of nitrogen source on alkaline lipase production was investigated using various organic and inorganic nitrogen sources. The highest lipase activity was attained in the medium containing yeast extract although statistically at par with soybean meal (Fig. 4). Similarly, yeast extract was the best organic source in *Bacillus subtilis* (8) and in *Bacillus pumilus* (10). Indeed, yeast extract apart from acting as an adequate nitrogen source; it also supplies B complex vitamins and minerals to the fermentation medium that act as activators in the bacterial growth and lipase production. All the organic nitrogen sources tested supported lipase production than the inorganic nitrogen sources (Fig. 4). Likewise, the organic nitrogen source such as peptone and yeast extract was preferred by *Bacillus* species than inorganic nitrogen source (5-7,9). This may be ascribed to the presence of some vitamins and accessory growth factors in them (3).

Effect of varying nitrogen source concentration on alkaline lipase production by Bacillus flexus XJU-1: The effect of varying yeast extract concentration on enzyme production was studied. A gradual increase followed by a decrease in lipase production was observed with maximum production at 1.5% as par with 2% (Table 1). 0.75% yeast extract (in combination with 0.75% peptone) led to a higher yield in Bacillus sp. RSJ1 (9). Contrastingly, a low yield in lipase was obtained when yeast extract (1%, w/v) was used as nitrogen source in Bacillus tequilensis NRRL B-41771 (24). A decrease in lipase activity seen at higher nitrogen source concentration may be probably attributed to nitrogen metabolite repression.

Effect of surfactants on alkaline lipase production by Bacillus flexus XJU-1: The effect of surfactants on lipase production may depend on microorganism, type of surfactant and its concentration (26). The different surfactants were analyzed to evaluate their capacity to support alkaline lipase production. The maximum lipase production was attained with Tween-80 (Table 1). This may be ascribed to the modification of plasma membrane by Tween-80 promoting both uptake and release of compounds. Lipase biosynthesis in *Bacillus subtilis* was stimulated by Tween-80 (8). Similarly, an increase in lipase production in *Bacillus licheniformis* MTCC-10498 was observed when Tween-80 was used as surfactant (12). Contrastingly Bisht et al. (23) tested various surfactants; the highest lipase yield was recorded with Triton-X-100 whereas a decrease in yield was observed with Tween-80 and SDS. In the present study, the difference in lipase activities was not statistically significant for other surfactants when compared to control (Fig. 8). An increase in lipase production in presence of a surfactant is not always observed (27).

Time course study of the alkaline lipase production by Bacillus flexus XJU-1: The influence of incubation period was investigated to find optimal time for maximum lipase production under optimized fermentation conditions. The highest enzyme production was achieved at 36 h of incubation and declined thereafter (Fig. 5). The decline may be ascribed to the accumulation of fatty acids from cottonseed oil hydrolysis or presence of protease in the sample. The decrease in enzyme activity was due to the accumulation of fatty acids (and glycerol) resulted from hydrolysis of olive oil (28) or proteolytic degradation (6,11,21). The maximum incubation time in the range of 24 h to 72 h was reported for various Bacillus species (6,8,10,11,24). All the fermentation flasks were shaken at 100 rpm. Moderate shaking allows a proper mixing of constituents thereby favoring lipase production. Likewise, low agitation rates (for example 100 rpm) were repeatedly reported to give maximum yields for most Bacillus species (3,10).

After optimizing the various nutritional and physicochemical parameters, a lipolytic activity of 18.31 U/ml was observed. An activity of 55.3 MLU/liter at pH 9.0 by titrimetrically using olive oil emulsion in Pseudomonas alcaligenes M-1 was noted and the lipase was able to remove fat fatty

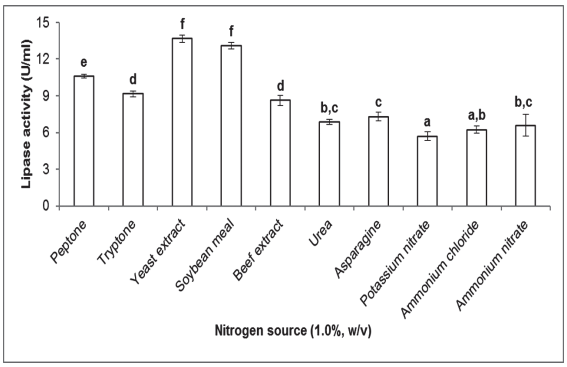

Fig. 4. Influence of different organic and inorganic nitrogen sources on alkaline lipase production by Bacillus flexus XJU-1. The cultivation media of pH 11.0 were inoculated with 2% inoculum and incubated at 37 °C with shaking at 100 rpm for 48 h. The control medium contained peptone. The different letters on the error bars show significant difference at $P_{0.05}$.

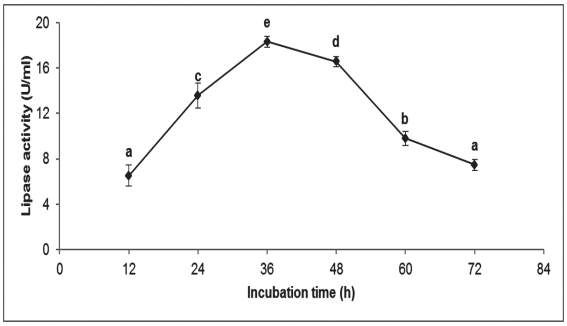

Fig. 5. Time course of alkaline lipase production by *Bacillus flexus* XJU-1. The cultivation media of pH 11.0 were inoculated with 2% inoculum and incubated at 37 °C with shaking at 100 rpm. The enzyme activities were noted at regular intervals after exactly 12 h. The different letters on the error bars show significant difference at $P_{0.05}$.

stains under modern washing conditions (29), 2 U/ml at pH 8.0 was observed in *Bacillus licheniformis* MTCC-10498 using p-NPP as substrate (12), 4.83 U/ml at pH 8.5 using p-NPP was recorded in *Bacillus pumilus* F3 (10), 5.0 U/ml at pH 8.0 by titrimetrically using olive oil emulsion in *Bacillus* sp. SS-1 (11), 18 U/ml at pH 9.0 using using p-nitrophenyllaurate (p-NPL) was seen for *Bacillus* sp LBN 2 (6), 41.7 U/ml at pH 8.0 using p-NPP was recorded for *Bacillus licheniformis* MTCC 6824 (4), 44 U/ml at pH 8.0 with p-NPL in *Bacillus* sp. (8), 730 U/ml at pH 9.0 was noted by titrating the fatty acids liberated from olive oil with 1 M NaOH solution in *Bacillus licheniformis* (15). It is therefore difficult to compare the enzyme activities as the conditions and substrates used to determine lipase activities are different.

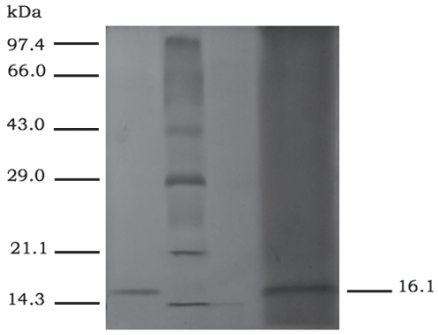

Fig. 6. SDS-PAGE pattern of purified alkaline lipase of *Bacillus flexus* XJU-1. Lane 1 (left): purified alkaline lipase, lane 2: protein molecular weight marker [phosphorylase b (97.4), bovine serum albumin (66.0), ovalbumin (43.0), carbonic anhydrase (29.0), soyabean trypsin inhibitor (20.1) and lysozyme (14.3)], lane 3 (right): crude alkaline lipase.

Table 2. Purification profile of the alkaline lipase of Bacillus flexus XJU-1

Purification step activity (U/mg)	Total activity (U) Purification fold		Total protein (mg) Recovery (%)		Specific
Crude extracellular extract	6231.4	273.0	22.8	1.0	100.0
Acetone precipitation	4717.2	46.8	100.8	1.3	75.7
Ion exchange chromatography	2499.0	8.0	312.4	2.5	40.1

Vol. 7 (3) 793-803 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Purification of alkaline lipase of Bacillus flexus XJU-1: The Bacillus flexus culture supernatant was subjected to acetone precipitation and ion exchange chromatography, a recovery in enzyme activity was 40.1% with 2.5-purification fold (Table 2). Two steps were thus sufficient to purify enzyme. Indeed minimum purification steps are preferable since a loss of about 10 % enzyme yield is seen at each processing step. In addition, as enzyme purification is expensive, lesser steps are prerequisite to reduce the enzyme cost in industries (6). Using DEAE-cellulose packed column, a recovery of 25% with 4250 U/mg was observed in *Bacillus licheniformis* (15). A specific activity of 520 U/mg with a yield in lipase activity of 8.36% was also recorded in *Bacillus licheniformis* MTCC 6824 (4), whereas 398 U/mg and 48% were noted in *Bacillus* sp LBN 2 (6).
Table 2. Purification profile of the alkaline lipase of *Bacillus flexus* XJU-1

***SDS-PAGE and determination of molecular weight*:** With the help of medium range standard molecular weight markers, the molecular mass of the purified alkaline lipase was estimated to be 16.1 kDa on SDS-PAGE (Fig. 5). It was found to be a monomer protein as shown by a single band. Similarly, 16 kDa was obtained for lipase of *Bacillus thermocatenulates* (30). Bora and Bora (6) reported that lipases are in general monomeric proteins with molecular weights ranging from 16 kDa to 90 kDa. For example, 19 kDa was found for lipase of *Bacillus subtilis* 168 (16), 33 kDa in *Bacillus* sp LBN 2 (6), 35 kDa in *Bacillus licheniformis* (15) and 74.8 kDa in *Bacillus licheniformis* MTCC 6824 (4). The molecular weights of *Bacillus* alkaline lipases are thus different from one another and this may be ascribed to the genetic differences.

Conclusion

In the present investigation, the *Bacillus flexus* XJU-1 was able to secrete higher amount of extracellular alkalophilic moderate thermostable lipase after 36 h using cottonseed oil. A short incubation period and use of a cheap substrate make the submerged fermentation cost-effective. The bacterium can thus be exploited for use in detergent industries and other biotechnological applications.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 804-807 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Evaluation of Hematological and Biochemical Parameters of Magnetic Field Exposed Albino Rats

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Abstract

The present investigation was carried out by exposing the adult male albino rats to the magnetic field of 202μT against the control group and both were analyzed for haematological and biochemical changes. The results showed that the magnetic field exposed (MF) animals showed significant increase in RBCs, WBCs, Hbs and platelet count as well as decrease in Red blood cell indices values of MCV, MCH and MCHC. In addition, the MF exposed group also showed significant increase of AST and ALT levels in plasma indicating the involvement of MF on liver cell membranes. The haematological parameters and liver enzymes were affected by the electromagnetic field exposure suggesting the possible induction of hazardous biological effects during the exposure to magnetic field.

Key words: Electromagnetic field, blood indices, liver enzymes, AST and ALT, Rats.

Introduction

Magnetic radiation is well associated with vast areas like industries, military and medical fields etc. The radio frequency portion of magnetic field is in the range of (0.5 MHz-100GHz) which can affect various organs and physiological systems (1,2). Generally, magnetic fields are generated by the physical movement of the electrical charge (or) current. The preponderance of evidence suggested that the low-power, low frequency, electromagnetic radiation associated with household current does not constitute a short or long term health hazard but some haematological and biophysical mechanisms will be affected and results in cancer (3).

Several haematological and biochemical variables are sensitive to exposure to magnetic field in animals as well as in humans which can act on various ions of all organs thus altering the cell membrane potential as well as the distribution of enzymes and cellular functions (4). It is also reported that magnetic field can also influence on the enzyme action, signal transduction, protein synthesis and gene expression (5, 6). On the other hand non- ionizing electromagnetic field on the human body are useful for various therapeutic purpose. The exposure of MF of 128 mT for 1h/ day for 10 consecutive days on albino rats induces an increase in hematocrit, hemoglobin (3) and tissue enzymes released within the blood. Moreover the high magnetic fields exposure also induces the behavioral and neural changes in rats (7).

The main damaging role of magnetic fields may be on the cellular membrane of living organisms which is complex process, but it is of more interest to give an insight into the expected hazards and the proper ways of its use and how organism can be protected from MF (8, 9). AST and ALT are the liver enzymes which are affected by the applied magnetic field. The elevation of AST and ALT in the plasma is an indicator of hepatotoxicity (6,10,11,12). Earlier studies have reported that the effects of chronic exposure of magnetic field and there is a few reports on the effect of acute exposure of magnetic field on

Vol. 7 (3) 804-807 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

albino rats. Hence this study was aimed to investigate the haematological and biochemical parameters on acute exposure of magnetic field on rats.

Material and Methods

Adult male albino rats weighing in the beginning of the experiment from 150 to 180 gm were taken for the study and were divided into two groups namely, control (n=6) and study group (n=6). The institutional animal ethical committee approved the experiment protocol of the study. All the animals were housed in plastic cages and normal ventilation, water and food were provided.

Exposure system: The exposure of magnetic field was done in specially made MF chamber consisting of two circular coils fixed with a small distance in between the coil whose diameter was 35cm. These coils having 1000 turns and was connected in series to avoid the maximum heat generation of coil and to provide the magnetic field of 202μT at the center of the coil where the rats were placed. The magnetic fields of the instrument were measured manually according to standard protocol. A uniform magnetic field was produced over a coil (Fig.1). In one cage, six rats were placed in the center of the coils. The experimental groups were exposed to MF for 6 hours (202μT) and the control animals were not exposed to MF.

Fig. 1. Magnetic Field exposure system

Biochemical analysis

Estimation of activity of Alanine Transaminase (ALT) (EC. 2.6.1.2) and aspartate transaminase (AST) (EC.2.6.1.1): Alanine Transaminase (L-Alanine: á-oxoglutarate amino transferase) and Aspartate transaminase (L-Aspartate: á-oxoglutarate amino transferase) levels was estimated by the method of Reitman and Frankel (1957). 500ml of ALT and AST substrate was incubated individually with 0.05 ml of serum or liver tissue homogenate along with standard tubes having standard pyruvate, taken at a concentration ranging from 0.2 to 0.8 ìM in separate tubes, at 37^{O}C for 30 min for ALT and 1h for AST. The reaction was arrested by addition of 1.0 ml DNPH and all the tubes were left at room temperature for 20 min. simultaneously, a control each for serum and liver tissue homogenate was prepared separately. These tubes contained ALT substrate alone and the serum and liver tissue homogenates were added after addition of DNPH. Finally, the colour was developed in all above tubes by addition of 5 ml of 0.4N NaOH and their absorbance was measured at 540 nm against blank using spectrophotometer. ALT levels in serum are expressed as IU/L and in tissue as IU/gm tissue.

Results and Discussion

The Fig. 2. Summarizes the blood parameters of RBC, WBC and Hb and shows the significant increases of RBC, WBC and Hb values when compared with the control group. Fig.3. shows the Red cell indices values where there is a significant decrease of MCV, MCH and MCHC which may be attributed to decreasing RBC. Fig.4 and 5.shows a significant increase of platelet

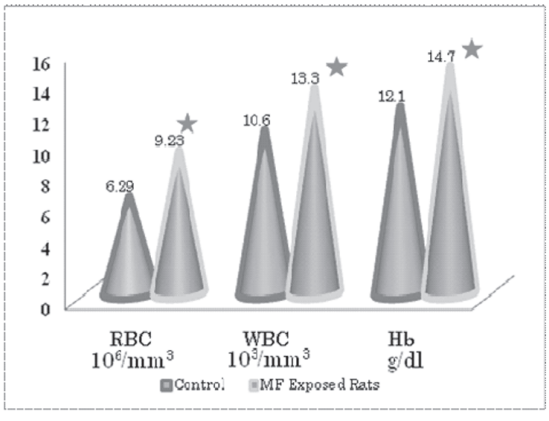

Fig. 2. Effect of electromagnetic field exposure on blood indices - RBC, WBC and Hb

count and plasma liver enzymes, respectively. The liver enzymes AST and ALT were increased in the study group of rats when exposed to the magnetic field as compared to the control groups. The acute exposure of rats to the magnetic field of 202 T showed a general increase in RBC, WBC and Hb. This increase in values can be due to harmful effect of magnetic field exposure which stimulates the haemopoietic system to release of RBC, WBC and Hb (3). There was a significant increase in Red cell indices values due to significant increase of RBC and Hb in the blood stream. The Mean corpuscular volume was decreased as the RBC level increases. Similarly the MCH and MCHC is also decreased. The above results concurs with the earlier studies of Robert *et al*., where they proved that the applied magnetic field affects erythropoietin levels in rats of the study groups (2,4).

The significant increase of the liver enzymes AST and ALT which are known biomarkers of liver were observed in our study thus indicating a form of biomagnetic injury in organs due to the effect of magnetic field (6,7,13,14). In general, this magnetic field having radiofrequency range of wavelength which can easily penetrate into the cell having the same inter atomic distance which affects the dipoles of the cells (13). This changes the membrane potentials and likewise produces injuries to the entire organs.

The AST and ALT concentration was increased in the plasma after acute exposure of magnetic field in our study when compared to the control (15). This magnetic field identifies itself as a type of acute stress in rats and during this stressful condition most of the biochemical parameters changes are well documented in many earlier studies. To summarize, the MF of strength 0.5 MHz-100 GHz can affect the hematological parameters and AST and ALT levels in albino rats and this type of acute magnetic stress can also induce behavior changes in these small animals.

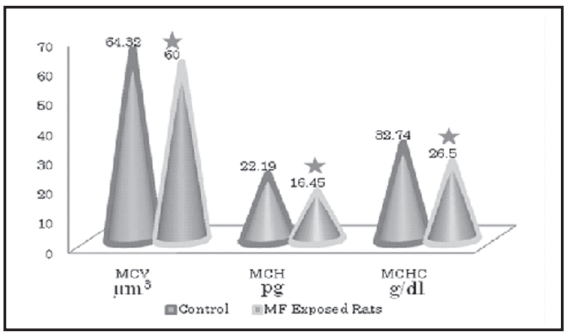

Fig. 3. Effect of electromagnetic field exposure on blood indices – MCV, MCH and MCHC

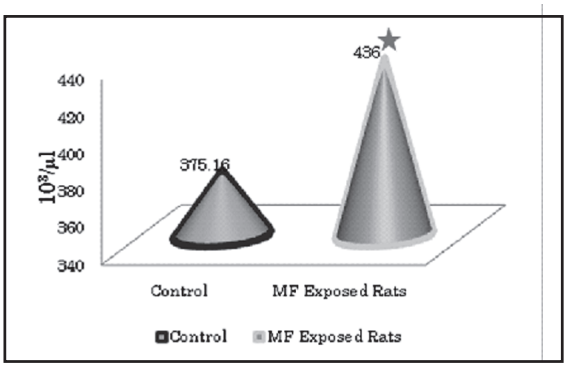

Fig. 4. Effect of electromagnetic field exposure on platelets

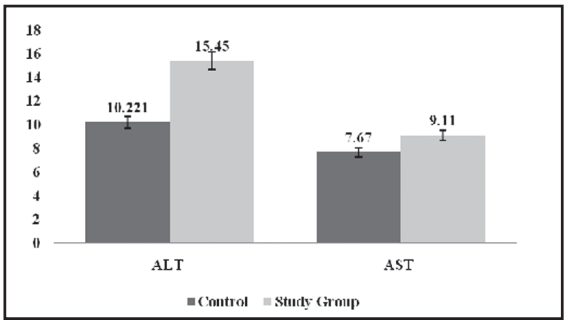

Fig. 5. Serum ALT and AST activities in liver of albino rats after exposure of electromagnetic field

Conclusion

Based on the above hematological and biochemical analysis, this study could suggest that similar changes in humans can also occur when they are exposed constantly to various

magnetic fields of various home electronic appliances.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) 808-824 July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Psoriasis: History, Present and Future Prospects

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Abstract

Psoriasis is a chronic condition which causes the inflammation of the skin due to the activation of immune system leading to abnormal keratinocytes proliferation. The keratinocytes mature within a week as compared to normal cycle of 27 to 28 days. The oldest of the treatment includes the use of coal tar, Dithranol, sunlight, Dead Sea salts and emollients. Dermatologists now have a host of new treatments for psoriasis, using immunobiologics to combat this disease. The first line treatment includes topical therapy with corticosteroids and phototherapy treatment. Immunomodulators have been use as an option for second line treatment, but most of them as off label therapies. The potent candidates include calcineurin inhibitors and cytotoxic agents. Methotrexate is considered as a gold standard against which the efficacies of other biologics are being evaluated. Novel drug delivery systems are also on their way for the treatment like methotrexate niosomal gel.

Keywords: psoriasis, corticosteroids, biological agents, calcineurin inhibitors, immuno-suppressants

Introduction

Psoriasis is one of the most common skin diseases affecting both men and women. The word has been derived from Greek word psora meaning itch. Psoriasis is not a contagious disease as it looks like. Psoriasis is mainly an autoimmune disease in which body's own immune system leads to excessive proliferation of keratinocytes. Recent findings indicate the involvement of cell mediated immune system in the epidemiology of psoriasis. The excessive proliferation leads to physical characteristics involving multilayered, thickened silver colored, scaling plaques. The squamous keratinocytes in psoriasis release very less amount of extracellular lipids which play an important role in adhesion of corneocytes, which results in scales and flakes. The active plaques are encircled by white vasoconstricted skin which results from local overproduction of prostaglandins. The disease has a much larger psychological impact which substantially affects the quality of life. Psoriasis is also associated with increased risk of many other diseases. At least 10% to 20% of the population suffering from psoriasis develops arthritis also known as psoriatic arthritis (1). The disorder is chronic and reoccurring which vary in severity in different genetically susceptible populations. An attempt has been made in the present manuscript to deliver information about the types of treatments available over the last few decades for the treatment of psoriasis. The disadvantages of the drugs used earlier that led to the development of newer strategies for treating psoriasis, have also been highlighted in the current article.

Epidemiology : The prevalence of psoriasis is almost equal in both the genders and is irrelevant of the age group. The most common occurrence is in the age of 15-35 years. 10-15 % of the cases have been diagnosed in children who are below

the age of 10 years of age (2). It has been rarely observed from birth. The mean age of occurrence is estimated to be 33 years. It affects mainly 3% population of America (25% having severe symptoms) and 2-3% in United Kingdom. The disease is most prevalent in Caucasians having a rate of 60 cases per 100000 in a year. The psoriasis has least occurrence in African and Asian countries with least value of 0.1%. China has only 0.3 % occurrence of psoriasis among total population. The least prevalence has been found in some ethnic groups of Japan and Indian natives from South America (3).

Psoriasis and psoriatic arthritis are ailments with genetic components as well. In the genetic analysis of patients suffering with psoriasis, many susceptibility loci have been identified in the recent findings. Family studies on genetic basis provide sufficient evidence that psoriasis have a strong genetic basis. In case of twins, the probability in monozygotic twins was 62-70% as compared for 21-23% dizygotic twins (4). The major gene locus PSORS1 has been mapped on chromosome 6p21.3 and is mainly responsible showing susceptibility to psoriasis and psoriasis arthritis.

Environmental factors have shown their direct effect in induction of psoriasis along with the quality of life geographically. A large number of endogenous and exogenous factors contribute to psoriasis such as stress, trauma, injury, infections, smoking, drugs and many more. Higher rates of incidence have been found in eastern Africans than in western Africans. It has been demonstrated by the fact that western Africans lack the main susceptibility loci as compared to eastern Africans. The regions having high humidity have recorded low cases of psoriasis as compared to dry hot areas. Low incidence of psoriasis have been observed in people having diet rich in proteins and low in carbohydrates such as the population of Canada, Greenland and Siberia (5).

Pathogenesis : Today psoriasis comes under the category of most prevalent autoimmune disease occurring due to inappropriate and unwanted activation of cellular immune system. Human skin is a primary lymphoid organ which is well equipped with keratinocytes, endothelial cells, epidermal T cells, different macrophages, draining nodal cells and other cells which form a part of human immune system. In case of psoriasis, an unknown antigen causes the Antigen Presenting Cells to activate in the epidermis resulting in whole cascade of inflammation. The maximum T–lymphocyte infiltration includes CD4+ in the dermis region and CD8+ in the epidermis region (3). The adhesion molecules which are responsible for leukocyte adherence and inflammatory pathway are expressed at higher levels in psoriasis. The activated T cells increases secretion of mRNA for interleukins (IL2), resulting in increased synthesis of IL-2 receptors as well. The cytokine production is also elevated, which includes TNFá, IFN-Ò, IL-4, IL-10 and IL-11, each down regulating and modifying others responses. The up regulation of the genes, which code for vascular endothelial growth factor increases vascularization and angiogenesis which is observed in psoriasis (Fig.1). The inflammatory response of body alters the normal epidermal cycle of 28 days to 2-4 days, leading to scaly plaques.

Types of Psoriasis : The severity, location, and appearance of psoriasis depend on the kind of psoriasis a person has. Plague psoriasis is the most common and widely spread form of psoriasis. The patches in psoriasis may appear as small red spots or may be in the form of scales and patches. Psoriasis has been classified into pustular and non pustular depending upon the presence of pustules. In pustular psoriasis, white blood cells are present in pus present in blisters. Guttae and inverse psoriasis have least occurrence among all types of psoriasis.

Novel Drug Delivery Approach for Psoriasis : The topical route always remains the first choice for producing a local effect. A survey reported that about 70% of the patients remain unsatisfied with the treatment from conventional dosage forms and other routes of administration (40). So, novel

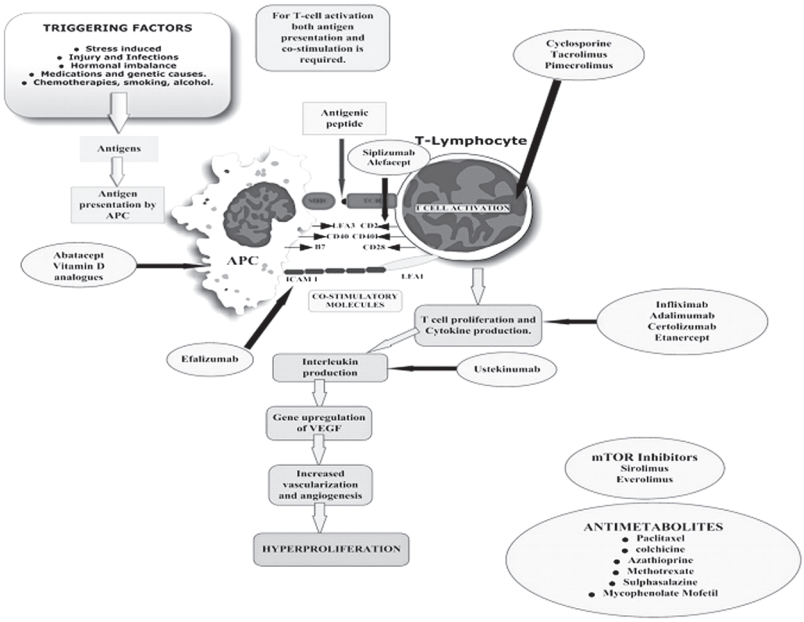

Fig. 1. Overview of the processes involved in the activation of immune system in psoriasis and the drug targets.

Table 1. Various types of psoriasis as defined clinically

Pustular (3,6): Pustular psoriasis appears as raised eruptions which consist of non infectious pus.	Von Zumbusch or Generalized pustular	The pustules appear on a larger part of skin and mainly consist of white blood cells.
	Palmoplanter psoriasis	Several patterns of pustules appear on palms of hands and feet.
	Acropustolosis	The pustules appear on the finger tips and toes
Non pustular (3,6) : In non pustular there are no signs of pustules or eruptions.	Inverse psoriasis or flexural psoriasis	It affects the body folds. Due to its area of occurrence, they are much worsened by friction and are mostly uncomfortable.
	Nail psoriasis	Mainly affects the nails which lead to discoloration of nail plate and formation of pits and crumbling of nails. Onycholysis and ridging of nails occur.
	Guttae psoriasis	Highest occurrence has been observed in children and teenagers. It causes water drop like sores on the body
	Scalp psoriasis	Mainly affects back of the head (scalp). White scaly patches are developed leading to itching and hair loss.

Table 2. Various agents used before 19th century

Therapeutic agents and Formulations	Information regarding therapeutic agents	Adverse drug reactions and contradictions.
Arsenic: Argue drops, Fowler's solution and Asiatic pills (7)	Arsenic was used as a sulphide derivative in paste form for skin disorders A tasteless formulation of arsenic known as Argue drops was preached.	Arsenic poisoning, nerve injury and cancer
Ammoniated Mercury: Ammoniated mercury ointment	Ammoniated mercury was used as an ointment. Mercury salts were best suited for psoriasis of palms and soles. Now days it is occasionally prescribed due to cases of heavy metal poisoning and skin deposition (7).	Mercury poisoning, skin rashes. Contraindicated with sulphur and iodine.
Anthralin derivatives: Ingram regimen	Anthralin (Dianthrone) was used as a bright yellow cream. The structure activity relationship of Anthralin was used to develop new derivatives like hydroxyl anthrones. The patients, treated were wrapped in stockings for about 24 hours and the procedure was repeated every day.	Local irritation, burning and staining Contraindicated in hypersensitivity and eruptive psoriasis.
Salicylic acid: Creams, ointments and cleansing soaps	Topically used 2-3 times a day earlier in the form of a paste but now a day's it is available as shampoos and lotions which are FDA approved for psoriasis (8). Salicylic acid acts by loosening and softening the keratinocytes and thus easy to remove scales.	Irritation, itching, peeling, redness, Folliculitis and salicylism. Contraindicated with other topical skin care products like Tretinoin.
Coal tar: Scytera®, Oxipor VHC® psoriasis lotion.	The treatment includes application of coal tar in the evening, allowing it to remain throughout the night and then removed next day. Coal tar was used individually or in combination with UV light. Tars consist of high benzene content and aromatic hydrocarbons which have carcinogenic potential (9).	Skin irritation, acne type eruptions and increased sensitization to sunlight. Contraindicated in pregnancy.
Dead sea salts	It includes bathing in sea water having high concentration of salts of mineral origin. These salts reduce the activation of T cells in the skin (10). This treatment is also termed as Balenotherapy and Climatotherapy.	Sensitivity reactions to mineral salts, promote skin allergy and ageing. Contraindicated in pregnancy, hypertension, and tuberculosis.
Tazarotene: Tazorac cream and gel (0.1%)	Tazarotene contains a chemical similar to vitamin A which slows cell proliferation. It belongs to a wide class known as retinoids. Tazarotene is a prodrug of tazarotenic acid which is a receptor selective acetylenic retinoid (1, 11). It is most commonly prescribed in concentrations of 0.05% and 0.1%.	Bleeding, cracks in skin, dry skin irritation, skin discoloration. Contraindicated in skin cancer and especially pregnancy.

Table 3. Various agents used from 1920-1970

Therapeutic agents and Formulations	Information regarding therapeutic agents	Adverse drug reactions and contradictions
Geokerman regimen	The therapy consists of application of coal tar, followed by exposure of skin to UV light. Coal tar is applied to the patient once or twice a day and skin was exposed to UVB radiation. It minimized the time of application of tar and amount.	Sunburn, freckling, and skin aging. Contraindicated in patients with tar sensitivity and Thrombophlebitis
Topical and oral steroids: Betamethasone valerate (Betnovate® cream/ointment) Clobetasol propionate (Etrivex shampoo®).	The corticosteroids directly affect the immune response by preventing phospho lipids release required as a primary precursor in synthesis of inflammatory mediators. Corticosteroids prevent the sequestration of CD4+ T-lymphocytes and prevent proliferation by inhibiting cytokines and lymphokines (12). Other mechanisms include inhibition of delayed hypersensitivity, antigen processing and inhibition of IL-2. The steroids have been classified in seven classes according to the potency and vasoconstricting properties. The Class I steroids are considered to be of highest potency (clobetasol propionate). Class VII indicates the lowest level of potency (hydrocortisone). A major problem with this therapy is that, the reemergence of the disease occurs if the treatment is stopped	Skin atropy, stretch marks, Prolonged use leads to Cushing's syndrome.
Hydroxyurea: Hydrea®(oral)	It was first recommended for psoriasis in 1960. Hydroxyurea is an antimetabolite used for treating chronic leukemia and cancer of cervix. The active form is converted into nitro oxide radical who selectively inhibits DNA synthesis in proliferating cells (12, 13). It is preferably recommended when other systemic agents like cyclosporine have failed.	Bone marrow toxicity, Neutropenia and leucopenia. Contraindicated in anemia and blood disorders.
Fumaric acid esters: Psori care plus®., Fumaderm®	They are marketed as mixture of dimethylfumarate and zinc salts of monoethylhydrogen fumarate (1). The mechanism includes shifting of Th1 type cytokine receptors to Th2 type pattern and inhibiting translocation of nuclear factors required for genetic regulation of inflammatory factors.	Allergic sensitizer, GIT stress and kidney disturbances. Contraindicated in chronic gastrointestinal disease, kidney disease and abnormal hematology counts.

Phototherapy Natural photo therapyArtificial ultraviolet BPsoralen+ Ultraviolet A(PUVA) Excimer laser therapy	A daily short non-burning exposure of sunlight has proved useful in treating psoriasis (14). UVB therapy includes a narrowband light source in which more than 83% of the UV rays is within the required range of 310-313 nm are considered to be most effective. Ultraviolet therapy having a range of 320-400nm along with a photo sensitizing agent such as 8-methoxy psoralen has been used for treating psoriasis (PUVA). Specific Excimer lasers which can produce a wavelength of 308 nm UVB provided localized effects (15).	Burning, skin tanning, skin cancer with prolonged exposure. Contraindicated in history of arsenic exposure, ionization radiation exposure and pregnancy.

Table 4. Various agents used from 1970 onwards

Therapeutic agents and Formulations	**Information regarding therapeutic agents**	**Adverse drug reactions and contradictions.**
T-Cell inhibitors/ Calcineurin Inhibitors **Cyclosporine**: Sandimmune®, Neoral®.	Cyclosporine is an endogenous peptide used in chronic conditions orally. Cyclosporine acts by inhibiting calcineurin and another protein cyclophillin A, forming a high affinity complex. The complex formation inactivates calcium stimulated calcineurin, preventing further activation of DNA binding proteins inhibiting transcription of interleukins (16). It is basically used in chronic conditions orally having a dose of 3-5mg/kg for about 10-12 weeks. A decrease in PASI score by 70-72% was observed when Cyclosporin was used for treatment of psoriasis.	Nephrotoxicity, hypertension, elevated serum lipids, gingival hyperplasia paresthesias. Contraindicated during breast feeding and hypersensitivity.
Tacrolimus: Prograf®, Advagraf®, Protopic®.	Tacrolimus is a macrolide antibiotic, which acts by inhibiting T cell activation. It binds to an intracellular protein FKBP-12 by forming a transcriptionally active NF-AT complex with them, preventing the dephosphorylation and translocation of activated T cells (17). It also targets mast cells and impairs histamine release. The major metabolite of Tacrolimus is 13-demethylated derivative, but 31-demethylated derivative shows immuno-suppressive effect. The systemic administration of Tacrolimus is associated with high liver toxicity and Nephrotoxicity, so topical application have been preferred	Nephrotoxicity, GIT upset, hypertension, insomniac conditions. Tacrolimus ointment is not approved for use in children. Contraindicated in pregnancy and pre existing hypertension.

Pimecrolimus: Elidel®	Pimecrolimus, another macrolide derivative which binds to macrophillin-12(FKBP-12) and inhibits calcineurin, preventing T cell activation and cytokine release (18). It is more selective than Tacrolimus and has no effect on dendritic cells which carry out the process of antigen presentation. The drug is being evaluated clinically for psoriasis and has shown improvements in PASI score.	Lymph node and skin malignancy, herpes infections. Cream 1% is contraindicated in individuals with a history of hypersensitivity
Sirolimus: Rapamune®	It blocks T cell activation later by binding with mammalian target of Rapamycin (mTOR) protein (19). It differs from Tacrolimus as it does not interact with calcineurin. It also inhibits platelet derived growth factor (PGDF) in smooth muscle cells. It also decreases T cell response, antibody production and thymocyte proliferation.	Lung toxicity, tachycardia, GI disturbances, hyperlipidaemia, peripheral edema, hypertension leuco-penia and angioedema. Contraindicated in pregnancy and lactation.
Everolimus: Zortress®, Certican®, Afinitor®.	Everolimus is 40-O-(2-hydroxyethyl) derivative of Rapamycin which is also an mTOR inhibitor. It has been approved for advanced kidney cancer and organ rejection but is under trial for psoriasis. Everolimus act specifically on mTOR protein, leading to hyper activation of kinase AKT via negative feed back loop mechanism of mTORc1. It has been observed in random controlled trials that the PASI score reduced to 60% after 4 weeks of therapy 3mg twice daily (20).	Back, arm or leg pain, constipation, diarrhea, dizziness, headache, loss of appetite, upset stomach, weakness, sleeping trouble. Contraindicated in lactating mothers and hypertension.
Efalizumab : Raptiva®	Efalizumab is a recombinant humanized monoclonal antibody which binds directly to CD11a subunit of LFA-1. The binding of LFA-1 to ICAM is hindered preventing co-stimulation (21). The recognition of ICAM-1 which is expressed on dermal surface promotes firm adhesion and subsequent migration of T lymphocytes into the cutaneous area, which is hindered. Due to its adverse effects including multifocal leucoencephalo-pathy it was withdrawn from market globally in 2009.	Headache, pale skin, dark colored urine, confusion. Contraindi-cated in known hypersensitivity and clinically important infections.
Infliximab (TNFá blocker): Remicade®	It is a chimeric monoclonal antibody (75% human+25% murine) which directly blocks TNFá. It neutralizes both soluble and membrane bound cytokines and lead to down	Pruritus, urticaria, dyspnoea, delayed hypersensitivity worsening heart

	regulation of IL-8. Infliximab is approved by FDA for psoriatic arthritis, moderate to severe plague psoriasis and Crohn's disease (22, 23).	failure. Contraindicated in active TB, moderate or severe CHF, severe infection, pregnancy and lactation.
Adalimumab: Humira®	Adalimumab is an engineered fully human monoclonal antibody (24). It binds to membrane bound TNFα based on concentration before TNFα can bind to p55 and p75surface receptors, inhibiting activity of cytokines. It has been administered along with phototherapy continuously or intermittently in patients with mild to severe psoriasis. It was approved in January 2008 for moderate to severe psoriasis.	Fatal blood disorders, re-occurrence of TB and associated infectious diseases.
Ustekinumab : Stelara®	Human monoclonal antibody which is directed against IL-12 and IL-23. It binds to p40 subunit of the interleukins. IL-12 is a key cytokine that stimulates T cell differentiation (25). Ustekinumab has been approved in Canada, Europe and USA for psoriasis.	Increased risk of infections, brain swelling, allergic reactions.
Siplizumab	Siplizumab is also a human monoclonal antibody with human IgG1 kappa in combination with rat antihuman CD2 monoclonal antibody (26). It binds to a conformational isotope on human CD2 receptors in T lymphocytes and natural killer cells.	Lymphopenia, anemia, chills, nausea, increased risk to infections.
Certolizumab, Golimumab, Briakinumab	Certolizumab, a pegylated Fc free anti TNF antibody has high affinity to TNFα. Another antibody golimumab is under phase III trial (27). It is fully human anti TNF IgG monoclonal antibody having affinity for both soluble and trans-membrane TNF. Briakinumab is fully human monoclonal IgG1 antibody.	Worsening of CNS demyelinating disease, neurological disorders and hematological disorders. Contraindicated in pregnancy and breast feeding
Etanercept : Enbrel®	Etanercept is a recombinant human TNF receptor p75 fusion protein which binds to TNFα specifically. It competitively interferes in interaction of TNFα with its cell bound receptor and so inhibits effect of cytokines. It was approved by FDA in May 2004 for treatment of moderate to severe psoriasis (28, 29).	History of recurrent infections or risk of infection, respiratory tract and abdominal pain, lymphoma, CNS demyelinating disorders. Contraindi

		cated in pregnancy, lactation, active severe chronic or localized infection and sepsis.
Alefacept: Amevive®	Alefacept is a fully human dimeric fusion protein, having extracellular CD2 binding portion of human leukocyte function antigen -3(LFA-3) linked to Fc portion of IgG1 (30). It reduces the circulating level of T memory cells by binding to CD2.It also induces apoptosis in CD4 memory effector T cells. Alefacept was approved in May 2002 by FDA for treatment of psoriasis. It is being recommended that a prior CD4 lymphocyte count should be carried out before initiating therapy.	Mild swelling, Bleeding at the injection site, pharyngitis, cough, nausea. Pruritus, myalgias and hepatic toxicity .Contraindicated in known hypersensitivity to Alefacept and in HIV patients.
Abatacept : Orencia®	It is recombinant fusion protein having extracellular domain of CTLA-4 which binds to CD80/86 receptor of APC (31). It inhibits the co-stimulation of T cells. The co stimulatory signal is generated by binding of T cell to B7 protein on Antigen Presenting Cells.	Chronic obstructive pulmonary disease (COPD), Signs of allergic reactions, including unexplained rashes. Concomitant Use with TNF Antagonists.
Vitamin D analogues : (Modify cytokine expression) Calcipotriene (Dovonex®) Calcipotriol (Rocaltrol®)· Calcitriol (Taclonex®)	Vitamin D analogues have shown to reduce the basic functioning of APC's (32). D3 is a hormone that binds to vitamin D receptors (VDR) and regulates calcium metabolism, and cell proliferation. They modify Th2 cytokine expression along with decrease in IL8 and decrease in IL10. Vitamin D analogues also reduce transcription of IL2 by inhibiting nuclear factor-kappa B thus affecting genetic regulation of cells (33).	Pruritus, burning sensation, erythema, rashes, photosensitivity and hypersensitivity reactions. Contraindicated in suspected disorders of calcium metabolism, Patients with erythrodermic, exfoliative and pustular psoriasis.
Retinoids (RAR receptor blocker) **Acitretin** (Soriatane®or Neotigason®)	Retinoids act directly by normalizing epidermal proliferation and cell differentiation. Acitretin is a FDA approved systemic and oral retinoid which has been used in combinations. Isotretinoin is a systemically active weak retinoid which is a metabolite of Acitretin (34).	Conjunctivitis, dry sore mouth, Hypervitaminosis A, hypercholesterolemia, benign intracranial hypertension; photosensitivity. Contraindicated in Pregnancy, lactation. Hepatic and renal impairment. Hyperlipidaemia.

Antimetabolites Methotrexate: Trexall®, Rheumatrex®.	Methotrexate is a synthetic analogue of folic acid which is a competitive inhibitor of enzyme dihydrofolate reductase, responsible for the conversion of dihydrofolate to tetrahydrofolate (35). So indirectly it inhibits the replication of T and B cells and suppresses cytokine secretion (IL-1, IFa and TNF±[3]). hepatic impairment,	Thrombocytopenia, Hepatotoxicity, GIT disturbances, mucosal ulceration, Stomatitis, malaise, pulmonary toxicity.Contraindicated in Severe renal or bone marrow suppression and lactation.
Azathioprine: Imuran®, Azasan®.	Azathioprine is a synthetic analogue of purine bases which are incorporated in RNA and DNA (36). Oral Azathioprine is used for systemic therapy for severe psoriasis. Azathioprine is a prodrug of 6- mercaptopurine which is formed after metabolism.6-MP is further anabolized via HPGRT pathway to a purine analog that inhibits DNA and RNA synthesis and repair leading to immunosuppression.	Carcinogen, Myelosuppression, veno-occlussive liver disease, anorexia, arthralgias. Contraindicated in Hypersensitivity, previous treatment with alkylating agents, pregnancy and lactation.
Mycophenolate mofetil: CellCept®, Myfortic®, Baxmune®.	It was in 1995, mycophenolate mofetil received approval for prevention of acute renal grafts and as a treatment for immune mediated diseases. Currently it is under trials for psoriasis, but often used off-label. MMF non-competitively binds to enzyme inosine monophosphate dehydrogenase in de novo purine synthesis pathway which inhibits the purine synthesis, thus overall inhibiting genetic expression (17, 37).	Hypomagnesaemia, hypocalcaemia, neoplasia, Myelosuppression. Extreme caution when MPA is used in patients with childbearing potential and in lactating mothers.
Others Sulphasalazine, Paclitaxel, Colchicine. .	Suphasalazine plays an important role in inflammatory bowel disease especially Crohn's disease. The mechanism of action has not been established properly still it has been found to act upon 5- lipooxygenase (38). Paclitaxel is a chemotherapeutic agent which is recommended for breast and ovarian cancer. Due to its anti-inflammatory properties, it has been used at a dose of 75mg/m^2 every two weeks in psoriasis patients. Colchicine has shown leukocyte suppression, antimitotic and cell mediated immune response inhibition activity (39). It has been used against palmoplantar and pustular type psoriasis.	Depression , infertility, thrombocytopenia, bone marrow suppression, Neutropenia, Hyperinhibition of mitosis

drug delivery always provides a better prospective in such cases. The base is to incorporate drug in specifically designed carriers which can provide specificity, act on target and reduce the side effects. The conventional dosage forms need to be incorporated in recent carrier systems to limit side effects of drugs.

Drug delivery to patients suffering from psoriasis need to be categorized in two categories:

Topical drug delivery limited to skin to provide a local effect.

Drug delivery to body's immune system to limit its over expression

Drug carriers such as nanoparticles can be an effective option in delivery of drug topically and systemically. It has been proved that nanoparticles smaller than 10 nm can directly penetrate through intracellular route and enter systemic circulation where they are recognized by reticuloendothelial system. Non-stealth nanoparticles are considered foreign and internalized by phagocytosis, where they are acted upon by various enzymes leading to their breakdown and releasing drug eventually. Particles larger than 10 nm accumulate in the hair follicle openings, interact with skin lipids and release the drug slowly. The small particle size of the nanoparticles enables its close contact with the horny layer of the skin and increases the amount of encapsulated compounds penetrating into the skin.

Transferosomes, one of the recent carriers, have a capability to penetrate corneocytes junctions due to their ultra deformable nature. Delivery of proteins and biological molecules with transferosomes provides a successful way to deliver high molecular weight molecules to skin.

Apart from advantages, novel drug delivery system has disadvantages like high cost of excipients, expertise in production and stability issues. The state of the hydration of skin is one of the most prominent factors influencing drug absorption. In psoriasis, the rigidity of skin occurs due to high concentration of cholesterol and low level of moisturizing factors. Apart from this excessive growth, aberrant differentiation of corneocytes leads to alteration in absorption properties (41).

***Liposome and Transferosomes**:* Transferosomes are highly deformable lipid carriers which can cross the skin barrier through intercellular pathways and can easily adapt during stress. Dexamethasone was approved by FDA in 1958 and mostly available in conventional formulations like topical sprays or ophthalmic drops. Earlier liposomes of corticosteroids have been prepared and reported but drug release has been found inconsistent. The preparation of corticosteroid loaded transferosomes showed spontaneous transport across the skin barrier better than other formulation. In vivo study of transferosomes of dexamethasone has shown to suppress ear edema than corresponding creams and topical formulations. The transferosomes of hydrocortisone and dexamethasone have showed faster onset, reduced drug abrasion and dose reduction, emerging as a potential carrier (42).

Dyphylline, a derivative of theophylline, inactivates cyclic AMP (cAMP), and therefore has been indicated in the treatment of psoriasis. An increase in dyphylline permeation has been observed across abdominal mice skin when liposomal delivery was targeted (43).

Temoporfin, a light sensitizer used in photodynamic therapy has been studied in the form of liposomal gels. Its poor aqueous solubility has limited its capacity for its topical delivery. Temoporfin loaded invasomes has showed positive results in topical delivery (40, 44).

An approved formulation for psoriasis known as Psorisome® Gel has also been formulated. Psorisome® is a novel liposomal formulation of Dithranol in gel base, which have enhanced drug application topically (45). The encapsulation of Dithranol in liposomes has reduced its irritant, burning and staining properties due to its oxidation during application. The micro carrier system provides a proper microenvironment and maintains hydration thus enhancing drug permeability.

Nanoparticles and Nanostructured lipid carriers:Nanoparticles are stable colloidal particles consisting of biodegradable polymers or lipids as a vehicle for delivery of drugs which have a size range of about 10-1000 nm. Nanostructured lipid carriers (NLCs) are lipid carriers in which liquid lipids have been used for increasing entrapment of the drugs (46).

Tacrolimus which is calcineurin inhibitor shows high inter-subject pharmacokinetic variability due to narrow therapeutic index and close drug monitoring is required during its administration. PEG–PLGA nanoparticles of Tacrolimus have been prepared for reduction in the adverse effects of systemic Immunosuppression and simultaneously enhancement in immunosuppressive efficacy by selectively transferring Tacrolimus into the lymphatic system (46). The pegylation of Tacrolimus showed positive results in lymphatic targeting.

Acitretin loaded nanostructured lipid carriers have been formulated and evaluated. Oral Acitretin is widely indicated and accepted for chronic adult psoriasis, but the application is restricted due to its systemic side effects and teratogenicity. The utilization of Acitretin is limited due to several disadvantages such as skin irritation, very low water solubility and high instability in the presence of light, air and water. Formulating with a suitable carrier has shown to lessen systemic toxicity and increasing local bioavailability (47). The results have shown remarkable decrease in erythema, scaling and betterment of symptoms.

Cyclosporine, another pioneer in the treatment of psoriasis has shown to treat psoriasis both by topical and systemic effects. Earlier cyclosporine was formulated in an oily vehicle and administered orally for graft rejections leading to incomplete absorption varying 6-60% and high degree of Nephrotoxicity. Polymeric nanoparticles of cyclosporine have been prepared using Eudragit RL100 and in-vivo and in-vitro studies have been carried out. The nanoparticles were highly taken up by reticuloendothelial system and thus their localization in lymph nodes and bone marrow (40, 48).

A combination study of calcipotriol and methotrexate was studied in the form of nanostructured lipid carriers or topical delivery. Calcipotriol has severe skin irritation reactions and so its dose needed to be minimized. During their combination study in the form of NLCs using different concentrations of a lipid precirol, a larger flux of drug through skin was observed as compared to control. The combination confirmed that NLC systems are a promising carrier for the topical delivery of ant psoriatic drugs as revealed by enhanced skin permeation, negligible skin irritation, and the compatibility of the two drugs (49).

Niosomes : Methotrexate has been studied due to its poor permeation and solubility characteristics. Topical methotrexate niosomal gel has been prepared and its efficacy has been estimated in humans using a double-blind placebo controlled study (50). It has been observed that topically applied niosomes can increase the residence time of the drug and reduce systemic absorption. The niosomes form a lipid layer on the skin which prevents the subcutaneous inter and intracellular hydration.

Dithranol and coal tar have also been used in niosomal and lipid coated microparticles form. Dithranol has not been fully accepted because of staining and irritant properties as well as poor permeation (42, 51). Dithranol therapy includes long term exposure of skin for proper treatment. The local topical delivery of drug have increased bioavailability and reduced dose dependent effects. Dithranol entrapped in niosomal form have shown better permeation properties as compared to conventional formulation.

Microemulsions: Microemulsions modify the skin properties which affect the permeation characteristics of drug. 8-methoxsalen which belongs to a category of furocoumarins has been widely used. The bioavailability of methoxsalen

Table 5. Biological agents under various trials for psoriasis

Molecule	Mechanism of action	Status
Apremilast	Phosphodiesterase 4 inhibitor	Phase 3 trials
Briakinumab	Phage display type antibody bind to p40 protein subunits	Phase 3 trials
Certolizumab Pegol	High affinity TNFá antibody prevents binding interactions	Phase 3 trials
Fezakinumab	IL-22 Inhibitor	Phase 1 trials
Golimumab	Anti TNFα antibody	Phase 3 trials
Sotrastaurin	Protein kinase C isoform inhibitor	Phase 2 trials
Valcosporin	Calcineurin inhibitor	Phase 3 trials
ABT-874	IL-12,IL-23 neutralization	Phase 2 trials
MEDI-545	IFN-α antagonist	Phase 1 trial

is highly variable due to poor solubility and first pass effect (52, 53). Microemulsion formulations containing 8-methoxsalen have been prepared and studied. The results obtained suggested the enhanced promotion and localization of 8-methoxsalen into skin and long term effects have been obtained using microemulsions as vehicle.

Other methods in drug delivery have been developed. Techniques such as iontophoresis, sonophoresis, alteration of skin permeability by mechanical perforation enhances the drug delivery of colloidal carriers (54). The improvement in topical delivery has been achieved by methods such as:

Use of hydrogels and ointment basis for preventing transepidermal water loss.

Disruption of stratum corneum using chemicals.

Use of permeation enhancers in formulations.

Enhancement of thermodynamic activity of drugs in formulations.

Novel biological therapies and their status : Apart from large number of therapies, cure has not been established for psoriasis. Since it is an immunological disorder, preference has been given in targeting bio-molecules which elicit the immune response. The biological agents have shown better targeting approach and higher clinical efficacy, as compared to other agents which have a large profile of adverse reactions. A large number of biological agents which were used in other immunological disorders are being evaluated clinically specifically in psoriatic conditions (Table 5).These synthetic proteins counteract one or more pathogenic pathway and specifically tackle various cytokine mediators (55,56). The biologicals have improved and better risk to efficacy ratio.

All of these novel drug delivery systems have provided better results than the conventional forms. Dithranol and coal tar gel is among the novel formulation that has been approved for psoriasis. Recent route of drug delivery includes transungual route which can be used to deliver medication in case of nail psoriasis. Till now no formulation have been approved for nail psoriasis but drugs incorporated and injected through suitable carriers can be used to administer drugs through nail plate. The stability of the formulations, their proper delivery techniques, proper safety profile and cost will be the targets to be achieved in the near future.

Herbal Treatments and Natural Remedies : The increasing resistance of newer therapies with immunosuppressants and antimetabolites still focuses the importance of natural and ayurvedic therapies in the treatment of psoriasis. Ayurvedic and Chinese herbal medicines have been used for centuries for the treatment of psoriasis. Herbal medicines involved in psoriasis involve *Psoralea corylifolia* (babchi), *Coleus forskohlii* and *Ammi visanga* (khella). Herbs such as aloe vera ,

chamomile, tea tree oil, turmeric, milk thistle, primrose oil, lavender, flaxseed oil, wintergreen oil, guggul and oregano oil have also found to be effective (1, 57). Psoraban® is an ayurvedic product which mainly reduces hyper proliferation and inflammation. This indicates that herbal treatments can offer safe, effective and inexpensive treatment for psoriasis.

Co-morbidities Associated with Psoriasis : Data collected all over the world have suggested the association of various co-morbidities with moderate to severe psoriasis. Co-morbidities like metabolic syndrome, cardiovascular diseases, Crohn's disease, chronic obstructive lung disease, diabetes mellitus, multiple sclerosis and insomnia are most prevalent. The metabolic changes include abdominal obesity, impaired glucose regulation and hypertension which have affected the normal population. It has been found that adipose tissue also have some immunological functions. The adipocytes release adipokines, resistin and leptin which mediates insulin resistance. Atherosclerotic plaque exhibits inflammatory infiltrates such as TNF á and interleukins (58). The cytokine levels are also associated with unstable angina and myocardial infarction, which can also be observed in psoriasis. In psoriasis, increased levels of Angiotensin Converting Enzyme (ACE), endothelin-1 and renin have been observed, thus promoting thrombotic state in the body. Most patients suffering from psoriasis get prone to depression and associate themselves with increased alcohol consumption, food intake and reduced physical activity, all aggravating the obesity and metabolic imbalance (59). Thus, if we consider the relation between psoriasis and co-morbidities the treatment plans must be adjusted so as to prevent the severe effects. Those patients which are already suffering from one or more diseases must be addressed by multidisciplinary approach before initiating a therapy for psoriasis.

Conclusion

Psoriasis has a large psychological impact on human health, as it is related to the appearance of the person. Although biological therapies with immune system modulators have modernized the psoriasis therapy, still proper therapy and agents need to be developed. The management of psoriasis is a problematic issue due to its continuous relapse and long duration of the disease. The utilization of initially used therapies such as arsenic and mercury derivatives has been suspended completely due to their potential side effects. Ultraviolet light therapy and Excimer lasers in combination with different oral and topical agents are widely used depending upon the severity of disease. Steroids became the drugs of choice in earlier seventies and still widely used, but possess very strong side effects. Cell growth inhibitors and antimetabolites along with biological therapies are recommended under complete medical supervision. Compiling the therapies and the molecules involved in treating psoriasis, it can be concluded that, biological therapies along with novel carrier systems providing selective targeting of molecules will be the most successful treatment in future. By understanding the mechanisms at molecular level and drug targeting based on a rationale approach will refine the psoriasis therapy. With the advancement and new approaches, wider options are being generated and these will help the patients to obtain an optimum level of treatment.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) i - vi July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

NEWS ITEM

US President Obama Recognized Biotechnology's Contribution to Renewable Energy

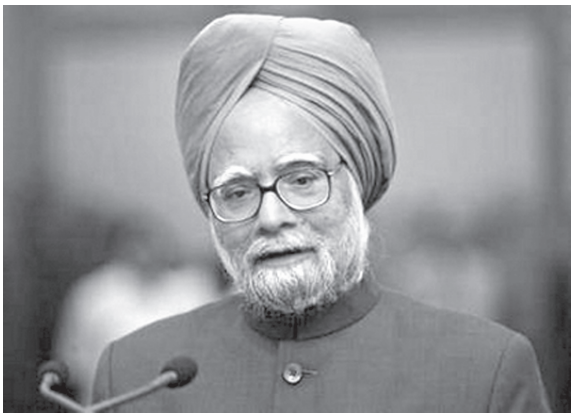
The Biotechnology Industry Organization (BIO) today applauded President Barack Obama's recognition of the contribution that biotechnology makes to the design of new biofuels and to agricultural productivity in the President's Climate Action Plan.Biotechnology enables energy efficiency and use of renewable resources in manufacturing and fuels as well as increased productivity on our agricultural lands," said BIO President & CEO Jim Greenwood. "Using our nation's scientific leadership, we can leave our children a healthier economy and environment by using renewable fuels and chemicals, adopting cleaner manufacturing processes, and producing more food on each acre while reducing pesticide applications, and water and on-farm fuel use. In addition, we are encouraged by the president's plan to use advanced biofuels to help reduce CO2 emissions in the transportation sector.

Hon'ble Prime Minister Dr.Manmohan Singh Inaugurates TERI's Delhi Sustainable Development Summit (DSDS) 2013 on 'Global Challenge of Resource-Efficient Growth and Development'

The Hon'ble Prime Minister of India, Dr.Manmohan Singh, inaugurated the 13th Delhi Sustainable Development Summit (DSDS) 2013 organised by The Energy and Resources Institute (TERI) today at the Taj Palace Hotel.

Over the years, Delhi Sustainable Development Summit (DSDS) has evolved as a global platform that addresses the key challenges of sustainable development and explores the means by which this can be attained. In its 13th edition, DSDS focusses on the theme, 'The Global Challenge of Resource-Efficient Growth and Development'. It is the first important meeting of global leaders following the June 2012, Rio+20 Summit. DSDS 2013 will take the Rio+20 deliberations forward, and examine what has been achieved and how awareness had been created; it will also attempt to define new directions to be followed; and set an agenda for further action.

Delivering the inaugural address, Hon'ble Prime Minister of India, Dr.Manmohan Singh congratulated TERI on organising this global forum which once again brought together eminent dignitaries, Nobel Laureates, CEO's and thought leaders from across the globe, Dr.Manmohan Singh said, "I am very happy to be present here today in the midst of such a distinguished gathering on the occasion of the inaugural session of the Delhi Sustainable Development Summit 2013. I would particularly like to extend a very warm welcome to the numerous foreign dignitaries who have come to Delhi from all over the world to attend this event.



Dr. Singh further added, "We in our India take due satisfaction in this development. Some 40 years ago, Prime Minister Indira Gandhi was one of the few leaders of the developing world to be present at the Stockholm Conference. Even then, she had made our commitment to environmental protection clear. But she had also pointed out that our challenge was ensuring development for all. It is a matter of some satisfaction to us that recent discourse has seen an implicit understanding that unless we find pathways for development that address the concerns of all, rather than the interests of a select few, our objective of global sustainable development will remain elusive.

In this context, the theme of this year's sustainable Summit, 'The Global Challenge of Resource-Efficient Growth and Development', has a particular resonance. Humanity has traditionally put its faith in advances of technology to resolve problems of resource scarcities. However, there is now a growing realization that there may be no easy alternatives for some resources, particularly environmental resources. Resource-efficiency is, thus, a necessary condition for sustainable development, and a key element of the economic pillar of sustainability."

"Indian Biotech Industry slated to be 100 Billion Dollars by 2025" Says DBT Secretary

With the recent breakthroughs by Indian Biotech Companies such as Bharat Biotech, Biocon making profits globally, Biotech Startups gaining good mileage, DBT Secretary Dr RenuSwarup has come out with the expectation that we will be cashing in dollar 100 Billion annually by the end of 2025. Currently the industry is grossing $ 4 Billion and has shown steady growth even in the recession Years. In 2001, DBT released its vision which emphasized on "attaining new heights in Biotechnology research, shaping Biotechnology into a premier precision tool of the future for creation of wealth and ensuring social justice-specially for the welfare of the poor"Over the past 4-5 years Biotechnoloy industry has witnessed a optimistic change in terms of more and more Industry and Academia interactions and encouragement to convert research projects in profitable business models. Additionally incorporation of BIRAC and Biotechnology Ignition Grant (BIG) has provided a catalytic role in provding financial as well Infra related support. With the continued & collaborative efforts of Biotech Companies, Academia, BIRAC, DBT today the country is expecting to break the 100 Billion dollar mark by 2025.

SCIENTIFIC NEWS

Scientist says GM crops can't be overlooked for long

The genetically modified (GM) crops were an important technological advancement which the country cannot overlook for long in view of its priorities and policies such as national food security, said Akhilesh Kumar Tyagi, Director, National Institute of Plant Genome Research (NIPGR), an autonomous body aided by Department of Biotechnology under the Union Ministry of Science and Technology. "This is an important technology to benefit the people and we should have a clear policy on this so that it is not restricted. At the same time, all quality controls should be in place as is done in the case of any new technology," On duplication and triplication of genes in plants, he said triplication of genes in tomato occurred at a time when dinosaurs disappeared from the earth. "In that period, there had been great changes in the environment and to adapt to those changes the plants got a duplicated genome. Then they diversified and this helped adoption and evolution of the plant types that we have today," he said. "If there is more than one gene, the pressure on the genes gets reduced and it paves way for them to diversify and occupy a different niche in order to provide something useful for the human beings," he said.

Nanoparticles help disrupt tumor blood supply, destroy tumors

In recent years, cancer researchers have been developing agents that destroy the blood vessels surrounding tumors with the goal of starving tumors to death. Some of these agents, such as tumor necrosis factor-alpha (TNF-α) have shown promising results, but often their toxicity has proven too great to be clinically useful. Using gold nanoparticles as a delivery vehicle for TNF-α has reduced this toxicity and the resulting construct has already completed a phase I clinical trial in humans.

Now, a team at the University of Minnesota headed by John Bischof, has shown that they can use this gold nanoparticle-TNF-α system to enhance the effects of either thermal therapy or cryosurgery. Moreover, the researchers demonstrated that they can use standard magnetic resonance imaging technology to visualize tumor destruction. Dr. Bischof and his colleagues reported their findings in the journal Molecular Pharmaceutics.

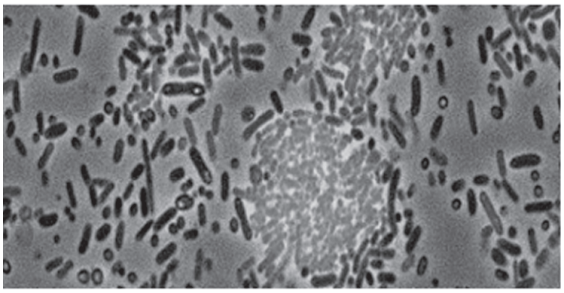
Experiments in a mouse model of human prostate cancer showed that the gold nanoparticle-TNF-α system disrupted blood flow into tumors within 90 minutes of injection, an effect that lasted up to six hours. Using a technique known as dynamic contrast-enhanced MRI, Dr. Bischof's team was able to clearly image the changes in blood flow into and around the tumor following nanoparticle treatment. The researchers note that in human patients, a simple five-minute MRI scan would be sufficient to detect a meaningful change in tumor blood vessel function.

Once the tumor blood vessels had been "preconditioned," Dr. Bischof and his collaborators

treated the animals with either thermal therapy or cryosurgery, both of which produced marked reductions in tumors. They noted that none of the animals treated with thermal therapy died, an important finding given that an equivalent dose of TNF-α with no gold nanoparticle attached followed by thermal therapy was found to be lethal in a large percentage of animals. The researchers also showed that nanoparticle-delivered TNF-α did not trigger inflammatory reactions associated with activated neutrophils, something that does occur when tumors are treated with native TNF-α.

***LuxR:*A new class of bacterial signaling molecules**

LMU microbiologist PD Dr. Ralf Heermann and Professor Helge Bode of the Goethe-University in Frankfurt have just reported the discovery of a previously unknown bacterial "language". Their findings are detailed in the latest issue of the journal Nature Chemical Biology. "Our results demonstrate that bacterial communication is much more complex than has been assumed to date," Heermann says.The bacterial communication system that is currently best understood uses N-acylhomoserine lactones (AHLs) as signals. These compounds are made by enzymes that belong to the group of LuxI-family synthases. Transmitting cells secrete the signal and neighboring cells recognize the concentration via a LuxR-type receptor. Signal perception changes the pattern of gene expression in the receiving cells, which results

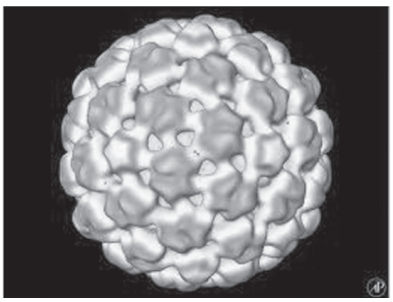


in alterations in their functional properties or behavior. However, many bacteria have LuxR receptors but lack any LuxI homolog, so that they cannot produce AHLs. These receptors are referred to as LuxR solos.Ralf Heermann and Helge Bode have now discovered a type of ligand that binds to LuxR solos. As model system, they chose the species Photo-rhabdusluminescens, a pathogenic bacterium that is lethal to insects. Novel bacterial 'language' discovered Bacteria communicate by means of chemical processes. LMU microbiologist PD Dr. Ralf Heermann and Professor Helge Bode of the Goethe-University Frankfurt have identified a novel bacterial cell-cell communication system that uses alpha-pyrones. It turns out that a LuxR solo of this bacterium responds to compounds called alpha-pyrones, specifically to photopyrones. Furthermore, the researchers have identified the pyrone synthase (PpyS) that catalyzes the biosynthesis of photopyrones. The pyrone-based signaling system allows the bacteria to recognize one another, whereupon they produce a surface factor that causes cell clumping. Heermann and Bode assume that this collective behavior makes the cells less vulnerable to the insect's innate immune system, and then allows them to kill their victims by the production of various of toxins. *P. luminescens* is a useful model organism, because it is related to many human pathogens, including coliform bacteria such as enterohemorrhagic*E. coli* (EHEC) and well as plague bacteria,Heermann points out.

Hidden Regulators: Viral Gene in Commercial GMO Crops

In the course of analysis to identify potential allergens in GMO crops, the European Food Safety Authority (EFSA) has belatedly discovered that the most common genetic regulatory sequence in commercial GMOs also encodes a significant fragment of a viral gene. This finding has serious ramifications for cropBiotechnology and its regulation, but possibly even greater ones for consumers and farmers. This is because there are clear indications that this viral gene (called Gene VI) might not be safe for human consumption. It also may disturb the normal functioning of crops, including their natural pest resistance.

What Podevin and du Jardin discovered is that of the 86 different transgenic events (unique insertions of foreign DNA) commercialized to-date in the United States 54 contain portions of Gene VI within them. They include any with a widely used gene regulatory sequence called the CaMV 35S promoter (from the



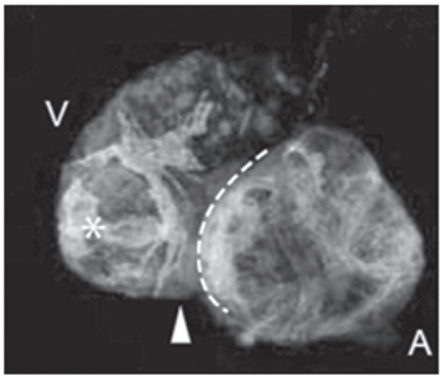
cauliflower mosaic virus; CaMV). Among the affected transgenic events are some of the most widely grown GMOs, including Roundup Ready soybeans (40-3-2) and MON810 maize. They include the controversial NK603 maize recently reported as causing tumors in rats (Seralini et al. 2012).

The researchers themselves concluded that the presence of segments of Gene VI "might result in unintended phenotypic changes". They reached this conclusion because similar fragments of Gene VI have already been shown to be active on their own (e.g. De Tapia et al. 1993). In other words, the EFSA researchers were unable to rule out a hazard to public health or the environment.

In general, viral genes expressed in plants raise both agronomic and human health concerns (reviewed in Latham and Wilson 2008). This is because many viral genes function to disable their host in order to facilitate pathogen invasion. Often, this is achieved by incapacitating specific anti-pathogen defenses. Incorporating such genes could clearly lead to undesirable and unexpected outcomes in Agriculture. Furthermore, viruses that infect plants are often not that different from viruses that infect humans. For example, sometimes the genes of human and plant viruses are interchangeable, while on other occasions inserting plant viral fragments as transgenes has caused the genetically altered plant to become susceptible to an animal virus (Dasgupta et al. 2001). Thus, in various ways, inserting viral genes accidentally into crop plants and the food supply confers a significant potential for harm

Migration of Heart Atrium cells to Repair Ventricle

Scientists from the Max Planck Institute for Heart and Lung Research in Bad Nauheim, together with U.S. colleagues, have now observed in the embryo of the zebrafish that muscle cells migrate from the undamaged atrium into the ventricle and thus significantly contribute to regeneration. This could serve as the basis for novel therapeutic approaches.If clinicians fail to reopen occluded coronary arteries after a heart attack within an appropriate time frame, the heart muscle is permanently damaged because of the long-term interrupted oxygensupply. The result is, among other things, a lifelong restriction of cardiac function, or even heart failure.



For many years, scientists worldwide have been searching for ways to stimulate the regeneration of damaged heart tissue. The working group of Didier Stainier from the *Max Planck Institute* for Heart and Lung Research has now, together with scientists from the University of San Diego, identified in zebrafish a novel mechanism, at which muscle cells from the atrium actively migrate into damaged parts of the heart muscle in the ventricle, thus forming new ventricular tissue.For their study, the Max Planck researchers used genetically modified fish larvae, in which the targeted muscle cells of the heart chamber were destroyed by the administration of a substance. This was done at a point in time, at which the heart was already functional and active. The damage of the muscle was followed by a reduction in heart function and ventricular size.To monitor the behavior of the different cell types, the heart muscle cells were furthermore altered by genetic engineering in such a way that cells from the atrium and the ventricle lit up differently.In this way we were able to track, the behavior of the individual cell types continuously in a confocal microscope, explains Didier Stainier, the director of the Developmental Genetics department at the MPI.A few hours after ablation only few red cells were remaining in the ventricle. Furthermore, it had been significantly shrunk, both references to the death of muscle cells," says Stainier. Just 24 hours later, much more fluorescing cells were back, indicating the initiation of cell division activity of surviving cells within the ventricle.In further studies, the scientists then found clear evidence of a so-called transdifferentiation of cells: The muscle cells of the atria of the heart of the fish lost their characteristic properties and subsequently transformed into ventricular cells in the course of regeneration. With the progression of heart regeneration, these cells were permanently installed in the muscle tissue and made their contribution to the restoration of cardiac function.The Max Planck researchers in their study see potential for future therapy. "Although, a atrial cell population is known to

be present in humans with comparable properties, it is questionable whether the human heart possesses a similar self-healing ability," says Stainier. One solution might be, however, using gene therapy to stimulate such reprogramming of cells and thus to strengthen the self-healing abilities of the heart.

SEMINARS/WORK SHOPS/CONFERENCES

BioDownstream Technology Course India 2013"conducted by VIT, Vellore: BioDownstream Technologies (BTC)-India will be jointly organized by CBST, India and COBIK, Slovenia. To Enhancing both theoretical and practical knowledge of current downstream processes technologies for biomolecules and looking in their future. A 5 day work shop will conduct during30th Sept – 4th Oct 2013 at Centre for Bioseparation Technology (CBST), VIT University, Vellore, Tamil Nadu, India. Pin: 632014 for People from industry engaged in downstream processing, and academic members who want to increase their theoretical and practical knowledge of modern downstream processing. Participants will receive anExtensive overview of the current state of the art in downstream processing of biomacromolecules, Theoretical aspects of commonly applied techniques including chromatography, precipitation, liquid-liquid extraction, ultrafiltration, and crystallization. Special topic will be on the scale-up of each technique. Features of specific groups of biomacromolecules especially, monoclonal antibodies,DNA, and viruses.Modern in-process analytics.Downstream process design based on collected results.Confronting opinions of participants, experts from industry and academiaca.Registration : There is INR 15000 /- fee for BTC-India which includes participation in lectures and hands-on training, workshop materials, coffee and snacks. Registrations are open until 10th August, 2013 Space is limited to 20 participants, so sign-up now! Your registrations will be processed on a first-come-first-serve basis. All participants should take care of their travel expenses and accommodation. The international participants are also requested to provide a suitable insurance. There is INR 2000/- fee for BTC-India to attend only the lectures (no limitation).For further Information contact :Prof. M A Vijayalakshmi .

Hands-on Workshop on Molecular Biotechnology and Bioinformatics : A 5 day Workshop at ICSCCB, R.H. 2, Ujwal Regalia, Near Prabhavee Tech Park, Baner Road, Pune – 411045, India Only 10 seats per batch so please register early to confirm your participation. We will update on the website if a batch is full.We have planned a series of five days basic workshops with practical hands-on training on the topics from molecular biology and bioinformatics. The biology and bioinformatics contents are interwoven in such a manner that it would help a molecular biologist to find solutions to real time problems in lab and a bioinformatician to understand the laboratory aspects of bioinformatics. *All topics below will be covered in each workshop by practical hands-on training:* Recombinant DNA Technology & Genetic Engineering, Bioinformatics part and Genomics Data VisualizationRegistration Fees (food & accommodation is not included), Rs. 6000/- (US$ 150 for International candidates). Accommodation can be booked in nearby hotels/guesthouses directly, we will provide the information by email. All UG/PG/PhD students, faculty, scientists as well as people working in industry in the field of Biotechnology, Bioinformatics, Life Sciences, Medical Sciences, Pharmaceutical Sciences, Chemical Sciences and related subject areas

OPPORTUNITIES

Ph.D. fellowship in the development and characteri-zation of induced haemarthrosis in the FVIII knockout rat as a model of haemarthrosis in human haemophilia Aat the LIFEPHARM *Centre:* Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen is offering afellowship commencing 1 October 2013 or soon thereafter. The application deadline is 7 August 2013. Project description: Haemophilia A is a genetically inherited X-linked bleeding disorder caused by abnormalities in the coagulation factor VIII (FVIII) gene located on the X chromosome. Despite aggressive therapy regimens, detrimental effects of recurrent haemarthrosis are well-known. The purpose of the project is to investigate the use of the Factor VIII knockout rat as a translational model for such haemarthrosis by characterising the interplay between inflammation and haemostasis to understand the different levels of the development of the haemarthrosis and if possible identify potential ways to dampen the inflammatory response and thereby improve the mobility and life quality of the patients. The specific project goals are to visualize the temporal relationship between bleeding, inflammation, and tissue remodeling, to map the time course of changes in circulatory inflammation markers in relation to development of the haemarthrosis , to investigate the inflammatory processes locally at different time points following haemarthrosis, and to explore pharmacological opportunities to dampen the

development of acute haemarthrosis and chronic haemophilic arthropathy. Further contact Principal supervisor-Professor Axel Kornerup Hansen.

PhD fellow in Analytical Biosciences @ University of Copenhagen: The Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen is pleased to announce that a fellowship will be available from 1 October 2013 or as soon as possible thereafter. The application deadline is 15 August 2013. Applications are invited for the three-year fellowship from applicants who hold or expect to hold a master´s degree in a field relevant to the following project: Pre-clinical studies of absorption, distribution, metabolism, and excretion (ADME) of new drug candidates are required to evaluate both the drug safety and to obtain the kinetics of drug plasma concentration after drug administration. In the body the drugs are being metabolized and converted into metabolites in which some are pharmacologically active while others may have toxic effects. One group of toxic metabolites is called reactive metabolites that have a very short life span due to their instability and high reactivity. Traditional methods used for studying drug metabolism are time consuming and may not be able to detect the presence of reactive drug metabolites. Likewise, current methods for analysis of large molecule protein drugs and their metabolism lack sensitivity and are very labour-intensive.

Postdoctoral Position in Molecular Microbiology is Vacant @ University of Copenhagen: A 2-year postdoctoral position for a researcher with a strong background in molecular is available in the Biomolecular Regulation Research Group at the Department of Biology, University of Copenhagen.Area of research**,** Our research focuses on fundamental regulatory and mechanistic aspects of gene expression in bacteria. A common interest among the group members is the role of RNA molecules as regulators and mediators of protein synthesis. The position is part of a project funded by the Danish Council for Independent Research, which aims to characterize a new class of bacterial noncoding RNAs suggested to mediate the degradation of cellular tRNA following nutritional stress. The tRNA degradation pathway will be determined using a combination of genetic, biochemical, and deep RNA sequencing approaches More information about the research group can be found on our webpage:www.bio.ku.dk/english/research/bv/biomolecular-regulation. Qualifications: Candidates should hold a -degree within the area of molecular and have documented extensive experience with bacterial genetics and and bacterial growth physiology will be preferred. Excellent written and spoken English is a requirement for the position.

PhD in Separation of Maillardglycated proteinsat Wageningen University:a candidate for the project "Glycation of proteins through the Maillard reaction and separation of reaction products". The position is part of a project with two positions, aimed at understanding and controlling the Maillard reaction between reactive carbohydrates and proteins in order to obtain tailored glycated proteins with specific functionalities. Using carbohydrates with different chemical structures and reactivities, intermediate- and final Maillardglycated products will be analysed and involved reaction mechanisms revealed. In this part of the project, separation techniques will be developed to fractionate the complex mixtures of (un)reacted proteins and mechanisms of modification will be investigated by analyses of distinct populations of modified proteins obtained. requirements: An MSc degree in Chemistry, Food Technology, Experience with electrophoresis, or chromatography and mass spectrometry. Experience with carbohydrates and proteins will add to the suitability of a candidate for this post;Excellent research skills;Ability to work in a multi-disciplinary project;Excellent communication skills; The selected fellow should have passed for an English proficiency test (e.g. TOEFLL or IELTS) before the employment starts. We offer you a temporary position for a period of 1.5 years with extension of 2.5 years after successful evaluation. Gross salary per month • 2083,- in the first year rising up to • 2664,- per month in the fourth year. We not only offer a competitive salary but also good (study) leave and a pension of the ABP Pension Fund. For more information about this position, please contact dr. ir. Peter Wierenga, assistant professor Food Chemistry, telephone number +31 317 483 786.

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Current Trends in Biotechnology and Pharmacy
Vol. 7 (3) July 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

7th Annual Convention of ABAP
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- Disease diagnosis and Molecular imaging
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- Other Related Areas

For further details contact

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Printed, Published and owned by Assocaition of Biotechnology and Pharmacy # 6-69-64; 6/19 Brodipet, Guntur - 522002, Andhra Pradesh, India. **Printed at :** Don Bosco Tech. School Press, Ring Road, Guntur - 522007, A.P., India. **Published at :** Association Biotechnology and Pharmacy # 6-69-64: 6/19 Brodipet, Guntur - 522002, Andhra Pradesh, India. **Editors :** Prof. K.R.S. Sambasiva Rao and Prof. Kannam S. Murthy.