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Exploring Microbes for the Production of Viral Antigens

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ABSTRACT

Purified protein serves as a basic starting material for understanding its functions and for structural characterization. However, it is one of the most challenging step compared to all other proteomic techniques because of the fact that each protein differs in its characteristics in terms of chemical building blocks ie, amino acids, hydrophobicities, secondary and tertiary structures and functions. Thus each protein or group of proteins requires a specific tailored purification procedure. This poses a need to develop either a new reliable and faster purification technique or modify the existing protocols to suit similar groups of proteins. To obtain a purified protein of interest, the source can be from natural system where protein is abundant or in most cases over expressed protein using bacterial or mammalian expression system. Once the source of the protein is understood, the purification procedure can broadly be classified into three categories, namely cytoplasmic periplasmic, membrane embedded and inclusion bodies-based on the localization of the protein. In this paper, we discuss about exploring the use of *E.coli* bacteria for the production of viral antigens from Dengue.

Key words: *E. Coli,* Membrane protein, viral antigen

INTRODUCTION

The release of microbial and human genome sequences (Doolittle, 2002) and the development of bioinformatics tools like BLAST (Altschul *et al.,* 1990) have led to identification of novel genes of putative functions (Florent *et al.,* 2010).This increased the demand for high-throughput cloning procedures and expression of recombinant proteins for structural and functional characterization using bio-chemical, bio-physical and structural biology techniques. Understanding the structural and functional relationship of proteins involved in disease and disorders, initiated the development of structure-based designed drugs against specific groups of proteins. For example, small molecule inhibitors for blocking ion channels, disrupting protein-protein interactions and modulating signal transduction and anti-peptides for targeting specific transmembrane helices (verkman and calietta, 2009; Bollati *et al.,* 2010). In this paper the different methods to obtain proteins for structural and functional studies especially membrane protein from virus expressed in prokaryotic system in *E. coli* is discussed. In the later part, improved purification methods for membrane proteins specifically the proteins which are targeted to the inclusion bodies when expressed in prokaryotic system are presented. The optimized procedure for obtaining purified protein from inclusion bodies using dengue virus (serotypes 1-4) envelope protein domain III as a model is demonstrated.

STRUCTURAL AND FUNCTIONAL STUDIES OF MEMBRANE PROTEINS

Integral membrane proteins (IMPs) constitute a third of prokaryotic and eukaryotic proteomes (Arkin and Brunger, 1998). IMPs span the lipid bilayer at least once, and are involved in almost every cellular process like: receptors to transduce signals across the membrane (Kolanus and seed 1997; Faull

and ginsberg, 1996), transporters (Kim, 2006), pores, pumps and channels. Inactive IMPs results in a range of diseases and modulation of its activity is one of the main therapeutic strategies (Torres *et al.,* 2003; Drews, 2000). For example, G protein-coupled receptors (GPCRs), a key player of signal transduction, are a major group of drug targets for the pharmaceutical industry. Currently in the pharmaceutical market about 25 % of the top 200 best-selling drugs are targeted against GPCRs (Fredriksson *et al.,* 2003).

Membrane proteins still pose a great challenge to structural biologists, by way of not yielding to over-expression, solubilization, purification, crystallization or structure determination (Tate and Krishammer, 1996). Although one third of the whole proteome comprises of membrane proteins and more than 40 % of them are pharmacologically important drug targets, only a minute percentage of the atomic structures of membrane proteins are available in the Protein Data Bank (PDB) when compared to the soluble proteins (>50,000 structures) (www.rcsb.com). To date, there are about 665 coordinate files of membrane proteins in the PDB with 217 unique structures.

Homologous and heterologous overexpression of membrane proteins such as channels, transporters and porins is a challenge as they become toxic to cells due to altered membrane properties (Laage and longosch, 2001; Liao *et al.,* 2004). Further, the absence of post-translational modification that is essential for the proper function also hinders structural studies (Mancia and Hendrikson, 2007). Presence of proper translocation machinery, e.g. prokaryotic Sec machinery or eukaryotic Sec61 complex are required for folding and insertion of membrane proteins to membranes (Schnell and Hebert, 2003), and therefore improper folding leads to intracellular protein aggregates (Miroux and Walker, 1996).

STRATEGIES FOR EXPRESSION AND PURIFICATION OF MEMBRANE PROTEINS

Although we have quite powerful techniques such as X-ray crystallography, nuclear magnetic resonance (NMR) and electron microscopy to study the structure of membrane proteins (Torres *et al.,* 2003), we do need to have a reasonable amount of high quality and stable protein samples to elucidate their structure. This is one of the major bottlenecks in membrane protein structural biology field (Junge, 2008). In order to obtain homogeneous and high concentration of membrane protein samples, there are now quite a lot of new expression strategies. These include vector designing with novel fusion proteins, cultivation conditions, optimized protocols for purification, choice of detergents and buffer conditions, that has significant impact on final yield and sample quality (Junge *et al.,* 2008; Wagner *et al.,* 2006).

In spite of these barriers in expression and purification, few membrane proteins structures have been solved by isolation from their highly abundant natural sources. Examples include bacteriorhodopsin from *H. salinarium* (Luecke *et al.,* 1999), aquaporin-1 extracted from red blood cells (Walz *et al.,* 1997) and opsin derivatives such as bovine rhodopsin isolated from the rod outer segments of bovine lenses (Palczewski *et al.,* 2000). The drawback in this method is that the production of mutants and labeling for NMR studies is not feasible.

To overcome such problems, efficient recombinant systems are highly preferable. Among the reported structures in PDB (Protein Data Bank), *E. coli* is the most commonly used expression system for homologous proteins such as OmpA (Arora *et al.,* 2001) and heterologous KcsA potassium channel (*S. lividans*) (Zhou *et al.,* 2001). Recently *E. coli* has been used efficiently for the heterologous overproduction of 24 membrane proteins from the human pathogen *Legionella pneumophilia* (Gordon *et al.,* 2008). In addition to *E. coli* expression system, *Lactococcus lactis* has been used in the hetero-expression of eukaryotic membrane protein and has been shown to have high level expression as in the case of human KDEL receptor (Kunji *et al.,* 2005). When eukaryotic proteins are synthesized in their natural host systems, they undergo post-translational modifications (PTMs). In contrast to bacterial expression systems, PTMs are absent because the respective enzymes are lacking (Manica and

Hendrickson, 2007). To obtain PTM proteins, one need to use an eukaryotic expression systems like *Schizosaccharomyces pombe*, *Pichia pastoris,* Sf9 insect cells and mammalian cell lines. However, such systems are still challenging (Midgett *et al.,* 2007). In addition to these expression systems, cell free expression systems is an emerging technique for efficient production of membrane proteins with the PTMs (Klammt *et al.,* 2007; Schwarz *et al.,* 2007).

Similar to expression strategies of cytosolic proteins (Sheibani, 1999), fusion technologies are often employed in order to optimize expression, detection and purification of membrane proteins. Popular fusion proteins used for expression are maltose binding proteins, glutathione S transferase, FLAG octapeptide and Streptavidin tags (Lichty *et al.,* 2005). Purification tags can dramatically influence expression, targeting, translocation or folding processes to the attached membrane protein (Woestenenk *et al.,* 2004). Larger fusion proteins have to be removed from membrane protein in order to proceed with structural and/or functional studies. Recognition sites for specific proteases [e.g. TEV (Tobacco Etch Virus) protease, enterokinase, thrombin or Factor-Xa] should be inserted within the linker region in between the fusion protein and the target membrane protein. At times the restriction site might not be accessible for the cleavage processes due to the region being folded or inserted into the membrane.

In spite of all these challenges, the field of membrane structural biology is marching towards a different dimension in this decade. Recently a number of membrane protein consortiums have been established. To name a few MPSi, NYCOMPS, E-MEP and SGC (Structural Genomics Consortium, Karolinska Institut.They comprise of integrated research activities from expertise groups ranging from protein expression, purification, crystallization, NMR, biophysical techniques and functional studies. These consortiums take advantage of using high through-put screening, expression and purification using automated techniques and latest robotic technologies for setting up crystallization trials which make membrane structural biology field very successful.

CHARACTERISTICS OF DENGUE VIRUS (DENV)

Among the membrane proteins one interesting class are the envelope proteins from dengue virus. Dengue virus (DENV) is an enveloped, RNA virus belonging to the flavivirus genus within the *Flaviviridae* family. DENV comprises of four antigenically distinct serotypes (DENV1-DENV4), which causes a wide range of diseases ranging from mild dengue fever to severe dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (Alvarez *et al.,* 2006; Wilder smith and Schwartz, 2005). It has been estimated that more than 2.5 billion people in over 100 countries are at risk of dengue infection (Whitehorn and Farrar, 2010) with several hundred thousand cases of life threatening DHF/DSS occurring every year (Gubler, 1998; Gubler, 2002). Presently there is no effective vaccine or antivirals for DENV infection and intensive research is still progressing for the development of vaccines (Murrell and Butler, 2011).

The dengue virus genome is one single-stranded, positive-sense RNA molecule and consists of a single large open reading frame of 10,200 nucleotides which encodes 3 structural and 7 non-structural proteins. The gene order is C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The viral envelope consists of lipid bilayers where envelope (E) and membrane (M) proteins are embedded. The E protein is 495 amino acids in length and is glycosylated in the dengue virus. The functional roles of E protein has been shown to be involved in virus attachment to cells and also involved in membrane fusion (Clyde *et al.,* 2006; Modis *et al.,* 2004). It has also been demonstrated to be highly immunogenic and is able to elicit production of neutralizing antibodies against wild-type virus (Gromowski and Barret, 2007; Rajamanonmani *et al.,* 2009). From the structural point of view, the envelope protein is important because to date the structure of full length protein has not been resolved because of the difficulties in obtaining it. Only the soluble part excluding the transmembrane domain part has been crystallized (Modi *et al.,* 2004; Bressanelli *et al.,* 2004). It is thus a challenge to obtain purified full length E protein. This

can be accomplished by using high-throughput expression, refolding, improved membrane protein purification and screening different crystallization conditions.

The dengue envelope protein comprises of 3 regions: Domain-I (DI), Domain-II (DII) and Domain-III (DIII). DI is the central domain; DII is the dimerization and fusion domain, while DIII is an immunoglobulin-like receptor binding domain (Mukhopadhyay *et al.,* 2005; Rey *et al.,* 1995). It has been proven that DIII domain is a receptor recognition and binding domain (Bhardwaj *et al.,* 2001; Zhang *et al.,* 2007). It is therefore interesting to exploit DIII herein for diagnosing dengue infections and in vaccine development. In order to obtain purified DIII protein for the above mentioned studies; we expressed, purified and characterized the DENV1 to DENV4 envelope protein DIII. The recombinant DENV1 to DENV4 envelope protein domain III (r-DIII) when expressed in *E. coli* is targeted to inclusion bodies. Here we discuss the methods for the isolation of purified protein from inclusion bodies using this model.

INCLUSION BODY FORMATION

The most commonly used system for expressing recombinant proteins is *E. coli* as it produces high level expression and the purification is relatively easy. However, most of the expressed proteins that are hydrophobic and mostly membrane protein form insoluble intracellular aggregates. These aggregates are known as inclusion bodies and are localized in the cell cytoplasm or periplasm (Hartley and Kane, 1988). These inclusion bodies can be observed under a light microscope due to their dense and refractile properties. They are also generally resistant to proteolytic breakdown but degradation products may still be observed. Inclusion bodies do not have biological activity and thus it is crucial to extract, solubilise and refold the protein to their native conformation. Formation of inclusion bodies has advantages, as separating them from the crude cell homogenate is easier and they contain high levels of the expressedprotein of interest (Burgess, 2009). Aggregation of expressed proteins occurs through different mechanisms; 1) The exposure of the hydrophobic regions of protein intermediates which are not properly folded and 2) self-association or chemical interaction with other similar proteins; and chemical degeneration (Wang *et al.,* 2010). Sample processing steps such as freeze-thawing and vigorous shaking could potentially destabilize the protein and expose their hydrophobic domains, thus enhancing aggregation. Moreover, variables such as temperature, light, pH and ionic strength of the solution are also critical factors to take into consideration.

ISOLATION OF INCLUSION BODIES FROM *ESCHERICHIA COLI* CELLS

Most methods for isolating inclusion bodies include breaking down the cells mechanically using French press or by sonication. In this case, a combined treatment of gentle enzymatic lysis using lysozyme followed by sonication is employed. During the process of cell disintegration via sonication, it is essential to keep the samples cool in ice to avoid heat denaturation of the target protein. The inclusion bodies, being higher densities, are sedimented from other cellular components by high speed centrifugation at 12,000 g for 10 min. To remove contaminating impurities, the isolated inclusion bodies are washed in buffer containing detergent Triton X-100. In addition to its washing properties for removal of non-specific bound cell proteins, Triton X-100 also promotes efficient protein folding into its native form. EDTA, a chelating agent is also a component included in the buffer system that helps to prevent metal-catalyzed air oxidation of cysteines present in the target protein. Thus avoids the formation of disulfide bridges between incorrect residues that would induce protein aggregation or misfolding (Dechavanne *et al.,* 2011). In addition, (Singh and Panda, 2005) described the use of sucrose gradient centrifugation as a preparative method for obtaining inclusion bodies of higher purity. Gel filtration is used to exclude inclusion bodies while the rest of the components of the crude homogenate penetrate the gel which composes of highly cross-linked agarose matrix with an exclusion limit (Gu *et al.,* 2002). An alternative to centrifugation for the isolation of inclusion bodies is cross-flow microfiltration (Batas *et al.,* 1999).

SOLUBILIZATION AND EXTRACTION OF INCLUSION BODIES

To distintegrate the inclusion bodies, various methods of solubilization are employed. These include highly concentrated chaotropic agents such as guanidium hydrochloride and urea, reducing agents such as dithiothreitol and detergents like sodium dodecyl sulfate and Triton-X-100. High concentration of these agents can denature the native-like secondary structures of inclusion body proteins and lead to random coil formation, thereby rendering the solubilized protein more prone to aggregation during the refolding process. It is thus of importance to apply an optimized method to obtain the bioactive target protein in its native structure when one isolates the protein from inclusion bodies. Other alternative procedures for isolating proteins from inclusion bodies can be achieved by mild solubilization of inclusion bodies at alkaline pH and 2 M urea (Singh and Panda, 2005; Patra *et al.,* 2000). Residual detergent may affect the purity of the refolded protein, therefore removing it thoroughly through extensive washing or using lower concentration of the denaturant would be a better option. Here, after extensive washing with the Triton-X-100, the small, white pellet of inclusion body was resuspended for 1 hour in 8 M urea in 20 mM Tris and 300 mM NaCl buffer (pH 8.0) which is sufficient to completely solubilize them.

METHODS OF AFFINITY PURIFICATION

After the protein is extracted from the inclusion bodies, it is subjected to Ni-NTA affinity purification as the r-DIII of envelope protein form DENV1-DENV4 has been cloned with an N-terminus Hexa-histidine tag. Three different methods namely Membrane adsorber, Histrap and packed bed column for affinity purification were used (Fig. 1).

Immobilized metal-ion affinity chromatography (IMAC) adsorber

The expressed recombinant proteins localized in the inclusion body are extracted and the sample is passed through an IMAC membrane adsorber (IMAC Adsorber, Sartobind- IDA 75, and Sartorius, Germany). The schematic representation of the membrane adsorber is shown in (Fig 1A). The membrane adsorber is immobilized with chelating ligand IDA (Iminodiacetic acid), charged with nickel ions and equilibrated with binding buffer. The target protein binds to the column through affinity tag (e.g. Histidine tag). To remove other contaminating proteins the column is washed with buffer consisting of low concentrations of imidazole. Elutions are carried out by using 0.2 or 0.5 M imidazole or decreasing pH gradient of 3.5 to 4. Further use of EDTA, an effective chelating agent, removes immobilized Nickel ion and can be regenerated again by passing Nickel sulfate through the membrane.
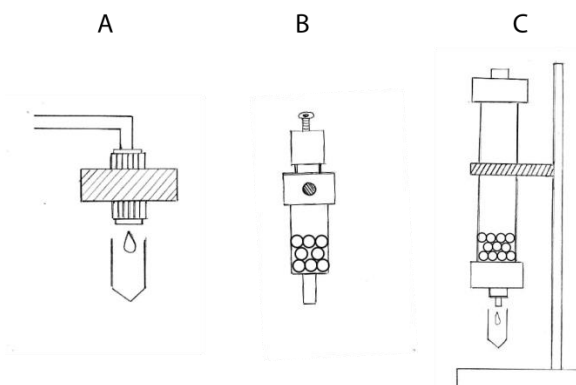
Fig. 1.Three different types of affinity purification for Histag proteins. (A) IMAC-Membrane adsorber (B) Histrap column and (C) Gravity flow-packed bed resin column.

The advantages of the membrane adsorber is, this system does not require a FPLC system for purification, using a positive displacement peristaltic pump the protein sample can be pumped in to bind the membrane (Tan *et al.,* 2010). Then the membrane can be washed to remove the non-specific bound proteins followed by elution of the target protein. The ability to control the speed of buffer passing through the membrane adsorber thus making the process fast and in addition it can be upgraded to industrial scale. But the refolding efficiency is under debate. This system can be useful when we purify proteins in larger quantities for raising antibodies in mice models or initial screening of diagnostics, where refolding and the purity (<90-95) is sufficient (Tripathi *et al.,* 2008). For achieving higher purity proper refolding conditions has to optimized followed by Fast Protein Liquid Chromatography (FPLC) using Gel filteration Chromatography (GFC) or Ion Exchange Chromatography.

After the Ni-NTA purification the eluted fraction corresponding to the DENV1-rDIII is analysed by SDS-PAGE to check the purity (Fig 2A). Although the procedure seemed to be faster, the final product showed that the proteins formed oligomers and aggregates. The Ni-NTA purified elutes are dialysed against PBS to remove imidazole and purified by Size Exclusion Chromatography (SEC) using Superdex G 75 5/150 GL gel filtration column using an Akta purifier FPLC system (GE Healthcare, UK) . The FPLC spectrum corresponding to DENV1-rDIII is shown in (Fig 2A) here it was observed that the protein forms mostly aggregates and multimers and only a small proportion existed as monomers. Using this procedure the yield of monomer obtained was 0.3 to 0.5 mg per liter of bacterial culture.
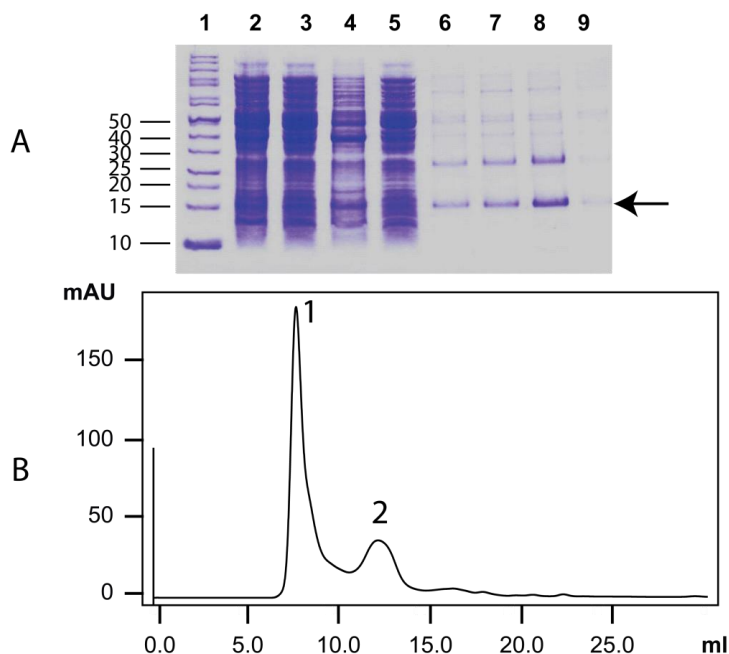
Fig. 2. Purification profile for Histag-DENV1-rDIII using membrane adsorber. (A) Coomassie stained SDS-PAGE gel corresponding to the flow-through (lane 2), wash (lanes 3-5) and Elutes (lanes 6-9). The expected molecular mass ~15 KDa is indicated by arrow. (B) FPLC-GFC profile for elutes from membrane adsorber showing the aggregates/multimers (1) and monomers (2). The black trace represents the UV absorbance at 280 nm.

Histrap columns are composed of extensively cross-linked agarose beads that are charged with nickel ions. The beads have a high binding capacity of 40 mg of His-tag recombinant protein per ml of medium (HisTrap FF crude from Ge Healthcare, UK). This system can purify as well as refold

recombinant proteins with histidine tags. The crude lysate sample can be loaded onto the column without pre-centrifiguation. Not only does the column has high binding capacity, it is also compatible with chaotropic agents like 8 M urea and 6 M guanidium chloride and additives such as 1 mM EDTA and 50 % glycerol. With the Histrap column, purification time is also reduced by connecting with Akta purification system which can be automated.
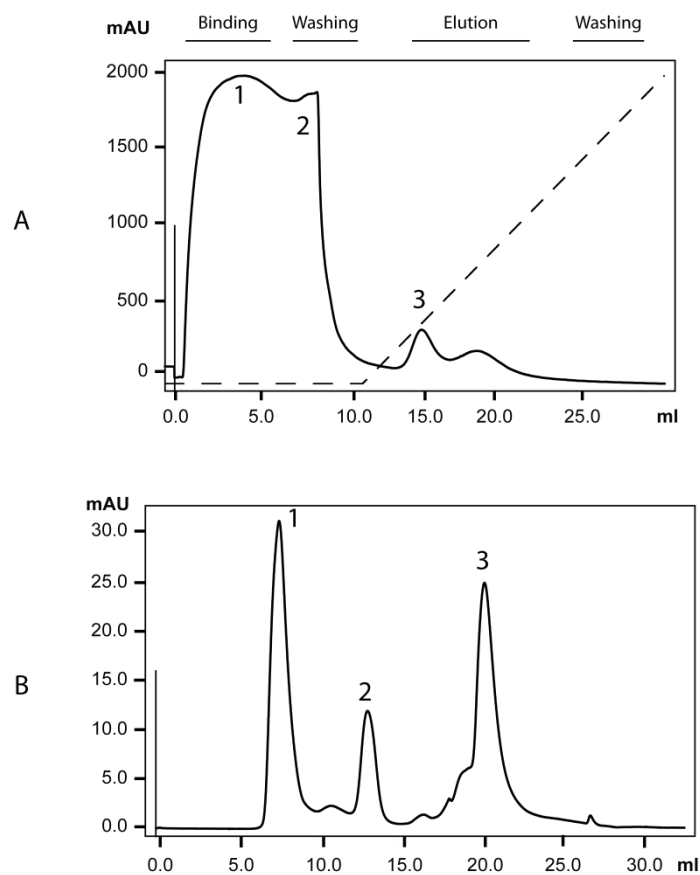
Fig. 3.Purification profile for histag-DENV1-rDIII using Histrap column. (A) FLPC chromatogram using Histrap column showing the different steps involved using the Histrap column are shown at the top. (B). FPLC profile for the eluates from His-trap column showing the aggregates/multimers (1), monomers (2) and imidazole (3). The black trace represents the UV absorbance at 280 nm (A&B). The broken line indicates the concentration of imidazole used in the various steps of purification (A) (20 mM for binding, washing and 500 mM for elution).

Elution is performed using 0.5 M imidazole which acts as a competitor ligand. The purification profile using the Histrap column is shown in (Fig 3A) The different steps involved are binding of the protein to the resin, washing the resin to remove non-specific binding proteins, elution of the target protein using imidazole and finally equilibrating or washing the column. Advantages in this technique are all the steps are automated, the flow rates of the buffers are controlled and the elution profile can be directly monitored using an Akta purifier system. The eluted sample is then subjected to gel filtration chromatography using FPLC and the spectrum is shown in (Fig 3B) The rDIII protein forms aggregates and multimers (60 %) and monomers (40 %). Using this procedure the yield of monomers obtained for DENV1-rDIII protein was 0.6 to 0.8 mg per liter of bacterial culture used. Previous reports showed that

Histrap columns have been used to obtain the DENV-rDIII protein from inclusion bodies although the analysis of aggregation/multimerization has not been analysed using gel filteration chromatography (Batra *et al.,* 2010).

Gravity-flow chromatography

Another one-step refolding and purification system tested is the use of Nickel-Nitriloacetic acid (Ni- NTA) column (Qiagen, USA). This system affinity purifies recombinant proteins with a polyhistidine tag. The mechanism works by introducing the lysate to the column containing the affinity matrix Ni-NTA agarose. Nickel ions which are bound to NTA bind tightly with the target protein in the lysate. The suspension column is then incubated on a rotator few hours to overnight in a cold room (4-8 °C) to maintain protein stability. The resin is then washed with a buffer containing low imidazole (20-30 mM) concentration and NaCl (300 mM). Lastly, the target protein is eluted using the same methods employed in IMAC adsorber as previously described using imidazole (0.5 M) or at low pH 4- 5 (Crowe *et al.,* 1995). Although this technique is dependent on gravity, the benefits are two-fold. Firstly, the Ni-NTA resin is reusable and can be re-equilibrated following a simple protocol. Secondly, there is high affinity and selectivity for recombinant proteins containing the polyhistidine tag, thereby achieving high purity of the target protein (binding varies from 5 to 15 mg per ml medium). The disadvantages are that if the lysate contains too high total protein concentration, extensive washing is needed and elution takes extremely long (5-6 hours) as it is gravity-dependent. Also, both the column and the Ni-NTA agarose are costly. Although reusable, the affinity resin has a limit to its usage. Using this procedure the yield of total rDIII protein obtained was 10 to 14 mg per liter of bacterial culture used. Further these purified proteins were subjected to various refolding conditions followed by gel filtration chromatography to obtain monomers. Comparison of the different properties for the three purification methods used for dengue envelope r-DIII protein is compiled and shown in (Table 1).

Table 1. Comparison of results obtained from IMAC-membrane adsorber, Histrap and Gravity flow-packed bed resin column (+ Low; ++ Medium; +++ High).

	Time	Efficiency	On-Column Refolding	Scale up	Aggregation	Reproducibility
IMAC-Membrane	Fast	++	+	++	+++	+
Histrap	Fast	++	++	++	++	++
Gravity flow	Slow	+++	+++	+++	+	+++
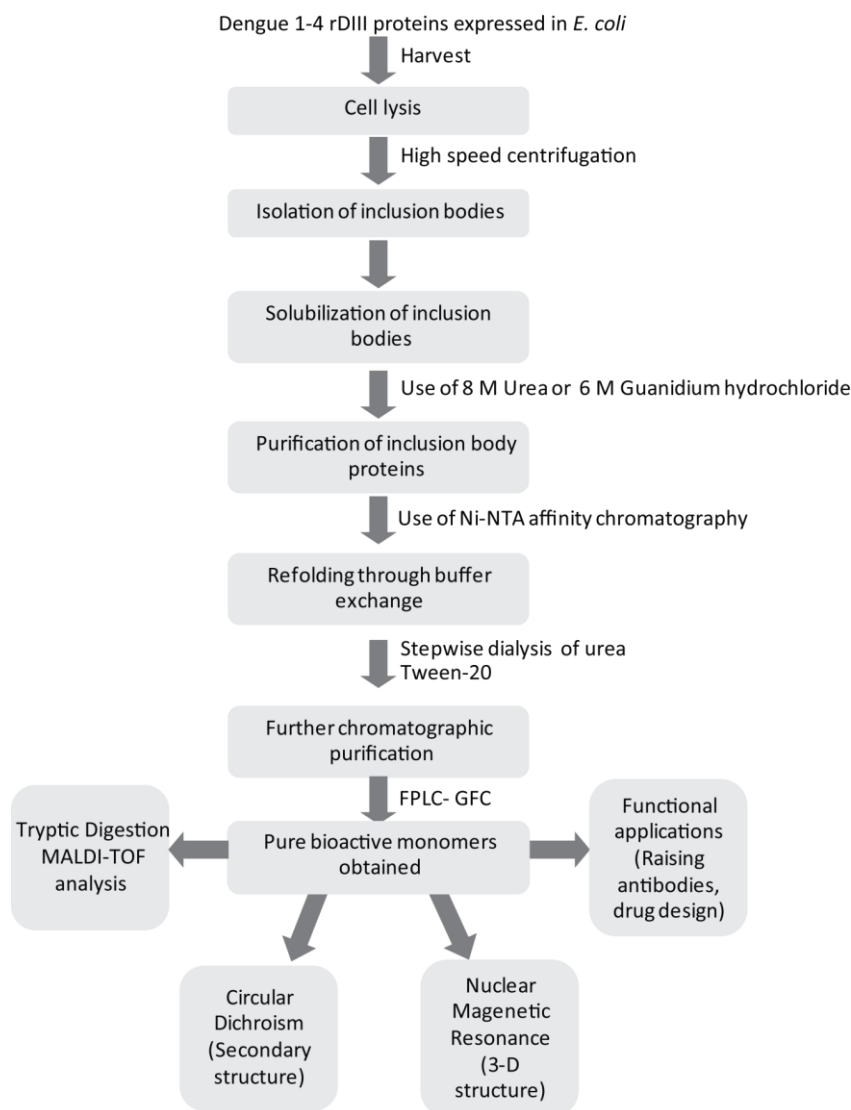
Fig. 4. Schematic diagram representing the different steps involved in the purification of DENV1-DENV4- rDIII from inclusion bodies.

CONCLUSION

With rapid advances in protein purification technologies as well as the understanding of protein folding pathways and nature of aggregation, it becomes easier to design experiments to obtain purified monomeric forms of the desired protein. Of course, optimizing conditions for maximum yield and purity is an absolute necessity. This is a trial and error process where environmental and solution variables are tempered with until a successful method is found. In our case, all dengue virus 1 to 4 r-DIII envelope proteins are subjected to rounds of tests in which they are highly expressed in inclusion bodies of *E. coli* cells. The r-DIII are isolated, extracted, purified by Ni-NTA affinity chromatography, refolded by stepwise dialysis and finally re-purified by FPLC to obtain bioactive monomeric proteins of maximum purity (Illustrated in Fig 4). The techniques described here for protein purification can also be further extended to the envelope proteins for the different members of *Flaviviridae*. The existing problem of aggregation is also drastically minimized by replacing harsh and highly concentrated denaturing or

chaotropic agents with mild solubilizing agents, additives and through one step refolding and purification methods. A challenge now posed is the up-scale industrial production of these pure products. Still, by obtaining the final refolded and purified protein, this takes us one step closer to exploit it in the field of therapeutics and development of a commercial vaccine that is safe and effective against dengue serotypes. Structural characterization and identification of small molecule inhibitors are under progress for the purified dengue viral proteins

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Production of Cyclosporine-A by Submerged and Solid State Fermentation Using Marine Fungus *Fusarium oxyforum*

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ABSTRACT

Fungi from the marine environments have been reported to be important sources of bioactive metabolites. Metabolites from fungi have attracted great deal of attention due to many healthy benefits such as immunomodulation, anticancer activity, prevention and treatment of cardiovascular diseases, antiviral and antimicrobial effects. Immunosuppressants are a class of drugs which are capable of inhibiting the body's immune system which include Cyclosporine A (CyA), tacrolimus, rapamycin and mycophenolate mofetil. Among these immunosuppressant's CyA is the first new generation of drugs with a specific site of action on the immune system. The present study involved the production and purification of CyA using *Fusarium oxysporum* isolated from mangrove sediments and investigated the effects of different fermentation media in cyclosporine-A (CyA) using Submerged Fermentation (SmF)and Solid State Fermentation (SSF). In addition, various carbon and nitrogen sources, fermentation time, temperature, pH and different concentrations of sea water along with different solid substrates were also optimized for the maximum production of CyA. Production of CyA was found to be 196mg/l and 1404 mg/kg by submerged and solid state fermentation respectively which leads to a conclusion that higher yields of CyA can be obtained by SSF. The wheat bran was found to be a supportive substrate for the production of CyA. Purification of CyA was carried out by silica gel column chromatography and characterization was confirmed by FT-IR and NMR spectroscopy.

Key words: Cyclosporine A, fermentation, *Fusarium oxysporum*

INTRODUCTION

Microorganisms are being used for thousands of years to supply useful products such as amino acids, nucleotides, vitamins, organic acids, solvents, vaccines and polysaccharides. A major segment of these products are represented by secondary metabolites such as antibiotics. Many antibiotics have been used for purposes other than killing or inhibiting the growth of bacteria and/or fungi. These include hypocholesterolemic agents, immunosuppressants, anticancer agents, bioherbicides, bioinsecticides, coccidiostats, animal growth promoters, and ergot alkaloids (Demain, 2000). Immunosuppressants are a class of drugs which are capable of inhibiting the body's immune system. Which have gained considerable importance in the world market include CyA, tacrolimus, rapamycin and mycophenolate mofetil (Survase *et al.,* 2011). Among these immunosuppressant's CyA is the first new generation of drugs with a specific site of action on the immune system. It is a family of neutral, highly lipophilic, cyclic undecapeptides containing unusual 11 amino acids. In addition to the immunosuppressive property, it has anti- inflammatory, antifungal, and antiparasitic properties (Dreyfuse, 1976 and Abd-Elsalam *et al.,* 2011). It is also used as a second-line drug in autoimmune diseases like rheumatoid arthritis, uveitis, bronchial asthma, and inflammatory bowel disease, including AIDS. Owing to its superior T-cell specificity, low levels of myelotoxicity and it play a major role in various types of cancers (Wei *et al.,* 2007).

The organisms that are known to produce CyA include T. inflatum (Agathos *et al.,* 1986), Fusarium solani (Sawai *et al.,* 1981), Neocosmospora varinfecta (Nakajima *et al.,* 1989), *Aspergillus* terreus (Sallam *et al.,* 2003) and some other rare actinomycetes (Park *et al.,* 2005). CyA is reported to be

produced by submerged fermentation (Agathos *et al.,* 1986), static fermentation (Balaraman and Mathew, 2006) and also solid state fermentation (SSF) (Survase *et al.,* 2009a). A number of fungi from the marine environments have been reported to be important sources of bioactive compounds (Biabani and Laatsch, 1998; Faulkner, 2002; Bugni and Ireland, 2004). Fungi, when cultivated on a solid substrate, under the conditions that are similar to their natural habitat could produce metabolites more efficiently than in liquid fermentation (Holker *et al.,* 2005). Most of the species belong to filamentous fungi, as these are best suited because of their ability to spread over and to penetrate inside the solid-substrate. The other advantage of using filamentous fungi is that the fungal mycelia synthesize and release large quantity of extra-cellular hydrolytic enzymes. Fungi and yeast seem to be the favorable one than bacteria because of their ability to grow on the substrates, which are low in water content. Due to high water activity requirement, bacterial cultures might not be suitable for solid state fermentation (Banerjee *et al.,* 2005). SSF has been used for the production of metabolites using several species of fungi including *Aspergillus, Rhizopus, Alternaria, Fusarium, Monilia, Mucor, Trichoderma* and *Penicillium.* Among these genus, Fusarium has great industrial and pharmaceutical applications for its richness in proteins and peptides (Verma *et al.,* 2003 and Khan *et al.,* 2007).

Cyclosporins are generally produced by *T. inflatum* in submerged fermentation (SmF) process and are reported to have low yield (Robinson *et al.,* 2001). To increase the product yield different approaches were made. To the best of our knowledge, there is just one single report (Nisha *et al.,* 2007) on comparative study on the production of CyA in SmF and SSF. Therefore, the present study was undertaken to screen a potential fungal species isolated from mangrove sediments and to find out the effects of different fermentation media in cyA using SmF and SSF. In addition, various carbon and nitrogen sources, fermentation time, temperature, pH and different concentrations of sea water along with different solid substrates were also optimized for the maximum production of CyA.

MATERIALS AND METHODS

Microorganisms

A total of 98 species were isolated and identified from the water and sediment samples collected from Pichavaram mangroves. Out of them, eight fungal species (*Aspergillus niger, A. terreus, A. flavus, A. fumigatus, Penicillium digitatum, P. fellutanum, Cladosporium herbarum* and *F. oxysporum)* were to be most dominant, which were screened for the production of CyA and fungal biomass. Maximum drug producing fungi were used for both solid and submerged fermentation.

Optimization of culture conditions for submerged fermentation

A fungal agar disc (6mm dia) of 7 days old culture was inoculated into a 250 ml of flask containing 100 ml of the autoclaved production broth medium. The culture flask were incubated at 30°C for 10 days and the necessary changes in the culture condition such as temperature (15, 20, 25, 30 and 35°C), pH (3.5, 4, 4.5, 5.0, 5.5 and 6.0), different concentrations of NaCl (0%, 1%, 1.5%, 2, 2.5% and 3%), different carbon sources (Glucose, Sucrose, Maltose, Mannitol, Fructose and Lactose) and nitrogen sources (Yeast extract, Peptone, Ammonium sulphate, Sodium nitrate, soybean meal and casein) were optimized to select the best one for the maximum production of CyA. The production medium containing (g/l) glucose 50.0g/l, Peptone 10.0g/l, KCl 2.5g/l, KH2PO4 5g/l, Distilled water 1000 ml and the range of pH 5.3 was used in this study.

Optimization parameters for solid state fermentation

Screening supports

Different solid supports in this study such as wheat bran, rice bran, gingili cake and groundnut cake were tested for the maximum production of CyA. These substrates were obtained from local flour mill.

Preparation of inoculums

The 5ml of sterile saline containing 0.1% tween 20 was added to the spores (approx. 2.5×10^7 spores/ ml) which were scrapped from 14 days slant, and mixed thoroughly. Then it was added to 10 ml of medium (glucose 2%, casein acid hydrolysate 1%, peptone 1%, malt extract 1%, pH 5.4) in a 50 ml flask and incubated in a rotary shaker at 150 rpm for 48 hrs at 24 °C. This was then inoculated to 200ml of medium in 1000 ml flask at 10% level (v/v) and incubated for 2 days under same condition. This was used as the inoculum for solid state fermentation process.

Preparation of solid substrates

100 grams of each substrate were taken in a 1000 ml flask and 70 ml of 0. 2 N HCl was added and mixed well and was autoclaved for 60 min at 121°C/ 15 psi. The flasks were then cool to room temperature and then 40% (v/w) inoculum was added, mixed thoroughly and incubated at 24 °C for14 days in a slanting position

CyA extraction

Submerged fermentation

The CyA extraction from the culture broth was carried out according to the method of Agathos et al. (1986). The fully grown mycelium was filtered through muslin cloth. The filtered mycelium was ground using culture filtrate in a cell homogenizer at 4oC and was subsequently kept frozen at −20°C until extraction time. Before extracting the sample, a concentrated solution of NaOH was added to reach the concentration of 1N and heated at 60°C for 30 min. This homogenized culture broth was extracted with equal volume of n-butyl acetate. The mixed sample was kept at 27°C in a rotary shaker at 250 rpm for 24 hours. The organic layer was separated and dried under vaccum, then dissolved in methanol to undergo the further analysis.

Solid State Fermentation

On the 14th day of incubation periods, 300 ml of ethyl acetate was added to each flask and were kept in a shaker at room temperature for overnight extraction. This was repeated on every alternative day up to give a colorless extract. This extracts were filtered through a Whatman No.1 filter paper to get a clear brown colored extract.

Analytical methods

Determination of Dry Cell Weight

Culture broth was filtered through pre weighed Whatman No.1 filter papers. The cells were then dried at 80°C to a constant weight prior to measuring dry weight.

Qualitative Determination of CyA by TLC

CyA was determined qualitatively according to the method described by Dreyfuse *et al.,* (1976) using pre-coated thin layer chromatographic (TLC) plates with silica gel GF-254. The methanol extract samples and standard CyA were spotted on the chromatograms and developed in the solvent system of hexane: chloroform: methanol (10:9:1). The TLC plates were removed from the solvent before they reach the end of the slide. The reaching point of the solvent marked and allowed to air dry for few minutes. Spots were visualized under vapors of iodine crystals. Then, the Rf value was calculated using this formula:

Rf = Distance traveled by components / Distance traveled by solvent

Purification of CyA by column chromatography

After filtration the crude ethyl acetate extract was evaporated to dryness and the dried residue was suspended in methanol and extracted twice with petroleum ether. During this process, all the oily

residues came into the petroleum ether layer. The methanol layer containing CyA was then evaporated to dryness and loaded on to a silica gel column. The silica gel column was washed with 500ml of hexane followed by 500ml of a hexane: chloroform (1:1) mixture. CyA was finally eluted with 1L of a mixture of hexane: chloroform: methanol in the ratio of 10:9:1. The TLC positive fractions containing CyA were pooled, evaporated to dryness and rechromatographed on fresh silica gel, maintaining the same sequence eluents. CyA was eluted with methanol, evaporated to dryness and recrystalized in -15ºC. The CyA obtained was subjected to FT- IR and NMR spectroscopy.

FT-IR spectroscopy

FTIR used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of CyA, glucosidic bonds and functional groups can be analyzed using FTIR spectroscopy. Samples (2mg) were mixed with KBr and pressed into pellets of 13 mm size and infrared spectrum was recorded using Perkin - Elmer IR spectrophotometer (Model IR 577).

NMR spectroscopy

The proton spectra at 400 MHZ and proton decoupled 13C NMR spectra at 100 MHZ were recorded at room temperature on DR x 400 NMR spectrometer using 10 mm sample tube. Sample were prepared by dissolving about 10 mg of the sample in 0.5 ml of chloroforml-d containing 1% TMS for 1H and 0.5 ml of sample in 2.5 ml of chloroform-d a few drop as TMS for 13C. The solvent chloroform-d also provided the integral field frequency lock signal.

RESULTS

Screening for CyA and biomass production

Totally eight different fungal species such as *A. niger, A. terreus, A. flavus, A. fumigatus, P. fellutanum, P. digitatum, Cladosporium herbarum* and *F. oxysporum* were screened for the maximum of CyA and fungal biomass production. Among these eight species were tested, *F. oxysporum* was produced maximum yield of CyA (196 mg/l) followed by A. terreus (124mg/l) and P. fellutanum (96mg/l) but other species like *P. digitatum, A. niger, A. flavus, C. herbarum* and *A. fumigatus* support only the biomass production but not in CyA production (Fig. 1). Based on both biomass and CyA production *F. oxysporum* was selected for further studies.
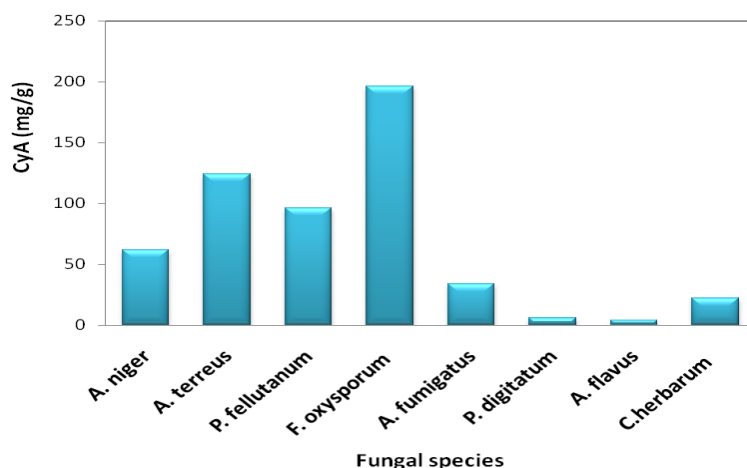
Fig. 1: Screening for CyA producing fungal species

Production of CyA by submerged fermentation

Results of submerged fermentation showed that the maximum production of CyA from *F. oxysporum* was 196mg/l and dry cell weight was 1.52 g/l of production medium. Different parameters like temperature, pH, incubation time, different concentrations of NaCl and various carbon and nitrogen sources were used to optimize the production of CyA and the results are as follows in table 1.

Table 1: Production of CyA

Functional groups	Position	^{1}H chemical shifts(ppm)	^{13}C chemical shifts(ppm)
Me Bmt	C=O	-	172.92
	α(CH)	4.70	62.77
	β(CH)	4.15	68.90
	γ (CH)	1.36	36.17
	δ (CH2)	2.31, 2.04	26.42
	6(CH)	5.36	130.24
	7(CH)	5.36	127.91
	8(CH3)	1.30	14.20
	9(CH3)	0.85	14.10
	V (OH)	2.18	-
Abu	C=O	-	173.38
	α(CH)	4.53	62.77
	β(CH2)	2.04	25.65
	γ (CH3)	0.85	12.60
	V-H	8.10	-
San	C=O	-	172.92
	α(CH2)	4.19, 4.14	42.84
	N-CH3	2.77	32.22
Me Leu 4,6,9& 10	C=O	-	172.92
	α(CH)	4.30	62.70
	β(CH2)	1.25	34.21
	γ (CH)	1.63	25.65
	δ,δ2(CH3)	0.85	29.70
	N-CH3	2.77	32.22
Val	C=O	-	173.34
	α(CH)	4.30	62.70
	β(CH)	2.68	31.95
	γ1,γ2 (CH3)	1.01	27.20
	N-H	8.10	-
Ala 7& 8	C=O	-	172.92
	α(CH)	4.30	62.77
	β(CH3)	1.30	23.40
	N-H	8.10	-
Me Val	C=O	-	173.34
	α(CH)	4.30	62.77
	β(CH)	2.31	31.95
	γ1,γ2 (CH3)	0.85	27.20
	N-CH3	2.77	32.22

Effect of different incubation temperature on the production of CyA

Results on effect of different temperature (15-35°C) on CyA production and dry cell growth was shown in Fig. 2. The optimum incubation temperature for maximum production of both dry cell growth (1.87 g/l) and CyA (319 mg/l00 ml) was observed at 25°C. After which a gradual decreases of both biomass and CyA were obtained with the increase of incubation temperature to 30 and 35°C.

Effect of different pH on the production of CyA

The initial pH of the medium was adjusted to a range of 3.5-6.5 by adding varying amounts of 1 N HCl and 1 N NaOH. It was observed that the values of dry cell weights (1.82g/l) and CyA (410 mg/l) were gradually increased up to pH 5.5 and decreased at the higher pH values was shown in Fig. 3. pH 5.5 was selected for further investigation throughout this study.

Effect of different incubation periods on the production of CyA

The time course profile of CyA production was shown in Fig.4. During the incubation period, the increase in fungal biomass was accompanied by hyper secretion of CyA. The higher values 1.51g of dry cell weights and 296mg/l of CyA were recorded at 9th day of incubation. Prolonged incubation hinders the production of biomass and CyA. So, the experimental fungus was grown for 9 days in the subsequent experiments throughout the present work.

Effect of different NaCl concentrations on the production of CyA

The effect of salinity on the mycelial growth and bioactive metabolite production was carried out by incubating the test fungus with various concentrations of NaCl ranging from 1 g/l to 3g/l into the production media. NaCl concentration of 2.5 g/l was recorded as optimal for maximum mycelial growth (1.67g/l) and improved active CyA (428 mg/l) production and above the concentrations of NaCl decreased both CyA and fungal biomass production (Fig. 5).

Effect of different carbon sources on the production of CyA

The effect of different carbon sources on production of CyA was investigated (Fig. 6). It was observed that, mannitol found to be the most effective substrate among the carbon sources were used and the highest CyA concentration 382 mg/ l was obtained with biomass production of 1.24 g/l. This was followed by glucose which supported maximum production of CyA (290 mg/l). But, Glucose supported maximal cell mass (1.92 g/l) when compared to mannitol as a carbon sources. Sucrose and maltose also supported satisfactory amount of CyA production. It was also observed that fructose and lactose supported only the biomass and were not able to create physiological conditions to produce CyA.

Effect of different nitrogen sources on the production of CyA

Various organic and inorganic nitrogen sources were added separately to the production medium. Of all the nitrogen sources were tested, peptone (426mg/l) supported the maximum CyA production followed by yeast extract (323mg/l) and ammonium sulphate (218mg/l). All though the experimental fungus produced low level of CyA when sodium nitrate (164mg/l) and soybean meal (108mg/l) were used as a nitrogen source, but it gave satisfactory level of cell mass. The production of biomass (0.58g/l) and CyA(117mg/l) was least supported by casein (Fig. 7).

FT-IR Spectroscopy

Infrared (IR) spectrum of this compound exhibited absorption at 3421 cm^{-1} indicating the presence of N-H group. The adsorption at 2926 and 2855 cm^{-1} are characteristics of aliphatic C-H stretch indicating the presence of methyl group. The adsorption at 1689 cm^{-1} stretching vibration was indicating the presence of amide carbonyl carbon (Fig. 8).
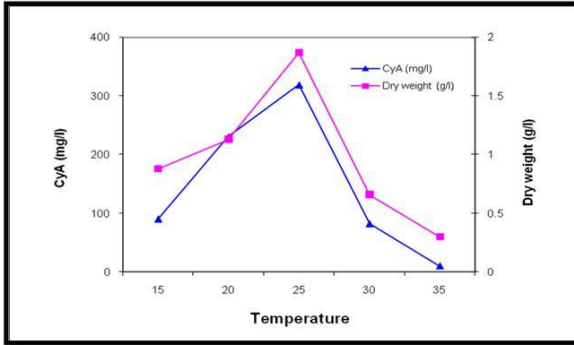
Fig. 2: Effect of different incubation temperature on the production of CyA
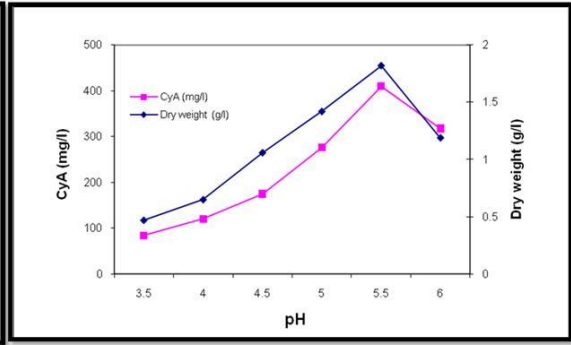
Fig. 3: Effect of different pH on the production of CyA
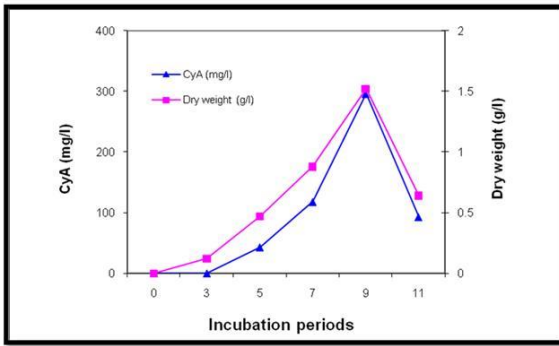
Fig. 4: Effect of different incubation period on the production of CyA
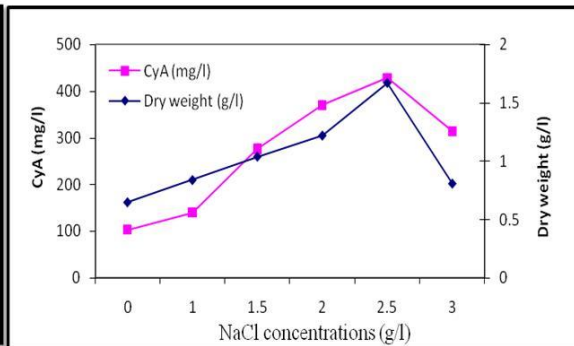
Fig. 5: Effect of different NaCl concentrations on the production of CyA
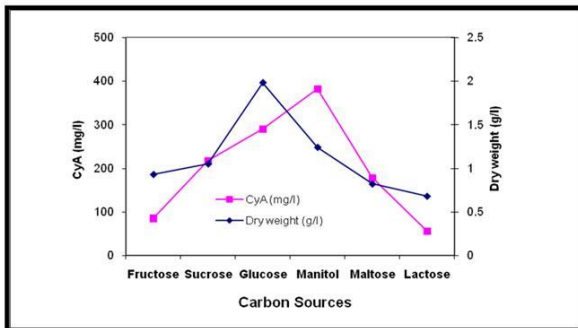
Fig. 6: Effect of different carbon sources on the production of CyA
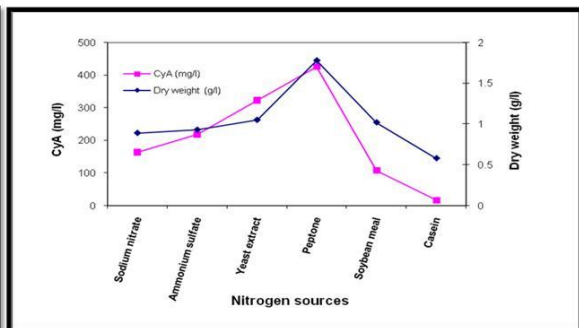
Fig. 7: Effect of different nitrogen sources on the Production of CyA
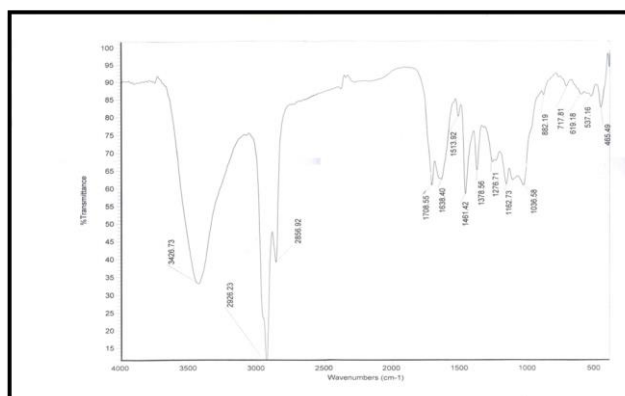
Fig. 8: FT-IR spectrum of CyA

NMR spectroscopy

^{1}H NMR (CoCl3): 1- MeBmt: δ 4.70(H-2), 4.15(H-3), 1.30(H-4), 2.31, 2.04(H-5), 5.36(H6, 7), 1.30(H-8), 0.85(H-9), and 2.18(OH); 2-Abu: 4.53(H-2), 2.04(H-3), 0.85(H-4), and 8.10(N-H); 3- San: 4.19, 4.14(H-2), 2.77(N-CH3).4, 6, 9 & 10- MeLeu: 4.30(H-2), 1.25(H-3), 1.63(H-4), 0.85(H-5), 2.77(N-CH3); 5- Val; 4.30(H-2), 2.68(H-3), 1.01(H-4), 8.10(N-H), 7, 8- Ala; 4.30(H-2), 1.30(H-3), 8.10(N-H), 11-Me Val; 4.30(H-2), 2.31(H-3), 0.85(H-4),2.77 (N-CH3). Peak values of ^{1}H NMR has showed in (Fig. 9).

The proton spectra at 400 MH 2 and proton decoupled ^{13}C NMR spectra at 100 MH 2 were recorded at room temperature on DRX 400 NMR spectrometer of CyA in CDCl3-d6. The signals in the ^{1}H NMR spectrum were assigned based on their positions, intervals and multiplicities. The sharp signals at 8.10 ppm (interval corresponds to three protons) is assigned to amide proton (N-H). Low frequency signals centered at 2.77 ppm is assigned to methyl proton attached to the nitrogen atoms (N-CH3 in Me Leu 4, 6, 9 &10 Me Val group). The up field singlet 2.18 ppm is assigned to OH proton appears around 1.25- 4.15 ppm, γ and δ protons appears in the range 0.85-2.31ppm respectively. In Me Bmt group H-6 and H-7 protons resonate at 5.36 ppm (integral corresponds to two protons) (Fig 10).
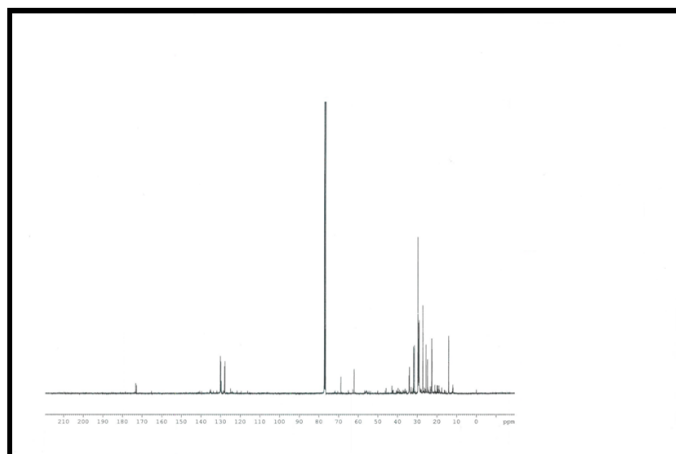
Fig. 9: ^{1}H NMR spectrum of purified CyA
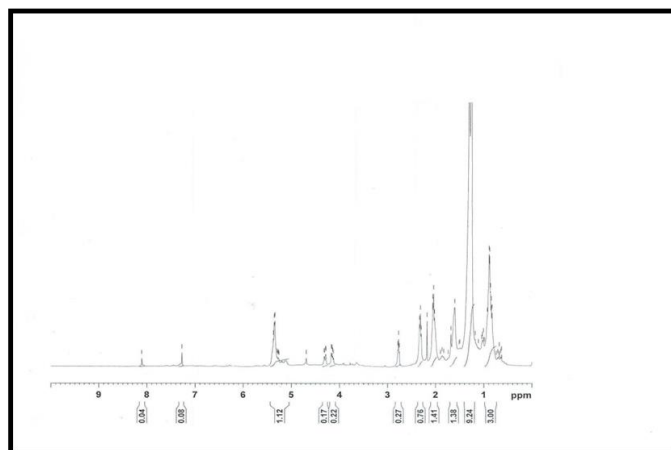
Fig. 10: ^{13}C NMR spectrum of purified CyA

The ^{13}C NMR spectrum downfield signals at 172.92 173.34 and 173.38 ppm are assigned to carbonyl carbons in the CyA moiety (Fig. 11). The frequency signal at 32.22 ppm is assigned to methyl carbon attached to nitrogen atom (N-CH3) in MeLeu 4, 6, 9 & 10 and MeVal groups. The α carbons appears around at 23.40- 68.90 ppm, γ and δ carbon observed in the range 12.60-36.17 ppm respectively. In MeBmt group C-6 and C-7 carbons resonate at 130.24 and 127.91 ppm. The ^{1}H and ^{13}C chemical shifts obtained in this manner are listed.

Effect of different solid substrates on the CyA production

In this study, locally available cheaper materials like wheat bran, rice bran, groundnut cake and gingili cake were used as solid substrate. Among the four solid substrates were tested, wheat bran was found to be best supportive in terms of biomass (24.4 g/kg) and CyA production (1404 mg/kg). In case of rice bran showed good fungal growth (20.6 g/100g) as well as drug production (1064 mg/kg), but it was comparatively less than the wheat bran. Whereas, groundnut cake and gingili cake enhanced only the fungal biomass of about 17.21 g/kg and 15.87 g/kg respectively, but not enhanced the CyA production (Fig. 11).
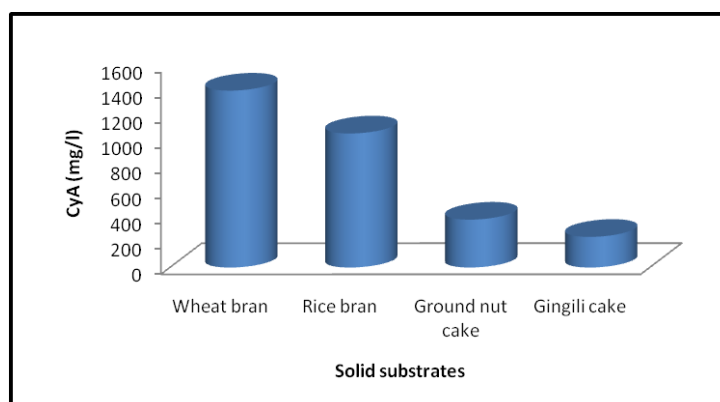
Fig. 11: Effect of different solid substrates on the production of CyA

DISCUSSION

The microbial production of cyclosporin has received little attention and developments in cyclosporin fermentation technology have been very few (Agathos *et al.,* 1987). SSF has gained importance currently due to its several advantages over submerged fermentation. SSF is being observed

as technique for producing higher yields of desired products. Various workers have shown interest for the production of high value microbial metabolites including antibiotics through SSF route (Survase *et al.,* 2011). From this study, it is clear that SSF has a distinct advantage over submerged fermentation for the production of CyA because of its increased yield. The selection of substrates and optimization of its concentration plays an important role in yielding the higher growth rates of microbes. Though there are many publications for the production of CyA using submerged fermentation (Nisha *et al.,* 2007), but very few reports are available on SSF.

It is evident from the result of fermentation studies much importance has been given to physical factors such as the temperature, pH, period of incubation and shaking etc., Variation in the type of carbon and nitrogen sources besides changes in pH, temperature, incubation period, shaking and inoculum size of the antagonistic fungal strain can greatly influence antibiotic biosynthesis (Thakur *et al.,* 2009). Antibiotic productivity can also decrease when media deficient in metal ions are used and culture vessels are incubated at high temperatures for long periods. Therefore, optimization and maintenance of proper culture conditions are necessary criteria to achieve maximum production of bioactive metabolites by an antagonistic microbial strain. Temperature is one of the most important factors influencing growth, sporulation and survival of microorganisms. On a certain optimum temperature microorganisms will grow normally and produce antibiotic. Our results showed that the maximum yield of CyA (319mg/l) and fungal dry weight (1.87g/l) at a temperature of 25ºC. This may be due to CyA synthesis was catalyzed by the enzyme cyclosporin synthetase, the activity of this enzyme was reported to be higher at 24ºC and thus may one of the reasons for higher production of CyA at 24ºC. Our results more less similar to the findings of Abdel-Fattah *et al*. (2007); Abd-Elsalam *et al*. (2011) and Lee *et al*. (2008) adjusted cultivated medium for maximum production of CyA from *T. inflatum* incubation at 27ºC. In general, fungus possessed a narrow range of temperature tolerance. The optimum conditions for growth, sporulation and secondary metabolites was occurred at 25°C, as reported by Sood (2011).

In the present study, maximum yield of CyA (410mg/l) was achieved at pH 5.5. Whereas Abdel-Fattah *et al.,* (2007) and Survase *et al.,* (2010 b) adjusted cultivated medium to pH 5.9 for maximum production of CyA from *T. inflatum*. In earlier studies the best yield of CyA was obtained at pH 5.7 (Lee *et al*. (2008); Sallam *et al*. (2005: Survase *et al*. (2009 b) and Ismaiel *et al*. (2010)). According to Survase *et al.* (2010 a) and Xiao-xian *et al*. (2009), lower pH resulted in higher yields, and higher pH of the medium resulted in lower yield of CyA. In general, most of the fungus grows well and maximum yield of secondary metabolites produced in the pH range of 4.5–5.5 (Sood, 2011). Additionally, Kobel and Traber (1982), Chun and Agathos (2001) and Manuela *et al*. (1996) reported that the best yield of Cyc A was obtained at pH 5.7.

In the present study, *F. oxysporum* was tested for the production of CyA at different time intervals between 1 to 11 days. The production of CyA was slow for the first five days of incubation period followed by a rapid increase in the 7 to 9th day of incubation. The maximum production was achieved on 9th day of the incubation period and after that analysis of the fungal mycelium has set in. Cyclosporin being an intracellular metabolite would be released into the culture medium if the fungus is permitted to grow beyond 9 days due to autolysis of the fungal cell wall. In the case of biomass, a rapid increase was observed upto 9th day of incubation which slowed down after 9th day of incubation period. The maximum CyA production has been reported to vary with respect to the fermentation time course, where Sakamoto *et al*. (1993) found that the best time span for CyA production was 10 days. Isaac *et al*. (1990) found that 12 days of growth is a good period for CyA production from *T. Inflatum* UAMH 2472. On the other hand, Traber *et al*. (1989) found that 14 days is the best period for maximal production of CyA from *T. inflatum* NRRL 8044. Effect of incubation period on the production of bioactive compound (Fumonisin B1) by *F. moniliforme* was investigated by Alberts *et al*. (1990).They observed that the

production of metabolite commenced after 12 days. The concentration of the metabolite in the medium continued to increase during the stationary phase and decreased after 9 days of incubation.

The present study proved that 2.5% of NaCl concentrations gave more yield of CyA (428mg/l) followed by 2% (276mg/l) and 1.5% (210mg/l) of concentrations. Our study also revealed that above the 2.5% of NaCl affect the CyA and fungal biomass production. This may be due to the fungus *F. oxysporum* was isolated from estuary sediment which required moderate amount of salts concentration for their growth as well as biomass production might be ultimately leads to the production of CyA. Similar observations were also made by Gogoi *et al.,* (2008) and they reported that NaCl concentration of 2.5–3.0 % was found to be optimum for maximum growth (4.3 mg/ml, 3.8 mg/ml) and production of bioactive metabolite (10.6l g/ml, 10.11 g/ml) by an antagonist fungus, *Fusarium* sp.

Impact of several carbon sources on biomass and CyA production was shown in. Among the six carbon sources were tested, mannitol supported maximum yi eld of CyA (382mg/l) followed by glucose (290mg/l), sucrose (217mg/l) and maltose (178mg/l). CyA production was least supported by fructose (85mg/l) and lactose (46mg/l). Dreyfuse *et al.,* (1976) used glucose (40 g/l) as carbon source and found that it produced 180mg/l of CyA using industrial strain of *T. inflatum*. Most of them reported to be glucose as a better carbon source for CyA and fungal biomass production (Sallam *et al.,* 2003) and Survase *et al.,* 2010b) as it was contrary to our results. Margaritis and Chahal (1989) developed fructose based medium for the production of CyA using *B. nivea*. They used fructose to minimize the catabolite repression and oxygen limitation in the pellets formed during the production stage to get maximum CyA yields. Survase *et al.,* (2010) reported that addition of maltose (2%) after 8 days of fermentation improved the CyA production. Abdel-fattah *et al.* (2007) used glucose (10 g/l), sucrose (20 g/l) and starch (20 g/l) in combination to give maximum CyA (110 mg/l) production using *T. inflatum* DSMZ 915.

Fungi mycelial dry weight and CyA production was obtained in submerged cultures in the study containing different complex nitrogen sources. Among the nitrogen sources were tested peptone supported the maximum CyA production (426mg/l) followed by yeast extract (323mg/l) and ammonium sulphate (218mg/l). The production of CyA was least supported by sodium nitrate, soybean meal and casein. In earlier studies reported that peptone was the most optimum nitrogen source for the production of CyA from *Tolypocladium* species (Agathos *et al.,* 1987 and Survase *et al.,* 2009b). However, Agathos *et al.* (1987) screened different organic nitrogen sources such as bacto peptone, soytone, and corn-steep liquor at various concentrations and reported bactopeptone at 10g/l to give the maximum production of CyA. Balaraman and Nisha (2006) used three nitrogen sources as casein avid hydrolysate (30g/l), malt extract (20g/l) and peptone (10g/l) in static fermentation to produce maximum CyA production after 21 days fermentation using *Tolypocladium* sp (VCRC F21 NRRL No.18950). Because of peptones are derived from animal milk or meat digested by proteolytic digestion and contains small peptides along with fats, metals, salts, vitamins and many other biological compounds. As peptone can provide vitamins and metals along with amino acids and it proved best supplement for drug enhancement in comparison to others (Tanseer *et al.,* 2011)

In the present study, wheat bran was found to be a best supportive solid substrates for biomass (24.66g/kg) and drug production (1404mg/kg) followed by rice bran (1064mg/kg), groundnut cake (380 mg/kg) and gingili cake (243 mg/100g). These results are more or less similar with the findings of Nisha *et al.,* (2008) screened different indigenously available and cost effective solid substrates and to found that wheat bran support maximum CyA (179mg/100g) and biomass production (22g/100g). This may due to that wheat bran is reported to contain high levels of polysaccharide but low levels of nitrogen. Apart from the presence of important nutritional components, physical characteristics of wheat bran also play vital role in fermentation process. Since polysaccharides have much higher moisture absorption potential

than lignin, wheat bran is able to retain higher moisture levels (De Souza and Peralta, 2001). Among the various solid substrates tested, wheat bran was observed to be a complete solid medium for the fungal proliferation and cyclosporin production in the SSF process. Cyclosporin being an intracellular metabolite is released into the medium during the stationary phase after the autolysis of the fungal cell wall. The particles of wheat bran have the ability to attract and retain the moisture levels mainly because of the hydrophilic functional groups in its organic matter are able to form hydrogen bonds with water molecules (Holker *et al.,* 2005). This ability of wheat bran promotes the fungal growth just as in the natural environmental conditions. Wheat bran proved a suitable substrate for the growth of filamentous fungi by SSF (Cavalcante *et al.,* 2008). Therefore, it was inferred from this study there was no need of additional nutrients in wheat bran medium for the production of CyA from *F. oxysporum.* It is inferred from the present study, it is possible to produce CyA by SSF at higher yields and this can be good alternative technology to submerged fermentation. The studies on the production of secondary metabolites confirm the use of wheat bran as the best choice of solid substrate both in terms of biomass and the secondary metabolite production in SSF bioprocess. Use of wheat bran for the production of this drug could economize the bioprocess will help in determining the economy of the wheat bran substrate for CyA production. A proper comparison of economics can be made only when scale up data are available.

ACKNOWLEDGEMENT

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Antioxidant, Antimicrobial Potential of Caulerpa racemosa Collected From Gulf of Mannar Marine Biosphere Reserve

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ABSTRACT

Macroscopic marine algae come into focus as the search for antioxidants increase. Here in we report the antioxidant activity of green seaweed *Caulerpa raemosa* extract derived using methanol extract method. The antioxidant parameter was evaluated by DPPH, hydroxyls radical scavenging, FRAP, and total antioxidant. The compounds present within the extract were identified using FTIR and GC-MS. Antimicrobial activity was analyzed by MIC assay. The extract shows 86.174 ± 2.1397 % scavenging activity in DPPH assay, antioxidant activity in FRAP assay was 86 % and 26.62 ± 0.15mg (AAE/g) activity in total antioxidant assay. The total phenol content was recorded as 48.8 ± 4.92 mg (GAE/g). In antimicrobial activity assay the extract showed more inhibition of Gram-negative than the Gram-positive bacteria. GC-HRMS spectroscopy showed the presence of 3, 7, 11, 15, Tetramethyl-z-hexadecan -1-ol, n-Hexadecanoic acid, Ethanol-2-(9-octodeceyroxy (Z)) and Sigmasterol, which are reported to have antioxidant and antimicrobial activities. FTIR analysis showed that the extract was high in phenolic content. From the biochemical and spectrum analysis of the *C. racemosa* CrF3 fraction, this green seaweed is a potential source of antioxidants and antimicrobial agents.

Keywords: Antioxidants, antimicrobial, *Caulerpa raemosa,*

INTRODUCTION

The (ROS) reactive oxygen species containing peroxides and oxygen ions were synthesized during electron transport chain, normal cellular respiration and excessive environmental stress (2013). Overload ROS, likely hydroxyl radicals (-OH), hydrogen peroxide (H_2O_2), superoxide anions (O_2-), singlet oxygen ($^1O_2$) were highly reactive to cause molecular oxidation, results in cancer, inflammation, diabetes, arthiritis, genotoxicity, mutation and impacts of cell damages leads to cirrhosis, necrosis, cellular death (Kourounakis, 1999; Gulcin, 2002; Buyyukokuroglu, 2001). The cells naturally overcome oxidative stress by antioxidant management system such as Superoxide dismutase (SOD), catalase, glutathione peroxide, alpha-1-microglobulin, Vitamin - C and Vitamin -E as primary scavengers (Aruoma, 1998; Sullivan *et al.*, 2011). To lever the problem various synthetic antioxidants like propyl gallate, butylated hydroxyl toluene (BHT), butylated hydroxy anisole (BHA), butyl hydroquinone used to scavenge excess ROS level (Devi, 2008; Sullivan *et al.,* 2011).Though the chemical synthesized antioxidant compounds are functionally active, they causing adverse side (kumar *et al.*, 2013).

As alternative, Numerous secondary metabolites includes alkaloids, polyphenolic compounds, terpenoids, tocopherols, carotenoids from seaweeds reported for chemo protective property as free radical scavenging, chelating activity (Heo *et al.*,2009; Hu *et al.,* 2008), protection against liver injury (Wong *et al.*,2000), anti-proliferative activity (Yuan *et al.,* 2006), antimicrobial agents (Devi *et al.,*2008). Thus, biocompatible Biomolecule compounds from natural source attract researchers to explore novel bioactive products.

About 15 million metric ton seaweed production was recorded in 2004, among that nearly 15-20% is contributed by Indian Ocean region (need recent status). Interestingly, the compounds extracted from seaweed are biodegradable, non-toxic, non-pollutant, and non-hazardous to higher animals (Chatterji *et al*, 2004; Dhargalkar and Pereira, 2005). Seaweeds extensive traditional use in folk medicine to reduce blood pressure, rheumatism, antibacterial, antiviral and natural dietary supplement among people of coastal livelihood particulate in South East Asian countries were recorded (Novaczek, 2001; Reine and Trono, 2001). *Caulerpa racemosa* a green algae has been recorded to grow in subtropical region, and rarely in few subtropical regions (Reine and Trono, 2001). The extensive report on *Caulerpa racemosa* compounds is scanty in recent research, present study targets on active fraction to demonstrate *invitro* antioxidant activity, bacteriocidal activity, and the GC-HRMS profiling of active fractions.

MATERIALS AND METHODS

Sample collection

The green seaweed C. racemosa samples were collected from the intertidal zone in mandapam coast, Gulf of Mannar reserve region (Lat $9^{0}27.776$ 'N, Long $79^{0}15.793$ 'E), Tamilnadu, India. The seaweed taxonomically identified, authenticated by morphological and molecular 18s characterization. The samples were washed with deionized water to remove surplus materials, shadow dried for extraction purpose.

Methanol extraction and fraction preparation

About 100 g of dried seaweed was mixed in 500 ml methanol and the mixture was incubated at 50 °C in an orbital shaking incubator for 24 h, under dark condition, followed with the supernatant extraction done using soxhlet apparatus for 12h at 37° C. The soxhlet materials were concentrated under rotary pressure evaporator to final as low as 5 ml. Further the crude concentrated dried, weighed and maintained at 4 °C for analysis. The crude concentrate mixture developed for fraction using silica gel column chromatography with increasing solvent polarity in DCM, chloroform and Methanol gradient system (Sullivan *et al.,* 2011) with modifications. All the fractions were analyzed for antioxidant, antibacterial property and the active fraction analyzed for GC-HRMS profiling.

FTIR analysis

The FT-IR spectrum was performed with range of 4000 to 400 cm-1 using automated Perkins Elmer infrared spectroscopy (USA). The functional chemical groups identified with mixture containing methonic concentrate and potassium bromide (KBr) pellet (Kumar *et al.,* 2013).

Antioxidant activity

DPPH radical scavenging

The 1,1- Diphenyl-2-picrylhydrazyl,(DPPH) free radical scavenging assay was carried out in triplicate with slight modification (Brand-Williams *et al.,* 1995). Different volumes (10, 20, 50, 100, 150 and 200µl) of 10 mg/ml crude extract were mixed with 100 µl of 0.15mM methanolic solution of DPPH. The preparation incubated for 30 minutes under dark and absorbance was measured at 500nM using ELISA microplate reader.

Radical scavenging activity (%) = [(Control + Blank – Sample) / Control] * 100

Ferric reducing antioxidant power (FRAP)

The Ferric reducing antioxidant power (FRAP) was determined with slight modification (Chu *et al.,* 2000). 0.1 M potassium phosphate buffer (pH 6.6) (2.5 ml) and 1% w/v potassium ferricyanide (2.5 ml) were mixed with 1ml of extracts of varying dilutions. The reaction mixture was incubated at 50 °C for 20 minutes, after which 10% w/v trichloroacetic acid (2.5 ml) was added. 2.5 ml of water and 0.5 ml

of 0.1% w/v $FeCl_3$ were then added to the reaction mixture. The solution was incubated at room temperature for 30 minutes and observed for color change. Later the absorbance was measured at 700 nm (Shimadzu, UV-160, Japan). The FRAP value was expressed in gallic acid equivalents per gram (GAE/g).

Hydroxyl scavenging activity

OH^- scavenging ability was measured according to a slightly modified literature procedure (Smirnoff and Cumbes, 1989). The reaction mixture contained 250 µl $FeSO_4$ (1.5 mM), 175 µl H_2O_2 (6 mM), 300 µl sodium salicylate (20 mM) and varying concentrations of the extracts. After allowing reaction for 30 minutes at 37 ^{o}C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm (Shimadzu, UV-160, Japan). OH^- scavenging ability was calculated from the log dose inhibition curve. All determination was carried out in triplicates. Ascorbic acid was used as a positive control.

Total phenolic content

Seaweeds, crude methanol extracts were estimated for total phenolic content with modified protocol (Sullivan *et al.,* 2011). Around 0.1 ml aliquot of sample was mixed with 2.0 ml of 2% Na_2CO_3 and allowed to stand for 2 minutes at room temperature in dark. After incubation 0.1ml of 50% Folin-Ciocalteau's phenol reagent was added and the reaction mixture was mixed thoroughly, allowed to stand for 30 minutes at room temperature under dark conditions. The absorbance of the samples was measured at 720 nm using UV-spectrophotometer (Shimadzu, UV-160, Japan). Total phenolic contents are expressed as gallic acid equivalents per gram (mg of GAE/g).

Test organisms

The test organism was obtained from Annamalai university medical hospital. Tamilnadu, India. The bacterial species includes *Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Vibrio cholera* were isolated in Nutrient agar medium and selectively cultured at 37 ^{o} C for 24 hrs. All the strains were confirmed by various biochemical and standard antibiogram tests adopted by Bergy's Manual for Determinative Bacteriology.

Antimicrobial assay by disc diffusion

To determine the antimicrobial activity of *Caulerpa racemosa* extract, four concentrations of each fraction extracts were prepared (50mg/ml, 25mg/ml, 12.5mg/ml, and 6.25mg/ml) and assessed by disc diffusions. From each concentrate, the fraction extract volume of 20 µl being impregnated on circular disc of 6 mm (whatman filter paper no 1) and placed on the MHA plate seeded with lawn culture of test organisms (0.5 Mc Farland turbidometry), under aseptic condition. All the plates were incubated in an upright position at 37ºC for 24 hours and the sensitivity of bacterial strains were assessed by measuring the diameter zone of incubation. Antibiotic susceptibility testing for measuring sensitivity and resistance of pathogenic bacteria was tested with the standard procedure (Kirby-Bauer method). The standard control antibiotics include ciproflaoxacin (10mcg) and streptomycin (10 mcg) was used in order to determine the possible inhibitory effect of crude extract on test organisms, also disc containing MeOH were also tested as negative control. Diameters between 12 and 16 mm were considered moderately active, and those with >16mm were considered highly active. The CLSI (formerly NCCLS) standard method was employed to determine antimicrobial susceptibility to reflect the resistant rate of pathogens (Saad *et al*., 2011).

Antibacterial activity by MIC assay

The minimum inhibitory concentration of active fraction tested against all pathogenic bacteria and MTCC strains (Indian institute of microbial technology, Chandigarh, India) using micro titer plate.

The active fraction compound was dissolved in DMSO and performed two fold dilutions to get final concentration of 0.25 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml, 0.156 mg/ml and 0.078mg/ml, in LB broth. About 50 µl of selected bacterial suspension was distributed eventually in 96-well plates, along with 100 µl of LB broth. Also the LB culture broth with ciprofloxacin, streptomycin was used as antibiotic control and DMSO as a solvent control system. The microtiter plates covered aseptically and incubated at 37°C for 24 h. The MICs were determined in duplicate with NCCLS standard guidelines and the results were expressed in mg/ml (Skov *et al*, 2000).

GC-HRMS analysis

Methanol extract was filtered using 0.2 micron filter and analyzed in GC-HRMS (Sophisticated instrumental facility, Indian Institute of Technology, Mumbai). The injection was carried out at 210°C in split mode and the helium as Carrier gas with constant flow rate of 1 ml / min. the GC oven temperature performed with start at 80° C for 2 min followed to final temperature of 280°C. Mass range analyzed between m/z 10 – 600 amu The methanol extract was analyzed under ionizing mode Ei+ and Ei- module in quadrupole analyzer, mass range from 10 - 350 amu. Identification of the individual components was carried out by comparison of mass spectra with literature data and by comparison of their retention indices (RI) (Yong, 2008).

RESULTS AND DISCUSSION

The *Caulerpa racemosa* were obtained from Gulf of Mannar, being extracted for bioactive compounds using different solvent system of increasing polarity. Among all fractions extract the MeOH Cr Fraction 3 exhibits potential activity for antioxidant and antimicrobial property. For the antioxidant analysis, DPPH assay has been widely used to evaluate the reducing activity of the substances (Cotelle *et al.,* 1996). The entire fractions were subjecting to the free radical scavenging activity. Among the assayed, 150 µl of 10 mg/ml concentrate *C. racemosa* fraction showed maximum DPPH radical scavenging activity of 86.174 ± 2.1397 GAE/10g (Fig. 1). In a similar study, Wang *et al.,* 2008 reported for free radical scavenging of compounds. Also the FRAP was determined by the modified method of Chu *et al.,* 2000. The FRAP value is expressed as Gallic acid equivalents (GAE/g). In the FRAP Assay the total antioxidant activity of *C. racemosa* extract was estimated as 89±1.98 GAE/g. The OH-scavenging effect of *C. racemosa* CrF3 extract was investigated using Fenton reaction and the results are shown as percentage inhibition. 50 µl of 10mg/ml *C. racemosa* exhibited the maximum activity of 64.329± 2.4961% as seen. Sullivan et al., 2011 reported that The Ferric reducing antioxidant potential with β-carotene bleaching activity of brown seaweed methanolic extract confirms the effective prevention of H_2O_2 mediated SOD depletion in cells along with DNA protective effects. There are several reports that these lipids show antioxidant activity, free radical scavenging (Purabi *et al.,* 2011; Meechaona *et al.,* 2007; Praveenkumar *et al.,* 2007). Subramaniam *et al.,* 2010 represents for the study on chemical synthesis of these similar compounds with compelling antioxidant activity.

The antibacterial activity of bioactive fraction CrF3 from *C. racemosa* extract was effective against the following gram positive and negative bacterial strains includes *Staphylococcus aureus, Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris* and ineffective against *Klebsiella pneumonia, Vibrio cholerae*. Among the tested the highest zone of inhibition observed against *Salmonella typhi* with 21 mm followed by 19 mm against *Staphylococcus aureus* and 18 mm against *Pseudomonas aeruginosa*, while the *E.coli* and *Proteus vulgaris* were moderately inhibited with zone of inhibition of 16mm and 15mm respectively (Table 3). From the results, it's evident that the *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa* were most susceptible strains of all tested pathogen. Following with minimum inhibitor assaying at different concentration ranging from 250µg/ml to minimum of 7.8 µg/ml, the CrF3 fraction analysis showed potent bacteriostatic and

bactericidal property. The results indicate the MIC value of bioactive fraction CrF3 as 15.6 µg/ml against *Salmonella typhi* and 31.2 µg/ml against *Staphylococcus aure*us exhibiting the highly potent antibacterial activity. While the *E.coli* and *P.aeurignosa* was moderately inhibited with MIC of 125 µg/ml and 62.5 µg/ml (Table 4). In a similar study the crude methanol extract concentrate shows moderate to good antimicrobial activity was against both gram positive and gram negative bacterial strains (Heo *et al.,* 2011). The total phenolic content of the *C. racemosa* methanol extract was measured via spectrophotometry using the Folin-Ciocalteau method and the results were expressed as gallic acid equivalents (GAE). The total phenolic content of the *C. racemosa* extract was estimated to be 48.8± 4.92 mg (GAE/10g).

In Table 2, the FTIR analysis of CrF3 confirms for possible biomolecules, exhibiting more number of alcohols and phenols with intensity of 92.438 followed by phenyl group and amino hydro halides (Fig. 2). The presence of phenyl, alkenes, amides and lactones provide evidence that the Crf3 have potential antioxidant and antimicrobial compound. Similarly, Polterait *et al*., 1997 reports for plant extract phenolic content for the antioxidant activity on active scavenging of free radical (Hafidh *et al.,* 2009; Chanwitheesuk *et al*., 2005). In early report that the lipids of crude and fraction concentrates were active scavenger of antioxidant activity (Meechaona *et al.,* 2007; Praveen Kumar *et al.,* 2007; Subramaniam *et al.,* 2010; Purabi *et al.,* 2011)**.**

To confirm the identity of successive eluted active fraction CrF3, the GC-HRMS coupled with NIST, WILEY8 and FAME library search were performed. The compounds were observed in the GC-HRMS analysis as shown in Fig. 3. The active methanol fraction extract showed high content of lipid group includes 3, 7, 11, 15, Tetramethyl-z-hexadecan -1-ol, n-Hexadecanoic acid, Ethanol-2-(9-octodeceyroxy (Z) and Sigmasterol (Table 1). From the results it was observed that the above compounds of CrF3 of MeOH extract exhibited greatest FRAP Values, potent antioxidant and displaying effective bactericidal activity on MIC assay. Therefore, it's possible that the antioxidant and antibacterial activity attribute to bioactivity of CrF3 of *C. racemosa extract.*

Table 1: GC-HRMS analysis of C. racemosa methanol extract

RT (min)	Compounds
923	3,7,11,15, Tetramethyl-z-hexadecan -1-ol
819	3-eiosyene
917	3,7,11,15, Tetramethyl-z-hexadecan -1-ol
741	Ethanol 2-(9-otodecenyloxy (Z)
759	1-(T)Asorbic acid Z- Dihhexadeanoate
765	n-Hexadecanoic acid
869	Sigmasterol

Table 2: FTIR spectrum shows Functional groups present in methanol extract of *C. racemosa*

S. No	Peak	Intensity	Functional Group identified
1.	472.56	84.693	Phenyl group c=c
2.	553.57	77.71	Nitriles C N
3.	590.22	76.338	Carboxylic acid
4.	624.94	73.773	Nitro
5.	677.01	70.086	Phosphines
6.	769.6	65.628	c-cl chloroform
7.	1026.13	47.615	Ether

8.	1114.86	72.921	C-NH2 primary alkenes
9.	1226.73	88.01	Ether
10.	1415.75	58.365	Carboxylic acid
11.	1456.26	56.855	Alkenes
12.	1647.21	50.052	Amides and Lactones
13.	2038.76	80.956	Unknown
14.	2198.85	80.005	Alkynes
15.	2331.94	84.072	Amino hydrohalides
16.	2360.87	82.143	Amino hydrohalides
17.	2520.96	73.987	Carboxylic acid
18.	2592.33	77.216	Thiols
19.	2835.36	37.144	Methyl
20.	2949.16	34.349	t-Butyl
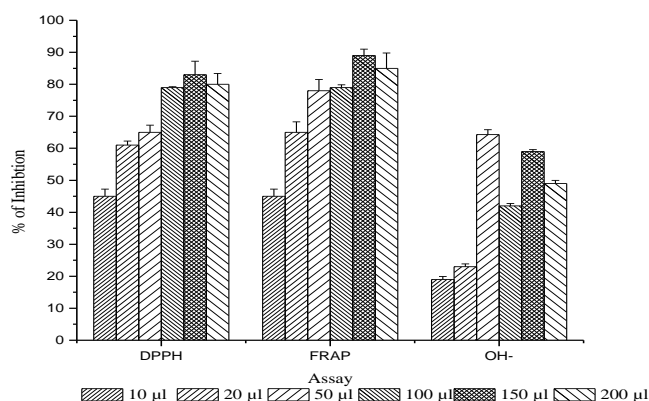
Fig. 1: The DPPH, FRAP and OH- Scavenging potential of *C. racemosa* methanol fraction extract. The activity is represented in percentage. Results are in Mean ±SD (N=6).
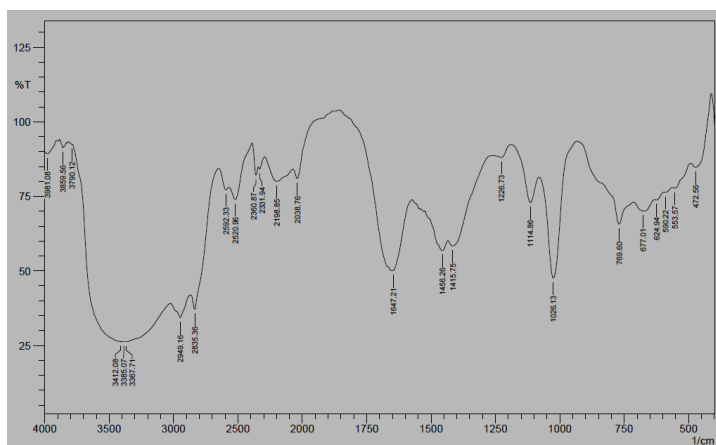
Fig. 2: FTIR spectrum of Methanol fraction extract of *C. racemosa*

Table 3: Minimum inhibitory Concentration assay of *C. racemosa* CrF$_3$ active fraction extract

S. No.	Test Organisms	Minimum inhibitory Concentration (µg/ml)		
		Active Fraction Cr F 3	Standard antibiotic control (Cip[+])	(Strp[+])
1	*Stap. aureus*	31.2 µg/ml	≤1	≤1
2	*Salmonella typhi*	15.6 µg/ml	≤1	≤1
3	*E.coli*	125 µg/ml	≤1	≤1
4	*P.aeurignosa*	62.5 µg/ml	≤2	≤2
5	*Kleb. Pneumonia*	NA	≤1	≤1
6.	*Proteus vulgaris*	≥250 µg/ml	≤2	≤2
7	*Vibrio cholera*	NA	≤1	≤1

NA*: Noacticity.

CONCLUSION

From the above results it is evident that *Caulerpa racemosa MeOH fractio* does possess high antioxidant and antimicrobial activity, it can be cultured and harvested using mariculture techniques to support the growing demand for antioxidant requirement in the food and pharmaceutical industry and potential source for novel bioactive agent.

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In Vitro Antibacterial Efficacy and GC-MS Profile of *Ocimum gratissimum* an Aromatic Medicinal Plant

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ABSTRACT

The prime aim of present investigation was focused on in vitro antibacterial, preliminary phytochemical screening and GC-MS analysis of O. gratissimum. The aqueous and four different solvents (Acetone, Chloroform, Hexane and Methanol) extracts were tested against both gram negative and gram positive bacterial strains (*Salmonella typhi, Shigella boydii, Shigella dysentriae, Staphylococcus aureus, Bacillus cereus, Lactobacillus acidophilus and Enterococcus faecalis*) by agar well diffusion method. The results highlights that the acetone extract exhibited significant antibacterial activity than other solvents. Preliminary phytochemical screening of indicates that the presence of saponins, phenols, tannins, flavanoids, glycosides, fixed oils. GC-MS analysis identified 45different bio-active compounds, among them three compounds are reflecting the highest peak values.

Key words: *Ocimum gratissimum*, antibacterial tests, phytochemicals, GC-MS

INTRODUCTION

Medicinal plants are gifts of nature to cure limitless number of diseases among human beings (Bushrabeegum and Gangadevi, 2003). The use of medicinal plants for the treatment of diseases has been practiced by human beings for many years and is still being widely practiced even today for prevention of various diseases (Kokwaro, 1993).Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects. So, antimicrobials of plant origin can be used as an alternative to synthetic drugs. Medicinal plants have enormous therapeutic potential to heal many infectious diseases by avoiding many side effects (Iwu *et al.,* 1999).Plants are rich source of wide variety of secondary metabolites (bioactive compounds) viz., tannins, phenols, terpenoids, alkaloids and flavonoids, which possess enormous antimicrobial properties (Suresh *et al.,* 1992). One such plant, *Ocimum gratissimum* L. belongs to Lamiaceae family; Clove basil, African basil, wild basil, tree basil and vernacular name is *Elumitchan tulsi*) is used for this study. The essential oil of *O. gratissimum* contains eugenol and shows some evidence of antibacterial activity (Silva *et al.,* 2010). Leaf extracts of *O. gratissimum* showed antidiabetic (Mohammed *et al.,* 2007), antitumor, anti-cancer (Veronica *et al.,* 1999), antidiarrhoeal (Obianime *et al.,* 2010), hepatoprotective (Ekpo *et al.,* 2009) and analgesic properties (Oparaocha *et al.,* 2010). Hence, the present study was planned to investigate antibacterial, phytochemical and GC-MS analysis of *O. gratissimum* leaf extracts.

MATERIALS AND METHODS

Collection of plant material

The healthy and fresh leaf material was collected from the highest altitudes of Yercaud hills, Salem district, Tamil Nadu and the plant name was identified by Dr. D. Natarajan, Assistant Professor, Department of Biotechnology, Periyar University, Salem. The herbarium specimen was deposited at the

Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem. The plant leaves were washed, air dried and powdered.

Preparation of Extract

100g of plant material was separately extracted with different solvents (acetone, chloroform, methanol, hexane and water) using soxhlet apparatus. The extract was filtered through Whatmann no.1 filter paper. The filtrate was concentrated in vacuumat 40ºC and it was allowed for evaporation under room temperature. After that, the extract was weighed to find the total yield of extracts per 100 gram of plant sample. The extracts were stored at -4º C for further analysis.

Sources of Organisms

The bacterial strains were collected from clinical labs in Salem district, Tamil nadu. Three Gram –ve organisms (Salmonella typhi, Shigella boydii, Shigella dysentriae) and four Gram +ve organisms (Staphylococcus aureus, Bacillus cereus, Lactobacillus acidophilus, Enterococcus faecalis) were used. The collected strains were sub-cultured in 30 days intervals on Muller Hinton Agar (MHA) slants. All the mother cultures were stored at 4º C refrigerator. The stock cultures of seven bacterial strains were inoculated in Muller Hinton broth and incubated at 37ºC for 12 to 14 hours prior to use.

Antibacterial activity

The antibacterial activity of leaf extracts of O. gratissimum were determined by agar well diffusion method with slight modifications (Natarajan *et al.,* 2005). About 30 ml of Muller Hinton agar was poured in a sterile Petri dish and it was allowed to solidify. Test organisms were swabbed on the surface of MHA plates using sterile cotton swab. A sterile cork borer (5mm diameter) was used to make 7 wells on each plate. In each plate different concentrations (50-250 mg/ml) of extracts (50 µl) were introduced into the wells. Standard antibiotic Ciprofloxacin (10µg/ml) was used as positive control. Sterile DMSO served as negative control. The plates were kept in incubator at 37ºC for 24 hours. After incubation, the diameter of inhibition zone was measured and recorded.

Preliminary Phytochemical Screening

Phytochemical screening of different solvents leaf extracts of Ocimum gratissimum were carried using standard procedures to identify the constituents as described by (Harborne, 1984, Trease and Evans, 1989).

GC- MS Analysis

The bioactive acetone extract of O.gratissimum was analyzed using GC-MS (GC Clarus 500 Perkin Elmer) with capillary column Elite-5(5% Phenyl 95% dimethylpolysiloxane). The injector temperature was set at 290ºC. The acetone extract (1mL) was injected and analyzed with a column length up to 30 m and column id 250 µm. Helium was used as carrier gas (1ml/min). The relative amount of individual components of the total extract expressed as percentage peak area relative to total peak area.

RESULTS

Antibacterial activity of *O. gratissimum*

The results of antibacterial activity of different concentrations of solvent extracts from *O. gratissimum* were done by agar well diffusion method (Table 1). All the extracts exhibited good activity at single concentration (250 mg/ml). Acetone extract showed maximum activity on *S. aureus* (18mm) and *L.acidophilus* (15mm) and minimum effect on *B. subtilis* (9mm). Chloroform extract showed the maximum activity on *E. faecalis* (12mm) and *B. subtilis* (11mm) and minimum effect on *L. acidophilus* (9mm). Methanol extract shows maximum activity on *S. aureus* (14mm) and *S. boydii* (13mm) and the minimum activity was notified on *S.typhi* (10mm). The maximum activity of hexane extract was reported on *S.typhi* (13mm) and *S.boydii* (11mm) and it followed by *S. aureus* (9mm). The results of water extract

shows the maximum activity on *S. typhi* (14mm), it followed by *S. boydii* (13mm) and minimum activity was observed on *S. dysentriae* (10mm). Ciprofloxacin was used as a positive control for all tested bacteria and DMSO was used as negative control. The significant antibacterial activity was noticed in acetone extract against *S. aureus* at 250mg/ml. Celso *et al.,* (1999) reported that the essential oil (EO) of *O. gratissimum* inhibited *S. aureus* at 0.75 mg/ml. Similar results were observed by (Prabhu *et al*., 2009) from the steam distillation extract of this plant had inhibitory effects on the selected bacteria. Previous studies reported the antimicrobial essential oils isolated from four *Ocimum* species grown in Rwanda, i.e. *O. canum*, *O. gratissimum*, *O. trichodon* and *O. urticifolium* respectively (Janssen *et al.,* 1989).

Table 1: Antibacterial activity against solvent extracts of dried leaves of *O. gratissimum*

Concentration of Extract (mg/ml)		Name of the organisms (Zone of growth inhibition (Diameter in mm)						
		S. aureus	*B. subtilis*	*S. Dysentriae*	*S. Typhi*	*L. acidophilus*	*E. faecalis*	*S. boydii*
Acetone	50	14	8	6	8	8	7	7
	100	15	8	7	8	8	8	7
	150	16	8	8	8	8	9	8
	200	17	9	9	9	9	9	9
	250	18	9	9	9	15	10	10
Chloroform	50	5	6	6	5	6	10	8
	100	5	8	7	6	7	11	8
	150	6	9	8	7	7	12	9
	200	7	9	8	8	7	12	9
	250	8	11	9	8	9	12	9
Methanol	50	7	6	6	6	8	6	6
	100	8	8	7	7	10	8	8
	150	10	9	8	8	11	10	12
	200	11	10	8	10	12	11	13
	250	14	11	10	10	13	14	13
Hexane	50	6	5	5	9	6	6	6
	100	7	7	6	11	7	8	7
	150	8	8	6	11	7	8	8
	200	9	9	8	12	8	9	9
	250	9	11	9	13	10	10	11
Water	50	9	8	7	7	7	8	6
	100	10	8	10	8	9	9	8
	150	11	11	10	11	9	10	10
	20	11	12	12	12	11	12	12
	250	12	13	10	14	12	13	13
Positive control	10μg	25	22	25	23	23	23	24
Negative control	50μl	-	-	-	-	-	-	-

Positive control – Ciprofloxacin, Negative control – DMSO

Qualitative Analysis of Phytochemicals

The results of qualitative phytochemicals analysis of *O. gratissimum* show all the extracts have some phyto constitutents (Table 2). The methanol and water extracts only reported to contain saponin and it was lack in other extracts (acetone, chloroforms and hexane). The presence of phenolic compounds was observed in extracts of acetone, chloroform and methanol except hexane and water. The phytosterol was found in extracts of acetone, chloroform, methanol and hexane but it was lack in water extract. All the

extracts did not contains protein, alkaloids, steroids and carbohydrates. Tannin was found in all the extracts except hexane. Flavanoid was obtained only in acetone, chloroform and hexane extracts. All the extracts contain glycosides and extracts of acetone, methanol and water contain fixed oils. The results of preliminary phytochemical analysis indicate the presence of saponins, phenols, tannins, flavanoids, glycosides and fixed oils. Similarly, identified alkaloids, glycosides, steroids and terpenoids from *O. sanctum* extracts (Himal *et al.,* 2008). The results were revealed that the Preliminary phytochemical screening revealed the presence of alkaloids, tannins, glycoside, saponin, resins, cardiac glycoside, steroidal terpens and flavonoids. These are believed to be responsible for the observed antibacterial effects (Nweze *et al.,* 2004).

Table 2: Phytochemical screening of different extracts of *O. gratissimum*

Phytochemicals	Acetone	Chloroform	Methanol	Hexane	Water
Proteins	—	—	—	—	—
Saponins	—	—	+	—	+
Phenols	+	+	+	—	—
Carbohydrate	—	—	—	—	—
Tannins	+	+	+	—	+
Alkaloids	—	—	—	—	—
Steroids	—	—	—	—	—
Flavanoids	+	+	—	+	—
Glycosides	+	+	+	+	+
Fixed oils	+	—	+	—	+

+ = Present, - = Absent.

GC-MS Analysis

The results of GC-MS analysis of *O.gratissimum* reflected 45 compounds. The name of the compounds, retention times and their percentages are listed in table 6.The peak values and structural elucidation of each compound was depicted in the figures 1. The major compounds were identified as phenol, 2- methoxy -3 (-2- propenyl), Estrogole, Squalene (peak values are 59.3316, 6.8218 and 4.7581 respectively) and rest of them are minor compounds. Similarly (Ankur *et al*., 2011), reported major chemical compounds in Ocimum species by GC/MS as methyl chevicol; 70.04%, followed by linalyl acetate; 22.54%, Camphor; 56.07%, DL‐limonene;13.56% and camphene; 7.32% . The results from other studies were suggested that the volatile oil (mostly phenols, particularly thymol) of this plant is responsible for its reported antimicrobial action against microbes (Olivier, 1960, Sainsbury and Sofowora, 1971). Previous study has been reported various components like, Thymol, eugenol, methyl chavical (Nadkarni, 1999), Gratissimol (Satyavati et al., 1987), Pentoses, hexoses, uronic acid and lipids (Tharanathan and Shamanna, 1975), Eugenol, methyl eugenol, cis-ocimene, trans-ocimene, pinene, camphor, germacrene- D, trans-carypohyllene, farnesene and l-bisabolene (Matasyoh, 2007), Eugenol, bisaboline and thymol (Janine et al., 2005), isolated from Ocimum species. The overall outcome of this study highlights that the acetone extract of this plant having better antibacterial activity.

Table 3: List of Chemical components identified by GC-MS analysis

S. No.	Peak name	Retenti-on time	Peak Area	%Pea k Area	Formula	Molecula r weight
1.	Terpineol, cis-á	8.15	4476065	0.6173	C10H18O	154
2.	2,6-Dimethyl-1,3,5,7-octatetraene, E,E	9.35	864124	0.1192	C10H14	134

3.	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]	13.78	1679358	0.2316	C15H24	204
4.	Phenol, 2-methoxy-3-(2-propenyl)	14.07	43021801 6	59.331 6	C10H12O2	164
5.	Copaene	14.32	6920956	0.9545	C15H24	204
6.	Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl)-, [1S-(1à,3aà,3bá,6aá,6bà)]	14.51	2814700	0.3882	C15H24	204
7.	Vanillin	14.85	12092452	1.6677	C8H8O3	152
8.	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]	15.17	2137530	0.2948	C15H24	204
9.	Phenol, 2-methoxy-4-(1-propenyl)	15.69	2125750	0.2932	C10H12O2	164
10.	Phenol, 2-methoxy-4-propyl	15.91	875506	0.1207	C10H14O2	166
11.	6-Methoxy-3-methylbenzofuran	16.23	1361998	0.1878	C10H10O2	162
12.	1-(4-Hydroxybenzylidene)acetone	16.36	523350	0.0722	C10H10O2	162
13.	Phenol, 2,4-bis(1,1-dimethylethyl)	16.72	1173815	0.1619	C14H22O	206
14.	1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclodecadiene	16.90	458001	0.0632	C15H26O	222
15.	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)	16.97	1563357	0.2156	C15H24	204
16.	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	17.19	2050806	0.2828	C10H12O3	180
17.	trans-Z-à-Bisabolene epoxide	17.55	2959138	0.4081	C15H24O	220
18.	Caryophyllene oxide	18.14	12625229	1.7412	C15H24O	220
19.	2-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl	18.30	2385054	0.3289	C10H16O	152
20.	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl	18.55	5620851	0.7752	C15H24O	220
21.	Aristolene epoxide	19.11	2896297	0.3994	C15H24O	220
22.	Megastigma-4,6(Z),8(Z)-triene	19.19	381823	0.0527	C13H20	176
23.	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl	19.26	2780751	0.3835	C15H24O	220
24.	Ledene oxide-(II)	19.79	10515224	1.4502	C15H24O	220
25.	4-Hydroxy-2-methoxycinnamaldehyde	20.53	12330986	1.7006	C10H10O3	178
26.	1-Nonadecene	21.09	748316	0.1032	C19H38	266
27.	Isoaromadendrene epoxide	21.68	1534674	0.2116	C15H24O	220

28.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	21.80	14398741	1.9857	C20H40O	296
29.	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1ar-(1aà,4á,4aá,7à,7aá,7bà)]	21.93	1703200	0.2349	C15H26O	222
30.	Aromadendrene oxide-(2)	22.46	11477791	1.5829	C15H24O	220
31.	Acetic acid, 2,6,6-trimethyl-3-methylene-7-(3-oxobutylidene)oxepan-2-yl ester	22.84	704293	0.0971	C16H24O4	280
32.	Phytol	25.85	1570295	0.2166	C20H40O	296
33.	Estragole	34.35	49465304	6.8218	C10H12O	148
34.	Acitretin	34.84	6385179	0.8806	C21H26O3	326
35.	Benzene, 1-methoxy-4-(1-propenyl)	35.92	1382952	0.1907	C10H12O	148
36.	Ethyl 2-(p-methoxyphenyl)-4,5-methylenedioxycinnamate	37.40	31248926	4.3096	C19H18O5	326
37.	Eugenol	38.06	4740368	0.6537	C10H12O2	164
38.	Squalene	38.93	34501652	4.7581	C30H50	410
39.	Papaveroline, 1,2,3,4-tetrahydro-3-O-methyl-	39.21	2472769	0.3410	C17H19NO4	301
40.	3-Hydroxy-4-methoxyphenethanamine, N-phenacetyl	39.33	5151019	0.7104	C17H19NO3	285
41.	4-(2,2,6-Trimethyl-7-oxabicyclo[4.1.0]hept-4-en-1-yl)pent-3-en-2-one	39.64	2060000	0.2841	C14H20O2	220
42.	Acetic acid, 5-(2,2-dimethyl-6-oxocyclohexylidene)-3-methyl-pent-3-enyl ester	40.16	1732052	0.2389	C16H24O3	264
43.	Vitamin E	43.05	6054980	0.8350	C29H50O2	430
44.	Stigmasterol	45.29	5248208	0.7238	C29H48O	412
45.	á-Sitosterol	46.41	18696142	2.5784	C29H50O	414
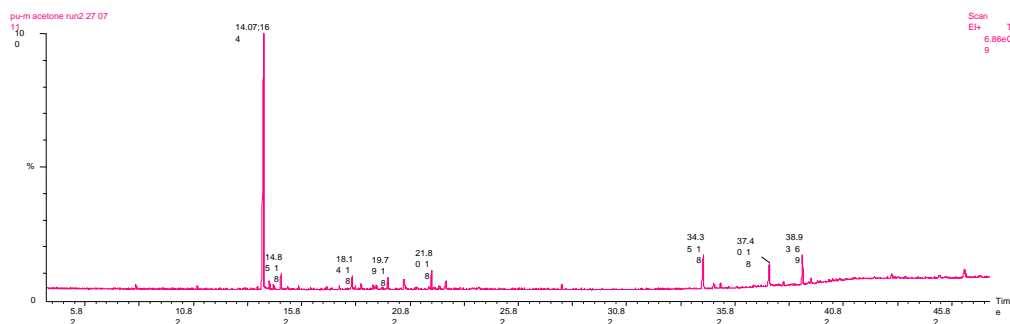
Fig. 1: GC-MS Chromatogram of acetone extract of *O.gratissimum*

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Microbial fuel cells: An Actinobacterial Mediated Novel Approach For Power Generation

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ABSTRACT

Microbial fuel cell (MFC) is a novel method for treating waste water by microbes to produce electrical current. In that microbes acted as a catalyst, which can promote biodegradation of organic matters and simultaneously it produce an electrical current. A microbial fuel cell is a bioreactor that converts chemical energy in organic compounds to electrical energy. The main objectives of this study was to utilize waste water and waste product rich in ions or organic content as a potent source for generating electrical current by using actinobacterium. The electrodes used for this MFC reactor are zinc electrode in anode chamber and copper electrode in cathode chamber. In this study, nearly 1.5 mV of current generated at the end of 5th day reaction, which can be measured by digital volt meter. The optimization study on the ionic exchangers could be carried out. In the anode, actinobacterial biomass are attaching on to it and forming the bio-films, which play an essential role in this MFC system to generate electrical current. The potent actinobacterial strain has identified as, *Streptomyces silaceus* (KBA8) based on the physico-chemical and molecular studies. The present investigation is a novel attempt to generate current by using microbial system as an electron donor.

Keywords: Microbial fuel cell, *Streptomyces silaceus*, bio-film, electrical current.

INTRODUCTION

A fuel cell is a tool that converts the chemical energy from a fuel into electricity through a chemical reaction with oxygen or another oxidizing agent. The first fuel cells were invented in 1838. They were used to it in power fuel-cell vehicles, including forklifts, automobiles, buses, boats, motorcycles and submarines. Energy saving programs is being implemented as the global demand for energy is rapidly increasing. Energy demand has been projected to grow more than 50 % by 2025. The idea of using microorganisms as catalysts in an MFC have been explored since the 1970s and 80s by Suzuki *et al.,* (1978) and Roller *et al.,* (1984). Microbial fuel cells (MFCs) are an electrochemical system that derives current from bacteria or mimic bacterial communication found in nature. It can be grouped into two categories; mediator and mediator-less. It is a device that used bacteria as the catalyst to oxidize organic and inorganic matter, resulting with generation of electrical current (Singh *et al.,* 2010). The chemical reactions transfer electrons from bacteria to anode. The first MFC was demonstrated in 20th century by using a mediator. The MFC bacteria typically have electrochemically active redox protein such as cytochromes. On outer membrane that can move electrons directly to anode. In most MFCs, the electrons that reach the cathode combine with protons that diffuse from the anode through a separator and oxygen provided from air; the resulting product is water (Rabaey*, 2005). However, these devices have recently become attractive again for electricity generation, providing opportunities for practical applications (Schroder *et al*., 2003; Liu and Logan*, 2004; Lu *et al.,* 2009). MFC is considered to be a promising sustainable technology to meet increasing energy needs, especially using wastewaters as substrates, which can generate electricity and accomplish wastewater treatment simultaneously, thus may offset the operational costs of wastewater treatment plant (Lu *et al*., 2009). The use of microbial fuel cells (MFCs) is a promising approach for direct production of electric energy or other energy carriers such as

hydrogen gas from various organic substrates (Logan, 2009; Pant et al., 2010). Sewage sludge has also been studied in MFCs for electricity generation. Based on the views, the present study is a novel attempt to generate electrical current by using microorganisms in general; actinobacterium in particular.

MATERIALS AND METHODS

Sample collection

The bauxite soil samples were collected from Kolli Hills (*Lat 11° 20' N; Long 75° 20' E*), Eastern Ghats, and Tamil Nadu. The samples were collected in polythene bags in aseptic condition. The soil samples were immediately transported to laboratory for isolation of actinobacteria and physico-chemical characterization (Radhakrishnan *et al.,* 2007).

Isolation of actinobacteria

Air dried, one gram of soil was dissolved in 10 ml of sterile distilled water and mixed thoroughly. Then the soil sample suspension was serially diluted upto 10^{-7}. The each diluted suspension spread over Starch Casein agar (SCA) plates incorporated with Nystatin and Nalidixic acid for avoiding fungal and bacterial contamination at the pH of 8.0 (Radhakrishnan *et al.,* 2007). All the plates were incubated at 28°C for seven days. The different suspected colonies were identified and inoculated on ISP2 medium for further studies (Shriling and Gottileb, 1966).

Oxidative fermentation test

The oxidative stress (aerobic or anaerobic) of actinobacterial strains were tested by Oxidative fermentation test. The OF basal medium was prepared in test tubes, and stabbed the actinobacterial cultures into each tubes. After stabbing, the paraffin wax was overlaid on the test tubes to avoid aeration and incubated at 28°C for 3 to7 days. The medium colour was changed green to yellow indicates the presence of facultative anaerobes (Lemos *et al.,* 1985).

Designing and working of MFC reactor

Two chamber MFC was constructed by using sterile plastic containers, which is acted as anode and cathode chambers. Each chamber consists of 1000 ml working volume. These two chambers were joined by a pipe which contains 50 ml of salt bridge. The joints were covered with M-seal to avoid leakage from the chambers. Two similar sized cylindrical copper and zinc electrodes were used in anode and cathode compartments respectively and the approximate length of each electrode was 6.5 cm. These two electrodes were connected with copper wires, which in turned connected to external digital multi meter. The anode chamber consists of 100 ml starch waste water (Rice wastewater and potato waste, 100 ml of phosphate buffered saline, 5 g of dextrose, pH 7.5). The cathode chamber contains, 5 g of 100 mM potassium ferricyanide prepared in 100 ml of phosphate buffer saline. The rice wastewater was inoculated aseptically with 10 g of actinobacterial strain (Holmes *et al.,* 2004).

Measurement and analysis of MFC reactor

The generated electrical current was measured using a digital multimeter (MASTECH M-830B, Russia) and converted to power according to the following formula, $P = iV$, where P = power (W), i = current (A), and V = voltage (V). The influent and effluent characteristics such as, COD, pH were monitored according to Standard Methods.

Optimization of ionic exchangers

The optimization of anode and cathode exchangers could be studied by suitable method described by Liu and Logan, (2004). In that, different concentration of actinobacterial biomass (5 to 25 g) and potassium ferricyanide (2.5 to 12.5 g) could be taken for the optimization study.

Identification of potential actinobacteria

Phenotypic characteristics such as micromorphology, cultural, physiological characteristics and molecular characteristics were studied by adopting the standard methods (Shiriling and Gottlieb, 1966; Hasegawa *et al.,* 1983; Radhakrishnan *et al.,* 2013). In molecular characterization, the genomic DNA of the isolate was extracted by using phenol chloroform extraction method and agarose gel electrophoresis used to verify the DNA quantity and quality, then the 16S ribosomal RNA was amplified by PCR technique with taq DNA polymerase and primers 27f (5''AGAGTTTGATCMTGGCTCAG3'') and 1492R (5'' TACGGYTACCTTGTTACGAC TT 3''). The conditions for thermal cycling were as follows, denaturation of target DNA was 95ºC at 4 minutes, followed by 30 cycles at 94ºC for one minute, primer annealing at 50ºC at one minute, and primer extension at 62ºC at one minute. At the end of cycling the reaction mixture was held at 72ºC for ten minutes and then cooled to 4ºC. The PCR product was sequenced by automated sequencer (ABI3730, eppendrof master cycler personal). The sequence was compared with similarity with the reference species of actinobacteria containing in genomic databases using the NCBI BLAST available at http://www.ncbinlminh.gov/ and the phylogenetic tree was constructed for identification of family distances using MEGA 4 software.

RESULTS

In the present study, totally 13 actinobacterial strains were isolated from the three bauxite mine soil samples. The phenotypic characteristics of actinobacterial strains were studied (Table 1).

Table 1: Phenotypic characteristics of actinobacterial strains

S. No	Strains	Appearance	Aerial mass colour	Aerial mycelium	Substrate mycelium	Reverse side pigment	Soluble pigment
1	KBA1	Powdery	White	+	+	+	-
2	KBA2	Powdery	Pale white	+	+	+	-
3	KBA3	Powdery	Yellow	+	+	+	-
4	KBA4	Powdery	Grey white	+	+	+	-
5	KBA5	Powdery	White	+	+	+	-
6	KBA6	Powdery	Grey	+	+	+	-
7	KBA7	Powdery	Grey white	+	+	+	-
8	KBA8	Leathery	White	+	+	-	-
9	KBA9	Leathery	Grey	+	+	+	-
10	KBA10	Powdery	Pink	+	+	-	-
11	KBA11	Powdery	Orange	+	+	-	-
12	KBA12	Leathery	Grey	+	+	+	-
13	KBA13	Powdery	Pale white	+	+	+	-

+: Present, - : No result

Oxidative fermentation test

In oxidative fermentation test, only one actinobacterial strain KBA8 showed colour change from green to yellow. It confirms the anaerobic nature of that particular actinobacterial strain. Based on this property, the strain KBA8 selected for further studies. Other 12 strains could not show any colour change in medium. It concludes that other strains are strict aerobic in nature (Table 2).

Table 2: Oxidative fermentation results selected actinobacterial strains

S. No	Strains	Result
1	KBA1	-
2	KBA2	-
3	KBA3	-
4	KBA4	-
5	KBA5	-
6	KBA6	-
7	KBA7	-
8	KBA8	+
9	KBA9	-
10	KBA10	-
11	KBA11	-
12	KBA12	-
13	KBA13	-

+: Positive, - : Negative

Measurement and COD analysis of MFC reactor

The microbial fuel cells inoculated with actinobacterial biomass was connected with digital multimeter and the value of voltage was calculated by the standard formula $P = iV$. On the 3rd day of set up the voltage was noted that about 0.4 mV. Finally, the 5th day of observation, nearly 1.5 mV of current was noted. Total COD average concentration was observed during the reactions is about 1125 ±70 mg/L (Figure 1 & 2).

Fig.1: MFC on 3rd day of observation   **Fig.2: MFC on 5th day of observation**

Optimization of ionic exchangers

Based on the optimization results, the concentration of ionic exchangers plays a main role in MFC. When the concentration of exchangers increased in Anode and Cathode chambers, the current generation also increased gradually. At the 25 g concentration of biomass and 12.5 g concentration of potassium ferricyanide in chambers, the current production was observed as 2.5 ± 0.3 mV (Fig.3a & 3b).
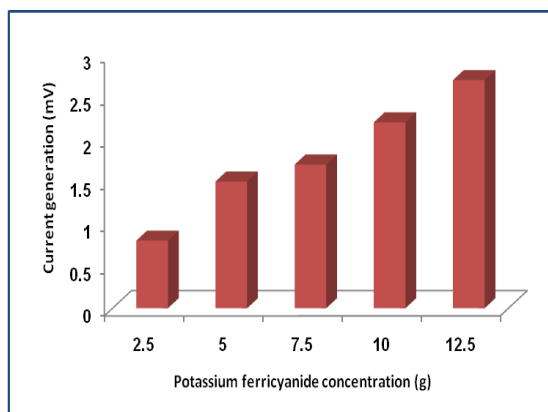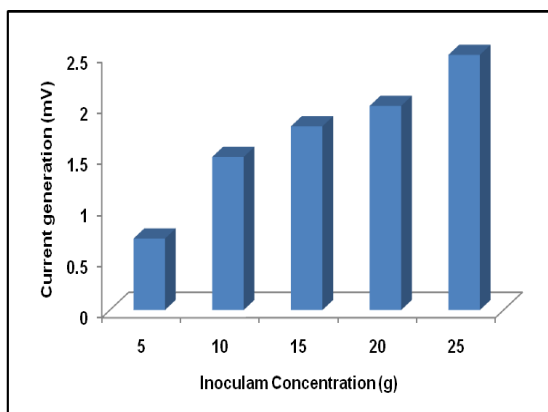
Fig 3a: Optimization of anode ion exchanger   **Fig 3b: Optimization of cathode ion exchanger**

Cultural characteristics

      The cultural characteristics of the potent strain KBA8 showed powdery aerial mycelium. Spore chain morphology was observed as Rectinoflexible (RF) in nature (Fig. 4a & 4b). The growth characteristics and carbon utilization nature of this strain KBA8 showed in Table 3. Among the various medium tested, ISP-1, ISP-2, and ISP-6 showed good growth. Additionally, glucose, sucrose, mannitol, cellulose and L-arabinose are the better carbon source for the growth of potent strain.

Table 3: Cultural Characteristics of potent actinobacterial strain KBA8

Characteristics	Results
Micro morphology	
Aerial mycelium	+
Substrate mycelium	+
Spore surface	Smooth (Sm)
Spore Chain Morphology	RF
Cultural Characteristics	
Colony consistency	Powdery
Aerial mass colour	Grey
Reverse side pigment	+
Soluble pigment	-
Growth On Different ISP Media	
ISP-1	Good
ISP-2	Very good
ISP-3	Moderate
ISP-4	Poor
ISP-5	Poor
ISP-6	Good
ISP-7	Moderate
Assimilation of carbon source	
Glucose	+
Sucrose	+
Dextrose	+

Maltose	+/-
Mannitol	+
Raffinose	-
L-arabinose	+
Xylose	-
Cellulose	+
Mannose	-

+: Positive, - : Negative
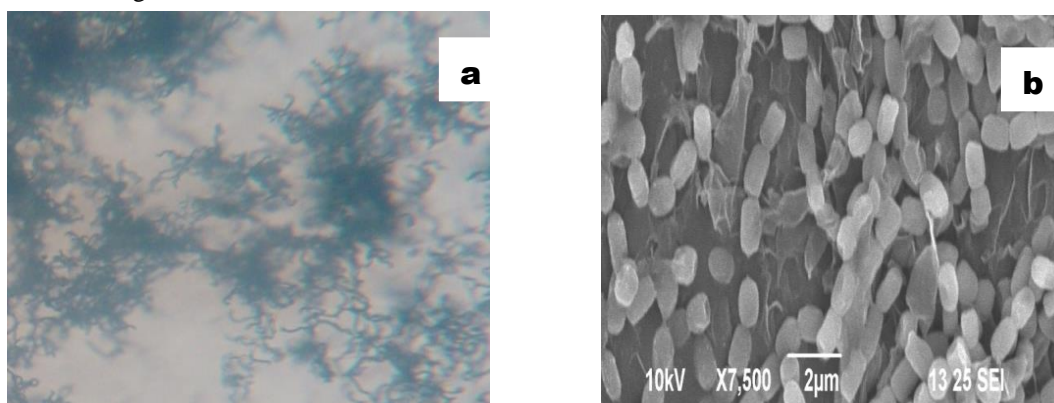
Fig 4: Micromorphology of KBA8 strain under (a) Light microscopy at 400X and (b) Scanning Electron Microscopy at 7,500X

Molecular identification of potent strain (KBA8)

The 16S rRNA partial sequence obtained from sequencer were aligned and compared with other sequence of DNA databases or gene bank which is availed in NCBI database. Based on the comparison, the potential strain KBA8 closely associated with the members of the diverse *Streptomyces* spectrum showing sequence similarities of 99% with *Streptomyces silaceus* was observed (Figure 5).
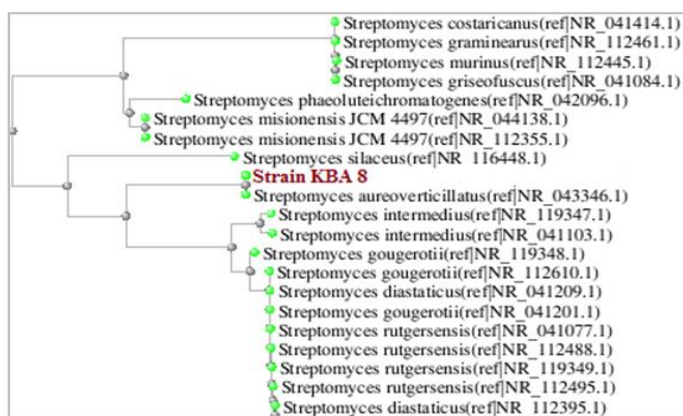
Fig 5: Phylogenetic analysis of potent actinobacterial strain

DISCUSSION

The Bauxite soil and their fertility status created an ecosystem which considered as rich source for isolation of different extremophilic microorganisms. The organism having capability to survive with these

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kinds of ecological niches due to the sun heated alkaline soil rich in bauxite, which was not well-studied upto date by scientific community. A microbial fuel cell is an electrochemical device capable of continuously converting chemical energy into electrical energy for as long as adequate fuel and oxidant are available. MFCs also used to treat domestic wastewater and it is considered a important technology to meet increasing electrical energy needs, especially using wastewaters as substrates, which can generate electrical current and achieve wastewater treatment simultaneously (Lu *et al.*, 2009).

Many microorganisms, particularly bacteria possess the ability to transfer the electrons derived from the metabolism of organic and inorganic matters to the anode. Marine sediments, different soils, wastewater, fresh water sediments and activated sludge are all rich sources for these kind of microorganisms (Niessen *et al.,* 2006; Zhang *et al.,* 2006). A numerous recent publications discussed the screening and characterization of microbes and the construction of a chromosome library for microorganisms that are able to generate electrical current from degrading organic and inorganic matters (Logan *et al.,* 2005; Holmes *et al.,* 2004; Back *et al.,* 2004).

Based on these views, the current study was undertaken to evaluate the effects of bauxite actinobacteria for the development of microbial fuel cells. The results of the present investigation showed that totally 13 actinobacterial strains (KBA1 to KBA13) were isolated and characterized from the Bauxite mine soil samples. Similarly, Mathiyazhagan and Natarajan, (2011) reported that bacterial species from bauxite mine soil are rich source of bioactive metabolites. Before going to MFC screening, the oxidative stress was observed among all the isolates. In this test, only one strain KBA8 showed positive colour change with anaerobic nature.

In the present study, for MFC, the selected actinobacterial strain was inoculated with sterilized rice wastewater in batch mode. The current production of 0.4 mV was observed at external circuit of 100Ω for first three days of continuous operation and later gradually increased in current production was observed. Finally, 1.5 mV of current have been observed after five days of incubation, because of the sterilized waste water contains the higher amount of actinobacterial biomass than earlier one. Similarly, the anodic reaction in mediator-less MFCs developed with metal reducing bacteria belonging to the families of *Shewanella, Rhodoferax*, and *Geobacter* is similar to that in this process because the anode acts as the final electron acceptor just like the solid mineral oxides (Lovley *et al.,* 2004; Vargas *et al.,* 1998; Holmes *et al.,* 2004). In this study, the optimization of ionic exchangers could be carried out. Based on the results, the concentration of exchangers could play a vital role in current generation. Additionally, *S. putrefaciens, G. sulferreducens, G. metallireducens* and *R. ferrireducens* transfer electrons to the solid electrode (anode) using a system, which are very similar to the present study. Mediators shuttle between the anode and the microorganisms transferring the electrons. They take up the electrons from microbes and discharge them at the surface of the anode. Normally, *Actinobacillus succinogenes, Desulfovibrio desulfuricans, E. coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas fluorescens* need extraneous mediators, while some bacteria can provide their own mediators. For example, *Pseudomonas aeruginosa* produces pyocyanin molecules as electron shuttles, during biofuel development.

The ML-MFC, inoculated with septic tank sludge, in batch mode. The electric current production of 0.3 mA was observed at external load of 10 Ω for first five days of continuous operation, but latter gradual decrease in current production was observed and it was 0.1 mA after 15 days because, the growth of methanogens in the anode chamber, reducing the availability of electron and proton, hence reducing current (Habermann and Pommer, 1991). Electricity can be generated by beer brewery wastewater using single chamber MFCs. Temperature affected the power output, and the maximum power densities of 435 (11 W/m^3) and 483 mW/m^2 (12 W/m3) were achieved at 20°C and 30°C respectively. Similar voltage outputs (467 ± 13 mV, 30°C; 443± 10 mV, 20°C) were got over a COD-

concentration ranging from 400 to 1,400 mg/L, but the CE decreased from 38 to 15%. Ionic strength is more important than wastewater strength in so far as this affected power outputs (Logan, 2009). In this study, two chambered MFC could be generated electrical current from anode compartment. At the temperature of about 28°C, the voltage was measured about 1.5 mV.

In this study, the potential strain was identified in species level by 16S rRNA sequencing method. Based on the available data, the identified potential strain *Streptomyces silaceus* KBA8, have a better antimicrobial properties. Additionally, the selected actinobacterial strain act as a potential candidate for development of biofuel cells in fuel cell technology. This is the novel and first report in this particular aspect in general, actinobacterial fuel cells in particular in worldwide. The *Streptomyces silaceus* used in nowadays for many applications like antibiotic production, enzyme production, etc. Therefore, it is probably that the *Streptomyces silaceus* (KBA8) has a high commercial value for the treatment of clinically resistant microbes and used to development of biofuel cells in future.

CONCLUSION

Based on the availed literature supports, MFC is an ideal way of generating electrical current, since it is also not a renewable resource but it could be treated waste water. It can also be used for the production of secondary fuel as well as bioremediation of toxic compound. However improvements and optimization are needed to achieve better results for electricity generation and pollutant removal. With continuous improvements in microbial fuel cell, it may be possible to increase power generation rates and lower their production and operating cost. Thus, the combination of wastewater treatment along with electricity production may help in saving of millions of rupees as a cost of wastewater treatment at present.

ACKNOWLEDGEMENT

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Spectrum of Bacterial Infections in Diabetic Foot Ulcers

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ABSTRACT

Diabetic foot ulcers are a major complication of diabetes mellitus resulting in a substantial morbidity and mortality. It is one of the major causes of social and economical problem resulting in long term complications like gangrene and amputation. This present study involves determining the clinical characteristic, spectrum of aerobic microbial flora and assesses their invitro susceptibility pattern of these bacterial isolates to commonly used antibiotics. This study was conducted as pus samples which were taken from 50 patients with diabetics foot inspection over a period of Jan2013 to Sep2013. The clinical specimens were processed by standard microbiological techniques. The anti microbial pattern was also studied by the Kirby Baver Method. Staphylococcus aureus was the predominant isolate recovered from 77% of the cases. The other organisms included *Pseudomonas aeruginosa* (14%), *Bacillus* spp. (5%) *Escherichia coli* (2%) and *Pneumococci* spp. (2%). Methicillin-resistant S. aureus (MRSA) (29%), Vancomycin Resistant *S. aureus* (VRSA) (11%) and extended spectrum of Beta lactamase (ESBL) (6%) were also be present in foot infection. Most of the Gram positive cocci were highly sensitive to amikacin, clindamycin, ciprocloxacin and vancomycin. Gram negative bacilli were highly resistant to antibiotics such as Cefepime, cefixime, rifampicin, cefoxitin and amoxyclav. Hence, Meropenem, piperacillin, piperacillin/ tazobactam and amikacin were found to be most effective antimicrobial agents. The anti microbial susceptibility data from our study suggest that would be the appropriate choice for antibiotic therapy.

Key words: Foot infection, Bacteriological profile, MRSA, ESBL

INTRODUCTION

Worldwide diabetic foot lesions are a major economic problem and are the leading cause of hospitalization for patients with diabetes. Patients with chronic diabetes mellitus are prone to development of foot ulcers, with a life time risk of 10-15% (Frykberg, 1998). The actual cost of diabetic foot disease is difficult to assess (Knox *et al.,* 2000). The consequences and considering the bacteria as contamination or commensals may result in sepsis and amputation (Mike, *et al.,* 2004)

The micro flora of leg and foot ulcers is usually polymicrobial (Davies, 2003). The diabetic wounds are mostly formed by pus forming microorganism like *Enterococci* sp. *Staphylococcus aureus*, *Klebsiella* sp and *Proteus* sp (Revathi, 1998). In addition, in many clinics (especially in developing countries), highly resistant strains of gram negative bacilli, especially Pseudomonas aeruginosa, are an increasing problem (Tascini *et al.,* 2006). Methicillin Resistant Staphylococcus aureus, Extended Spectrum of Beta Lactamase represents plays a major group currently being identified worldwide (Rodriguer, et.al. 2004). The prevalence of ESBL producing organisms is increasing and several outbreaks have reported worldwide (Jasser, 2006).

Choosing an antibiotic would depend on clinical diagnosis, Gram nature of the organisms, the aetiology of the organism and patient allergies to antibiotics, renal and hepatic insufficiency, local antibiotic susceptibility data, gastro intestinal absorption impairment and most importantly the severity of

the infection (Dharod , 2010). Once culture and sensitivity results are available, therapy should be targeted specifically to the pathogens present to prevent long term use of broad-spectrum antibiotics (Tahawy *et al.,* 2000). Since, some organisms are resistant to all approved antibiotics and they can only treat with experimental and potentially toxic drugs (Todar *et al.,* 2008). The challenge is, therefore, to use an appropriate antibiotic and it is worth noting that the attack of these virulent bacterial pathogens on diabetic wound ulcers (Guppta, 2000).

MATERIALS AND METHODS

This prospective study was carried out on 50 diabetic patients with foot ulcers during the period of Jan2013 to Sep2013, at a tertiary care hospital in Salem, Tamilnadu. The study protocol was approved by the Institutional Ethics Committee prior to the study.

Collection of diabetic foot wound swabs

A total of 50 pus samples were collected using sterile swabs from the foot ulcers presenting at Tertiary care hospital in and around Salem, Tamil Nadu, India. Study protocol and procedure had got approved by the Institutional Ethics Committee and all the study participants had given the written informed consent prior to participating in the study. A questionnaire was developed to record the medical history, examination details and investigation reports. Details regarding Age, sex, duration of diabetes, types of diabetes, site of ulcer, smoking habits, duration of the hospital stay, hyperglycemic level A1 and other associated co − morbid conditions like hypertension, retinopathy, nephropathy, Peripheral Vascular Disease, neuropathy and Osteomylitis were recorded. On local examination, foot ulcers in diabetic patients were categorized in to six grades (grade 0 − grade 5) based on Wagner Classification System. (Wagner 1981). They were

Grade 0 −Absent skin lessions, hyperkeratosis and bony deformity.

Grade I - Ulceration involving only the dermis.

Grade II - Ulceration involving tendon and capsule

Grade III- Extending to bone usually causing osteomyelitis

Grade IV - Localized gangrene (forefoot)

Grade V - Gangrene involving a major part of the foot.

Processing of wound samples

The pus samples were inoculated on Brain heart infusion agar, nutrient agar, blood and Mac Conkey agar plates. The streaked plates were incubated at 37∘C for 24 hrs. Identification of isolates were done based on standard biochemical test.

Antibiotic sensitivity test for bacterial isolates

All the clinical isolates were subjected to antimicrobial susceptibility testing on Mueller Hinton agar using the Kirby-Bauer method. Antimicrobial disk were Aztreonam (30μg), Amoxyclav (30μg), Cefpodoxime (10μg), Cefepime (30μg), Cefoperazone (75μg), Cefoperazone / sulbactam (75/10μg), Cefixime (5μg), Piperacillin (100μg), Ceftazidime (30μg), Ceftazidime/clavulanic acid (30/10μg), Ceftriaxone (30μg), Amikacin (30μg), Rifampincin (5μg), Meropenem (10 μg), Cefoxitin (30μg), Ticarcillin/Clavulanic acid(75/10μg), Piperacillin/Tazobactam (100/10μg), Erythromycin (15 μg), Methicillin (5 μg), Chloramphenicol (30μg), Clindamycin (10μg), Vancomycin (30μg), Tetracycline(30μg) and Ciprofloxacin (5μg). All discs were obtained from Hi-Media labs, Mumbai, India. Inter-pretative criteria for each antimicrobial tested were those recommended by manufacturer's guideline (Hi-Media labs, Mumbai, India)

RESULTS

About 50 patients (Male 48% and Female 52%) were considered to Type II diabetes. The duration of diabetic patients was more than 10 to 19 years (54%) followed by less 10 years (42%) and more than 20 years (4%). The duration of foot infection was more than 3 months (94%) whereas few of them had less than 3 month (6%). The foot ulcers were graded according to Wagner grade classification. Out of 50 patients, 42 patients were in Grade I (84%) and 8 patients were in Grade II (16%).The size of ulcer was more or less similar to 4 cm^2(92%) while few had less than 4 cm^2 (8%) (Table 1).

Table 1. Wagner's Grade Classification

Wagner's Grade	No. of patients	Percentage
Grade 0	-	0%
Grade 1	42	84%
Grade 2	8	16%
Grade 3	-	0%
Grade 4	-	0%
Grade 5	-	0%
Total	50	100%

Among 50 patients, 37 patients had an infection of right foot (74%) and left foot was infected in 13 patients (26%). In these, fore foot was infected in 22 patients (44%), 19 patients in mid foot (38%) and 9 patients with hind foot (18%) infection. Although, 24 (48%) were males and 26 (52%) were females. Out of 50 patients, thirteen (26%) were between the age of 30 – 40 years, nine (18%) were between the age of 41-50 years. From 51-60 years, twelve (24%) patients were infected, fourteen (28%) were between 61-70 years and remaining two (4%) were between above 71-80. In this, the patients above the age group of 61- 70 years were most significantly infected with diabetic foot infections followed by 30 - 40 years and 51-60 years. In associated co – morbid conditions, 2 patients had retinopathy (4%) and 7 patients had neuropathy (14%). The habits of smoking were seen in 10 patients (20%), tobacco chewing in 5 patients' (10%) and alcohol in 3 patients (6%) (Table 2).

Table 2. Cliniacal features of the patients

Feature	Number of Patients
Age(mean+/- SD years)	52.58 +/- 11.65
Sex	
Male	24 (48%)
Female	26 (52%)
Types of Diabetes	
Type 1	-
Type 2	50 (100)
Duration of foot ulcers	
≤ 3	47 (94%)
> 3	3 (6%)
Duration of diabetes mellitus	
< 10	21 (42%)
10 -19	27 (54%)
≥ 20	2 (4%)
Ulcer Localisation	
Fore Foot	22 (44%)
Mid Foot	19 (38%)

Hind Foot	9 (18%)
Associated diseases	
Hypertension	-
Retinopathy	8 (16%)
Nephropathy	
Neuroparthy	1 (2%)
Peripheral Vascular Disease	-

Out of 50 pus samples, 44 (88%) samples yielded pathogenic organisms and the remaining six 6(11%) showed no growth. Poly microbial present in 11% and mono microbial showed 85% (fig.1).
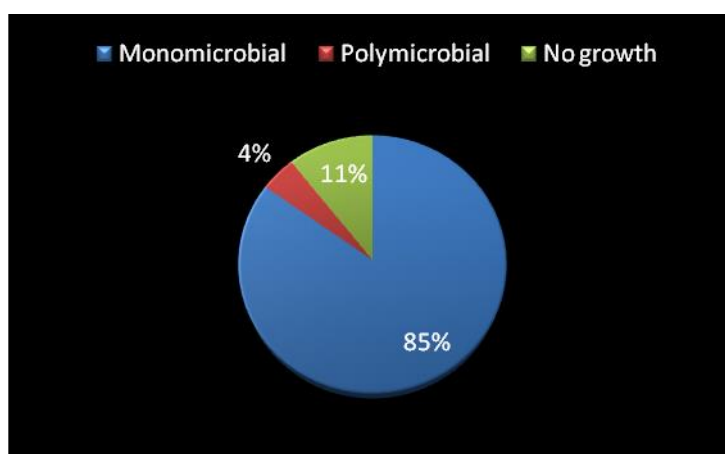
Fig. 1 Microbial Growth

The Bacterial pathogens isolated during this study included Gram positive cocci namely *Staphylococcus aureus* (77%), *Pneumococci* (2%) and *Bacillus* (5%) followed by Gram-negative bacilli namely *Pseudomonas* aeruginosa (14%) and *Escherichia coli* (2%). Gram positive organism were present in greater number than Gram negative organism. *Staphylococcus aureus* was the predominant organisms present in diabetic foot infection (Table 3).

Table 3. Bacteria isolated from diabetic foot infections

Organism isolated	No. of isolates	Percentage
Staphylococcus aures	34	77%
Bacillus	2	5%
Pseudomonas aeruginosa	6	14%
Escherichiacoli	1	2%
Pneumococci	1	2%
Total	44	100%

The antimicrobial susceptibility testing was done on the bacterial isolates isolated during this study by the Kirby Bauer disc diffusion method as per the CLSI guidelines, 2012. Most of the Gram positive cocci were found to be highly sensitive to amikacin, clindamycin,

ciprocloxacin and vancomycin. Methicillin-resistant *S. aureus* (MRSA) was present in 10 patients (29%) and Vancomycin Resistant *S. aureus* (VRSA) present in 4 patients (11%) (Fig. 2).
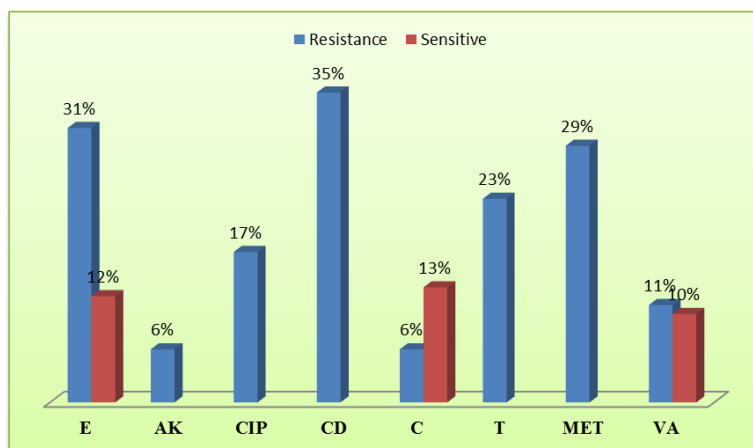
Fig.2. Antibiotic resistant and sensitive pattern of gram positive organism

The Extended spectrum of Beta lactamase (ESBL) was present in 3 of cases. Most gram negative bacilli were highly resistant to antibiotics such as Cefepime, cefixime, rifampicin, cefoxitin and amoxyclav. Hence, Meropenem, piperacillin, piperacillin/ tazobactam and amikacin were the most effective antimicrobial agents for the gram negative bacterial species (Fig. 3).
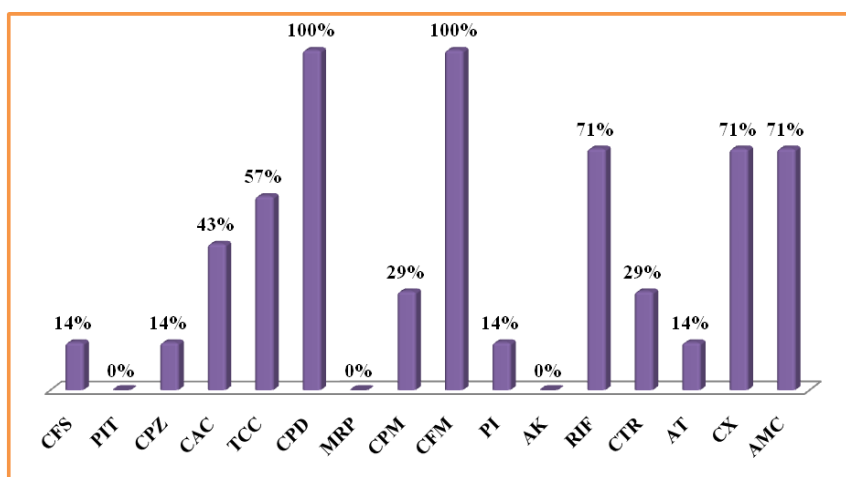
Fig.3. Antibiotic resistant pattern of gram Negative organism

DISCUSSION

Foot ulcers and their complications were an important cause of morbidity and mortality in patients with diabetes (Moulik *et al.,* 2003). All diabetic foot ulcers were superficially colonized by the plethora of microbes (Kumar and Veelakund, 2004). Several recent studies were now shedding new light on the bacterial populations associated with chronic wounds (James *et al.,* 2008; Wolcott *et al.,* 2009)

In our study, the patients enrolled were first time to the hospitals for the foot infection and did not receive any antibiotics. Most of the patients had mild to moderate degree of severity especially Grade 1 and 2 ulcers were predominantly seen in all patients. Grades 1 and 2 ulcers, which represent the majority of wounds treated at nonsurgical clinics, usually do not develop deep pockets or undermined edges that lead to the proliferation of anaerobic bacteria (Abdulrazak *et al.,* 2005). Right foot (74%) was more infected than left foot (26%). In that, fore foot was most predominantly infected in all patients. This study co related with the study of Oyibo *et al.,* 2002, which the majority of patients were referred with a forefoot ulcer and had some clinical evidence of both micro vascular and macro vascular complications. There was no significant difference in the age at diagnosis, sex, duration of diabetes and DFU and level of diabetic control. Female was predominant in this study. The patients above the age group of 61- 70 years were most significantly infected with diabetic foot infections followed by 30 - 40 years and 51-60 years. Similarly the study of Ribu *et al.,* 2006 reported that a mean age of 61 years was highly preferable for foot infection. The association of co – morbid conditions was always related with the diabetic foot infections. Neuropathy (14%) was mostly present in DFU patients than the retinopathy (4%). Neuropathy predisposes a diabetic to unrecognized injury due to loss of sensation. Our finding was co related with the results of Vinod Kumar *et al.,* 2011. When comparing with the other habitats, smoking habit (20%) plays a major role among the men patients. The study of Zubair *et al.,* 2010 stated that the patients who had the habits of smoking, tobacco chewing and alcoholism had significantly caused severe of foot infection which leads to amputations.

Infectious agents are associated with amputation of the infected foot if not treated promptly (Gadepalli *et al..,* 2006). Diabetic foot infection was known for poly microbial infection but our study observed that mono microbial infection 42% and poly microbial 5% respectively. Our findings were in accordance with those of another similar study of Tiwari *et al.,* 2011 and Dhanasekaran *et al.,* 2003. Staphylococcus aureus was the most predominant isolate followed by *Pseudomonas aureginosa* (14%), *Bacillus* spp. (5%), *Escherichia coli* (2%) and *Pneumococci* spp. (2%). Similarly the study co related with the studies of Abdulrazaka *et al.,* 2005, Tahawy, 2000; Lianes et al 2001; and Pathare *et al.,* 1998.

Antibiotic susceptibility test revealed that amikacin, clindamycin, ciprocloxacin and vancomycin were most effective for gram positive organisms whereas meropenem, piperacillin, piperacillin/ tazobactam for gram negative bacterial species. Similarly the results of Rajalakshmi and Amsaveni, 2012 stated that imipenem, meropenem, amikacin, piperacillin and tazobactam were the most effective antimicrobial agents for diabetic foot pathogens.

CONCLUSION

Patients with Diabetic mellitus had severe complications than the patients without diabetes. Staphylococcus aureus and Pseudomonas aeruginosa were found as predominant bacterial pathogens found in Diabetic Foot ulcers. Multidrug resistant organisms alarming high in present days and increase the rate of amputation. For better outcomes, a larger study population for a longer period of time should be undertaken to know the bacteriology and to the select the effective drugs of choice for diabetic foot infections. However, a study of bacteriology and its antimicrobial therapy of diabetic foot infection would be very fruitful in the future.

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Screening of Potential Indigenous Cellulolytic Bacteria from Paper Mill Effluent from Dindigul District

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ABSTRACT

Paper industries are the sixth largest toxic effluent generating industries of the world which play a significant public health hazard. The effluent of pulp and paper mill is darkly colored. Apart from having oil, grease, resins, detergents, acids, diterpen alcohol and polychlorinated diphenyl, they are usually laden with lignin and cellulose degradation products. All these chemical substances are already known to drastically change the natural quality of water. The effluent is very alkaline with high chemical oxygen demand and biological oxygen demand. The organic and inorganic contents of the effluent provide ample opportunity to the flourishing of a variety of microorganism and especially those of cellulose degrading organisms. Since the paper mill effluents are rich in cellulose content, the indigenous microbial flora must have the adaptation for degrading cellulose using cellulase enzymes. In the present study an attempt was done to isolate indigenous cellulose degrading microbes from paper mill effluents. The predominant microbes were isolated and screened for cellulase activity. Nearly fifty different organisms were isolated among which seven bacterial colonies were found to have detectable cellulase activity in plate assay method. The organism S7 was found to be an efficient strain with the zone of clearance of upto 27mm. The organism was confirmed to be Bacillus amyloliquefaciens by molecular gene sequencing studies. The organism may be used for commercial cellulase enzyme production. The BOD and COD of microbe treated effluent water also showed drastic reduction value suggesting the possible inclusion of this strain in the microbial consortium for treating any cellulose loaded effluents.

Key words: *Bacillus amyloliquefaciens*, Cellulose, Cellulase, Paper mill effluent treatment

INTRODUCTION

In the history of papermaking Egyptian papyrus and Chinese silk cloths were the first writing materials. The first true paper, prepared from fibrous cellulose-containing plant material, was manufactured in China in the year 105 A.D. In earlier times Chinese clothing was made largely from China grass (Boehmeria nivea), a common fibrous plant. Worn-out clothing was collected by rag merchants and sold to paper makers, who recycled the cellulose content into paper. Technological improvements and chemical bleaching methods have greatly helped the industry to flourish well. Paper was concurrently introduced in Japan sometime between the years 280 and 610 (DeVinne, 1876). After the defeat of the Chinese in the Battle of Talas in 751, the invention spread to the Middle East (Meggs, 1998). The technology of paper-making slowly crossed the Arab world and by the year 1150 made its way to Europe through Moorish Spain and thereafter spread worldwide. From 1150 to the middle of the 19th century the feedstock for paper-making remained recycled cellulosic materials, such as rags, rope, fish nets and burlap.

Depending upon the nature of raw materials used in the manufacturing process adopted, the effluent is very alkaline with high chemical oxygen demand and biological oxygen demand (Gomathi *et al.,* 2012).The untreated effluents from paper mills discharged into water bodies, damages the water

quality and living organisms therein. The waste water gets into natural source and may causes change in physico-chemical composition of water and ultimately becomes unsuitable for use.

The papermaking process produces an effluent which contains a substantial quantity of cellulose "fines" and other additives. This can be up to 50% of the total mass. This contaminated water is frequently referred to as ``whitewater''. Cost of effluent treatment for all water assigned to drain is found to be too expensive and hence ignored in many places, but it would also involve a loss of large amounts of raw materials and energy. Though a proportion of the water is recycled to the beaters for use in dilution or other processes, reclamation of the effluent is economically essential as the gross usage of water in the industry is very high (Thompson *et al.,*2001).

Due to the modern trend towards enclosing processing of water systems, paper machine waters have become richer in nutrients for microbial growth in addition to the suitable temperatures (30° – 500^oC) and a neutral pH which favor the growth bacteria. Up to now, very few studies have addressed the characterization of the microflora of paper sludge and their industrial importance. Since the paper mill effluents are rich in cellulose content, the indigenous microbial flora must have the adaptation for degrading cellulose enzymatically using cellulose enzymes.

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Aside from ruminants, most animals (including humans) do not produce cellulase and are therefore unable to use most of the energy contained in plant material. Cellulases are widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms. The three types of reaction catalyzed by cellulases:1. Breakage of the no covalent interactions present in the amorphous structure of cellulose (endocellulase) 2. Hydrolysis of chain ends to break the polymer into smaller sugars (exocellulase) 3. Hydrolysis of disaccharides and tetrasaccharides into glucose (beta-glucosidase).
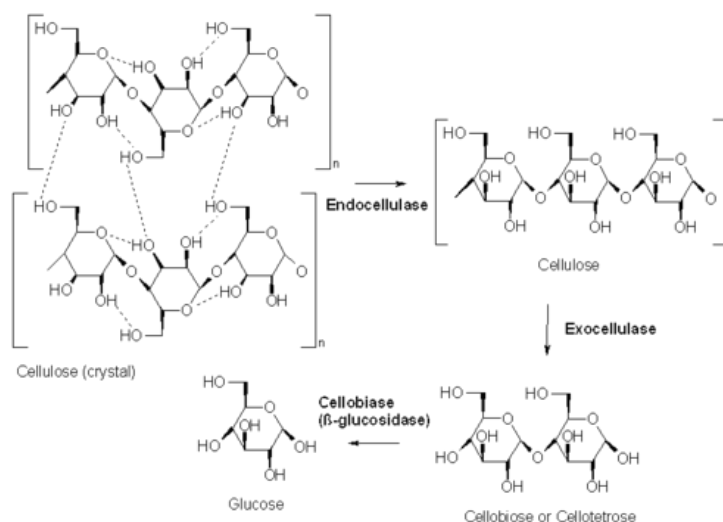
Cellulase is a very important enzyme because the human body cannot produce it on its own. Cellulase digests fiber. It helps a remedy for digestive problems such as mal absorption. Cellulase is used as a treatment for Phytobezoars, a form of cellulose bezoar found in the human stomach. Cellulase is used in animal healthcare as a feed supplement for better Milk yield. Cellulase is used in textile industry as a fading agent. Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present.

MATERIALS AND METHODS

Sample collection

The paper mill effluent was collected from Nilakotai, Dindigul Dt, Tamilnadu. The sample was collected using a sterile plastic container which was rubber-corked and transported to the laboratory in sterile zip-lock covers for further processing. The samples were filtered through ordinary filter paper to remove large suspended particles and impurities. The untreated and treated effluent was analyzed for pH, BOD and COD as described in APHA (1975).

Isolation and identification of microorganisms

The total microbial load of the effluent was determined with help of the serial dilution method. The effluent samples were serially diluted upto seven folds. From the dilution 10-1, 1 ml was serially transferred to reach serial dilutions up to 10-7. 0.1ml of sample of each dilution was transferred to each prepared sterile nutrient agar plate. The plate was rotated gently for uniform distribution of sample in the nutrient agar using a sterile L-rod. The plates were incubated at 37°C for 24 hours. After incubation the colonies are identified using colonial morphology, staining techniques, motility test and biochemical characterization (Garrity *et al*., 2005). The species confirmation will be done by gene sequencing method.

Screening of baterial isolates for cellulose degradation

Cellulase activities of the bacterial isolates were checked by plate assay. The isolated bacterial cultures were inoculated on sterile presterilized mineral media plates by spot inoculation method and the plates were incubated at 37°C for 24 hrs (Jeffrey *et al.,* 2008). After incubation, the plates were observed for the zone formation which was accomplished by the addition of iodine solution.

Isolation of bacterial genomic DNA

Genomic DNA was isolated by CTAB Method. The bacterial cells were grown in LB CMC broth. The broth was centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded. The cells were resuspended in TE buffer. The OD was adjusted by TE buffer to 1, at 600n. 740 µl of cell suspension was transferred to a clean centrifuge tube. 20 µl of lysozyme was added. It was then incubated for 5 minutes at room temperature. 40 µl of 10% SDS and 10 µl of proteinase k were added. The mixture was incubated for 1 hour at room temperature. 100 µl of 5M NaCl was added and it was mixed well and heated at 65˚C. 0.5 ml of chloroform: isoamyl alcohol (24:1), were added and mixed well. The mixture was spun at 10000 rpm for 10 minute at room temperature. Upper aqueous phase was transferred to clean eppendrof tube. 0.5ml Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to aqueous phase. This mixture was centrifuged at 10000 rpm for 10 minute at room temperature. Upper aqueous phase was transferred to clean eppendrof tube and add 0.6 volume of isopropanol (-20˚C). It was incubated at room temperature for 30 minute. The spun was given at 12000 rpm for 15 minute. The pellet was washed with 70% ethanol and again spun at 12000 rpm for 10 minute. The supernatant was discarded and the pellet was dried at room temperature for 5-10 minute. The pellet was resuspended in the mixture TE buffer and RNase (99 µl and 1 µl). The separated DNA sample was amplified using PCR method (Kary Mullis, 1983) and subjected to 16S r RNA sequencing method to identify the organism.

The PCR primers used are:

27F- 5' AGAGTTTGATCMTGGCTCAG 3'

1492R – 5' TACGGYTACCTTGTTACGACTT 3'

Sequencing primers employed are:-

518F- 5' CCAGCAGCCGCGGTAATACG 3'

800R- 5' TACCAGGGTATCTAATCC 3'

RESULTS

The paper mill effluent sample collected from the primary settling tank of Nilakotai, Dindigul Dt, Tamilnadu.was brownish black liquid with a woody odour. Physico-chemical characteristics of the paper mill effluent showed a pH of 7.8 with a BOD and COD of 162 and 392 mg l-1 respectively before treatment and showed a pH value of 6.7 with BOD and COD values of 103 and 273 mg l-1 respectively.

Isolation of microorganisms

The aliquots from the serially diluted effluent sample were spreaded on sterile nutrient agar plates which after incubation showed the presence of isolated bacterial colonies. Fifty morphologically different bacterial colonies were selected and screened for cellulose degradation by using minimal salt agar medium with cellulose as substrate.

Screening of selected isolates for maximum cellulase production

The 50 isolated bacterial strains were tested in mineral salt medium (MSM) for the degradation of cellulose by spot inoculation method in the MSM medium. The plates were observed for the formation of clear zone around the bacterial colonies (Fig.1). The zones were measured and the results were tabulated (Table 1). The results showed that only 7 strains had the potential to degrade the cellulose with zone formation. The S7 strain produced maximum zone (27 mm) followed by S3 and S5 (23 mm) and S1, S4 (22 mm). The bacterial isolates which had the lowest potential to degrade cellulose was S2 (17 mm) and S6 (11 mm) respectively. Hence for further bacterial identification procedure and confirmation sequencing techniques the strain S7 was selected.

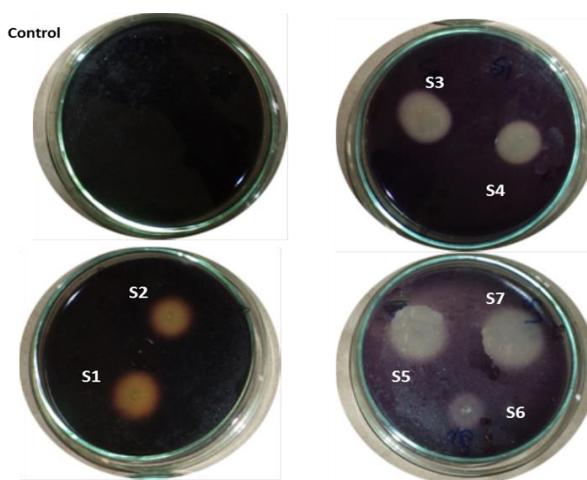**Fig. 1: The cellulase enzyme production zone of the strains S1 –S7**

Table 1: Cellulose Degradation of 7 Bacterial strains indicated by Zone Size (mm in diameter)

S.No	Bacterial Strains	Zone Size (mm in diameter)
1.	S1	17
2.	S2	23
3.	S3	22
4.	S4	23
5.	S5	22
6.	S6	11
7.	S7	27

Biochemical test

Based on the colony morphology and staining results it had been suggested that the selected organism with maximum cellulose production in the present study was the Bacillus genus. By further biochemical test results (Table 2) and comparing them to Bergey's Manual of Determinative Bacteriology the test organism had been identified to be B. amyloliquefascience. To confirm the organism species further, the pure culture colony of S7 strain was subjected to 16s RNA Sequencing and the results conformed to the above findings.

Table 2: Biochemical tests for Strain S7

S.No	Biochemical Test	Result
1.	Oxidase	+
2.	Catalase	+
3.	Indole	-
4.	MR	-
5.	VP	+
6.	Citrate	+
7.	Urease	-

16s RNA Sequencing of the S7 Strain

The 16S RNA sequencing revealed that the strain showing maximum cellulose degradation was found as *Bacillus amyloliquefaciens*, which was previously confirmed by conventional biochemical test also. The results of BLAST Alignment were shown in Fig.2.

3/29/13   NCBI Blast:Nucleotide Sequence (1496 letters)

Descriptions

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus amyloliquefaciens FZB42 strain FZB42 16S ribosomal RNA, complete sequence	2712	2712	100%	0.0	99%	NR_075005.1
Bacillus vallismortis strain DSM11031 16S ribosomal RNA, partial sequence	2689	2689	100%	0.0	99%	NR_024696.1
Bacillus subtilis subsp. subtilis strain DSM 10 16S ribosomal RNA, partial sequence	2684	2684	100%	0.0	99%	NR_027552.1
Bacillus atrophaeus 1942 strain 1942 16S ribosomal RNA, complete sequence	2678	2678	100%	0.0	99%	NR_075016.1
[Brevibacterium] halotolerans strain DSM 8802 16S ribosomal RNA, complete sequence	2678	2678	100%	0.0	99%	NR_042638.1
Bacillus mojavensis strain IFO15718 16S ribosomal RNA, partial sequence	2678	2678	100%	0.0	99%	NR_024693.1
Bacillus atrophaeus strain JCM9070 16S ribosomal RNA, partial sequence	2678	2678	100%	0.0	99%	NR_024689.1
Bacillus amyloliquefaciens strain NBRC 15535 16S ribosomal RNA, partial sequence	2660	2660	98%	0.0	99%	NR_041455.1
Bacillus licheniformis DSM 13 = ATCC 14580 strain ATCC 14580; DSM 13 16S ribosomal RNA, complete sequence	2551	2551	100%	0.0	97%	NR_074923.1
Bacillus subtilis subsp. spizizenii strain NRRL B-23049 16S ribosomal RNA, partial sequence	2529	2529	94%	0.0	99%	NR_024931.1
Bacillus stratosphericus strain :41KF2a 16S ribosomal RNA, partial sequence	2507	2507	100%	0.0	97%	NR_042336.1
Bacillus aerophilus :28K 16S ribosomal RNA, partial sequence	2507	2507	100%	0.0	97%	NR_042339.1
Bacillus pumilus SAFR-032 strain SAFR-032 16S ribosomal RNA, complete sequence	2501	2501	100%	0.0	97%	NR_074977.1
Bacillus aerius strain :24K 16S ribosomal RNA, partial sequence	2486	2486	98%	0.0	97%	NR_042338.1
Bacillus altitudinis strain :41KF2b 16S ribosomal RNA, partial sequence	2473	2473	98%	0.0	97%	NR_042337.1

Fig. 2: BLAST Analysis of S7 Strain

DISCUSSION

The waste waters from paper mills have dissolved wood derived substances which contain substantial quantities of cellulose 'fines'. In the present study since the Paper Mill effluent had rich cellulose content, there had been seven bacterial cellulose degraders. Isolation of cellulose degraders suggested that the organisms of an environment get acclimatized to utilize the available carbon source as a source of energy for their growth and development. Such isolated organisms can be further mass cultured either to be utilized for commercial cellulose enzyme synthesis or for environmental reclamation studies. The latter can be carried out by active or passive methods. The potential strain Bacillus amyloliquefascience from the present study was capable of reducing the BOD and COD values by 35 % and 30% respectively. As per literature Bacillus amyloliquefascience has not been considered as a toxic organism in the environmental bodies. Hence they can be treated with cellulose contaminated water for reducing the cellulose contents. On the other hand they can be used for passive treatment methods by way of immobilization techniques for treating the cellulose polluted water bodies.Bioremediation of paper mill effluent using cellulose degrading microbes would be a better way to treat the effluent (Thompson *et al.,* 2001).

From the literatures it is known that many kinds of fungi are capable of producing cellulose enzyme when cellulose was provided as the substrate. The fungi like *Aspergillus* and *Trichoderma* sp. though had reported with efficient cellulose production by formation of larger zones of cellulose clearance (50mm and 30mm respectively (Mahdi *et al.,* 2011), the same quantum of enzyme production and activity may be reached by using bacterial cultures as screened in the present study, since the organism growth time and enzyme production time are short when compared to slow growing fungi. Since the *Bacillus* sp. can be easily grown under lab condition with cellulose supplementation, the cellulose enzyme production can be enhanced if the growth conditions were optimized. The diameter of zone of clearance observed in plates predicted the amount of cellulose activity produced by the strains (Kaur and Arora, 2012).

In nature, cellulose, lignocellulose and lignin are major sources of plant biomass; therefore, their recycling was indispensable for the carbon cycle. Each polymer was degraded by a variety of microorganisms which produce a battery of enzymes that work synergically. The characterization of cellulose degrading bacterial species was one of the very good strategies to obtain energy from this abundant cellulose molecule present in the paper mill and pulp effluent. Finding naturally occurring cellulose degrading bacteria from the environment is important in the field of biorefining to help overcome costly hurdles in the biorefining process. Isolation and characterization may provide a good starting point for the discovery of such beneficial enzymes (Maki *et al.,* 2011).

CONCLUSION

Industrially important byproduct namely cellulase enzyme can be commercially synthesized by using the efficient cellulase producing bacteria Bacillus amyloliquefaciens screened by this study. The present study can be also be useful for researches planning to treat cellulose contamination in the environment. By way of enzymatic cellulose degradation using microbial cellulases, the effluent water quality can be increased. In the near future, processes that use lignocellulolytic enzymes biosynthesized by microorganisms could lead to new, environmentally friendly technologies.

ACKNOWLEDGEMENT

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Bioemulsification of Crude Oil in Contaminated Soil through Lab Scale Process

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ABSTRACT

Crude oil serves as the mainstay and the bedrock of economy of Organization of Petroleum Exporting Countries (OPEC) and some other countries exporting crude oil. The oil industry unavoidably generates large quantities of oily and viscous residue called oily sludge, which is formed during various production, transportation and refining processes. Petroleum hydrocarbons are among the most common and widespread environmental contaminants, adversely affecting human health and posing environmental problems. The hydrocarbon degrading microorganisms occur in most environments, where hydrocarbons may serve as organic carbon sources. Bioremediation is based on the use of microorganisms or microbial processes to degrade environmental contaminants. The present study aimed to elaborate a strategy of biosurfactant process by the application on soil decontamination process. Based on the sand absorption method, 80% of the crude oil removal in artificially contaminated sand.

Key words: Crude oil, biosurfactant, Sand adsorption, bioremediation, *Aspergillus* sp.

INTRODUCTION

Petroleum exploration and commercialization continues to increase, routine and accidental spills are causing greater damage to the environment. The persistence of these contaminants can cause irreversible damage to the soil, air, rivers, seas and groundwater (Vieira *et al.,* 2009). Leaks and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum and petroleum products. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year.

The oil industry unavoidably generates large quantities of oily and viscous residue called oily sludge, which is formed during various production, transportation and refining processes. Petroleum hydrocarbons are among the most common and widespread environmental contaminants, adversely affecting human health and posing environmental problems (Boulding, 1996; Kingston, 2002).

The hydrocarbon degrading microorganisms occur in most environments, where hydrocarbons may serve as organic carbon sources. Bioremediation is based on the use of microorganisms or microbial processes to degrade environmental contaminants and offers several advantages over the conventional chemical and physical technologies. It can be a cost effective, environmental friendly technology. Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds (Alexander, 1994; Pepper *et al.,* 1996). Bioremediation is the degradation of toxic pollutants through biological means. Biosurfactants are attractive for bioremediation because they are biodegradable and relatively non-toxic, making it an attractive compound to be released in bulk at a remediation site (Singh and Cameotra, 2004; Rahman and Gakpe, 2008).

It would be interesting to develop the bioremediation process further using fungi, because of their capacity to incorporate rapidly into the soil matrix. Furthermore, they have the ability to grow in environments with low nutrient concentrations, low humidity and acidic pH (Potin *et al.,* 2004; Mollea *et*

al., 2005). Several different bioremediation techniques have been developed, but biostimulation is the most often used (Head, 1998). This consists of the activation of native soil microorganisms through the addition of nutrients (Providenti *et al.,* 1993) reported that an efficient removal of contaminants requires $1x10^3$ CFU g-1 of soil, although other factors to be considered are the molecular structure and bioavailability of the contaminants (Juhasz andNaidu, 2000). The microbial culture must have the ability to withstand different soil environmental condition and to survive in the presence of other microorganisms (Roberts, 1998). Biosurfactants act by emulsifying hydrocarbons, increasing the solubilization of crude oil and subsequent availability for microbial degradation (Menezes *et al.,* 2005; Zhang *et al.,* 2005).

The term surfactant was derived from the phrase 'surface active agents' and describes the activity of these amphiphilic molecules at the interfaces between different phases, gas, liquid and solid. Nowadays, the huge demand of surfactants is currently provided by chemical surfactants derived from petroleum, but these compounds have the problem of being toxic to the environment and non-biodegradable. Surfactants are able to act as detergents, wetting agents, emulsifiers, dispersants and foaming agents and form major ingredients of many product formulations ranging from household detergents, shampoos, personal care products and pharmaceuticals to paints. The worldwide use of surfactants is enormous, estimated in 2008 to be 13 million tonnes per annum (p.a.) (Reznik *et al.,* 2010), with a predicted increase in use of approximately 2% p.a.

Biosurfactant is a surfactant produced extracellularly or as part of the cell membrane by bacteria, yeasts and fungi from various substrates including sugars, oils, alkanes, among others (Mulligan, 2005). They are amphiphilic compounds with considerable potential in commercial applications within various industries, such as health care and food processing industries, as well as enhancing oil recovery, crude oil drilling lubricants, bioremediation of water-insoluble pollutants (Peypoux *et al.,* 1999; Fox and Bala, 2000).

Microbial biosurfactants exert some influence on interfaces in both aqueous solutions and hydrocarbon mixtures. These properties cause micro-emulsions in which micelle formation occurs where hydrocarbons can solubilize in water, or water in hydrocarbons. Generally, biosurfactants are classified in five major groups, viz. glycolipids, phospholipids and fatty acids, lipopeptides (lipoprotein), polymeric and particulate biosurfactant.

Nowadays, biosurfactants are used in industries as a cosmetic and special chemical substances, food, pharmaceutics, agriculture, cleansers, enhanced oil recovery and bioremediation of oil-contaminated sites (Makkar and Cameotra, 2002; Mukherjee *et al.,* 2006). They are potential alternatives of chemically synthesized surfactant in a variety of application because of their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelleconcentration, each of production, ability to be synthesized from renewable resources, higherfoaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Desai and Banat, 1997; Mukherjee *et al.,* 2006). In thisrecent year, the biosurfactants have been placed on the environmental impacts of chemicalsurfactants and new surfactants for use in any field (Surachai *et al.,* 2007).

In ecosystems, fungi plays an important role during their ability in removing hazardous compounds from the water, whereas sediment particles contaminated with crude oil from oil spills is one of the desired ecological niche to fungi which inhabits such substrate and use carbon source from hydrocarbons in polluted sediment particles to biodegrade crude oil from the sediments in the beaches. Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria and although hydrocarbon degraders may be expected to be readily isolated from a petroleum oil- associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment (Batelle, 2000; Ojo, 2005).

Recently, many researchers studied the role of fungi in biodegradation process of petroleum products and the most common fungi which have been recorded as a biodegrades belongs to following genera: *Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Pleurotus, Polyporus, Rhizopus, Rhodotolura, Saccharomyces, Talaromyces and Torulopsis*(Saraswathy *et al.,* 2002; Adekunle *et al.,* 2004; Atagana *et al.,* 2006; Adekunle *et al.,* 2007; Gesinde *et al.,* 2008; Husaini *et al.,* 2008; Obire *et al.,* 2009; Hadibarata *et al.,* 2009; Hadibarata *et al.,* 2009; Romero *et al.,* 2010). In present study aimed to produce suitable biosurfactant for emulcification of crude oil in contaminated soil.

MATERIALS AND METHODS

Fungal strain and screening

Aspergillus sp. (PN1) was obtained from the of the Bioremediation laboratory in Department of Microbiology, Periyar University, Salem. The microorganism was maintained at $4^{o}C$ on Sabrouad Dextrose Agar slants containing Mycological Peptone, 10g/l; Dextrose, 40g/l and agar, 15g/l. Transfers were made to fresh agar slants each month to maintain viability. The fungal strains were identified and confirmed by using LCB wet mount, Tap method and slide culture technique. For screening, the fungal strain was transferred to 100 ml flasks containing 50 ml of sterile mineral salts medium with 1% crude oil. The tubes were incubated under shaking (200 rpm) at $30^{o}C$ for 7 days. After incubation, the strain was enumerated by spread plate technique using Sabroaud Dextrose Agar plates (Rahman *et al.,* 2002).

Preliminary determination of surfactant production

The fungal strain was inoculated in sterile BH broth amended with 1% crude oil and dextrose. The flasks were kept in agitation at 120 rpm for 96 hrs. After incubation the broth was centrifuged and the cell free supernatant was used to determine the surfactant character by oil spread assay drops collapse method and emulsification index (E_{24}).

Emulsification index (E_{24})

The emulsification index was determined using the method described by Cooper and Goldenberg (1987), whereby 3 ml of a hydrocarbon (Petrol, Diesel, Kerosene, Used engine oil and Fresh engine oil) were added to 3 ml of the cell-free culture broth in a graduated screw-cap test tube and vortexed at high speed for 2 min. Emulsion stability was determined after 24 h. The emulsification index was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100.

Oil spread assay

The oil spread assay was done by 20 ml of distilled water was added to a petri dish with a diameter of 15 cm. Then, 20μl crude-oil was dropped onto the surface of the water, followed by the addition of 10 μl of cell culture supernatant. After 30 sec, the visualized clear zone was observed under visible light (Rodrigues *et al.,* 2006).

Foaming index

The foaming index done by 5 ml of cell free supernatant in a graduated tube vortexed at high speed for 1 min. Foam activity was detected as duration of foam stability and foam height was determined in a graduated cylinder (Dehghan *et al.,* 2003).

Effect of various carbon sources on the production of biosurfactant

There are six different carbon sources such as dextrose, sucrose, cellulose, fructose, glucose and starch were screened for the production of biosurfactant. The optimum carbon sources were selected by using gravimetric analysis, oil spread assay and drops collapse method.

Mass production of biosurfactant

The mass production was carried by using mineral salts medium enriched with 1% crude oil. The enrichment was continuously stirred by using magnetic stirrer and the setup was maintained for 120 hrs. On final day of this study, the surfactant was extracted by solvent extraction method.

Extraction of biosurfactant

The cell-free culture broth was acidified with 6M HCl to pH 2.0 and precipitated with two volumes of methanol. After 24 h at 4°C, samples were centrifuged at 5000 rpm for 30 min, washed twice with cold methanol and dried at 37°C for 24-48 h. Biosurfactant yield was expressed as g/l. Known amounts of crude precipitate were re-suspended in distilled water and used for the determination of the characterization and further studies.

Preliminary characterization of biosurfactant

Biosurfactant composition

The protein concentration in the isolated biosurfactant was determined using the method developed by Lowry *et al.* (1951), with bovine albumin as the standard. Total carbohydrate content was determined using the phenol-sulphuric acid method (Hanson et al., 1981). Lipids were quantified based on Manochaet al. (1980): 0.5 g of the isolated material were extracted with chloroform: methanol at different proportions (1:1 and 1:2, v/v). The organic extracts were then evaporated under vacuum conditions and the lipid content was determined through gravimetric estimation.

Total protein estimation (Lowry's Method)

The Sample was mixed in suitable solvent system. Different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ml) and water. The final volume in each of the test tubes is 5 ml. the BSA range is 0.05 to 1 mg/ml. from these different dilutions, pipette out 0.2ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well. This solution was incubated at room temperature for 10 mins. Then add 0.2 ml of regent folinciocalteau solution (reagent solution) to each tube and incubate for 30 mins. Measure the absorbance at 660 nm with spectrophotometer.

Total carbohydrate estimation

A standard curves was prepared by dispensing various volume of 50μg/ml solution of standard glucose solution into labeled tubes. The varied volumes of each tube were then made up to 0.4 ml by adding distilled water. 0.2 ml of phenol was added to each tube. 1ml of concentrated sulphuric acid was then carefully dispensed to each tube. The solution was allowed to stand for 20 min before taking the reading at 490nm.

Determination of ionic charge

The ionic charge of the biosurfactant was determined using the agar double diffusion technique (Meylheuc *et al.,* 2001). Two regularly spaced rows of wells were made in an agar of low hardness (1% agar). The wells in one row were filled with the biosurfactant solution and those on the other side were filled a pure compound of known ionic charge. The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 mM and the cationic substance was barium chloride 50 mM. The appearance of precipitation lines between the wells (indicative of the ionic character of the biosurfactant) was monitored over a 48 h period at room temperature.

Antibiotic characteristics of biosurfactant

The extracted compound was tested for antimicrobial activity using well diffusion method and area of the zone was calculated (Cappucino, 1999). Extracted active compounds were tested against human pathogens such as E. coli, Proteus mirabilis, Staphylococcus aureus and Klebsiellapneumoniae.

Mueller Hinton Agar (beef infusion solids 4.0 g, starch 1.5 g, casein hydrolysate 17.5 g, agar 15.0 g, dis.H$_2$O 1000 ml and the final pH 7.4±0.2 at 37°C) plates were prepared and swabbed with appropriate pathogen. Using a sterile cork borer well was made and 50 µl of extracted compound was added in wells, incubated at 30°C for 24 h. After incubation, the clear zone was measured and calculated.

Stability studies

Effect of temperature

The cell-free broth was obtained by centrifuging the cultures at 5000 rpm for 20 min. About 40 ml of the cell-free culture broth were heated at 70, 100 and 120°C for 1 h and cooled to room temperature, after which the emulsification index were determined by using five different hydrophobic phases (petrol, diesel, kerosene, used engine oil and fresh engine oil). Similar procedure was also followed in the study carried out at 5°C.

Effect of pH

The cell-free broth was obtained by centrifuging the cultures at 5000 rpm for 20 min. The pH of the cell-free broth was adjusted to different values (2.0–12.0) and the emulsification index was determined.

Effect of salt concentration

The cell-free broth was obtained by centrifuging the cultures at 5000 rpm for 20 min. The NaCl concentration (2.0–10.0%) of the cell free broth was adjusted and the emulsification index was determined.

Effect of biosurfactant on the removal of motor oil from contaminated sand under batch mode condition

The suitability of the biosurfactant for enhanced oil recovery was determined using 60g of beach sand impregnated with 5.0 ml of motor oil. The biosurfactant produced by A. fumigatus cultivated in the optimized medium was used in the removal tests. Fractions of 20g of contaminated sand were transferred to 250 ml Erlenmeyer flasks, followed by the addition of 40 ml of the cell-free broth. Control was also maintained by the addition of 40 ml distilled water. The samples were incubated on a rotary shaker (150 rpm) for 24 h at 27°C and centrifuged at 5000 rpm for 10 min for separation of the laundering solution and sand. The amount of oil in the sand after contact with the biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane (Sobrinho *et al.,* 2008).

RESULTS

Culture collection and confirmation

The fungal strain *Aspergillus* sp. (PN1) was collected from bioremediation laboratory, department of microbiology, Periyar University Salem. This strain was identified and confirmed the species by lactophenol cotton blue method and physical morphology in SD agar plate. This strain was produce colonies show typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores. The microscopic appearance shows uniseriate and columnar conidial heads with the phialides limited to the upper two thirds of the vesicle and curving to be roughly parallel to each other (Fig. 1).

Toxic resistant study

The toxic resistant study was carried out for the identification of tolerance of the specific fungal strain. Based on this study monitored the BH broth with oil and same broth without oil, the carbon source were given as 1% dextrose. Result shows the more or less same colony forming units in control and test.

Fig. 1: Colony morphology and microscopic observation of *Aspergillus fumigatus* (PN1) on SDA plate

The strain *A. fumigatus* (PN1) was having crude oil tolerant character and also this strain was able to utilize the oil by gravimetric analysis. In a previous review, Bartha and Atlas listed 14 genera of fungi isolated from an aquatic environment which had been demonstrated to contain members which utilize petroleum hydrocarbon. The evolution of the hydrocarbon mixture depends on the nature of the oil, microbial community and environmental factors which impact microbial activities.

Preliminary determination of surfactant production

Most of the oil degrading microbes were produce biosurfactant in for emulsifies the oil content in polluted area. In this study can prove the *A. fumigates* (PN1) able to produce very efficient biosurfactant based on the results of emulsification index, foaming index and oil spread assay. The emulsification index was done by comparing biosurfactant (cell free supernatant (CFS) with synthetic surfactants (SDS and TritonX100) and water using different hydrophobic phases (Petrol, Diesel, Kerosene, Used engine oil and fresh engine oil). The CFS emulsifies 50% of petrol and fresh engine oil,and 23% of Kerosene and used engine oil, 16 % in Diesel. The synthetic surfactants gave a very good results compare to the biosurfactant, but most of the authors said the synthetic surfactants were toxic to environment, so we can't apply to environmental remediation processes.

Optimization of carbon source, mass production and extraction of biosurfactant

The carbon source was very important to any microbial products. But the need of specific carbon sources for production of specific product will help to increase the product quantity and also the efficiency. Here, six (Glucose, Dextrose, Fructose, Starch, Cellulose and Sucrose) different carbon sources were used for biosurfactant production and increase the biosurfactant efficiency (Fig. 3). Based on this result, the dextrose was give a 97.7% of oil removal from medium and for efficiency, 2 cm clear zone was produced in oil spread assay (Fig. 2). So using dextrose the mass production was carried out in large scale (1000 ml flasks) using continuous stirring with magnetic stirrer. After incubation period the biosurfactant was extracted by solvent extraction method using separating funnel. Approximately, get the biosurfactant 3 g/L ratio (Fig. 4). According to Wei *et al.* (2004), hydrophobic substrate like crude-oil could induce much higher production of biosurfactant as compared to glucose and glycerol. In the present study, biosurfactant production was studied starting with 1% (v/v) crude-oil as the sole source of carbon because the strain exhibited a high yield of biosurfactant when compared with higher concentration of crude-oil.

Preliminary characterization of biosurfactant

The preliminary analysis demonstrated that the biosurfactant isolated from *A. fumigates* (PN1) is a glycoprotein, consisting of 70% protein and 15% carbohydrates. There are numerous reports on the

isolation and biosurfactant production of different species of the genus bacteria, fungi and yeast. The biosurfactants produced by this genus can differ widely from one species to another, lipid–carbohydrate complexes (Gallert, 2002), protein–carbohydrate complexes (Cirigliano and Carman, 1984), long-chain fatty acids (Kappeli, *et al.*, 1978) and protein–carbohydrate-lipid complexes (Sarubbo, 1999) when grown on either hydrophobic or water-miscible substrates. The agar double diffusion test revealed the appearance of precipitation lines between the biosurfactant produced by *A. fumigates* (PN1) and the ionic compound used. While no lines formed between the biosurfactant and the ionic compounds. Under the experimental conditions of the present study, this simple test demonstrated the non-ionic character of the biosurfactant produced. Most of the other biosurfactants produced by yeast and fungal species also display a nonionic character when submitted to the same test (Sobrinho*et al.*, 2008).

One useful property of many biosurfactant that has not been reviewed extensively is their antimicrobial property. Other medical relevant uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as therapeutic agents (Singh and Comeotra, 2004). The antimicrobial property of biosurfactants to cellsurfaces caused determination in the integrity of cell membrane and also breakdown in the nutrition cycle (Hingley*et al.,* 1986). Biosurfactant isolated from *A. fumigates* (PN1) showed a wide activity against the pathogenic strains. The partial purified biosurfactant showed activity against *S. aureus*. According to Tsuge*et al.* (1996), lipopeptide surfactants are potent antibiotics mainly the surfactin, streptofactin and gramicidin produced by the microorganism and had the wide antimicrobial activity (Peypoux*et al.,* 1999; Richter *et al.,* 1998) compared to the glycolipid producing strain. A glycolipid surfactant from the *C. antartica* has demonstrated antimicrobial activity against Gram-positive bacteria.

Stability study: Effect of temp, pH and NaCl concentration

The good emulsification property is critical for biosurfactant to be promising in different environmental and industrial applications. In earlier report, Ron and Rosenberg (2001) demonstrated that most of the bacteria utilize insoluble hydrocarbons by producing biosurfactant that promote substrate solubilisation and emulsification, thus allowing the cells to get into direct contact with the oil phase. In the present study, the ability of biosurfactant obtained from *A. fumigates* (PN1) as to emulsify crude-oil in cell-free supernatant was investigated at different environmental conditions. The effects of pH, salinity and temperature on the emulsification activity of the cell-free supernatant against crude-oil are shown in (Fig. 5) respectively. Initially, when the pH was set to 2-12 the emulsification activity of cell-free supernatant was done with six different hydrocarbon sources. This suggested that the activity of this biosurfactant is limited to acidic pH.

The highest emulsification activity (E_{24}-83.3%) (Fig. 5) was observed at pH 8 against fresh engine oil. A linear decrease in emulsification index from pH 10-12 was observed which might be the result of some alteration of the biosurfactant under extreme condition (Thavasi*et al.*, 2007). The results obtained from the present work suggests that the strain was moderately halophilic showed a maximum emulsification activity (E_{24}-83.3 and 73.3%) at 2 and 4% (w/v) NaCl concentration. Also, it was observed the biosurfactant was not precipitated between 8-12% (w/v) NaCl solutions. The results are in concurrence with Shavandi*et al.* (2011). They stated that this special ionic strength tolerance offers the biosurfactant more suitability for oilrelated application. The temperature was one of the critical parameter that has been controlled in bioprocess. The result in the present study revealed there is no notable changes biosurfactant activity reached highest 70, 100 and 120ºC (E_{24}-83.3%) and this clearly indicates moderately thermostable in nature. The competence of the biosurfactant produced from strain for emulsification of crude-oil indicates that it can act as a potent tool for application in microbial enhanced oil recovery.

Fig. 4. Massproduction of biosurfactant from *A. fumigatus* **(PN1) and extraction of biosurfactant by solvent extraction method**
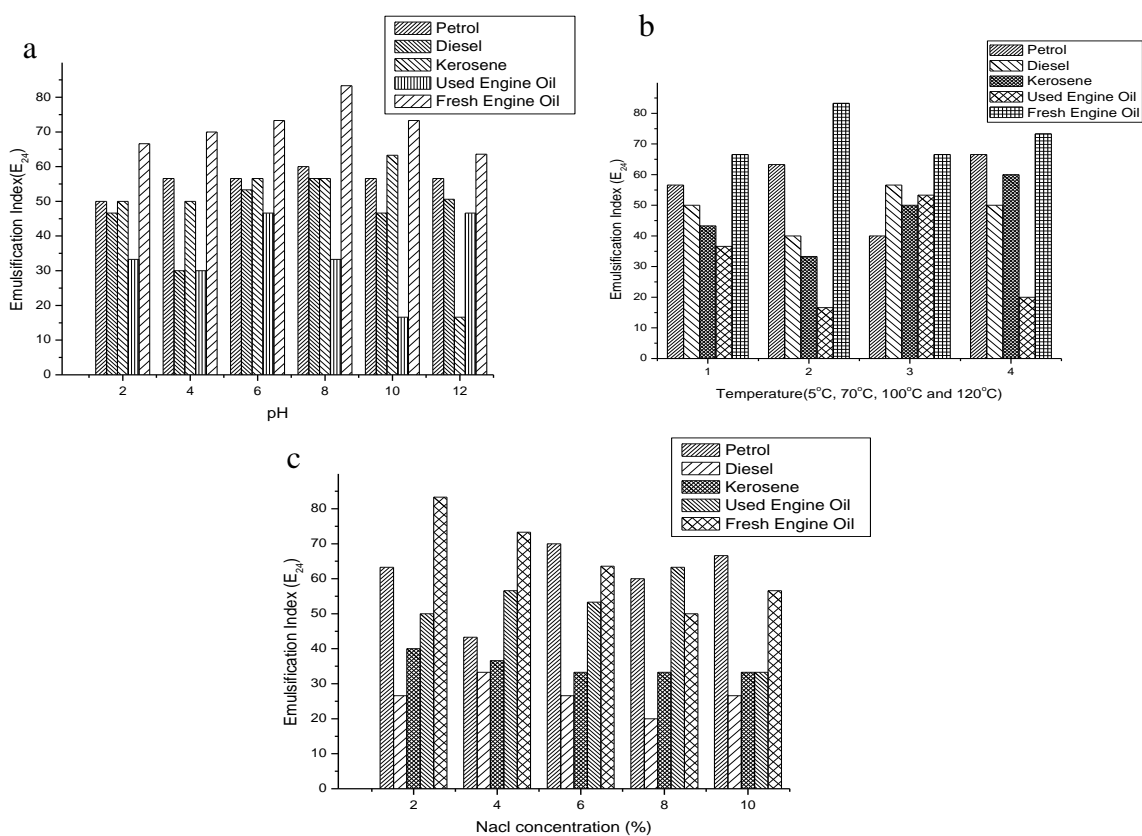
Fig. 5. Stability Study: a) Effect of pH, b) Effect of temperature and c) Effect of NaCl concentration on emulsification activity of biosurfactant

Application of biosurfactant in motor oil removal from contaminated sand

Biosurfactants emulsify hydrocarbons by enhancing their water solubility, decreasing surface tension and increasing the displacement of oil substances from soil particles. Satisfactory results were obtained for the removal of motor oil adsorbed to sand samples by the cell-free broth (crude

biosurfactant) from *A. fumigates* (PN1) in comparison to the control (distilled water), with removal rates of 80% (fig. 6). The same result reported by Batista *et al.*, (2010) found an 80% recovery rate of residual crude oil adsorbed to sand. Coimbra *et al.*, (2009) demonstrated the considerable ability of biosurfactants produced by *C. guilliermondii* and *C. lipolytica,* to remove motor oil and petroleum adsorbed to sand. A biosurfactant from *C. antarctica* was found to remove about 50% of oil adsorbed to sand (Joshi, 2008).

Fig. 6: Application of biosurfactant in motor oil removal from contaminated sand

CONCLUSION

Based on the above observation, concluded that potential nature of the of *Aspergillus fumigatus (PN1)* on biosurfactant production. Dextrose was selected as a suitable carbon source for biosurfactant production and remediation of oil contamination on the base of optimization result. After optimization the biosurfactant production was highly increased in 0.1 to 3 g/l. In all the parameters revealed that there are no notable changes in biosurfactant activity. In specifically the pH 8, Temperature 70oC and NaCl at a concentration of 2% shows high emulsifying (83.3%) activity in fresh engine oil. The sand absorption method also displays 80% of the crude oil removal in artificially contaminated sand. Based on the observation, it could be concluded that the biosufactant produced by *Aspergillus fumigatus* (PN1) is a potential biological resource can be used for remediation of soil contaminated with crude oil pollution.

ACKNOWLEDGEMENT

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Bacteriological Profile and Antibiogram of Acute and Chronic Dacryocystitis

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ABSTRACT

In this study the demographic details of patients presenting with acute and chronic dacryocystitis, their predominant agents and the antimicrobial susceptibility pattern were studied. Fifty- five lacrimal swab materials collected from patients aged above 10 years suffering from both acute and chronic dacryocystitis. *Staphylococcus aureus* was the predominant bacterial isolate accounting for 36.3% followed by *Staphylococcus epidermidis* (26.0%), Coagulase negative Staphylococci (CONS) (10.87%), *Bacillus subtilis* (9%), *Micrococcus* (5.4%), *Klebsiella* species (3.6%), *Pseudomonas aeruginosa* (3.6%), and *Corynebacterium* species (1.8%). Females (50.8%) were more predominantly affected than men (49%). The antimicrobial susceptibility pattern, showed the entire Gram positive and Gram negative isolates as sensitive to ofloxacin and ciprofloxacin. *Staphylococcus aureus* was the most frequently isolated bacteria than the *Staphylococcus epidermidis*. Ofloxacin was the most effective drug of choice for both acute and chronic dacryocystitis. Knowledge of the bacteriology of dacryocystitis and the susceptibility of the bacteria towards antibiotics will better guide a clinician in the choice of the medication for the most appropriate drug for the treatment.

Key words: Acute dacryocystitis, chronic dacryocystitis, nasolacrimal duct, *Staphylococcus aureus*.

INTRODUCTION

Dacryocystitis is an inflammation of the lacrimal sac which usually occurs because of the obstruction of the nasolacrimal duct. The obstruction may be an idiopathic inflammatory stenosis infection, neoplasm or mechanical obstruction (Chaudary *et al*., 2010). Dacryocystitis has long been noted to occur more frequently on the left side than on the right side. Obstruction of the nasolacrimal duct leading to stagnation of tears in a pathologically closed lacrimal drainage system can result in clinical condition described as dacryocystitis (Bharathi *et al*., 2007).The microbiology of dacryocystitis may differ in acute and chronic conditions. A delay in management may lead not only to secondary infection in the remaining years of life but also ultimately to blindness. Though various regional studies on microbial analysis of dacryocystitis and their sensitivity pattern towards different antibiotics are available, there is still a considerable scope in these studies to document the change in pattern, if any, of the pathogens in dacryocystitis according to the age group, gender, type of dacryocystitis; and this will help reduce the unnecessary load of antibacterial agents. Further, this will also help in enhancing the understanding of the interrelationships between humans and microorganisms and of the virulence of specific microbial pathogens (Shah *et al*., 2011). Hence, this present study was planned to compare the bacterial aetiology of acute and chronic dacryocystitis cases presenting at an eye care Hospital, Salem. To determine the *in vitro* antibacterial susceptibility of bacterial pathogens to commonly used antibacterial agents. The results of this study would contribute substantially to the understanding of dacryocystitis and their choice of effective therapy.

MATERIALS AND METHODS

A total of 55 samples suspected clinically of dacryocystitic were enrolled in this study. The patients were examined on the Slit lamp biomicroscope by an ophthalmologist and the cases of dacryocystitis were identified based on their signs and symptoms. Acute dacryocystitis was diagnosed in patients with pain, swelling, redness and chronic dacryocystitis was diagnosed with persistent epiphora painless or recurring swelling over the lacrimal sac and extensive purulent material. Sample collections were done by using sterile cotton swabs dipped in saline were used to collect discharge from the lacrimal punctum of infected lacrimal sac, ensuring that the lid margin or conjunctiva was not touched. Two samples were taken and used for microbiological evaluation.

The materials obtained were subjected to microscopy by Gram's staining. The swabs were plated on to Nutrient agar, Sheep Blood agar, and Sabouraud Dextrose agar (obtained from HiMedia, Mumbai, India) plate respectively. The culture plates were incubated at 37ºC for bacterial and 28 to 30 ºC for fungi. The identification of the bacterial organisms was done by the standard microbiological technique which involves colony morphology, staining reaction and different biochemical properties (Forbes *et al*., 2008). The antibiotic susceptibility testing was done by Kirby Bauer Disc diffusion method (Monica, 2000).

Statistical analysis

The significance of the difference between two proportions was calculated by Chi square (X^2) test. Tables were mostly 2x2 tables with one degree of freedom. If P>0.05, the difference observed were deemed not significant. If P<0.05, the differences were deemed significant.

RESULTS

A total of 55 patients with dacryocystitis were referred for microbiological evaluation, of which, 26 (45.4%) patients had acute onset and the remaining 29 (51.0%) had chronic onset dacryocystitis. Single eye was infected in all 55 patients. The percentage of chronic dacryocystitis was found to be higher than that of acute dacryocystitis and the difference was statistically non significant (p > 0.05). (Table 1). Among 55 patients, 17 (49%) were males and 18 (50.8%) were females. Males had acute (26 of 12; 21%) and chronic infection (29 of 15; 19%). Female predominance was seen among both acute (26 of 14; 18.5%) and chronic infection (29 of 14; 20.7%). The probability P value was significant (p < 0.05) (Table 2). The highest percentage of infection was seen in patients above the age groups of 41- 50 years were significantly infected with both acute and chronic infection (Table 3). The most frequent bacteria associated with both acute and chronic dacryocystitis were *Staphylococcus aureus* followed by *Staphylococcus epidermidis,* and *Corynebacterium* species (Table 4). Out of 55 patients, 39 patients were infected with left eye and the remaining 16 were infected in right eye. The probability P value was found to be non significant (P>0.05) (Table 5). A total of 29 chronic cases, 28 were found to be culture positive. The mucopurulent discharge from 12 patients (4.1%), extensive purulent 5 (1.7%) and clear fluid from 12 patients (4.1%) (Table 6).

The antimicrobial susceptibility testing showed all the Gram positive isolates were 100% sensitive to Methicillin, Ciprofloxacin, Ofloxacin, Amikacin, Tobramycin, Norfloxacin and Erythromycin and Gram negative isolates sensitive to ciprofloxacin, ofloxacin and norfloxacin and least sensitive to amikacin, tobramycin and erythromycin. There was one strain of   Methicillin Resistant *Staphylococcus aureus* (MRSA) in this study.

Table 1: Distribution of types of Dacryocystitis

Type of Dacryocystitis	No. of Patients	Total No. of Isolates	Percentage of Isolation
Acute dacryocystitis	26	25	45.4%
Chronic dacryocystitis	29	28	51.0%
No growth	2	-	3.6%
Total	**55**	**53**	**100%**

Table 2: Distribution of Gender

Type of Dacryocystitis	Gender	Number of Cases	Percentage of Isolation
Acute	Male	12	21.8%
	Female	14	25.4%
Chronic	Male	15	27.2%
	Female	14	25.4%

Table 3: Age wise Distribution Pattern of Acute and Chronic Dacryocystitis

Type of Dacryocystitis	Age (years)				
	0-20	21-30	31-40	41-50	Above 50
Acute	3 (1.1%)	4 (1.5%)	8 (3.0%)	8 (3.0%)	3 (1.1%)
Chronic	3 (1.0%)	5 (1.7%)	5 (1.7%)	8 (2.7%)	9 (3.1%)

Table 4: Pattern of Bacterial Isolates among Acute and Chronic Dacryocystitis

Name of the Isolates	Acute Dacryocystitis	Percentage of Isolation	Chronic Dacryocystitis	Percentage of Isolation
Gram Positive organisms				
Staphylococcus aureus	8	14.5%	12	21.8%
Staphylococcus epidermidis	10	18.8%	4	7.2%
Micrococcus	2	3.6%	1	1.8%
Bacillus subtilis	-	-	5	9%
Corynebacterium species	-	-	1	1.8%
Coagulase negative Staphylococci (CoNS)	4	7.27%	3	3.6%
Gram Negative organisms				
Klebsiella species	-	-	2	3.6%
Pseudomonas aeruginosa	-	-	2	3.6%

Table 5: Growth Pattern in Unilateral and Bilateral cases of Dacryocystitis

Infected Eye	Total	Growth	Growth percentage (%)
Left eye	39	38	97.4%
Right eye	16	15	93.7%

Table 6: Growth Pattern of Bacterial IsolatesiIn Different types of Discharge from cases of Chronic Dacryocystitis

Name of the Bacterial Isolate	Type of discharge		
	Mucopurulent	Extensive Purulent	Clear fluid
Staphylococcus aureus	4	2	4
Staphylococcus epidermidis	1	-	4
Micrococcus	1	-	-
Bacillus subtilis	4	-	1
Coagulase negative staphylococci (CoNS)	1	-	2
Klebsiella species	-	1	-
Pseudomonas aeruginosa	-	1	-

DISCUSSION

Dacryocystitis is an inflammation of the lacrimal sac because of the obstruction of the nasolacrimal canal with the most common site being nasolacrimal duct (Francisco *et al.,* 2007). Obstruction of the nasolacrimal duct leading to stagnation of tears in a pathologically closed drainage system can result in dacryocystitis. The present study is one such which demonstrated more prevalence of chronic dacryocystitis than acute dacryocystitis. The percentage of culture positivity was found to be higher among eyes with chronic dacryocystitis (51%) than among eyes with acute dacryocystitis (45.4%) the findings of this study is correlated with Imtiaz *et al*., 2000. Among 55 patients, 17 (49%) were males and 18 (50.8%) were females. Males had acute (26 of 12; 21%) and chronic infection (29 of 15; 19%). Female predominance was seen among both acute (26 of 14; 18.5%) and chronic infection (29 of 14; 20.7%) and females were significantly higher among chronic dacryocystitis. The probability P value was significant. This may be either due to obliteration of lumen (Chaudhary *et al.,* 2010) or use of cosmetic especially kajal in their eyes which may lead to partial or complete blockage of drainage system and the predilection of females may be due to smaller nasolacrimal canal diameter than men. Similar results were observed by other authors (Janssen *et al.*, 2001).The most frequent bacteria associated with the chronic dacryocystitis was *Staphylococcus aureus.* These results are similar to other findings (Hartikainen *et al.,* 1997).

Dacryocystitis usually involve one eye but can be bilateral. Out of 55 patients, 39 patients were infected with left eye and the remaining 16 were infected in right eye. The growth percentage of left eye showed 97.4 and 93.7% for right eye. The probability P value was found to be non significant (P>0.05). This study correlated with Brook *et al.,* 1998 in which left lacrimal sac was involved in 36 patients (58%). In general, the disease has predilection to left side because of narrow bony canal. The nasolacrimal duct and lacrimal fossa formed a greater angle on the right side than on the left side. In the antimicrobial susceptibility testing all the Gram positive isolates were 100% sensitive to Methicillin,

Ciprofloxacin, Ofloxacin, Amikacin, Tobramycin, Norfloxacin and Erythromycin but Gram negative isolates highly sensitive to ciprofloxacin, ofloxacin and norfloxcin and least sensitive to amikacin, tobramycin and erythromycin. The results of this study correlated with the studies of Bhuyan *et al.,* 2010.

CONCLUSION

The results of the study suggest that both Gram positive and Gram negative organism are associated with dacryocystitis. However, it is essential to study a larger population for a longer period of time to know the exact clinical picture of these infections which will be very helpful in the design and selection of appropriate therapy of choice.

ACKNOWLEDGEMENT

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Production, Optimization and Partial Purification of Protease by *Bacillus megaterium* MP8 Isolated from Marine Water

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ABSTRACT

Proteases have characteristics of biotechnological interest and have thus become important industrial enzymes. We report the production of thermostable protease and its characterization in *Bacillus* species, which is a thermotolerant bacterium; *Bacillus megaterium* is widely used for isolating protease enzyme. Water sample were collected and screened for protease producing bacteria. The selected organism was identified based on Bergey's manual and molecular characterization was performed. Physico chemical parameters were optimized for selected strain then it was observed that maximum enzyme production seen at 24 hours (120 U/ml), temperature at 30 °C for (134 U/ml), pH at 6.5 for (142 U/ml). Enzyme was partially purified using ammonium sulphate precipitation technique and SDS–PAGE analysis. Efficacies of enzyme are removals of blood stain were observed.

Key words: *Bacillus megaterium*, protease, optimization, 16S rRNA

INTRODUCTION

Proteases constitute a class of enzymes which occupy pivotal positions with respect to their physiological roles and their commercial applications. They perform both degradative and synthetic functions. They occur ubiquitously in a wide diversity of sources such as plants, animals and microorganisms. Proteases are essential for synthesis of proteins, controlling protein composition, size, shape, turn over and ultimate destruction. Their actions are exquisitely selective, each being responsible for splitting very specific sequences of amino acids under a preferred set of environmental conditions.

The exopeptidases act only near the ends of polypeptide chains. The chains based on their site of action at the N or C terminus, they are classified as amino and carboxypeptidases, respectively. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi. In general aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular peptidase produced by *A. oryzae*. (Rao *et al.,* 1998) The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Proteases of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent applications in industry and biotechnology. Microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Gupta *et al*., 2002).

The aroma and texture of these milk products are a result of fat, protein, and lactose metabolism in milk (Gerhartz *et al.,* 1990). Genckal and Tari (2006) isolated *Bacillus* strains under extreme alkaline conditions (Izmir, Turkey), were screened and identified for high alkaline protease activity. Strains with high protease yields were optimized with respect to inoculum concentration, temperature, agitation speed, initial medium pH and incubation time.

MATERIALS AND METHODS

Samples were collected in a sterile container from the Besant nagar beach Chennai. The collected sample was serially diluted in sterile saline water and the dilutions were plated in nutrient agar

plates with 5% sodium chloride and kept for incubation at 37 °C. Colonies were picked based on divergence in morphology, size and colour.

Production of protease enzyme

The enzyme production was carried out by shake flask fermentation using production medium which comprising of yeast extract as carbon source and amended with peptone as a proteinaceous substrate with pH 7. Five hundred ml of sterile production broth was prepared in one-liter conical flask and 5% inoculum was transferred aseptically in to the production medium. The inoculated medium was incubated at 37 °C for 48 h at 250 rpm for better aeration and growth of the organism.

Bacterial genomic DNA isolation using CTAB

CTAB method can be used to extract large quantities of large molecular weight DNA from bacteria. The bacterial suspension was transferred to centrifuge tube and again centrifuged at 10,000 rpm for 5 min, the supernatant was discarded. The cells were resuspended in 875 µl of TE buffer then 20 µl of lysozyme (100 mg/ml) was added to it and mixed well and incubated at room temperature. After 5 mins of incubation add 40 µl of 10% SDS mixed well. Then add 8 µl Proteinase K (10 mg/ml) to it and incubate it for 1 hr at 37 °C. To the mixture 100 µl 5 M NaCl was added and mixed well. 100 µl of CTAB/NaCl (heated to 65 °C) was added. Mixed well and incubated at 65 °C for 10 min. 0.5 ml chloroform: isoamyl alcohol (24:1) was added and mixed this was centrifuged at max speed for 10 min in room temperature. The aqueous phase was transferred to clean micro centrifuge tube, and 0.5 ml phenol: chloroform: isoamyl alcohol (25:24:1) was added and was centrifuged. The aqueous phase was transferred and added 0.6 volume of isopropanol (-20 °C) was added and incubated at room temperature for 30 min. The mixture was centrifuged at maximum speed for 15 min. The pellet was washed with 70% ethanol, and centrifuged at maximum speed for 5 min. The supernatant was discarded and the pellet was dried for 5–10 min at room temperature. The pellet was resuspended in 20 µl of RNAse (10 mg/ml) containing TE buffer and transferred to a sterile micro centrifuge tubes and incubate at 37 °C for 20 min.

PCR amplification

The PCR amplification of 16S ribosomal RNA gene was carried out based on the methodology of (Elizabeth *et al.,* 1990) in Thermocycler (PTC – 100 TM Programmable Thermal Controller, USA)**.**

Primers: **Forward:** 5' AACGGCTCACCAAGGCGACG 3'

                             Reverse: 5' GTACCGTCAAGGTGCCGCCC 3'

Gene sequencing

The gene sequencing was carried out using Beckman Coulter CEQ 8000 auto analyzer. (The original sequence was done by Delhi University South Campus). The amplified products were cleaned up using QIAQuick (Qiagen) Spin column. The cycle sequencing was carried out using DTCS quick start Dye terminator kit (Beckman Coulter).

16S rRNA sequence analysis

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. BLAST Search Tool (http://www.ncbi.nlm.nih.gov/blast/) provides a method for rapid searching of nucleotide and protein databases.

Phylogenetic tree analysis

This method is useful to determine whether a group of genes are related through a process of divergent evolution from a common ancestor or the result of convergent evolution. In the present study a phylogenetic tree was constructed using BLAST tree tool.

Assay of protease enzyme production by the isolates

Plate assay

The plate assay was performed using skim milk agar medium, plates were prepared using skim milk along with casein substrate, sodium chloride and agar. The pH was adjusted to 7.2 to 7.4 by using 0.1 N NaOH. Wells of around 10 mm diameter was cut out aseptically with the help of cork borer. The wells were filled with culture filtrate of different concentrations ranging from 20 µl to 50 µl and incubated at 37 °C over night. After incubation the plates were observed for the formation of clear zone.

Chemical assay

Proteolytic activity of protease was measured by the hydrolysis of protein using the method followed by Mazumdar and Majumdar, 2003. Crude culture filtrate was used as an enzyme sample; about 1 ml of culture filtrate was boiled in a water bath (100 ºC) for 20 minutes. Boiled sample was cooled suddenly using ice cubes for 5 minutes. Both killed and live samples were taken for the assay. 2% casein substrate was prepared freshly by addition of 2 g of casein in Tris buffer pH-8. The reaction mixture containing 0.1 ml of substrate (Casein), of enzyme solution was mixed and incubated at 30 °C for 2 h for enzymatic reaction. After incubation, the enzymatic reaction was terminated by addition of 2 ml trichloroacetic acid solution (5%,v/v) and precipitate was removed by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was collected neutralized using 4 ml of 1 N NaOH to alkalize the contents of the tube. To the reaction mixture 500 µl of 1 N Folin's-phenol reagent was added and allowed to develop the colour. The absorbance was read at 750nm (Rahman *et al.,* 2005) in UV-VIS Spectrophotometer (Beckman DU 40).

Optimization of physico chemical parameters for production of protease enzyme

The effect of different concentrations of incubation time, temperature and pH on protease production was done and was plotted graphically. After incubation, around 20 ml of culture was aseptically with drawn periodically at 6 h intervals up to 60 h. After incubations the culture filtrate was examined for the protease activity.

Dialysis and SDS-PAGE analysis

The pretreatment of the dialysis membrane was done by immersing the membrane into the warm 0.1 M Tris-HCl (pH 7) for 10 min. One end of the membrane tube was closed by tightly by fixing the clip. The precipitated protein was then transferred in the dialysis tube and other end was tied with a thread. The pack was freely left into a large beaker, which contains around 500 ml of 0.1 M Tris HCl buffer pH 7. The buffer was stirred slowly using magnetic stirrer. The entire setup was placed in the cold room for 48 h.

The lyophilized protein sample was dissolved in 0.1M Tris (pH 9) and around 100 ml of dissolved sample was mixed with equal volume of protein gel loading buffer. The sample mixer was heated at 90 °C for 10 min. The 10 ml sample and known molecular weight protein marker were loaded at separate wells into the polymerize gel 12% (polyacrylamide). Tris-Glycine (pH 8.3) buffer was used as a electrode buffer. When the tracking dye reached the opposite end the running was terminated. The gel was stained with Coomassie Brilliant Blue (CBB) and destaining was done using ethyl alcohol-acetic acid mixture solution.

Efficacy of protease are removal of blood stain

Washing test with protease preparation

Application of protease as a detergent additive was studied on white cotton cloth pieces (4×4 cm) stained with blood stain. Visual examination of various pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with blood stain were taken as control.

RESULTS

Sample collection and isolation of protease producing bacteria

In this study, bacterial strains were isolated from marine water samples collected in Chennai Besant nagar beach. The samples were serially diluted and plated on nutrient agar medium (Fig.1a). 17 individual isolates were selected for further screening.

Screening for protease enzyme

The individual isolated from nutrient agar plate were further plated on skim milk agar medium to screen protease producing organism. Among 17 bacterial isolates that have been initially screened among that only 4 strains showed positive result for protease enzyme (Fig.1b).
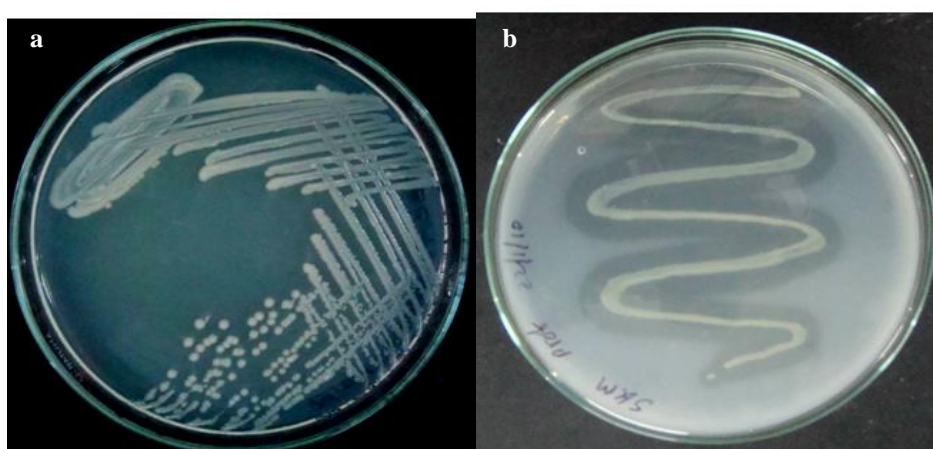
Fig.1: Growth and protease activity of *B. megaterium* MP8

Molecular identification of *Bacillus megaterium*

The potential producing bacteria, *Bacillus megaterium* was ascertained its systematic position based on 16S rRNA sequence analysis and with the aid of computational programme, BLAST homology analysis was also carried out to compare with other 16S rRNA sequences available in the GenBank of NCBI. It revealed that the sequence of *Bacillus megaterium* MP8.

### 16S ribosomal RNA gene, partial sequence (726 bps) for *Bacillus megaterium strain* MP8

TCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGT
GGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGATAGGAT
CTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCG
GTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAAACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTT
CGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTAACTGCTCGTACCTTGAC
GGTACCTAACCAAAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTG
AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGA
AAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA
AGGCGGCTTCGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
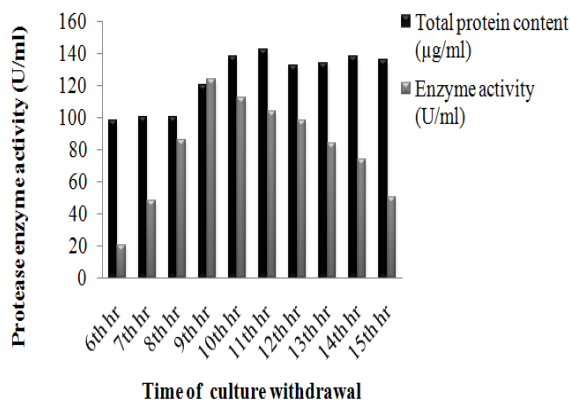
Plate assay

Skim milk agar plates were used for protease (Beselin and Rosenau, 2006). The production of protease enzyme was indicated by the appearance clear zone formed. It was visually observed (Fig.2).

Effect of incubation on protease production

The isolate was cultivated in Zobell medium. The various incubation times were analyzed and tabulated (Fig.3). The maximum amount of protease enzyme produced by these isolate was 124 U/ml. It was observed at 24 h.
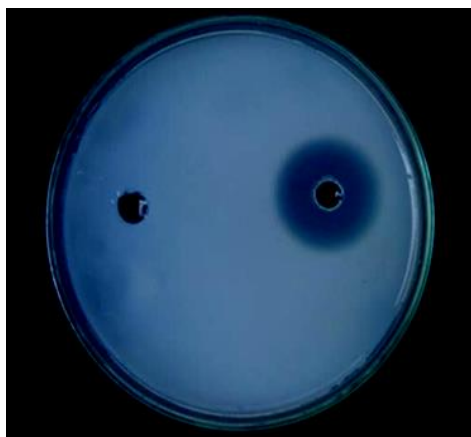
Fig.2: Zone of clearance in plate assay   **Fig.3: Effect of incubation on protease production**
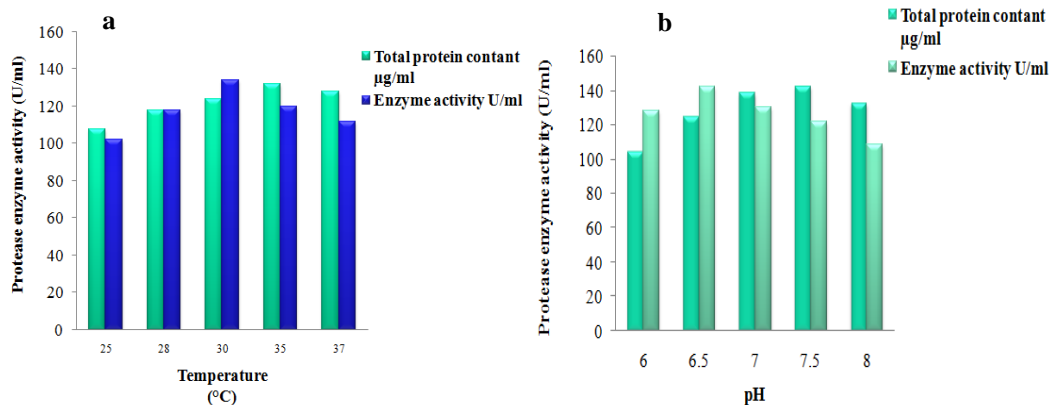
Effect of Temperature on protease

The isolate was cultivated in Zobell medium and incubated at various temperatures for 48 h. The amount of enzyme produced at different temperature was analyzed and tabulated (Fig.4a). The maximum amount of protease enzyme produced by these isolates was 134 U/ml. It was observed at 30 ℃.

Effect of pH on protease

The isolate was cultivated in Zobell medium and incubated at various pH for 48 h the amount of enzyme produced different pH were analyzed and tabulated (Fig.4b). The maximum amount of protease enzyme produced by these isolate was 142 U /ml. It was observed at pH 6.5.

Partial purification of enzyme

The enzyme was precipitated by ammonium sulphate precipitation method and purified using dialysis membrane. The partially purified enzyme was separated on SDS PAGE and molecular weight was carried 50 to 75KDa (Fig.5).

Fig.4. Effect of temperature and pH on protease production

Destaining of blood stain

The blood stain was complete removal and observed after 30 minutes. The test-4 contains both detergent and enzyme blood stain was complete removal and observed after 60 minutes. It was visually observed (Fig.6).
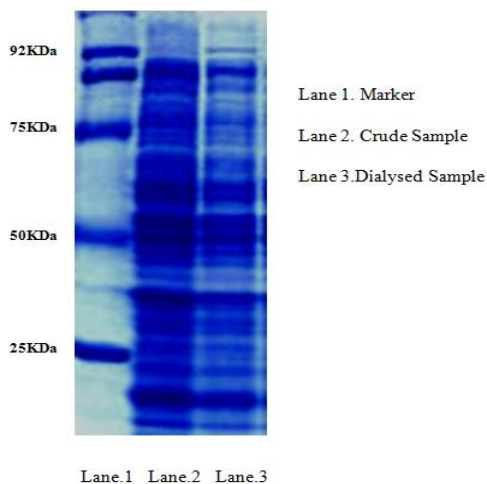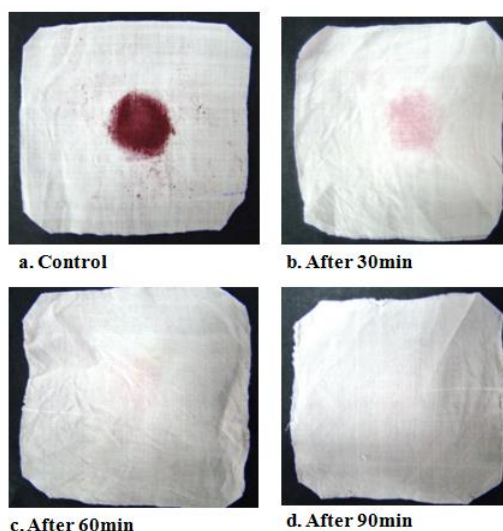

Lane 1. Marker

Lane 2. Crude Sample

Lane 3.Dialysed Sample

Fig.5. Protein profile in SDS-PAGE

a. Control

b. After 30min

c. After 60min

d. After 90min

Fig.6. Destaining of blood stain

DISCUSSION

The majority of the protease was secreted within the first 24 h, and this result indicates that protease production was related to the exponential growth phase of the bacteria. Similar results have been reported in previous investigations (Kaur *et al.,* 2001; Uyar and Baysal, 2004). Comparing the results to the literature there is a broad incubation time ranging from 24-120 h reported for *Bacillus* strains (Singh *et al.,* 2001; Mabrouk *et al.,* 1999; Gupta and Beg, 2003). Based on these data our strains fit in this interval. The results showed close resemblance with alkaline protease of thermophilic *B. licheniformis* (Sinha and Satyanarayana, 1991). The results on the effect of temperature on protease production showed maximum activity at 30 °C. Comparable results were obtained for *B. alcalophilus*, isolated from Lonar lake, India (Kanekar *et al.,* 2002). With this regard our strains are in agreement with the literature reported data (Takami *et al.,* 1989; Mabrouk *et al.,* 1999; Banerjee *et al.,* 1999; Çalık *et al.,* 2002; Puri *et al.,* 2002; Kanekar *et al.,* 2002; Joo *et al.,* 2003). The optimum temperature for protease production by *Pseudomonas* sp. strain S5 was at 37 °C (Baharum *et al.,*2003). Many researchers have reported on the optimization of protease production. However, only few reports are available in literature on the optimization for the production of organic solvent-stable proteases (Gupta and Khare, 2007). Obviously, because the medium contain soul source of protein as a substrate, so unutilized protein also will be present in the exhausted medium. But the Presence of protein band nearing the molecular weight 50–75 kDa confirms the presence of enzyme.

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Deciphering the Diversity of Microalgal Bloom in Wastewater - An Attempt to Construct Potential Consortia for Bioremediation

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ABSTRACT

The present study documents the biodiversity of microalgal bloom in a waste water pond located near Mogambikkai College of Engineering at Kalamavur, Keeranur, Tamil Nadu, India. Algal mats and plankton cells were collected from four different sites during the month of August 2014. The most dominant microalgae was found to be *Chlorella* sp. along with which other organisms like *Oscillatoria* sp, *Anabaena* sp, *Spirulina subsalsa* and *Pandorina* sp. were found in less numbers. Further physicochemical parameters of the waste water was also analysed to study the factors influencing bloom formation. The analysis chemical parameters showed the presence of elevated amounts of nitrate (855 mg L^{-1}) and chloride (439.86 mg L^{-1}). Mg and Silicate were also present at the concentrations of 132 mg L^{-1} and 99 mg L^{-1}. On the whole, results of this study throw light on utilizing these parameters for developing artificial blooms, a strategy of recent interest for raising algal biomass, which is the need for numerous industries.

Keywords: Microalgae; waste water; physico-chemical; *Chlorella* sp.; bloom formation.

INTRODUCTION

Microalgae are large and diverse group of prokaryotic and eukaryotic photosynthetic, microscopic organisms found dwelling in both fresh and marine water environments. The simple structure of microalgae is the reason for their high growth rate and high photosynthetic efficiency (Prescott, 1954 and 1962; Suresh *et al.,* 2012; Khan and Mohammad, 2014). They are the rich source for lipids, proteins and starch and also they are economically important because of their numerous biotechnological and industrial products (Thajuddin and Subramanian, 2005; Praveenkumar *et al.,* 2012; Baldev *et al.,* 2014). The availability of light and sufficient nutrient in aquatic habitat aids the formation of blooms either by a single or group of organisms. Microalgae have 50 times higher growth rate when compared to that of the switch grass - a fastest growing terrestrial plant (Nakumara, 2006; Demirbas, 2006).

Removal of waste water nutrient is necessary to decrease the risk of pollution and eutrophication in the environment. Enormous discharge of domestic waste from industries and households provide a convenient and cheap source of growth media for microalgae. Domestic waste water is the one of the most nutritious source of growth media for microalgae (Wiley *et al.,* 2009; Bare *et al.,* 1975). The formation of algal bloom is mainly supported by the presence of nitrogen and phosphate along with suitable temperature and pH. Cultivation of microalgae in the waste water could be handy to solve issues related to waste water discharge in the environment. There are several hurdles for the production of algal biomass, among which requirement of water source and cost effective nutrients are the major one (Barbosa, 2003; Ferrell and Sarisky-Reed, 2010). Therefore exploring the ability of microalgae to grow in domestic waste water could be a valuable attempt to address all issues like bioremediation and biomass production simultaneously.

Previous reports regarding the exploration of microalgal diversity in waste water blooms are very few. Therefore exploring the factors required for bloom formation in waste water could pave way

towards easy and effective method of algal biomass production integrated with waste water treatment. The present study focusses on the criteria required for the formation of algal bloom in domestic waste water. Physicochemical characterization of the domestic waste water was performed and the microalgal diversity present in that habitat was also recorded.

MATERIALS AND METHODS

Collection of sample

Waste water was collected from the domestic sewage pond of Mogambikkai College of engineering situated at Kalamavur, Keeranur, Tamil Nadu, India. The exact Latitude is 10º383333 N and Longitude is 78º816667 E (Figure 1). Along with the waste water, algal sample were also collected from the respective sites.
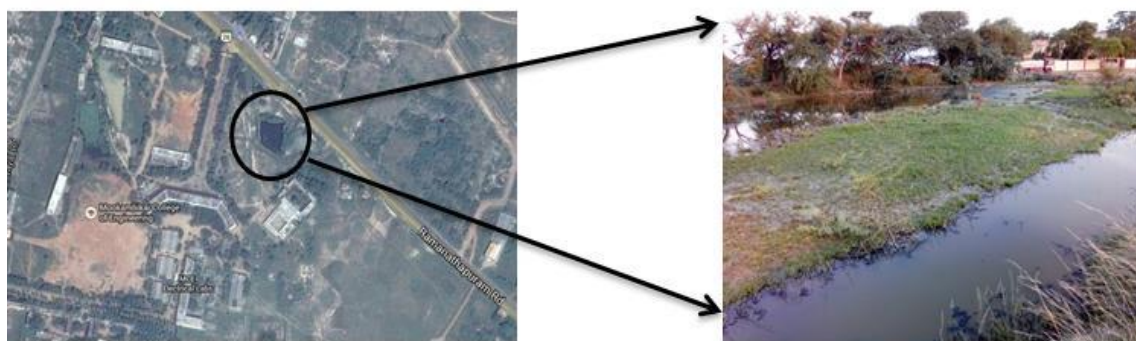

Fig. 1: Sampling site from the domestic waste water was collected (Mogambikkai College of Engineering, Kalamavur, Keeranur, Tamil Nadu, India)

Analysis of Physicochemical parameters

The water samples collected from 4 different sites were subjected to physicochemical parameter analysis. Physical parameters like temperature, pH, light intensity in terms of photon intensity (μmol m^{-2} sec^{-1}) using Solar quantum meter (Spectrum technologies, USA) were measured. Electric conductivity (EC), Chemical parameters like Total solid (TD), Total Dissolved solid (TDS), Dissolved solid (DO), Chemical oxygen demand (COD), alkalinity, phosphate, sulphate, silicate, chloride, nitrate, nitrite, calcium and magnesium were also measured using the standard method (APHA, 1989).

Identification and Documentation

The microalgae isolated from the waste water pond were subjected to microscopic identification based on standard monographs and available literatures (Taylor et al., 2007; Desikachary, 1959; Prescott 1954, 1962). Microphotographs of the microalgal samples were taken and documented using camera coupled microscopic system (Micros, Austria). Further the microalgal samples were subjected for purification in respective media like BG 11+ (Rippka et al., 1979) and Chu 10 medium (Chu, 1942).

RESULTS AND DISCUSSION

The term 'microalgae' apply to a diverse group of microscopic eukaryotic and prokaryotic organisms. They occur in both unicellular and filamentous forms. Microalgae refer to both Cyanobacteria (Blue green algae) and green algae. They occur in freshwater, marine water and damp environments, and their size ranges from minute phytoplankton to giant marine kelp (Van Vuuren *et al.,* 2006; Horn and Goldman 1994; Suresh *et al.,* 2012).Microalgae are the primary producers of organic matter which higher animals depended on, either directly or indirectly through the food chain. The regular dumping of drainage has maintained the dominant growth of microalgal flora in particular sites. The nutrients available in waste water body favour the luxurious growth of microalgae present. The success of eutrophication is due to the presence of weeds and algal bloom. Waste water bodies are the large and

cheapest source of media for the growth of microalgae (Bosman and Hendricks, 1980). The water sample collected from the waste water pond showed more number of *Chlorella* sp. Along with which, other microalgae like *Oscillatoria* sp, *Pandorina* sp, *Spirulina subsalsa*, *Anabaena* sp, *Selenastrum* sp, *Phacus* sp., *Phacus caudatus* and *Scenedemus quadricauda* were also recorded at the sampling site (Figure 2).
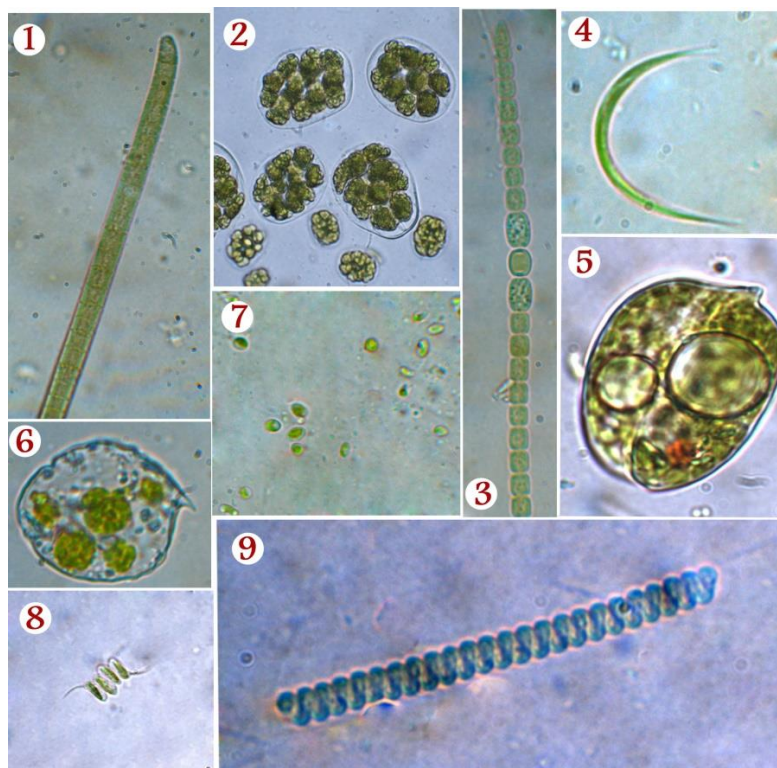

Fig. 2: Microphotographs of Microalgae 1.*Oscillatoria* sp. 2. *Pandorina* sp. 3. *Anabaena* sp 4. *Ankistrodesmus* sp. 5. *Phacus caudatus* 6. *Phacus* sp. 7. *Chlorella* sp. 8. *Scenedesmus quadricauda* 9. *Spirulina subsalsa*

The study site selected has not yet been explored for microalgal diversity and the nutrient profile of this site is also not known. Considering this, physicochemical parameters of the waste water sample were recorded. Temperature during sampling was 28-34 ºC (30-36 ºC is the atmospheric temperature), pH of the water was 6.25, light intensity in terms of photon was 830-1900 µmol m^{-2} sec^{-1} and EC was 1.737 µsec cm^{-1}. The exposure of the waste water microalgal flora to suffice light, temperature and pH plays crucial role in eutrophication and formation of algal blooms in the waste water bodies. The acidic pH and temperature could be the factors favouring rapid growth of *Chlorella* sp. in the study site.

The measurement of physicochemical parameters showed the following: alkalinity - 100 mg L^{-1}, TS - 0.0159 mg L^{-1}, TDS - 0.126 mg L^{-1}, Dissolved oxygen - 3.05 mg L^{-1}, COD - 8.76 mg L^{-1}, Phosphate - 0.023 mg L^{-1}, Sulphate - 0.198 mg L^{-1}, Silicate - 99 mg L^{-1}, Chloride - 439.86 mg L^{-1}, Nitrite - 0.8 mg L^{-1}, Nitrate - 855 mg L^{-1} and Calcium - 40 mg L^{-1} (Table 1). Among the nutrients determined, the concentrations of Nitrate and Chloride in the waste water were found significantly higher. P, N and C are the three primary nutrients required for algal productivity. Previous report suggested that nitrogen source is plays crucial role in accelerating algal growth and a major nutrient required for increasing algal biomass (Oliver and Ganf, 2002; Praveenkumar *et al*., 2012).

Table 1: Physicochemical parameters of the waste water sample

S.No	Physicochemical parameters	mg/L
1	pH	6.25
2	EC- µs/cm	1.737
3	Alkalinity	100
4	TS	0.0159
5	TDS	0.0033
7	DO	3.05
9	COD	8.76
10	Phosphate	0.023
11	Sulphate	0.198
12	Silicate	99
13	Chloride	439.86
14	Nitrite	0.8
16	Calcium	40
18.	Nitrate	855
19	Magnesium	132.62

Other than N and Cl, the concentration of the micronutrient Mg was also found high. The availability of micronutrients has been shown to positively influence the growth of algae. Micronutrients such as calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), iron (Fe), manganese (Mn), sulfur (S), zinc (Zn), copper (Cu) and cobalt (Co) are found responsible for the enzymatic activities in algae (Goldman and Horne, 1983). In a study by Finkle and Appleman (1952), the role of magnesium in cell division of *Chlorella* sp. was reported. When the concentration of Mg decreases, the reproduction of algae stops or significantly reduced. Therefore the study concluded that the presence of Magnesium was indispensible for algal growth.

The development of algal biomass has become difficult due to the limitations of water resources and absence of cheaper growth media. Microalgae utilize the nutrients of waste water and can grow under variety of stress conditions. The present study clearly documents the diversity of microalgae in waste water which has high concentrations of nitrate, chloride and magnesium. Other factors like pH, temperature and light intensity supported algal growth and rapid bloom formation in the sampling site. Further cultivating microalgae in waste water for the removal of nutrients could pave way towards finding an integrative technique of raising algal biomass while significantly purifying the waste water.

ACKNOWLEDGMENTS

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