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Potential Pharmaceutical Compounds from Dioxygenase-Derived Chiral Metabolites

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Abstract

Aromatic and chlorinated aromatic compounds are used in large quantities as herbicides, pesticides, and solvents, and distributed in the biosphere due to these industrial activities contamination. A broad range of peripheral reactions results in a restricted range of central intermediates, which are subject to ring-cleavage and funneling into the Krebs cycle. Key enzymes in aerobic aromatic degradation are oxygenases, preparing aromatics for ring-cleavage by the introduction of hydroxyl functions and catalyzing cleavage of the aromatic ring. The regio- and stereo-specificity of dioxygenase enzymes revealed that these enzymes are an important class of biotechnology. These Enzymes that are capable of catalyzing the insertion of oxygen into aromatic substrates have many potential applications in pharmaceuticals manufacturing, production of chemicals and also in medicine. The regio- and stereospecific oxidation of an unactivated aromatic compound is very difficult to accomplish using conventional chemical techniques, which typically produce an array of byproducts that must be separated and destroyed. Their potential for derivatization through arene functionalities makes *cis*-dihydrodiols valuable synthetic building blocks for the synthesis of biologically important pinitols, conduritols, and acyclic as well as the drugs indinavir and pancratistain, screening methods for Dioxygenase enzymes, a product of an oxidation reaction is converted into a phenol or a catechol, which is easily detected by a Gibbs assay. This conversion allows for a sensitive and efficient assay. Also methods for detecting phenolic ether-products and sulfhydryl products from oxidation reactions by using a Gibbs assay.
.

Keywords: Ring hydroxylating dioxygenase, *cis*-dihydrodiol, Indinavir, Indigo.

Introduction

Most of building blocks of biomass are aromatic compounds, which are widely distributed from low-molecular mass compounds to polymers in nature, and mostly are found as aromatic amino acids, xenobiotic compounds and lignin components in higher plants, which is second most abundant polymer in nature after cellulose, comprising about 25% of the land-based biomass on Earth (1). The recalcitrant organic matter formations in soils is due to lignin degradation, aromatic compounds from other plants and decomposition process. The aromatic compounds are the most stable and persistent organic pollutants which comes from aromatic amino acids and extensive use of natural and xenobiotic aromatic compounds in industrial processes, in addation to inadequate waste management strategies.

The degradation of aromatic polymers is an important component of global biogeochemical cycles and is accomplished almost exclusively by microorganisms which play

important roles in the degradation and mineralization of xenobiotic and aromatic compounds in natural environments and such capabilities can be used for the clean up of contaminated environments (bioremediation). Bioremediation is considered as a relatively low-cost technology, which usually has a high public acceptance and can often be carried out on site. Bacteria have evolved diverse strategies to degrade aromatic compounds using its huge catabolic diversity relaxed substrate specificity of some of the catabolic pathways, and thereby derive carbon and energetic benefit from them. Two key steps for the bacterial degradation of hydrophobic aromatic pollutants is usually initiated by dioxygenases, which utilize molecular oxygen as a required substrate adding both atoms of O_2 to the aromatic ring. In general, this reaction is the most difficult in the degradation of aromatic compounds, and the addition of hydroxyl groups to the highly stable aromatic ring structure activates the molecule for the further second step which is oxidation and eventual ring cleavage. The activation of aromatics is usually catalyzed by members of the super family of Rieske non-heme iron oxygenases.

Rieske non-heme iron oxygenases **:** Members of this super family are known to overall oxidize hundreds of substrates including linked and fused aromatic, aliphatic olefins, and chlorinated compounds and are distributed among a variety of Gram-negative and Gram-positive bacteria capable of degrading key classes of aromatic pollutants. Rieske non-heme iron oxygenases are soluble, multicomponent enzyme systems comprising two or three separate proteins, and require oxygen, ferrous iron (Fe^{2+}) and reduced pyridine for catalysis. These enzymes consists of an electron transport chain (Fig. 1), that channels the electrons from NAD(P)H to the catalytic terminal oxygenase component where substrate transformation take place (2, 3, 4). These terminal oxygenase component and different electron transport proteins, usually catalyze the incorporation of two oxygen atoms into the aromatic ring to form arene-*cis*-dihydrodiols (Fig. 2) a reaction which is followed by a dehydrogenation catalyzed by *cis*-dihydrodiol dehydrogenases to give (substituted) catechols. Since decades, Members of the Rieske non-heme iron oxygenases are known to be involved in benzoate degredation (5), then 1-carboxy-1,2-*cis*-dihydroxycyclohexa-3,5-diene (benzoate-*cis*-dihydrodiol) was formed (6). Similar two-component enzyme systems (Fig. 1) are responsible for 1,2-dioxygenation of anthranilate (7), an intermediary metabolite of tryptophan degradation and a precursor for the

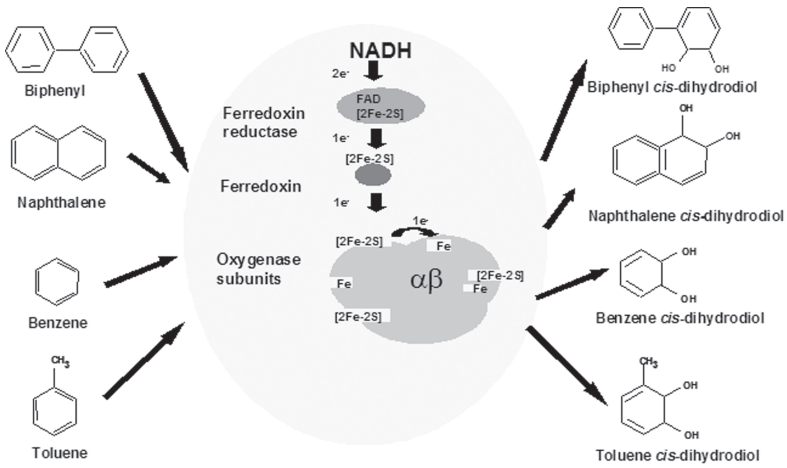

Fig. 1. The bacterial degradation of hydrophobic aromatic pollutants is usually initiated by dioxygenases, which utilize molecular oxygen as a required substrate adding both atoms of O2 to the aromatic ring to form cis-dihydrodiol.

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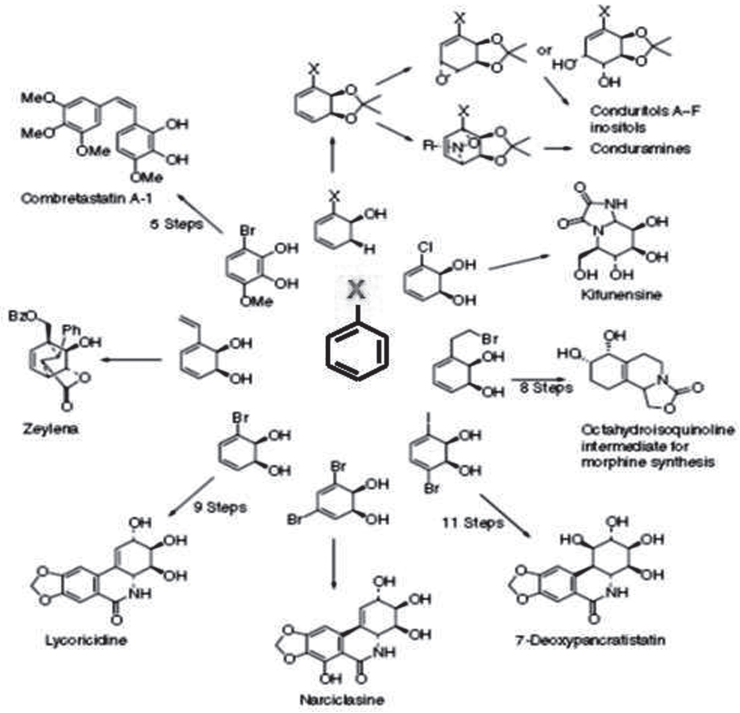

Fig. 2. The potential of cis-dihydrodiols for derivatization through arene functionalities makes them valuable synthetic building blocks for chiral drugs and specialty chemicals.

Pseudomonas quinolone signal (8). Many Rieske non-heme iron oxygenases have been characterized, and classified, its classification system was based on the components of the electron transfer chains present in the Rieske non-heme iron oxygenase systems either two or three, this classification system was rather suitable as long as only a small number of enzymes were known. Two-component (reductase, oxygenase) and three-component (reductase, ferredoxin, oxygenase) enzyme systems could be differentiated and these classes were further subdivided based on the number of proteins comprising the oxygenase, the type of flavin moiety (FAD or FMN) present in the reductase, the presence or absence of an iron-sulfur center in the reductase, and the type of iron-sulfur center present in the ferredoxin. However, with the increasing number and diversity of enzyme systems characterized and the presence of new enzymes with unusual redox partners that do not fit into the original classification it became obvious that such classification was not useful anymore. Moreover, it became clear, that the components of the electron chain are not determining substrate specificity, but was to a certain extent interchangeable between different Rieske non-heme iron oxygenase systems. Werlen et al (9) proposed a classification system based on sequence alignments of the oxygenase á subunits, differentiating four families (naphthalene, toluene/benzene, biphenyl, and benzoate/toluate). Since the oxygenase is the catalytic component and the α subunit plays a major role in determining substrate specificity (10, 11) these classifications are based on the catalytic activity of the enzymes. Further analysis based on α-subunit sequence comparisons confirmed that the grouping of the oxygenases largely correlates with the respective substrate preferences (2, 12). Also Gibson and Parales

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distinguished four families. Group I, or the phthalate family, comprises Rieske non-heme iron oxygenases that contain only α subunits. Substrates for this diverse group of enzymes include several aromatic acids such as phthalate, *p*-toluate, and phenoxybenzoate, but also carbazole and 2-oxo-1,2-dihydroquinoline. Group II, or the benzoate family represents a cluster of enzymes with activities toward various aromatic acids. Naphthalene, phenanthrene, and nitroarene dioxygenases clustered as group III and were termed naphthalene family. Biphenyl, toluene and benzene dioxygenases were observed to be highly similar in sequence and thus grouped as one cluster (Group IV or the toluene/biphenyl family). With the increasing interest in microorganisms capable to degrade aromatic pollutants as well as naturally occurring aromatics, however, various enzymes were characterized in the recent years, which were only distantly related to above described oxygenases (4). Some initial dioxygenases and other catabolic genes share the chain for the transport of electrons as in case of the naphthalene dioxygenase and and salicylate 5-hydroxylase (13).

Pharmaceutical applications of dioxygenase: Aromatic-ring-hydroxylating dioxygenases have proven useful in a number of biotechnology applications. The basis for most applications depend on the stereoselective cis-dihydroxylation of nonactivated aromatic compounds, which is unique to this enzyme while they catalyze an impressive array of different reaction types. Examples include dioxygenase-catalyzed synthesis of chiral intermediates for the preparation of natural products, polyfunctionalized metabolites, and pharmaceutical intermediates; expression of recombinant naphthalene dioxygenase (NDO) in an engineered bacterial strain for the production of indigo from glucose; and target-specific biodegradation of environmental pollutants.

Enantioselectivity of the dioxygenase: The fine chemicals, natural products, pharmaceutical intermediates, and biologically active compounds can be prepeared using Dioxygenase-derived chiral metabolites (Fig.2). The use of enzymatically formed *cis*-diols in enantioselective synthesis has been the subject of a comprehensive review (14, 15) a wide range of cyclitols, conduritols, conduramines, inositols, heteroatom carbohydrates, alkaloids, and a variety of natural products were synthetic designed via dioxygenase-catalyzed *cis*-dihydroxyation and their application in asymmetric methodology for the synthesis of the cis-dihydrodiols of dictamnine and 4-chlorofuroquinoline yielded phenolic derivatives from which a range of furoquinoline alkaloids were synthesized (16). A summary of recent progress in the synthesis of morphine alkaloids included the use of several metabolites derived via dioxygenase biocatalysis. Cyclohexadiene cis-dihydrodiols of phenethyl bromide and bromobenzene, as well as 3-bromocatechol (produced by a strain overexpressingTDO and DDH), have been employed as synthons in two separated synthetic strategies to produce advanced intermediates for the synthesis of morphine alkaloids (17). The biooxidation of 4-bromoanisole by recombinant E. coli expressing TDO and DDH yielded p-methoxybromocatechol. This functionalized catechol was coupled with trimethoxyphenylacetylene in convergent syntheses of combretastatins A-1 and B-1, members of a class of oxygenated natural products with potent cytotoxic activity (18). An efficient chemoenzymatic synthesis of strawberry furanone (4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one), a naturally occurring flavor compound, was enabled through directed evolution of TDO and tetrachlorobenzene dioxygenase operons that yielded improved enzymes for the conversion of p-xylene to the requisite diol synthon, *cis*-1,2-dihydroxy-3,6-dimethyl-3,5-cyclohexadiene (19).

Indinavir production: *Cis*-(1S)-amino-(2R)-indanol formation as a result of biocatalytic production of enantiopure (-)-*cis*-(1S, 2R)-indandiol, which is a precursor for Merck's HIV-

1 protease inhibitor Indinivir Sulfate (Crixivan) (20). *Pseudomonas putida* F39/D or *E. coli* harbouring tolune dioxygenase (TDO) revealed that wild-type TDO oxidized indene to (-)-*cis*-(1S, 2R)- indandiol (~30% ee) and (1R)-indenol as the main products, with traces of 1-indenone formed (21, 22). As a result of indene conversion by wild-type *P. putida* F1 about 98% of (-)-*cis*-(1S, 2R)-indandiol was obtained (20), also (-)-cis-(1S, 2R)-indandiol was obtained as coexpression of dihydrodiol dehydrogenase (DDH) together with Tolune dioxygenase in E. coli (22). As a result of kinetic resolution catalyzed by DDH that is selective for the undesired (þ)-*cis*-(1R, enantiopurity of (-)-*cis*-(1S, 2R)-indandiol was increased at the expense of total indandiol yield (22). Directed evolution was used to select the variants of TDO that produced. The reduced amounts of the indene by-products 1- indenol and 1-indenone, while maintaining high (-)-*cis*-(1S, 2R)-indandiol enantiopurity was selected in variants of TDO using directed evolution (23). The variants that produced significantly more cis-indandiol relative to the undesired by-product indenol were obtained after three rounds of mutagenesis. To favor the production of the undesired (+)-cis-indandiol enantiomer, the stereoselectivity was altered (23). The elimination of indene formation of indene by-products with the limited yield about 60% (-)-*cis*-(1S, 2R)-indandiolwas not achieved either with these strategies or the application of oxygenases from *Rhodococcus* strains (24). TDO-catalyzed enantioselective monohydroxylation of 2-indanol to (-)-*cis*-(1S, 2R)-indandiol is the alternative route to the vicinal aminoindanol. Preparation of chiral 1-hydroxy-2-substituted indan intermediates by this reaction is the basis for this process (25). *P. putida* strains UV-4 (26) and F39/D expressing TDO oxidized 2-indenol to (-)-*cis*-(1S, 2R)-indandiol in >98% ee and >85% yield; minor products included trans-1,2-indandiol (<15%) and 2-hydroxy-1-indanone (<2%) (27).

Indigo production: *E. coli* strain expressing NDO from *Pseudomonas* sp. NCIB 9816-4 was for the first time discovered that it can oxidaize of indole to indigo was first shown in recombinant (28). NDO oxidized indole to an unstable *cis*-dihydroindolediol that dehydrates to indoxyl, and subsequently undergoes spontaneous oxidation to indigo. Dioxygenases catalyze this reaction and by colorimetric test has been widely utilized for detection and isolation of strains expressing mono- and dioxygenases, and in screening for mutants of these strains (Fig. 3). Commercial interest in the reaction led Genencor International to genetically engineer a cost-competitive, multistep pathway for the production of indigo from glucose in *E. coli* (29). The process for indigo production was based on a recombinant *E. coli* strain in which the tryptophan pathway was modified to allow a high level of indole production and cloned NDO from *P. putida* was expressed (29). Numerous modifications were made to the strain to improve metabolite flux, eliminate the formation of the by-product isatin, and ultimately increase the production of indigo to levels exceeding 18 g/L. Despite the technical success of the process, the commercial production of indigo has not been implemented at an industrial scale.

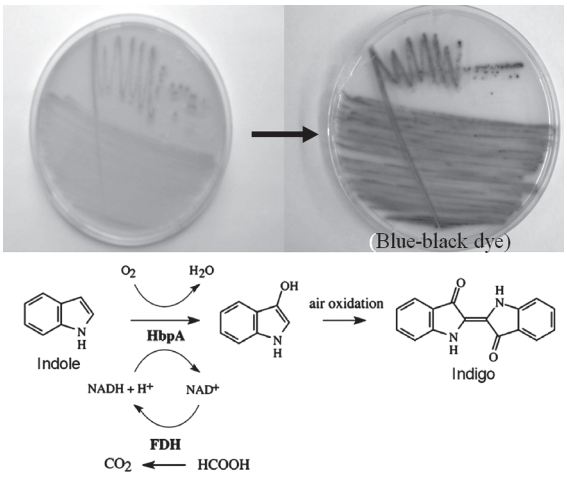

Fig. 3. Production of Indigo using Naphthalene dioxygenase from Indole

Dioxygenase and new biodegradation pathways: The engineering new pathways for the degradation of recalcitrant compounds have been used by aromatic hydrocarbon dioxygenases. Cloning of Genes encoding TDO from *P. putida* F1 and cytochrome P450cam into the genome of *Deinococcus radiodurans*, in the presence of high levels of radiation this recombinant strain was able to degrade toluene and related aromatic hydrocarbons (30). An engineered strain *Deinococcus radiodurans* has obtained as results of cloning and expression of mercury resistance gene (merA) together with the TDO genes, this strain has the ability to remediate mixed radioactive waste containing aromatic hydrocarbon pollutants and the heavy metal mercury. Genetic engineering was used to modify many bacterial isolates, that have the ability to degrade few number of aromatic hydrocarbon pollutants to increase the ability of these isolates to degrade a broad range of aromatic pollutants. When a constructed cassette, carrying genes for the conversion of styrene to phenylacetate, was introduced into *P. putida* F1 carrying the TOL plasmid, the engineered strain was capable of growth on an expanded range of aromatic hydrocarbons, including benzene, toluene, ethylbenzene, m-xylene, p-xylene, and styrene (31). The preferred gene cassette, which harbour the interested genes system was applied to a mini-transposon to increase the expression of the function genes caste and eliminate the undesirable expression of horizontal transfer among bacteria in the environment through genetic engineering. Such a strain could prove useful in the bioremediation of low molecular weight aromatic hydrocarbon pollution.

Conclusion

Multicomponent aromatic-ring-hydroxylating dioxygenases are class of enzymes holds significant promise for oxidize aromatic hydrocarbons to vicinal arene *cis*-diols are of paramount importance in providing the scientific foundations necessary for the development of bioremediation technology and in green chemistry by the ability of many of these enzymes to form pharmaceutical compounds in high enantiomeric purity are of great interest. To achieve this target either by identification an enzyme that oxidizes aromatic compound substrate to produce a specific product, the important thing firstly one can attempt to isolate a new bacterial strain with the desired ability, secondly screen the large number of well-characterized dioxygenases that are currently available, thirdly modify an available enzyme known to have activity by random mutagenesis methods (13, 32, 33) or by rational design, taking advantage of the growing number of available dioxygenase crystal structures (34, 35, 36). Finally, If you did not need to isolate and characterize the host bacterium, you can screen by metagenomic libraries (37, 38), especially with samples from diverse environments, may allow the identification of new dioxygenases with useful activities. Many steps are required to develop viable and economical commercial processes, once an enzyme with the desired selectivity is obtained. Efficiency can be realized through high protein expression coupled with process development. Overall productivity may also be increased by improving the activity or thermostability of the enzyme, or by reducing product inhibition. To get more product and determine which material will be used either purified enzymes or whole-cells, the well-designed process must take into consideration two things, firstly the multicomponent enzyme and the NADH requirement

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References

1. Kirk, T.K. and Farrell, R.L. (1987). Enzymatic “combustion”: the microbial degradation of lignin. Annu Rev Microbiol 41: 465-505

2. Gibson, D.T. and Parales, R.E. (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr Opin Biotechnol 11: 236-243

3. Pieper, D. H., González, B., Cámara, B., Pérez-Pantoja, D. and Reineke, W. (2009). Aerobic degradation of chloroaromatics in Hydrocarbons, oils and lipids (K. N. Timmis, ed). Vol. 2 Microbiology of utilization of hydrocarbons, oils and lipids, Springer Verlag

4. Yuji Ashikawa, Zui Fujimoto, Yusuke Usami, Kengo Inoue, Haruko Noguchi, Hisakazu Yamane and Hideaki Nojiri (2012). Structural insight into the substrate- and dioxygen-binding manner in the catalytic cycle of rieske nonheme iron oxygenase system, carbazole 1,9a-dioxygenase BMC Structural Biology 12:15

5. Gibson, D.T., Koch, J.R. and Kallio, R.E. (1968). Oxidative degradation of aromatic hydrocarbons by microorganisms. 1. Enzymatic formation of catechol from benzene. Biochemistry 7: 2653-2662.

6. Reiner, A.M. (1972). Purification and properties of the catechol-forming enzyme 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (NAD+) oxidoreductase (decarboxylating). J Biol Chem 247: 4960-4965.

7. Bundy, B.M., Campbell, A.L. and Neidle, E.L. (1998). Similarities between the antABC-encoded anthranilate dioxygenase and the benABC-encoded benzoate dioxygenase of Acinetobacter sp. strain ADP1. J Bacteriol 180: 4466-4474.

8. Farrow, J.M. and Pesci, E.C. (2007). Two distinct pathways supply anthranilate as a precursor of the Pseudomonas quinolone signal. J Bacteriol 189: 3425-3433.

9. Werlen, C., Kohler, H. P. and van der Meer, J. R. (1996). The broad substrate chlorobenzene dioxygenase and cis-chlorobenzene dihydrodiol dehydrogenase of Pseudomonas sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. J Biol Chem 271, 4009-4016.

10. Beil, S., Mason, J. R., Timmis, K. N. and Pieper, D. H. (1998). Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. J Bacteriol 180, 5520-5528.

11. Parales, J. V., Parales, R. E., Resnick, S. M. and Gibson, D. T. (1998). Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from Pseudomonas sp. strain JS42 is determined by the C-terminal region of the alpha subunit of the oxygenase component. J Bacteriol 180, 1194-1199.

12. Nam, J. W., Nojiri, H., Yoshida, T., Habe, H., Yamane, H. and Omori, T. (2001). New classification system for oxygenase components involved in ring-hydroxylating oxygenations. Biosci Biotechnol Biochem 65, 254-263.

13. Zhao, H., Chockalingam, K. and Chen, Z. (2002). Directed evolution of enzymes and pathways for industrial biocatalysis, Curr. Opin. Biotechnol., 13:104–110.

14. Hudlicky, T., Gonzalez, D. and Gibson, D.T. (1999). Enzymatic dihydroxylation of aromatics in enantioselective synthesis: expanding asymmetric methodology, Aldrichimica Acta, 32: 35–62

15. Eduardo Busto, Vicente Gotor-Fernández and Vicente Gotor (2012) Asymmetric Chemoenzymatic Synthesis of Ramatroban Using Lipases and Oxidoreductases J. Org. Chem. 77, 4842

16. Boyd, D.R., Sharma, N.D., O'Dowd, C.R., Carroll, J.G., Loke, P.L. and Allen, C.C.R. (2005). cis- Dihydrodiol, arene oxide and phenol metabolites of dictamnine: key intermediates in the biodegradation and biosynthesis of furoquinoline alkaloids,

Chem. Commun., 31: 3989–399

17. Zezula, J. and Hudlicky, T. (2005). Recent progress in the synthesis of morphine alkaloids, Synlett, 3, 388–405.

18. Bui, V.P., Hudlicky, T., Hansen, T.V. and Stenstrom, Y. (2002). Direct biooxidation of arenes to corresponding catechols with E. oli JM109(pDTG602). Application to synthesis of combretastatins A-1 and B-1, Tetrahedron Lett., 43:2839–284.

19. Newman, L.M., Garcia, H., Hudlicky, T., and Selifonov, S.A. (2004). Directed evolution of the dioxygenase complex for the synthesis of furanone flavor compounds, Tetrahedron, 60, 729–734.

20. Connors, N., Prevoznak, R., Chartrain, M., Reddy, J., Singhvi, R., Patel, Z., Olewinski, R., Salmon, P., Wilson, J. and Greasham, R. (1997). Conversion of indene to cis-(1S, 2R)-indandiol by mutants of Pseudomonas putida F1, J. Ind. Microbiol. Biotechnol., 18: 353–359.

21. Wackett, L.P., Kwart, L.D. and Gibson, D.T. (1988). Benzylic monooxygenation catalyzed by toluene dioxygenase from Pseudomonas putida, Biochemistry, 27: 1360–1367.

22. Reddy, J., Lee, C., Neeper, M., Greasham, R. and Zhang, J. (1999). Development of a bioconversion process for production of cis-1S, 2R-indandiol from indene by recombinant Escherichia coli constructs, Appl. Microbiol. Biotechnol., 51: 614–620.

23. Zhang, N., Stewart, B.G., Moore, J.C., Greasham, R.L., Robinson, D.K., Buckland, B.C. and Lee, C. (2000). Directed evolution of toluene dioxygenase from Pseudomonas putida for improved selectivity toward cis-indandiol during indene bioconversion, Metab. Eng., 2: 339–348.

24. O'Brien, X.M., Parker, J.A., Lessard, P.A. and Sinskey, A.J. (2002). Engineering an indene bioconversion process for the production of cis-aminoindanol: a model system for the production of chiral synthons, Appl. Microbiol. Biotechnol., 59, 389–399

25. Blacker, A.J., Boyd, D.R., Dalton, H. and Bowers, N. (1996). Preparation of hydroxyl compounds by conversion with dioxygenase. WO 96/37628, May 20.

26. Boyd,D.R.,Sharma,N.D.,Bowers, N.I.,Goodrich, P.A.,Groocock, M.R., Blaker, A.J., Clarke, D.A., Howard, T. and alton, H. (1996). Stereoselective dioxygenase-catalyzed benzylic hydroxylationat prochiral methylene groups in the chemoenzymatic synthesis of enantiopure vicinalamino-indanols, Tetrahedron Asymmetry, 7: 1559–1562.

27. Lakshman, M.K., Chaturvedi, S., Zajc, B., Gibson, D.T. and Resnick, S.M. (1998). A general chemoenzymatic synthesis of enantiopure cis b-amino alcohols from microbially derived cis-glycols, Synthesis, 9:1352–1356.

28. Ensley, B.D., Ratzkin, B.J., Osslund, T.D., Simon, M.J., Wackett, L.P. and Gibson, D.T. (1983) Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo, Science, 222:167–169.

29. Berry, A., Dodge, T.C., Pepsin, M. and Weyler, W. (2002). Application of metabolic engineering to improve both the production and use of biotech indigo, J. Ind. Microbiol. Biotechnol., 28:127–133.

30. Lange, C.C., Wackett, L.P., Minton, K.W. and Daly, M.J. (1998). Engineering a recombinant Deinococcus radiodurans for organopollutant degradation in radioactive mixed waste environments, Nat. Biotechnol., 16, 929–933.

31. Lorenzo, P., Alonso, S., Velasco, A., Diaz, E., Garcia, J.L. and Perera, J. (2003) Design of catabolic cassettes for styrene

biodegradation, Antonie Van Leeuwenhoek., 84:17–24.

32. Cirino, P.C. and Arnold, F.H. (2002). Protein engineering of oxygenases for biocatalysis, Curr. Opin. Chem. Biol., 6, 130–135.

33. Jaeger, K.E. and Eggert, T. (2004). Enantioselective biocatalysis optimized by directed evolution, Curr. Opin. Biotechnol., 15, 305–313.

34. Kauppi, B., Lee, K., Carredano, E., Parales, R.E., Gibson, D.T., Eklund, H. and Ramaswamy, S. (1998). Structure of an aromatic ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase, Structure, 6:571–586.

35. Furusawa, Y., Nagarajan, V., Tanokura, M., Masai, E., Fukuda, M. and Senda, T. (2004). Crystal structure of the terminal oxygenase of biphenyl dioxygenase from Rhodococcus sp. strain RHA1, J. Mol. Biol., 342: 1041–1052.

36. Martins, B.M., Svetlitchnaia, T. and Dobbek, H. (2005). 2-Oxoquinoline 8-monooxygenase oxygenase component: active site modulation by Rieske-[2Fe-2S] center oxidation/reduction, Structure, 13: 817–824.

37. Daniel, R. (2004). The soil metagenome a rich resource for the discovery of novel natural products, Curr. Opin. Biotechnol., 15: 199–204.

38. Lorenzo, P., Liebeton, K., Niehaus, F. and Eck, J. (2002). Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space, Curr. Opin. Biotechnol., 13: 572–577.

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T cell response to oncoprotein E7 of Human Papillomavirus genotype 16 (HPV 16) delivered by *Salmonella typhi* strain Ty21a (*S. typhi* Ty21a) in mice.

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Abstract

Immunotherapy is intensely being considered for either a stand-alone or supplemental treatment of HPV associated pre-cancers and cancers. The aim of the study was to enquire whether *S. typhi* Ty21a engineered to express the HPV 16 E7 (Ty21a-E7) elicits T cell response to the oncoprotein. The codon optimized synthetic HPV16 E7 gene was cloned in an expression vector under a constitutive promoter (P_{tac}). Female mice of strain C57BL/6 were immunized intra nasally with Ty21a-E7. The cell mediated immune response was evaluated employing the IFNγ-Elispot assay. Splenocytes were stimulated with the affinity purified recombinant proteins, either GST-E7 or GST expressed in *E. coli* BL21. The HPV 16 E7 specific IFN secreting- spot forming cells (SFCs) obtained on stimulation with purified GST-E7 in the Elispot assay was 240 (±6.92) cells/million splenocytes, significantly higher ($p< 0.01$) than the number for splenocytes stimulated with GST (67.3 ±26.40 cells/million). The antigen specific T cells were found to be predominantly of the CD4+ T cell type. The results show E7 specific CMI response in the mouse model of study. To our knowledge this is the first study reporting CMI response in mice to an HPV oncoprotein delivered by *S. typhi* Ty21a- the live-attenuated, oral, typhoid vaccine strain.

Keywords : HPV 16 E7, Elispot, T cell response, CD4+ T cells, Ty21a-E7.

Introduction

Infection of the high-risk genotypes of human papilloma virus (HPV) is a pre-requisite for cervical cancer (1, 2). The high-risk HPV genotype 16 (HPV16) contributes to nearly 50-56% of all cervical cancers (3). The latest consolidated global cancer statistics, GLOBOCAN 2008 ranks cervical carcinoma, the third most occurring cancer in women. Approximately 80% of these cases occur in the developing nations where, including India, it is the biggest cause for cancer related mortality among women (4). The world-wide implementation of the recently licensed prophylactic vaccines is projected to bring down incidence by 70% but has no impact on the existing cervical cancer burden (5).

The disease management strategy of mandatory screening, follow-up and prompt treatment has substantially brought down the cancer burden and mortality in the developed world (5, 6). But economical, social and logistical constraints have impeded its implementation in India and most of the developing world (6, 7). Novel, non-invasive treatment strategies such as therapeutic vaccines therefore hold much promise. The emerging view is that effective therapy is possible by employing, in concert, the different approaches- therapeutic vaccines, blockers of immunosuppressive mechanisms and conventional therapies (8, 9, 10).

The unique features of the HPV associated cancers present a perfect model for the development of an effective therapeutic cancer vaccine (8, 11). The long latency from infection to cancer coupled with distinct stages of the disease progression provides the right window to monitor vaccine efficacy (8, 11). HPV early genes E6 and E7 play a central role in the induction and progression towards cancer (11, 12). The oncogenes are more often than not integrated into the host genome and are expressed in nearly all stages of the disease (8, 11, 12). Thus, E6 and E7 present cancer specific, non-self, target antigens for the development of therapeutic vaccines. Several candidate therapeutic vaccines based on E6 and E7 are in different stages of development (13).

The live-attenuated *Salmonella enterica* serovar Typhi strain Ty21a (*S. typhi* Ty21a) has been in use as an oral typhoid fever vaccine for nearly three decades (14). The proven safety record and immune efficacy of Ty21a has led to its extensive evaluation for the oral delivery of heterologous antigens (14, 15). The present study dwells on construction of the HPV16 E7 recombinant *S. typhi* Ty21a and evaluation of cell mediated immune response (CMI) in the mouse model.

Materials and Methods

Plasmids and Bacterial Strains: The live-attenuated vaccine strain *Salmonella typhi* Ty21a and the constitutive expression vector pFS14nsd was obtained from Dr. Denise Nardelli, CHUV-Laussane, Switzerland. Codon optimized HPV16 E7 synthetic gene was procured from GeneArt, Life Technologies, USA cloned in a plasmid. The *E.coli* cloning host Top 10 was procured from Invitrogen Corporation, USA.

Cloning: The insertion of E7 in the pFS14nsd vector to generate the pFS14nsd/E7 clone was performed using standard cloning techniques (16). E7 gene was introduced in the pFS14nsd vector between the Nco I and Hind III sites. The vector-insert ligation was performed with the T_4 DNA ligase based Rapid Ligation Kit™ from Roche, USA as per kit directions. Plasmids were screened for E7 gene insertion by restriction enzyme analysis with Nco I and Hind III. The pFS14nsd/E7 clone was confirmed by DNA sequencing.

Transformation of Salmonella typhi Ty21a: The *S. typhi* Ty21a competent cells were prepared following the standard procedure (16). Electroporation of competent *S. typhi* Ty21a (100µl cell suspension) with 5 µg of pFS14nsd/E7 was performed in the 2mm cuvettes (BTX Harvard Appartus, USA) using standard procedures (16) at- voltage-2.5KV, capacitance-50µF and resistance- 200Ω. The cell suspension, subsequent to electroporation and a brief incubation (1hr) was plated in LB agar containing kanamycin sulfate (50µg/ml).

HPV16 E7 expression in S. typhi Ty21a: The HPV 16 E7 recombinant *S. typhi* Ty21a whole cell lysates for overnight cultures were electrophoresed on a pre-cast, Precise Tris-HEPES, SDS-PAGE system (Thermo Scientific). The proteins in the pre-cast gel were then blotted on to the PVDF membrane using the Hoefer Protein blotting apparatus (Hoefer, USA). The blot was probed with the monoclonal antibody specific to HPV16 E7 sourced from Santa Cruz Biotechnology, Inc, USA.

Mice: Specific pathogen free, C57BL/6 female mice were used in the study in accordance with the guidelines laid out by the Institutional Animal Ethics Committee (IAEC). Mice were housed in ventilated cages. Each treatment group contained ten numbers of mice. Feed and water were provided *ad libitum*. Clean bedding and sanitation was maintained at all times during the course of the experiment.

Preparation of Inoculum: Ty21a-E7 was cultured in flasks overnight at 37ºC in a gyratory shaker. The culture at OD_{600} of 1.8 to 2.0 was harvested by centrifugation (2500g/20min) at 4ºC in a table top centrifuge (Eppendorf AG, Germany). Cell pellet was washed once with phosphate buffer saline, pH 7.4 (PBS), centrifuged, re-suspended in sterile PBS in 1/

100th of initial culture volume. Viable bacteria were determined by the standard, spread plate culture of serial dilutions. Hundred micro litre volumes of the dilution of the inoculum *viz.,* 10^{-10}, 10^{-9}, and 10^{-8} in PBS were plated in Luria-Bertani agar containing kanamycin sulphate (50µg/ml). Plates were incubated at 37ºC for 36h for the enumeration of colonies.

Anesthetization and Immunization of Mice: Mice were anesthetized by intra peritoneal administration of a mixture containing ketamine hydrochloride, xylazine hydrochloride and diazepam at 100µl/mouse (each 100µl anesthetic dose constituted 30µl of ketamine (stock=50mg/ml), 1.0µl of xylazine (stock=20mg/ml) and 4.0µl of diazepam (stock=5mg/ml) in sterile distilled water). Ty21a-E7 inoculum was administered at 15 µl /mouse. Anesthetized mice were administered in the nostrils 15µl/mouse of Ty21a-E7 inoculum with the aid of a micropipette. Sham immunized mice were administered 15µl of PBS/mouse. Immunizations were performed in four doses. The first booster dose was administered 30 days after the primary immunization; subsequent two boosters were given 15 days apart.

Isolation of splenocytes: Mice were sacrificed by spinal dislocation 6 days after the final booster. Splenectomy and isolation of splenocytes were performed in aseptic conditions according to the standard procedures (17). Briefly, Spleens were disrupted mechanically with the aid of syringe plunger while in RPMI-1640 (Life Technologies Corp., USA.) and passed through a 70 µm Cell Strainer (BD Corp., USA) to obtain single cell suspensions. Splenocytes suspensions were treated with RBC lysis buffer (5 ml/spleen; Sigma-Aldrich Chemical Co., USA) and subjected to density-gradient centrifugation, with Lymphoprep™ (Axis-Shield PoCo, Norway) at 1:2 v/v ratio of Lymphoprep™ and splenocytes. The splenocyte enriched "buffy-coat" layer at the junction was retreived using a micropipette and washed with RPMI-1640 containing 10% fetal bovine serum (FBS, Life Technologies Corp., USA.). Viable cells were enumerated under microscope using a haemocytometer (Improved Neubauer, Rohem India). Splenocytes were resuspended in the cryo-preservant medium-FBS containing 10% di-methyl sulfoxide (DMSO; Sigma Aldrich), and aliquots transfered into screw capped cryo vials (Nalgene® Cryoware, Thermo Fisher) at 20million cells/ml/ vial in cryo cans (liquid Nitrogen) until further use.

CD4+ T cell and CD8+ T cell isolations with Magnetic activated cell sorting (MACS): Cryo-vials containing splenocytes were thawed at 37ºC for 2 min in a water-bath; splenocytes were washed and enumerated before sorting. The CD4+ enriched T cell population from mouse splenocytes were isolated using the CD4+ T cell isolation kit-II and the autoMACS™ Separator from Miltenyi Biotec Gmbh, Germany. About 40 million viable splenocytes were passed through the autoMACS™ column (Miltenyi Biotec) in the autoMACS™ separator following the manufacturer's instructions. The flow-through contained the CD4+ enriched T cells. The cells bound to the column were eluted as per the manufacturer's instructions to obtain the CD4+ depleted preparation. Similarly, the CD8+ T cell enriched and depleted preparations were obtained using the CD8+ T cell isolation Kit-II (Miltenyi Biotec). Cells from all fractions were enumerated before the Elispot assays.

Elispot assay: The Elispot assays were performed with the Mouse IFN gamma Elispot Ready-SET-Go!® kit from eBiosciences® in the 96 well MultiScreen HTS™ IP sterile plates (Millipore, USA). The coating (with capture antibodies) and blocking with fetal bovine serum (10% FBS in RPMI-1640) were performed as per the instructions in the kit. Cryo-vials containing splenocytes were thawed at 37ºC for 2min in a water-bath. Splenocytes were then washed in RPMI -1640 containing 10% FBS (cRPMI) and enumerated. Viable splenocytes at 0.5 X 10^6 cells/100µl/ well in cRPMI were seeded on to the wells of the Elispot plates in triplicates for the determination of E7 specific IFNγ secreting T cells. Similarly, the T cell enriched (either CD4+ T cell or CD8+ T cell) splenocyte preparations

and the T cell depleted fractions were also plated at a viable cell concentration of 0.3 X 10^6 cells/ well in triplicates. Splenocytes were stimulated with either affinity purified recombinant GST or GST-E7 at 1µg/well. Assay controls were stimulated with 2µg of ConA. Non-stimulated controls were maintained in all assays. Stimulations were performed in the Hera Cell 240 incubator at 37ºC with 5% CO_2 for 24h. After stimulation, cells were discarded; wells washed and incubated with biotinylated detection antibodies for 1h. Wells were washed again and incubated with 100µl/well of Streptavidin-ALP (Mabtech Gmbh) at 1:1000 in PBS containing 0.05% Tween 20. Finally the wells were developed with BCIP/NBT (Mabtech Gmbh, Germany).

Enumeration of Spots: Air-dried Elispot plates were scanned in the automated ImmunoSpot® Series 5 UV Reader from Cellular Technology Limited (CTL), USA. Spots were analyzed and enumerated in the reader using the Immunocapture® Version 5.0 software tool according to the recommended, default, user-independent SmartCount® settings.

Statistical analysis: The one-way Student's t-test was employed for determining the test of significance of the spots obtained on stimulation with GST-E7 over that obtained by stimulation with GST at 99% confidence limit ($p<0.01$)

Results and Discussion

Considering the socio-economic setting of the cervical cancer scourge a therapeutic vaccine is ought to be cost-effective, and more importantly easy to administer (7, 13). An efficacious oral vaccine is therefore an attractive proposition. The mode of infection and morphological features of Ty21a is said to augment immune responses to heterologous antigens. *S. typhi* naturally colonizes the macrophages and the infection activates the naïve cells into efficient antigen presenting cells (APCs). The involvement of APCs is likely to evoke strong, T_H1 cell dependant, T and B cell responses to antigens expressed in Typhi strain Ty21a (15). The flagellin and monophosphoryl lipid-A present on the bacterial surface are known ligands for toll-like receptors (TLRs) in the APCs (18). These pathogen associated molecular patterns (PAMPs) therefore would have an adjuvanting effect in eliciting a strong immune response to the heterologous antigens (18, 19).

Intra nasal mouse model for oral S. typhi Ty21a derived vaccines: *Salmonella typhi* is an enteric pathogen of humans and do not infect any other mammalian species. A transient, non-pathogenic infection occurs on intra nasal delivery of Ty21a in mice. Immune response to either Ty21a or the Ty21a delivered heterologous genes and proteins are evaluated in the intra nasally immunized mice (20). Hence the immune evaluation of recombinant Ty21a-E7 was conducted in intra nasally immunized female mice. The C57BL/6 mice strain were chosen for their T_H1 dominant phenotype that is ideally suited for the development of cell mediated immune response (21).

Cloning and expression: Immune response to heterologous antigens delivered by *Salmonella* is subject to, among other factors the level and stability of expression in the bacterial delivery vehicle (19, 22, 23). HPV genomes in contrast to the prokaryotes are AT rich and show a strong codon bias towards 18 codons (24). Hence, expression of the native HPV genes in the heterologous expression hosts has often been proven a tall order (24). Therefore, the synthetic E7 gene codon optimized for expression in *S. typhi* (obtained from GeneArt, USA) was used for cloning in to pFS14nsd. Figure 1 shows the restriction enzyme analysis for screening putative pFS14nsd/E7 clones. The plasmid clones were further sequence verified before introduction of the authenticated pFS14nsd/E7 construct into the live-attenuated Ty21a cells. The codon modified E7 cloned in Ty21a showed stable expression under the constitutive P_{tac} promoter in Ty21a (Fig. 2).

Evaluation of CMI response using **Elispot** ***assay:*** The cell mediated immune response to

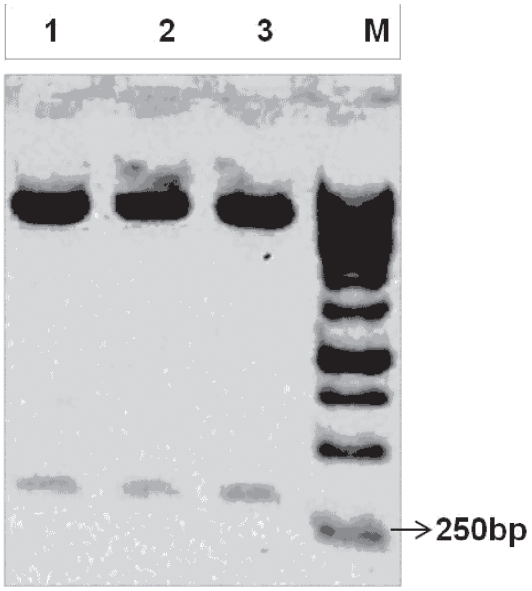

Fig. 1. Restriction enzyme analysis of the codon optimized HPV 16 E7 clone in the constitutive expression vector pFS14nsd. Lane 1, 2, 3- putative plasmid clones digested with Nco I and Hind III depicting ~297 bp products; Lane M- DNA molecular weight marker.

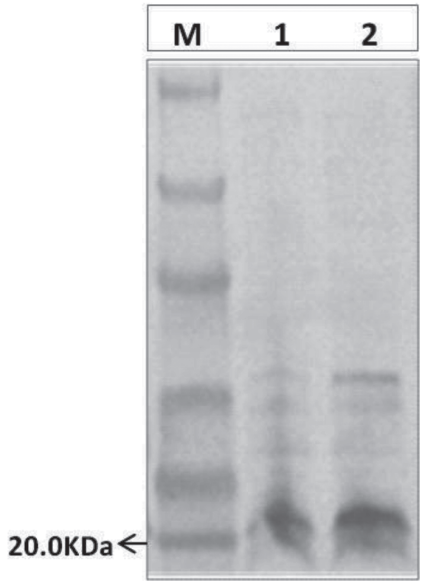

Fig. 2. Immunoblot probed with the HPV16 E7 specific monoclonal antibody for confirmation of E7 expression in Salmonella Typhi Ty21a. Lane 1, 2- Bacterial cell-lysate from HPV16 E7 recombinant *Salmonella* Ty21a colony cultures; Lane M: Pre-stained Protein molecular weight marker.

an antigen may be evaluated as a function of IFN_a secretion by T cells on antigenic stimulation *in vitro*. The E7 specific T cell response in Ty21a-E7 delivered mice, in this study, was determined using the Elispot assays. A well standardized Elispot assay scores over other alternative systems in its simplicity, ease of use and reproducibility (25, 26, 27). Since the assay is based on viable cells it is acknowledged to give a more realistic estimate of the immune response in vivo (27). Generally, oligopeptides (14 to 22 amino acids) that contain defined T cell epitopes of the antigen are used for T cell stimulations (28, 29). In the absence of well defined T cell epitopes, over lapping peptides spanning the full antigen are used. Synthetic peptides of high purity although preclude non-specific stimulation are expensive. The lack of well characterized T cell epitopes in HPV16 E7 apart from the CD8+ T cell epitope, RAHYNIVTF (30) prompted us to choose the affinity purified GST-E7 for splenocyte stimulations. We reasoned that stimulation with only the GST, expressed in the same *E.coli* host and purified by identical procedures, would serve as an adequate control to address possible non-specific stimulation. Further, the full length E7 in the GST-E7 comprises the full-range of epitopes specific for both the CD4+ and CD8+ T cell types. The T cell response detected therefore represents a comprehensive profile of E7 specific immune response.

T cell response to HPV16 E7: Stimulation of splenocytes from immunized mice showed significant E7 specific T cell response in the Elispot assays upon antigenic stimulation ($p<0.01$, one way Student's t-test). The IFNã secreting cells are represented as spot forming cells (SFCs) per million splenocytes (Fig. 3). SFCs obtained on stimulation with purified GST-

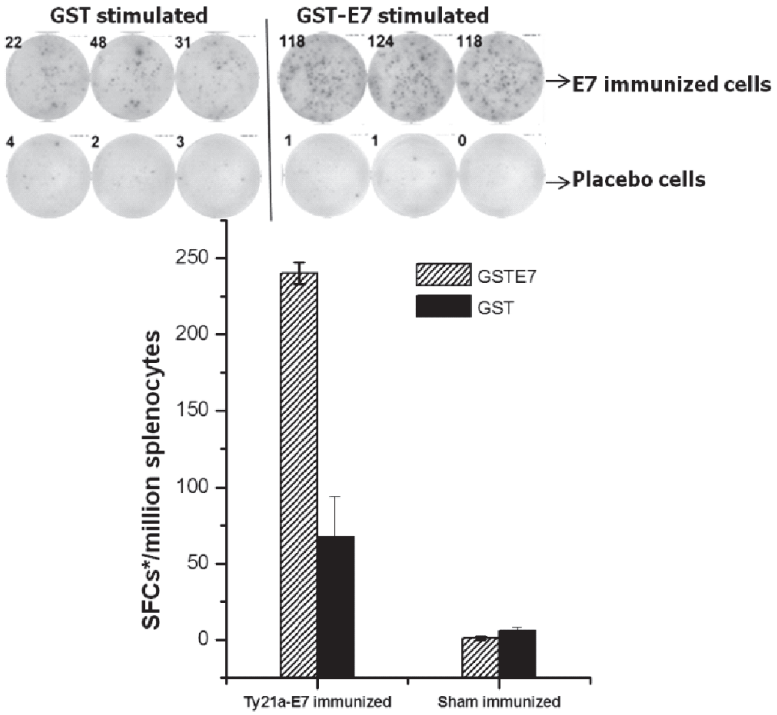

Fig. 3. IFNγ secreting spot forming T cells on stimulation of splenocytes with recombinant protein in the Elispot assay. Splenocytes were stimulated with either glutathione-S-transferylase - HPV16 E7 fusion protein (GST-E7) or GST. E7 immunized mice- Splenocytes from HPV16 E7 recombinant *S. typhi* Ty21a immunized mice; Sham immunized mice- Splenocytes from PBS administered mice.

E7 in the Elispot assay was 240 (±6.92) cells/ million splenocytes and significantly higher ($p< 0.01$) than the number for splenocytes stimulated with GST (67.3 ±26.40 cells/million). The results indicate E7 specific CMI response on intranasal immunization of mice. The non-immunized placebo control mice splenocytes showed barely any spots when stimulated with either the GST-E7 or GST thereby validating the results of the assay.

T cell subsets in the E7 specific cell mediated immunity: The immune response to E7 was further analysed to delineate the T cell subsets involved in the CMI. The CD4+ T cells were separated from the splenocytes using the MACS® technology. The Elispot assays of the CD4+ T cell enriched and CD8+ enriched T cell population indicate that the E7 specific response is predominantly that of the CD4+ T helper type (Fig. 4). The SFCs in CD4+ T cell enriched samples were 86.58 (±6.93) whereas only scanty spots (6.67; ±1.93), were visible in the CD8+ T cell enriched samples. The E7 specific, SFCs for the CD4+ T cell fractions were significantly higher than obtained on GST stimulation ($p<0.01$).

The predominance of CD4+ T helper subset in the IFNγ response to E7 was further confirmed by the Elispot results for the CD4+ T cell depleted and CD8+ T cell depleted fractions. The CD4+ depleted fraction of the splenocytes would contain CD8+ T lymphocytes apart from other lymphocytes and monocytes; and similarly the CD8 + T depleted fractions would contain mostly

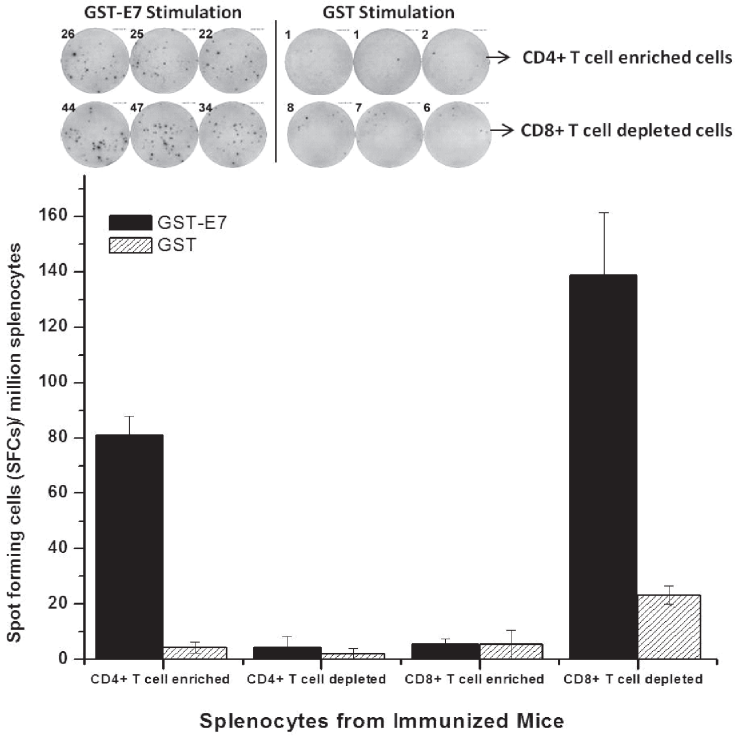

Fig. 4. Subset of T cells secreting IFNγ on stimulation with either GST-E7 or GST.

the CD4+ T cells. The Elispot results for the CD8+ depleted fraction that contain CD4+ cells showed higher number of SFCs (138.75, ±22.67) than in CD4+ T cell enriched population (86.58 ±6.93). This is perhaps indicative of the significance of APCs in processing recombinant protein for the stimulation of T cells to secrete IFNγ. The CD4+ depleted fraction (comprising CD8+ T cells and monocytes) did not show significant E7 specific SFCs (2.22 ± 1.92). Figure 4 illustrates the results in a bar graph.

***Significance of CMI in therapeutic efficacy*:** The protective and therapeutic efficacy of a vaccine against the HPV associated cancer is shown to be directly dependant on the strength of the T cell response. Several published reports on candidate therapeutic vaccines suggest that both CD4+ and CD8+ T cell response against the oncogenes E6 or E7 are vital for tumor protection and regression in the tumorigenic mouse models (8, 13, 28). Ty21a-E7 in the intra nasal mouse model seems to elicit CD4+ T helper response but little CD8+ T cell response as determined in the *ex vivo* Elispot assay. It is however premature to conclude the absence of CD8+ T cell response. Since, recombinant proteins are known to have limited ability in stimulating CD8+ T cells (27).

Pre-invasive cancer, the high grade cervical intra epithelial lesions (CIN-III), caused by recurrent HPV infection spontaneously regresses in many individuals (2, 11). The immune profile in such individuals is characterized by a robust CD4+ T cell response irrespective of the CD8+ T cell component in the lymphocyte milieu (11, 29). Therefore, even if E7 specific CD8+ T cells are lacking, the presence of specific CD4+ T cell response holds significance in the therapeutic efficacy of Ty21a-E7. The safety of the live-attenuated Ty21a strain, ease of administration

of an oral vaccine, and the T cell response to the expressed antigen are encouraging factors to continue the efforts in the development of the Ty21a-E7 candidate vaccine.

Conclusion

The HPV16 E7 recombinant Ty21a delivered through the intra nasal route in mice elicits E7 specific cell mediated immune response. The IFNγ secreting splenocytes are pre-dominantly CD4+ T helper cells. To our knowledge this is the first report on T cell response to HPV oncogenes delivered by *Salmonella typhi* Ty21a. The results are encouraging to further pursue efforts towards the development of the Ty21a based oral therapeutic vaccine for HPV associated cancers.

References

1. Zur Hausen, H. (2009). Papillomaviruses in the causation of human cancers - a brief historical account, 384(2):260-265.
2. Muñoz. N., Castellsagué, X., de González, A. B. and Gissmann, L. (2006). Chapter 1: HPV in the etiology of human cancer, Vaccine, 24 Suppl 3:S3/1-10.
3. Clifford, G., Franceschi, S., Diaz, M., Muñoz, N.and Villa, L. L. (2006). Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases, Vaccine, 24 Suppl 3:S3/26-34.
4. Arbyn, M., Castellsagué, X., de Sanjosé, S., Bruni, L., Saraiya, M., Bray, F. and Ferlay, J. (2011). Worldwide burden of cervical cancer in 2008. Annals of Oncology, 22(12):2675-86.
5. Hakim, A. A. and Dinh, T. A. (2009). Worldwide impact of the human papillomavirus vaccine. Current Treatment Options in Oncology, 10(1-2):44-53
6. Sellors, J., Lewis, K., Kidula, N., Muhombe, K., Tsu, V.and Herdman, C. (2003). Screening and management of precancerous lesions to prevent cervical cancer in low-resource settings. Asian Pacific Journal of Cancer Prevention, 4(3):277-80.
7. Vallikad, E. (2006).Cervical cancer: the Indian perspective. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer, International Journal of Gynaecology and Obstetrics, 95 Suppl 1:S215-33.
8. van der Burg, S. H. and Melief, C. J. (2011).Therapeutic vaccination against human papilloma virus induced malignancies. Current Opinion in Immunology, 23(2):252-257.
9. Durrant, L. G., Pudney, V., Spendlove, I. and Metheringham, R. L. (2010). Vaccines as early therapeutic interventions for cancer therapy: neutralising the immunosuppressive tumour environment and increasing T cell avidity may lead to improved responses. Expert Opinion in Biological Therapy, 10(5):735-48.
10. Gray, A., Raff, A. B., Chiriva-Internati, M., Chen, S. Y. and Kast, W. M. (2008). A paradigm shift in therapeutic vaccination of cancer patients: the need to apply therapeutic vaccination strategies in the preventive setting, Immunological Reviews, 222:316-27.
11. Stanley, M. A. (2009). Immune responses to human papilloma viruses. Indian Journal of Medical Research, 130(3):266-76
12. Yugawa, T. and Kiyono, T. (2009) Molecular mechanisms of cervical carcinogenesis by high-risk human papillomaviruses: novel functions of E6 and E7 oncoproteins. Reviews in Medical Virology, 19(2):97-113.
13. Su, J. H., Wu, A., Scotney, E., Ma, B., Monie, A., Hung, C. F. and Wu, T. C. (2010). Immunotherapy for cervical cancer: Research status and clinical potential. BioDrugs, 24(2):109-129
14. Kopecko, D.J., Sieber, H., Ures, J.A., Fürer, A., Schlup, J., Knof, U., Collioud, A., Xu D,

Colburn, K. and Dietrich G. (2009). Genetic stability of vaccine strain Salmonella Typhi Ty21a over 25 years. Int J Med Microbiol.; 299(4):233-46.

15. Gentschev, I., Spreng, S., Sieber, H., Ures, J., Mollet, F., Collioud, A., Pearman, J., Griot-Wenk, M.E., Fensterle, J., Rapp, U.R., Goebel, W., Rothen, S.A. and Dietrich, G. (2007). Vivotif--a 'magic shield' for protection against typhoid fever and delivery of heterologous antigens. Chemotherapy.; 53(3):177-80.

16. Sambrook, J.and Russell, D. W., (2001). Chapter 1. Plasmids and their usefulness in cloning In Molecular Cloning: A laboratory manual. (3rd edition) Cold, Spring Harbor Laboratory Press, New York, pp 1.31-1.103.

17. Kruisbeek, A. M. (2001). Isolation of mouse mononuclear cells In. Chapter 3 In vitro Assays for mouse lymphocyte function, Current Protocols in Immunology, Wiley Online Library.

18. Steinhagen, F., Kinjo, T., Bode, C. and Klinman, D. M. (2011). TLR-based immune adjuvants.Vaccine, 29(17):3341-3355.

19. Fraillery, D., Baud, D., Pang, S.Y., Schiller, J., Bobst, M., Zosso, N., Ponci, F. and Nardelli-Haefliger, D.(2007). Salmonella enterica serovar Typhi Ty21a expressing human papillomavirus type 16 L1 as a potential live vaccine against cervical cancer and typhoid fever. Clinical Vaccine and Immunology, 14(10):1285-1295.

20. Pickett, T. E., Pasetti, M. F., Galen, J. E., Sztein, M. B. and Levine, M. M. (2000). In vivo characterization of the murine intranasal model for assessing the immunogenicity of attenuated Salmonella enterica serovar Typhi strains as live mucosal vaccines and as live vectors.Infection and Immunity, 68(1):205-13

21. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., Hill, A. M. (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm J. Immunol. 164,6166-6173.

22. Baud, D., Ponci, F., Bobst, M., De Grandi, P. and Nardelli-Haefliger D. (2004) Improved efficiency of a Salmonella-based vaccine against human papillomavirus type 16 virus-like particles achieved by using a codon-optimized version of L1. Journal of Virology; 78(23): 12901-12909.

23. Gustafsson, C., Govindarajan, S. and Minshull, J. (2004). Codon bias and heterologous protein expression. Trends in Biotechnology, 22(7):346-53.

24. Zhao, K. N. and Chen, J. (2011). Codon usage roles in human papillomavirus. Rev Med Virol21(6):397-411.

25. Applications and advantages of ELISPOT. (2007). at http://www.immunospot.com/fileadmin/Documents/Documentations/Applications%20 and%20 Advantage% 20of% 20ELISPOT.pdf accessed on 23/06/2012.

26. Kalyuzhny, A. E. (2005). Chemistry and biology of the ELISPOT assay. Methods in Molecular Biology (Clifton, N.J.), 302:15-31.

27. Janetzki, S., Cox, J. H., Oden, N. and Ferrari G. (2005). Standardization and validation issues of the ELISPOT assay. Methods in Molecular Biology (Clifton, N.J.), 302:51-86.

28. Ren, F., Xu, Y., Mao, L., Ou, R., Ding, Z., Zhang, X., Tang, J., Li, B., Jia, Z., Tian, Z., Ni, B. and Wu, Y.(2010). Heat shock protein 110 improves the antitumor effects of the cytotoxic T lymphocyte epitope E7(49-57) in mice. Cancer Biology and Therapy, 9(2):134-141.

29. Coleman, N., Birley, H. D., Renton, A. M., Hanna, N. F., Ryait, B. K., Byrne, M., Taylor-Robinson, D. and Stanley, M. A. (1994). Immunological events in regressing genital warts, American Journal of Clinical Pathology, 102(6):768-74.

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Curcumin Induces Human Colon Cancer Cell death via p62/SQSTM1 Degradation, Phospho-ERK Up-regulation and Ceramide Generation

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Abstract

Curcumin is a natural yellow phenolic compound extracted from the Indian spice, turmeric (*Curcuma longa*). Several studies demonstrated the ability of curcumin to inhibit events associated with the promotion of cancer. We investigated the effects curcumin on human colon cancer cells *in vitro* and further examined the molecular mechanisms of curcumin induced cell death. The signaling adapter p62/SQSTM1, a multifunctional protein implicated in autophagy, apoptosis, cell signaling pathways and tumorigenesis, is one of the potential targets for anti-cancer therapy. In this study, we demonstrate a dose and time-dependent down-regulation of p62/SQSTM1 expression by curcumin that correlates with increase in the loss of viability of human colon cancer cells. We also found that curcumin enhanced phospho-ERK expression and ceramide (Cer) generation in human colon cancer cells. However, the present study also shows that, curcumin-induced p62/SQSTM1 degradation, up-regulation of ERK phosphorylation, Cer generation and cell death can be reversed by extracellular anti-oxidants such as glutathione (GSH) and N-acetyl cysteine (NAC). Overall, our results suggest that down regulation of p62/SQSTM1 and up-regulation of phospho-ERK and Cer generation may contribute to the anti-proliferative effects of curcumin against human colon cancer cells.

Keywords: p62/SQSTM1, phospho-ERK, Curcumin, Ceramide, Apoptosis, GSH

Introduction

Curcumin, a well-known chemo-preventive agent, has been shown to possess anti-inflammatory and anti-oxidant activities (1). It also has been reported as a potent inhibitor of mutagenesis and carcinogenesis (2). The anti-cancer property of curcumin has been extensively investigated in various cancer cells and in different laboratory animal models of cancer. Curcumin was found to inhibit cellular proliferation, and enhance apoptosis in a variety of human cancer cell lines *in vitro* (3). Currently, curcumin is in clinical trials for the treatment of cancers of pancreas, colon and multiple myeloma (3). The proposed mechanism of anti-tumor action of curcumin in majority of these studies involves suppression of NF-kB related gene products expression (4). Previously, we have shown that curcumin induces generation of reactive oxygen species (ROS) which leads to caspase dependent and independent apoptosis (5). We have also reported the modulation of curcumin-induced apoptosis by using PI3K inhibitor in breast carcinoma cell lines (6).

The signaling adapter p62/SQSTM1 is a multifunctional protein implicated in autophagy, apoptosis, cell signaling pathways and

tumorigenesis (7,8). p62/SQSTM1 was initially believed as interacting partner of atypical protein kinase C (aPKC). Recent studies reveal that p62/SQSTM1 act as a prime signaling molecule through its ability to recruit and oligomerize important signaling proteins in the cytosolic speckles to control cell survival and apoptosis (9,10)]. Recently, it has also been reported that elimination of p62/SQSTM1 is critical for the autophagy mediated suppression of tumorigenesis. These discoveries shed light on the significant role of p62/SQSTM1 as a central player in the life and death decisions of the cell.

Ceramide (Cer), a tumor suppressor lipid has been shown to exert potent growth suppressive effect on a variety of cell types (11). It has been reported that diverse array of stressors, including TNF-α (12), Fas ligation (13), irradiation (14), heat shock, (15) and anti-cancer drugs (16) were able to increase intracellular Cer level leading to the induction of apoptosis. Ceramide is produced by *de novo* synthesis in the endoplasmic reticulum or by the hydrolysis of sphingomyelin by acid sphingomyelinases (localized in the acidic compartment) and neutral sphingomyelinases (localized in the plasma membrane and mitochondria). Recently, we have reported curcumin induced caspase dependant Cer generation and apoptosis in human leukemic cells (17).

Extracellular signal-related kinase (ERK) is a family of mitogen-activated protein kinases (MAPKs) that are activated through a sequential phosphorylation cascade that amplifies and transduce signals from the cell membrane to the nucleus. Depending on the cell types and stimulus, ERK activity will mediate different tumor suppressive events, such as apoptosis, autophagy and senescence *in vitro* and *in vivo* (18). It has been reported that ERK can promote both intrinsic and extrinsic apoptotic pathways (19). Moreover, it has been established that sustained activation of ERK can induce autophagic cell death (20,21).

In the present study, we investigated the molecular mechanism of the anti-tumor potential of curcumin towards human colon tumor cell lines. The present study demonstrates the effects of curcumin on p62/SQSTM1, ERK and Cer generation *in vitro*. The results indicate that curcumin is potent regulator of p62/SQSTM1 expression. Curcumin induces degradation of p62/SQSTM1, up-regulation of ERK phosphorylation and Cer generation, which are the prime cellular signaling regulatory molecules. We also showed that curcumin-induced p62/SQSTM1 degradation, up-regulation of ERK phosphorylation, Cer generation and cell death were inhibited by the extracellular administration of glutathione (GSH) and N-acetyl cysteine (NAC).

Materials and Methods

Cell line, cell culture conditions and drug treatment: The HT-29 cells (ATCC, Rockville, MD, USA) were grown in McCoy's 5A containing GlutaMAX medium supplemented with 10% (V/V) heat inactivated fetal bovine serum (FBS). Cells were grown without antibiotics in an incubator containing humidified atmosphere of 95% air and 5%CO2 at 37ºC. Curcumin (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mM and was stored in a dark colored bottle at -20ºC. The stock was diluted to the required concentration with DMSO when needed. Prior to curcumin treatment, cells were grown to about 80% confluence, and then exposed to curcumin at different concentrations (0-100 μM) and for a different period of time (0-24 h). Cells grown in a medium containing an equivalent amount of DMSO served as control. Glutathione, NAC, U0126 and TPA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell viability Assay: Cell viability assay was carried out as described elsewhere with slight modifications (22). Cells were grown in 96 well microtiter plates (10,000 cells/well) and incubated for 24 h with or without different concentrations of curcumin. At the required time point, 100 μl media were removed and 25 ml of MTT (5 mg/ml) was added to each well. The plates were incubated for further 4 h at 37°C. After incubation

the plates were centrifuged at 1500 rpm for 5 min and the media were removed from all the wells. The formazan crystals were then solubilized in a 200 µl of DMSO. The colored solution was quantified at 570 nm by using 96 well plate reader (Perkin Elmer spectro-fluorometer, Victor 3X). The viability was expressed as a percentage over control.

Protein lysate preparation and western blot analysis: Cells were washed twice with phosphate buffered saline (PBS) and lysed in a RIPA lysis buffer [50 mM Tris HCl (pH 7.4), 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO4, 1 mM phenylmethylsufonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT) and EDTA-free protease inhibitor tablets per 20 ml buffer]. The cell lysates were centrifuged at 14000 rpm for 15 min. Total protein, determined by Bio-Rad protein assay, were mixed with 6X loading buffer and boiled at 100ºC for 3 min. SDS-PAGE and Western blot analyses were carried out as described previously [23]. The following antibodies were used: Anti-actin, anti-ERK1/2, anti-p62 and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-PARP and anti-phospho ERK1/2 were from Cell Signaling Technology. Anti-rabbit IgG and anti-mouse IgG were purchased from Sigma chemicals Co (St. Louis, MO, USA).

Intracellular Cer measurement: Intracellular Cer measured as described previously with little modification (24). After treatment cells were washed in PBS and lysed 50 mM Tris (pH-7.4) containing 0.4% IGEPAL CA 630 by freeze and thaw method. The final concentration of IGEPAL CA 630 in the assay was 0.2%. The lysate were heat at 70°C for 5 min in a water bath and centrifuged at 12000 rpm for 10 min at 4°C. The reaction was started by adding 10 µl of supernatant in the tube containing 20 ng recombinant human neutral ceramidase enzymes (10 µl) for 1 h at 37°C. The reaction was stopped by adding 55 µl of stopping buffer (1:9, 0.07 M potassium hydrogen phosphate buffer: methanol). The released SPH was derivatized with o-phthaladehyde (OPA) reagent. After stopping the reaction add 25 µl of freshly prepared OPA reagent (12.5 mg OPA dissolved in 250 µl ethanol and 12.5 µl 2-β-mercaptoethanol and made up to 12.5 ml with 3% (w/v) boric acid) was added. The mixture was allowed to stand for 30 min. An aliquot of 25 µl was injected in the HPLC. HPLC analysis was done using *Waters 1525* binary pump system. Waters XTerra C18 column (5 µm, 3 mm x 250 mm) was equilibrated with a mobile phase (20% methanol, 80% 1:9, 0.07 M potassium hydrogen phosphate buffer: methanol) at a flow rate of 0.5 ml/min. The fluorescence detector (Waters 2475) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Results

Curcumin induces degradation of p62/SQSTM1, Cer generation and cell death in HT-29 cells: Recently, it has been reported that p62/SQSTM1 is a multi-domain protein plays a central role in life and death decisions of the cell [7]. Therefore, we first examined the involvement of p62/SQSTM1 in human colon cancer cell line, HT-29 up on curcumin treatment. Curcumin treatment of HT-29 cells resulted in a dose (0-100 µM) dependent reduction in the expression of p62/SQSTM1 (Fig 1A). We then examined the optimum time required for the down-regulation of p62/SQSTM1 and found that 6 h incubation with curcumin was sufficient for the maximum suppression p62/SQSTM1 (Fig 1B).

Ceramide has been suggested as a tumor suppressor lipid, and its generation is induced exclusively by apoptotic insult and not during growth stimulation. Therefore, we next examined the involvement of Cer generation in HT-29 cells up on curcumin treatment. Curcumin treatment of HT-29 cells resulted in a dose (0-100 µM) dependent accumulation of Cer (Fig. 1C). The time required for Cer generation following curcumin treatment was also investigated. HT-29 cells when treated with 75 µM curcumin, an increase in the cellular Cer level was observed

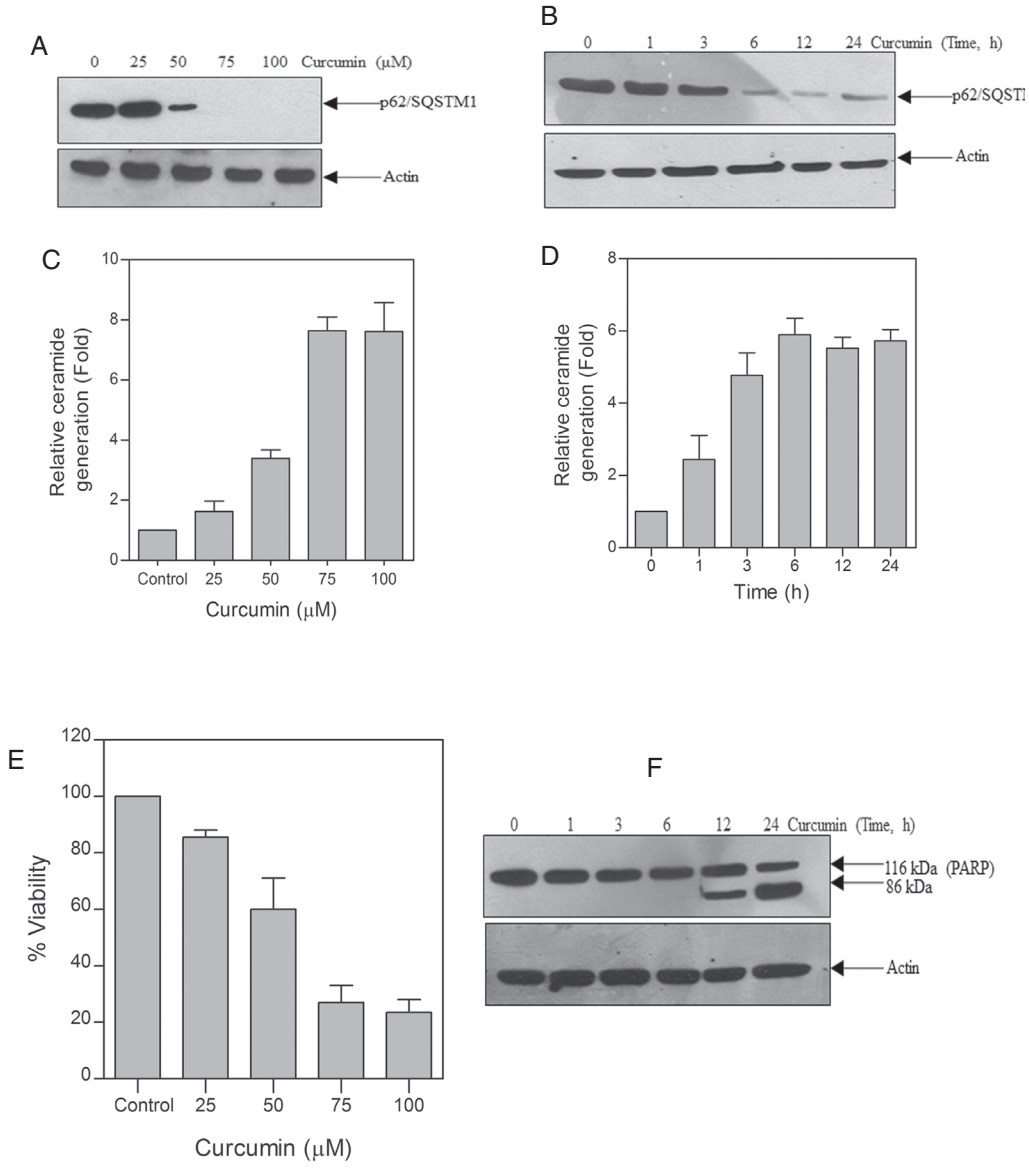

Fig. 1. Curcumin induces p62/SQSTM1 degradation, Cer generation and apoptosis in HT-29 cells. (A) HT-29 cells were treated with 0-100 μM concentration of curcumin for 24 h, (B) HT-29 cells were treated with 75 μM curcumin for indicated time period. The expression of p62/SQSTM1 was measured by using Western blot analysis. Actin was used as the loading control. Blots shown here are representative of three independent experiments. (C) HT-29 cells were treated with 0-100 μM concentration of curcumin for 24 h, (D) HT-29 cells were treated with 75 μM, curcumin for indicated time period. The Cer levels were measured as described in the "materials and methods". Data represent the mean ± S. D (*n=3*). (E) HT-29 cells were treated with 0-100 μM concentration curcumin for 24 h and cell viability was measured using MTT assays as described in the "materials and methods". Data represent the mean ± S. D (*n=3*). (F) HT-29 cells were treated with 75 μM curcumin for indicated time period and PARP cleavage was measured by Western blot analysis. Actin was used as the loading control. Blots shown here are representative of three independent experiments.

1h after treatment and sustained up to 24 h (Fig 1D).

In order to check whether p62/SQSTM1 degradation and rapid Cer generation induced by curcumin would lead to loss of cell viability, MTT assay was performed. Curcumin caused a dose dependent reduction in cell viability (Fig 1E). The cytotoxic effect of curcumin was, at least in part, attributable to apoptosis, as evidenced by PARP cleavage in HT-29 cells (Fig 1F).

Curcumin induces sustained ERK activation in HT-29 cell lines: The ERK signaling pathway has been shown to be activated in response to certain cellular stresses (18). Hence, we checked the effect curcumin on the ERK activation. HT-29 cells were exposed to curcumin and activation of ERK was determined by Western blot analysis using antibodies against phosphorylated form of ERK. Curcumin induces both dose and time dependent strong activation ERK (Fig 2A and B). The activation was apparent 1h after curcumin

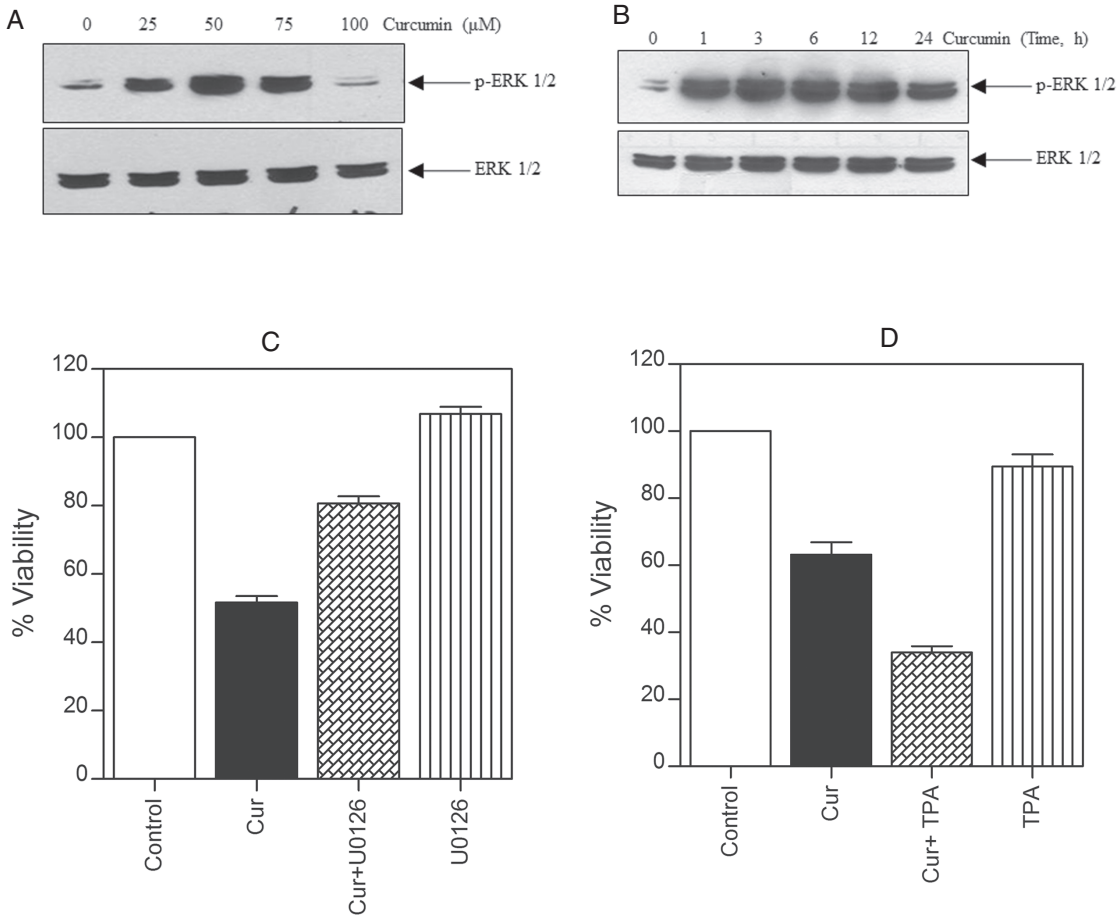

Fig. 2. Curcumin induces up-regulation of phospho-ERK in HT-29 cells. (A) HT-29 cells were treated with 0-100 μM concentration curcumin for 24 h, (B) HT-29 cells were treated with 75 μM concentration of curcumin for indicated time period and the expression of phospho-ERK was measure using Western blot analysis. Blots shown here are representative of three independent experiments. (C) HT-29 cells were pre-treated with 20 μM concentration of U0126 and (D) 50nM TPA for 1h, followed by incubation with 50 μm curcumin for 24h and cell viability assay was performed as described in the "materials and method".

treatment and sustained up to 24 h (Fig 2B). The increase in phospho-ERK did not result from the increased ERK expression, as total ERK levels were not altered relative to untreated cells.

In order to check whether ERK activation is required for curcumin induced apoptosis, we used U0126, a selective inhibitor of ERK (25). HT-29 cells were pre-treated with 20 μM concentration of U0126 for 1h prior to the addition of curcumin and viability assay was performed using MTT assay. As shown in the Fig 2C, exposure of curcumin alone to the cell resulted in a significant decrease in the viability and its effect was partially reversed by the U0126. Next, we have used phorbol ester TPA, an agent that capable of stimulating ERK signaling pathway (26, 27). HT-29 cells were pre-treated with 50 nM concentration of TPA for 1h prior to the addition of curcumin and viability assay was performed using MTT assay. As shown in the Fig 2D, TPA pre-treated cells were much more sensitive to curcumin. These results suggest that ERK activation plays an important role in curcumin induced HT-29 cell death.

Glutathione and NAC block curcumin induced down-regulation of p62/SQSTM1 and cell death in HT-29 cells: The antioxidants GSH and NAC play an important role in scavenging reactive oxygen species and the detoxification process (5). The exact molecular mechanism of the protective effects of these antioxidants against curcumin-induced apoptosis is not known. Therefore, we investigated whether GSH and NAC can protect cells against curcumin-induced p62/SQSTM1 down regulation and cell death in HT-29 cells. Pre-treatment of HT-29 cells with 10 mM GSH and NAC strongly suppressed the curcumin-induced degradation of p62/SQSTM1 (Fig. 3A) and cell death (Fig. 3B). These results indicate that the involvement oxidative stress in curcumin-induced HT-29 cells death.

Effects of GSH and NAC on curcumin induced up-regulation of phospho-ERK and Cer generation in HT-29 cells: In order to evaluate

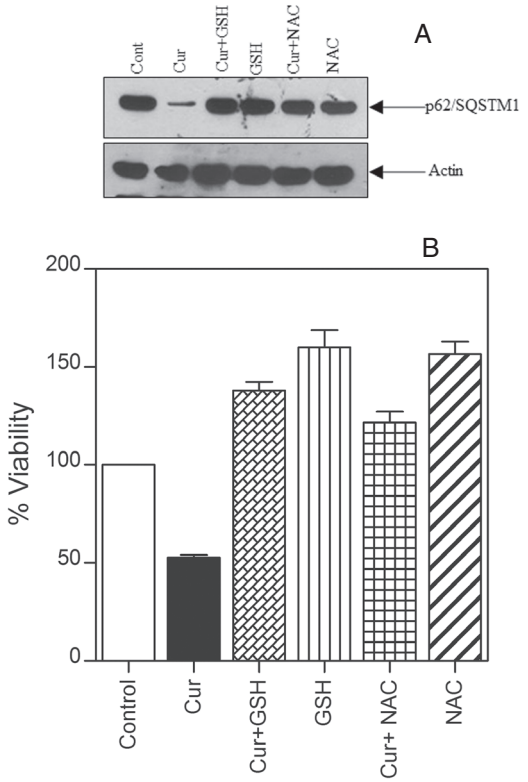

Fig. 3. Extracellular supplementation of GSH and NAC prevents curcumin induced p62/SQSM1 degradation and loss of viability. HT-29 cells were pre-treated with 10 mM concentration GSH and NAC for 1h, followed by incubation with 75 μM concentration of curcumin. (**A**) Expression of p62/SQSTM1 was measured as described in the "materials and method". (B) Viability was measure by MTT assay.

the protective mechanism(s) of GSH and NAC on curcumin-induced cell death, the influence of GSH and NAC on curcumin-induced ERK phosphorylation and Cer generation was examined. As shown in the Figure 4A and 4B, pre-treatment of the cells with GSH and NAC inhibited curcumin-induced ERK phosphorylation and Cer generation. These findings indicate that GSH and NAC may, indeed, mediate its anti-apoptotic effects via inhibition of ERK phosphorylation and Cer generation in curcumin-induced HT-29 cell death.

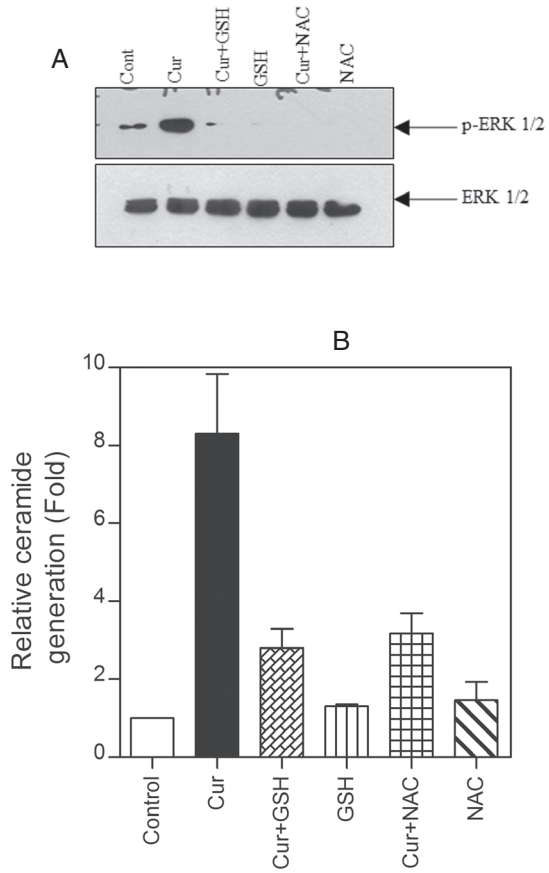

Fig. 4. Effects of GSH and NAC on curcumin induced up-regulation of phospho-ERK and Cer generation. HT-29 cells were pre-treated with 10 mM concentration GSH and NAC for 1h, followed by incubation with 75 μM curcumin. (A) Expression of phospho-ERK was measure using Western blot analysis. Blots shown here are representative of three independent experiments. (B) Cellular lipids were extracted and assayed for Cer by HPLC method as described in the "materials and method".

Discussion

Curcumin is one of the major anti-cancer drugs widely used in the treatment of wide variety of cancers. However, the signalling pathways triggered by curcumin in human colon cancer cells are not completely understood. The present study was designed to investigate the mechanism by which curcumin mediates its anti-proliferative effects in human colon cancer cells. In this study, we specifically focused on p62/SQSTM1, ERK and Cer, which are some of the prime cellular signalling regulatory molecules. Our data shows that, the ability of curcumin to inhibit the proliferation of human colon cancer cells correlated with the down-regulation of p62/SQSTM1 expression. Curcumin induced down-regulation of p62/SQSTM1 and cell death was reversed by anti-oxidants such as GSH and NAC. We found that curcumin enhanced the expression of phospho-ERK and the generation of tumor suppressor lipid, Cer. In sum, these results suggest that down-regulation of p62/SQSTM1, up-regulation of phospho-ERK and Cer generation contribute to the antiproliferative effect of curcumin in HT-29 human colon cancer cells.

This is first report to show that curcumin can induce the degradation of p62/SQSTM1. p62/SQSTM1 is a ubiquitin-binding multifunctional protein, which promotes survival-critical signals including proliferation, differentiation, and induction of anti-apoptotic genes (28). p62/SQSTM1 interacts with a central component of autophagy of the autophagy machinery, LC3, and transports ubiquitinated proteins to degradation by the autophagosome (29). It has been reported that p62/SQSTM1 is a selective substrate for autophagy. Abnormal expression of p62/SQSTM1 has been documented in various cancers including gastro-intestinal, prostate and breast cancers (30,31). Moreover, knock down of p62/SQSTM1 sensitizes SKOV3/DDP ovarian cancer cell to cisplatin (32). Previously, we have shown that anti-oxidants such as NAC and GSH tightly controls curcumin induced cell death in mouse fibroblast L929 cells (5) and human leukemic cells such as Jurkat, Molt-4 and K562 (17). Reversal of the effect of curcumin on p62/SQSTM1 by anti-oxidants suggests the role of oxidative stress in this pathway.

We found that curcumin up-regulated the expression of phospho-ERK in human colon cancer cell. Our data demonstrate that, the activation of ERK plays an important role in

curcumin induced cell death in HT-29 cells. Curcumin treatment resulted in high and sustained activation of ERK in these cells. Several other studies also reported that, the activation of ERK leads to increased sensitivity of cancer cells to chemotherapeutic drugs (19,33). Utilizing U0126 and TPA, to modulate ERK activity, we found that down regulation of ERK resulted in partial protection against curcumin-induced cell death, whereas, enhancement ERK activity sensitized it. In our study, the ERK inhibitor did not completely prevent curcumin induced cell death. This may be due to the involvement of other signaling pathways that are independent ERK activation. In the present study, the antioxidants GSH and NAC inhibited curcumin-induced activation ERK, suggesting that, the curcumin-induced activation of ERK is mediated by oxidative stress dependent mechanisms.

In the present work, we demonstrate that Cer production parallels the sensitivity of curcumin, based on the loss of cell viability levels. Consistent with our results, sensitivity of curcumin to various cancer cells including HCT 116 colon cancer cells (34), Jurkat leukemic cells (17), PC3 prostate cancer cells (35) was directly related to the Cer generation. Moreover, recently it has been reported that, C_6-Cer sensitizes melanoma calls to curcumin-induced cell death (36). Furthermore, a direct relationship has been observed between resistant to radiation induced apoptosis and defective Cer generation (37). Our results are in agreement with the fact that the kinetics of cell death and Cer generation parallel each other, suggests an involvement of Cer in curcumin-induced cell death in HT-29 cells.

Conclusion

In conclusion, our results provide an additional mechanism through which curcumin may mediate its antiproliferative effects in HT-29 cells, via down-regulation of p62/SQSTM1, up-regulation of phospho-ERK and Cer generation. These results suggest that p62/SQSTM1 could be a novel therapeutic target for cancer treatment using curcumin.

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References

1. Azuine, M. A., Kayal, J.J. and Bhide, S.V. (1992). Protective role of aqueous turmeric extract against mutagenicity of direct-acting carcinogens as well as benzo [alpha] pyrene-induced genotoxicity and carcinogenicity. J Cancer Res Clin Oncol, 118: 447-452.

2. Rao, C. V., Rivenson, A., Simi, B. and Reddy, B.S. (1995). Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res, 55: 259-266.

3. Aggarwal, B.B., Kumar, A. and Bharti, A.C. (2003). Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res, 23: 363-398.

4. Aggarwal, S., Ichikawa, H., Takada, Y., Sandur, S.K., Shishodia, S. and Aggarwal, B.B. (2006). Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IkappaBalpha kinase and Akt activation. Mol Pharmacol, 195: 195-206.

5. Thayyullathil, F., Chathoth, S., Hago, A., Patel, M. and Galadari, S. (2008). Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. Free Radic Biol Med, 45: 1403-1412.

6. Kizhakkayil, J., Thayyullathil, F., Chathoth, S., Hago, A., Patel, M. and Galadari, S. (2010). Modulation of curcumin-induced Akt phosphorylation and apoptosis by PI3K

inhibitor in MCF-7 cells. Biochem Biophys Res Commun, 394: 476-481.

7. Moscat, J. and Diaz-Meco, M.T. (2009). p62 at the crossroads of autophagy, apoptosis, and cancer. Cell, 137: 1001-1004.

8. Nezis, I.P. and Stenmark, H. (2012). p62 at the Interface of Autophagy, Oxidative Stress Signaling, and Cancer. Antioxid Redox Signal. doi:10.1089/ars.2011.4394.

9. Sanz, L., Diaz-Meco, M.T., Nakano, H. and Moscat, J. (2000). The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. EMBO J, 19: 1576-1586.

10. Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R. and Ashkenazi, A. (2009). Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell, 137: 721-735.

11. Dbaibo, G.S., Pushkareva, M.Y., Rachid, R.A., Alter, N., Smyth, M.J., Obeid, L.M. and Hannun, Y.A. (1998). p53-dependent ceramide response to genotoxic stress. J. Clin. Invest, 102: 329-339.

12. Kim, M.Y., Linardic, C., Obeid, L.M. and Hannun,Y.A. (1991). Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation. J. Biol. Chem, 266: 484-489.

13. Cifone, M.G., De Maria, R., Roncaioli, P., Rippo, M.R., Azuma, M., Lanier, L.L., Santoni, A. and Testi, R. (1994). Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J. Exp. Med, 180: 1547-1552.

14. Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R.S., McLoughlin, M., Fuks, Z. and Kolesnick, R.N. (1994). Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J. Exp. Med, 180: 525-535.

15. T. Yabu, T., S. Imamura, S., M. Yamashita, M. and T. Okazaki, T. (2008). Identification of $Mg2^{+}$ -dependent neutral sphingomyelinase 1 as a mediator of heat stress-induced ceramide generation and apoptosis. J. Biol. Chem, 283: 29971-29982.

16. Thayyullathil, F., Chathoth, S., Kizhakkayil, J., Galadari, A., Hago, A., Patel, M. and Galadari, S. (2011). Glutathione selectively inhibits Doxorubicin induced phosphorylation of p53Ser¹u , caspase dependent ceramide production and apoptosis in human leukemic cells. Biochem Biophys Res Commun, 411: 1-6.

17. Kizhakkayil, J., Thayyullathil, F., Chathoth, S., Hago, A., Patel, M. and Galadari, S (2012). Glutathione regulates caspase dependant ceramide production and curcumin-induced apoptosis in human leukemic cells. Free Radic biol and Med, 52: 1854-1864.

18. Cagnol, S. and Chambard, J.C. (2010). ERK and cell death: mechanisms of ERK-induced cell death—apoptosis, autophagy and senescence. FEBS J, 277: 2-21.

19. Wang, X., Martindale, J.L. and Holbrook, N.J. (2000). Requirement for ERK activation in cisplatin-induced apoptosis. J Biol Chem, 275: 39435-39443.

20. Ogier-Denis, E., Pattingre, S., El Benna, J. and Codogno, P. (2000). Erk1/2-dependent phosphorylation of Galpha-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. J Biol Chem, 275: 39090-39095.

21. Pattingre, S., Bauvy, C. and Codogno, P. (2003). Amino acids interfere with the ERK1/2-dependent control of macroautophagy by

controlling the activation of Raf-1 in human colon cancer HT-29 cells. J Biol Chem, 278: 16667-16674.

22. F. Thayyullathil, F., S. Chathoth, S., A. Hago, A., U. Wernery, U., M. Patel, M. and S. Galadari, S. (2008). Investigation of heat stress response in the camel fibroblast cell line Dubca. Ann N Y Acad Sci, 1138: 376-384.

23. Thayyullathil, F., Chathoth, S., Shahin, A., Kizhakkayil, J., Hago, A., Patel, M. and Galadari, S. (2011). Protein phosphatase 1-dependent dephosphorylation of Akt is the prime signaling event in sphingosine-induced apoptosis in Jurkat cells. J Cell Biochem, 112: 1138-1153.

24. Thayyullathil, F., Chathoth, S., Hago, A., Patel, M., Szulc, Z.M. and Hannun, Y.A., Galadari, S. (2011). Purification and characterization of a second type of neutral ceramidase from rat brain: a second more hydrophobic form of rat brain ceramidase. Biochim Biophys Acta, 1811: 242-252.

25. Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A. and Trzaskos, J.M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem, 273:18623-18632.

26. El-Shemerly, M.Y., Besser, D., Nagasawa, M. and Nagamine, Y. (1997). 12-O-Tetradecanoylphorbol-13-acetate activates the Ras/extracellular signal-regulated kinase (ERK) signaling pathway upstream of SOS involving serine phosphorylation of Shc in NIH3T3 cells. J Biol Chem, 272: 30599-30602.

27. He, H., Wang, X., Gorospe, M., Holbrook, N.J. and Trush, M.A. (1999). Phorbol ester-induced mononuclear cell differentiation is blocked by the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059. Cell Growth Differ, 10: 307-315.

28. Moscat, J., Diaz-Meco, M.T. and Wooten, M.W. (2007). Signal integration and diversification through the p62 scaffold protein. Trends Biochem Sci, 32: 95-100.

29. Jaakkola, P.M. and Pursiheimo, J.P. (2009). p62 degradation by autophagy: another way for cancer cells to survive under hypoxia, Autophagy. 5: 410-412.

30. Qian, H.L., Peng, X.X., Chen, S.H., Ye, H.M. and Qiu,J.H. (2005). p62 Expression in primary carcinomas of the digestive system. World J Gastroenterol, 11: 1788-1792.

31. Thompson, H.G., Harris, J.W., Wold, B.J., Lin, F. and Brody,J.P. (2003). p62 over-expression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells, Oncogene. 22: 2322-2333.

32. Yu, H., Su, J., Xu, Y., Kang, J., Li, H., Zhang, L., Yi, H., Xiang, X., Liu, F. and Sun, L. (2011). p62/SQSTM1 involved in cisplatin resistance in human ovarian cancer cells by clearing ubiquitinated proteins. Eur J Cancer, 47: 1585-1594.

33. Hayakawa, J., Ohmichi, M., Kurachi, H., Ikegami, H., Kimura, A., Matsuoka, T., Jikihara, H., Mercola, D. and Murata, Y. (1999). Inhibition of extracellular signal-regulated protein kinase or c-Jun N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line. J Biol Chem, 274: 31648-31654.

34. Moussavi, M., Assi, K., Gómez-Muñoz, A. and Salh, B. (2006). Curcumin mediates ceramide generation via the de novo pathway in colon cancer cells, Carcinogenesis. 27: 1636-1644.

35. Hilchie, A.L., Furlong, S.J., Sutton, K., Richardson, A., Robichaud, M.R.,

Giacomantonio, C.A., Ridgway, N.D. and Hoskin, D.W. (2010). Curcumin-induced apoptosis in PC3 prostate carcinoma cells is caspase-independent and involves cellular ceramide accumulation and damage to mitochondria, Nutr Cancer. 62: 379-389.

36. Yu, T., Li, J. and Sun, H. (2010). C6 ceramide potentiates curcumin-induced cell death and apoptosis in melanoma cell lines in vitro. Cancer Chemother Pharmacol, 66: 999-1003.

37. Chmura, S.J., Nodzenski, E., Beckett, M.A., Kufe, D.W., Quintans, J. and Weichselbaum, R.R. (1997). Loss of ceramide production confers resistance to radiation-induced apoptosis. Cancer Res, 57: 1270-1275.

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Solid State Fermentation (SSF) for the Production of Sophorolipids from *Starmerella bombicola NRRL* Y-17069 using glucose, wheat bran and oleic acid

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Abstract

The present investigation describes the exploratory work on the production of sophorolipids (SL) by solid-state fermentation (SSF) technique. The ability of *Starmerella bombicola NRRL* Y-17069 to produce sophorolipids by solid state fermentation was investigated. In this study, glucose was used as the hydrophilic carbon source while three different substrates were investigated as potential lipid carbon sources. 1) Blend of oleic acid with wheat bran, 2) Blend of oleic acid with isabgol husk (wheat bran and isabgol husk were used as inert support for adsorbing and distributing oleic acid) 3) Soya seed powder and 4) Peanut seed powder. Maximum yield of sophorolipids obtained was 18g per 100g of the substrate (18% conversion) using glucose and oleic acid (blended with wheat bran). The structural identity of the synthesized sophorolipids was confirmed by FTIR and ^{1}H NMR spectroscopy. This work has indicated that the production of sophorolipids by solid state fermentation is also possible.

Keywords : Solid state fermentation (SSF), Sophorolipids, Starmerella bombicola, Biosurfactant.

Introduction

Sophorolipids (SL) are surface-active glycolipid compounds synthesized by few of the non-pathogenic yeast species like *Candida bombicola* (Starmerella bombicola) (1-3), *Wickerhamiella domericqiae* (4,5), *Rhodotorula bogoriensis* (6) etc. Sophorolipids and their derivatives also possess spermicidal, virucidal, and anti-cancer activity (7-10). Sophorolipids also may be used as an active biodegradable surfactant in formulations for enhanced oil recovery, cosmetics, germicidal preparations and in other detergent formulation (11). Surfactant properties of low-cost sophorolipids have been identified to be an appropriate method for environmental applications, such as heavy metal removal from soil sediments, and degradation of insoluble aromatic compounds (12,13). A lot of research has been performed on the optimization of the sophorolipid by submerged fermentation using various substrates and fermentation conditions. However to the best of our knowledge there are no reports on sophorolipid production by solid state fermentation (SSF). Because of simplicity and being economically more attractive, solid-state fermentation has been used by many workers for the production of various enzymes (14-19). In fact, some of the bio-molecules give better yield with SSF, compared to conventional submerged fermentation. For example it has been reported that solid-state fermentation (SSF) with fungal strains results in much higher productivity than submerged fermentation (20) and it also offers many other advantages (21).

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Thus we found it to be worthwhile to test the possibility of sophorolipids production by *Starmerella bombicola NRRL* Y-17069 using SSF. The present investigation describes the exploratory work on the production of sophorolipids by solid-state fermentation technique at shake flask scale.

Material and Methods

***Microorganism*:** The yeast *Starmerella bombicola NRRL Y-17069*, was obtained from ARS Culture Collection, USA. The organism was maintained at 4°C on Potato Dextrose Agar (PDA) slants and was sub-cultured monthly.

***Preparation of Pre-inoculum*:** 15 to 20 ml of autoclaved solution containing 39 g/L of potato dextrose agar (PDA) and 2.0 g/L of yeast extract was poured in sterilized petri plates and was allowed to cool and set as a solid surface. The prepared petri plates were inoculated by spread plate technique using 0.4ml of organism (prepared by adding 10 ml of saline to PDA slant culture) and were incubated at 30°C for 48 hr to produce the pre-inoculum.

***Preparation of inoculums*:** The suspension for inoculation was prepared by adding 20 ml of sterile saline (0.9%w/v of NaCl in distilled water) to the pre innoculum i.e 48 h grown petri plate culture, and gently shaking the same for 1 min using sterile spatula for extraction of the cells.

***Substrates and chemicals*:** Different substrates viz., peanut seeds, soya seeds and wheat bran, were purchased from local markets and all other reagents used were of analytical grade. Peanut seed and soya seeds were ground to a coarse powder before use.

***Screening of different lipid sources for the production of sophorolipids*:** A total of 10g of solid substrate containing 2 g of glucose and 8 g of different lipid source (Table 1) were taken in 250 ml Erlenmeyer flasks, moistened with 8 ml of 0.1 M citrate buffer of pH: 3 and was sterilized using autoclave (121°C, for 15 min). After cooling, the flasks were inoculated with 2 ml of the inoculum; further an additional quantity of buffer was added to increase the moisture content up to 65%. And then the contents, after mixing, were incubated at 30°C for 10 days.

***Effect of the initial moisture content*:** To check the influence of moisture activity on sophorolipid production during SSF, the media containing 2 g glucose, 2 g oleic acid and 6 g of wheat bran powder was moistened with different amounts of distilled water to obtain moisture content of 50%, 65% and 80% by weight of the total mass (% w/w) prior to fermentation.

***Effect of the pH of the moistening agent*:** To check the influence of the pH of the buffer used as the moistening agent on the sophorolipid production during SSF, the media containing 2 g glucose, 2 g oleic acid and 6 g of wheat bran powder was moistened (50% w/w) with 0.1M citrate buffer of pH 3,4,5 and 6.

***Time course of sophorolipid production and the effect of the incubation temperature*:** To check the influence of the incubation temperature and fermentation time on the sophorolipid production during SSF, the media containing 2 g

Table 1. Various lipid source used for solid state fermentation of *S.bombicola* for sophorolipid production.

	Lipid source	Inert support	Sophorlipid yield* (g/100g of carbon source)
Medium 1	Soya seed powder (8g)	-	0.56
Medium 2	Peanut seed powder (8g)	-	0.3
Medium 3	Oleic acid (2g)	Wheat bran (6g)	14.8
Medium 4	Oleic acid (2g)	Isabgol husk (6g)	4.5

* Values indicate mean of triplicate observations.

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of glucose, 2g of oleic acid and 6g of wheat bran powder was moistened (50% w/w) with 0.1M citrate buffer of pH 4 and were incubated at various temperatures (25, 30, and 35°C) for different time periods (2 to 10 days).

***Isolation of sophorolipids*:** Sophorolipids were extracted by mixing fermented substrate of each flask with 40 ml ethyl acetate and then shaking the mixture in an orbital shaker at 250 rpm for 1h. The suspension was then centrifuged at 5000×g for 15 min for separating the extract from the fermented solid substrate. The solvent was then removed from the extract by rotary evaporation. The amber colored, honey like semi-crystalline sophorolipids (Fig. 1) was washed twice with 20 ml of n-Hexane to remove the unused oily residue, and was stored at 4ºC.

Structural Characterization of isolated Sophorolipids: Structural identity of the synthesized sophorolipids was confirmed by FTIR and ^{1}H NMR spectroscopy.

Results and Discussion

Production of sophorolipids: Out of the various lipid sources tested; when soya seed powder (Medium 1) and peanut seed powder (Medium 2) were used as source of lipids, there was no need to use additional inert solid support. However, when free oleic acid was used as lipid source (Medium 3 and Medium 4), because of its liquid state of oleic acid, there was a need to use a solid support. Thus, two different solid supports were tested 1) wheat bran powder (Medium 3) and isabgol powder (Medium 4). It was observed that the maximum sophorolipid yield (14.8 g/100 g of the substrate) was obtained when free oleic acid was used as a lipid source and wheat bran powder was used as solid support (Table 1 & Fig 2). However when isabgol husk was used as solid support, the yield of sophorolipids decreased substantially (4.5 g/100 g of the substrate) compared to the use of wheat bran powder as solid support (Table 1; Fig. 2). A possible explanation for the above results could be the high absorbing and swelling capacity of isabgol husk, which could lead to the absorption of the most of the water from the medium and thus the actual freely available water, could be very less, making the conditions unfavorable for the production of sophorolipids.

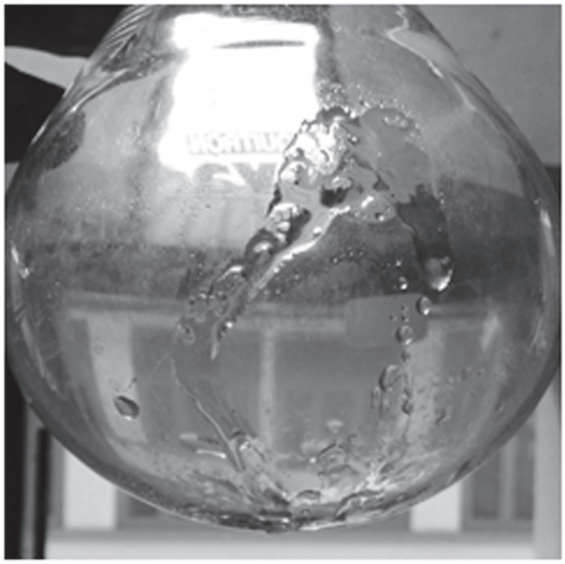

Fig. 1. Amber colored, honey like sophorolipids.

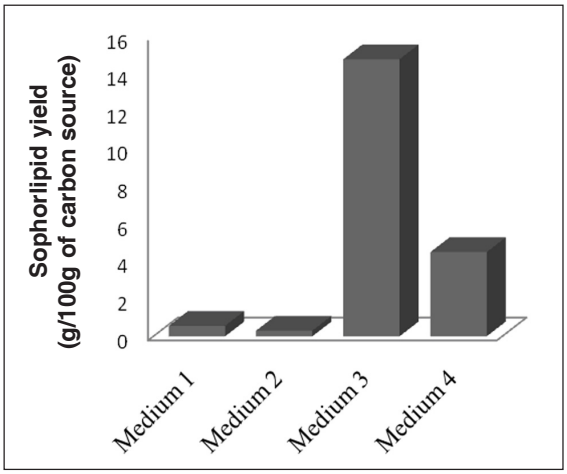

Fig. 2. Effect of lipid sources on the production of sophorolipids

A possible reason for the poor yields of sophorolipids using soya seed powder and

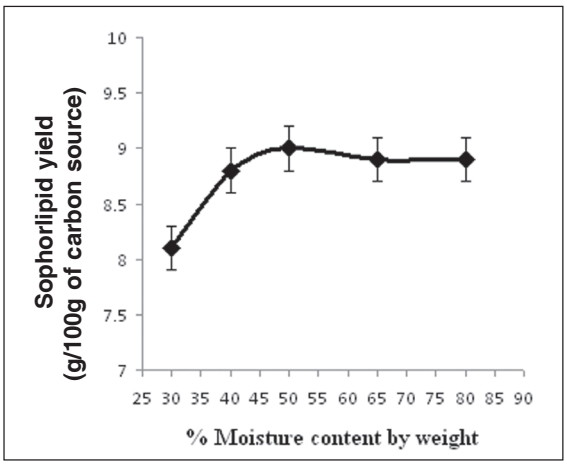

Fig. 3. Effect of moisture content on sophorolipid yield

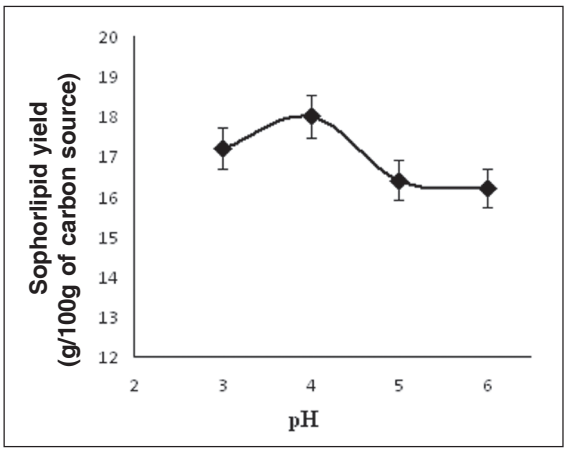

Fig. 4. Effect of pH on sophorolipid yield

peanut seed powder as lipid sources (0.56 g of sophorolipids/100 g of total substrate and 0.30 g of sophorolipids/100 g of the total substrate respectively) could be due to the fact that oily components (lipids) of the powdered seeds are not freely available as they are present in intracellular location. It is possible that the organism can synthesize sophorolipids only if lipids are freely available and cannot utilize intracellular lipids present in the vegetable sources.

From this initial screening it was found that blend of oleic acid with wheat bran was the better lipid source and support respectively amongst the others for the sophorolipids production and thus it was standardized for use in further experiments.

The moisture content, pH and the incubation temperature are few of the critical factors which affects the growth and production of various secondary metabolites during solid-state fermentation. However it was observed that the production of sophorolipids was only marginally affected by pH and the initial moisture content. Moisture content of 50%w/w and pH of 4.0 was found to be optimum (Fig. 3 and Fig.4 respectively) for the sophorolipid production. Incubation temperature significantly affected the production of sophorolipids. Maximum production of sophorolipids occurred at incubation temperature of 30°C (18g/100g of the substrate) and the sophorolipids yield substantially decreased at lower and higher incubation temperature. (Fig. 5) Further it was observed

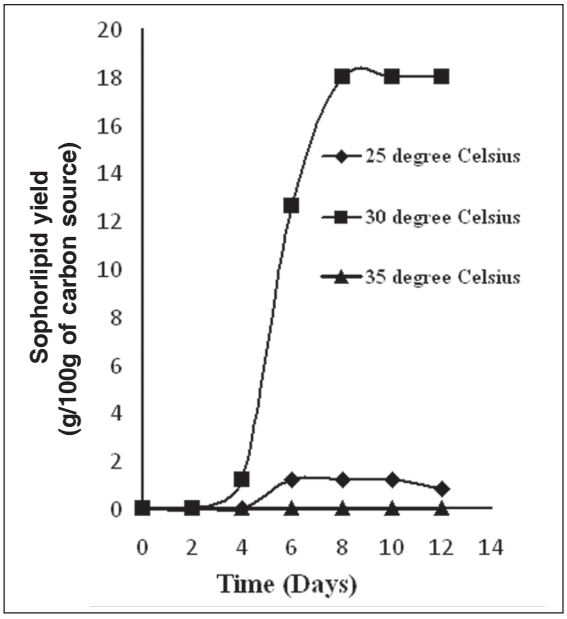

Fig. 5. Effect of temperature and fermentation time on sophorolipid yield.

that the production of sophorolipids begins on approximately the 4th day and it reaches to its maximum value by 8th day (Fig 5).

***Structural Characterization of Synthesized Sophorolipids*:** Structural identity of the isolated viscous honey like compound was confirmed to

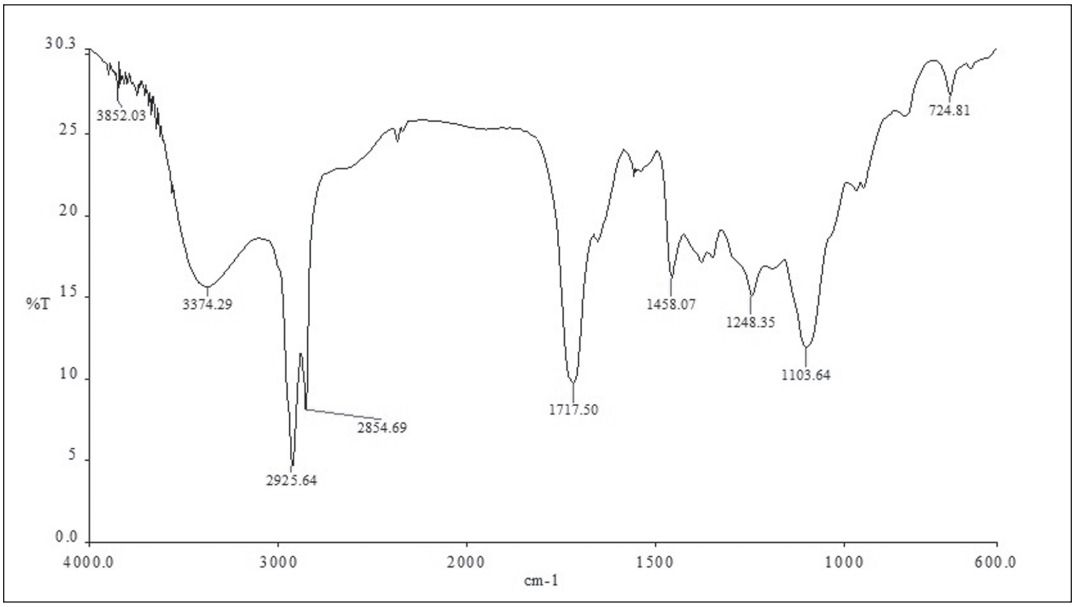

Fig. 6. FTIR spectrum of the synthesized sophorolipids

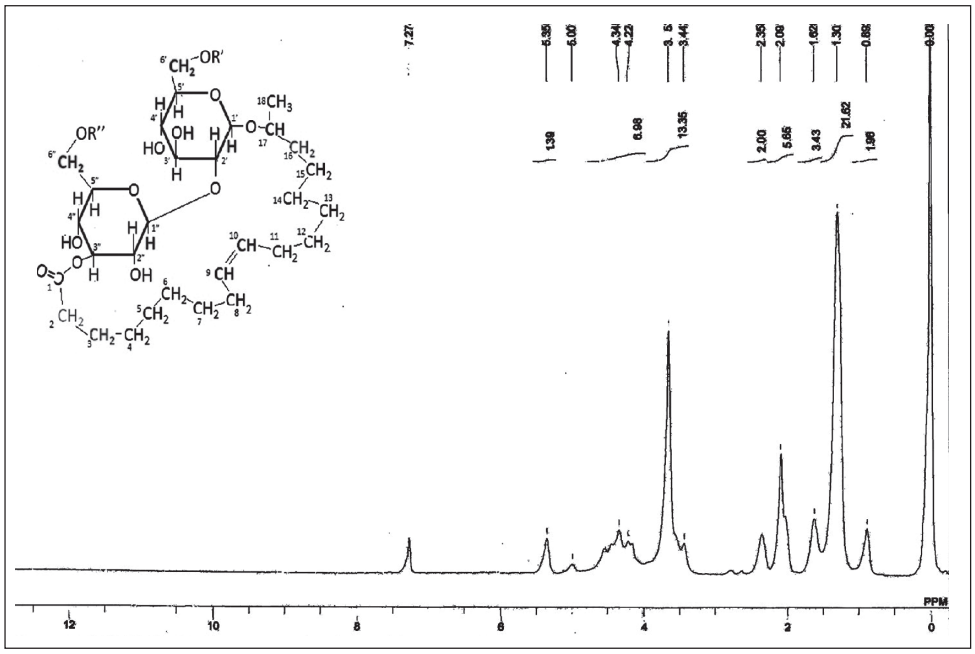

Fig. 7. 1H NMR spectrum of the synthesized sophorolipid.

be that of crude sophorolipid by FTIR and ^{1}H NMR spectroscopy.

***FTIR results*:** FT-IR measurements of the synthesized sophorolipids (SL) showed (Fig. 6) broad absorption band at 3374 cm”1 (corresponds to the OH stretch). The asymmetrical stretching and symmetrical stretching of methylene (CH2) was observed at 2925 and 2854 cm”1 respectively. The C=O absorption band at 1717 cm”1 may include contributions from that of lactones, esters, or acids, while that from the acetyl esters was observed at 1248 cm”1. Sugar C-O stretch of C-O-H groups was observed at 1103cm”1.

***Results from ^{1}H NMR study*:** NMR spectrum was taken in $CDCl_3$. The ^{1}H NMR spectrum (Fig. 7) indicated a typical glycolipid-type structure. The Protons on the carbons C2, C3, C4 and C5 of both the glucose gave chemical shift at 3.4-3.6 ppm. The other protons on carbons C1 and C6 of both the glucose gave chemical shift at 4.2-4.3. The signals of –CH=CH– group were obtained at 5.35 ppm, –CH3 group of sugar acetate at 2.09 ppm, and multiple signals of protons at 1.30 ppm.

Conclusion

To the best of our knowledge, this is the first report on sophorolipid production by SSF technique. This investigation indicated that the production of sophorolipids by SSF technique is possible. Further we have obtained a considerable yield without any media optimization, thus it is likely that sophorolipid production by SSF method can be further increased by the application of various techniques of media optimization, use of different substrates and even use of other microbes known for producing sophorolipids in submerged fermentation. Considering the advantages of SSF and the increasing importance of microbial biosurfactants, this study will be an encouraging lead towards the solid state production of microbial biosurfactants.

Acknowledgements

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References

1. Felse, P.A., Shah, V., Chan, J., Rao, K.J. and Gross R.A. (2007). Sophorolipid biosynthesis by Candida bombicola from industrial fatty acid residues. Enzyme Microb. Technol, 40: 316-323.
2. Parekh, V.J., Pandit A.B. (2011). Optimization of fermentative production of sophorolipid biosurfactant by starmerella bombicola NRRL Y-17069 using response surface methodology. Int. J. Pharm.Bio.sci, 1(3): 103-116.
3. Takahashi, M., Morita, T., Wada, K., Hirose, N., Fukuoka, T., Imura, T. and Kitamoto, D. (2011). Production of sophorolipid glycolipid biosurfactants from sugarcane molasses using Starmerella bombicola NBRC 10243. J. Oleo Sci, 60(5): 267-273.
4. Jing, C., Xin, S. Hui, Z. and Yinbo, Q.U. (2006). Production, structure elucidation and anticancer properties of sophorolipid from Wickerhamiella domercqiae, Enzyme Microb. Technol, 39: 501–506.
5. Li, H., Ma, X., Shao, L., Shen, J. and Song, X. (2012). Enhancement of Sophorolipid Production of Wickerhamiella domercqiae var. sophorolipid CGMCC 1576 by Low-Energy Ion Beam Implantation. Appl Biochem Biotechnol, 167(3): 510-523.
6. Nunez, A., Ashby, R., Foglia, T.A. and Solaiman, D.K.Y. (2004). LC/MS analysis and lipase modification of the sophorolipids produced by Rhodotorula bogoriensis, Biotechnol. Lett, 26: 1087–1093.
7. Shah, V., Doncel, G.F., Seyoum, T., Eaton, K.M., Zalenskaya, I., Hagver, R., Azim, A.

and Gross, R. (2005). Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrobial Agents and Chemotherapy, 49(10): 4093-4100.

8. Fu, S.L., Wallner, S.R., Bowne, W.B., Hagler, M.D., Zenilman, M.E., Gross, R. and Bluth, M.H. (2008). Sophorolipids and Their Derivatives Are Lethal Against Human Pancreatic Cancer Cells. Journal of Surgical Research, 148(1): 77-82.

9. Chen, J., Song, X., Zhang H. and Qu Y. (2006). Production, structure elucidation and anticancer properties of sophorolipid from Wickerhamiella domercqiae. Enzyme and Microbial Technology, 39(3): 501-506.

10. Shao, L., Song, X., Ma, X., Li, H. and Qu, Y. (2012). Bioactivities of sophorolipid with different structures against human esophageal cancer cells. J Surg Res, 173(2): 286-291.

11. Singh, S.K, Felse, A.P., Nunez, A., Foglia, T.A., and Gross, R.A. (2003). Regioselective enzyme-catalyzed synthesis of sophorolipid esters, amides, and multifunctional monomers. J Org Chem, 68:5466–77.

12. Mulligan, C.N., Yong, R.N. and Gibbs, B.F. (2001). Heavy metal removal from sediments by biosurfactants. J Hazard Mater, 85:111–25.

13. Schippers, C., Gessner, K., Muller, T. and Scheper, T. (2000). Microbial degradation of phenanthrene by addition of a sophorolipid mixture. J Biotechnol, 83:189–98.

14. Singh, H. and Soni, S.K. (2001). Production of starch-gel digesting amyloglucosidase by Aspergillus oryzae HS-3 in solid state fermentation. Process Biochemistry, 37(5): 453-459.

15. Liming, X. and Cen, P.(1999). Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. Process Biochemistry, 34(9):909-91.

16. Rodríguez, C.S. and Sanromán, M.A. (2005). Application of solid-state fermentation to ligninolytic enzyme production. Biochemical Engineering Journal, 22(3): 211-219

17. Archana, A. and Satyanarayana T. (1997). Xylanase production by thermophilic Bacillus licheniformis A99 in solid-state fermentation. Enzyme and Microbial Technology, 21(1):12-17.

18. Kamini, N.R., Mala, J.G.S. and Puvanakrishnan, R. (1998). Lipase production from Aspergillus niger by solid-state fermentation using gingerly oil cake. Process Biochemistry, 33(5): 505-51.

19. Sangeetha, R., Geetha, A. and Arulpandi, I. (2011). Pongamia pinnata seed cake: a promising and inexpensive substrate for production of protease and lipase from Bacillus pumilus SG2 on solid-state fermentation. Indian J Biochem Biophys.48(6):435-439.

20. Hesseltine, C.W. (1972). Biotechnology report: solid state fermentations. Biotechnology and Bioengineering, 14: 517-532.

21. Lonsane, B. K., Ghildyal N. P., Budiatman S. and Ramakrishna, S. V. (1985). Engineering aspects of solid state fermentation. Enzyme and Microbial Technology, 7: 258-265.

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Evaluation of Antioxidant Power of Stone fruits for Development of Functional Food

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Abstract

Stone fruits have been reported to possess various health promoting activities due to their high antioxidant levels. In the current study eight plum, six sweet cherry, five apricot and five peach cultivars grown in Kashmir valley of India were harvested at maturity stage and evaluated for their total antioxidants in terms of total phenols, carotenoids, anthocyanin and vitamin C content as well as their respective antioxidant activities on fresh weight basis (fw). Among the plum cultivar, "Green Gauge", demonstrated the highest phenolic content (210mg/100g fw) as well as total antioxidant activity (476µmole/g) and in case of sweet cherries, "Siah Gole" cultivars exhibited the highest total phenol content (350mg/100g fw) and respective antioxidant activity (446µmole/g). In case of apricot, though "Quetta" variety showed the highest phenol content but "Hercott" demonstrated the highest antioxidant activity. Likewise in peach "July Elberta" possessing lesser phenol content than "Saharanpuri" cultivar showed highest antioxidant activity. Such type of results suggests that quantity of phenolic content alone does not impart high antioxidant activity to such fruits but the type of phenolic compound equally governs such characteristics. The current investigation suggests that such type of stone fruits rich in phenolic antioxidants comprises a promising functional food group to be used against various oxidative stress related diseases.

Key words: Stone fruits, Phenolics, Vitamin C, Anthocyanin, Carotenoids, Antioxidant activity.

Introduction

Functional Food are foods or dietary components that may provide a health benefit or desirable physiological effects beyond basic nutrition and thus allows us to take greater control of our health through the food choices we make (1). Currently, functional attributes of many traditional foods are being discovered, while new food products are being developed with beneficial components. Rapid advances in science and technology, increasing healthcare costs, changes in food laws affecting label and product claims, an aging population and rising interest in attaining wellness through diet are among the factors fueling whole world's interest in such functional foods (2). Credible scientific research indicates that there are many clinically demonstrated potential health benefits from food components, specifically to reduce the risk of oxidative stress related diseases and help the body combat such metabolic processes that lead to degenerative conditions (3,4). Examples of foods possessing such active ingredients include fruits and vegetables, whole grains, fortified or enhanced foods and beverages, and some dietary supplements that usually, look, smell and taste the same as their regular counterparts and supplementing the diet with such beneficial phytonutrients may reduce the risk of degenerative diseases during aging.

Historically, fruits and vegetables have been used as medicinal agents and until recently the practice of western medicine involved the prescription of specific plants and foods (5). It is

now well established fact that fruit and vegetable products especially those that are rich in secondary metabolites (frequently called phytochemicals) are gaining increasing interest (6). Epidemiological studies have shown that diet rich in such foods significantly reduce the incidence and mortality rates of degenerative diseases caused by oxidative stress. The protective effect of such fruit and vegetable based foods has been attributed to the fact that they may provide an optimal mix of phytochemicals including natural antioxidants that comprise one of the most common components in functional food production. During recent past in food industry, these antioxidants are widely being used to prevent rotting and help long storage, transportation and easy marketing without deteriorating the quality of the product. Thus, interest on use of antioxidants from natural sources in the form of phytochemicals is remarkebaly increasing due to their important role in disease prevention of both plants as well as animals (7). In humans, these naturally occurring compounds act by scavenging harmful free radicals implicated in the most common cancers as well as in other degenerative diseases including poor brain function. (8,9). In addition, they are also responsible for induction of enzymes that detoxify carcinogens and also block the progression of cancer by deactivating at least 30 types of agents that may cause cancer (10). In contrast, the synthetic forms of antioxidants have been seen to have entirely different role to play with most of them possessing toxic and carcinogenic effects (11).

During recent years, stone fruits are acquiring new interest mainly due to the fact that they contain some components in different quantities in the form of phenolic compounds and carotenoids with potent antioxidative effects and are thus known to play an important role in human health. Phenolic compounds from such fruits are becoming of great interest as researchers are discovering their functional activities in the form of drugs, colorants, flavors, and antioxidants. Some phenolics share certain biological and chemical properties that might be effective inhibitors of chemical mutagens and/or carcinogenesis. Thus, one of the best approaches to increase the intake of such beneficial compounds is to screen the potent cultivars and increase their concentration inside these fruits by breeding and selection. To best of our knowledge, none of the stone fruit cultivar till date from the Kashmir valley has been analyzed for such important compounds. Thus, in the current study, an attempt has been made to evaluate health promoting secondary metabolites as well as antioxidant activities in different cultivars of plum, cherry, apricot and peach grown in Kashmir valley of India.

Materials and Methods

Chemicals: All chemicals and reagents used in the current study were of analytical grade and mostly purchased from Sigma chemicals (India).

Sample collection: In this study eight plum, six cherry, five apricot and five peach cultivars were procured from various fields of SKUAST (K), Shalimar, Srinagar, Kashmir as well as from local market at fresh maturity stage. The fruits were selected according to uniformity of size, shape and colour and then transported to the Biochemistry and Molecular Biotechnology Laboratory, Division of Post Harvest Technology, SKUAST (K), Shalimar Campus, Srinagar within an hour for analysis. All the measurements were conducted in triplicates.

Estimation of Vitamin C, Anthocyaninn and Carotenoids : Estimations were performed as per methods of Rangana (12). For Vitamin C estimation 10 ml of sample extract was taken in volumetric flask and made upto 100ml volume with metaphospheric acid and filtered. 10 ml of filtrate was pipetted into conical flask and titrated against the standard dye solution to a pink end point. Total anthocyanin content in samples was determined by extracting with ethanolic HCl and measurement of colour determined at the wavelength of maximum absorption. The content was calculated by making use of the λ max (molecular extinction coefficient) value as 98.2.

For estimation of carotenoids, the samples were extracted in acetone and transferred to petroleum ether phase. Total carotene was read colorimetrically using petroleum ether for baseline correction.

Total phenolic content analysis: Total phenolic content was determined according to Singleton and Rossi method (13). Ten grams of sample was thoroughly crushed and homogenized in mortar pestle with10 ml of 80% ethanol. The extract was centrifuged at 10,000 rpm for 15 min at 4^0C and supernatant preserved. The pellet following was resuspended in 5 ml of 80% ethanol, centrifuged and the resulting supernatant combined with initial extract. Triplicate supernatant extractions were made for each sample. The pooled ethanolic extracts were evaporated to dryness. The evaporated extracts were solublised in 5ml distilled water and used for the estimation of total phenolics. 500µl of the sample extract was combined with 2.5ml of double distilled water and 0.5ml of Folin cioucalteau reagent. After 3min of incubation period, 20% sodium carbonate was added to each sample ,vortexed and boiled in a water bath for exactly one min. The absorbance was measured at 650nm against reagent blank A standard curve was established using catechol. Absorbance values were converted to milligram of phenolics per 100g of fresh tissue. For each cultivar three replicates were analyzed.

Determination of total antioxidant activity : The total antioxidant potential of samples were determined using using FRAP assay of Benzie and Strain (14) as a measure of antioxidant power and extracts were prepared as described above for estimation of total phenols. The assay was based on the reducing power of a compound (antioxidant). A potential antioxidant reduces the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) that forms a blue complex (Fe^{2+}/TPTZ). FRAP reagent consisted of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40mM HCL, 300 mM sodium acetate buffer (pH 3.6) and 20mM ferric chloride in distilled water in the ratio of 1:1: 10 (v/v). A 100 ml extract was added to 3ml of FRAP reagent and mixed thoroughly. After standing at ambient temperature (20°C) for 4 min, absorbance at 593 nm was noted against reagent blank. Calibration was against a standard curve (50-1000 mmol ferrous ion) produced by the addition of freshly prepared ammonium ferrous sulfate. Values were obtained from three replications and expressed as mmol FRAPg^{-1} fresh weight

Statistical analysis: Three replicates of each sample were used for statistical analysis. Analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) and regression analysis. Differences at $P < 0.05$ were considered to be significant.

Results and Discussion

Recent reports have shown that a healthy diet containing high plant based antioxidants could prevent approximately 30% of all cancers (15). No doubt genetic manipulations can increase such antioxidant constituents of fruits and vegetables, however there is a limit beyond which increased concentrations may cause undesirable levels of astringency in these crops (16). Therefore, plant breeders and food producers are increasingly identifying specific genotypes and varieties of fruits and vegetables rich in functional ingredients comprising of nutritive and non-nutritive antioxidants e.g. anthocyanin, carotenoids, phenols etc. Among different types of fruits, stone fruits such as plums, peaches and apricots, have been found to be successful in killing cancer cells and are known to play an important role in human health due to the range of antioxidant rich phytochemicals especially phenolic compounds. However, before recommending such fruits as functional foods, further studies including rapid selection procedures, secondary effects of phenolics on fruit quality and postharvest traits, and the bioactive properties of selected fruit genotypes are needed. (17). In the current study different cultivars of stone fruits grown in Kashmir valley of India, were screened for their health promoting effects in terms of their antioxidant activities as well as various antioxidants present in their respective extracts. As previous reports

have shown (18) that phenol extraction from plant tissue in ethanol is preferred over extraction in water due to the presence of water-soluble antioxidant vitamins and sugars that may mask the antioxidant activity of of polyphenols therefore, all the extracts in the current study were prepared in ethanol.

Amoung the stone fruits large number of plum cultivars have been introduced into India and it has been found that European plums perform better in the hills, while as Japanese plums adopted more in sub-mountainous lower elevations. In order to develop a database of plums grown in Kashmir valley of India, eight different cultivars were analyzed for their secondary metabolites including total phenols, vitamin C, carotenoids and anthocyanin in addition to their respective antioxidant activities. As per previous reports the anthocyanin content of plums range from 4.41 to 23.12 mg/100g fresh weight (f.w.) and total phenolic content from 298 to 563 mg/100g f.w.. Further, anthocyanin content and phenolic content were reported to be well correlated with the antioxidant activities (19). In the current study the anthocyanin and phenolic content were found to be variable in red and yellow flesh plum varieties. It was observed that anthocyanin content slightly increased with the red colour intensity, which ranged from 15.25 mg100/g f.w for "Wickson "to 45.8 for "Satsuma" (Table 1). Interestingly, our results clearly demonstrated that most of the plum varieties evaluated in this study possess higher levels of anthocyanin content than those reported previously and thus put them in the category of other fruits rich in anthocyanin content.

Previously it has been reported that total phenol content of different fruits and vegetables, can vary from 2-500 mg/100g f.w. and in case of plum it falls in the range of 125.0 to 372.6 mg/100 g f w. (20). Interestingly, in this current study the total free phenolic content of most of the selected cultivars was found to fell in the range of that found in apple varieties i.e. 117- 430 mg/100g f.w (21). It was note worthy that "Green Gauge" variety that is very unpopular in Kashmir valley recorded significantly highest phenol content (210 mg/100gm fw) followed by "Santa Rosa" (205 mg/100gfw), Burbank (200 mg/100gfw), Grand Duke (198mg/100gfw), Wickson (180 mg/100gfw), Satsuma (165g/100gfw), Warwick (112mg/100gfw) and Reine Claude-de-Bary (78 mg/100gfw) respectively.

As large number of reported scientific data indicates a strong correlation between total phenol content and antioxidant activity of fruits and vegetables (18, 22). Therefore, rather than measuring only the antioxidant contents, there is an increasing interest in the measurement of total antioxidant activity of crops by using various assay methods like ferric reducing antioxidant

Table 1. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh Plum cultivars

Plum Cultivar	Vitamin C (mg/100g)	Total carotenoids µg/100g)	Anthocyanin (mg/100g)	Total Phenols (mg/100g)	Antioxidant activity (µmole/g)
Burbank	5.01	320	17.8	200	442
Green Gauge	7.2	433	22.9	210	476
Wickson	4.8	560	15.25	180	382
Satsuma	5.30	820	45.8	165	326
Grand Duke	3.8	520	35.6	198	441
Warwick	6.2	300	29.5	112	191
Reine Claude-de-Bary	4.9	400	20	78	209
Santa Rosa	6.3	850	33.5	205	335

Table 2. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh cherry cultivars

Cherry Cultivar	Vitamin C (mg/100g)	Total carotenoids µg/100g)	Anthocyanin (mg/100g)	Total Phenols mg/100g)	Antioxidant activity (µmole/g)
Makhmali	3	1400	159	190	307
Awal No	2	1650	134	190	288
Siah Gole	5	1800	182	350	446
Double	4	2100	134	180	300
Misri	2.3	1700	122	325	394
Tontal	1.9	1870	102	400	201

Table 3. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh Apricot cultivars

Apricot cultivars	Vitamin C (mg/100g) µg/100g)	Total carotenoids	Anthocyanin (mg/100g)	Total Phenols (mg/100g)	Antioxidant activity (µmole/g)
Charmagz	8	840	9.2	300	149
Gilgiti sweet	10	600	12	303	202
Hercot	8.5	450	8	379	276
Quetta	7.6	620	6.9	385	165
Halman	6.12	110	6.3	346	240

Table 4. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh Peach cultivars

Peach cultivars	Vitamin C (mg/100g) µg/100g)	Total carotenoids	Anthocyanin (mg/100g)	Total Phenols (mg/100g)	Antioxidant activity (µmole/g)
Saharanpuri	8	600	6	952	160
July Elberta	7.5	652	5	361	452
Elberta	6.90	430	7	287.4	241
Quetta	6	940	5	538	150
Awal No	4	441	7	309	132

power (FRAP) assay developed by Benzie and Strain (14). In this study the antioxidant activity measured by FRAP assay in red/purple-flesh varieties of plum was found to be higher as compared to light colored yellow flesh plums. **Interestingly,** Green Gauge cultivar possessing highest phenolic content also demonstrated maximum value of antioxidant activity (476 mmol /g f.w.) followed by Burbank (442mmol/g f.w.), Grand Duke (441mmol/ 100g f.w.), Wickson (382mmol/ g f.w.), Santa Rosa (335mmol/ g f.w.), Satsuma (326mmol/ g f.w.), Reine Claude-de-Bary (209mmol/ g f.w.) and Warwick (191mmol /g f.w.) . The correlation developed between total antioxidant activity (Y) and total phenolic content (X) of these selected plum varieties had a high

correlation coefficient of R^2=0.790. Whereas in contrast to earlier reports, total anthocyanin and total antioxidant activity showed least correlation with the correlation coefficient of only R^2= 0.013. It can be suggested that green guage cultivar of plums is a promising fruit with rich antioxidant composition (especially phenolic content) as well as antioxidant activity and can be better exploited to be used as functional food.

Likewise, sweet cherries have been reported to possess many health promoting activities due to presence of many secondary metabolites including phenols. Prvulovic *et al* (23) reports that phenolic composition of sweet cherries is genotype dependent as well as influenced by climatic conditions. In the Kashmiri Agricultural market, fresh sweet cherries represent an important, but fragile, commodity. As evident from table 2, the antioxidant composition and antioxidant activities of selected six cherry cultivars viz Makhmali, Awal Number, Siah Gole, Double, Misri and Tontal were found to be highly variable. It was observed that total phenol content in the extracts of the freshly harvested cherry samples varied from ~180 - 350 mg/100 g fw. Amoung the selected cultivars "Siah Gole" cultivar exhibited the highest antioxidant activity (446 μmole/g f w) as compared to other varieties. In this type of stone fruit also strong correlation (R^2 = 0.782) was observed between total phenolic content and total antioxidant activities. These results are very well in accordance to previous reports that indicate anthocyanins did not seem to be the only important antioxidant to influence the antioxidant activity of the fruits, when correlating with DPPH data (23)

Among stone fruits apricot possesses very high carotene content that plays an important role in maintenance of human health. Due to presence of carotene and lycopene this fruit has been reported to have antipyretic, antiseptic, emetic, and ophthalmic properties and also protect heart and eyes (24*).* As the levels of antioxidant compounds especially phenolic compounds have been reported to be different in apricot varieties (25). Therefore, genotype variations in terms of antioxidant composition and antioxidant activities of five apricot cultivars grown in Kashmir valley were also evaluated in the current study. The ethnolic extracts from these cultivars demonstrated variable levels of antioxidant composition with Gilgit Sweet demonstrating the highest Vitamin C content and Halman the lowest (Table 3). Likewise Charmagz showed the highest Carotenoid content and Halman the lowest. Highest phenolic content was found in Quetta and lowest in Charmagz variety. Interestingly, in this type of fruit the Hercot variety possessing intermediate phenol content showed the highest antioxidant activity as compared to "Quetta" and though a good correlation (R^2 = 0.782) was found between total phenol content and total antioxidant activity in almost all the fresh apricot varieties. Such type of results suggests that quantity of phenolic content alone does not impart high antioxidant activity to such fruits but the type of phenolic compound equally governs such characteristics.

Previous reports have shown that carotenoid content in yellow-flesh peaches is higher (2-3 mg carotene/100 g fw-fresh weight) than as found in white or red-flesh one (0.01-1.8 mg carotene/100 g fw). In contrast antioxidant activity has been reported to be about 2-fold higher in red-flesh varieties than in white/yellow-flesh peach varieties that correlated best with their respective phenolic content (19). In our study as shown in table 4, Saharanpuri peach cultivar showed the highest Vitamin C content and Quetta the highest carotenoid content. Saharanpuri cultivar possessed the highest phenol content and Elberta the lowest. Interestingly, in these selected peach cultivars also, inspite of demonstrating a good correlation (R^2 = 0. 648) between total phenolic content and total antioxidant activities, "July Elberta" cultivar possessing intermediate phenol content demonstrated the highest antioxidant activity as found in case of apricot cultavers.

Conclusion

It is suggested from the present study that these stone fruits grown in Kashmir valley comprise a promising group with wide range of genetic variability in terms of antioxidant composition (especially phenolic content) as well as antioxidant activities. The fruits were found to show better correlation between total phenolic content and their respective antioxidant activities. In plum it is Green Gauge, in cherry it is Siah Gole, in apricot it is Hercott and in peach it is July Elberta that are recommended to all age groups for better consumption per day due to their highest antioxidant activities. The current study signifies that such fruits due to high antioxidant power can be further exploited to be used as strong antidegenrative food as well as health promoting functional foods. Commercial value added powder can be formulated from them for designer foods that can also be included in breakfast cereals, snacks, confectioneries, backed goods and pet foods as health promoting food. In addition, it is suggested that such cultivars could be incorporated into the breeding programmes for development of antioxidant rich germplasm of stone fruits in the area.

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References

1. Sembries, S., Dongowski G., Mehrländer, K., Will, F. and Dietrich, H. (2006). Physiological effects of extraction juices from apple, grape, and red beet pomaces in rats. Journal of Agricultural Food Chemistry, 54(26):10269-80.

2. Sarin, R., Sharma, M., Singh, R. and Kuma, R. S. (2012). Nutraceuticals:A review. International Journal of Pharmacy, 3(4): 95-99.

3. Shipp, J. and Abdel-Aal E. S. M. (2010). Food Applications and Physiological Effects of Anthocyanins as Functional Food Ingredients. The Open Food Science Journal, 4: 7-22.

4. Carlsen, M. H., Halvorsen, B. L., Holte, K., Bøhn, S. K., Dragland, S., Sampson, L., Willey, C., Senoo, H., Umezono, Y., Sanada, C., Barikmo, I., Berhe, N., Willett, W. C., Phillips, K. M., Jacobs and D. R., Blomhoff, R. (2010). The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. Nutrional Journal, 9:1-11.

5. Iriti, M., Vitalini S., Fico, G. and Faoro, F. (2010). Neuroprotective Herbs and Foods from Different Traditional Medicines and Diets. Molecules , 15: 3517-3555

6. Kaur, C. and Kapoor, H.C. (2002). Anti-oxidant activity and total phenolic content of some Asian vegetables. International Journal of food Science and Technology, 37: 153-162.

7. Ou, B., Huang, D., Hampsch-Woodil, M., Judith, A., Flanagan and Elizabeth, K. D. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. Journal of Agriculture and Food Chemistry, 50: 3122-3128.

8. Dillard, C. J. and German, J.B. (2000). Phytochemicals: nutraceuticals and human health. Journal of Science Food and Agriculture, 80: 1744-1756.

9. Soleas, G.J., Tomlinson, G., Diamandis,E.P. and Golidberg, D.M. (1997). Relative contributions of polyphenolic constituents to the antioxidant status of wines-development of predictive model. Journal of Agriculture and Food Chemistry, 45: 3995-4004.

10. Percival, M. (1997). Phytonutrients and detoxification. Clinical Nutrition and Insigh, 5:1-4.

11. Gazzani, G., Papetti, A., Massolini, G. and Daglia, M. (1998). Antioxidative and prooxidant activity of water-soluble components of some common diet vegetables and effect of thermal treatment. Journal of Food Chemistry, 6: 4118-4122.

12. Ranganna, S. (1997). Manual of analysis of fruit and vegetable products, (9th edition). Tata Mc Graw Hill, New Delhi.

13. Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology Viticulture, 16: 144-158.

14. Benzie, I.F. and Strain, J. (1999). Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and asdcorbic acid concentration. Methods in Enzymology, 299: 15-27.

15. Boyer, J. and Liu, R. H. (2004) Apple phytochemicals and their health benefits. Nutrition Journal , 3: 121-134.

16. Drewnowski, A. and Gomez-Carneros, C. (2000). Bitter taste, phytonutrients, and the consumer: a review. American Journal of Clinical Nutrition;72:1424–35.

17. Vizzotto, M., Cisneros-Zevallos L. and. Byrne, D.H. (2007).Large Variation Found in the Phytochemical and Antioxidant Activity of Peach and Plum Germplasm Journal of American Sociecty of Horticulture Science, 132(3):334–340.

18. Kaur, C. and Maini, S. B. (2001). Fruits and vegetables—Health foods for new millennium. Indian Horticulture, 8: 29-32.

19. Vizzotto, M L., Cisneros-Zevallos, D.H., Byrne, D.W., Ramming, W.R. and Okie (2006). Total Phenolic, Carotenoid, and Anthocyanin Content and Antioxidant Activity of Peach and Plum Genotypes Acta Horticulture, 713: 453 -445

20. Bravo, I. (1998). Polyphenols: Chemistry, dietary sources metabolism and nutrition significance. Nutritional Review, 56: 317-333.

21. Imeh, U. and KhoKhar, S. (2002). Distribution of conjugated and free phenols in fruits: Antioxidant activity and cultivar variations. Journal of Agriculture and Food Chemistry, 50: 6301-6306.

22. Patthamakanokporn, O., Puwastien, P., Nitithamyong, A. and Sirichakwal, P.P. (2010) Neuroprotective Herbs and Foods from Different Traditional Medicines and Diets. Molecu les , 15: 3517-3555.

23. Prvulovic, D., Malenèic, D., Popovic, M., Ljubojeviæ, M. and Ognjanov, V. (2011). Antioxidant Properties of Sweet Cherries (*Prunus avium* L.) role of Phenolic Compounds. World Academy of Science, Engineering and Technology, 59: 1149-1152.

24. Ghasemnezhad, M., Shiri M. A. and Sanavi, M. (2010). Effect of chitosan coatings on some quality indices of apricot (*Prunus armeniaca* L.) during cold storage Caspian Journal of Environmental Science, 8 (1): 25-33.

25. Sochor, J., Zitka, O., Skutkova, H., Pavlik, D., Babula, P., Ales Horna, B. K., Adam, V., Provaznik, I. and Kizek, R. (2010). Content of Phenolic Compounds and Antioxidant Capacity in Fruits of Apricot Genotypes. Molecules, 15: 6285-6305.

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Cloning, Expression and Characterization of Matrix protein (M1) of highly pathogenic avian influenza H5N1 in *Escherichia coli*

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Abstract

Avian influenza is highly contagious disease of avian species. Vaccination is considered as one of the efficient tool for controlling the disease. Recombinant avian influenza vaccines comprising haemagglutinin and neuraminidase genes expressed in various platforms has already been investigated and is demonstrated to offer protection in animals. However, because of the high variable nature of these antigens, the less variant matrix protein could be an efficient vaccine candidate against avian influenza. But the applicability of M1 protein as a vaccine is not studied much in animals. Hence, in an effort to develop a matrix protein based sub-unit vaccine against avian influenza, the M1 protein from Eschericia coli was cloned and expressed in BL21-DE3 pLysS. The expression levels were found to be better when the cells were induced with 0.5 mM IPTG and incubated for 3 hrs at 25°C. The rM1 protein was purified using Ni-NTA chromatography under denaturing conditions. The protein was refolded in a reducing buffer conditions which resulted in soluble form of the protein. The total yield of rM1 protein was estimated to be 12-14 mg / liter bacterial culture. Since, this method does not involve complicated purification methods and higher yield can be produced in less than week time, this method of producing rM1 antigen may provide useful insights for the development of faster, cheaper and bulk vaccines for avian influenza pandemic.

Key words : Matrix protein1, Highly pathogenic avian Influenza, Escherichia coli, Expression, Purification.

Introduction

Avian influenza is a highly contagious disease of avian species. Sporadic transmission of highly pathogenic avian influenza from avian species to humans in the recent past has demonstrated that the potential thread is just not restricted to avian species but can be extended to humans as well (1). Vaccination has been the most effective method to combat the disease. The current influenza vaccine is an egg based vaccine which involves the use of whole virus antigen propagated in embryonated eggs which offer complete protection in animals. However, this method of vaccine manufacturing pose many practical challenges since live virus has to be used for manufacturing which necessitates certain strict bio-safety measures to be maintained. This method, however, becomes impractical in many places where the required biosafety could not be maintained (2). In addition to these challenges, this method of vaccine manufacturing is time consuming and often limits the applicability because millions of eggs will be required to manufacture a pandemic vaccine (3). On the other

hand, recombinant vaccines of avian influenza virus have been demonstrated to be effective against the infection and offered protection in animals.

The avian influenza virus consists of three main structural antigens namely neuraminidase (NA), haemagglutinin (HA) and the matrix (M) protein. Majority of the recombinant vaccine strategies against avian influenza aims at the HA (4, 5) antigen either alone or in combination with other avian influenza antigens (6) which offer complete protection in animals. Antibodies against HA antigen plays a major role in protection against the infection while antibodies to NA helps reducing the disease spread (7, 8). However, these antigens will not protect against different subtype variants of avian influenza.

This is mainly due to the frequent variations in the HA and NA antigenic subtype and the newer subtype can consist of one amongst 16 HA subtypes accompanied by one among 9 NA subtypes which almost is highly unpredictable. Hence, the less variant structural antigen such as matrix proteins may be an attractive alternative candidate to the HA and NA antigens. The matrix protein, one of the abundant proteins of avian influenza virus demonstrated to elicit protective immune response in mice (9). It has already been described that DNA vaccines comprising the matrix protein either alone or in a combination with other structural antigens elicits protective immune response in animals (10). On the other hand, the DNA vaccine may not help controlling the disease in a pandemic scenario because of difficulties in mass vaccination as it requires efficient delivery methods and large quantities of DNA. Other methods of recombination vaccine development for avian influenza which were under various development stages are the expression of one or more subunit antigens in baculovirus (4), yeast (5) and bacterial systems (11, 12). The bacterial system may be more appropriate compared to baculovirus and yeast expression system as the earlier is easier and quicker which may help in controlling the pandemic.

The present paper reports cloning and expression of the matrix protein of a highly pathogenic avian influenza virus. The expression of the matrix protein was standardized and the recombinant protein was purified through Ni-NTA affinity chromatography.

Materials and Methods

Strains and plasmids: The cDNA of Avian Influenza virus structural gene M1 of H5N1 strain (A/Hatay/2004/H5N1) was obtained from the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. Escherichia coli TOP10 (F– mcrA Δ(mrr-hsdRMS-mcrBC) F80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG) cells were used for all the plasmid propagation work and BL21(DE3) pLysS (F– ompT hsdSB(rB–, mB–) gal dcm (DE3) pLysS (CamR) was used for the expression of rM1 protein. The bacterial cells were grown and maintained in LB broth and/or LB agar containing appropriate antibiotic.

Cloning of M1 into E.coli expression vector pRSETA: The gene encoding M1 protein was amplified from cDNA (Genbank accession No: AM040045) of Avian Influenza virus structural gene M1 of H5N1 strain (A/Hatay/2004/H5N1) by PCR using forward primer (5'-ATCGGC TAGCATGA GTCTTCTAACCGAGGT-3') and a reverse primer (5'- ATCGAAGCTTTTATTA CTTGAATCGCTGCATCTGC-3') consisting NheI and HindIII restriction endonuclease sites (underlined) respectively. The PCR amplicon was purified using QIAquick PCR Purification Kit following manufacturer's instructions. The purified PCR amplicon was digested with NheI and HindIII and cloned into a bacterial expression vector pRSETA (Invitrogen, USA). The positive clones were confirmed by restriction enzyme analysis using NheI and HindIII followed and DNA sequencing.

Expression of rM1 in E.coli : The positive clones were transformed into BL21 (DE3) pLysS cells and grown to an optical density 0.6 at 600 nm. The cells were then induced with 0.5 mM of

Isopropyl ß-D-1-thiogalactopyranoside (IPTG) and the cells were incubated for four hours. Four hours post induction, the cells were harvested by centrifugation and the expression of rM1 was analyzed in SDS-PAGE and western blot using HisProbe-HRPO.

Optimization of rM1 gene expression: The positive transformants were grown at 37ºC till the optical density at 600nm reaches 0.4-0.6. The cells were then induced with 0.125 mM, 0.25 mM, 0.5 mM and 1 mM to find out the appropriate concentration of IPTG to be used to get maximum level of expression. An un-induced cell control was maintained to compare the difference. All the samples were adjusted to an optical density 5 at 600 nm and analyzed in 12% SDS-PAGE and western blot using HisProbe-HRPO (Thermo scientific, USA). The SDS-PAGE and the western blot were observed for any visual increase in the expression of rM1 protein.

The influence of different growth temperatures after induction with IPTG was analyzed. Briefly, the cells were grown at 37ºC till the optical density at 600 nm reaches 0.6 and the cells were then transferred 37ºC, 30ºC, 25ºC and 16ºC. The cells were incubated for four hours and after the induction period the cells were harvested. All the samples were adjusted to an optical density 5 at 600 nm and analyzed in 12% SDS-PAGE and western blot using HisProbe-HRPO. The SDS-PAGE and the western blot were observed for any visual increase in the expression of rM1 protein.

To optimize the post induction time periods, the cells grown at the optimized temperature and were induced with appropriate concentration of IPTG. After induction the cells were collected at different time points as 1hr, 2hrs, 3hrs and 4hrs post induction. All the samples were adjusted to an optical density 5 at 600 nm and analyzed in 12% SDS-PAGE and western blot using HisProbe-HRPO. The SDS-PAGE and the western blot were observed for any visual increase in the expression of rM1 protein.

Purification of rM1: The clones expressing the rM1 protein were scaled up to 500 ml in shaker culture flasks and induced with 0.5mM IPTG when the OD reached 0.4-0.6 at 600 nm. After three hours post induction at 37ºC, the cultures were centrifuged at 4000 rpm for 20mins at 4ºC and the pellet was resuspended in 50 mM sodium phosphate buffer (pH-8). The cells were lysed in French press cell disrupter (Constant systems, UK) at 40000 psi, 2 cycles. The cell lysate was centrifuged at 12000 rpm for 30 min at 4ºC. The supernatant was filtered through 0.2µm syringe filter and transferred to a sterile container. Fraction of the supernatant was analyzed on a 12% SDS-PAGE with appropriate negative control. An immunoblot was performed using HisProbe-HRP following standard protocols.

The rM1 protein was purified using Ni-NTA chromatography under denaturing conditions. The lysate was mixed with equal volume of 8 M urea. NaCl was added to a final concentration of 300 mM. The lysate was filtered through a 0.2 µM syringe filter and passed through a pre-equilibrated Ni-NTA Superflow resin (Qiagen, USA). The column was washed with 50 mM sodium phosphate buffer containing 4M urea, 300mM NaCl and 30mM imidazole (Sigma, USA) and the bound M1 protein was eluted with 50mM sodium phosphate buffer containing 300mM imidazole, 4M urea and 300mM NaCl. The fractions were analyzed on 12% SDS-PAGE and the fractions containing target protein were pooled. The protein was refolded by dialyzing against 50mM sodium phosphate buffer containing 2M urea, 50mM glycine, 0.5mM EDTA and 1mM DTT (pH-8) at +4ºC. The concentration of urea in the dialysis buffer was changed for every 6 hours through 2, 1, 0.5 and 0.25M till no urea. The protein concentration of the dialyzed solution was determined using BCA protein assay reagent (Pierce, Rockford, IL). The purified rM1 protein was concentrated through a vivaspin20 (10,000 MWCO) and analyzed on 12% SDS-PAGE. The protein was dialyzed with 50mM sodium phosphate (pH-8) and the protein concentration was estimated using BCA reagent.

Results

Cloning of M1 gene into E.coli expression vector pRSETA: The M1 was amplified from the cDNA using specific primers consisting NheI and HindIII at their 5'end. The resultant amplicon was analyzed on 1% agarose gel which showed a 756bp M1 product (Fig.1). The PCR product was purified using QIAquick PCR purification kit. The purified M1 gene was digested with NheI and HindIII and was cloned into similarly digested pRSETA. The positive clones carrying the M1 gene were identified by restriction digestion with NheI and HindIII and the clones releasing a 756bp product (Fig.1) was considered positive clones.

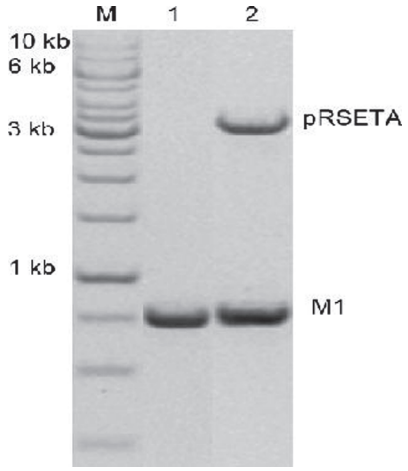

Fig. 1. Cloning of M1 gene. The M1 gene was amplified from the cDNA (lane1) and was cloned into the bacterial expression vector pRSETA (lane2). The clones were confirmed by restriction digestion using NheI and HindIII enzymes which released a 756 bp M1 gene.

The positive clones were analyzed by automated DNA sequencing using vector specific (T7 forward) primer and the results were compared with the cloned M1 gene sequence which was found to be 100% homologous.

Expression of rM1 gene in E.coli: The positive clones were transformed into BL-21(DE3) pLysS cells. The expression of M1 gene was analyzed by SDS-PAGE and western blot (Fig. 2) using HisProbe-HRP which showed a 30kDa M1 protein in induced cells.

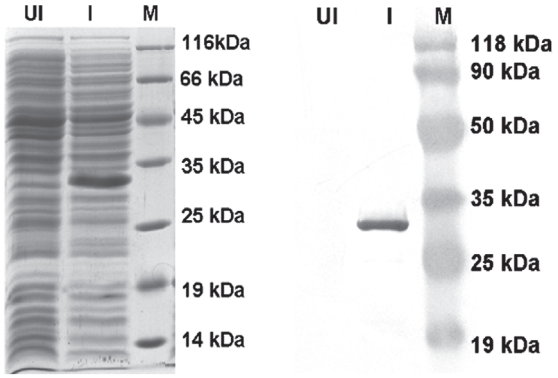

Fig. 2. Expression of rM1 in E.coli. The positive clones were transformed into BL21-DE3-pLysS. The cells were induced 1mM IPTG and incubated for four hours. After four hours, the cells were harvested and analyzed in 12% SDS-PAGE (left) and western blot using HisProbe-HRP (right) which showed a 30kDa M1 protein. UI – Uninduced; I – Induced; M – Marker.

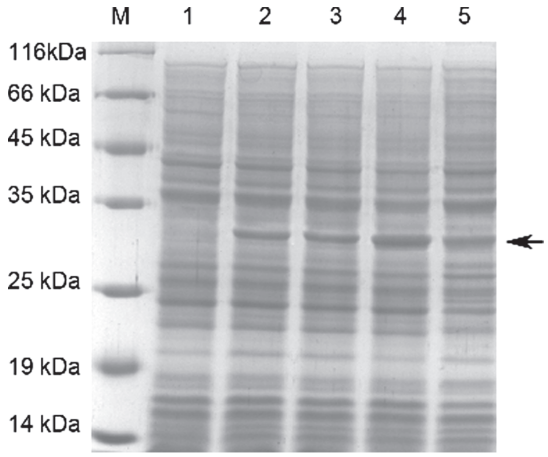

Fig. 3. Optimization of IPTG concentration. BL21-DE3-pLysS cells carrying the pRSETA-M1 plasmid was grown in LB media at 37°C and induced with different concentrations of IPTG such as 0.12mM, 0.25mM, 0.5mM and 1mM. The cells were incubated for four hours and then analyzed in 12% SDS-PAGE. Induction with 0.5mM IPTG yielded maximum expression as visibly seen in the gel. Arrow indicates target protein. Hence, further optimization was performed with 0.5mM IPTG. 1 – Un-induced control, 2 – 0.12mM, 3 – 0.25mM, 4 – 0.5mM, 5 – 1mM IPTG.

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Optimization of rM1 gene in expression

The level of rM1 gene expression was analyzed with varying concentrations of IPTG, temperature and post induction periods to optimize the level of rM1 expression without compromising the cell yield. The expression of rM1 protein was not seems to be differing significantly with different concentrations of IPTG (Fig. 3) when the cell number was normalized to equal optical density. However, at higher concentrations, typically at 1mM IPTG concentration, the cells started lysing immediately post induction. This indicates that higher concentration of IPTG is creating toxicity which may be associated with the high level expression of rM1 gene. Conversely, the final cell mass was significantly lesser compare to cells induced with other IPTG concentrations. Hence, 0.5mM was used as optimized IPTG concentration in all further optimization.

The effect of different temperatures on the level of rM1 expression was analyzed by incubating the cells at different temperature after induction with IPTG. Cells grown at 25ºC were showing better expression level than 16ºC, 30ºC and 37ºC temperature (Fig. 4) when normalized to equal optical density. However, the total cell mass was better for cells grown at 37ºC compared to cells grown in other temperatures. Hence, the cells were incubated at 37ºC and induced with 0.5mM IPTG for further optimization.

To optimize the post induction period, the cells grown at 37ºC were induced with 0.5mM IPTG. At different time points after induction, the cells were analyzed. The expression of rM1 was better at three hours post induction as visualized by the SDS-PAGE (Fig. 5).

Purification of rM1 protein: The clones expressing rM1 protein was scaled up to 2 liter cultures in a shake flask and the expression was carried out with above optimized parameters. After induction, the cells were harvested and were lysed using French press. The rM1 was purified using Ni-NTA chromatography under denaturing conditions (Fig. 6) as majority of the expressed

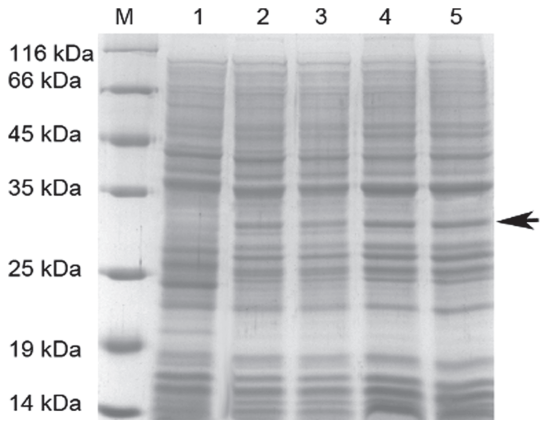

Fig. 4. Optimization of growth temperature. BL21-DE3-pLysS cells carrying the pRSETA-M1 plasmid was grown in LB media at 37°C and induced 0.5mM. After induction, the cells were incubated for four hours at different temperatures such as 16°C, 25°C, 30°C and 37°C and then analyzed in 12% SDS-PAGE. Cells grown at 25°C showed significantly better expression as seen in the gel. 1 – Un-induced control, 2 – 16°C, 3 – 25°C, 4 – 30°C, 5 – 37°C. Arrow indicates target protein. Hence, further optimization was performed at 25°C.

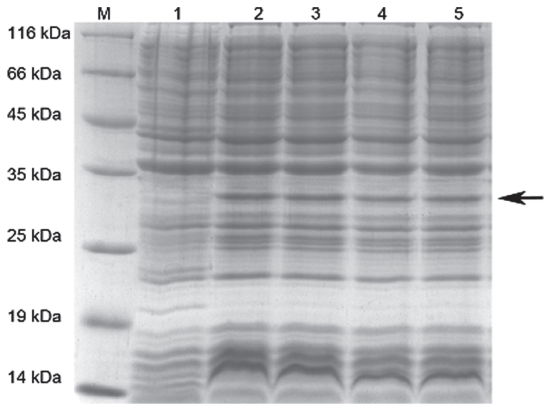

Fig. 5. Optimization of post induction period. BL21-DE3-pLysS cells carrying the pRSETA-M1 plasmid was grown in LB media at 37°C and induced 0.5mM. After induction, the cells were incubated at 25°C and the cells were harvested at different time post induction and analyzed in 12% SDS-PAGE. 1 – Un-induced control, 2 – 1hour, 3 – 2 hours, 4 – 3 hours, 5 – 4 hours. There was no significant difference when the cell number was normalized to equal optical density. But considering the cell mass and expression 3 hours post induction gave better expression yields. Arrow indicates target protein.

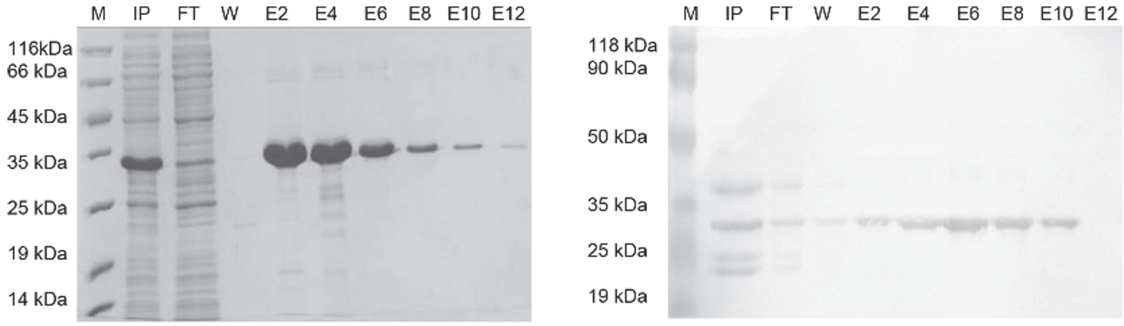

Fig. 6. Purification of rM1 protein. The cells expressing rM1 was scaled up. The cells were induced with 0.5mM IPTG, incubated for 3 hours at 25°C. The cells were harvested, lysed and the rM1 protein was purified through Ni-NTA chromatography under denaturing conditions. The purified protein was pooled and refolded by dialyzing in reduced buffer conditions. M – Marker; IP – Input; W – Wash; E2-E12 – alternative elution fractions.

rM1 protein was found in the insoluble fractions. The protein was eluted with 300mM imidazole and the fractions containing pure rM1 protein were pooled. The pooled fractions were dialyzed to remove the imidazole and urea so as to bring the rM1 protein in to soluble form. After refolding in reduced buffer conditions, the protein came into soluble form and was stable in 4ºC. The total yield of rM1 protein was estimated to be 12-14 mg / liter culture when the cells were incubated at 37ºC and induced with 0.5mM IPTG. The protein was finally dialyzed in 50mM sodium phosphate (pH-8) and the concentration was adjusted to 1mg/ml and stored at -80ºC.

Discussion

The avian influenza is long standing potential thread to many species including avian species and humans. Vaccination seems to be the efficient method to combat the disease. Several reports suggest the use of recombinant HA (4, 13, 14) and NA (15, 16) either alone or in combination offer protection against high pathogenic avian influenza in animals. The virus like particles comprising the whole set of structural antigens have also been reported to provide either partial or complete protection in animals (6, 17). However, not a single or group of antigens belongs to single subtype can offer complete protection in animals against circulating strain because of the extremely varying antigenic nature of the highly pathogenic avian influenza. The future avian influenza pandemic is inevitable, but at the same it is highly impossible to predict the antigenic nature of the virus as it can be any combination of one among 17 HA subtypes and one among 9 NA subtypes. On the other hand, the less variant matrix proteins could be a better antigenic candidate for controlling avian influenza. But the expression, purification and use of matrix protein as a potential vaccine candidate have not been studied in detail.

In this study, the M1 protein of highly pathogenic avian influenza was cloned into a bacterial expression vector and expressed in E.coli. Although the expression of rM1 protein led to some amount of toxicity in bacteria which was visible by the expression induced lysis, the expression parameters were optimized to suit better expression levels without compromising the final cell mass. The high level expression of the rM1 protein also led the protein to accumulate in insoluble fractions and hence, the protein was purified under denaturing conditions. However, after refolding, the protein was found to be stable at 4ºC. The expression in bacterial system can be promising as bulk vaccines can be produced in no time which is a pre-requisite for such a rapidly spreading disease. The total yield of rM1 protein was estimated to be 12-14 mg / liter bacterial culture. This concentration can be significantly increased with further optimization in large scale fermentation as higher cell mass can

be generated. Bacterially expressed avian influenza matrix protein (18), HA (11) and NA (19) genes were already demonstrated to offer partial or complete protection against infection in animals. But a detailed study on the use of rM1 against various subtypes of avian influenza will be necessary to evaluate the applicability of M1 protein as a broad-spectrum antigenic target against avian influenza. M1 alone may not be sufficient to offer protection in animal since, antibodies to M1 neither blocks virus entry nor help preventing virus dissemination. However, conserved epitopes within M1 can induce CD8+ T cells and can contribute to protection against morbidity and mortality from influenza. Furthermore, the rM1 in combination with other structural antigens such as HA and NA should also be evaluated. The development of quicker and cheaper vaccines is a must for effective control of circulating strain of avian influenza. The egg based vaccine development takes a minimum of five to six weeks for bulk vaccine manufacturing. However, the bacterial system helps reducing time as more amount of vaccine can be produced in less than a week time. Although the baculovirus and yeast expression system comprise many advantages over the bacterial expression system in terms of its post translational modifications, the bacterial expression system is easy to handle. Hence, this method of manufacturing the matrix protein of avian influenza virus can pave a way for the development of faster, safer and cheaper vaccines against avian influenza.

Acknowledgement

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References

1. Amendola, A., Ranghiero, A., Zanetti, A. and Pariani, E. (2011). Is avian influenza virus A (H5N1) a real threat to human health? Journal of preventive medicine and hygiene 52: 107-110.
2. Wright, P.F. (2008). Vaccine Preparedness — Are We Ready for the Next Influenza Pandemic? New England Journal of Medicine 358: 2540-2543.
3. Singh, N., Pandey, A. and Mittal, S.K. (2010). Avian influenza pandemic preparedness: developing prepandemic and pandemic vaccines against a moving target. Expert reviews in molecular medicine 12: e14.
4. Crawford, J., Wilkinson, B., Vosnesensky, A., Smith, G., Garcia, M., Stone, H. and Perdue, M.L. (1999). Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine 17: 2265-2274.
5. Athmaram, T., Saraswat, S., Santhosh, S., Singh, A.K., Suryanarayana, V., Priya, R., Gopalan, N., Parida, M., Lakshmana Rao, P. and Vijayaraghavan, R. (2011). Yeast expressed recombinant Hemagglutinin protein of Novel H1N1 elicits neutralising antibodies in rabbits and mice. Virology Journal 8: 524.
6. Bright, R.A., Carter, D.M., Crevar, C.J., Toapanta, F.R., Steckbeck, J.D., Cole, K.S., Kumar, N.M., Pushko, P., Smith, G., Tumpey, T.M. and Ross, T.M. (2008). Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. PloS one 3: e1501.
7. Qiu, M., Fang, F., Chen, Y., Wang, H., Chen, Q., Chang, H., Wang, F., Zhang, R. and Chen, Z. (2006). Protection against avian influenza H9N2 virus challenge by immunization with hemagglutinin- or neuraminidase-expressing DNA in BALB/c mice. Biochemical and biophysical research communications 343: 1124-1131.
8. Johansson, B.E., Bucher, D.J. and Kilbourne, E.D. (1989). Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. Journal of virology 63: 1239-1246.

9. Okuda, K., Ihata, A., Watabe, S., Okada, E., Yamakawa, T., Hamajima, K., Yang, J., Ishii, N., Nakazawa, M., Ohnari, K., Nakajima, K. and Xin, K.Q. (2001). Protective immunity against influenza A virus induced by immunization with DNA plasmid containing influenza M gene. Vaccine 19: 3681-3691.

10. Park, K.S., Seo, Y.B., Lee, J.Y., Im, S.J., Seo, S.H., Song, M.S., Choi, Y.K. and Sung, Y.C. (2011). Complete protection against a H5N2 avian influenza virus by a DNA vaccine expressing a fusion protein of H1N1 HA and M2e. Vaccine 29: 5481-5487.

11. Aguilar-Yanez, J.M., Portillo-Lara, R., Mendoza-Ochoa, G.I., Garcia-Echauri, S.A., Lopez-Pacheco, F., Bulnes-Abundis, D., Salgado-Gallegos, J., Lara-Mayorga, I.M., Webb-Vargas, Y., Leon-Angel, F.O., Rivero-Aranda, R.E., Oropeza-Almazan, Y., Ruiz-Palacios, G.M., Zertuche-Guerra, M.I., DuBois, R.M., White, S.W., Schultz-Cherry, S., Russell, C.J. and Alvarez, M.M. (2010). An influenza A/H1N1/2009 hemagglutinin vaccine produced in Escherichia coli. PloS one 5: e11694.

12. Bommakanti, G., Citron, M.P., Hepler, R.W., Callahan, C., Heidecker, G.J., Najar, T.A., Lu, X., Joyce, J.G., Shiver, J.W., Casimiro, D.R., ter Meulen, J., Liang, X. and Varadarajan, R. (2010). Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. Proceedings of the National Academy of Sciences of the United States of America 107: 13701-13706.

13. Vanlandschoot, P., Maertens, G., Jou, W.M. and Fiers, W. (1993). Recombinant secreted haemagglutinin protects mice against a lethal challenge of influenza virus. Vaccine 11: 1185-1187.

14. Biesova, Z., Miller, M.A., Schneerson, R., Shiloach, J., Green, K.Y., Robbins, J.B. and Keith, J.M. (2009). Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. Vaccine 27: 6234-6238.

15. Kilbourne, E.D., Pokorny, B.A., Johansson, B., Brett, I., Milev, Y. and Matthews, J.T. (2004). Protection of mice with recombinant influenza virus neuraminidase. The Journal of infectious diseases 189: 459-461.

16. Martinet, W., Saelens, X., Deroo, T., Neirynck, S., Contreras, R., Min Jou, W. and Fiers, W. (1997). Protection of mice against a lethal influenza challenge by immunization with yeast-derived recombinant influenza neuraminidase. European journal of biochemistry / FEBS 247: 332-338.

17. Mahmood, K., Bright, R.A., Mytle, N., Carter, D.M., Crevar, C.J., Achenbach, J.E., Heaton, P.M., Tumpey, T.M. and Ross, T.M. (2008). H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses. Vaccine 26: 5393-5399.

18. Na nakorn, J., Lekcharoensuk, P., Boodde, O., Chumsing, W. and Wajjwalku, W. (2008). Cloning and Expression of M1 Protein of Influenza A Virus in Escherichia coli. KKU veterinary Journal 18:97-108.

19. Jones, I.M. and Brownlee, G.G. (1985). Differential expression of influenza N protein and neuraminidase antigenic determinants in Escherichia coli. Gene 35(3): 333-42.

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Use of Unconventional Bioresources for Production of Prodigiosin from *Serratia marcescens* NRRL B-23112

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Abstract

The present study investigates the use of conventional and unconventional bioresources for the production of an anticancer biomolecule, prodigiosin using *Serratia marcescens* NRRL B-23112 which also produces serratiopeptidase (SRP). Initially, both conventional and unconventional bioresources were screened for maximum production of prodigiosin. Jatropha seed powder, an unconventional bioresource, gave highest production of 5331 mg/l of prodigiosin and 850 EU/ml of SRP. Further, evaluation of the effect of concentration of seed powder, effect of seed oil and the effect of seed protein on production of prodigiosin and SRP showed that jatropha seed powder at 3% supported highest production of 6000±200 mg/l of prodigiosin, but not that of SRP.

Keywords: Prodigiosin, Serratiopeptidase, Unconventional Bioresouces, *Serratia marcescens*, Jatropha.

Introduction

Prodigiosin is a multifaceted secondary metabolite. It is produced by *Serratia marcescens*, *Pseudomonas magneslorubra*, *Vibrio psychroerythrous* and other bacteria (1). The prodigiosin group of natural products is a family of tripyrrole red pigments that contain a common 4-methoxy-2,2 bipyrrole ring system (2). Prodigiosin is thought to have potential for antibacterial, antimalarial, anticancer, cytotoxic and immunosuppressive activities (3-7).

Typical media used for the fermentative production of prodigiosin by *S. marcescens* strains are complex media that are rich in a variety of nutrients (1,8-9). Certain nutrients such as thiamine and ferric acid (10) are particularly crucial for prodigiosin production, whereas phosphate (11), adenosine triphosphate, and ribose (12) have inhibitory effects on prodigiosin yield. From an industrial point of view it is necessity to obtain a suitable medium to simultaneously enhance the growth of *S. marcescens* and the pigment production. Giri et al. (1) tested a series of media and discovered peanut seed broth and sesame seed broth to give significant enhancement of prodigiosin production.

Traditionally, hydrocarbons and non-hydrocarbon substrates (fats, oils, glycerol and carbohydrates) are used for the growth of microorganism. The choice of inexpensive raw materials is important to the overall economics of the process and accounts for 50% of the total product cost. Inspired from the study of Giri et al. (1), we screened several oil seed powder and its oilcakes such as peanut seed powder, castor seed powder, coconut seed powder, sunflower seed powder, soybean seed powder, sesame seed powder (black and white variety) and its oilcakes as a conventional economical media source for the production of prodigiosin. An attempt was also made to use several unconventional bioresources which are either toxic to human and animals or a complete waste,

for production of prodigiosin. The unconventional bioresources substrates are used in this study were i) jatropha seed powder, *Jatropha curcas* (has a phorbol ester which is toxic to human and animals), ii) *Leucaena leucocephala* (subabul) seed powder contains mimosine, a toxic amino acid, which precludes it from use in human food and animal feed, iii) *Delonix regia* (gulmohar) seed powder (rich source of protein and oil), and iv) trash fish powder (a waste) (13).

The unconventional bioresources were selected in the present study since they are unsuitable for human and animal consumption due to health hazard. However, it could have potential as a carbon/nitrogen source for the production of microbial metabolites, in this case, serratiopeptidase (SRP) and prodigiosin from *Serratia marcescens* NRRL B-23112.

Materials and Methods

Peanut seed powder (PnSP), castor seed powder (CsSP), coconut seed powder (CcSP), sunflower seed powder (SfSP), soybean seed powder (SbSP), sesame seed powder (SsSP) (black and white variety) and its oilcakes (OC), and jatropha seed were purchased from Hakim Chichi, Surat, Gujarat, India (a famous ayurvedic shop) and local market of Mumbai. *Leucaena leucocephala* (subabul) seed and *Delonix regia* (gulmohur) seed were collected from ICT campus, Mumbai. Trash fish was collected from local fishermen of Mumbai. All the chemical and reagents used were of AR grade unless specified.

S. marcescens NRRL B-23112 was a gift sample from ARS culture collection, USA. It was maintained on soybean casein digest agar medium and subcultured after every 15 days. The slants of *S. marcescens* NRRL B-23112 were stored at 6°C in a refrigerator.

Conventional sources for fermentative production of prodigiosin: Conventional sources such as peanut seed powder, castor seed powder, coconut seed powder, sunflower seed powder, soybean seed powder, sesame seed powder (black and white variety) and their oilcakes (OC) were collected and crushed in a mixer and sieved to fine particles before preparing the broth. Fine particles of oilseed powders and oilcakes were stored at 4°C until further use. For the preparation of broth, 2% powder of conventional sources in distilled water was used for prodigiosin production using *S. marcescens* NRRL B-23112. The pH of all the above broth was adjusted to 7.0. All the media were autoclaved at 121°C for 20 min. All experiments were carried out in triplicates.

The conventional sources which gave higher production of prodigiosin such as peanut and sesame seed powder were further screened for their concentration in the range of 1 to 5%.

Unconventional bioresources for fermentative production of prodigiosin: Jatropha seed powder (devoid of shell), *subabul* seed powder and *gulmohur* seed powder were collected, crushed in a mixer, and then sieved to fine particles before preparing the broth. Trash fish was first sun dried and crushed to fine particles. Fine particles of unconventional bioresources were stored at 4°C until further use. For preparation of broth, 2% fine powder of these unconventional bioresources was used in distilled water and evaluated for prodigiosin production using *S. marcescens* NRRL B-23112. The pH of all the above broth was adjusted to 7.0. The media was autoclaved at 121°C for 20 min. The experiments were conducted in triplicates.

The unconventional bioresources which gave higher production of prodigiosin such as jatropha seed powder was further screened in the concentration range of 1 to 5%.

Use of defatted jatropha seed powder for production of prodigiosin: Fat and oil content in powdered jatropha seed was determined according to AOAC methods (14) by soxhlet extraction using 100 ml petroleum ether/g of powder. Defatted jatropha seed powder 3% and defatted jatropha seed fortified equivalent amount of oil in distilled water was used for production of prodigiosin.

Use of protein-free jatropha seed powder for production of prodigiosin: To solublize protein,

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3% jatropha seed powder in distilled water was taken and heated to 70°C for 30 min to solubilize protein. Suspension of seed powder was cooled and centrifuged to separate insoluble matter and kept aside. To the soluble matter, 5% TCA was added drop wise to precipitate proteins. Precipitated protein was removed by centrifugation at 6000 x g for 15 min. Insoluble matter and supernatant left after protein precipitation was mixed and adjusted to pH 7 by using 2 N NaOH and protein-free jatropha seed powder fortified with equivalent amount of protein was used for production of prodigiosin.

Analytical methods

Analysis of prodigiosin: The quantitative determination of prodigiosin was done by measuring the absorbance at 535 nm using Double Beam UV-Visible spectrophotometer (á-Helios, Thermo electron corporation) (15,16). To the culture broth (0.5 ml), 4 ml of methanol was added to the test tube and the mixture was vigorously vortexed for 2 min. The solution was then centrifuged at 6000 x g for 10 min. A fixed amount (0.8 ml) of the supernatant was further mixed with 0.2 ml of 0.05 N HCl:methanol mixture (4:1 v/v). The optical density of the resulting solution was measured at 535 nm (OD_{535}). The OD_{535} was converted to mass concentration *via* appropriate calibration using in-house purified prodigiosin as the standard (16).

Analysis of SRP: Determination of SRP activity is based on its caseinolytic property. The method was adapted from (17). To the substrate solution (0.75 ml, consisting of 1.0% w/v casein in 100 mM Tris/HCl, 1 mM $MgCl_2$, at pH 8.0), sample solution (0.1 ml) was added and incubated at 40°C for 30 min. The reaction was quenched with 0.5 ml of 10% (w/v) trichloroacetic acid (TCA) to precipitate the unhydrolyzed casein. After 15 min, the reaction mixture was centrifuged at 6000 x g for 10 min and the absorbance of the supernatant was determined at 280 nm. One unit of enzyme activity (EU) is defined as the amount of enzyme that produces an increase in absorbance of 0.1 at 280 nm under the conditions of the assay. For blank, 0.5 ml of 10% (w/v) TCA was added to 0.1 ml of sample solution and mixed; further 0.75 ml of the substrate solution was added and allowed to stand for 30 min at 40°C and proceeded as above (18).

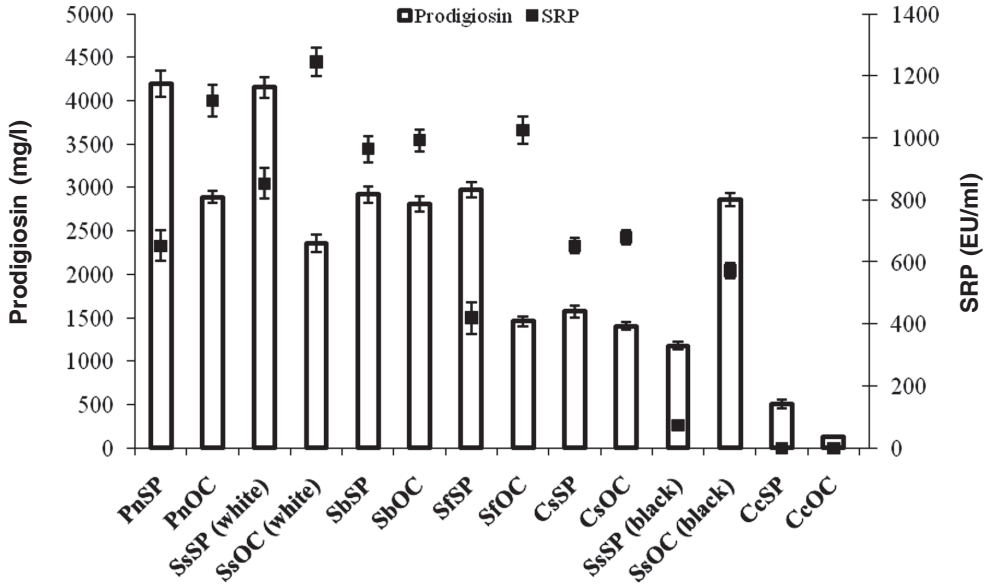

Fig. 1. Evaluation of conventional sources for the production of prodigiosin and SRP

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Results and Discussion

Conventional sources for the production of prodigiosin: Among the various conventional sources screened for production of prodigiosin, peanut seed powder and sesame seed powder produced almost similar amount of prodigiosin of 4190±150 and 4150±120 mg/l, respectively (Fig. 1). Higher production of prodigiosin was reported by Giri et al. (1) using peanut seed powder and sesame seed powder. In all the conventional sources screened, oil seed powders produced higher amount of prodigiosin than its oilcakes, whereas the oilcakes supported higher production of SRP as compared to its seed powder. It is clear from the result that oil content in the medium inhibited the induction of metalloprotease from *S. marcescens* NRRL B-23112. Highest SRP production of 1025±43 EU/ml was observed with sunflower oilcake, whereas coconut power and its oilcake did not support SRP production. Coconut powder and its oilcake supported poor production of prodigiosin, which clearly indicates that high oil content alone is not suitable for production of prodigiosin, but there is also need for adequate protein in the substrates. We also observed a great variation in production of prodigiosin between black and while varieties of sesame used in this study. Siva et al (19) used spoiled coconut for the production of prodigiosin. They reported maximum production of 10 mg/l using *Serratia rubidaea*.

Serratia species is known to produce lipase which helps in utilization of fatty acids present in oily seed powder (20). Besides, fatty acids are known to induce stress in the microorganism which could induce the production of secondary metabolites like prodigiosin. When peanut seed powder and sesame seed powder was mixed with distilled water, they produced a milky white emulsion. Due to this, *S. marcescens* NRRL B-23112 can easily access nutrients present in seed powder. We have also observed an interesting phenomenon which is usually not seen with standard medium. Prodigiosin produced by *S. marcescens* NRRL B-23112 was excreted in the surrounding media. This could be due the hydrophobic nature of medium which helped in extraction of prodigiosin present in the vesicles near cell wall (21).

Further different concentration of peanut seed powder and sesame seed powder were screened for their effect on prodigiosin production (Fig. 2). 4% peanut seed powder and 3% sesame seed powder gave highest production of prodigiosin 5020±120 and 5010±100 mg/l, respectively. In case of peanut seed powder, the SRP production decreased with an increase in its concentration. However no such phenomenon is observed with sesame seed powder.

Unconventional bioresources for the production of prodigiosin: Surprisingly, we found that jatropha seed protein supports highest production of prodigiosin followed by gulmohur seed powder and subabul seed powder at 5331±130, 2244±100 and 1010±98 mg/l, respectively (Fig. 3). Trash fish powder supported lowest prodigiosin among the unconventional bioresources screened in this study. The jatropha seed powder also produced a milky white suspension when added with distilled water and eventually converted to dark pink coloured suspension on growth of *S. marcescens* NRRL B-23112.

Jatropha seed powder is a good source of fatty acid as well as protein (22). Besides, it also contains high amount of proline, an amino acid which is a precursor for biosynthesis of prodigiosin. Giri et al. (1) reported the role of saturated fatty acid for enhanced production of prodigiosin. However, jatropha is reported to contain approximately 80% of oleic (monounsaturated) and linoleic acid (diunsaturated). Hence, it can be speculated that a mixture of saturated and unsaturated fatty acids could play a role in enhancing production of prodigiosin.

Further jatropha seed powder was screened for different concentration and 3% was found to support highest production of 6000±200 mg/l (Fig. 4). The production of SRP was found to decrease with an increase in seed powder concentration.

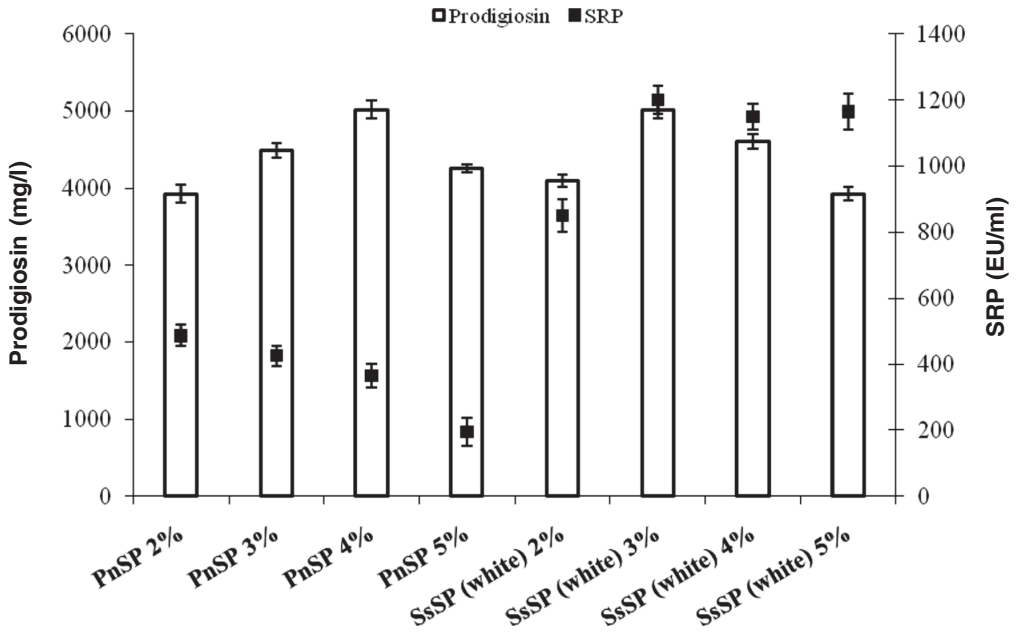

Fig. 2. Effect of concentration of peanut seed powder and sesame seed powder for production of prodigiosin

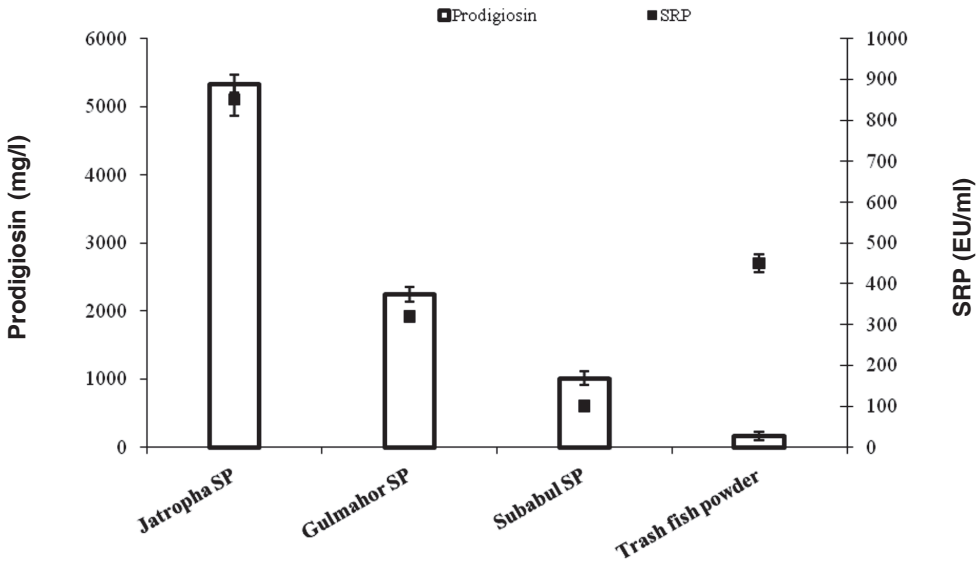

Fig. 3. Evaluation of unconventional bioresources for the production of prodigiosin and SRP

Unconventional Bioresources for prodigiosin production

An attempt was made to find out which component of the jatropha seed powder is essential for prodigiosin production. Jatropha seed powder was defatted which gave a yield of 58±5% w/w (Table 1). Fermentation was carried out using defatted 3% jatropha seed powder and defatted 3% jatropha seed powder fortified with equivalent amount of oil. Removal of oil drastically decreased prodigiosin production to 2560±100 mg/l, but increased the SRP production to 2010±150 EU/ml. Fortification of defatted seed powder with equivalent amount of oil did not improve the prodigiosin production significantly. We observed most of fortified oil to remain in the supernatant of fermentation media and hence not easily available for growth of *S. marcescens* NRRL B-23112. In case of protein-free 3% jatropha seed powder and protein-free 3% jatropha seed powder fortified with equivalent amount of protein, we observed same phenomenon. Hence, it can be concluded that natural form of jatropha seed powder effectively supports the production of prodigiosin. Both oil and protein content is necessary for higher production of prodigiosin. Wang et al (23) reported production of 1116 mg/l of prodigiosin *S. marcescens* TKU011 when squid pen powder (a fishery processing waste) was used as substrate.

Conclusions

Unconventional bioresources would be a valuable research tool and could become a viable source for industrial processes. Conventional as well as unconventional media sources were found to be promising source for production of prodigiosin. Jatropha seed powder medium supported a production that was much higher than the conventional medium. Therefore, it can be expected that use of unconventional bioresources for secondary metabolite production will become more routine, especially for high-value-added products. The reason for such high production with jatropha seed powder remains unclear and needs detailed investigation. Its role in newer applications such as natural dye

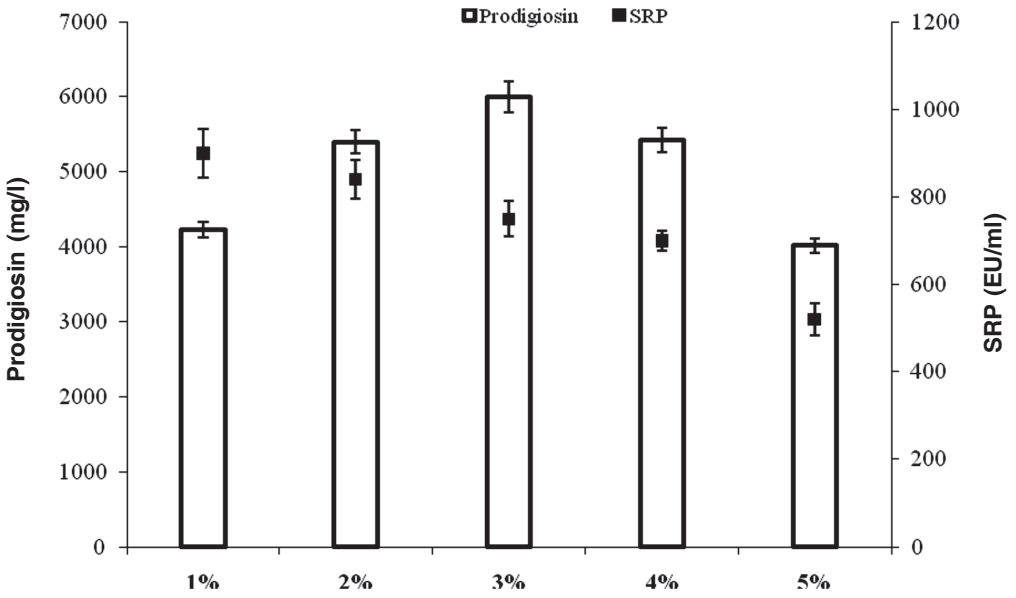

Fig. 4. Effect of concentration of jatropha seed powder on production of prodigiosin

Table 1. Different fermentation conditions for unconventional bioresources for prodigiosin production

Fermentation conditions	Oil /or protein content (% w/w)*	Prodigiosin (mg/l)*	SRP (EU/ml)*
Defatted 3% jatropha seed powder	58±5	2560±100	2010±150
Defatted 3% jatropha seed powder fortified with equivalent amount of oil	-	3360±79	1090±90
Protein-free 3% jatropha seed powder	20±3	4520±210	590±35
Protein-free 3% jatropha seed powder fortified with equivalent amount of protein	-	4750±150	620±55
3% jatropha seed powder	-	5940±200	720±50
Standard Optimized media	-	4209±150	-

*Results are mean ± SD of atleast three determinations

in textiles will make good market in future. Such waste utilization will result in cost effective fermentation process development for mass production of prodigiosin. These unconventional bioresources can further be studied for production of other secondary metabolites.

Acknowledgement

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References

1. Giri, A.V., Anandkumar, N., Muthukumaran, G. and Pennathur, G. (2004). A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. BMC Microbiology, 4: 1–10.
2. Bennett, J.W. and Bentley, R. (2000). Seeing red: the story of prodigiosin. Advances in Applied Microbiology, 47: 1–32.
3. Han, S.B., Kim Kim, H.M., Lee, Y.H., Jang, C.W., Son, E.S., Kim, K.H. and Kim, Y.K. (1998). T-cell specific immunosuppression by prodigiosin isolated from *Serratia marcescens.* International Journal of Immunopharmacology, 20: 1–13.
4. Montaner, B., Navarro, S., Piqué, M., Vilaseca, M., Martinell, M., Giralt, E., Gil, J. and Pérez-Tomás, R. (2000). Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. British Journal of Pharmacology, 131: 585–593.
5. Montaner, B. and Pérez–Tomás, R. (2001). Prodigiosin–induced apoptosis in human colon cancer cells. Life Sciences, 68: 2025–2036.
6. D'Alessio, R., Bargiotti, A., Carlini, O., Colotta, F., Ferrari, M., Gnocchi, P., Isetta, A., Mongelli, N., Motta, P., Rossi, A., Rossi, M., Tibolla, M. and Vanotti, E. (2000). Synthesis and immunosuppressive activity of novel prodigiosin derivatives. Journal of Medicinal Chemistry, 43: 2557–2565.
7. Diaz-Riuz, C., Montaner, B. and Pérez-Tomás, R. (2001). Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cell line HGT-1. Histology and Histopathology, 16: 415–421.

8. Furstner, A. (2003). Chemistry and biology of roseophilin and the prodigiosin alkaloids: a survey of the last 2500 years. Angewandte Chemie International Edition, 42:3582-3603.

9. Yamashita, M., Nakagawa, Y., Li, H. and Matsuyama, T. (2001). Silica gel-dependent production of prodigiosin and serrawettins by *Serratia marcescens* in a liquid culture. Microbes and Environments, 16: 250–254.

10. Goldschmidt, M.C. and Williams, R.P. (1968). Thiamine-induced formation of the monopyrolle moiety of prodigiosin. Journal of Bacteriology, 96: 609–616.

11. Witney, F.R., Failia, M.L. and Weinberg, E.D. (1977). Phosphate inhibition of secondary metabolism in *Serratia marcescens.* Applied and Environmental Microbiology, 33: 1042–1046

12. Lawanson, A.O. and Sholeye, F.O. (1975). Inhibition of prodigiosin formation in *Serratia marcescens* by adenosine triphosphate. Experientia, 32: 439–440.

13. Oseni, O.A. and Akindahunsi, A.A. (2011). Some Phytochemical Properties and Effect of Fermentation on the Seed of *Jatropha curcas* L. American Journal of Food Technology, 6: 158-165.

14. Association of Official Analytical Chemists (AOAC). (1998). Official methods of analysis of the association of analytical chemists, sixteenth ed. Washington DC: Association of Official Analytical Chemists.

15. Wei, Y.H. and Chen, W.C. (2005). Enhanced production of prodigiosin–like pigment from *Serratia marcescens* SMÄR by medium improvement and oil-supplementation strategies. Journal of Bioscience and Bioengineering, 99: 616-622.

16. Pansuriya, R.C. and Singhal, R.S. (2010). A process for the production of prodigiosin using *Serratia marcescens* by solid state fermentation, Indian Patent Application No. 103/MUM/2010.

17. Salamone, P.R. and Wodzinski, R.J. (1997). Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens*, Applied Microbiology and Biotechnology, 48: 317–324.

18. Pansuriya, R.C. and Singhal, R.S. (2010). Evolutionary operation (EVOP) to optimize whey independent serratiopeptidase production from *Serratia marcescens* NRRL B–23112. Journal of Microbiology and Biotechnology, 20: 950-957.

19. Matsuyama, T., Murakami, T., Fujita, M., Fujita S. and Yano, I. (1986). Extracellular Vesicle Formation and Biosurfactant Production by *Serratia marcescens*. Journal of' General Microbiology, 132: 865-875.

20. Akatsuka, H., Kawai, E., Omori, K. and Shibatani, T. (1995). The three genes *lip*B, *lip*C and *lip*D involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N–terminal signal peptide. Journal of Bacteriology, 177: 6381–6389.

21. Siva, R., Subha, K., Bhakta, D., Ghosh, A.R. and Babu, S. (2012). Characterization and Enhanced Production of Prodigiosin from the Spoiled Coconut. Applied Biochemistry and Biotechnology. 166: 187–196.

22. Martinez-Herrera, J., Siddhuraju, P., Francis, G., Davila-Ortiz, G. and Becker, K. (2006). Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chemistry, 96: 80–89.

23. Wang, S.L., Wang, C.Y., Yen, Y.H., Liang, T.W., Chen, S.Y. and Chen, C.H. (2011). Enhanced production of insecticidal prodigiosin from *Serratia marcescens* TKU011 in media containing squid pen. Process Biochemistry. doi:10.1016/j.procbio.2011.07.010.

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Impact of Nutritional factors verses Biomass and Serralysin Production in isolated *Serratia marcescens*

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Abstract

A high proteolytic enzyme producing marine isolate has been evaluated for its extracellular protease production with respect to different major nutritional sources. The phylogenetic analysis (16S rRNA ribotyping) of the isolated strain revealed that the strain belongs to *Serratia marcescens* and the sequence has been submitted to EMBL under the accession No. HE613732. Carbon source utilization profile indicated that the isolate has potential to use wide range of carbohydrates ranging from monosaccharides to polysaccharides. This isolate produces cellulase, amylase and chitinase extracellularly. Among tested carbon sources, dextrose among monosacchardies, maltose among disaccharides and starch among polysaccharides supported the best growth as well as enzyme production. Maltose among all carbon sources revealed high ratio of biomass growth *Vs* enzyme yield. Type of complex nitrogen source influenced the enzyme and biomass production independently. Tryptone supported more than 95% improved enzyme yields compared to combination of yeast extract and peptone. Present study revealed that growth and enzyme production in this isolate is differently regulated by type of complex nitrogen source. Yeast extract supplementation supported for maximum biomass development while other complex nitrogen sources supported equally for biomass and enzyme production. To the best of our knowledge, this strain revealed higher enzyme yields compared to literature reports suggesting commercial potential of this isolate.

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

Introduction

Microbial relation with human development is well recognized as these biological tiny particles significantly improved biotechnological and pharmaceutical products (1 - 7). Among different microbial products, proteases have been investigated for their possible role in human health care sector for the treatment of inflammation and inflammatory disorders and recognized that certain proteolytic enzymes from a variety of sources have shown significant contribution in reduction of inflammation especially resulting from sickness or injury (8) or breast engorgement (9).

Serralysin is a proteolytic enzyme initially isolated from *Serratia marcescens*, a potentially pathogenic bacterium, found in the gut of the Japanese silkworm. The isolated protein belongs to alkaline metalloprotease and known to activate the Hageman factor-kallikrein-kinin systems of mammals and directly involve in degradation or inhibition of IgG and IgA immune factors as well as regulatory proteins such as 2-macroglobulin, 2-anplasmin and anti-thrombin III (10, 11). Because of these functionalities, it is administered in dietary supplements for the treatment of assorted inflammatory disorders in Asia and Europe, it gained wide clinical usage as cardiovascular, anti-inflammatory, respiratory, or immune support agent and as an adjunct to antibiotic therapy (12) and to treat other chronic

inflammatory diseases, like atherosclerosis, arthritis, bronchitis, carpel tunnel syndrome, fibrocystic breast disease, and sinusitis (13). Recent studies have even suggested the use of oral serratia peptidase, aid in the prevention of viral infections, such as AIDS and hepatitis B and C (14). Several microbial strains belonging to *Serratia marcescens* have been well documented in the literature for production of Serratia protease/peptidase (15 - 19). Subsequently, this enzyme also reported from different bacterial strains including *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia freundii* (20). Much attention has been focused on serralysin production by *Serratia marcescens* due to its potential for higher enzyme yields compared to literature reports (1). The production yields in these strains regulated by nutritional parameters, fermentation growth conditions especially organic nitrogen sources (21, 22). The growing interest in the world wide in use of this protease in dietary supplement and along with high price for this enzyme in the are some of the powerful appeals that lead to search for new protease producing sources and subsequent bioprocess development. In this context, authors reported isolation and biochemical characterization of bacterial strain belongs to *Serratia* sp (1). Since, the isolated strain's revealed characters similar to genus *Serratia*, further studies would offer a better understanding of the strains potential at industrial scale, hence in the present investigation, efforts have been made to characterize this isolate based on phylogenetic analysis for identifying the same at species level followed by understanding the culture conditions role on improvement of proteolytic enzyme yields. The data revealed that the isolated strain belongs to *Serratia marcescens* and type of nitrogen source play a regulatory role in ratio of biomass and serralysin yields.

Materials and Methods

***Organism identification*:** Previously isolated *Serratia* sp. (1) from marine habitat was used in this study. This isolated strain was grown on nutrient agar slants at 30°C and sub-cultured regularly in the same respective medium. For growth and inoculum development the nutrient broth was used.

Phylogenetic analysis: Phylogenetic characterization of *Serratia* sp. was performed based on 16S rRNA amplification and nucleotide sequencing. The obtained nucleotide sequence was used as query to search for homologous sequence in the nucleotide sequence databases by running BLASTN program. Using Gene bank database, the high scoring similar to 16S rRNA gene sequences were identified and aligned using Clustal W Alignment option followed by phylogenetic tree construction using the neighbour-joining method in MEGA 5.0 program (5). Bootstrap analysis was carried out of the neighbour-joining data, using 1000 representative samplings, to evaluate the validity and reliability of the tree topology.

***Production media and culture conditions*:** Medium consisting of (%, w/v) Yeast extract - 1%, Peptone - 1%, Dextrose - 1%, $MgSO_4$ - 0.02%, KH_2PO_4 - 0.05%, NaCl - 0.25%, $CaCl_2$ - 0.002% adjusted to pH 7.0 was used for the enzyme production. For all experiments, inoculum developed by growing the isolate in nutrient broth for 18 h was used after adjusting the optical density to 0.8 (OD_{600nm}). For bulk production of enzyme, 1% inoculum was added to 50 ml production medium in 250ml conical flasks and then incubated at 30°C for 3 days. Samples withdrawn at specific time intervals were centrifuged at 10,000 rpm for 10 min and the supernatant used as enzyme source for assay. All the culture conditions were same unless otherwise mentioned.

***Protease assay*:** Protease activity was analyzed according to modified (1) method of Anson (23). A suitable blank was run simultaneously, in which TCA was added to the enzyme solution, followed by substrate addition. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions.

***Effect of carbon and nitrogen sources*:** The effect of carbon and nitrogen sources on protease production by this strain was studied by replacing the respective medium components with the selected sources. The samples were withdrawn aseptically every 3h, where cell density was monitored using UV-Visible spectrophotometer at 600nm and enzyme activity measurement as described above.

Results and Discussion

Proteolytic enzymes are ubiquitous in nature and are distributed in all living organisms. They have biological significance especially in maintenance of cell growth and differentiation. However, their production yields differ considerably in microbial strains and mainly influenced by nutritional status of the growth medium or environmental nitch and enzyme yield values differ with biological source. This warrants selection of microbial strain depending on the application. In view of the isolated strain potential in enzyme yield compared to literature cited (Table 1). In the present study *Serratia marcescens* RSPB11 was characterized phylogenetically and investigated in detail with respect to biomass and enzyme yield regulatory role of different carbon and nitrogen sources.

***Molecular characterization of the isolate*:** Initial biochemical, physiological and morphological evaluation, revealed that the isolated serralysin producing strain belongs to *Serratia* sp RSPB11 (1). In view of this *Serratia* sp. RSPB11 with higher enzyme production potential to that of various strains reported in the literature (Table 1), further identification of this isolate was investigated phylogenetically based on molecular characterization. In order to evaluate the same the genomic DNA of the isolate were amplified and analyzed for molecular-based identification. The purified gene sequence revealed that it contains 1506 base pairs. Blast analysis denoted 99% similarity to

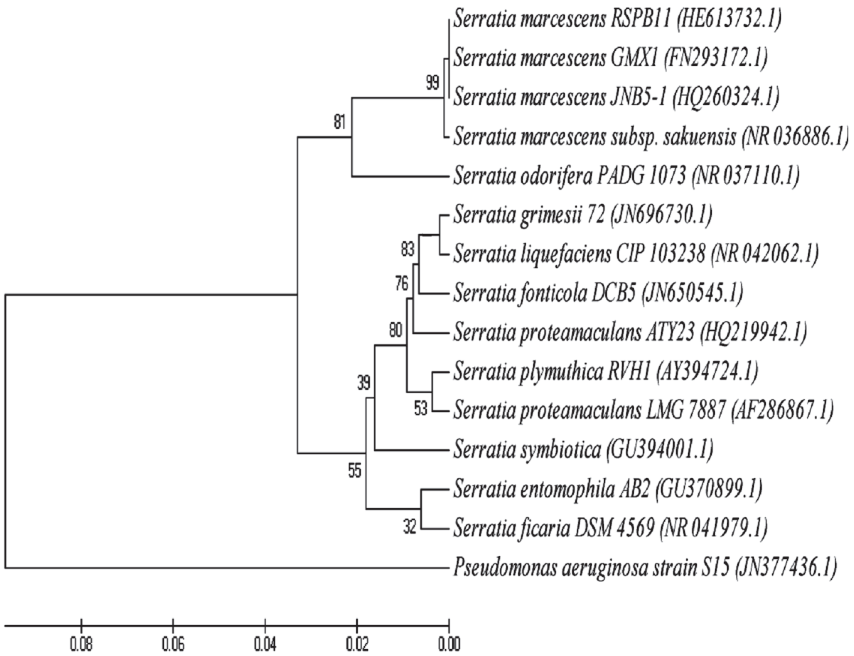

Fig. 1. Neighbor joining phylogenetic tree constructed according to Kimura two parameter models is showing phylogenetic relationship of *Serratia marcescens* RSPB11 with the members of the genus *Serratia*

Serratia marcescens family and so the isolate was designated as *Serratia marcescens* RSPB11. Phylogenetic tree was constructed by taking the sequences obtained in the BLAST search, using *Pseudomonas aeruginosa* (JN377436.1) as an outer group. (Fig. 1) shows the phylogenetic tree representing the similarity of *S. marcescens* RSPB11 with other group members. The obtained 16S rRNA gene sequence of 1506bp length was deposited at to EMBL database under accession number HE613732.1.

***Enzyme yield improvement studies*:** Any microbial metabolite/enzyme yield highly depends on the metabolic functions of the selected strain which is generally regulated by growth environment (nutritional, physiological and biological growth parameters) and genetic nature. Hence, desired product yields could be achieved by understanding the influence of above factors on biomass growth and monitoring of product yields with the function of fermentation time. Literature supports could be evidenced for several microbial products on supplementation of nitrogen and carbon source as well as variation of concentration. Subba Rao *et al.,* (6) demonstrated that extracellular alkaline protease production in *Bacillus subtilis* is regulated by glucose as carbon source and other growth conditions. The authors improved the protease production more than 400% by optimizing the bioprocess conditions. In view of the above, experiments are planned to evaluate different bioprocess parameters on serralysin production by isolated *S. marcescens* RSPB11.

Effect of Carbon sources: Carbon source is one of the macronutrients and a constituent of most of macromolecules of any living organisms and also required for any microbial metabolism and associated product production in addition to biomass growth. In general, glucose is considered as the best metabolizable carbon source hence, extensively used as major carbon source for microbial based compound/ metabolite/enzyme production by various investigators (24). However, variations do exist and carbon source mediated regulations in enzyme yields have been reported in several microbial strains (2 - 7). Hence, impact of different carbon sources on serralysin production by this isolated microbial strain was studied by replacing the dextrose in the fermentation medium with selected carbon source in the same concentration i.e. 1.0% (w/v) and measuring the enzyme production after 48 hours of bioprocess. The data presented in Fig. 2a revealed that serralysin production differed with type of carbon source indicating the metabolism of microbial strain differs with the carbon source availability for growth of organism. Dextrose as carbon source supported maximum enzyme production among all test carbon sources while, least serralysin yield observed when medium supplemented with starch (3055 U/ml) and chitin (3350 U/ml) as carbon sources. Further, among different disaccharides such as sucrose, galactose, and maltose used as sole carbon sources, maltose and galactose supplementation showed 25% higher serralysin production compared to sucrose. It is interesting to observe that arabinose, fructose, mannose and xylose also serve as carbon source and supported serralysin production 3730, 3975, 3755 and 3870 U/ml, respectively (Fig. 2a) in this isolate suggesting that this isolate has potential to utilize different carbon sources and support metabolism mediated enzyme production. Based on above results dextrose, fructose, galactose and maltose were selected to further evaluate their impact on serralysin production with respect to incubation time. The data presented in this ms is contradict with literature reports where an increased alkaline protease production reported by several other studies with the use of different carbohydrates such as sorbitol and starch (25), sucrose (26) and maltose (18) indicating that the best carbon source for enzyme production is different and influenced by microbial genetics. Mohan Kumar (27) working with *S.marc*escens reported that dextrose is the best carbon source. Dextrose at 1% level supported for optimized serralysin production with 5465 U/ml (Fig. 2b), followed by

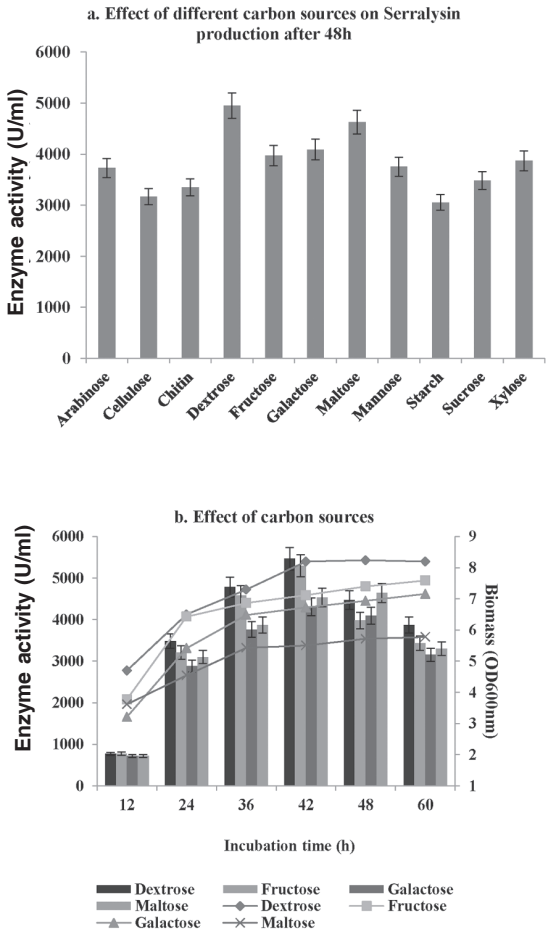

Fig. 2. Influence of different carbon sources on serralysin production by *Serratia marcescens* RSPB11 with the function of fermentation time

fructose (5300 U/ml), maltose (4640 U/ml) and galactose (4310 U/ml) at 42h of fermentation. Highest biomass yield was also observed in case of dextrose (Fig. 2b) followed by fructose, galactose and maltose. A stationary phase has been attained after 36hours for all the sources and showed stable growth till 72hours. In case of maltose even though the biomass was less, a higher enzyme production was achieved compared to fructose and galactose after 48 hours.

Critical evaluation of relation between carbon source and enzyme production by this isolated strain revealed that this microbe has potential to metabolize different carbon sources ranging from polysaccharides to monosaccharides. However, there is no trend is noticed. Among all tested carbon sources, monosaccharides regulated positively on metabolism based enzyme production. The observed enzyme production with the utilization of cellulose, starch and chitin do suggested that this isolate has potential to produce these polysaccharides degrading enzymes and subsequently convert to monomeric carbon compounds and utilize for metabolism associated serralysin production. This was further confirmed by analyzing the cellulase, amylase and chitinase production of the isolate and noticed that these polymer degradation enzymes were produced in the medium during fermentation process (Fig. 3). This character is most essential for commercial production of serralysin using this isolate which will reduce the bioprocess economics. However, the noticed difference in serralysin enzyme production yields with cellulose, starch and chitin as sole carbon source may be attributed to variation in polymeric carbon source degrading enzyme production.

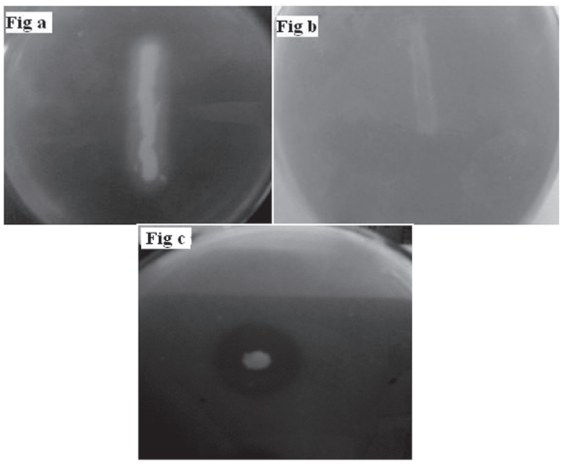

Fig. 3. Petri plates showing the *Serratia marcescens* RSPB11 with a potential to produce extracellular amylase (a) and cellulase (b) and chitinase (c)

Effect of nitrogen sources: In microorganisms, nitrogen plays a significant role in metabolism. Nitrogen is one of the integral elements of bio-molecules like amino acids, nucleic acids, proteins and cell wall components. Generally microbes absorb nitrogen from growth environment either in organic or in inorganic forms. Alkaline protease production depends heavily on the availability of nitrogen sources in the medium, which has regulatory effects on enzyme synthesis (9). Although complex nitrogen sources were usually recommended for proteases production, the requirement for a specific organic nitrogen supplement differs from organism to organism. In the present study, protease production ranged between 5005 and 9845 U/ml indicating the influence of type of nitrogen source on metabolism of isolated strain. Tryptone gave a maximum production of 9845 U/ml followed by Casaminoacids (9320 U/ml), Casein enzyme hydrolysate (8910 U/ml), Casitone (6710 U/ml) and Yeast extract-Peptone (5005 U/ml) after 48h (Fig. 4). Mohankumar and Raj working with soil isolate *S.marcescens* reported that optimized enzyme production with the supplementation of tryptone (27) while Patil et al reported maximized enzyme production with the support of casamino acid (28).

Chemically, casitone and tryptone are rich sources of amino acids. The presented data indicated that replacement of casitone and tryptone by yeast extract and peptone, improve enzyme yields. In fact, other complex nitrogen sources like beef extract, meat extract, malt extract and soya-peptone, though supported the growth however not improved protease production to that of casamino acids (Fig. 4a). The observed higher support of yeast extract for growth and lower enzyme production in this strain compared to other selected complex nitrogen sources may be attributed to compositional

Table 1. Serralysin production from different sources at various cultivation conditions

Organism	Production medium conditions	Growth	Enzyme activity	Comments	Ref
Serratia marcescens NRRL B-23112	Maltose 4.5%, Soybean meal 6.5%, K_2HPO_4 0.8%, NaCl 0.5%	pH 6.0, 25°C, 180rpm 1% inoculum	7,333 EU/ml at 48h	Optimized through EVOP design	(14)
Serratia marcescens SB08	Beef extract 0.3%,Yeast extract 0.3%,NaCl 0.5%, Peptone 0.5%	pH 6.0, 30°C, 100rpm 1% inoculum	281.23 U/ml at 51h	Plackett-Burman and RSM designs applied	(26)
Serratia marcescens ATCC27117	Casein 1%, Soybean meal 2%, $(NH_4)_2HPO_4$ 1%, NaCl 0.1%, KCl 0.05%,$CaCl_2$ 0.02%, $MgSO_4$ 0.02%, Soybean oil 0.3%	25°C, 200rpm	9100 U/ml	Recombinant strain	(30)
Serratia ureilytica	Squid pen powder 1.5%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%	pH 7.5, 25°C,150 rpm, 72h	195 U/ml	Two types of proteases detected	(31)
Serratia marcescens RSPB11	Glucose 1%, Tryptone 2%, $MgSO_4$ 0.02%, K_2HPO_4 0.05%, NaCl 0.5%, $CaCl_2$ 0.002%	pH 7.0, 30°C, 150 rpm	9845 U/ml at 48h	Shake flask fermentation	Present work

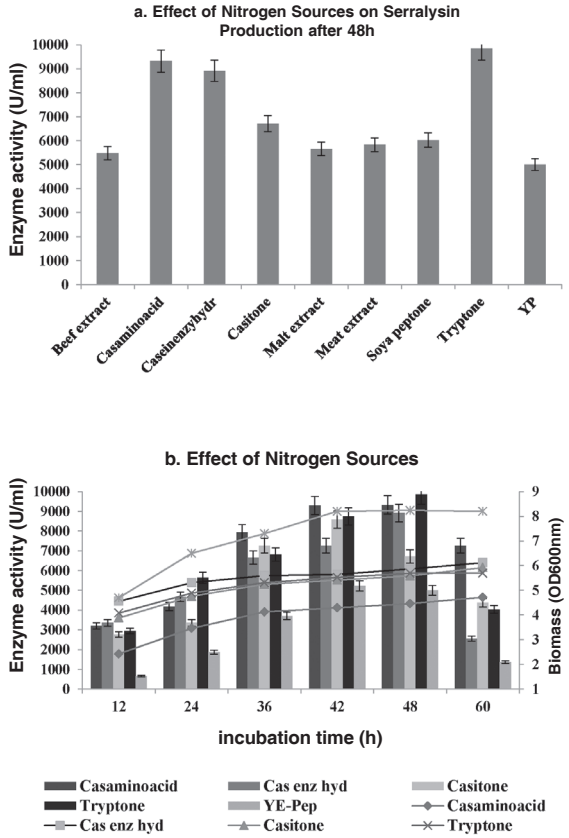

Fig. 4. Influence of different nitrogen sources on serralysin production by *Serratia marcescens* RSPB11 with the function of fermentation time

nature as yeast extract known to contain several key nutrients such as vitamin, nucleic acid, lipid. etc. Though these nutrients may also present in beef, malt and soya peptone, the ratio of biomass and serralysin yield was higher in above complex nitrogen sources supplementation compared to yeast extract. This further may be attributed to the ratio of above nutrient composition might be the regulating factor for biomass and metabolism mediated protease production in this isolate. Further analysis suggested that all tested organic nitrogen sources supported biomass development however, maximum biomass growth was observed when combination of peptone and yeast extract was used (Fig. 4b).

Analysis of biomass production in the present study denoted that the medium containing yeast extract and peptone supported the growth and maximum cell density was attained in 42 hours of growth (Fig. 4b) compared to all other nitrogen sources however, the enzyme production was very low. Similar effect was shown for *Bacillus* sp., Vel, (28) and other marine isolate *S. marcescens* (29) where growth was best supported by a combination of peptone and yeast extract, while the optimum protease production was with casaminoacid. This data is in support with observed protease production results in the present study. Whereas Venil and Lakshmanperumalsamy reported that higher concentration of nitrogen sources suppress the protease synthesis in *S.marcesc*ens SB08 (26). The observed growth and enzyme production in isolated *S. marcescens* RSPB11 further suggested that casaminoacid as nitrogen source support higher biomass and stable enzyme production throughout the stationary phase. Since this nutrient is a hydrolysed form of casein it might help in supplying the essential aminoacids required for protein degradation and a stationary growth. From this study it has been observed that optimization of nitrogen source resulted in a further increase in serralysin production of about 4.475 fold compared to the basal medium under un-optimized conditions. From the results it is interesting to note that higher biomass may not be necessary for higher enzyme production.

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References

1. Bhargavi, P.L. and Prakasham, R.S. (2012). Proteolytic enzyme production by isolated *Serratia* sp RSPB11: Role of environmental parameters. Current Trends in Biotechnology and Pharmacy. 6: 55-65.

2. Mahalaxmi, Y., Sathish, T., Rao, C.S. and Prakasham, R.S. (2010). Corn husk as a novel substrate for the production of rifamycin B by isolated *Amycolatopsis* sp. RSP 3 under SSF. Process Biochemistry. 45: 47–53.

3. Prakasham, R.S., Rao, C.S., Rao, R.S., Lakshmi, G.S. and Sarma, P.N. (2007a). L-Asparaginase production by isolated *Staphylococcus* sp. – 6A: Design of experiment considering interaction effect for process parameter optimization. Journal of Applied Microbiology. 102: 1382–1391.

4. Prakasham, R.S., Rao, C.S., Rao, R.S. and Sarma, P.N. (2007b). Enhancement of acid amylase production by an isolated *Aspergillus awamori.* Journal of Applied Microbiology. 102: 204–211.

5. Sambasiva Rao, K.R.S., Tripathy, N.K., Mahalaxmi, Y. and Prakasham, R.S. (2012). Laccase- and peroxidase-free tyrosinase production by isolated microbial strain. Journal of Microbiology and Biotechnology. 22: 207–214.

6. Subba Rao, Ch., Sathish, T., Mahalaxmi, Y., Suvarna Laxmi, G., Rao, R.S., and Prakasham, R.S. (2008). Modelling and optimization of fermentation factors for enhancement of alkaline protease production by isolated *Bacillus circulans* using feed-forward neural network and genetic algorithm. Journal of Applied Microbiology. 104: 889–898.

7. Suvarna Lakshmi, G., Bhargavi, P.L. and Prakasham, R.S. (2011). Sustainable bioprocess evaluation for xylanase production by isolated *Aspergillus terreus* and *Aspergillus fumigatus* under solid - state fermentation using oil palm empty fruit bunch fiber. Current Trends in Biotechnology and Pharmacy. 5: 1434-1444.

8. Ryan, R.E. (1967). A double-blind clinical evaluation of bromelains in the treatment of sinusitus. Headache. 7: 13-17.

9. Snowden, H.M., Renfrew, M.J. and Woolridge, M.W. (2001). Treatments for breast engorgement during lactation. Cochrane Database System Review. 2: CD000046.

10. Maeda, H. and Molla, A. (1989). Pathogenic potentials of bacterial proteases. Clinical Chemistry Acta. 185: 357-368.

11. Molla, A. (1989). Activation of hageman factor and prekallikrein and generation of kinin by various microbial proteinases. Journal of Biological Chemistry.264: 10589-10594.

12. Rothschild, A.J. and Locke, C.A. (1991). Reexposure to fluoxetine after serious suicide attempts by three patients: the role of akathisia. Journal of Clinical Psychiatry. 52: 491-493.

13. Klein, G. and Kullich, W. (2000). Short-term treatment of painful osteoarthritis of the knee with oral enzymes, a randomized, double-blind study versus diclofenac. Clinical Drug Investigation. 19: 15-23.

14. Pansuriya, R.C. and Rekha, S.S. (2010). Evolutionary operation (EVOP) to optimize whey-independent serratiopeptidase production from *Serratia marcescens* NRRL B-23112, Journal of Microbiology and Biotechnology. 20: 950–957.

15. Aiyappa, P.S. and Harris, J.O. (1976). The extracellular metalloprotease of *Serratia marcescens:* I. Purification and characterization. Molecular Cell Biochemistry. 13: 95-100.

16. Decedue, C.J., Broussard, E.A., Larson, A.D. and Braymer, H.D. (1979). Purification and characterization of the extracellular proteinase of *Serratia marcescens.* Biochemistry Biophysical Acta. 569: 293-301.

17. Miyata, K., Tomoda, K. and Isono, M. (1971). *Serratia protease.* Part III. Characteristics of the enzyme as a metalloenzyme. Agricultural and Biological Chemistry. 35: 460-467.

18. Pansuriya, R.C. and Rekha, S.S. (2011). Effects of dissolved oxygen and agitation on production of serratiopeptidase by *Serratia marcescens* NRRL B-23112 in stirred tank bioreactor and its kinetic modeling. Journal of Microbiology and Biotechnology. 21: 430-437.

19. Salamone, P.R. and Wodzinski, R.J. (1997). Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens.* Applied Microbiology and Biotechnology, 48: 317-324.

20. Maeda, H. (1996). Role of microbial proteases in pathogenesis. Microbiology and Immunology.40: 685-699.

21. Braun, V. and Schmitz, G. (1980). Excretion of a protease by *Serratia marcescens.* Archives of Microbiology, 124: 55-61.

22. Bromke, B.J. and Hammel, J.M. (1979). Regulation of extracellular protease formation by *Serratia marcescens.* Canadian Journal of Microbiology. 25: 47-52.

23. Anson, M.L. (1938). Estimation of Pepsin, papain and cathepsin with haemogloblin. Journal of General Physiology. 22: 79-89.

24. Ustariz, F.J., Laca, A., Garcia, L.A. and Diaz, M. (2008). Fermentation conditions increasing protease production by *Serratia marcescens* in fresh whey. Revista tecnica de la facultad de ingenieria universidad del zulia. 31: 79-89.

25. Nguyen, T.T. and Quyen, D.T. (2011). Over production of an extracellular protease from *Serratia sp.*DT3 just using soybean powder. World Journal of Agricultural Sciences. 7: 29-36.

26. Venil, C.K. and Lakshmanperumalsamy, P. (2009). Application of response surface methodology in medium optimization for protease production by the new strain of *Serratia marcescens* SB08. Polish Journal of Microbiology. 58: 117-124.

27. Mohankumar, R., and Raj, H.K. (2011). Production and characterization of serratiopeptidase enzyme from *Serratia marcescens.* International Journal of Biology. 3: 39-51.

28. Patel, R., Mittal, D. and Singh, S.P. (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. Process. Biochemistry. 40: 3569-3575.

29. Daatsellar, M.C.C. and Harder, W. (1974). Some aspects of the regulation of the production of extracellular proteolytic enzyme by a marine bacterium. Archives of Microbiology.101: 21–34.

30. Kim, K.S., Park, K.S., Byun, S.M., Pan, J.G. and Shin, Y. (1995). Overproduction of *Serratia marcescens* metalloprotease (SMP) from the recombinant *Serratia marcescens* strains. Biotechnology Letters. 17: 497-502.

31. Wang, S.L., Lin, C.L., Liang, T.W., Liu, K.C. and Kuo, Y.H. (2009). Conversion of squid pen by *Serratia ureilytica* for the production of enzymes and antioxidants. Bioresource Technology. 100: 316-323.

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Production of Pharmaceutically Important Saponins from *in vitro* Regenerated plants of *Chlorophytum borivilianum*

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Abstract

Chlorophytum borivilianum belonging to the family Liliaceae is commonly called safed musli. It is a perennial rhizomatous herb widely distributed in the pan tropical regions which contains pharmaceutically important saponins. Among the saponins, stigmasterol and hecogenin as the major secondary metabolites are responsible for the various biological activities viz. aphrodisiac, antioxidant, anticancer and immune booster. Due to the immense medicinal uses the convemtional propagation is not sufficient to meet the commercial demand. So, micropropagation of such conventiaonally propagated plants is very essential to meet the commercial demand as well as to ensure easy storage and transportation of disease free stocks.

The leaf sheath from *in vitro* raised plants was used for induction of callus on MS basal media fortified with 1mg/l of 2,4-D. Somatic embryogenesis and plant regeneration was observed from the callus obtained from MS basal media. Stigmasterol and Hecogenin were quantified from the callus and *in vitro r*egenerated plants of *C. borivilianum*. Maximum stigmasterol production (3.265 mg/gm dry weight) was observed from plants regenerated from somatic embryos, which is 5.4 fold higher than the amount of stigmasterol in undifferentiated callus cultures (0.6 mg/gm). Maximum hecogenin production (43.55 mg/g) was observed in plants regenerated from somatic embryos, which is 27.9 fold higher than the amount of hecogenin in callus cultures (1.56 mg/gm). The present study reports higher production of medicinally important stigmasterol and hecogenin in regenerated plants in comparision to the callus cultures.

Key words: *Chlorophytum borivilianum* (Safed musli), Stigmasterol, Hecogenin, Somatic embryogenesis, Quantification

Introduction

Plants synthesize an extensive array of secondary metabolites, with highly complex structures. Since chemical synthesis of most important secondary metabolites of pharmaceutical importance is not economically feasible, they are isolated from cultivated or wild plants (1). Nutraceutical industry in India is at a blooming stage (2,3,4). The world health organization has estimated that more than 80% of the world population in developing countries depends primarily on herbal medicines for basic healthcare needs (5).To meet the barrier between demand and supply biotechnological production in plant cell cultures is an alternative which gained limited success because of lack of knowledge in understanding the synthesis of these metabolites. Severe bottlenecks are identified for the production of medicinal compounds on a commercial scale, which include poor biomass productivity due to insufficient knowledge on

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biosynthetic pathways or sensitiveness to shear stress (1). *Chlorophytum* is considered as the major herbal plant which has high commercial importance, thirteen species of *Chlorophytum* have been reported from India (4,6,7) among which *C. borivilianum* is having the highest saponin content. *C. borivilianum,* commonly known as Safed musli, belongs to family Liliaceae. In India *C. borivilianum* is mainly distributed in Southern Rajasthan, North Gujarat and Western Madhya Pradesh (3). Major biochemical constituents of Safed musli are Saponins (3), among which stigmasterol and hecogenin are two major pharmacologically important saponins. *C. borivilianum* has therapeutic applications in ayurvedic system of medicine (4,3,7). Fasciculate roots of *C. borivilianum* are used as tonic and constitute important ingredient of many ayurvedic and unani preparations. Roots are used for the preparation of nutritional tonic used in general sexual weakness and enhanced antioxidant property. Further, plant cell/organ cultures are restricted/ altered by the environmental, ecological and climatic conditions hence cells can proliferate at higher growth rates in comparison to the conventional methods. The ability to produce morphologically and developmentally normal embryos and whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis are the potential models for studying early events in plant embryo development. Somatic embryos are induced from *in vitro* grown callus cultures by a relatively simple manipulation of the culture conditions. In this technique limited initial explants and space for multiplication was used through phenomenon based on totipotency concept. This research work was undertaken to study the secondary metabolite production under *in vitro* condition in *C. borivilianum* from cultures of various differentiation stages.

Materials and Methods

Chemicals and Reagents: MS media, Indole-3-Butyric acid, agar were from Himedia, India. Methanol of analytical/ HPLC grade, Acetic acid of HPLC grade were procured from Merck. Standard stigmasterol was supplied by Tokyo chemical industry Co. Ltd, Japan and hecogenin by MP Biochemicals, LLC, France.

Plant material: Plant material of *Chlorophytum borivilianum- santapau and fernandes* was collected from Agro farms of Nandan Biomatrix ltd., Hyderabad.

In vitro culture establishment : Different explants (viz, leaf tip, leaf sheath, rhizome) were cleaned and surface sterilized with 0.1% mercuric chloride for 7 minutes and then rinsed with sterile distilled water thoroughly. The sterile explants were cultured onto Murashige and Skoog (MS) agar medium supplemented with different concentrations of 2, 4-D (0.5, 1.0, 1.5, 2.0 mg/l). The pH of this media was adjusted to 5.88 before autoclaving (121°C for 15 min at 15 lbs pressure). The cultures were incubated under controlled environmental conditions at 25±2° C with 16/8 hrs light/dark regime at 3,000 lux. These cultures were sub-cultured at an interval of 28 days.

Estimation of saponins: Samples of undifferentiated callus, differentiated callus and regenerated plant from callus were collected at different stages of development and the amount of stigmasterol and hecogenin (8).

Phytoconstituent extraction from Chlorophytum borivilianum : The dried tissue samples were ground and extracted in methanol using a soxhlet apparatus. The extract was filtered, dried and the dried powder was extracted with 1ml of HPLC grade methanol. The samples were filtered using (0.22µm) Millipore filters, followed by quantification. Two major saponins stigmasterol and hecogenin were quantified using HPLC (8).

Quantitative estimation of stigmasterol and hecogenin : HPLC analysis was carried out using the Shimadzu—LC-10AT VP series HPLC system equipped with a Supelco column (250x4.6 mm, C18, ODS with particle size of 5 µm) with a flow rate of 1ml/min.

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The mobile phase for stigmasterol was methanol, water and Acetic acid in the ratio of 70:30:1, and for hecogenin methanol and water in the ratio of 90:10. Stigmasterol was detected at 254 nm and hecogenin was detected at 210 nm (8).

Statistical analysis: The experiments were done in triplicate. Statistical analysis of data was carried out using ANOVA. The data was compared by the least significance difference (Pd"0.05).

Results and Discussion

***Initiation of callus*: Different explants viz :** leaf, leaf sheath, rhizome were tried for callus initiation on MS media supplemented with different concentrations of 2,4-D (0.5—2.0mg/l). Only leaf sheath explant responded for callus induction rest of the explants did not show any sign of dedifferentiation (Table-1). Among different concentrations of 2,4D, 1mg/l proved to be the best (Fig. 1, 2a and 2b).

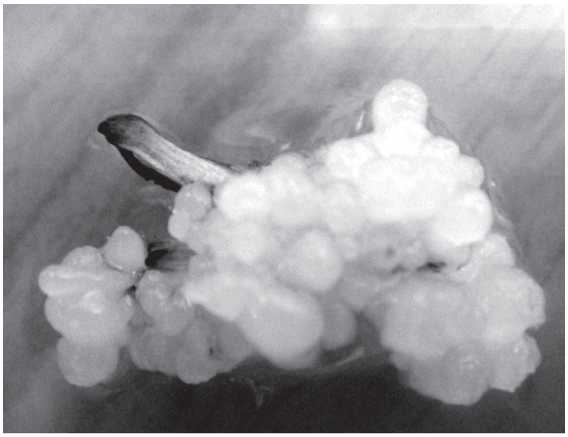

Fig. 1. Formation of callus from leaf sheath, explant on MS medium supplemented with 1mg/l 2,4-D.

Table 1. Response of different explants and 2,4 –D concentrations for callus induction.

Phytohormone	Concentration (mg/l)	Explants	% response
2,4 – D	0.5	--	Nil
	1.0	Leaf sheath	43.6± 0.45
	1.5	Leaf sheath	46.9 ± 0.30
	2.0	Leaf sheath	45.2 ±0.35

The callus was further maintained on the same media for few subculture (4-5) passages. When transferred to media devoid of 2, 4-D the callus gave rise to somatic embryos after 21 days of inoculation. These somatic embryos on further growth gave rise to plantlets on MS basal media (Fig. 3a and 3b).

Production of Stigmasterol at different stages of development in *Chlorophytum borivilianum:* Stigmasterol a pharmaceutically important saponin of *Chlorophytum borivilianum* was quantified at different stages of differentiation from callus to regenerated plant. Maximum amount of stigmasterol was observed from plants regenerated from somatic embryos (3.265 ±0.13 mg/gDCW) whereas (0.6±0.1mg/gDCW) was

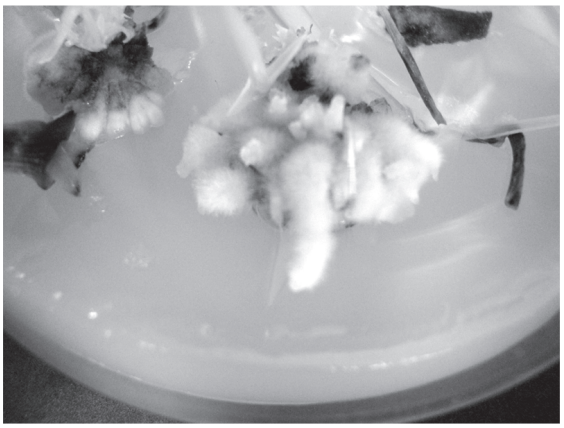

Fig. 2a

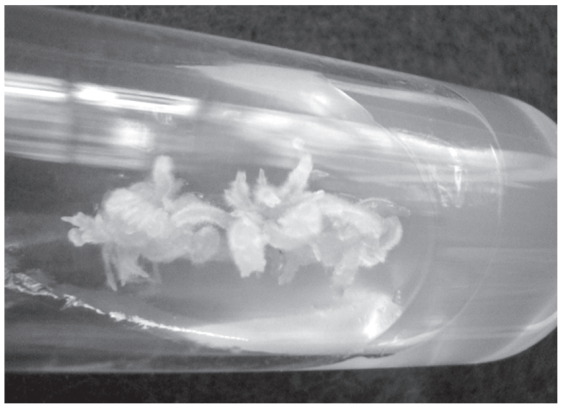

Fig. 2b

Fig. 2 a and 2b. Somatic embryogenesis from callus on MS basal media devoid of 2,4- D.

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Fig. 3a


Fig. 3b

Fig.3a and 3b. Plant regeneration from somatic embryos on MS basal media devoid of 2,4-D.

obtained from early callus and (0.92±0.1 mg/gDCW) from differentiating callus (Fig. 4).

Production of Hecogenin at different stages of development in *Chlorophytum borivilianum:* Hecogenin a pharmaceutically important saponin of *Chlorophytum borivilianum* was estimated at different stages of differentiation from callus to regenerated plant. Among the callus extracts tested for the presence of hecogenin, a maximum of 43.55±0.52 mg/gDCW was observed in plants regenerated from somatic embryos when compared to the early callus (1.56±0.49 mg/gDCW) and differentiating callus (3.28±0.32 mg/gDCW) (Fig. 5).

This is the first report of production of stigmasterol and hecogenin at different stages of differentiation from callus cultures of *Chlorophytum borivilianum*. The amount of stigmasterol and hecogenin was found to be very low in case of young (Undifferentiated) callus (0.6±0.2 mg/gDCW stigmasterol and 1.56±0.07 mg/gDCW of hecogenin), which increased with further differentiation of callus to plantlets. Callus after 48 days accumulated 0.92±0.03 mg/gDCW stigmasterol and 3.28±0.12 mg/gDCW hecogenin. However, maximum stigmasterol (3.265±0.02 mg/gDCW) and hecogenin (43.55±0.41 mg/gDCW) could be observed from plants regenerated from somatic embryos. The stigmasterol content from regenerated plants was 5.4 fold higher ($P < 0.05$) when compared to the

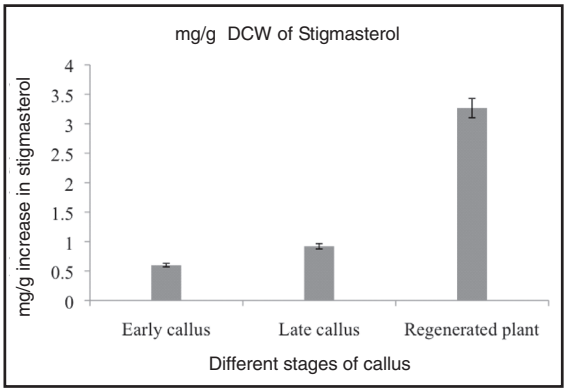

Fig. 4. Production of stigmasterol at various stages of differentiation

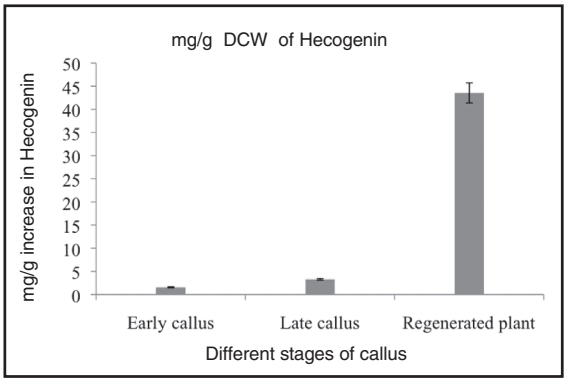

Fig. 5. Production of Hecogenin at various stages of differentiation

early callus and that of hecogenin is found to be 27.91 fold higher ($P < 0.05$) when compared to the early callus.

The first product shikonin of tissue culture produced from *Lithospermum erythrorhizon* which was economically viable (1). Ansari and Asghari (8) in 2008 reported on the production of gingerol and zingiberene along the differentiation process. This study indicates that accumulation of secondary metabolites result from complex metabolic changes accompanying the differentiation of cells in plants wherein biosynthetic pathways are initiated.

The positive role of 2,4-D for callus induction has been reported in other species (9,10,11). In *Ceropegia candelabrum L.* 2,4-D and BAP/Kn combinations were efficient in callus induction from internode and leaf explants (12). Higher concentrations of NAA and BAP stimulated callusing at basal end in case of *Clitoria ternatea L*. (13). Moreover, the regenerated plantlets obtained via the callus phase were shown to accumulate higher amounts of secondary metabolites in comparision to the normal *in vitro* multiplicated shoots and the *in vivo* plants (14)

This shows that different plant species exhibit differences towards phytohormones and explants for the callus induction.

References

1. Ashwani Kumar. (2009). Secondary metabolites are currently being obtained commercially by extraction from whole plants. Scientific Blogging.
2. Afsharipour, S., Asgary, S. and Blookwood, G.B. (1996). Constituents of the essential oil of Achillea wilhelmsii from Iran. Planta Medica, 62: 77-78.
3. Maiti, S. and Geetha, K.A. (2005). Characterization, genetic diversity and cultivation of Chlorophytum borivilianum- an important medicinal plant of India. Plant Genet Resour, 3, 264-72.
4. The Wealth of India (2000)- a dictionary of Indian raw material and industrial products. First supplement series (Raw material) National Institute of Science Communication, CSIR, New Delhi, vol 1:A-Ci, 247.
5. Agretious, T.K., Martin, K.P. and Hariharan, M. (1996). *In vitro* colonal multiplication of *Alpinia calcarata* Rose. Phytomorphology, 46,133138.
6. Toth, K., Haapala, T. and Hohtola, A. (1994). Alleviation of browning in oak explants by chemical pretreatments. Biologia Plantarum, 36: 511-517.
7. Oh, C.S., Kim, J.C. and Han, K.S. (1994). Effects of polyamines on senescence in Lactuca sativa L. Stabilization on leaf protoplasts. Journal of the Korean Society for Horticultural Science, 35: 318-322.
8. Gayathri, Bathoju. and Archana, Giri. (2012). Production of medicinally important secondary metabolites (stigmasterol and hecogenin) from root cultures of Chlorophytum borivilianum (Safed musli). Recent Research in Science and Techonology, 4(5):45-48.
9. Anasori, P. and Asghari, G. (2008). Effects of light and differentiation on gingerol and zingiberene production in callus culture of Zingiber officinale Rosc. Research in Pharmaceutical Sciences, Vol 3, No 1.
10. Yasuda, K., Tsuda, T., Shimizu, H. and Sugaya, A. (1988). Multiplication of Curcuma species by tissue culture. Planta Med, 54:75-79.
11. Salvi, N.D., George, L. and Eapen, S. (2001). Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant Cell Tiss. Org. Cult, 66: 113-119

12. Shirgurkar, M.V., Naik, V.B., Von, Arnold, S., Nadgaida, R.S. and Clapham, D. (2006). An efficient protocol for genetic transformation and shoot regeneration of turmeric (Curcuma longa L.) via particle bombardment. Plant Cell Reports, 25: 112-116.

13. Beena, M.R. and Martin, K.P. (2003). *In vitro* propagation of the rare medicinal plant Ceropegia candelabrum L. through somatic embryogenesis. In vitro Cell. Dev. Biol.-Plant, 39:510-513.

14. Gyana, Ranjan, Rout. (2004). Effect of cytokinins and auxins on micropropagation of *Clitoria ternatea* L. Biol. LETT, 41(1):21-26.

15. Kiranmayee, Rao., Bhuvaneswari, Chodisetti., Suryakala, Gandi., Lakshmi, Narasu, Mangamoori. and Archana, Giri. (2011). Direct and Indirect Organogenesis of Alpinia galanga and the Phytochemical Analysis. Appl Biochem Biotechnol, 165:1366–1378.

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Antibacterial Efficacy of Bark extracts of an Ethnomedicinal plant *Trema orientalis* Blume

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Abstract
Background: Traditional systems of medicine are often a valuable source of novel antimicrobials. Dima Hasao Hill district is endowed with rich cultures of traditional system of medicine and this study is the first of its kind from the area. The aim of the study is to to assess the antimicrobial efficacy of *Trema orientalis* Blume (Ulmaceae) on six selected bacterial strains. The minimum inhibitory concentrations were determined with the aqueous extract to validate the application of the plant species in traditional medicine.

Methods: Plant materials were collected after prior informed consent and processed using standard herbarium technique. Antimicrobial activity was determined by Kirby-Bauer Agar Disc Diffusion method with slight procedural modifications. Minimum inhibitory concentration of the aqueous extract was determined with standard antibiotics as positive control.

Result: The selected bacterial strains were highly susceptible to the test material. Aqueous extracts showed fairly good activity. The zones of inhibition of all the test materials ranged from 11 to 15 mm. MIC of aqueous extract showed inhibition of bacterial growth at a concentration as low as 0.625 mg/ml.

Conclusion: *T. orientalis* is a potentially good source of antibacterial agent. The efficacy against the selected bacterial strains and the resultant MIC values corroborates with its application in traditional medicine.

Keywords: Antimicrobial; Dima Hasao Hill district; *Trema orientalis*; plant extracts.

Introduction

According to the World Health Organization (WHO), infectious diseases are the primary cause of deaths worldwide and they account for more than 50 % of the death in tropical countries (1). To combat such diseases, a number of antibiotics have been produced by pharmacological industries worldwide, but the resistance of microbes has also increased in parallel. Further, bacterial strains are becoming increasingly resistant to most of the antibiotics available in the market. This has resulted in multiple drug resistant microbial strains, and to combat them, copious number of synthetic drugs especially in the developing countries (2). Moreover, some antibiotics have serious undesirable side effects that limit their application.

There is a resurgence of interest in herbal medicines due to the increased awareness of the limitations of synthetic drugs and their undesirable side effects and the need to discover new molecular structures as lead compounds from plants. The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Thus, any phytochemical investigation of

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given plant will reveal only a very narrow spectrum of its constituents (3). Historically, pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics (4, 5). Even now, contrary to common belief, drugs from higher plants continue to occupy an important niche in modern medicine. On a global basis, at least 130 drugs, all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons (6). Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as micro-organisms, animals, and plants. One of such resources is traditional medicines. Systematic screening of them may result in the discovery of novel effective compounds (7).

Dima Hasao Hill district, a small district of Assam, North-East India, located between 92º37/ E - 93º17/ E longitudes and 23º30/ N - 25º47/ N latitudes, lies in one of the world's 12 mega biodiversity hotspot regions. It is a living anthropological museum of many ethnic tribes, such as *Dimasa, Zeme-Naga, Hmar, Kuki, Biate, Hrangkhol, Khelma, Jaintia, Karbi, Vaiphei* etc., each with their own unique cultures and traditional system of healing. The small hill district has a total population of 1, 86,189 with a density of 38 persons per square kilometer, the lowest in the state of Assam (2001 census). The tribal villagers have considerable knowledge on the use of both conventional and non conventional plants for curing many common as well as severe ailments. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (8-12). Much work has already been done in India also (13-19) but very few reports exist from North-East India and such works has never been taken up from Dima Hasao Hill district of Assam.

A perusal of the available literature reveals that although reports on the application of plants from the northeastern region of India has exceeded 1350 species with ethnomedicinal uses, 665 as food plants and 899 species for miscellaneous uses (20), the small hill district still remains virtually unexplored except some sporadic reports by Tamuli *et al*., Sajem et al., and Rout *et al* (21-26). Set in this backdrop, *Trema orientalis* Blume., (Fig. 1), an evergreen tree, commonly found in the district, has been selected for antimicrobial screening (Table 1). The present paper describes the antibacterial activity of the bark extracts of *Trema orientalis* Blume., against six different bacterial strains viz., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*.

Methodology

Plant material: The plant species (Fig. 1) was selected on the basis of its reported use against cuts and infected wounds by the *Hmar* and *Zeme Naga* tribes (Table 1) from the study area. Plant materials were collected from the traditional healers after prior informed consent. The species is identified using relevant literature (27-29) and

Fig. 1. *Trema orientalis* plant used by the *Hmar* and *Zeme* tribes of North Cachar Hills district of Assam for the treatment of infected cuts and wounds.

Table 1. Method of use of *T. orientalis* Blume. by the *Hmar* and *Zeme Naga* tribe of N.C.Hills district.

Botanical Name (Collector's initial/Herbarium Voucher No)	Family	Local Name	Part Used	Disease	Method of use	Other use
Trema orientalis Blume (EGY/AS/109)	Ulmaceae	Hatou (*Hmar*) Kedubang (*Zeme*)	Barks	Infected cuts and wounds	Fresh barks are collected, washed in clean water and then pounded to pulp. It is then applied as a poultice in the affected area. Dressings are changed everyday till the wound is cured (which according to the Zeme traditional healer, would take approximately 3-6 days depending on the severity of the wound)	The barks are collected in bulk. It is pounded together with Potassium permanganate ($KMnO_4$) and other ingredients using traditional method to prepare gun powder which is used for hunting.

in consultation with the Botanical Survey of India, BSI/APC (ARUN Herbarium,) Itanagar and BSI (Kanjilal Herbarium), Eastern circle, Shillong. The voucher specimen has been processed through standard herbarium techniques (30-31) and submitted in the Department of Ecology and Environmental Science, Assam University, Silchar, Assam, India.

Preparation of Extracts: Barks of healthy plants were collected from the study sites, washed thoroughly in tap water and dried in a dark room at normal temperature for 15 days. They were then grounded to fine powder using an electric blender. The powdered samples were then extracted following standard procedures with slight modification (32). An amount of 5g of powdered material were soaked separately in 40 ml each of four different solvents namely distilled water, ethanol, methanol and acetone by keeping in a shaker for 3 days. The extracts were filtered with Whatman filter paper no.1 and reduced to 10 percent of their original volume by concentrating in vacuum using a rotary evaporator.

Inoculums: The test microorganisms namely, *Klebsiella pneumoniae* ATCC-13588, *Pseudomonas aeruginosa* ATCC-1037, *Proteus* vulgaris ATCC-128, *Staphylococcus aureus* ATCC-0016, *Bacillus subtilis* ATCC-9372 and *Escherichia coli* ATCC-0127, were obtained from the Department of Biotechnology, Assam University, Silchar. The organisms were inoculated in to Mueller Hinton broth and incubated at 37ºC overnight to bring them into their mid-logarithmic phases of growth. The bacterial cells were harvested by centrifuging at 500g for 15 minutes. The pellets formed were washed twice with phosphate buffer saline (PBS) and the cells were counted by a haemocytometer (33). The bacterial cells were then diluted at approximately 10^5 CFU (colony forming unit) per milliliter before use (33).

Determination of antibacterial activities: Determination of the antibacterial activity of the extracts was performed by Kirby-Bauer Agar Disc Diffusion method with slight procedural

modifications (34). The agar plates were prepared by pouring 15 ml of molten Mueller Hinton agar media into sterile petriplates. The plates were allowed to solidify for 5 minutes and the agar medium was inoculated with test microorganisms by pour plate method. Discs (5mm diameter) were punched in Whatman number 1 filter paper. The dried and sterilized disc was then impregnated with known amount of the plant extract (50mg/ disc). The loaded disc was placed on the surface of the medium and the compound was allowed to diffuse for five minutes. The plates were then kept for incubation at 37ºC for 24 hours. The antibacterial activity was assessed by measuring the diameter of zone of inhibition of the respective extracts with the help of a transparent ruler in millimeter (Table 1).

Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration of the aqueous extract was determined as described by Kabir *et al* (35). The test was performed by serially diluting the extracts to various concentrations ranging from 10 mg/l to 0.02 mg/l. Each volume of each extract and nutrient broth were mixed in a test tube and the inoculum size was adjusted so as to deliver a final inoculum of approximately 10^5 CFU per ml. Triplicates were maintained along with two control tubes for each test batch-tubes containing the growth medium, physiological saline and the inoculum (organism control) and tubes containing extract and the growth medium without inoculum (antibiotic control). The tubes were inoculated at 37°C for 24 hours. The lowest concentration of extract that produced no visible bacterial growth (no turbidity when compared with control tubes was regarded as MIC. Minimum inhibitory concentrations of standard antibiotics against the bacterial strains taken were also determined as positive control.

Results and Discussion

The profile of the plant used in this study is shown in Table-1. The Table-2 demonstrates the antibacterial activity of the plant extracts in different solvents. The study showed that all the bacterial strains used in the present study were highly susceptible to the test material. Ethanolic and methanolic extracts against *S. aureus* showed maximum activity against the microorganisms studied. The zones of inhibition of all the test materials against the gram positive bacteria ranged from 12 to 15 mm and that of the Gram negative bacteria ranged from 11 to 14 mm showing that Gram negative bacteria is marginally more resistant in agreement with previous reports (36-40).

Table-3 shows that the aqueous extract of the test material presented similar MIC's against *Pseudomonas aeruginosa* and *Proteus vulgaris* at a concentration of 1.25 mg/ml. *S. aureus* and *E.coli* were found to be inhibited at an MIC of 2.5 mg/l. Lowest MIC (0.625 mg/ml) was observed against *K. pneumoniae* and *B. subtilis.* However no significant trend was noticeable for the different solvents.

The present finding on the antibacterial activity of the present test material against different strains validates the traditional use of these species by the two tribes against infected wounds. Pertinent here is to mention that the traditional use of the plants by the tribes always involve aqueous extracts. The study showed that aqueous extracts also showed fairly good activity against the bacterial strains. Considering the inherent toxicity of the non aqueous solvents, the aqueous extract holds significant promise for useful phytochemicals. Infections caused by *P.aeruginosa* especially those with multi drug resistance are among the most difficult to treat with conventional drugs (39). In the present study, growth of *P.aeruginosa* was inhibited by the aqueous extract of the test material at a concentration as low as 1.25 mg/ml (Table 3).

Choudhury and Islam (40) worked on the antimicrobial efficacy of ethyl acetate, n-hexane and methanolic extracts of the root of *T. orientalis* and found that no extract was active against *Klebsiella sp*, *P. aeruginosa*, *B. subtilis* and *S. aureus* even at a dose of 500 mg/disc. Only methanolic extract showed significant activity against *E. coli*. Our present study on the aqueous

extract of bark of the same plant species, on the other hand is in contrast to the above report. Our test material showed significant activity against all the bacterial strains at a dose of 50 mg/disc giving an inhibition zone ranging from 11 to 14 mm against *K. pneumoniae*, 12 to 13 mm against *P. aeruginosa*, 12 to 14 mm against *B. subtilis*, and a the best zones ranging from 14 to 15 mm against *S. aureus* (Table-2). MIC of aqueous extract further revealed that the bacterial strains are inhibited at a concentration as low as 0.625 mg/ml (Table-3).

Conclusion

The results of the present study have shown that *T. orientalis* Blume is a potentially good source of antibacterial agent. Significant efficacy against the selected bacterial strains and the resultant MIC values of aqueous extract corroborates the traditional medicinal application of the plant species. The study area with its vast ethnobotanical wealth deserves extensive exploration of their potentials in the discovery of newer active principles which could lead to the development of newer and safer drugs.

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Table 2. Antibacterial activity of *Trema orientalis* Blume in different solvent extracts.

Species	Part used	Concentration	Solvent	Zone of Inhibition (mm)					
				Ec	Kp	Pa	Pv	Sa	Bs
				Gram (-)				Gram (+)	
T. orientalis Blume.	Bark	50mg/disc	D	11	12	13	12	14	12
			E	13	14	13	12	15	13
			M	12	12	12	14	15	14
			A	14	15	13	14	11	12

D - Distilled water; E - Ethanol; M - Methanol; A - Acetone.
Ec- *Escherichia coli*; Kp- *Klebsiella pneumoniae*; Pa- *Pseudomonas aeruginosa*;
Pv- *Proteus vulgaris*; Sa- *Staphylococcus aureus*; Bs- *Bacillus subtilis*.

Table 3. Minimum inhibitory concentration of aqueous extract of *Trema orientalis* Blume.

Bacterial strains	Minimum Inhibitory Concentration (mg/l)		
	Aqueous extract of *Trema orientalis* Blume	Antibiotics	
Klebsiella pneumoniae	0.625	Ciprofloxacin	0.21
Pseudomonas aeruginosa	1.25	Ciprofloxacin	0.12
Proteus vulgaris	1.25	Cefpodoxim	2.56
Staphylococcus aureus	2.5	Vancomycin	1.36
Bacillus subtilis	0.625	Ampicillin	0.007
Escherichia coli	2.5	Ciprofloxacin	0.008

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References

1. Dabur, R., Gupta, A., Mandal, T.K., Singh, D.D., Bajpai, V., Gurav, A.M. and Lavekar, G.S. (2007). Antimicrobial activity of some Indian medicinal plants. African Journal of Traditional Complementary and Alternative Medicine, 4(3):313-318.

2. Hart, C.A. and Karriuri, S. (1998). Antimicrobial resistance in developing countries. British Medical Journal, 317:421-452.

3. Mahesh, B. and Satish, S. (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. World Journal of Agricultural Science, 4 (S): 839-843.

4. Gerhartz, W., Yamamota, Y.S., Campbell, F.T., Pfefferkorn, R. and Rounsaville, J.F. (1985). Ullmann's Encyclopedia of Industrial.

5. Kroschwitz, J.I. and Howe-Grant, M. (1992). Kirk- Othmer encyclopedia of chemical Technology, 2: 893.

6. Newman, D.J., Cragg, G.M. and Snader, K.M. (2000). The influence of natural products upon drug discovery. Natural Product Research, 17:215-234.

7. Tomoko, N., Takashi, A., Hiromu, T., Yuka, I., Hiroko, M., Munekazu, I., Totshiyuki, T., Tetsuro, I., Fujio, A., Iriya, I., Tsutomu, N. and Kazuhito W. (2002). Antibacte-rial activity of extracts preparated from tropical and subtropical plants on methicillin-resistant Staphylo-coccus aureus. Journal of Health Science, 48: 273–276.

8. McCutcheon, A.R., Ellis, S.M., Hancock, R.E.W. and Towers, G.H.N. (1992). Antibiotic screening of medicinal plants of the British Columbian native peoples. Journal of Ethnopharmacology, 37:213-223.

9. Nascimento, G.G.F., Locatelli, J., Freitas, P.C. and Silva, G.L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Brazilian Journal of Microbiology, 31: 247- 256.

10. Inouye, S. and Yamaguchi, H. (2001). Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. Journal of Antimicrobial Chemotherapy, 47: 565-73.

11. Islam, M.J., Barua, S., Das, S., Khan, M.S. and Ahmed, A. (2008). Antibacterial activity of some indigenous medicinal plants. Journal of Soil and Nature, 2(3):26-28.

12. Karim, A., Nouman Sohail, M., Munir, S, and Sattar, S. (2011). Pharmacology and Phytochemistry of Pakistani Herbs and Herbal Drugs Used for Treatment of Diabetes. International Journal of Pharmacology, 7 (4): 419-439.

13. Aswal, B. S., Goel, A. K. and Patnaik, G .K. (1996). Screening of Indian medicinal plants for biological activity. Indian Journal of Experimental Biology, 34: 444 – 467.

14. Bhaskarwar, B., Itankar, P. and Fulke, A. (2008). Evaluation of antimicrobial activity of medicinal plant Jatropha podagrica (Hook). Roumanian Botanical Letters, 13(5):3873-3877.

15. Ramya, S., Jepachanderamohan, P.J., Alaguchamy, N., Kalayanasundaram, M. and Jayakumararaj, R. (2009). In vitro antibacterial prospective of crude leaf extracts of Melia azedarach Linn. against selected bacterial strains. Ethnobotanical Leaflets, 13:254-258.

16. Alagesaboopathi, C. (2011). Antimicrobial screening of selected medicinal plants in

Tamilnadu, India. African Journal of Microbiology Research, 5(6): 617-621.

17. Jalajakumari Mithrajaa, M., Irudayarajb, V., Kirubac, S., Jeevad, S. (2012). Antibacterial efficacy of Drynaria quercifolia (L.) J. Smith (Polypodiaceae) against clinically isolated urinary tract pathogens. Asian Pacific Journal of Tropical Biomedicine, 2(1): S131–S135.

18. Vijayarathna, S., Zakaria, Z., Chen, Y., Yoga Latha, L., Kanwar, J.R and Sasidharan, S. (2012). The Antimicrobial Efficacy of Elaeis guineensis: Characterization, in Vitro and in Vivo Studies. Molecules, 17: 4860-4877.

19. Savitharamana, N. Ankanna, S., Linga Rao, N, and Saradvathi, J. (2012). Studies on antimicrobial efficacy of medicinal tuberous shrub Talinum cuneifolium. Journal of Environmental Biology, 33:775-780.

20. Dutta, B.K. and Dutta, P.K. (2005). Potentials of ethnobotanical studies in North East India : An overview. Indian Journal of Traditional Knowledge, 4:7-14.

21. Tamuli, P. and Saikia, R. (2004). Ethno-medico-botany of the Zeme tribe of North Cachar Hills District of Assam. Indian Journal of Traditional Knowledge, 3:430-436.

22. Sajem, A.L. and Gosai, K. (2006). Traditional use of medicinal plants by the Jaintia tribes in North Cachar Hills district of Assam, northeast India. Journal of Ethnobiology and Ethnomedicine, 2:33.

23. Sajem, A.L., Rout, J. and Nath, M. (2008). Traditional tribal knowledge and status of some rare and endemic medicinal plants of North Cachar Hills district of Assam, Northeast India. Ethnobotanical Leaflets, 12: 261-275.

24. Rout, J., Sajem, A.L. and Nath, M. (2010). Traditional medicinal knowledge of the Zeme (Naga) tribe of North Cachar Hills district, Assam on the treatment of diarrhoea. Assam University Journal of Science and Technology. 5 (1):63-69.

25. Rout, J., Sajem, A.L. and Nath, M. (2010) M. Traditional Medicinal Knowledge of the Dimasa tribe of North Cachar Hills district of Assam (North East India). In Indian Journal of Traditional Knowledge, 11(3):520-527.

26. Rout, J., Sajem, A.L. and Nath, M. (2011) Traditional Medicinal Knowledge of the Zeme tribe of North Cachar Hills District of Assam, India. In Status and Conservation of Biodiversity in North East India (Ed Choudhury M.D et al). Swastik Publications, New Delhi, 417-440.

27. Hooker, J.D. (1989). The Flora of British India. Volume 1-7. L.Reeve & Co. London.

28. Kanjilal, U.N., Kanjilal, P.C. and Das, A. (1982). Flora of Assam,Vol I and II. Avon Book Company, Delhi-6.

29. Kanjilal, U.N., Kanjilal, P.C., De, R.N. and Das, A. (1982). Flora of Assam, Vol III&IV. Avon Book Company, Delhi-6.

30. Jain, S.K. and Rao, R.R. (1977). A handbook of field and herbarium methods. Today and tomorrow's printers and publishers. New Delhi.

31. Alexiades, M.N. (1996). Collecting ethnobotanical data: an introduction to basic concept and techniques. In Alexiades, M.N. (Ed.), Selected guidelines for Ethnobotanical Research: A Field Manuel. The New York Botanical Garden, New York, 53-94.

32. Essawi, T. and Srour, M. (2000). Screening of some Palestinian medicinal plants for antibacterial activity. Journal of Ethnopharmacology,70:343-349.

33. Owais, M., Sharad, K., Shehbaz, A. and Saleemuddin, M. (2005). Antibacterial

efficacy of Withania somnifera, an indigenous medicinal plant against experimental marine Salmonellosis. Journal of Phytomedicine, 12:229-235.

34. Perez, C., Pauli, M. and Bazevque, P. (1990). An antibiotic assay by agar well diffusion method. Acta Biologiae Medicine Experimentalis, 15:113-115.

35. Kabir, O.A., Olukayode, O., Chidi, E.O., Cristopher, C. and Fasure, K.A. (2005). Screening of crude extracts of six medicinal plants used in south-West Nigerian unorthodox medicine for anti-methicillin resistant Staphylococcus aureus activity. African Journal of Traditional, Complementary and Alternative Medicines, 5:6.

36. Valsaraj, R., Pushpangadan, P., Smitt, U.W., Andersen, A. and Nyman, U. (1997). Antimicrobial screening of selected medicinal plants from India. Journal of Ethnopharmacology, 58:75-83.

37. Perumalsamy, R., Ignacimuthu, S. and Raja, D.P. (1999). Preliminary screening of ethnomedicinal plants from India. Journal of Ethnopharmacology 66:235-240.

38. Srinivasan, D., Nathan, S., Suresh, T. and Lakshmanaperumalsamy, P. (2001). Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of Ethnopharmacology, 74:217-220.

39. CDC NNIS System. (1999). Natural Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1990-May 1999. American Journal of Infection Control, 27:520-532.

40. Choudhury, A.A and Islam, M.S. (2004). Antimicrobial activity of Trema orientalis The Dhaka University Journal of Pharmaceutical Sciences,Vol 3(1-2).

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A Comparative analysis of Long PCR and Standard PCR technique in detecting the *Wolbachia* Endosymbiont

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Abstract

Allele specific polymerase chain reaction (Standard PCR) is being widely used to amplify *Wolbachia* DNA from arthropods. Preliminary tests with Wolbachia allele-specific polymerase chain reaction suggested that the assays were prone to false negative results in insects. Standard PCR frequently produced false negative results, perhaps due to low titer *Wolbachia* DNA mixed with host genomic DNA. On the other hand, another PCR protocol, Long PCR which uses proof reading enzyme consistently amplified *Wolbachia* DNA and revealed that 49% of 35 arthropod species tested positive for *Wolbachia* which is considerably higher than the rate of 31% obtained in Standard PCR. An attempt has been made in this study to compare both Standard and Long PCR and to evaluate Long PCR as a technique for amplifying *Wolbachia* DNA from a diverse array of arthropods.

Key Words: Polymerase Chain Reaction (PCR), Long PCR, Standard PCR, *Wolbachia*, Proof reading, Insects.

Introduction

Numerous invertebrate species form long lasting symbiosis with bacteria (1). One of the most common of these bacterial symbionts is *Wolbachia pipientis* which has been estimated to infect anywhere from 15-70% of all insect species (2-3). *Wolbachia* have an impressive host range. Infections have been detected in all major orders of insects, arachnids, terrestrial crustaceans and filarial nematodes (4-5). This extreme diversity of hosts makes *Wolbachia* one of the most ubiquitous intracellular symbiont yet described. In most arthropod associations, *Wolbachia* act as reproductive parasites manipulating the reproduction of their host to enhance their own vertical transmission. There appears to be little direct fitness cost to the infected host besides the cost arising from the reproductive manipulations. However instances have been reported where *Wolbachia* can be either deleterious (6-7) or beneficial to their hosts (8-12).

Endosymbiotic bacteria of the genus *Wolbachia* are wide spread in insect species. The success of *Wolbachia* is best explained by the variety of phenotypes they induce, which ranges from mutualism in nematodes to various reproductive alterations in arthropods, such as cytoplasmic incompatibility (13), parthenogenesis (10), feminization of genetic males (7), male killing (14), and its obligations for the oogenesis of certain insect species (15). The molecular targets used by *Wolbachia* and the mechanisms involved in their effects are not known, and the absence of any correlation between *Wolbachia* phylogeny and effects they induce in hosts have led several authors to speculate on the evolution of *Wolbachia* induced phenotypes (16-17).

Previous work have indicated that the Standard PCR produced a high frequency of false positive detection of *Wolbachia* endosymbiont in arthropods (18). The Long PCR protocol has been shown to more consistently amplify DNA than Standard PCR because two DNA polymerases (*Taq* and *Pwo*) are integrated, one of which has proof-reading activity (19). Long PCR improves the probability of detecting *Wolbachia* DNA when mixed with host insect DNA, a condition comparable to the situation in which plant, psyllid, or parasitoid DNA would be mixed with greening DNA. They found that the Long PCR protocol was approximately six orders of magnitude more sensitive than Standard PCR when amplifying *Wolbachia* DNA (18).

Wolbachia cannot be cultured in defined media and detection within infected gonad cells may be time consuming. Therefore detection of *Wolbachia* infection has been based largely by amplification of *Wolbachia* DNA using allele specific polymerase chain reaction (PCR) using standard techniques. The failure of standard PCR analysis prompted the need to evaluate a different PCR based procedure called Long PCR. The Long PCR is a technique for amplifying *Wolbachia* DNA from a diverse array of arthropods **(**18**)**. Present analysis is also used to compare the relative efficiency of the Long PCR and Standard PCR protocols.

Materials and Methods

***Insects collection and preservation*:** Insect pests collected from various regions of country were frozen at –80ºc (Table 1) until further use for DNA isolation and subsequent screening for *Wolbachia* allele specific polymerase chain reaction.

***DNA Isolation*:** The DNA from a minimum of an individual insect was extracted following the standard phenol: chloroform: isoamyl alcohol (24:24:1) extraction and purification method (20).The isolated genomic DNA were subjected to RNAase-A treatment and quantified on 0.8% agarose gel.

***Wolbachia diagnosis in Standard PCR*:** A PCR assay based on the amplification of the published *Wolbachia* specific sequence primers, were used to detect A and B supergroup *Wolbachia* in individual insect and pests. The *ftsZ* sequence of the A supergroup is *ftsZ Adf* 5'-CTC AAG CAC TAG AAA AGT CG-3'; *ftsZ Adr*5'-TTA GCT CCT TCG CTT ACC TG-3'; and *ftsZ* sequence of the B supergroup is *ftsZ Bf* 5'-CCG ATG CTC AAG CGT TAG AG-3'; *ftsZ Br* 5'-CCA CTT AAC TCT TTC GTT TG-3'. These primers were designed for the detection of *Wolbachia* which amplifies the gene from 940 bp. Standard PCR was carried out with PTC 200 of MJ Research Thermocycler in reaction mixture containing 2.5ìl of 10X PCR buffer, 0.5ìl of dNTP's (10mM each), 2.5 ìl of 25mM $MgCl_2$ and 0.5 Units of *Taq DNA* polymerase, 1ìl of 26 ìM forward primer and 1ìl of 35 ìM reverse primer, 30 ng template DNA, millipore water was added to a final volume. PCR was carried out with a cyclic condition of initial denaturation step at 94ºC for 5 minutes (min) followed by 35 cycles with denaturation step at 95ºC for 1 min, primer annealing at 59ºC for 1min and primer extension in the presence of *Taq DNA* polymerase at 72ºC for 1min and final extension at 72ºC for 10 mins for both the primers.

Wolbachia diagnosis in Long PCR: The Long PCR was carried out using three linked profiles over 35 cycles; (1) 1cycle of denaturation at 94^0 C for 2 mins, (2) 10 cycles each consisting of denaturation at 94^0 C for 10 s, annealing at 59^0 C for 30 s and extension at 68^0 C for 1 min, and (3) 25 cycles each consisting of denaturation at 94^0C for 10 s, annealing at 59^0 C for 30 s and extension at 68^0 C for 1 min, plus an additional 20 s added for every consecutive cycle between 11 and 36 for both the primers. The amplified PCR products were separated on a 1.5% agarose 1X TBE gel stained with 0.5ìg/ml of Ethidium bromide. Documentation was done with the gel documentation system.

Results and Discussion

Comparing the sensitivity of Standard PCR and Long PCR protocols in detection of

Table 1. Wolbachia infection status in insects and insect pests of sericulture based on ftsZ A and B gene primer.

Sl. No.	Name of the insects and pests of sericulture and their family	Amplification of fts Z-A primer		Amplification of fts Z-B primer	
		Std PCR results	Long PCR results	Std PCR results	Long PCR results
1	*Exorista sorbillans* , Wiedemann,(Tachinidae),(Laboratory population)	+	+	+	+
2	*Exorista sorbillans,* Wiedemann,(Tachinidae)(Bangalore population)	+	+	+	+
3	*Exorista sorbillans,* Wiedemann,(Tachinidae)(Tumkur population)	+	+	+	+
4	*Exorista sorbillans,* Wiedemann,(Tachinidae)(Mandya population)	+	+	+	+
5	*Exorista sorbillans,* Wiedemann, (Tachinidae)(Kolar population)	+	+	+	+
6	*Exorista sorbillans,* Wiedemann, (Tachinidae)(Kollegal population)	+	+	+	+
7	*Exorista sorbillans*, Wiedemann, (Tachinidae)(Tamilnadu population)	+	+	+	+
8	*Exorista sorbillans,* Wiedemann, (Tachinidae)(Andhra population)	+	+	+	+
9	*Bombyx mori* Linnaeus, (Bombycidae)(Nistari)	-	-	-	-
10	*Maconellicoccus hirsutus.* Green (Pseudococcidae)	-	+	-	+
11	*Bombyx mori* Linnaeus, (Bombycidae)(Mysore princes)	-	-	-	-
12	*Bombyx mori* Linnaeus, (Bombycidae)(Tamilnadu white)	-	-	-	-
13	*Bombyx mori* Linnaeus, (Bombycidae)(Pure mysore)	-	-	-	-
14	*Diacrisia oblique,* Walker, (Arctidae)	-	-	-	-
15	*Margaronia pulverulentalis,* Hampson, ((pyrallidae)	-	-	-	-
16	*Diaphania pyloalis* Walker (Pyraustidae)	-	+	-	+
17	*Myllocerus discolor* Boheman, (Curculionidae)	-	-	-	-
18	*Baris deplanata* Roelofs (Curculionidae)	-	+	-	+
19	*Dermestid ater, (* Dermestidae)	-	-	-	-
20	*Nesolynx thymus* Girault, (Eulophidae)	-	+	-	+
21	*Osmia lignaria propinqua* Cresson, (Megachilidae)	-	-	-	-
22	*Adalia decempunctata* Linnaeus, (Coccinellidae)	-	-	-	-
23	*Empoasca Spp.* Walsh, (Jassidae)	-	-	-	-
24	*Sitophilus oryzae*	-	-	-	-
25	*Saissetia nigra* Nietm, (Coccidae)	-	-	-	-
26	*Pluvinaria maxima,* Green, (Coccidae)	+	+	+	+
27	*Udonga montane, Distant, (* Pentatomidae)	+	+	+	+
28	*Empoasca Spp.* Walsh, (Jassidae)	+	+	+	+
29	*Neorthacris acuticeps nilgiriensis* Uvarov, (Acrididae)	-	-	-	-
30	*Acanthacris spp.,(* Acrididae)	-	-	-	-
31	*Tetigonia viridissima, Savignone(* Tettigoniidae)	-	-	-	-
32	*Aleyrodicus disperses, Russel (* Aleyrodidae)	-	+	-	+
33	*Hierodulla spp.,(* Mantidae)	-	-	-	-
34	*Neoperla spp, (* Perlidae)	+	+	+	+
35	*Tetranychus Spp, (* Tetranichidae)	+	+	+	+
36	*Lasioderma sericorne*	-	-	-	-
37	*Ephestia cautella*	-	-	-	-
38	*Tribolium confusum*	-	-	-	-

Comparative analysis of Long and Standard PCR

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Wolbachia: Standard and Long PCR were used to amplify *Wolbachia* DNA from the insects associated with sericulture (Table 1). The Standard PCR sometime produced false negatives. However, Long PCR assays based on specific amplification of the *ftsZ-A and B* super group *Wolbachia* gene fragments, showed that 49% of the insects were positive for a *Wolbachia* infection (Fig. 2). In contrast, 31% of the insects were positive for *Wolbachia* infection when Standard PCR was used (Fig. 1).

Although standard allele specific PCR has been widely accepted as an efficient method for amplifying *Wolbachia* DNA, very little information is available regarding the frequency of false negatives and false positive results. Researchers compared Standard PCR and cross-breeding tests with *Wolbachia*-infected and -uninfected *D. simulans* lines. They were able to detect Standard PCR positive individuals using 16S rDNA primers from infected lines identified by the cross-breeding test (21). When attempts were made to amplify *Wolbachia* DNA by Standard PCR from single individual of *T. urticae, M. occidentalis* and *A. suspense* known to be infected with *Wolbachia* (22), few produced PCR products, suggesting that false negatives were obtained frequently (23).

On the contrary, the Long PCR procedure consistently amplified *Wolbachia* DNA from insect DNA even when there were as few as 100 copies of the plasmid DNA present. The differences in the two PCR protocols could be the reason for the increased sensitivity. Long PCR uses long primers (30-mers recommended minimum); template denaturation for only 10 s; a buffer with higher pH (9.2), a slightly higher Mg2+ ion concentration (1.75 mM), higher concentrations of dNTPs (350 mM each), a mixture of two different DNA polymerases at a higher concentration (5 units of *Taq* and 1 of *Pwo*) in a 50ml reaction volume, lower annealing (65 °C) and extension (68 °C) temperatures, and a linked PCR profile for thirty-five cycles with each of the last twenty-five cycles having an additional 20 s added to each extension segment (19).

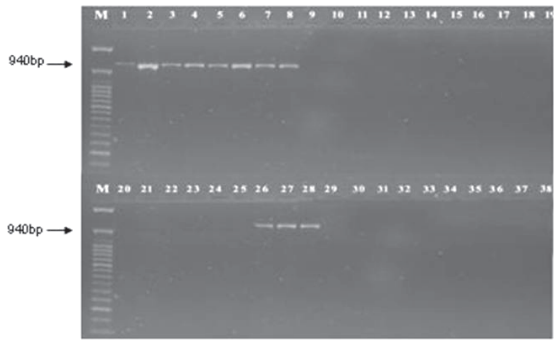

Fig. 1. Standard PCR product amplified from the 35 insects of sericulture Using ftsZ –B sequences. (Lane M-50bp ladder,Lane1 to 35-insects of sericulture).

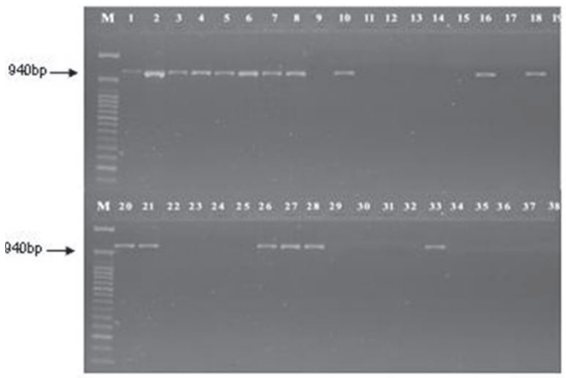

Fig. 2. Long PCR successfully amplified ftsZ-B sequences from the same insects of sericulture used for Standard PCR. (Lane M-50bp ladder,Lane1 to 35-insects of sericulture) .

Factors affecting the failure of Standard PCR: The following some of the factors could be responsible for the failure of Standard PCR to amplify *Wolbachia* DNA consistently. It is likely that relatively small amounts of *Wolbachia* DNA are mixed with large amounts of arthropod genomic DNA, which could result in a non optimal primer and DNA template ratio. Further prolonged denaturation could cause breakage of the DNA template and depurination of bases, which could stop the extension by *Taq* (24). Low buffer pH (8) also can enhance depurination (25). Compared to other DNA polymerases such as T4, *Taq* is more error-prone. Introducing one base-pair substitution for every 9000 bases amplified. In contrast, T4 DNA polymerase

introduces one substitution for every 160000 bases (26). *Taq* has terminal deoxynucleotidy ltransferase activity and adds the nucleotide 'A' to the 3' end of the amplified DNA (27). If incompletely synthesized DNA strands containing mismatches or with added A' s at the 3' end can accumulate during early PCR cycles. These incomplete products (also called as 'mega primers') could interfere with the annealing of the primers to the DNA template during subsequent PCR cycles. Additionally, *Taq* completely lacks any DNA editing ability and cannot correct mismatches or remove the added A from mega primers (28). The mega primers also could sequester and deplete the Mg2+ ions from the reaction buffer. The long PCR procedure utilizes both *Taq* and a thermostable DNA polymerase (e.g. *Pfu, Vent or Deep Vent*) which exhibits a 3'- to 5'-exonuclease activity (19). The *Pwo DNA* polymerase from *Pyrococcus woesei* shares 100% DNA sequence identity with *Pfu* from *P. furiosus* and exhibits similar exonuclease activity (29). The successful amplification by the long PCR may be due to the exonuclease activity of the *Pwo* polymerase. Evidence for this was provided by Barnes (19). When he showed successful amplification of the 1500 bp *CryV* insecticidal protein gene sequence from *Bacillus thuringiensis* by long PCR using a 384 bp mega primer with an added A on the 3' end and a 43-mer primer. The sequence obtained indicated that the added A was indeed removed from the mega primer by *Pfu.* All the above results indicate that long PCR could detect *Wolbachia* infections within individuals of arthropod population.

Conclusion

From the results we can conclude that only long PCR which includes a proof reading enzyme to correct errors in copying the DNA and gives consistent results. These results shows that the long PCR is not only more sensitive than the standard PCR but also provide a wealth of information to exploit this endobacterium for the management of insect pests and vectors of agriculture, veterinary, medical and also to explore the possibility of *Wolbachia* as a vector to transfer the gene of interest. Long PCR might be used to confirm that antibiotic or heat-treated individuals are actually free of *Wolbachia*. Detection of *Wolbachia* from so many arthropod species by Long PCR also suggests that some infections with *Wolbachia* might be ancient, involving mutualistic relationships with the host.

Acknowledgements

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References

1. Buchner, P. (1965). Endosymbiosis of animals with plant microorganisms (Interscience. NewYork).
2. Werren, J. H., Windsor, D. and Gao, L. (1995). Distribution of *Wolbachia* among neotropical arthropods. Proc R Soc Lond B, 262: 197-204.
3. Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. and Werren, J.H. (2008). How many species are infected with *Wolbachia*?- a statistical analysis of current data. FEMS Microbiol. Lett. 281: 215-220.
4 O'Neill, S. L., Hoffmann, A. A. and Werren, J. H. (1997). Influential Passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford University Press. UK.
5. Bandi, C., Anderson, T. J. C., Genchi, C. and Blaxter, M. L. (1998). Phylogeny of *Wolbachia* in filarial nematodes, Proc R Soc Lond B, 265: 2407-2413.
6. Min, K. T. and Benzer, S. (1997). *Wolbachia*, normally a symbiont of Drosophila, can be virulent, causing degeneration and early death. Proc Natl Acad Sci USA, 94: 1792-1796.
7. Bouchon, D., Rigaud, T. and Juchault, T. (1998). Evidence for widespread *Wolbachia* Infection in isopod crustaceans: molecular

identification and host feminization. Proc R Soc Lond B, 265: 1081-1090.

8. Girin, C. and Bouletreau, M. (1995). Microorganism- associated variation in host infestation efficiency in a parasitoid wasp *Trichogramma bourarachae*. Experientia, 52: 398–402.

9. Stolk, C. and Stouthamer, R. (1995). Influence of a cytoplasmic-incompatibility inducing *Wolbachia* on the fitness of the parasitoid wasp *Nasonia vitripennis*. Proc Sect Exp Appl Entomol Net. Entomol Soc*,* 7: 33–37.

10. Stouthamer, R., Luck, R. F. and Hamilton, W. D. (1990). Antibiotics cause parthenogenetic Trichogramma (Hymenoptera/ Trichogrammatidae) to revert to sex. Proc Natl Acad Sci USA, 87: 2424–2427.

11. Stouthamer, R. and Kazmer, D.J. (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. Heridity. 73: 317-327.

12. Guruprasad N.M., Mouton L. and Puttaraju H.P. (2011b). Effect of antibiotic, temperature curing of *Wolbachia* and seasonal variation on the reproductive fitness of the Uzifly *Exorista sorbillans* (Diptera: Tachinidae). *Symbiosis.* 54, 151-158.

13. O'Neill, S. L. and Karr, T. L. (1990). Bidirectional incompatibility between conspecific Populations of *Drosophila simulns.* Nature*,* 348: 178-180.

14. Jiggins, F. M., Hurst, G. D., Schulenurg, J. H. and Majerus, M. E. (2001). Two male killing *Wolbachia* strains coexist within apopulation of the butterfly *Acraea encedon*. Heridity, 86: 161-166.

15. Dedeine, F., Vavre, F., Fleury, F., Loppin, B. and Hochberg, M. E. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a Parasitic wasp. Proc. Natl Acad Sci USA, 98: 6247-6252.

16. Stouthamer, R., Breeuwer, J. A. and Hurst, G. D. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu Rev Microbiol, 53: 71–102.

17. Werren, J.H., Baldo, and Clark, M.E. (2008). *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6: 741-751.

18. Jeyaprakash, A. and Hoy, M. A. (2000). Long PCR improves *Wolbachia* DNA Amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol Biol, 9: 393- 405.

19. Barnes, W. (1994). PCR amplification of up to 35-kb DNA with high fidelity and high yield from ë bacteriophage templates**.** Proc Natl Acad Sci USA, 91: 2216–2220.

20. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

21. Turelli, M. and Hoffmann, A. A. (1995). Cytoplasmic incompatibility in *Drosophila simulans* Dynamics and parameter estimates from natural populations. Genetic*,* 140: 1319–1338.

22. Johanowicz, D. L. and Hoy, M. A. (2001). *Wolbachia* in a predator-prey system: 16S ribosomal DNA analysis of two phyotoseiids (Acari: Phytoseiidae) and their prey (Acari: Tetranychidae*).* Anul Entomol Soc America, 89: 435–441.

23. Guruprasad, N.M., Sumithra, Mouton, L. and Puttaraju, H.P. (2012). Long PCR: a sensitive PCR protocol for amplification of *Wolbachia* endosymbiont in Indian Honey Bees**.** Journal of Entomological Research**.** 36 (2): *119-122*

24. Chu, G., Vollrath, D. and Davis, R. W. (1986). Separation of large DNA-molecules by contour clamped homogeneous electric-fields**.** Science, 234: 1582–1585.

25. Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature, 362: 709- 715.

26. Kunkel, T. A. and Eckert, K. A. (1989). Fidelity of DNA polymerases used in polymerase chain reactions. Polymerase Chain Reaction (Erlich, H.A., Gibbs, R. and Kazazian, H.H., eds),. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York, USA, pp. 5–10.

27. Clark, J. M. (1988). Novel non-templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases**.** Nucl Acids Res, 16: 9677–9686.

28. Tindall, K. R. and Kunkel, T. A. (1988). The fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase**.** Biochemistry, 27: 6008–6013.

29. Dabrowski, S. and Kur, J. (1998). Cloning and expression in *Escherichia coli* of the recombinant His-tagged DNA polymerases from *Pyrococcus furiosus* and *Pyrococcus woesei***.** Prot Expres Purific, 14: 131–138.

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Standardization of an ELISPOT protocol for the evaluation of Human papillomavirus 16 (HPV16) E7 specific T cell response in mice immunized with the E7 recombinant *Salmonella typhi* strain Ty21a (*S.typhi* Ty21a)

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Abstract

The study aimed to develop a standard ELISPOT protocol for the evaluation of T cell responses in mice to HPV 16 E7. C57BL/6 female mice had been immunized with the HPV 16 E7 recombinant *S. typhi* Ty21a, a candidate therapeutic vaccine for HPV associated cancer. A commercial ready-to-use (RTU) IFNγ ELISPOT kit (supplied with pre-standardized capture and detection antibodies) was chosen; hence the effort in this study lay on the standardization of non-kit components (appropriate splenocyte and antigen concentration). The optimum splenocyte concentration determine by the assay using normal, non-immunized, mice splenocytes (stimulated with con A) was 0.5 million cells/well/100µl (373.3± 30.6 SFCs/million). Affinity purified recombinant protein (GST-E7) was chosen for stimulation of splenocytes. Despite using an RTU kit, initial assays were plagued with high background that hindered interpretation of data. Apparently, the heterogeneous immunogen (recombinant bacteria), the choice of heterogeneous antigen for stimulation (recombinant protein) and most importantly, the enzyme-conjugate/chromogenic substrate combination influenced the background. Streptavidin-ALP and its substrate (BCIP/NBT) but not the kit provided, Avidin–HRP and its substrate (TMB) showed vivid spots, reduced background and improved readability on development of the assay. Changing the enzyme-substrate combination enabled determination of the appropriate concentration (1.0µg/well) of GST-E7 for antigenic stimulation of splenocytes (290.67±8.1 SFCs/million cells). The ALP enzyme/BCIP NBT substrate seems a more ideal developing reagent for ELISPOT assays involving heterogeneous immunogens (*S. typhi* Ty21a) or/and stimulating antigens (recombinant whole proteins). The protocol with optimized splenocyte and recombinant antigen concentrations is of acceptable precision as determined in the intra assay %CV ranging from (7.3 to 14.1) and inter assay % CV of nearly 13%.

Keywords: RTU ELISPOT kit, enzyme-conjugate and substrate combination, Recombinant protein, SFCs..

Introduction

Human papilloma virus (HPV) infection in women can lead to the cancer of the reproductive tract mainly the uterine cervix (1). The HPV16 genotype is associated with nearly 70% of the cervical cancer cases (2, 3). A salient feature of the HPV infected epithelial cells, precancerous cells and tumorigenic cells is the constitutive expression of the viral oncoproteins E6 and E7 (4). Hence both E6 and E7 are candidate target

antigens for therapeutic vaccines (5). In this backdrop, the recombinant *S. Typhi* Ty21a expressing HPV16 E7 was constructed as an intended oral therapeutic vaccine against HPV16 associated cancers.

The live-attenuated *Salmonella enterica* serovar *Typhi* strain Ty21a has been widely studied for efficacious oral delivery of heterologous antigens (6, 7, 8). Serovar *Typhi* is extremely species specific infecting only the human gut. It can however establish a transient infection in the nasal passage of mice. Intra nasal immunization of mice with *Typhi* strain Ty21a is an established animal model for studying immune response to foreign antigens expressed in the bacteria (9). Our aim was to develop a standard ELISPOT protocol to evaluate the HPV16 E7 specific T cell response in mice intra nasally immunized with HPV16 E7 recombinant Ty21a-the candidate therapeutic vaccine against HPV16 associated cancers.

ELISPOT was originally developed to quantify antigen specific B cells (10). But the modified version to quantify the IFNγ secreting antigen specific T cells has found wider application (11). ELISPOT is conceptualized on the antibody sandwich format of the enzyme linked immunosorbant assay (ELISA) and is based on antibody pairs for capture and detection (12, 13). The two assays however differ functionally; while ELISA reports the presence and concentration of physiologically relevant soluble substances (eg. antibodies, cytokines, chemokines) ELISPOT is a cell-based assay that quantifies cells secreting the soluble molecule. The property of antigen specific T cells to secrete IFNα on *ex vivo* stimulation with the respective antigen has been ingeniously adopted in ELISPOT assays to determine the T cell response. In recent years ELISPOT has become a mainstay in evaluating cell mediated immune (CMI) response to vaccines owing to its sensitivity, simplicity and reliability (13, 14, 15).

ELISPOT assays require detailed standardization procedures to arrive at a reproducible protocol for evaluating the T cell response in an immunogenicity study. The performance of the assay depends on the following standard components in the assay *viz.,* (a) The affinity of capture and detection antibodies, (b) The choice of enzyme conjugates, (c) chromogenic substrates and (d) the antibody binding surface (membranes) in the wells of the plate (12). The standardization procedures, especially the selection of the best capture and detection antibody pairs, are laborious and time consuming. The pre-standardized commercial ready- to-use (RTU) ELISPOT kits offer a good, often validated alternative (12).

ELISPOT is a highly sensitive assay and therefore are also susceptible to minor variations (16). Even when an RTU kit is used, considerable effort is expected to go in to the standardization of the non–kit assay components. Lymphocyte concentration per well, either the splenocytes or peripheral blood mono-nuclear cells (PBMCs), and the antigen concentration used for *ex vivo* stimulation of T cells influence readability of the assay. The effect of antigen concentration is more acute when recombinant whole proteins rather than synthetic peptides of high-purity are used for lymphocyte stimulations. The choice of either a protein or peptides depend on factors such as cost, availability, knowledge of specific T cell epitopes and the assay design. We chose the recombinant fusion protein, glutathione-S-transferylase – HPV16 E7 (GST-E7) expressed in *E.coli* BL21, for the stimulation of splenocytes so as to determine the comprehensive profile of E7 specific T cell response (all possible epitope specificity). Apart from optimizing the splenocyte concentration, the article details the standardization of the IFNγ Elispot assay with respect to the antigen concentration for splenocyte stimulation and the choice of enzyme-conjugate/substrate to arrive at a reproducible ELISPOT protocol.

Materials and Methods

Recombinant Salmonella typhi and Mice: The live-attenuated Salmonella *typhi* Ty21a was provided by Dr. Denise Nardelli-Haefliger, CHUV,

Laussane, Switzerland. Recombinant Ty21a expressing HPV16 E7 constitutively was generated by electroporation with pFS14nsd-E7. Mice experiment was conducted at the Laboratory Animal Research Services (LARS) facility, Reliance Life Sciences Pvt. Ltd, Mumbai. Four to six week old C57BL/6 strain female mice were intranasally immunized with the recombinant Ty21a. Non-immunized, control, female mice of the same strain were also housed at LARS until the completion of the immunization schedule.

Reagents for splenocyte isolation and ELISPOT assay: RPMI-1640 medium and sterile fetal bovine serum (FBS) was procured from Life Technologies Corp., USA. The sodium diatrizoate and polysaccharide mixture, Lymphorprep™ for density gradient centrifugation was obtained from Axis-Shield PoCo, Norway. The Red Blood Cell Lysing Buffer (RBC Lysis buffer) and Tween-20 were from Sigma-Aldrich Chemical Co. GST-E7 was purified by glutathione affinity chromatography using the Glutathione Sepharose 4B matrix from GE, USA from *E. coli* BL21. Streptavidin conjugated with alkaline phospatase (Streptavidin –ALP) and chromogen solutions -Tetramethylbenzidine (TMB) and bromo-4-chloro-3-indolyl phosphate *p* toludine salt and Nitroblue tetrazolium chloride (BCIP/NBT) were procured from Mabtech AB.

Isolation of Splenocytes: Isolation and separation of splenocytes were performed in aseptic conditions according to the standard techniques (17). Briefly, Splenocytes were extracted by gentle disruption of the spleens placed in a 70 µm Nylon mesh (Cell Strainer from Becton Dickinson Corporation, USA) with a sterile syringe plunger into Petri-dish containing RPMI-1640. The splenocyte suspension was centrifuged at 300g for 7 min. Red blood cells were removed by incubation in the RBC lysis buffer. Cells were duly washed with RPMI-1640 (centrifugation at 300g/7min), resuspended and subjected to density gradient centrifugation with Lymphoprep™ (density- 1.77g/ml; 1:2 v/v to the cell suspension). The lymphocyte enriched fuzzy layer at the junction of Lymphoprep™ and RPMI-1640 was retrieved, washed twice with RPMI-1640, resuspended in FBS containing 10% DMSO; aliquots transferred into cryo-vials and stored in liquid nitrogen until further use.

ELISPOT assay for determination of optimum splenocyte concentration: The ELISPOT assays were performed with the Mouse IFN gamma ELISPOT Ready-SET-Go!® kit from eBiosciences® according to the manufacturer's instructions. Briefly, the PVDF backed plate (Sterile 96 well MultiScreen HTS™ IP plate, Millipore), after brief ethanol activation was coated with IFNγ capture antibodies overnight. Plates were washed with sterile phosphate buffer saline (PBS, pH 7.4) and blocked with 10% FBS in RPMI-1640 (cRPMI-1640). Cryo vial containing splenocytes (from non-immunized mice) were thawed at 37ºC, 2min, enumerated and viable splenocytes of counts 0.25 X 10^6, 0.5 X 10^6 or 1.0 X 10^6 in 100µl cRPMI /well were seeded in triplicate wells (three wells per specified count). The T cell mitogen, Concanavallin A (Con A), 2µg/100µl/well was added for non-specific T cell stimulation and IFNγ secretion. During stimulation plate was incubated in the humidified CO_2 incubator (Hera Cell 240 from Heraeus) for 24h at 37ºC. Plates were then washed with PBS containing 0.05% Tween-20 (PBST). The rest of the assay steps - addition of IFNγ detection antibodies, washing and addition of enzyme conjugate, Streptavidin conjugated with horse radish peroxidase (Streptavidin-HRP; kit reagent) were carried out as per the instructions in the kit. After the final wash the wells were developed with filtered TMB for 2 min. Wells in the plate were then washed thoroughly with copious water, air dried and the spots were analyzed and enumerated in the automated ImmunoSpot® Series 5 UV Reader (Cellular Technology Limited (CTL), USA) using Immunocapture® Version 5.0 software tool according to the recommended, default, user-independent SmartCount® settings. The antigen specific IFNγ secreting T cells were presented as spot forming cells (SFCs).

ELISPOT assays for determination of optimum antigenic concentration: The assays were performed as above except for the changes mentioned. All wells were added 0.5 X 10^6 viable splenocytes from either immunized or non-immunized mice. Triplicate wells were added with GST-E7 at concentrations- 10μg, 5μg, 2μg, or 1μg per well for splenocyte stimulation. The assay plates were either developed with TMB or BCIP/ NBT substrate. For the latter, prior to development the wells were incubated with Strepatavidn-ALP at 1:1000 dilution in PBST plus 10% FBS at 100μl/well. Positive and media controls were maintained in all assays.

Assays for determination of precision: Elispot assays with viable immunized mice splenocytes at 0.5 X 10^6cells/well were performed in triplicates. Splenocytes were stimulated with GST-E7 at a concentration1μg/well. Wells were developed with the Streptavidn-ALP/ BCIP/NBT enzyme substrate combination. The assays were performed on three different days. Assays performed on day 1 and day 2 involved splenocytes from the same batch of immunized mice. Assay on day 3 was performed with splenocytes from a different batch of mice. The immunization procedure and immunogen were identical. The age, strain, sex and body weight of the animal were also similar. Assay positive and media controls were maintained in all assays.

Determination of the linear fit of the assay protocol: ELISPOT assay of triplicate wells containing splenocyte concentrations, *viz.,* 0.25 X 10^6, 0.5 X 10^6 or 1.0 X 10^6 cells/well were performed as before. Wells were developed with Streptavidin-ALP and BCIP/NBT enzyme conjugate/ substrate combination.

Statistical Analysis: The Microsoft excel spreadsheet was used for all statistical calculations. The precision of the assay protocol was analyzed with respect to intra assay repeatability as percentage of coefficient of variability (Standard error/Mean X 100) across the wells in the ELISPOT assay performed in a day (3 wells in a day). For determination of inter assay precision the percentage of coefficient of variability was analyzed in the Elispot assays performed over the three different days (day 1, day 2 and day 3. i. e, 3wells X 3days=across 9 wells). For determination of the linearity of the protocol the linear regression analysis of SFCs over a range splenocyte concentrations were performed using the Origin 8 software tool.

Results and Discussion

Optimum splenocyte concentration: Murine Elispot assays are generally performed in the range of 0. 25 million splenocytes to 1.0 million cells per well (16, 18). Significant, mostly personnel and equipment (Elispot reader etc.) introduced variations can creep in an Elispot procedure due the elaborate steps involved from splenectomy, isolation, enumeration and seeding of splenocytes to analysis of spots (18). It is deemed necessary therefore that the optimum cells per well for an Elispot procedure is standardized for each laboratory before embarking on a specific study (16. 18). Besides, it is found that the readability of ELISPOT is influenced by the cell numbers. Too many cells are known to cause over-crowding and give rise to un-resolved spots and too little cells tend to exaggerate variations across replications; both interfering with accurate enumeration and interpretation of data (16)

Generally, splenocytes from immunized mice are limited and are required for various immunological analyses. Standardization of certain components of the ELISPOT assay such as splenocytes per well can readily be carried out with non treatment samples. Titration of splenocytes from normal, non-immunized mice would not be limiting and are enough to arrive at the appropriate concentration (16). Fig. 1 illustrates the result of standardizing the optimum splenocyte concentration per well using normal mouse splenocytes followed by stimulation with con A. Plating splenocytes at 0.5million cells/ well returned, well resolved enumerable spots with the least variation (373.3 SFCs/million ± 30.6) while the splenocyte concentration of 1million cells/ well was overwhelming (TNTC=

too numerous to count) and beyond the resolution of the ELISPOT reader (~600 cells/ well is the upper limit). At 0.25 million per well the number of enumerable spots were low (102.7) and varied across triplicate wells (std. error, σ ± 54.6).

Optimum concentration of GST-E7 for stimulation: Synthetic oligo-peptides of ~95% purity are the preferred reagents for stimulation of T cells. The advantage of using oligopeptides is the less possibility of non-specific stimulation and shorter stimulation times than with the whole antigen (16). But prior knowledge of T cell epitopes is necessary if the limited peptides are used for stimulation. Besides it limits the strength of the assay in reporting only the T cell response specific to the epitopes. In the event of little knowledge on T cell epitopes, synthesizing the complete range of overlapping peptides encompassing the whole antigen substantially adds to the cost.

Recombinant proteins purified to near homogeneity are a practical and economical alternative to synthetic oligo-peptide pools (16, 22, 23). They naturally comprise the entire set of T cell epitopes in the antigen. The presentation of full-range of epitopes is likely to stimulate the entire repertoire of T cells specific to the antigen. Therefore, the T cell response deduced from an ELISPOT assay involving recombinant antigen is more comprehensive than that derived from oligo-peptides. However stimulation of T lymphocytes requires the presence of APCs- the activated macrophages and dendritic cells, and longer incubation times for processing and presentation of epitopes (16, 22). Another frequently encountered problem is the chance of non-specific stimulation of T cells that compromise the specificity of the assay (16).

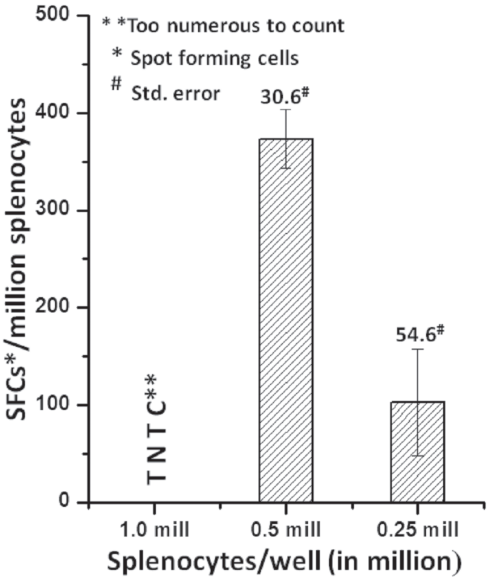

Fig. 1. Determination of optimal concentration of splenocytes. Elispot assay was performed in triplicate wells; Splenocytes were stimulated with 2.0µg/ well of Con A and developed with Avidin-HRP and TMB.

Considering the availability and the advantage of assaying the T cell response across all possible epitopes including the evaluation of CD4+ T_H1 response; we made the practical choice of using GST-E7 for stimulation of splenocytes from the recombinant Ty21 immunized mice. Splenocytes from both non-immunized and recombinant Ty21a immunized mice were stimulated with a range of GST-E7 concentration (10.0µg to 1.0µg per well) to find the appropriate concentration for use in the evaluation of T cell response. However, the assay performed with the mouse RTU ELISPOT kit according to the kit instructions at the standard splenocyte concentration (0.5 million cells/well) was inconclusive and showed enormous variation making data interpretation impossible (Fig.2). Even at the least concentration of GST-E7 (1.0µg per well) the deviations among triplicates were too high (σ ±182.2); besides a much higher number of SFCs were recorded than in wells where splenocytes were stimulated with higher GST-E7 concentration (2 and 5µg). The coefficient of variability (% CV), of 101.0 depicts the zero agreement in the data. High background staining in the wells of immunized splenocytes were seen in the scanned pictures of wells. However the corresponding picture in the non-immunized mice splenocytes showed neither appreciable number of spots (SFCs/million cells=

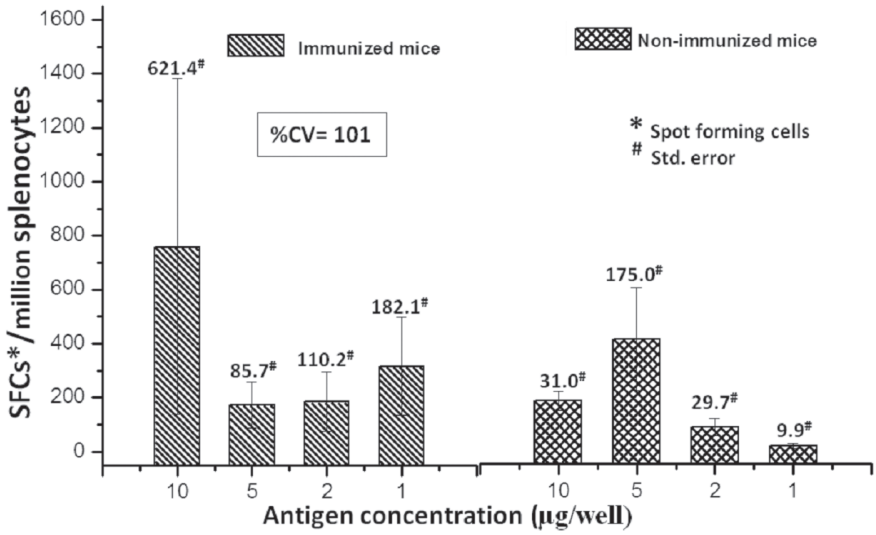

Fig. 2. Determination of optimal antigen (GST-E7) for stimulation of splenocytes. Splenocytes (from immunized or non-immunized mice) were plated at 0.5 million cells/ well; Plates were developed using Streptavidin-HRP and TMB.

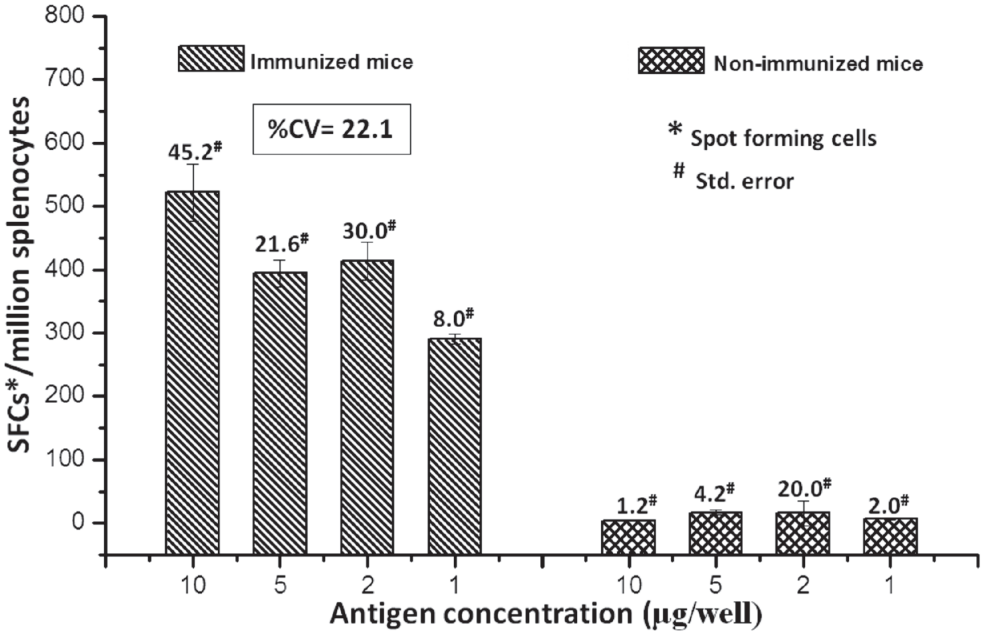

Fig. 3. Determination of optimal antigen (GST-E7) for stimulation of splenocytes. Splenocytes (from immunized or non-immunized mice) were plated at 0.5 million cells/ well; Plates were developed using Streptavidin-ALP and BCIP/NBT.

10.67) nor a high background which meant that the high background is not due to non-specific stimulation of splenocytes by GST-E7. On close observation of the scanned ELISPOT well pictures of immunized mice splenocytes (data not shown) it was apparent that using the least concentration of GST-E7 (1.0µg/well) showed relatively better resolution of spots. However the readability was still compromised in at least one well in which spot counts failed validation in the ELISPOT reader. These results were significant pointers to conclude that -

- The high background in the wells are not due to non-specific development of spots (Since a similar picture was not seen in wells of non-immunized splenocytes).
- A high portion of spots developed due to cross-stimulation of splenocytes by the residual *E.coli* proteins in the antigen (GST-E7) preparation; causing variation across triplicate wells.

Cross-stimulation of T cells: It is relevant to note that the immunogen in this study is the E7 expressing *Salmonella* strain Ty21a. The corollary that follows is that the immune response directed against the immunogen would comprise T cells against both the bacterial components as well as E7. *Salmonella* and *E. coli* share significant homology (21) alluding that remnants of *E. coli* proteins in the recombinant antigenic preparation although, seemingly devoid of contaminants, would cross-stimulate the T cells specific to Ty21a antigens hence accentuating the background staining in the ELISPOT assay. As observed, this would mean GST-E7 at higher concentrations show a higher background due to the corresponding increase in the *E. coli* contaminants it was surprising that even at a low concentration of GST-E7 (1.0µg/well) the background reduced only marginally, as seen in the scanned output of the readers (data not shown)

We reasoned that an enzyme substrate combination with a temporal linearity in colour development and adequate sensitivity might return lesser background than seen for HRP/TMB (12, 24). This view was bolstered by the past experiences for bovine lymphocyte ELISPOTS in the lab where assays returned well resolved spots with low background in the wells (20). The horse radish peroxidase enzyme mediates a high-turnover reaction. HRP is known to effect rapid development of spots by the H_2O_2 mediated oxidation of chromogenic substrates but has a propensity for higher back ground staining (12, 24). This prompted us to attempt optimizing the GST-E7 concentration using the RTU kit but developing with an ALP enzyme conjugate.

Effect of the enzyme conjugate reagent on background: The assay to determine the appropriate concentration of GST-E7 for stimulation was performed as before using the RTU kit but was developed using the Streptavidin-ALP and BCIP/NBT instead of Avidin-HRP reagent provided in the kit. Developing the assay with Strepatavidin-ALP had a dramatic effect on the readability of the assay (Fig. 3).

The background was significantly low at decreasing concentrations of GST-E7 used for stimulation of splenocytes from immunized mice. Although at higher concentrations (10µg, 5µg, 2µg) significant deviations were observed among triplicates (Std. error, $\sigma \pm$ 45.21, 21.63, 30.00 SFCs) respectively, the least variation among triplicates and statistically acceptable deviation ($\sigma \pm$ 8.02) was observed at the lowest antigenic concentration of 1.0µg/well of GST-E7 (Fig. 3) The mean SFCs/million for triplicates were 290.67 (Fig. 3). Well-spaced, uniform spots were observed in scanned outputs of the ELISPOT reader, especially for the wells with 1.0µg/well of GST-E7. The %CV across 9 wells of the ELISPOT was 22.1% (Fig. 3); showing better agreement across antigenic concentrations in the wells. The assay indicated that GST-E7 at 1.0µg/well can readily be used for evaluation of E7 specific T cell response avoiding tedious efforts to purify it further.

Precision of the assay protocol after optimized splenocyte and antigen concentration: Reproducibility of an ELISPOT protocol may be ascertained by determining the extent of intra assay variability. Assays performed on three different days in triplicates showed %CVs, 14.1, 10.8 and 7.3 on day 1, day 2 and day 3 (Fig.4). The intra assay precision was therefore acceptable for ELISPOT assay. An intra assay variability of < 20.0% for the highly sensitive ELISPOT assay is generally considered acceptable (23, 24). The assays here showed CV of less than 15% which we consider acceptable for inbred mice. The inter assay variability that accords reproducibility over multiple assays also showed good agreement. The %CV of the three assays was 12.7 (Fig. 4), under 15% much lesser than a general acceptability limit of < 25.0% (23, 24) a in at least triplicates.

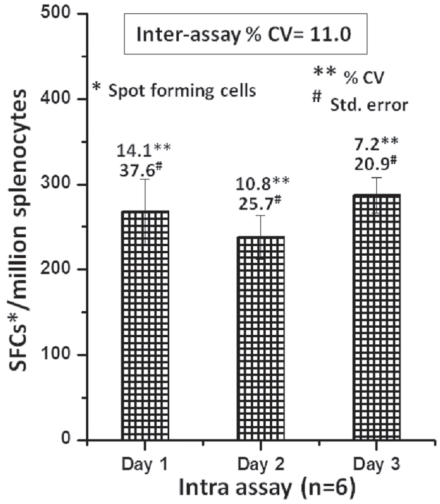

Fig. 4. Intra assay and inter assay variability of the standardized ELISPOT protocol. Immunized mice splenocytes were plated at 0.5 million cells/ well; splenocytes were stimulated at 1.0µg/well of affinity purified GST-E7; Plates were developed using Streptavidin-ALP and BCIP/NBT.

Linear fit of the assay protocol: Linear relationship of SFCs to the concentrations of serially diluted cells in an assay is a statistically accepted criterion for declaring ELISPOT results as positive for a T cell response (18). Having optimized the splenocyte and antigen concentration in the assay we sought to probe whether the standard ELISPOT protocol using the RTU kit along with the streptavidn-ALP enzyme conjugate would be statistically relevant.

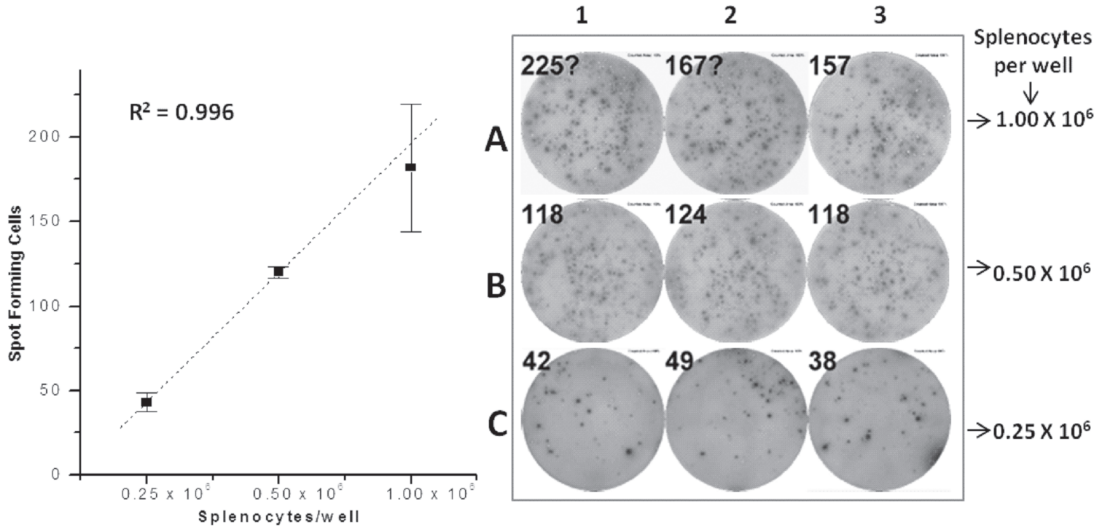

Fig. 5. Linear fit of spot forming cells in the two-fold serially diluted (row-A to C) ELISPOT of immunized mice splenocytes. Splenocytes were stimulated with 1.0µg/well of affinity purified GST-E7; Plates were developed using Streptavidin-ALP and BCIP/NBT. ?= Inconclusive number of spots.

Therefore an ELISPOT assay was performed with two-fold serial dilutions of splenocytes while the cells were stimulated with 1µg/well of GST-E7. The least variation among replicates at the concentration of 0.5 million cells/well and well resolved spots lends further credence to optimization of splenocytes for use in the evaluation of immune response (Fig. 5). The results confirm the linearity of the assay to the serial dilutions of splenocytes.

Conclusion

The study entailed standardizing an ELISPOT protocol for evaluation of immune response to HPV16 E7 delivered by recombinant *Salmonella* Typhi Ty21a. The choice of purified recombinant protein expressed in a bacterial host for *ex vivo* antigenic stimulation of splenocytes in combination with the heterogeneous immunogen, the *S.Typhi* Ty21a- contributed to background spots and interpretation of data. This was far more accentuated with the sensitive developing reagent, HRP/TMB. Developing the ELISPOT assay with Streptavidin-ALP enzyme conjugate showed marked difference in spot development, reduced background and improved readability compared to Avidin –HRP provided in the kit. The ALP enzyme and its substrates seem a better choice for ELISPOT assays in studies with heterogeneous immunogens and recombinant protein for *ex vivo* stimulation of T cells. The protocol with the necessary optimizations for concentrations of splenocytes and the antigen for stimulation is reproducible as determined by the intra and inter assay coefficients of variability.

References

1. zur Hausen, H (2009) Papillomaviruses in the causation of human cancers - a brief historical account. Virology, 20; 384(2):260-265.

2. Arbyn, M., Castellsagué, X., de Sanjosé, S., Bruni, L., Saraiya, M., Bray, F. and Ferlay, J. (2011) Worldwide burden of cervical cancer in 2008. Annals of Oncology, 22(12): 2675-86

3. Clifford, G., Franceschi, S., Diaz, M., Muñoz, N. and Villa, L. L. (2006) Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine, 24 Suppl 3:S3/26-34.

4. Boulet, G., Horvath, C., Vanden Broeck, D., Sahebali, S. and Bogers, J. (2007) Human papillomavirus: E6 and E7 oncogenes. International Journal of Biochemistry & Cell Biology, 39(11):2006-2011.

5. van der Burg, S. H. and Melief, C. J. (2011). Therapeutic vaccination against human papilloma virus induced malignancies. Current Opinion in Immunology, 23(2): 252-257.

6. Zhang, X. L., Jeza, V. T.and Pan, Q. (2008) *Salmonella* typhi: from a human pathogen to a vaccine vector. Cellular & Molecular Immunology, 5(2):91-97.

7. Galen, J. E., Pasetti, M. F., Tennant, S., Ruiz-Olvera, P., Sztein, M. B. and Levine, M. M. (2009) Salmonella enterica serovar Typhi live vector vaccines finally come of age. Immunology and Cell Biology, 87(5):400-412.

8. Gentschev, I., Spreng, S., Sieber, H., Ures, J., Mollet, F., Collioud, A., Pearman, J., Griot-Wenk, M. E., Fensterle, J., Rapp, U. R., Goebel, W., Rothen, S. A. and Dietrich, G. (2007). Vivotif—a 'magic shield' for protection against typhoid fever and delivery of heterologous antigens. Chemotherapy, 53(3):177-180

9. Pickett, T. E., Pasetti, M. F., Galen, J. E., Sztein, M. B. and Levine, M. M. (2000). In vivo characterization of the murine intranasal model for assessing the immunogenicity of attenuated Salmonella enterica serovar Typhi strains as live mucosal vaccines and as live vectors. Infection and Immunity, 68(1):205-13

10. Czerkinsky, C.C., Nilsson, L.A., Nygren. H., Ouchterlony, O., Tarkowski, A. (1983) A

solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. Journal of Immunological Methods, 65(1-2):109-21

11. Czerkinsky, C., Andersson, G., Ekre, H. P., Nilsson, L. A., Klareskog, L. and Ouchterlony, O. (1988) Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. Journal of Immunological Methods, 110(1):29-36
12. Kalyuzhny, A. E. (2005) Chemistry and biology of the ELISPOT assay. Methods in Molecular Biology (Clifton, N.J.), 302:15-31. Review
13. Mashishi, T. and Gray, C. M. (2002) The ELISPOT assay: an easily transferable method for measuring cellular responses and identifying T cell epitopes. Clinical Chemistry and Laboratory Medicine, 40(9):903-10
14. Cox, J. H., Ferrari, G. and Janetzki, S. (2006) Measurement of cytokine release at the single cell level using the ELISPOT assay, Methods, 38(4):274-82.
15. McCutcheon, M., Wehner, N., Wensky, A., Kushner, M., Doan, S., Hsiao, L., Calabresi, P., Ha, T., Tran, T. V., Tate, K. M., Winkelhake, J and Spack, E. G. (1997) A sensitive ELISPOT assay to detect low-frequency human T lymphocytes, Journal of Immunological Methods, 210(2):149-66.
16. Janetzki, S., Cox, J. H., Oden, N. and Ferrari G. (2005) Standardization and validation issues of the ELISPOT assay. Methods in Molecular Biology (Clifton, N.J.), 302:51-86
17. Peters, C. E., Woodside, S. M. and Eaves, A. C. (2005) Isolation of subsets of immune cells, Methods in Molecular Biology (Clifton, N.J.), 302:95-116
18. Cole, G. A. (2005) Interferon-gamma ELISPOT assay for the quantitative measurement of antigen-specific murine CD8+ T-cells, Methods in Molecular Biology (Clifton, N.J.), 302:191-204.
19. Rosenberg, E. S., LaRosa, L., Flynn, T., Robbins, G. and Walker, B. D. (1999) Characterization of HIV-1-specific T-helper cells in acute and chronic infection. Immunology Letters, 66(1-3):89-93.
20. Parthasarathy, S., Veerasami, M., Appana, G., Chandran, D., Das, D. and Srinivasan, V. A. (2012) Use of ESAT-6-CFP-10 fusion protein in the bovine interferon-gamma ELISPOT assay for diagnosis of Mycobacterium bovis infection in cattle, Journal of Microbiological Methods, doi: 10.1016/j.mimet.2012.06.001.
21. George, M.and Garrity, E. D. (2005). *The HYPERLINK "http://www.springer.com/life+sciences/book/978-0-387-24144-9"Gammaproteobacteria*. Bergey's Manual of Systematic Bacteriology. 2B (2nd edition), New York: Springer, pp. 1108.
22. Savage, M. D., Mattson, G., Desai, S., Nielander, G. W., Morgensen, S. and Conklin, E.J. (1992) Avidin-Biotin Chemistry: A Handbook. Pierce Chemical Co., Rockford, IL, p.467.
23. Maecker, H.T., Hassler, J., Payne, J. K., Summers, A., Comatas, K., Ghanayem, M., Morse, M. A., Clay, T. M., Lyerly, H. K., Bhatia, S., Ghanekar, S. A., Maino, V.C., Delarosa, C. and Disis, M. L. (2008) Precision and linearity targets for validation of an IFNgamma ELISPOT, cytokine flow cytometry, and tetramer assay using CMV peptides, BMC Immunol. 9: 9.

24 Xu. Y, Theobald. V., Sung. C., DePalma, K., Atwater, L., Seiger, K., Perricone, M. A. and Richards, S. M. (2008) Validation of a HLA-A2 tetramer flow cytometric method, IFNgamma real time RT-PCR, and IFNgamma ELISPOT for detection of immunologic response to gp100 and MelanA/MART-1 in melanoma patients.J Transl Med., 6:61

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Streptomyces albus var. *alkalis* var. nov

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Abstract

A new alkalophilic variety of *Streptomyces albus* capable of growing up to pH 10 is described. It was isolated from the soil sample collected from MIET campus, Meerut, India.

Key words: *Streptomyces albus* var. *alkalis*. Soil, MIET Campus, Meerut.

Introduction

Streptomyces albus the type species of *Streptmyces* is ubiquitous in nature and has been isolated from various substrates including soil, contaminated building materials (1) as hypermycoparasite on *Nectrea inverta* (2), as a causative agent of actinomycetoma (3) and marine sediments (4). An alkalophilic variety of *S. albus* var. i*ndicus* was reported from kothagudem coal mine soil (5).

No published data on microbial diversity of Meerut region is available, except the recent publications on rare thermophilic fungi (6, 7). Hence, an attempt has been made to study the microbial flora in this region.

The present isolate significantly differs from *S. albus* var. i*ndicus* in carbon utilization and few other characters and hence it is described as new variety .The name signifies its ability to grow under highly alkaline conditions.

Materials and Methods

Soil samples were collected into sterile vials and the samples were heated at 80°C for ten minutes. Serial dilutions were made and a loop full of diluted samples were streaked on Bennetts agar and incubated at 37°C. When small,discrete colonies appeared, each colony was transferred on to a fresh agar plate by streaking across the plate. Pure colonies were isolated and maintained on Potato dextrose agar.

Media used in culture characterization were those recommended by ISP (8) and were incubated at 37°C. Carbohydrate utilization was studied by using ISP medium 9 supplemented with 1% carbon source (9). Liquefaction of gelatin was investigated by the method of Waksman (10) Hydrolysis of starch was studied by the method of Gordon et al (11).

Culture Characters

Bennetts agar*:* Growth profuse; colonies off white and powdery; substrate mycelium brown; aerial mycelium well developed; reverse colony snuff colored in the center and brown towards periphery; diffusible pigment none; sporulation abundant.

Cellulose agar*:* Growth poor and thin; substrate mycelium white; aerial mycelium thin, white and poorly developed or scanty; reverse colony colorless; diffusible pigment absent.

Czapeks agar*:* Growth poor and transluscent; substrate mycelium white; aerial mycelium thin, white and poorly developed; reverse colony colorless; diffusible pigment not produced.

Emerson's agar*:* Growth excellent; substrate mycelium well developed and pale brown in color;

aerial mycelium off white, moderately developed; reverse colony dark brown; diffusible pigment dark brown.

Glucose asparaine agar: Growth moderate; substrate mycelium white; aerial mycelium off white but turns light brown with age and sporulation; reverse colony woody brown; diffusible pigment none.

Glucose peptone agar : Colonies well developed with good growth; substrate mycelium white; aerial mycelium moderately developed, white but changes to light brown with age; reverse colony pale brown; diffusible pigment pale yellow.

Glycerol asparagine agar : Moderately growing colonies dull white and powdery; substrate mycelium light brown; aerial mycelium dull white to white; reverse colony pale brown; diffusible pigment none.

Potato dextrose agar: Colonies compact and white; aerial mycelium white to begin with, but turns pale brown with age: reverse colony colorless; diffusible pigment none.

Tyrosine agar: Growth poor; tyrosine utilization poor to none

Yeast extract and Malt extract agar: Colonies thick and profusely growing; substrate mycelium white; aerial mycelium thin, white and moderately developed; reverse colony colorless; diffusible pigment none.

Carrot plug: Growth moderate; aerial mycelium white; diffusible pigment none.

Potato plug: Aerial mycelium abundantly developed, chalky white; diffusible pigment cream colored.

Casein hydrolysis: Strong

Gelatin liquefaction: Rapid and strong

H2S production: Negative

Melanin production: Negative

Nitrate reduction: Negative

Starch hydrolysis: Strong

Carbon utilization

Arabinose	+
Dextrose	+++
Dulicitol	+
Galactose	+/-
Glycerol	++
Inulin	+
Lactose	-
Levulose	+/-
Maltose	++
Mannitol	+
Mannose	-
Soluble starch	+++
Sarbose	-
Sorbitol	+/-
Xylose	++
Control	-

pH relations: It grows well in the pH range of 8-10 with an optimum at 9.0

Temperature relations: It grows from 24-40°C with 37°C optimum temperature.

Habitat: Soil, MIET Campus, Meerut, UP.

The culture is deposited at MIET culture bank, Meerut.

Isolation number: S55

Date of isolation: 23 September 2010

Identity: It is assigned to *Streptomyces albus* group due to the presence of white aerial mycelium bearing more or less oval spore chains on most of the growth media (12). It resembles *S. albus* in several respects including the utilization of carbon substances but differs from it in its ability to grow in alkaline conditions. Significant differences observed between *S. albus* var. *indicus* and *S. albus* var. *alkalis* are presented in Table 1.

Table 1. Difference between *S. albus* var. *indicus* (SAI) and *S.albus* var *alkalis* (SAA)

Potato plug	SAI	SAA
Diffusible	Pale	Cream
pigment	green	colour
Gelatin liquefaction	Negative	Rapid and
		Strong
Tyrosinse utilization	Strong	Poor to none
Optimum pH	8.O	9.0
Optimum		
temperature	24-28°C	37°C
Arabinose	++	+
Dulicitol	-	+
Galactose	++	+/-
Glycerol	+++	++
Inulin	++	+
Lactose	++	-
Maltose	+++	++
Mannitol	++	+
Mannose	+	-
Laevulose	++++	+/-
Sorbitol	+	+/-
Control	- -	-

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References

1. Rafal, L. Gorny, Gediminas Mainelis, Sergey A. Grinshpun, Klaus Willeke, Jacek Dutkiewicz and Tina Reponen (2003). Release of *Streptomyces albus* propagules from contaminated materials. Environmental Research, 91:45-63.
2. Tu, J.C. (1986). Hyperparasitism of *Streptomyces albus* on a destructive mycoparasite *Nectria inverta*. Phytopathology, 117: 71-76.
3. Martin, M Cruz, Manteca Angel, Vazquez Fernands and Mendez, F, J (2004). *Streptomyces albus* isolated from a human actinomycetoma and characterization by molecular techniques. Jour. Clin. Microbiol.42: 5957-5960.
4. Sunanda Kumari, K. and Vidavalur Siddaiah (2011).Taxonomy,-Identification and Biological activities of novel isolate of *Strptomyces albus*. Pharm. Resh. 4: 4678-4680.
5. Vijayalakshmi, B. and Subrahmanyam, A. (1993). A new alkalophilic variety of *Streptomyces albus*. H.A.Bull.35:208-211.
6. Subrahmanyam, A., Swathi Sri, A. and Raju, V.S. (2010). *Abradeosporangium*, a new genus of Mucorales JoTT, 2: 1219-1222.
7. Swathi Sri A, Subrahmanyam, A. and Raju, V.S. (2012). *Thermomucor abortosporangium*. Taiwania, 57: 67-70.
8. Shirling. E.B and Gottleib, D. (1966). Methods of characterization of *Streptomyces*. Int. Jour. Syst. Bact. 16: 313-340.
9. Pridham, T.G and Gottlieb, D. (1948). The utilization of carbon compounds by Actinomycetes as an aid for species determination. Jour. Bact. 56: 107-114.
10. Waksman, S.A. (1969). The Actinomycetes: Identification,class- ification and description of genera and species. Williams and Wilkins Comp. Baltimore, 2: 327.
11. Gordon, R.E, Barnett, D.A., Handerhan, J.E. and Pang, C.H. (1974). *Nocardia coeliaca Nocardia autotropica*. Int. Jour. Syst. Bact. 24: 54-61.
12. Pridham, T.G and Lyons, A.J. (1961). *Streptomyces albus* (Ross-Dorsai) Waksman et .Henrici. Taxonomic study of strains labeled *Streptomyces albus*. Jour. Bact. 81: 431-441.

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NEWS ITEM

Help India leapfrog in the future: Prime Minister Manmohan Singh tells scientists

Inaugurating the new campus of CSIR's Institute of Genomics and Integrative Biology (IGIB) in south Delhi, Prime Minister of India, Manmohan Singh asked scientists to seek newer frontiers of research, match capabilities of peers around the world and help India leapfrog. Pointing out that India has been overtaken in scientific research by other emerging powers, he asked scientists to redouble efforts in research and development, a field where India has been overtaken by other rapidly emerging powers. He added that India needs a new leap forward in science and had the good fortune of investing in science long before other developing countries did. Singh also said that it was the patriotism of the generation represented by scientists like C V Raman and J C Bose that had enabled the political leadership to help build research capabilities across the country. He asked the scientists to rediscover the patriotism of an earlier generation, match the enterprise and capability of peers around the world and help the country leap frog in the future.

Biodiversity still the key in difficult economic times: UK minister, Richard Benyon

Despite very difficult economic times and wider financial scepticism among the wider population, it is more important than ever to protect biodiversity, and at the same time allowing for economic growth and poverty reduction, said UK's minister of state for environment, Richard Benyon, who is representing UK at Hyderabad at the 11th meeting of countries signed up to protecting biodiversity targets. He said that the high level political ambitions come at a time when there is financial scepticism among the wider population. But every pound spent yields enormous economic dividends. Mr Benyon said that India is taking a clear leadership role in biodiversity protection, and working closely with the other government on various sectors. India and UK have jointly financed the high level panel, whose job it is to estimate the cost of biodiversity protection, and which will submit its preliminary findings at Hyderabad, giving governments and the international community some idea of what is needed in funding. Mr Benyon told that they are very much involved with the international community on protecting biodiversity. The main aim of the Hyderabad meeting is pragmatic, to outline the next practical steps for the global community to help protect biodiversity.

Nuclear energy is clean: Abdul Kalam

Former President A.P.J. Abdul Kalam said that the country needs nuclear energy as it is a clean energy unlike energy generated by burning fossil fuel. Dr. Kalam, a strong advocate of nuclear power, said about safety of nuclear power plant and that every watt of electricity was important for the country. He said that generation of electricity by burning the fossil fuel was not environmentally sensible as every one litre fossil fuel used for generating energy produces two kilograms of carbon dioxide. He told that Nuclear plants generates clean energy. About the safety concerns in the wake of the Fukushima nuclear plant accident in Japan last year, the former President said that that there are 546 nuclear reactors currently operating in different countries in the world and he added that the country had to focus on alternative energy sources such as wind and solar energy. Dr. Kalam said that in India all the nuclear reactors are Uranium-based. India is blessed with thorium as one-third of the total world thorium reserve is in the country, he said. Thorium, however, is not a fissile material, he said adding that the country needed fast breeders to convert thorium into a fissile material.

Need for high quality national health system used by poor, rich alike: President

President Pranab Mukherjee emphasized on the need to craft a high quality national health system

that is used by the poor and rich alike. Addressing the 40th annual convocation of All India Institute of Medical Sciences, the President expressed deep concern over the impoverishing impact of health and medical expenses on the vulnerable sections of the society. He said that it is unacceptable that almost 80 per cent of the expenditure on healthcare by our people is met by personal, out of pocket, payments and as many as four crore people plunge into poverty each year due to expenses on medical treatment. The President stressed on the health services for the poor. Progress in the health sector is key to India's future place of prominence in the world. He said that the nation's productivity depends on the health and well-being of its citizens and Economic growth that does not go hand in hand with reduction in avoidable mortality and ill health is neither sustainable nor desirable. Mr Mukherjee said the time had come for India to aim at attaining Universal Health Coverage in the next two to three Plan periods.

World should commit resources to meet biodiversity goals: Jayanthi Natarajan

The global community should agree to interim commitments and targets on resource mobilisation, failing which implementation of the biodiversity protection agenda would be severely impacted considering its time-bound nature, said Environment and Forests Minister of India, Jayanthi Natarajan. Speaking at opening of the 11th Conference of the Parties (CoP 11) to the Convention on Biological Diversity (CBD), she said resource mobilisation was the most important unfinished agenda inherited and was adopted for funding the means to achieve biodiversity targets. she said that we have been provided with another singular opportunity to collectively decide on committing resources to infuse confidence and generate momentum for implementation of biodiversity targets and If we miss this, it will be our collective failure to achieve the targets by 2020. Natarajan stressed the need to adopt new approaches and mechanisms, leveraging resources from existing sources through mainstreaming, adjusting economic instruments and further engaging the business sector.

SCIENTIFIC NEWS

Can Vaccines Be Delivered Via the Lungs Instead of by Injection?

Researchers from University of Groningen and National Institute for Public Health and the Environment (Bilthoven), Netherlands described the unique physiology and immune responsiveness of the respiratory track that make pulmonary vaccine delivery such an attractive alternative to traditional injections. Although pulmonary vaccination is still a young field, with much more research needed, evidence suggests administration of a vaccine to the lungs can induce a local immune response more effectively than conventional types of vaccine delivery, in addition to stimulating antibody production throughout the body. This could be especially important for combating pathogens that cause pulmonary diseases. In addition to the obvious benefit of eliminating the need for an injection, new vaccine delivery methods via the lungs offer particular advantages for protecting against infectious agents that enter the body through the respiratory track.

T.Ravali

Strengthening a Billion-Dollar Gene in Soybeans

Crop sciences researchers at the University of Illinois and the University of Wisconsin have found a way to strengthen plant resistance. Soybean cyst nematode (SCN) does hundreds of millions of dollars' worth of damage each year. An area on chromosome 18 called Rhg1 (Resistance to H. glycines) is known to be the location of the main source of SCN resistance. Rhg1 disrupts the formation and maintenance of potential nematode-feeding sites on plant roots. Using fine mapping, which is a technique that involves mapping genes in a very constrained area, researchers narrowed the search down to a few gene candidates. They found that nearly every soybean variety that is known to be SCN resistant has more than one set of these genes. The Wisconsin researchers used a technique called Fiber-FISH to show that the genes make soybeans nematode-resistant. . It allowed them to look into the DNA molecule and count the number of genes in a row. They also found that levels of expression of these genes were higher where there

were more copies of the genes. The results are interesting from a scientific point of view because having several genes next to each other that control the same trait is unusual in multicellular organisms. So is having an effect that is clearly due to multiple repeats of a stretch of DNA. The practical implication of the study suggests a way to engineer artificial resistance that is stronger than natural resistance.

B.Suraghavi

Scientists Use New Method to Help Reduce Piglet Mortality

Scientists from U.S. Department of Agriculture (USDA) have developed a new method that predicts animals' mortality and nursing ability which helps to increase the survival of newborn piglets. The measuring technique is called the "immunocrit," which determines whether preweaning piglets receive adequate colostrum from the sow. The colostrum produced by a sow after giving birth contains immunoglobulins, or antibodies, which help build immunity against bacteria, viruses and other foreign elements. Piglets that fail to nurse and receive enough colostrum from their mother within the first 24 hours after birth usually die. The immunocrit measures newborn piglet serum immunoglobulin in blood samples. Immunocrit results also show that the average measurement of piglets in a litter reflects the sow's ability to produce colostrum. In addition, scientists have found a connection between immunocrit measurements, piglets' weight and mortality. Pigs that weighed more were more likely to survive the challenge of not getting colostrum within the critical timeframe, as opposed to those that weighed less. The immunocrit recognizes piglets within a litter that have not eaten or had the chance to nurse. This provides an opportunity to save at-risk piglets by using intervention strategies.

E.Manoj Kumar

Even Our Fat Cells Need Sleep, According to New Research

Researchers from University of Chicago have found that not getting enough shut-eye has a harmful impact on fat cells, reducing by 30 percent their ability to respond to insulin, a hormone that regulates energy. Body fat, also known as adipose tissue, stores and releases energy. In storage mode, fat cells remove fatty acids and lipids from the circulation where they can damage other tissues. When fat cells cannot respond effectively to insulin, these lipids leach out into the circulation, leading to serious complications. Sleep deprivation has long been associated with impaired brain function, causing decreased alertness and reduced cognitive ability. The researchers performed a biopsy, removing abdominal fat cells and measured how these fat cells responded to insulin. The researchers assessed insulin sensitivity at the molecular level by measuring the phosphorylation of a protein called Akt within fat cells. Akt phosphorylation is a crucial early chemical step in the cell's response to insulin. The insulin sensitivity of fat cells decreased by 30 percent. They found that the sleep-deprived study participants had a decreased response to a range of doses of insulin. This study is "a valuable contribution to the understanding of the causal pathways by which reduced sleep duration may directly contribute to diabetes and obesity. These results point to a much wider influence of sleep on bodily functions, including metabolism, adipose tissue, cardiovascular function, and possibly more. The study suggests that sleep's role in energy metabolism is at least as important as it is in brain function.

K.Manideeep

EDUCATION

PhD/Post Doctoral Programs

Admission to Ph.D. in Biomedical Sciences: Applications are invited for admission to Ph.D programmes in Institute of Liver & Biliary Sciences (ILBS), D-1, Vasant Kunj, New Delhi, India in the in the field of Liver and Biliary diseases and allied disciplines. The selection is made on the basis of interview only. Candidates with M.Sc./M.B.B.S/ MS/ MD/DNB are eligible. Candidate should submit their application online at the ILBS website (http://www.ilbs.in). Application form duly filled in with all necessary documents attached should be submitted in person or sent by post to the Associate Dean, Institute of Liver & Biliary Sciences, D-1, Vasant Kunj, New Delhi-110 070, India on or before 30-11-2012. Application form must be accompanied by the application fee in form of Demand Draft for Rs. 2500, payable at New Delhi, in favour of the Director, ILBS New Delhi. All applications would be screened for eligibility and only the eligible candidates would be called for the entrance test. The schedule of entrance test is Written Examination at New Delhi on 09th December, 2012 and Departmental assessment on 10th December, 2012.

Admission to Post Doctoral Fellowships: Applications are invited from the eligible candidates

to pursue Post-Doctoral (PDF) / Doctoral Research(JRF) in the areas of Health and allied subjects including Life / Bio Sciences at Yenepoya University, University Road, Deralakatte, Mangalore - 575 018, India. The candidate have Written test followed by personal interview. The filled-in applications for the JRF and Curriculum Vitae with Research proposal (for PDF) should reach to The Registrar, Yenepoya University, University Road, Deralakatte, Mangalore - 575 018, India on or before 01-11-2012. Email : reachus@yenepoya.org. Web: www.yenepoya.edu.in.

OPPORTUNITIES

CSIR-Indian Institute Of Chemical Technology, Hyderabad - 500 607, India. Applications are invited from eligible candidates for position of Junior Research Fellow (JRF) to pursue AcSIR PhD Program at CSIR-IICT in the areas of Organic Chemistry (35 vacancies), Analytical Chemistry (2), NMR (1), Molecular Modelling (1), Polymers & Functional Materials (7), Chemical Engineering /Technology (1), Inorganic & Physical Chemistry (23), Biology/ Chemical Biology (12), Bioengineering and Environmental Sciences (1). Candidates with First class M.Sc. degree or equivalent in Chemistry/ Biology/ Physics or allied sciences with i) a valid rank in CSIR/UGC-NET JRF Examination, OR ii) a valid DST INSPIRE/ICMR/ Fellowship are eligible. Also candidates with First class BE/B.Tech in Chemical Engineering /Chemical Technology/ Biochemical/ Biotechnology/ Environmental Sciences with a valid GATE score / CSIR-UGC-NET rank are eligible. Candidates may apply online for PhD Admission to CSIR- IICT at www.iictindia.org on or before October 28, 2012. In addition, the candidates must also submit on-line applications for PhD registration in AcSIR at http://acsir.res.in on or before October 28, 2012. A soft copy of the receipt/ acknowledgement of AcSIR should be mailed to phdatiict@gmail.com. The interviews/written test are conducted on November 29 & 30, 2012.

Jawaharlal Nehru University, New Delhi, India. Applications are invited from eligible candidates for **one Senior Research Fellow (SRF)** position to work in a research project entitled "Study of Antiangiogenic and Anti-tumor Effects of Fisetin in Lung Cancer: Implications for Intervention of Cancer" sponsored ICMR. Candidates with M.Sc. in any branch of Life Sciences and had Qualified National Eligibility Test (CSIR / UGC / ICMR) with experience in Cancer Biology are eligible. The application on plain paper indicating name, date of birth / age, photo, address, essential / technical / professional qualifications, experiences, research work, list of published papers, should reach to Dr. Rana P. Singh, (Project Director), 104/105, Cancer Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, on or before 5th November 2012.

Annamalai University, Annamalai Nagar – 608 002, Tamil Nadu, India. Applications are invited from eligible candidates for one post of Project Fellow to work in UGC-Special Assistance Program awarded to the Department of Zoology, Annamalai University funded by UGC, New Delhi, India. Candidates with M.Sc. in Zoology with at least 55% marks (50% in case of SC/ST/PH) at post-graduate level or M.Phil. in the subject concerned are eligible. NET/GATE qualified candidates will be given preference. The interested candidates can send their applications to Dr.Mrs.).Selvisabhanayakam, Co-ordinator, UGC-SAP, Professor and Head, Department of Zoology, Annamalai University, Annamalai Nagar – 608 002, Tamil Nadu, India and attend the interview on 31.10.2012. Mobile: 9442441018, E-mail: drselvisabha@gmail.com.

Banaras Hindu University, Varanasi, India. Applications are invited from eligible candidates for the one Position of **Re**search Associate (RA) for 3 years to work on a project entitled "Thrombus modulation by metallic nanoparticles" funded by DST, New Delhi. Candidates with PhD in any branch of science with research experience/specialization in area of nanotechnology as evidenced from publications in peer-reviewed international journals are eligible. Application on plain paper giving biodata along with qualifications supported by attested documents should reach to Principal Investigator, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India.

Jawaharlal Nehru Technological University, Hyderabad, India. Applications are invited from eligible candidates for one Junior Research Fellow (JRF) position to work in a research project entitled "Inventory and bioprospecting of spirochetes of marine habitats of India" sponsored by the Department of Biotechnology, New Delhi, India. Candidates with Masters degree in any branch of Life/Chemical Sciences(Env. Biotech.)/PlantSciences(Botany)/ AnimalSciences (Zoology) /Microbiology/ Biotechnology/Genetics/Biochemistry/Env. Sciences/

Org. Chemistry are eligible. Candidates with UGC/ CSIR-JRF/NET or GATE are desirable. Candidates interested are requested to send their applications to the Principal Investigator and appear for an interview before the selection committee on 5-11-2012, 10.00 AM at the office of the Head, Centre for environment, Institute of science and Technology, JNTUniversity, Hyderabad. For further details contact: Prof. (Mrs). Ch. Sasikala, Principal Investigator, DBT project, Centre For Environment, IST, Jnt University Hyderabad, Kukatpally, Hyderabad – 500085. Telephone: [O] +91-40-23158661(3480). [R]: +91-40-27535462. [M]: 09000796341. Fax: +91-40-27531563. E_mail: sasi449@yahoo.ie, sasikala.ch@gmail.com.

SEMINARS/WORKSHOPS/CONFERENCES

International Conference on Environmental Impact on Human Health and Therapeutic Challenges (ICEHT - 2012) & 6th Annual Convention of Association of Biotechnology and Pharmacy (ABAP): An International Conference on "Environmental Impact on Human Health and Therapeutic Challenges" (ICEHT - 2012) & 6th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) was going to held on December 20-22, 2012 at Sri Venkateswara University, Tirupati, India organized by Department of Virology, Sri Venkateswara University, Tirupati, India. Abstract can be sent through Email: iceht2012@gmail.com on or before November 30th, 2012. Many Gold medals and awards like Lifetime Achievement award in Biotechnology and Pharmacy, Talented Industrial Biotechnologist award, ABAP-Senior Scientist Awards, ABAP-Young Scientist Award (Below 30 years and above 30 years category), ABAP-junior Scientist Award (for poster presentation) are available. For further details contact: Prof.DVR Sai Gopal, Chairman, ICEHT - 2012, Department of Virology, Sri Venkateswara University, Tirupati - 517502, A.P, India.

The Fifth AP Science Congress - 2012: The Fifth AP Science Congress - 2012 with a focal theme of "Innovations in Science, Technology & Mathematics" was going to held on 14th- 16th November, 2012 at Acharya Nagarjuna University, Guntur, A.P, India organized by Acharya Nagarjuna University and Andhra Pradesh Akademi of Sciences, India. Abstract can be submitted online by clicking the hyper link Abstract Submission on the home page http://www.apsc2012.in on or before October 25th, 2012. The abstracts also need to be submitted through email - apas1963@yahoo.co.in. For further details contact: Prof.K.R.S.Sambasiva Rao, Organizing Secretary, APSC-2012, Department of Biotechnology, Acharya Nagarjuna University, Guntur, A.P, India. Mobile:9440869477. Email: 2012apsc@gmail.com.

International Conference on Bioengineering (ICBE - 2012): An International conference on Bioengineering (ICBE - 2012) was going to held on January 2nd - 4th, 2013 at Rajalakshmi Engineering College organized by Department of Biotechnology, Rajalakshmi Engineering College, Rajalakshmi Nagar, Thandalam, Chennai – 602105, Tamil Nadu, India. Abstract can be submitted through E-mail: mohamedali.s@rajalakshmi.edu.in or icbe2013@rajalakshmi.edu.in on or before December 5th, 2012. For further details contact: Dr. S. Mohamed Ali, Organising Secretary, Department of Biotechnology, Rajalakshmi Engineering College, Rajalakshmi Nagar, Thandalam, Chennai – 602105, Tamil Nadu, India.
E-mail to: mohamedali.s@rajalakshmi.edu.in. Phone No: +919791186168, 9381981008.

International Conference on Advances in Free radicals, Redox Signaling and Translational Antioxidant Research & XII Annual Meeting of the Society for Free Radical Research, Lucknow, India: An International Conference on Advances in Free radicals, Redox Signaling and Translational Antioxidant Research & XII Annual Meeting of the Society for Free Radical Research was going to held on January 30 – February 1, 2013 at Hotel Clarks Awadh, Lucknow, India organized by CSIR- Indian Instiute of Toxicology Research (CSIR-IITR), P.B. 80, M.G. Marg, Lucknow – 226001, Uttar Pradesh, India. Abstract can be submitted online through E-mail: sfrrstar2013@gmail.com on or before November 30, 2012. For further details contact: Dr. Poonam Kakkar, Organizing Secretary, SFRR-STAR- 2013, CSIR-Indian Institute of Toxicology Research, Post Box No. 80, M.G. Marg, Lucknow-226001, (U.P.) India, E. mail: sfrrstar2013@gmail.com, Tel: (+91)-0522-2213786, 2627586 extn. 269; Fax : (+91)- 0522-2628227/2611547, Mob: +919335902630. Conference website: www.sfrrstar2013.org.

ALLIANCE INSTITUTE OF ADVANCED PHARMACEUTICAL AND HEALTH SCIENCES

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About Alliance

Alliance, located conveniently in the heart of Hyderabad, trains industry-ready graduates by bridging education with industry needs in pharmaceutical sciences. Alliance's visionary management built state of the art facilities and laboratories to provide quality education meeting national and international standards.

Collaboration with JNTUH, India

Alliance is having collaboration with **Jawaharlal Nehru Technological University, Hyderabad (JNTUH)**, which is a premier institution with academic and research–oriented programs, offered through the constituent and affiliated colleges. Alliance's syllabi, academic regulations and course structure are **approved by the JNTUH. JNTUH awards the degrees after fulfilling the degree requirements.**

Collaboration with University of the Pacific, USA

University of the Pacific, ranks in the top 100 among the 3000 national universities in the United States. Alliance has entered into research collaboration with Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific.

Alliance students have an option to do research work at the University of the Pacific to fulfill requirements for MS degree in India. Pacific faculty teaches Alliance students via live online classes. Pacific is also interested to offer admissions to Alliance students based on their performance at Alliance.

Programs offered

- MS in Industrial Pharmaceutics
- MS in Pharmaceutical Analysis & Quality Control
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For admissions, application forms and additional information visit online at www.jntuh.ac.in/alliance or www.allianceinstitute.org.

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