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SOLID STATE FERMENTATION OF COCONUT PITH FOR CELLULASE ENZYME PRODUCTION USING MICROBIAL CONSORTIUM

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ABSTRACT

Coconut pith, available in abundance, can be an excellent new substrate for the production of cellulase enzyme by solid state fermentation. Several studies have been carried out to produce cellulolytic enzymes from bio waste using degradation process by using microorganisms including fungi, such as *Trichoderma*, *Penicillium*, and *Aspergillus* spp. Cellulases are imperative enzymes not only for their potent applications in different sectors, like industries of food processing, animal feed production, pulp and paper production, detergent and textile, but also for the significant role in the bioconversion of agricultural wastes into sugar and bioethanol. The soil from compost area were aseptically collected and serially diluted and were spread on surface of Carboxy methyl cellulose agar and incubated for 5 days at 30ºC. Fungal colonies were picked and subcultured to obtain pure culture. Coir pith was sun dried for 1 h and hot air oven dried at 80 °C for 2 h, crushed and sieved to obtain particle size of 150 μm. The coir pith was further pretreated using NaOH. The pretreated samples were hot air oven dried at 80 °C and stored for further hydrolysis. Solid-state fermentation was performed in a solid-state fermenter employing untreated and pretreated coir pith with the help of microbial consortium. The enzyme produced is then subjected to purification. The residual coir pith obtained after the extraction of cellulase was mixed with *Azobacter chroococcum, Bacillus megaterium, Bacillus mucilaginosus* and stored in air-tight containers. This mixture acts as the microbial inoculum.

Keywords: Coir pith; Cellulase; CMCase; solid state fermentation; pretreatment; *Trichoderma, Penicillium,* and *Aspergillus* spp ; bio fertilizer; CWC.

INTRODUCTION

Coir pith is an organic matter which originates from the tropical hemisphere, especially from south-east Asia where coconut oil production is extensive. For professional oil winning companies, the husk of the nut is a waste product. These coconut husks mainly consist of coir

pith and coir fibers. Initially, coir pith was considered a waste but now research has established widespread applications for it. Being an agro-waste from the coir industry, coir pith serves as a renewable source [1]. Accumulation of coir pith near coir retting factories causes solid waste pollution problems mainly due to the ligno-cellulosic compounds present in them. The lignin (31 %) and cellulose (27 %) that they contain is responsible for their slow degradation [2]. Over the last few years, environmental concerns have increased attention to-ward using coir pith as an alternative substrate with orientation towards agricultural needs. Thus, the conversion of such agricultural wastes into useful products may decrease the problems they cause. This study deals with the utilization of coir pith for cellulase production. But the lignin present in the coir pith was observed to interfere with hydrolysis by irreversibly binding the hydrolytic enzymes, thereby blocking access to cellulose. Pretreatment of coir pith increases the crystallinity of cellulose, thus removing lignin and enabling its enzymatic degradation. In addition, pretreatment may increase the surface area of the cellulose thereby enhancing its reactivity with the enzyme and thus its transformation [3].

Cellulose is commonly degraded by an enzyme called cellulose [4]. Several studies have been carried out to produce cellulolytic enzymes from biowaste using degradation process by using microorganisms including fungi, such as Trichoderma, Penicillium, and Aspergillus spp [5]. The commercial use of cellulases is dependent on the following: high titer and good enzymatic activity, low production cost and feasible mass production. Cellulases are inducible enzymes and their synthesis is strongly repressed by soluble sugars. Cellulases are imperative enzymes not only for their potent applications in different sectors, like industries of food processing, animal feed production, pulp and paper production, detergent and textile, but also for the significant role in the bioconversion of agricultural wastes into sugar and bioethanol. Parameters such as control of pH and temperature are critical for the production and release of cellulases [6]. Many species of Trichoderma and Aspergillus are strongly cellulolytic [7]. Solid-state fermentation (SSF) is an attractive strategy to produce cellulase using a variety of lignocelluloses as substrates [7]. Alkaline pretreatment using NaOH is used to delignify the feed stock material (coir pith). The experiments were carried out under solid state conditions employing coir pith with 60 % moisture content, pH 5, temperature of 40 °C for 11 days. Cellulase obtained was purified and characterized. The extract drawn was purified using ammonium sulphate salt precipitation. The coir pith, after its utilization as substrate for cellulase

production, was modified and applied as a biofertilizer for tomato plantations (Solanum lycopersicum).

MATERIALS AND METHODS

a) Pretreatment and Characterization of Coir Pith

Coir pith obtained from Sakthi coir exports, Alappuzha was sun dried for 1 h and hot air oven dried at 80 °C for 2 h, crushed and sieved to obtain particle size of 150 μm. The coir pith was further pretreated using NaOH. The pretreated samples were hot air oven dried at 80 °C and stored for further hydrolysis.

b). Isolation of Fungi

Fungi *Penicillium citrinum, Aspergillus niger* and *Trichoderma viride* were isolated from soil by serial dilution technique using 10g soil [8]. Soil was dissolved in 0.85% saline water in Erlenmeyer conical flask (500ml capacity) having 95ml saline water and beads then shaken to mix soil properly, mark as 10-1 dilution. Obtain 10-3 dilution by serially transferred 10ml soil sample from 10-1 dilution to conical flask having 90ml saline water. 0.1ml sample was transferred from 10-3 dilution to sterilize Petri dish and poured melted Potato dextrose agar (PDA) medium. Plate was shaking gently to mix sample and medium then left at room temperature to solidify medium. The solidified plate was incubated at 30ºC for 5 days. After that plate was used for observation. After incubation period, few spores were taken by wire loop and streaked on PDA medium plate by zigzag method and incubated at 30°C for 5 day. Colonies were observed by light microscope using 10 and 40X objective lens. Pure colonies were transferred to PDA plate and used for identification.

c). Identification of Fungi

Fungal culture was stained with Lacto Phenol Cotton Blue stain (LPCB) and covered with glass cover slip. This was then observed under light microscope using low power (10X) and high power (40X) objective lens.

d). Fungal Screening

The isolated fungal cultures were screened for their ability to produce cellulase enzyme on selective media containing NaNO3– 2g, K2HPO4- 1g, MgSO4.7H2O– 0.5g, KCl – 0.5g, Carboxy methyl cellulose sodium salt– 2g, Agar agar- 17g and Distilled water- 1000ml. pH of the medium was adjusted to 5.0. After autoclaving at 121°C and 15 lb pressure, the medium was

poured into petri plates and was allowed to solidify. After solidification the plates is inoculated with fungal culture. These plates were then incubated at room temperature (30°C) for five days to allow fungal growth. After incubation, 10ml of 1% Congo red staining solution was added to the plate and kept for 15min to de-stain. Finally 1N NaCl was discarded and the stained plates were analyzed by observing the formation of clear zone around the fungal colonies. The high zone of clearance showing fungal isolates was used for cellulase production.

e).Fermentation Production Media

The fermentation media is prepared by adding K2HPO4 - 2g, CaCl 2 - 0.3g, MgSO4.7H2O - 1g, NH4NO3 - 2g, FeSO4.7H2O - 5mg, MnSO4.4H2O - 1.6mg, ZnSO4.7H2O - 3.45mg, CoCl2.6H2O - 2mg, Carboxy methyl cellulose salt - 5g and Distilled water - 1000ml. 100ml of this media is transferred to 4 clean and dried Erlenmeyer conical flask of 250ml capacity containing 10gm of pre-treated coir pith, and their mouths were plugged with cotton wools. All flasks were sterilized by moist heat at 15 lb pressure for 15min and cooled for inoculation of fungal species.

f).Enzyme Production

5ml sterilized water poured to slant of selected isolates and scrapped via nichrome wire loop. 1ml spore suspension was transferred aseptically to each flask and incubated at 30ºC for 11 days under stationary condition.

g).Enzyme Activity

Enzyme activity was assayed using carboxy methyl cellulose (CMC) as substrate [9]. The assay was performed on the basis of red brown color produced by dinitro salicylic acid (DNS) reagent [10]. In this assay, 1ml of 2% CMC solution in 0.05M sodium citrate buffer of pH 5.0 was taken in a glass test tube and added 0.5ml culture filtrate and was mixed using cyclomixer. All test tubes with reaction mixer was incubated in water bath at 30ºC for 15min and then add 2ml DNS reagent (DNS- 10g, Phenol- 2g, Sodium succinate- 0.5g, sodium hydroxide - 10g and Distilled water- 1000ml) to stopped enzyme - substrate reaction. All test tubes were incubated for 20 min in boiling water bath to developed red brown color. All test tubes were taken out from water bath and cooled. Then add 1ml of 1% Rochella salt (Sodium potassium tartrate- 10g and Distilled water- 1000ml). All test tubes were left at room temperature for 20min to stabilize red brown color. Color density was recorded by spectrophotometer at 550nm using spectrophotometer and compared with standard curve of

glucose (1mg/ml). Enzyme activity was determined in international unit (IU) as an amount of enzyme that produces 1µM glucose/min.

RESULTS AND DISCUSSION

Identification of the Fungi

Three fungi were isolated and screened from the samples collected from soil for cellulase production by congo red assay. The colony morphology and the microscopic observation by lacto phenol cotton blue stain confirmed the organisms to be Aspergillus, Trichoderma and Pencillium spp.

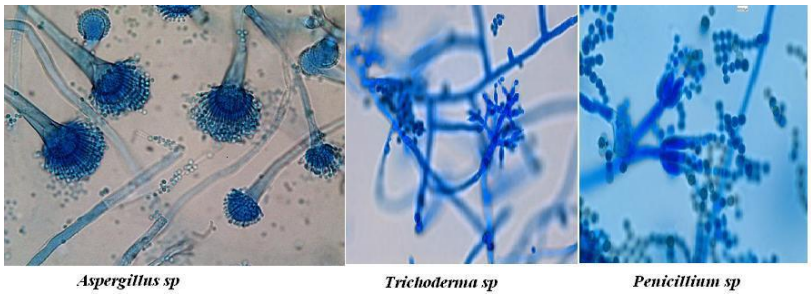


Figure 1: Microscopic features of isolated fungal strains stained with lactophenol cotton blue stain

Figure 2: Zone of clearance produced by isolated cellulolytic organisms on CMC agar after treating with Congo red and NaCl.

Enzyme Activity

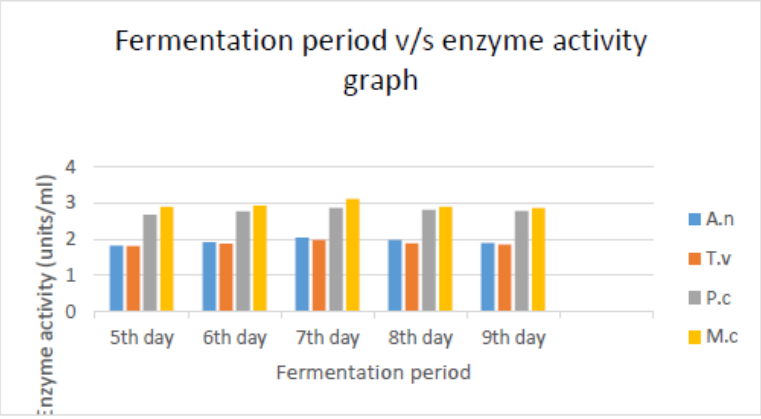
i. Preparation of crude enzyme

After incubation, the cultures were centrifuged and supernatant were used as a source of crude enzyme [11]. The crude enzyme solution was utilized for determination of enzyme activity.

ii.DNS method

Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, G.L., 1959) [12], through the determination of the amount of reducing sugars liberated from carboxy methyl cellulose (CMC) solubilized in 50 mM Tris–HCl buffer, pH 7.0. This mixture was incubated for 20 min at 70 °C. For crystalline cellulose substrates, incubation times were extended to 2hr and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 min, cooled in water for color stabilization, and the optical density was measured at 550 nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

DAY	CELLULASE ACTIVITY (Units/ml)			
	Aspergillus niger	*Trichoderma viride*	*Penicillium citrinum*	Mixed consortium
5	1.83	1.81	2.68	2.89
6	1.91	1.87	2.77	2.93
7	2.04	1.98	2.86	3.12
8	1.98	1.89	2.82	2.90
9	1.90	1.85	2.78	2.86



A.n- Aspergillus niger

T.v- Trichoderma viride

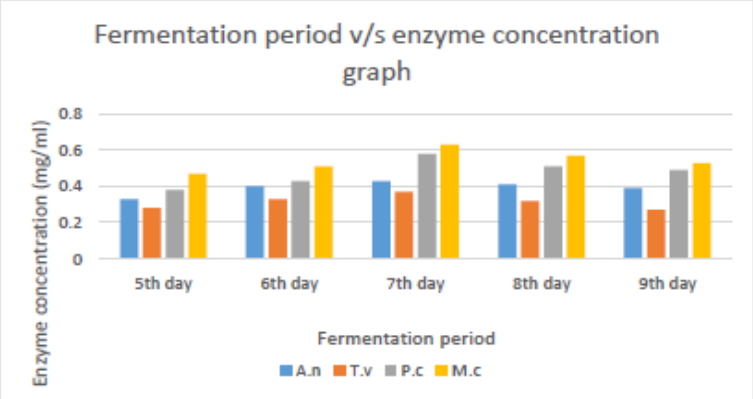
P.c- Pencillium citrinum

M.c- Mixed consortium

Protein determination

Protein concentrations in crude sample were determined by using a Lowry's method [13].

DAY	CONCENTRATION OF CELLULASE (mg/ml)			
	Aspergillus niger	*Trichoderma viride*	*Penicillium citrinum*	Mixed consortium
5	0.33	0.28	0.38	0.47
6	0.40	0.33	0.43	0.51
7	0.43	0.37	0.58	0.63
8	0.41	0.32	0.51	0.57
9	0.39	0.27	0.49	0.53



A.n- Aspergillus niger

T.v- Trichoderma viride

P.c- Pencillium citrinum

M.c- Mixed consortium

Partial Purification of Cellulase

i.Ammonium sulphate precipitation

About 20ml of the crude enzyme prepared was brought to 80% saturation with solid ammonium sulphate [14]. The mixture was left overnight at 4ºC in a magnetic stirrer. Centrifuge the mixture and the pellet was dissolved in 10ml of 50mM sodium acetate buffer (pH-5.5) for further purification.

ii.Dialysis

The pre-treated dialysis bag was used for the dialysis of the enzyme collected after the ammonium sulphate precipitation [15]. 8ml of the partially purified enzyme was dialyzed against 30mM sodium acetate buffer (pH-5.5) at 4ºC with three changes of buffer. The partially purified sample was assayed for enzyme activity and protein content.

iii.Determination of molecular weight

a) SDS- Polyacrylamide Gel Electrophoresis

SDS PAGE was carried out in order to determine the molecular weight of purified enzyme sample [15]. It was carried out in such a way that the standard protein marker was loaded next to the purified sample, followed by the dialyzed and the crude. The silver staining was used to separate proteins by SDS- PAGE analysis.

b) Native PAGE and Zymogram staining for checking cellulase bands

PAGE also called native gel electrophoresis was performed according to the standard procedure, using a discontinuous buffer system without SDS [16]. The gel was removed and placed on a CMC-agar gel (CMC 1% and agar 1%). Both the gels were incubated at 37ºC for overnight. The CMC-agar gel was treated with sodium chloride for 15 minutes and then stained with Congo red solution. The bands of cellulase were seen as a clearance zone under black background.

Optimization of process parameters

Optimization of process parameters like temperature, incubation time, pH, inoculum density, and substrate concentration was carried out to maximize cellulase enzyme production during degradation of lignocellulosic waste in mixed-culture fermentation.

Effect of incubation period on Cellulase production

The incubation period is directly related with the production of enzymes and other metabolite up to a certain extent. The production of cellulases increased with the increase in incubation period and reached maximum after 7th day of incubation. Further increase in the incubation period however, resulted in the gradual decrease in the production of cellulases. Therefore, incubation period of 7 days was found to be optimal for cellulases production by mixed cultures on coir waste. The decrease in the production of cellulases by mixed cultures after 7 days of incubation period might be due to the depletion of the nutrients and accumulation of other byproducts like proteases in the fermentation medium [17]. Their results showed that crude inducer was found to be very effective in inducing cellulases and reducing the incubation period.

Effect of temperature on Cellulase production

The effect of incubation temperature (20-80ºC) on the cellulase biosynthesis by mixed culture was studied. There was a gradual increase in the production of cellulase as the temperature was increased. But it showed maximum yield at 30ºC. As the temperature was

further increased, there was a gradual reduction in the enzyme production. This may be due to the fact that higher temperature denatures the enzymes. Results showed that cellulases production was maximum in flasks incubated at 30+ 2oC and decreased with high temperature. High temperature may also lead to inhibition of microbial growth.

Effect of initial pH

Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. The optimum pH for maximum enzyme production was 5.0 followed by pH 6. The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual fall in activity. Most microorganisms grow optimally within a wide pH range. At the pH value of 4.0, considerable amount of enzyme production was started, however, it started to increase as the initial pH of the growth medium was increased and reached maximum at pH 5.0. Further increase in pH resulted in a gradual reduction of cellulases biosynthesis by the organism. Hence, pH of 5.0 was optimized for the maximum cellulases biosynthesis by mixed fungal consortium. Enzyme production is greatly influenced by initial pH of the culture medium.

Results also showed that cellulase production was maximum at pH 5.4. Fungal metabolism progressed from imminent to actual exhaustation of carbon source which is indicated by increase in pH. This may lead to the situation in which at least part of the biomass started to sporulate, after which a return to the productive phase no longer occurred. After pH value of 5.0, the production of cellulases decreased which might be due to the fact that cellulases are acidic proteins and are greatly affected by the neutral pH values [18].

Effect of inoculums density on Cellulase production

The effect of concentration of inoculum on enzyme production was studied by inoculating different concentration of inoculums ranges from 2.0% to 10.0% in enzyme production media containing coir pith as substrate. The concentration of initial inoculums plays a critical role in enzyme yield in production media. The production of enzyme was minimum at 2% inoculum and reached maximum with 6% inoculum containing 3.1 × 108 conidia/ml. Further, increase in inoculum size resulted in the gradual decrease in production of cellulases by mixed cultures. At low inoculum size i.e., 2%, conidial cells were not enough to utilize the fermentation medium in a better way hence, resulted in less growth and cellulases biosynthesis. On the other hand, at high concentration of conidial cells, anaerobic condition of fermentation

medium, due to the tremendous growth of microorganism may lead to nutritional imbalance in medium, which resulted into gradual reduction of cellulases yield [19].

Effect of substrate concentration on cellulase production

Enzyme activity increased with the increase in substrate concentration. The maximum production of cellulase was observed at 35 % of substrate concentration and further increase in substrate concentration resulted in no significant increase of enzyme activity. Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of cellulose. Low substrate concentration normally increases the yield of enzyme and reaction rate of the hydrolysis. However, high substrate concentration could cause substrate inhibition, which lowers enzyme production [20].

Modification of residual coir pith into coir pith waste compost (CWC)

Residual coir pith obtained after the extraction of cellulase was stored in air tight containers. Amongst biofertilizers Azotobacter and Bacillus strains play a key role in improving fertility conditions of soil.18 The residual coir pith was oven dried at 70 °C for 48 h, ground and sieved to pass a 2-mm sieve. It was then autoclaved at 121 °C for 20 minutes. This residual coir pith obtained after the ex-traction of cellulase was mixed with Azobacter chroococcum, Bacillus megaterium, Bacillus mu ci-la ginosusand stored in air-tight containers. This mixture acts as the microbial inoculum. Preparation of coir pith waste compost (CWC)The entire mixture comprising of the residual coir pith and the microbial inoculum was composted in trapezoidal windrow piles (0.5 m height × 1 m width × 6 m lenght). Forced aeration was provided during the first 25 days, followed by a maturation period of 110 days. The piles were turned periodically, every 10 days, to maintain adequate O2levels by blowing air at the base of each pile during the initial stages of composting. At the end of the com-posting period, the prepared coir pith waste com-post or the biofertilizer was well dried and obtained in the form of powder. The biofertilizer was sealed in sterile plastic bags and stored at 4 °C for field trials.

Field trials using CWC

The study on the applicability of residual coir pith as biofertilizer was conducted under laboratory conditions, by setting up a model experiment with four pots, in which Solanum lycopersicum (tomato) plants were chosen to grow.19,20 Field trials were carried out in four pots, Pot A: Soil + biofertilizer + Seeds (A), Pot B: Soil + Seeds (B), Pot C: Soil + CF + Seeds

(C), Pot D: Soil + Autoclaved bio fertilizer + Seeds (D). Small pots with perforated bottom were used for growing the seeds.

Growth of Solanum lycopersicum seedlings (a) Pot A: Soil + biofertilizer + Seeds (after 12 d); (b)Pot B: Soil + Seeds (after 30 d); (c)Pot C: Soil + CF+ Seeds (after 18 d); (d)Pot D: Soil + Autoclaved biofertilizer + Seeds (after 24 d) (e)Pot E:Soil+CWC+Seed (after 45d); (f) Pot F:Soil+CWC+Seed (after 52 d)

CONCLUSIONS

Thus, this work showed that the delignification through pretreatment is one of the most important pathways to increase the enzymatic digestibility. Alkali pretreatment using NaOH on coir pith was seen to increase the feasibility of cellulose exposure and hence the production of cellulase with significant activity. This study exploits coir pith as a substrate for cellulase production and the residual coir pith as a carrier material for the preparation of bio fertilizer.

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MICROBIAL PECTINASE PRODUCTION FROM AGRO-WASTE SOIL ISOLATES AND ITS APPLICATIONS

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ABSTRACT

Pectinase are one of the upcoming enzymes of commercial sector. It has been reported that microbial pectinase accounts for 25% of global food sales. The aim of this study was to isolate and screen native bacterial and fungal isolates from agro-waste dump soil for pectinase activity and to standardize the conditions for cost effective production of enzyme. Among the screened isolates obtained, *Bacillus sps.*4 showed maximum pectinase activity and in case of fungi, *Aspergillus fumigatus* showed maximum activity.

Agricultural wastes containing pectin can be considered as an alternate substrate for the production of pectinase. In this study different agricultural wastes as well as pure chemicals were used for pectinase production. A comparative study on the production of pectinase under solid state and submerged fermentation systems as well as the effect of temperature, incubation time and various carbon sources were also analyzed. Higher production of pectinolytic enzymes was observed under solid state fermentation at 37^0C by bacterial and at 25^0C by fungal isolates. In the juice clarification, the juice treated with pectinase enzyme was found to be more clarified than those treated with culture. In case of seed germination, the seeds treated with the culture of pectinase producers germinate quickly than those with pectinase.

Key words: - Enzymes, Pectinase, Solid state fermentation, *Bacillus sps.*

INTRODUCTION

Enzymes are among the most important products required to meet human needs in the areas of industrial, environmental and food biotechnology (Chaudhri and Suneetha, 2012). Microbial enzymes are routinely used in many eco-friendly and economically cost effective industrial sectors. Over the past century there has been a tremendous increase in awareness of the effects of pollution, and public pressure has influenced both industry and government. There is increasing demand to replace some traditional chemical processes with biotechnological processes involving microorganisms.

Pectinase are a group of enzymes that break the glycosidic bonds of the long chains of galacturonic acid residues of pectic substances, which are structural polysaccharides of plant cells. The wide spread use of pectinases have placed it among the most important enzymes of great significance for biotechnology (Celestino *et al.*, 2006). Pectinase are among the major enzymes required in extraction of fruit and vegetable juices to increase yield; controlling clarity of juices; enzymatic peeling of fruits; improving the texture of fruits and vegetables; wine production; extraction of pigments and food colorings (Tochi *et al.*, 2009). They have also been applied in textile industry as well as coffee and tea fermentation (Jayani *et al.*, 2005). It is also used for waste water treatment containing pectin (Chaudhri and Suneetha, 2012). This enzyme accounts for approximately 25% of the world enzyme market. In recent years, interest in its microbial production has increased (Jayani *et al.*, 2005).

The present investigation aimed at the isolation and characterization of pectinase producing microbes from agro-waste dump soil and optimization of pectinase production under different fermentation methods such as solid state and submerged fermentation, extraction and partial purification of the enzyme and the evaluation of its potential in various sectors.

MATERIALS AND METHOD

- Isolation, identification and screening of pectinase producing microbes (Barkavi and Sankari, 2014)
- Isolation and identification of pectinase producing microbes were done from agro-waste dump soil samples collected from different localities of Puthenvelikkara, Ernakulam Dt.
- Screening of pectinase producing bacteria and fungi (Hitha and Girija, 2012) was done by flooding the plates with Lugol's iodine and incubated for 15minutes at room temperature, the clear zone around the colonies indicated pectinase activity.
- Pectin hydrolyzing isolates were identified by macroscopic, microscopic, morphological, cultural and biochemical characteristics (Torimiro and Okonji, 2013).
- Comparison of pectinase production under solid state and submerged fermentation (Torimiro and Okonji, 2013**).**

- ❖ Quantitative estimation of pectinase production :- Quantitative estimation of pectinase production was done using pectinase assay by DNSA method (Torimiro and Okonji, 2013)
- ❖ Optimization of process parameters (Mrudula and Anitharaj, 2011)
  - o The effect of temperature (such as 16^0C, 25^0, 37^0C, and 45^0C), effect of incubation time (such as 48hr, 72hr, 96hr and 120hr) and effect of substrate (such as orange peel, banana peel and wheat bran) on the production of the enzyme were carried out.
- ❖ Enzyme extraction, partial purification (Tariq and Reyaz, 2012) and determination of molecular weight of pectinase by sds-page **(**Vasanthi**,** 2012) with the standard protein marker (A7517, SIGMA-ALDRICH) were done.
- ❖ Application of pectinase in various sectors: Application of pectinase in various sectors such as juice clarification (Hitha and Girija, 2012) and seed germination (Tariq and Reyaz, 2012) were carried out.
- ❖ Statistical analysis: The data obtained in triplicates were statistically analyzed using Analysis Of Variance (ANOVA) INSTAT-3.

RESULT AND DISCUSSION

Isolation and screening of pectinase producing microbes: Six bacterial and six fungal isolates were screened as pectinase producers from agro waste dump soil. Similarily, Venkata Naga Raju *et al.*, 2013 isolated pectinase producers from dump yards of Bangalore market. They were identified on the basis of production of a clear halo zone (Plate1& 2). (Hitha and Girija, 2012). Clear zone produced by isolates on pectin agar medium

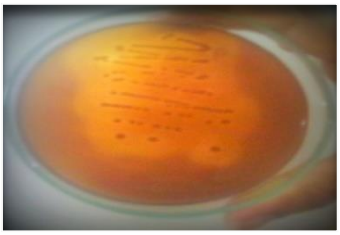

Plate 1: Pectinase producing bacteria, Plate 2: Pectinase producing fungi

Identification of isolates: The identity of the bacterial isolates was determined with reference to Bergey's Manual of Determinative Bacteriology as *Bacillus sps.* for all the 6 isolates and they were designated as *Bacillus sps.*1,*Bacillus sps.*2, *Bacillus sps.*3, *Bacillus sps.*4, *Bacillus sps.*5, and *Bacillus sps.*6 respectively. Kashyap *et al.,* 2001 reported the isolation of pectinase producing soil bacteria *Bacillus sps*.

According to the macroscopic and microscopic characteristics the fungal Isolates were identified as *Aspergillus fumigatus, Penicillium sps, Mucor sps., Aspergillus niger, Aspergillus nidulans* and *Exophiala spinifera*. The results are quite similar to the ones reported by Priya (2014) and Ramachandran (2013) who observed the pectinolytic activity of *Aspergillus sps., Penicillium sps., Mucor sps.* and *Exophiala spinifera*.

Comparison of pectinase production of the isolates under submerged and solid state fermentation: Pectinase assay of isolates under submerged (SMF) as well as solid state fermentation (SSF) was done with DNSA method (Table 1 & 2).

Table 1: Comparison of Pectinase production by *Bacillus sps*. under SMF and SSF

Bacterial isolates	Concentration of pectinase (mg/ml)	
	Under submerged fermentation (SMF)	Under solid state fermentation (SSF)
Bacillus sps. 1	0.083± 0.001	0.275± 0.001
*Bacillus sps.*2	0.125± 0.001	0.347± 0.001
*Bacillus sps.*3	0.041± 0.001	0.318± 0.001
***Bacillus sps.*4**	**0.25± 0.001**	**0.440± 0.001**
*Bacillus sps.*5	0.137± 0.001	0.275± 0.001
*Bacillus sps.*6	0.152± 0.001	0.194± 0.001

P<0.0001

Table 2: Comparison of Pectinase production by Fungal isolates under SMF and SSF

Fungal isolates	Concentration of pectinase (mg/ml)	
	Under submerged fermentation	Under solid state fermentation
Aspergillus fumigatus	**0.25± 0.001**	**0.416± 0.001**
Penicillium sps.	0.10± 0.001	0.222± 0.001
Mucor sps.	0.22± 0.001	0.318± 0.01
Aspergillus niger	0.125± 0.01	0.3± 0.001
Aspergillus nidulans	0.05± 0.001	0.275± 0.01
Exophiala spinifera	0.083± 0.01	0.275± 0.001

P<0.0001

Among bacterial isolates, *Bacillus sps.*4 was identified as the best pectinase producer under the solid state fermentation and it have the enzyme yield 0.44± 0.001 mg/ml (Table1). The results showed that selected *Bacillus sps* can produce and secrete large quantities of extracellular pectinase enzyme (Namasivayam *et al.,* 2011).

Among fungal isolates, *Aspergillus fumigatus* have more enzyme activity under solid state fermentation with 0.416± 0.001mg/ml and followed by *Penicillium sps.* with 0.222±0.001mg/ml (Table 2).The results are quite similar to the observations of Sukumaran *et al.,* 2005.

Based on the results of comparison of pectinase production of fungal and bacterial, it was found that bacteria and fungi have maximum pectinase production under solid state fermentation.

Optimization of process parameters: According to the optimized parameters of solid state fermentation, the bacterial isolates showed the maximum pectinase production at the

fermentation conditions such as 37^0C temperature, 48 hrs incubation time and orange peel as substrate. Fungal isolates have maximum production at 25^0C for 96 hrs when orange peel as substrate.

Table 3:-Pectinase activity by isolates under optimized process parameters

Isolates	Concentration of pectinase (mg/ml)
*Bacillus sps.*4	**0.5± 0.01**
Aspergillus fumigatus	**0.44± 0.01**

P<0.0018

Extraction, partial purification and determination of molecular weight of pectinase enzyme: Crude enzyme was obtained by centrifugation of filtered mass. It was then partially purified by ammonium sulphate precipitation. After the precipitation the purified enzyme was obtained as pellet on centrifugation (Tariq and Reyaz, 2012).

Determination of molecular weight of pectinase enzyme by SDS-PAGE: It was done by using a protein standard marker. The partially purified pectinase from *Bacillus sps.*4 and *Aspergillus fumigatus* exhibited two bands on SDS-PAGE. The bands were observed in between 31 and 45 kDa. These values fall within molecular weights values recorded for microbial pectinase (30- 80 kDa) **(**Vasanthi**,** 2012).

Evaluation of application of pectinase in various sectors:

- **Juice clarification**: In case of the clarification of juice using partially purified enzyme, 84.6% of clarification was obtained. (Hitha and Girija, 2012).
- **Effect of pectinase on seed germination:** In the seed germination, the seeds which socked in the culture were germinated quickly. These results are quite similar to the study of Tariq and Reyaz, 2012 who reported that the pectinase degraded the pectin content in the shells of seed; it helps in easy germination.

CONCLUSION

The present study made a successful attempt to optimize the production of industrially important pectinase by the potential bacterial and fungal strains from the agro-waste dump soil. Among the isolates *Bacillus sps.4* and *Aspergillus fumigatus* were found as efficient producers. Studies revealed that the enzyme production by the isolates was high in solid state fermentation when compared with submerged fermentation. Isolates were grown at different temperature, time and substrate and studies revealed that *Bacillus sps.4* requires temperature of 37^0C with an incubation period of 48 hrs and *Aspergillus fumigatus* requires temperature of 25^0C and an incubation period of 96 hrs with orange peel as substrate showed high pectinase production. Purified pectinase molecular weight was confirmed by SDS-PAGE and was found to be in between 31 and 45 kDa. In the juice clarification, the juice treated with pectinase enzyme was found to be clarified. And microbial culture speeds up the seed germination. The study concludes that agricultural wastes provide cost effective and ecofriendly method for pectinase production which have application in different sectors.

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EFFECT OF PHYSICO CHEMICAL PARAMETERS ON DISTRIBUTION OF MICROFLORA IN THE INNER SHELF SEDIMENTS OF CENTRAL WEST COAST OF INDIA

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ABSTRACT

The aim of this study was to investigate the benthic bacterial communities in different depths of the inner shelf sediment of central west coast of India**.** Vertical and horizontal distribution of culturable heterotrophic bacteria and fungi and physico-chemical parameters were characterized in sediment cores. Sediment were sliced into 3 cores of 4cm each were collected from five different stations. The heterotrophic bacterial distribution, diversity and activities are controlled by various hydrobiological factors and nutrient levels present in the aquatic environment. Distribution of bacteria depends on changes in water temperature, salinity and other physico-chemical parameters. Sediment plays a vital role in the benthic ecology of aquatic ecosystem. Biodiversity indexes and multivariate statistical analyses were used to characterize the spatial distributions of microbial diversity in response to the environmental parameters. Microbial abundance displayed a significant positive correlation with sediment texture and organic matter. The inner shelf of south has more percentage of coarse fractions than northern region. Besides texture, bacterial abundance is also found related to the organic matter concentration. Depth wise variation in organic matter was observed in the study. Microbial biomass can be assessed by analyzing the ATP concentration in the sediment community. The diversity was found to be high in southern region than in northern region. A total of 221 bacterial cultures were isolated, out of which 63% gram negative and 37% were gram positive. The study showed that there was no significant pattern of distribution of fungus among the sediment cores of 5 transects. Out of the 47 fungal isolates, 34% were belonging to genus Aspergillus and 25% to Scopulariopsis.

Keywords: Arabian Sea, Inner shelf sediments, Heterotrophic bacteria, Fungi, Organic matter.

INTRODUCTION

Microorganisms inhabiting aquatic ecosystems play an important role in biogeochemical cycling by way of decomposing a wide spectrum of organic compounds ranging

in molecular size from monomers to polymers [1]. Many of these microbes necessarily credit the oceans ability to sustain life on earth. Though a cosmopolitan distribution of free-living bacteria has been proposed to be the governing rule behind prokaryotic life, the knowledge on the distribution of marine sediment bacteria is still in its infancy [2]. The characteristics of the sediment greatly influence the microbial community [3].Within sediments, there is often a heterogeneous mixture of particles of different size, origin and surface features. These alterations influence the number and composition of the microbial community [4]. Nevertheless, benthic microbial community alters vividly over the course of a year [5]. At different redox depths microbial community structure is found to be significantly different. The structure of the microbial communities depends strongly on the sediment depth, organic carbon content, oxygen and salinity levels [6].

Sedimentary organic matter distribution across the Arabian Sea fuels an on-going debate over the controlling environmental factors [7]. The quantity and quality of organic matter in surface sediments are documented as major factors affecting benthic faunal dynamics [8]. Sediment microbial communities with bacteria as the primary mediators play a significant role in the decomposition of sinking organic matter from the water column and transport of dissolved inorganic matter to phytobenthos and phytoplankton [9].

Several studies have been undertaken to identify the microbial communities and dominant bacterial classes in diverse marine sediment environments. Studies conducted by Cavallo et al. [10] along the coastal sediments of Ionian Sea found gram positive bacilli as abundant genus together with *Aeromonas*, *Photobacterium* and *Pseudomonas*. The most abundant marine fungi encountered in various regions of the Sea of Japan belong to the genera *Penicillium, Aspergillus, Wardomyces, Trichoderma, Chrysosporium,* and *Chaetomium* [11]. Recent studies by Jacob et al. [12] on the distribution and diversity of bacterial diversity along the shelf sediments of Bay of Bengal determined *Bacillus*, *Vibrio* and *Alteromonas* as the dominant genera. Studies conducted by Ramya et al. [13] along the Arabian Sea shelf sediments determined *Bacillus*, *Alteromonas*, *Vibrio*, Coryneforms and *Micrococcus*, as the dominant heterotrophic bacterial groups. Investigating the distribution and diversity of microbial communities is of great importance for gaining a better understanding of aquatic ecosystem. With this objective we determined the vertical and horizontal distribution of bacterial and fungal communities along the inner shelf sediments of central west coast of India. Sediment characteristics that influence the distribution and diversity pattern were also identified.

MATERIALS AND METHODS

Study Area

Sediment samples were collected from central west coast of India (Arabian Sea), which includes 5 stations off Cochin, Kannur, Mangalore, Goa, and Ratnagiri (Fig. 1). These study area lies between the latitude 09˚55'99.5''N and longitude 75° 51'21''E to latitude 17˚ 00'40''N and longitude 73˚ 00'32''E.

Sample Collection

Samples for the present study were collected onboard Fisheries and Oceanographic Research Vessel (FORV) *Sagar Sampada*, Ministry of Earth Sciences (MoES), Govt. of India, during Cruise No. 258. Sediment samples were collected from 50m depth using Piston Corer and these sediments were sliced into 3 cores of 4cm each (0-4, 4-8, 8-12 cm). Bottom water was collected separately for analysis of abiotic factors such as temperature, salinity and dissolved oxygen from each station. Sediment samples were aseptically transferred into sterile polythene bags and were immediately preserved at -20°C in glycerol for further studies.

Grain size analysis

The sediment samples were dried overnight in a hot air oven at 60^0 C. 10g each of dried sample was accurately weighed and dispersed using sodium hexametaphosphate (10%) and kept overnight. The fine fractions of the sediment were separated by wet sieving. Grain size analysis was performed using a Laser Diffraction Particle Size Analyzer (SYMPA TECH, Germany).

Analysis of organic matter and microbial biomass

The sediment samples were homogenized and powdered well after drying in hot air oven at 60^0C overnight. 1g each of powdered sediment was ignited at 500^0C for 3 hours in a muffle furnace. The organic carbon content of the sample was determined by Loss on Ignition method (LOI) and was expressed as percentage of organic matter in sediment. ATP was extracted from the sediment sample to estimate the microbial biomass [14].

Microbiological Analysis

Standard plate count method was adopted. Sediment samples were subjected to serial dilution in sterile seawater and spread plated on to ZoBell's 2216e agar medium and Rose Bengal agar medium for the isolation of heterotrophic bacteria and fungi respectively. The plates

were incubated at 28 ± 2°C for 5-7 days, the colonies were counted and expressed as colony forming units (CFU) per gram dry weight sediment. Morphologically different bacterial colonies were isolated, purified and identified by gram staining, spore staining and biochemical tests. The isolates were identified up to generic level following Bergey's Manual of Systematic Bacteriology [15] and taxonomic scheme of Oliver [16].

Statistical Analysis

To study the variables that best explain the distribution of heterotrophic bacteria in sediments and their relation with environmental parameters, the spearman rank correlation was carried out using XLSTAT v.2012.6.01 (Addinsoft). Similarity between stations with respect to the generic composition and diversity indices were analyzed using PRIMER v 6 [17].

RESULT AND DISCUSSION

Physico-chemical Parameters

The horizontal and vertical distribution of microbial population in sediment is influenced by various factors, such as the physico-chemical nature of sediment and the presence of organic matter [18-19]. In the present study temperature and dissolved oxygen were found to fall from the southern to northern latitude (Fig. 2). The study revealed no significant effect of temperature on THB. Similar findings were reported by Velankar [20] from Gulf of Mannar and Palk Bay near Mandapam (India).Variation in salinity was not so prominent in stations from north to south during the sampling. Dissolved oxygen was negligible towards the northern transects, similar to the previous report from Arabian Sea [21].

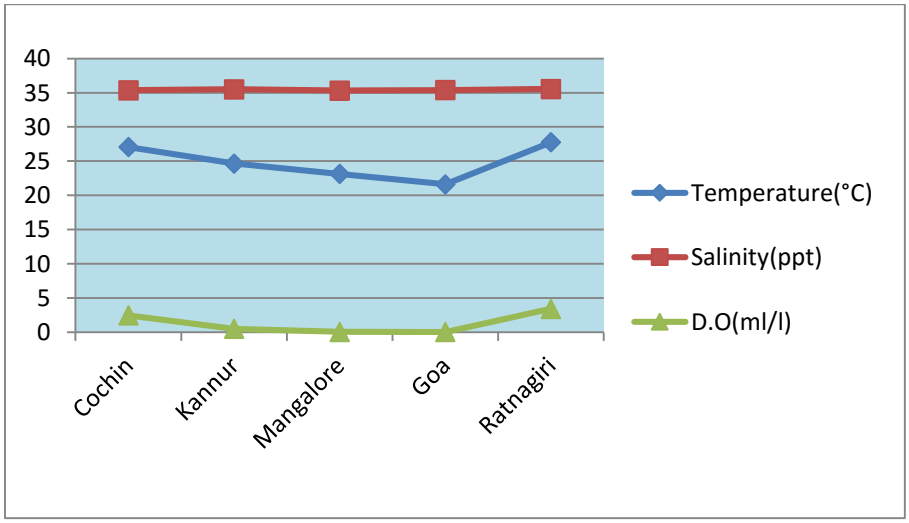

Fig 2: Temperature, Salinity and Dissolved oxygen profile of bottom water in the inner shelf regions of central west coast of India.

Particle Size Characteristics of the Sediment

Texture

The textural types of sediments recorded were silty sand, sandy silt and clayey silt (Fig. 3). The inner shelf of southern latitude has more percentage of coarse fractions than northern region. Studies along the west coast of India by Murthy et al. [22] also have revealed that there existed a distinct zonation with regard to their distribution.

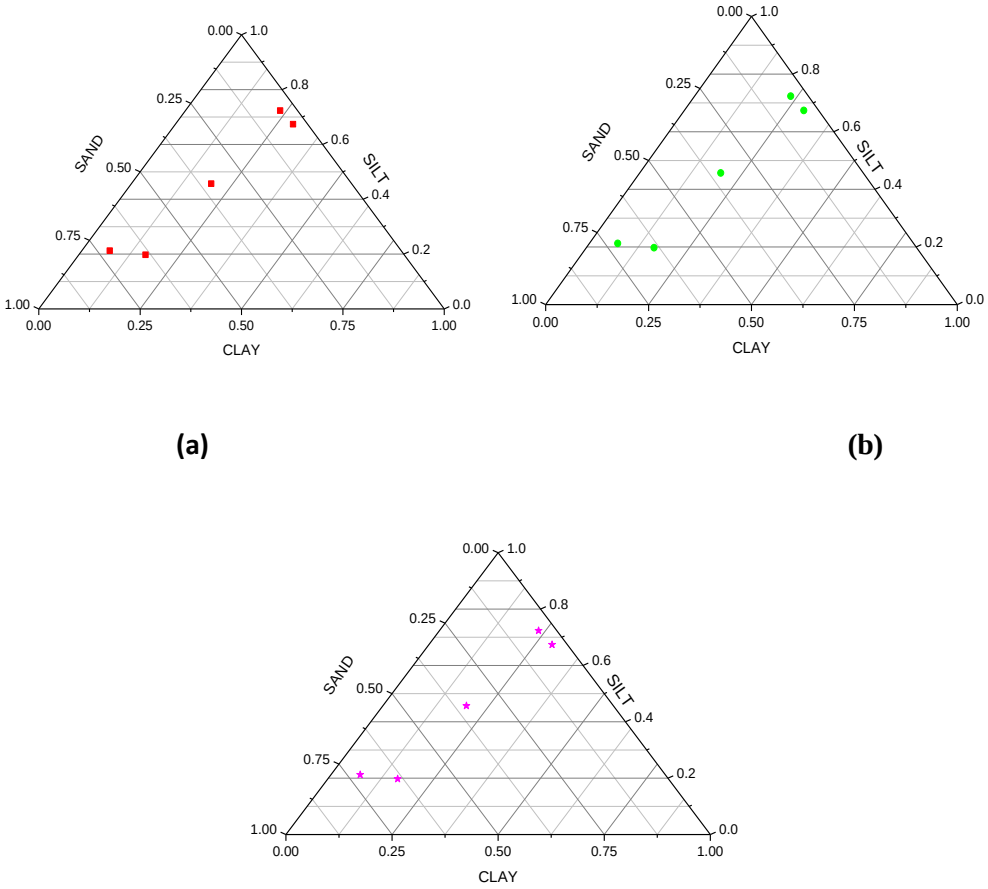

Fig 3: Ternary plot showing Sediment texture at (a) 0-4, (b) 4-8, (c) 8-12 cm cores in the inner shelf regions of central west coast of India.

Organic Matter

Organic production was very high along the west coast of India [23]. Organic matter along the inner shelf sediments ranged between 6-10% in 0-4 cm of the cores; 5-7% in 4-8 cm of the cores and 4-7% in 8-12 cm of the cores of various stations. The organic matter of sediment showed an increasing trend towards the north. Studies revealed that organic matter depends on the texture of the sediment and higher organic matter is associated with finer fractions than coarser ones [24]. Pearson correlation matrix showed that organic matter had significant positive correlation with clay (0.804, $p<0.05$); THB (0.892, $p<0.05$); and ATP (0.895, $p<0.05$). Along the west coast of India, sediment varied in their organic matter composition due to the existence of variable environmental conditions [25]. Organic matter in the sediment core decreased as the core depth increased.

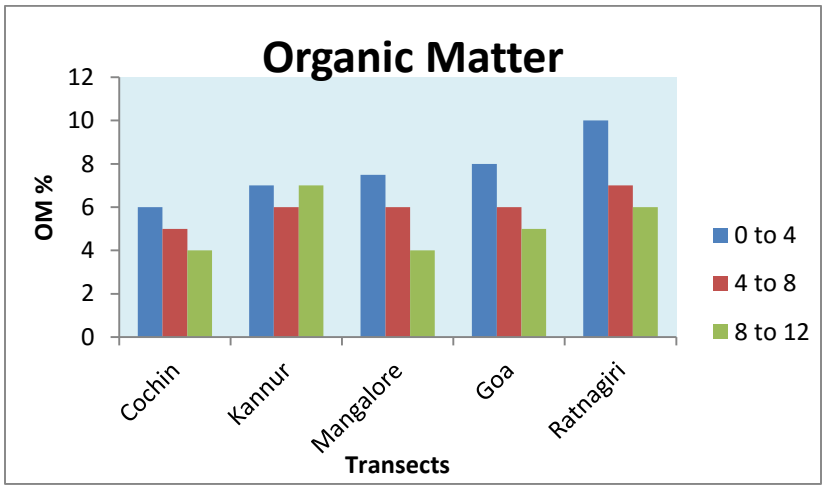

Fig 4: Organic matter (%) in the shelf sediments at various stations

Microbial Biomass Estimation by ATP Analysis

A marked depth wise variation in the microbial biomass was noticed in the sediment core. It is assumed that ATP content generally decreased with depth, but detectable amounts were always present even in the deepest sediments of Halifax Harbor (10 cm core) [26]. The present study illustrated that organic matter and bacterial biomass is positively correlated as reported by Dale [27] and Rublee [28]; a significant linear correlation between the organic matter of muddy sediments and the size of the microbial biomass. Microbial biomass showed a decreasing pattern as the core depth increased. Towards the north, the microbial biomass increased and Ratnagiri showed the highest concentration of ATP (Fig 5).

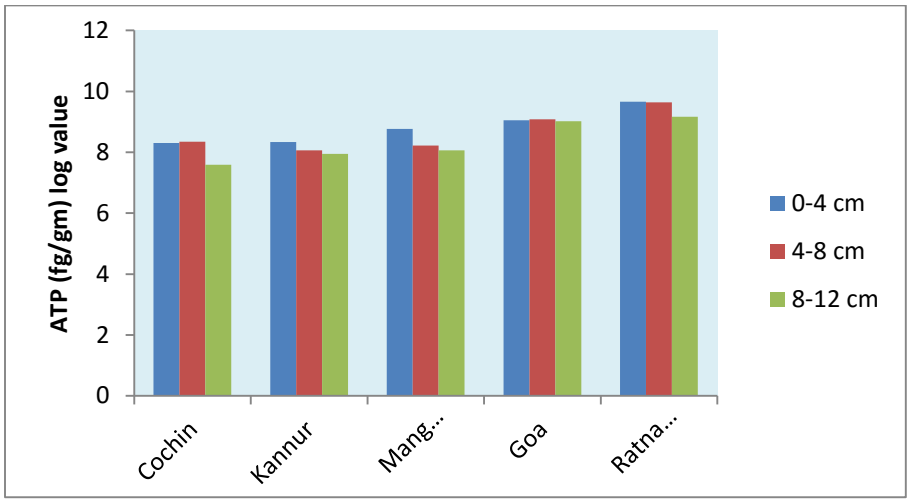

Fig 5: Concentration of ATP in the shelf sediments of central west coast of India

Culturable Heterotrophic Microbial Population

Total heterotrophic bacterial population

Cultivation dependent approach was adopted in the present study to describe the heterotrophic bacterial population of the sediment cores. The result showed that total heterotrophic bacteria had a significant positive correlation with organic matter (0.892, $p<0.05$); clay (0.629, $p<0.05$); silt (0.544, $p<0.05$); and ATP (0.890, $p<0.05$); whereas it showed negative correlation with sand (-0.596, $p<0.05$). Nair et al. [29] reported that bacterial population had a direct relationship with organic matter. Report by Raghukumar et al. [30] found that clayey sediment of the deep sea harbour higher bacterial numbers.

Total heterotrophic culturable bacteria showed a progressive increase from southern to northern region and were found to be highest at Ratnagiri followed by Goa. Culturable bacterial population showed a significant depth wise variation (Fig. 6). Present investigation on microbial abundance in the cores of sediments from 50m depth along the west coast of Arabian sea illustrated that the THB values were in the range 10^4-10^5 cfu g^{-1} dry weight of the sediment. These results were similar to the observation of other studies from different areas which range between10^2-10^9cfu g^{-1} [31-34].

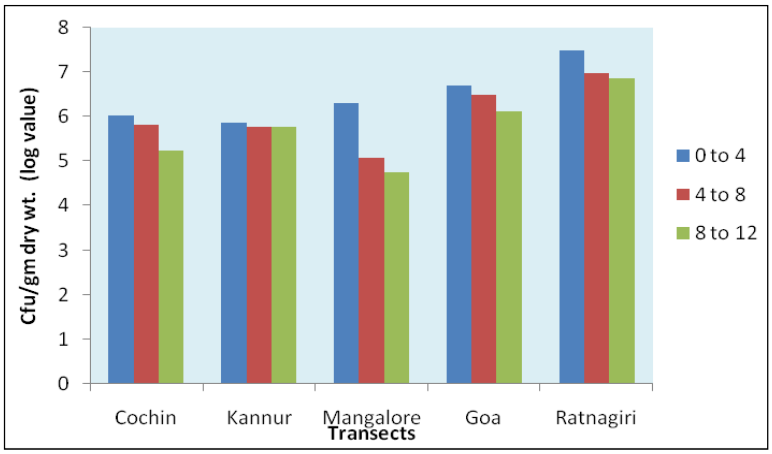

Fig 6: Total Heterotrophic Bacteria (culturable) in the inner shelf sediments of central west coast of India.

Fungal Population

The present study showed that there was no significant pattern of fungal distribution within the sediment cores with respect to the 5 transects. Fungus population did not show any significant correlation with OM, clay, sand or silt (Fig 7). The present study showed that fungal population ranged between 10^2-10^3cfu g^{-1}dry weight of the sediment.

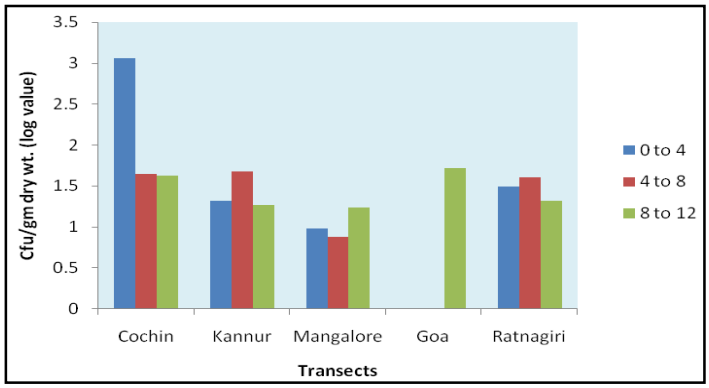

Fig 7: Fungal population in the inner shelf sediments of central west coast of India

Generic Composition of Heterotrophic Bacteria

The study revealed gram negative bacteria as the predominant group in different core depth at various transects compared to gram positive bacteria as was reported by Vasantha and co-workers [35]. Of the 221 isolates, 63% were gram negative and 37% gram positive (Fig.8). Among gram negative bacteria *Alteromonas* (29%) was the predominant group. Another

important group identified during the study was *Micrococcus* (18%) and its occurrence in the coastal waters has been earlier reported by other researchers [36-37]. The present study illustrated the presence of heterotrophic bacteria such as *Bacillus* (10%), *Psychrobacter* (10%), *Moraxella* (6%), *Acinetobacter* (5%), *Flexibacter* (5%), Coryneforms (5%), *Pseudomonas* (3%), *Alcaligenes* (2%), *Staphylococcus* (2%), Enterobacteriaceae (1%) and *Vibrio* (1%) (Fig.9). Generic diversity of bacteria was found to be decreasing from south to north and was found to be higher off Cochin and minimum off Ratnagiri. Studies showed that significant changes in the bacterial species composition occur at various temporal and spatial scales [38].

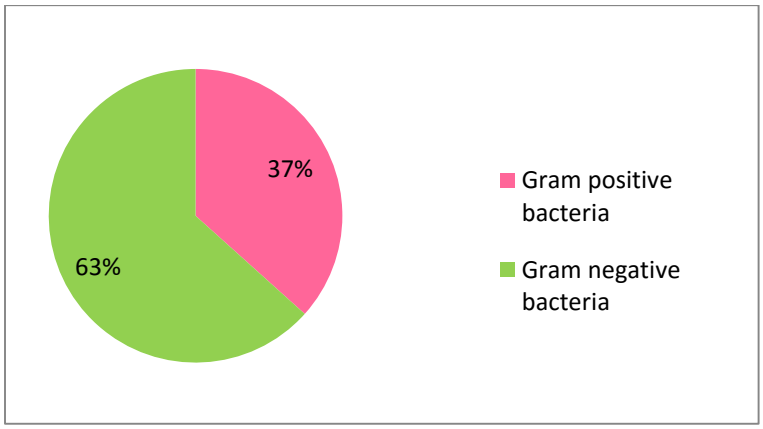

Fig 8: Percentage occurrence of gram positive and gram negative bacteria in the inner shelf sediments of central west coast of India.

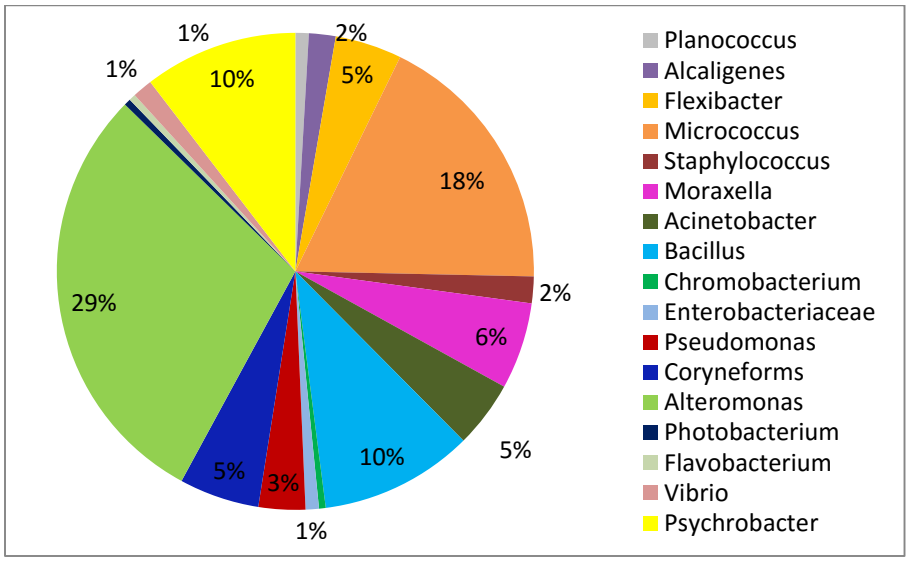

Fig 9: Percentage contribution of different genera isolated from the sediment cores of central west coast of India.

Generic Composition of Fungi

Isolates belonging to 5 different genera of fungi were obtained from the core sediments of inner continental shelf regions of the central west coast of India. Out of the 47 isolates, 34% belonged to genus *Aspergillus* and 25% to *Scopulariopsis*, *Penicillium* and *Cladosporium* (13% each), *Fusarium* (4%) and unidentified strains (11%) (Fig.11). Representatives of *Penicillium* and *Aspergillus* are reported to be versatile, ubiquitously distributed species capable of anaerobic denitrification [39]. The composition varied with different core depth. The fungal communities thriving under oxygenated conditions are distinctively different from fungal communities living under anoxic conditions [40].

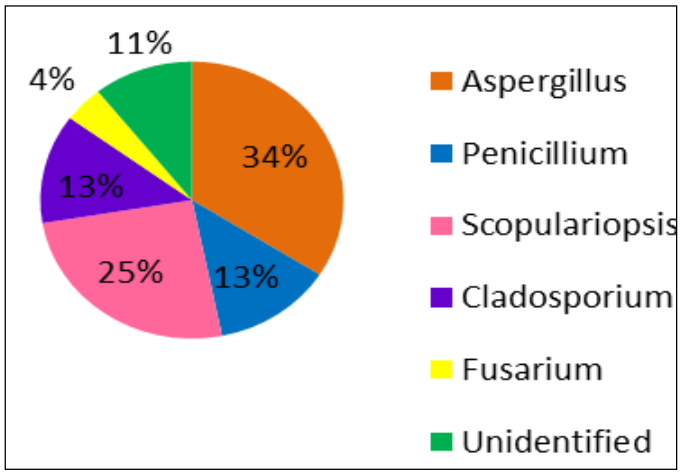

Fig 11: Percentage contribution of different genera of fungi isolated from the sediment cores of central west coast of India.

Statistical Analysis

The combined use of species richness and diversity estimates provide information that enables deeper understanding of microbial diversity. Diversity indices showed a decreasing pattern towards the northern transects. The species richness was higher off Cochin and Kannur where as it decreased towards northern transects off Mangalore, Goa and Ratnagiri. There was no significant pattern of variation in species richness as core depth increased. Off Goa, the core depth of 0-4 cm had the least species richness but a prominent increase could be noted in deeper

cores. Off Cochin and Kannur, the species evenness showed a decreasing pattern as core depth increased. In the case of Mangalore, Goa and Ratnagiri, it was found that the Pielou's Species evenness decreased as core depth increased. The diversity was found to be high in southern region than in northern region. Shannon diversity does not showed a significant pattern with respect to core depth. Highest degree of diversity was found in southern region. Species dominance showed similar pattern throughout the cores. Hierarchical clustering analysis delineates the bacterial communities of the study area into two main groups and these 2 major clusters showed a similarity below 60%. Within each cluster the sediment cores of different transects showed 70-80% similarities. First major cluster mainly showed the similarity between station 17 and 27. This major cluster was further grouped into sub clusters based on their similarity. Each cluster showed a similarity range between 60 and 80%. Second major cluster showed the similarity pattern between the different cores of transects 42, 47 and 32. Similarity pattern ranged between 60-85%. The transects 17 and 27 belonged to the southern region and stations 32, 42 and 47 are northern region of the study area.

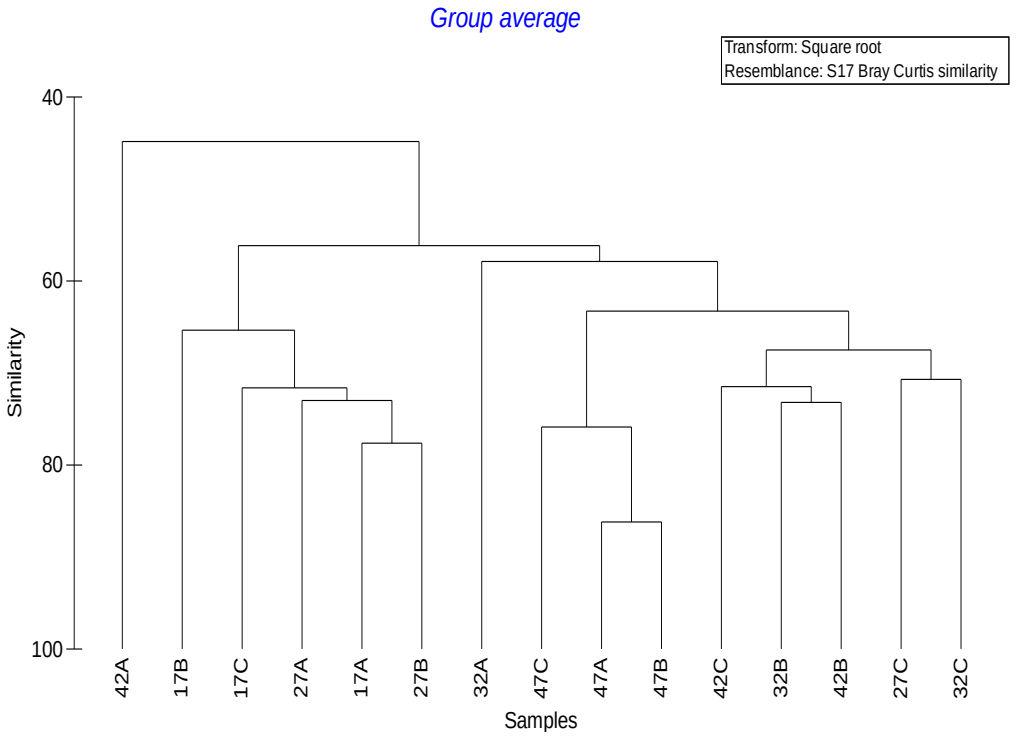

Fig 14: Dendrogram based on bacterial community recorded at various stations

17-COCHIN 27-KANNUR 32-MANGALORE 42-GOA 47-RATNAGIRI

A- (0-4 cm) B- (4-8 cm) C- (8-12 cm)

CONCLUSION

The study imparts knowledge on the microbial ecology of the inner shelf sediments of eastern Arabian Sea. Total heterotrophic (culturable) bacteria showed a progressive increase from southern to northern region and the study area could be demarcated into two ecosystems (southern and northern regions) in terms of the microbial diversity in the benthic realm of the region. The study revealed that the physico-chemical parameters and sediment texture greatly affected the total microbial population in the study area.

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CONTROL OF PATHOGENIC BACTERIA IN *Penaeus monodon* and *Metapenaeus monoceros* USING PROBIOTIC BACTERIA

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ABSTRACT

Shrimps are an economically important aquaculture products produced in many countries. Intensive researches have concentrated on maximizing shrimp production with alternative use of antimicrobials as an eco-friendly approach. Use of probiotic bacteria in aquaculture has tremendous scope. In the present study, the aquafarm shrimps *Penaeus monodon* and *Metapenaeus monoceros* were used for bacteriological examinations. Tissues of head, skin, muscle and alimentary canal were cultured on Nutrient Agar (NA) and Thiosulfate Citrate Bile Sucrose Agar (TCBS) plates after homogenization. Results showed that there was a significant variation in bacterial flora among the tissues. Five types of *Vibrio sps*. such as *V. cholerae, V. parahaemolyticus, V. harveyi, V. vulnificus* and *V. alginolyticus* and some of the non-*Vibrio sps*. (*Bacillus sps*., *E. coli*, *Staphylococcus aureus*, and *Proteus sps*.) were identified. During the study, *Pseudomonas sps.* and *Lactobacillus sps.* showed antibacterial activity against *Vibrio sps*. which can be used as a promising alternative to the use of antibiotics in shrimp aquaculture.

Key words: Shrimps, Aquaculture, Probiotics, *Vibrio sps*.

INTRODUCTION

Shrimp is one of the most delicious seafood and is part of almost every nation's traditional meal (Ehigiator *et al*., 2014). Aquaculture is one of the fastest growing food production sectors in the world. Culture of *Penaeus monodon*, tiger shrimp is one of the most profitable ventures in aquacultures sector in India and in many countries of the world (Maharajothi *et al*., 2012).

Currently, aquaculture industry in India and other part of the world has been facing serious problems due to microbial diseases. *Vibrio sps*. are the most common bacterial pathogens causing some of the most serious diseases, growth retardation and sporadic mortalities in Penaeid shrimp (Maharajothi *et al*., 2012).

Probiotic bacteria can prevent the damage or disease by pathogenic bacterium in the host by producing antimicrobial substances that inhibits the growth or attachment by harmful bacteria (Mary and Ammani, 2014). A wide range of probiotics have been examined for use in aquaculture such as lactic acid bacteria, *Pseudomonas sps*., *Shewanella algae* and *Bacillus sps*. (Balasundaram *et al*., 2012).

The current investigation aimed at the evaluation of antibacterial activity of probiotic bacteria against pathogenic organisms isolated from *Penaeus monodon* and *Metapenaeus monoceros.*

OBJECTIVES

- To isolate and identify the bacterial pathogens which exist in various tissues of (Head, Skin, Muscle and alimentary canal) prawns.
- To isolate and identify probiotic bacteria, which could be used for inhibiting pathogenic isolates in prawns.
- To test bacterial isolates with antagonistic activity against pathogenic *Vibrio sps*.

MATERIALS AND METHODS

- Isolation and identification of pathogenic bacteria from prawn samples (Maharajothi *et al*., 2012).

Fresh prawns of *Penaeus monodon* and *Metapenaeus monoceros* were collected from aquafarm in Thrissur District. One gram of tissues from head, skin, muscle and alimentary canal of prawns were taken aseptically and homogenized in sterile distilled water. Isolation was carried out using 10 fold serial dilutions in sterile distilled water. The serially diluted samples were subjected to spread plate technique on NA and TCBS agar. The plates were incubated at 37^0C for 24hrs. After incubation, the isolated colonies obtained were maintained as pure cultures. The isolated microorganisms were identified by morphological, cultural and biochemical characteristics.

- Isolation and identification of *Pseudomonas sps*. and *Lactobacillus sps*. from sediment and curd respectively, using aseptic procedures.

The samples were homogenized and serially diluted. Serially diluted samples (1ml) were subjected to spread plate technique on NA plates and MRSA plates. The plates were incubated at 37^0C for 24 hrs. After incubation, the isolated colonies obtained were maintained

as pure culture. The isolated microorganisms were identified by morphological, cultural and biochemical characteristics. The isolates were maintained on nutrient agar slants as stock cultures.

- Pathogenicity test of *Pseudomonas sps* .were monitored by detecting the survival rate of shrimps **(**Chythanya *et al*., 2002).

24 hrs. old culture of *Pseudomonas sps*. in peptone water was centrifuged. The sediment was washed in saline, centrifuged and the pellet was resuspended in saline. About 40 ml of this suspension was added to the two tanks containing 400 ml of sterilized aqua farm water. To each tank, two kinds of shrimp samples (*Penaeus monodon* and *Metapenaeus monoceros)* were introduced. Sterilized aqua farm water with shrimp samples were kept as control. The shrimp samples were monitored for survival rate up to 5 days.

- Biocontrol of pathogenic *Vibrio sps*.

In vitro antagonism assay of *Pseudomonas sps. against Vibrio sps.* by cross streak method (Chythanya *et al*., 2002) and *In vitro* antagonism assay of *Lactobacillus sps. against Vibrio sps.* by well diffusion agar assay (Vijaya Baskar and Kannan, 2009).

RESULTS

- Isolation and identification of pathogenic bacteria from prawn samples.

Different pathogenic bacteria were isolated on NA and TCBS plates. The isolates were identified based on cultural, morphological and biochemical characteristics. The isolates include *Vibrio sps*. such as *V. cholerae, V. parahaemolyticus, V. harveyi, V. vulnificus, V. alginolyticus* and non *Vibrio sps*. such as *Staphylococcus aureus, E. coli, Bacillus sps*. and *Proteus sps.*

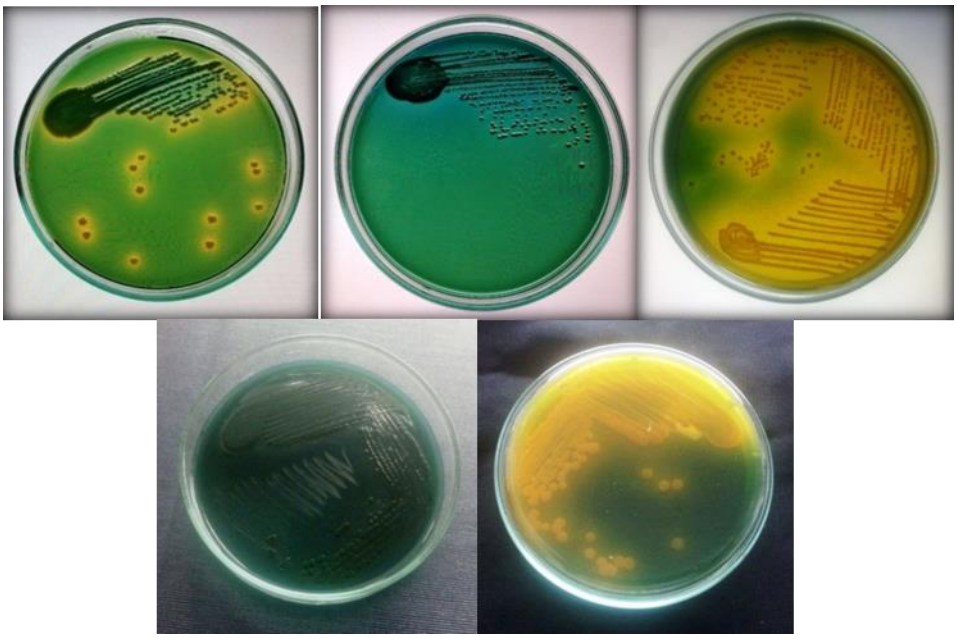

Plate 1: *Vibrio sps*. on TCBS agar

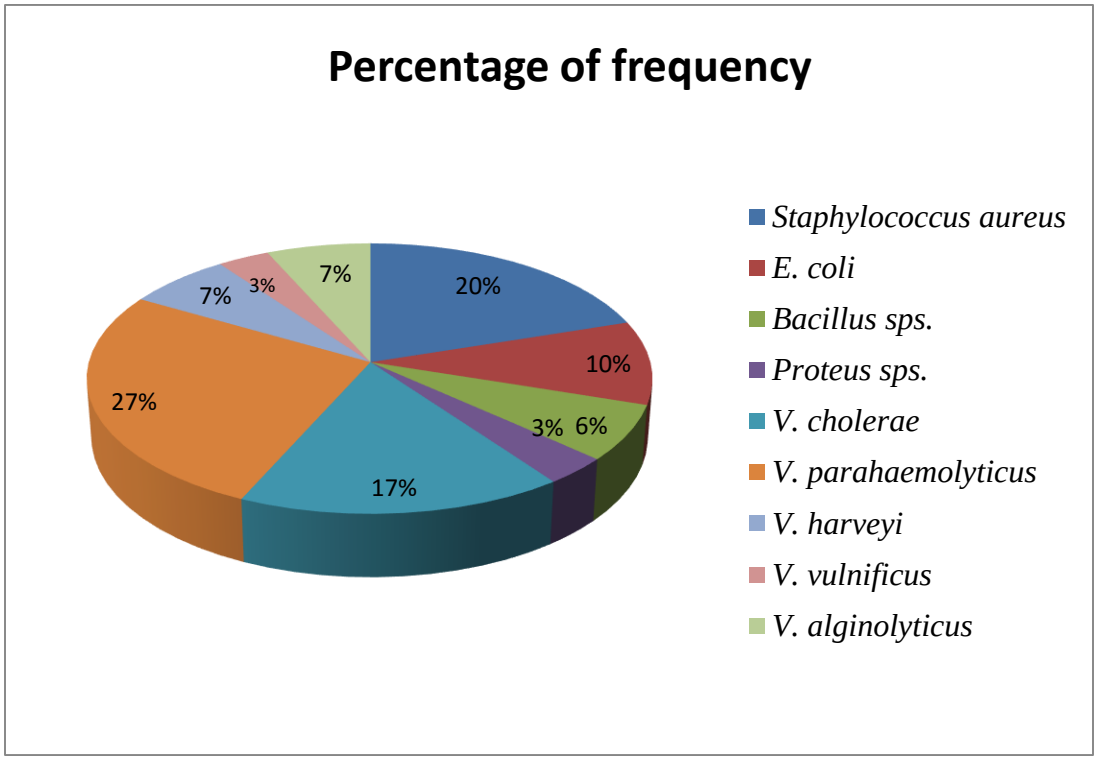

Fig 1: Frequency of occurrence of bacterial isolates from prawns

- Isolation and identification of *Pseudomonas sps*. and *Lactobacillus sps*. from sediment and curd respectively.

Pseudomonas sps. and *Lactobacillus sps*. were isolated from sediment and curd respectively. The isolates were identified based on cultural, morphological and biochemical characteristics.

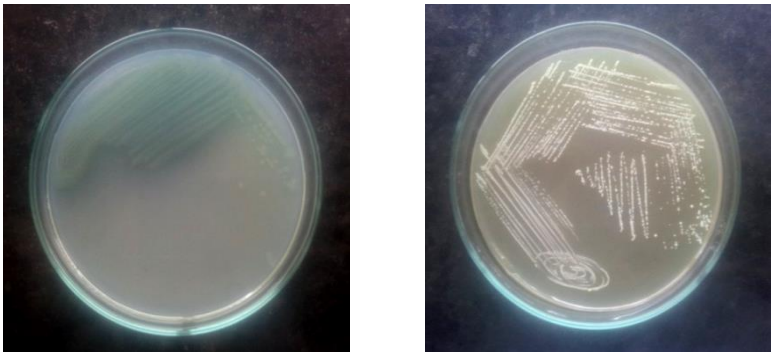

Plate 2: Probiotic isolates- *Pseudomonas sps.* and *Lactobacillus sps.*

- Effect of *Pseudomonas sps*. on survival of shrimps was studied and it was found that shrimp was not affected by the presence of *Pseudomonas sps*. in aqua farm water.

- Biocontrol of pathogenic *Vibrio sps*.

    - ❖ Inhibition by *Pseudomonas sps.*: The non pathogenic strain of *Pseudomonas sps*. isolated from sediment was tested for antibacterial activity against pathogenic *Vibrio sps*. by cross streak method.
    - ❖ Inhibition by *Lactobacillus sps*. : The antibacterial activity of *Lactobacillus sps*. against different pathogenic *Vibrio cholera* was performed by using well diffusion agar assay.

Plate 3: Inhibitory effect of *Pseudomonas sps*. against *V. vulnificus* (A), *V. harveyi* (B) and *V. cholera* (C)

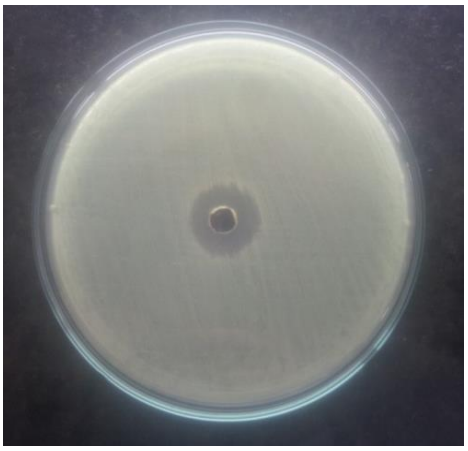

Plate 4: Zone of inhibition of *V. cholera* by well diffusion method

DISCUSSION

Penaeus monodon and *Metapenaeus monoceros* are the main aquaculture commodities in India. Presently, the biggest problem faced by the aqua culture industry worldwide is diseases caused due to various biological and non biological agents. Bacterial diseases, mainly due to *Vibrio*, have been reported in Penaeid shrimp culture systems (Maharajothi *et al*., 2012).So we attempted to carried out the biocontrol of pathogenic *Vibrio sps.* isolated from the prawns (*Penaeus monodon* and *Metapenaeus monoceros*).

During the study, the isolates obtained were *Vibrio sps.* such as *V. cholerae, V. parahaemolyticus, V. harveyi, V. vulnificus, V. alginolyticus* and non-*Vibrio sps*. such as *Staphylococcus aureus, E. coli, Bacillus sps*. and *Proteus sps. Staphylococcus sps*. and *Vibrio sps*. were predominantly isolated from almost all shrimps (Plate 1, Fig 1). The incidence of *Vibrio sps.* in the present study are almost similar with the findings of Bhasker *et al*., (1995) who reported an incidence of 16 % for *V. cholerae* and 28 % for *V. parahaemolyticus* in South India.

Vibrio cholera is water borne pathogen that causes gastrointestinal disorders with a wide range of clinical manifestations including vomiting and rice like diarrhoea. Sea food importing countries generally do not accept the presence of *Vibrio cholera* in any sea

food or sea food products. Bacteria showing antagonistic activity have potential application as biocontrol agents (Sugita *et al.,* 1998).

In the present study, *Pseudomonas sps*. and *Lactobacillus sps*. were isolated from sediment and curd respectively. They were examined for inhibitory effect against pathogenic *Vibrio sps*. The *Pseudomonas sps*. isolated in this study inhibited the pathogenic *Vibrios* tested (Plate 3). Antagonistic activity of *Pseudomonas* against a number of pathogens such as *Salmonella, Staphylococcus aureus and V. parahaemolyticus* has been reported in the literature, Oblinger and Kreft (1990). Results in this study have clearly shown that *Pseudomonas sps*. is non pathogenic to shrimps.

During the study, biocontrol of *Vibrio sps*.by using *Lactobacillus sps*., have been studied and was observed that *Lactobacillus sps* inhibited *Vibrio* (Plate 4). So It appears to be an effective way of controlling pathogen, which could substitute the negative impacts of the use of antibiotics in aquaculture.

CONCLUSION

Shrimps are often contaminated with pathogens. In the present study, there were 9 species of bacteria predominantly isolated and identified. The isolates were *Vibrio sps*., such as *V. cholerae, V. parahaemolyticus, V. harveyi, V. vulnificus, V. alginolyticus* and non *Vibrio sps.* such as *Staphylococcus aureus, E. coli, Bacillus sps*. and *Proteus sps. Pseudomonas sps.* and *Lactobacillus sps.* were isolated from sediment and curd respectively and they were used as probiotics for the biocontrol of pathogenic *Vibrio sps*. *Pseudomonas sps*. inhibited all the five pathogenic *Vibrios* tested by cross streak method. It showed maximum inhibition against *V. vulnificus, V. harveyi,* and *V. cholerae* and minimum inhibition against *V. parahaemolyticus* and *V. alginolyticus*. The pathogenicity test was done to prove that the *Pseudomonas sps*. is non pathogenic. For the biocontrol of *Vibrio sps*. by using *Lactobacillus sps*., well diffusion agar assay was employed. *V. vulnificus* was inhibited to the maximum extend (25 mm) followed by *V. harveyi* (20 mm) and *V. cholerae* (12 mm). In conclusion, it can be stated that the *Pseudomonas sps*. and *Lactobacillus sps*. have probiotic properties in terms of inhibitory activity against pathogenic *Vibrio sps*. for use in shrimp hatcheries and farms.

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MOLECULAR VERSUS MORPHOLOGICAL APPROACHES IN TERMITE TAXONOMY

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ABSTRACT

Termites (Order: Isoptera) are serious pests of agricultural, horticultural and plantation crops including forest trees, especially in the semiarid and sub-humid tropics and cause significant yield losses. Lack of taxonomic understanding has been a major impediment to the study and management of termites. Identification of termite species is a challenging task due to the ambiguity in their morphological characters and crypto-biotic social structure. Molecular tools have come in handy to complement the value of morphological taxonomy and to understand the evolutionary relationships among the species. Molecular taxonomy based on mitochondrial DNA has proved to be an efficient alternative to species identification and their phylogenetic relationships. DNA sequences of the mitochondrial genes cytochrome oxidase subunit II, ribosomal RNA (rRNA) large subunit (16S) and the rRNA small subunit (12S) have been extensively used for molecular diagnostics and to conduct comparative genetic analyses to study the taxonomy, gene flow, colony characterization and genetic variations. RAPD markers are also useful in studying Genetic diversity in subterranean termites. The use of molecular markers may be helpful in estimating phylogenetic relatedness between the insect species and estimating genetic differentiation among local populations within each species.

Key Words: Termites, taxonomy, morphology, mitochondrial DNA, 12SrRNA

INTRODUCTION

Insect molecular systematics has complemented and enhanced the value of morphological and ecological data, making substantial contributions to evolutionary biology in the process. During the past decade, our understanding of the relationship among organisms at various levels of taxonomy has advanced greatly with the aid of DNA molecular systematic techniques and phylogenetic theory. However, the advent of molecular taxonomy provides more sensitive

techniques for examining complex identification issues. Advances in method objects have led to the accumulation of large amounts of DNA sequence data from most major insect groups [1].

Molecular taxonomy based on mitochondrial DNA has proved to be an efficient alternative to species identification and their phylogenetic relationships. Infact, mitochondrial markers have been used with a number of insects for systematic and identification purposes [2,3]. The use of mitochondrial genome sequence is further supported by the occurrence of cladistically informative gene order rearrangement events. Mitochondrial sequence data have, therefore, been extensively used in the past 10 years to evaluate the population structure, gene flow, phylogeny, phylo-geography and taxonomy of termites [4]. Indeed, several studies based on mitochondrial genomesequences such as the cytochrome oxidase genes and the AT rich region have thrown a great deal of light on termite taxonomy.

Termites are also very important ecological players in tropical ecosystem, having been described as "ecosystem engineers", due to their important role in providing soil ecosystem services [5]. Termites adapt to arid environment and play an important role in decomposition, where common decomposers such as micro bacteria and fungi cannot function. Despite their importance, our understanding of a number of their basic biological processes in termites is extremely limited. Developing a better understanding of termite biology is closely dependent upon reliable species identification. The use of molecular markers may be helpful in estimating phylogenetic relatedness between the termite species and estimating genetic differentiation among local populations within each species. *12SrDNA* is highly conserved, and has been employed to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. In general, *12S* and 16S *rDNAs* are the most conserved regions among the mitochondrial genes. Mitochondrial *12S* and *16SrRNA* genes are useful for the phylogenetic studies because of their slow evolutionary rate, and the existence of universal insect primers and ease of reliable PCR amplification. In this paper procedure and application of ribosomal RNA based molecular taxonomy in termite phylogeny and taxonomy is discussed.

ADVANTAGES OF MOLECULAR METHODS

Perhaps the greatest advantage of molecular data is the extent of the data set. Because all heritable information of an organism is encoded in DNA, the set of morphological data with a

genetic basis is a small subset of molecular information.The maximum number ofindependent characters of an organism is limited by the number of nucleotide pairs in its DNA. This number ranges from about 5X 10^3 for the smallest viruses to nearly 4 X10^{11} for some eukaryote (6). However, except for a number of viruses, onlya small fraction of this sequence information has been examined in any organism.

In recent years, available DNA sequence information has been compiledin GenBank data base under contract with the US National Institutes of Health. Release 44.0 (August 1986) summerises 11,413 reports and lists approximately 4 X 10^6 base pairs (bp) from eukaryote nuclear genomes, 5 X 10^5bp from eukaryote organelles, 1X 10^6bp from bacterial genomes, and 2X 10^6bp from viral sequences. The information, however, is highly variable

By taxonomic group, and over half the sequence data has been obtained from fewer than 10 species. Of eukaryote nuclear sequences, 74% have been obtained from vertebrates; mammalian sequences alone account for 64% of the total and sequences from one species , *Homo sapiens*represent 30% of the eukaryotic base. Advances in DNA sequencing technology undoubtedly will result in a tremendous increase in the DNA sequene data base, which represent the largest possible set of characters for systemic analysis.

PHYLOGENETIC LIMITS

In principle, neither molecular nor non morphological methods are limited by phylogenetic scale in their application.In practice, however, few morphological characters are shared among major group of orgaisms (eukaryotes versus eubacteria, for instance).In contrast, biomolecules provide a phylogenetic record from very recent time to the origin of life on earth, because of the size and diversity in rates of change of different portions of the genome(7).

Among the most rapidly evolving DNA sequences are those found in the mitochondria of eukaryotes (mtDNA) (7). Studies of mitochondrial DNA have been useful for studying population phylogeny within species, as well as for recovering phylogenies of closely related complexes of organisms. In addition to mtDN Aallozymes have been widely used to detect cryptic species and to recover phylogenies of morphologically similar organisms. In some species reproductively isolated species aremorphologically nearly or completely indistinguishable; their reproductive isolation became clear to systematicsonly after they are studied by molecular systematics (8).

On the other end of the phylogenetic spectrum, some gene sequences that are involved in basic life processes evolve so slowly that homologies can be established throughout living organisms. Chief among these are the ribosomal RNA genes (rDNA), which has been used to reconstruct the basic outlines of organic evolution. Numerous other DNA sequences evolve at intermediate rates between mtDNA and rDNA, so that virtually any level of phylogenetic question can be addressed by choosing the correct molecular segments. In addition to the coding regions, some gene arrays contain a diversity of transcribed and non transcribed spacers that provide a record of evolutionary history from very recent to ancient times (9).

EXTENT OF NON HERITABLE VARIATION

In order for comparative data to be useful for phylogenetic reconstruction, the characters under study must represent heritable variation. For some groups, environment seems to have little influence o phenotype, but for other groups the effects ofenvironment are great. Although methods have long been in use for estimating heritability (10), in many cases the effect of environment are simply assumed to be minimal unless shown otherwise.

Although non heritable variation is primarily a problem of morphological characters, some molecular characters can be confounded by this problem as well. Although DNA sequence data are nearely or completely free of non heritable variation, the expression of gene products can be affected by environment. In addition, molecular analyses can be affected by degradation of the gene products over time due to inadequate or inappropriate storage(11). In general, however, biomolecular data are confounded less by environmental influences than morphological data.

Advantages of Morphological Methods

Applicability to Museum Specimens

One of the greatest advantages of morphological over molecular approaches to systematics is the much greater applicability of the former approach to the extensive collections of preserved specimens in museums. Although some molecular information can be obtained from traditionally preserved specimens, the majorityof molecular techniques require fresh or cryopreserved material(12). For many groups of poorly known organisms, the only known specimens of many species are represented merely by the holotype or type series. Collecting

additional material can be prohibitive because of rarity of the species, high costs of procurement or legal protection of the habitat or species, inaccessibility of the habitat, destruction of known collection localities. A high percentage of recently extant species have been exterminated in this century by human activities, especially through the destruction of tropical rain forests. Because of this high extinction rate, a majority (or at least a large fraction) of described species may never be collected again and will remain known only from traditionally preserved specimens.

Efforts to establish and maintain collections of cryopreserved specimens have increased dramatically. A recent review of frozen tissue collections lists nearly 100 such collections in 18 countries throughout the world (13). However, unlike traditional museum collections, material is rarely "loaned" from frozen tissue collections, primarily because the tissues are modified and usually destroyed during analysis. A more satisfying solution is beginning to appear with establishment of gene libraries that have been cloned into bacterial cultures. Gene libraries can be stored indefinitely and can be shared by any number of researchers. It is surprising that the systematics community has done virtually nothing to encourage the proliferation of such collections, considering that gene libraries can contain an in exhaustible record of virtually all genetic information about an organism.

Use of Ontogenetic Information

Two methods are commonly used to distinguish phylogenetically informative data (apomorphies) from the phylogenetic noise (plesiomorphies) in systematics: the out group criterion and the ontogeny. Much has been written about the relative merits of these two methods. Nelson in particular has argued for the superiority of the ontogeny criterion, primarily because the ontogenic states can be observed directly and the method need not make prior assumptions of relationships. Furthermore, Nelson has criticized the out-group method as indirect (in that transformations are not directly observable) and as requiring some prior knowledge of relationships. Others have addressed these criticisms (14), and many systematists favour the outgroup criterion even when ontogenetic information is available (14)

The argument over out group versus ontogeny criteria has not involved molecular systematists, because most molecules lac ontogenetic development. Gene sequences are either present or absent; timing of protein expression may vary, but for the most part proteins go through little ontogenetic change. Some proteins (eg. The components of haemoglobin) are

encoded by multiple genes that are expressed at different times during development, but this information is of limited use in character analysis. On the other hand,

Morphological ontogeny is under genetic and epigenetic control, so advances in developmental genetics should help clarify the ontogeny question. Nevertheless, use of ontogenetic information in systematics is likely to be largely restricted to morphological systematics for some time.

A major obstacle to increase the use of molecular techniques in systematics is cost. Although modern morphological methods can be expensive, some morphological data can be collected with minimal expenditures on supplies and equipment. The greatest barrier for molecular systematists probably is the initial set –up of a laboratory. Cost vary by specific discipline but most molecular laborotaries require tens of thousands to hundreds of thousands of dollars to establish and maintain. Because systematics is a relatively poorly funded sub disciple of biology (15), these cost can be prohibitive. However, the value and need for molecular data in systematics is recognised inspite of the expense.

CONCLUSION

Morphological and molecular systematic techniques each have distinct advantages for phylogenetic reconstruction. Morphological techniques are applicable to an enormous range of museum and fossil material, and a large portion of the Earths organisms will continue to be studied primarily or exclusively from morphological information. On the other hand, the potential molecular data set is incredible extensive and when fully utilized, should provide a detailed record of the history of life. Studies that combine the two approaches can maximize both information content and usefulness. However, it is important to select methods of analysis that are as assumption - free as possible and also are amenable to combination of such data sets. This requires rate independent methods of network construction and tree rooting, as well as use of character-state data rather than distant summaries whenever possible. Such combinations of molecular and morphological studies should provide a truly comprehensive view of biotic evolution.

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IN VITRO STUDIES ON THE INFLUENCE OF DUCKWEED (*Lemna minor* L) ON WATER QUALITY PARAMETERS WITH RESPECT TO PHYSICOCHEMICAL AND MICROBIOLOGICAL PARAMETERS

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ABSTRACT

Aquatic plants play a major role in maintaining the waste water quality by removing pollutants effectively. *Lemna minor* is a free floating macrophyte with reported capacity for heavy metal removal and nitrate removal from water. The present study focuses on the efficiency of Lemna to remove impurities from water with respect to the physicochemical and biological parameters. Temperature, pH, alkalinity, TSS, TDS, TS, COD, BOD, total plate count and MPN index were analysed. Physio-chemical parameters of Lemna incubated water sample shows high dissolved oxygen, alkalinity and pH, lower temperature, total dissolved solids, total solids, total suspended solids and chemical oxygen demand. The microbial population of the Lemna incubated water was also significantly lower than the control water. This indicates the unique capacity of the aquatic plant Lemna to remove contamination from water. At the present day condition in which the natural water sources are getting polluted day by day by means of various anthrapogenic activities, *Lemna minor* can be recommended as a natural, ecofriendly and effective method to solve the problem up to a considerable level.

Key words : *Lemna minor*, COD, BOD, water quality.

INTRODUCTION

Bioremediation is a waste management technique that involves the use of organisms to remove or neutralize pollutants from a contaminated site. Microorganisms are efficient agents of bioremediation. However, not all the contaminants are easily treated by bioremediation using microbes. For example, heavy metals such as cadmium and lead are not easily adsorbed or captured by microorganisms. Phytoremediation is useful in these circumstances because natural plants or transgenic plants are able to accumulate true toxins in their above ground parts, which are then harvested for removal. Phytoremediation (Greek word *phyto* means 'plant' and Latin word *remedium* meaning 'restoring balance') describes the treatment of environmental

problems through the use of plants that mitigate the environmental problems without the need to excavate the contaminant material and dispose of it elsewhere.

The principal sources of water for human use are lakes, rivers and relatively shallow groundwater basins. Water quality in rivers and lakes is subjected to natural degradation, process of eutrophication and impacts of human activities. Currently, additional sustainable ways to mitigate the degradation of water quality are researched all over the world. Phytoremediation, is one of the serious efforts towards water sustainability. Most of the aquatic macrophytes are naturally occurring and well adapted for their surroundings. Aquatic macrophytes have the capability to remove excessive nutrient load from water that otherwise cause eutrophication of the water body. They absorb nutrient mineral ions from water column and influence metal retention indirectly by acting as traps for particulate matter, by slowing the water current and favouring sedimentation of suspended particles. The use of aquatic macrophytes for treatment of waste water to mitigate variety of pollution level is one of the most researched issues all over the world. Aquatic plant species are very specific for the uptake of nutrients. Owing to this specificity, the selection of the aquatic plant species is one of the skilled tasks prior to the design of a water treatment facility.

Duckweeds show great promise for water containing nitrogenous pollution. There are a number of reports regarding the effectiveness of Lemna minor on removing heavy metal pollution (Basile *et al*., 2012). But the effect of the plant on physicochemical and biological parameters is a least concentrated area. So the present study is aimed at analyzing the influence of *Lemna minor* on water quality management by analyzing the physical chemical and biological parameters of the water treated with the plant.

MATERIALS AND METHODS

Plant Material

The plants were collected from a local pond near Irinjalakuda, Thrissur. The collected plant material was washed thoroughly and acclimatized in 32% Hoagland's nutrient solution (Garg and Chandra Prakash, 1994) for ten days.

Eperimental design

In the first stage, study on the original pond water sample was carried out to understand the different levels of water quality parameters studied in the present experiment. Second stage

involves the invitro studies in which a control and experiment groups were set. Control group was the pond water incubated for a period of 14 days under green house without incubating the plant material. Experiment group was the pond water incubated with plant material at a concentration of 1g (wet weight) per 500 ml of water for a period of 14 days under green house. The experiment was done in triplicate. After the period of incubation, water was subjected to analysis of various parameters.

Physico -chemical parameters like pH and Temperature were recorded at the time of sample collection by using Thermometer and pH Meter. Alakalinity, TSS, TDS, Dissolved oxygen (DO), Biochemical oxygen demand (BOD5) and Chemical oxygen demand (COD) were analysed according to standard procedures (APHA, 1993). Enumeration of total plate count (TPC) and most probable number (MPN) of coliforms were performed using methods described in the Bacteriological Analytical Manual online 2001.

RESULTS AND DISCUSSION

The analysis of Dissolved oxygen level in the present study, in Lemna treated water and controls revealed that a significantly ($p>0.05$) elevated level of DO was there in the test sample (Table 1). The polluted water has usually very low level of DO as the oxidation of organic matter present in water, by microorganisms consumes a significant amount of DO in the water. Here the increased photosynthetic activity of Lemna might have resulted in the increased levels of DO in test sample. Moorhead and Reddy (1988) observed such an increase in oxygen level after the culture of aquatic plants in domestic waste water due to exchange of oxygen from aerial tissue into root zone.

Biological Oxygen demand (BOD) is a measure of the oxygen used by microorganisms to decompose the organic matter present in water. If there is a large quantity of organic waste in the water supply, there will also be a lot of bacteria present working to decompose this waste. In this case the demand for oxygen will be high. So the capital BOD level also will be high. In phytodegradation, plant enzymes act on organic pollutants and catabolize, either mineralizing them completely to inorganic compounds (e.g.; carbon dioxide, water, chloride), or degrading them partially to a stable intermediate stored in the plant (McCutcheon and Schnoor, 2003). This enzymatic degradation of organics can happen in both root and shoot tissue. Lemna might be effectively utilizing this technique to reduce the BOD value as shown by the present

experiment. There was a significant reduction in the BOD value in the experimental groups when compared to that of control groups (Table 1).

Table 1

	Initial	Control	Test
DO (mg/l)	7.2±0.1	4±0.05	6.67± 0.33
BOD (mg/l)	3.2±0.03	3.03±0.08	1.33±0.03
COD (mg/l)	9.6±0.3	8.1±0.1	6.6±0.1
Alkalinity (ppm)	27±0.6	26±0.2	32.67±1.8
TSS (mg/l)	0.32±0.01	0.38±0.01	0.18±0.03
TDS mg/l	0.65±0.03	0.7±0.04	0.26±0.002
TS (mg/l)	0.97±0.06	1.08±0.02	0.44±0.01
Total plate count (CFU)	$208\pm6.4 \times 10^2$	$210\pm3.8 \times 10^2$	$74\pm1.3 \times 10^2$
MPN	91±3.9	93±6	36±2
Temperature (^{0}C)	28±1.2	28±1.0	26.67±0.9
pH	7.06±0.04	7.03±0.04	7.5±0.05

Chemical Oxygen Demand (COD) test is commonly used to indirectly measure the amount of organic compounds in water. Plants have the capacity to absorb pollutants from the soil or water and to transform them to less toxic compounds to store within the plant tissue. The reduction in the COD values of the test sample clearly indicates the potential of Lemna for such phytotransformation process. Plants are reported to have the capacity to reduce the COD of contaminated water (Dipu *et.al.,* 2010). The same is reported in the present study also. The Lemna treated sample showed reduction in the COD value (Table 1) which was significant statistically at 0.05 level.

Alkalinity is the natural buffering capacity of a stream that may mask the presence of acidic or basic pollutants. Alkalinity is measured to determine the ability of a stream to resist change in pH. Total alkalinity is the sum of hydroxides, carbonates and bicarbonates. Presence of

hydroxides was not recorded in any pond; bicarbonate was the major ion responsible for alkalinity in all the ponds. A significant increase ($p>0.05$) was observed in the values of alkalinity for the test samples than the initial level when compared to that of control groups (Table 1). The physical factors like temperature and pH analysed showed that a significant decrease in the temperature level and a slight increase in pH in test sample. Importance of pond water pH has been studied for long in India. Banerjee (1967) reported neutral to slightly alkaline pH range to be most favourable to fish. An increase in pH level supports the growth of aquatic plant (Vermant and Hanif, 1998). Temperature and light impact on water hyacinth was reported by Olga and Alenka, (1989).

Total dissolved solids are materials which are completely soluble in water. It includes dissolved minerals, gases and organic matters. They may deplete the dissolved oxygen in the water. High levels of TDS make water unfit for drinking. The total dissolved solids (TDS) and total suspended solids (TSS) in the test sample were less, comparing with control sample ($p>0.05$). Macrophytes create conditions for the sedimentation of suspended solids SS (Jatin, Won, and Jae, 2008). The ability of the plants to reduce the TSS, TS, and TDS was reported earlier (Sudarsan, Deetha, and Ashutosh, 2012). There is a direct relationship between TSS and bacterial population in the water sample (Timothy, Huvaj-Sarihan, and Li, 2001). This is consistent with our standard plate count method which showed increase in the viable microbes in the control sample when compared to test sample ($p>0.05$).

MPN index in the present study showed a significant reduction ($p>0.05$) in Lemna treated samples as shown in the table. Total coliform index is used for evaluating the microbiological suitability of freshwaters. A 40% reduction in the MPN index was observed in the treated groups when compared with control. Such a reduction in the MPN index was reported in the previous study using *Eichhornia* plant (Valipour, Raman, and Ghole, 2011). This indicates the effective rhizosphere remediation. Rhizosphere remediation occurs completely without plant uptake of the pollutant in the area around the root. Plants release a variety of photosynthesis-derived organic compounds in the rhizosphere that can serve as carbon sources for heterotrophic fungi and bacteria (Bowen and Rovira, 1991). In turn, rhizosphere microbes can promote plant health by stimulating root growth (some microorganisms produce plant growth regulators), enhancing water and mineral uptake, and inhibiting growth of other, non pathogenic microbes (Kapulnik, 1996)

SUMMARY AND CONCLUSION

Phytoremediation was very useful as it is an innovative, ecofriedly and efficient technology in which natural properties of the plant is used to remediate hazardous pollutants from the domestic waste water. Experiment results suggest that free-floating macrophytes, duckweed (*Lemna minor* L) have a high capability to improve water quality by removing pollutants. By comparing the physiochemical parameters of Lemna incubated test water sample and control sample, it was observed that Lemna has a unique, efficient capacity for the removal of contaminants. Physio-chemical parameters of Lemna incubated water sample shows high dissolved oxygen, alkalinity and pH, then lower temperature, total dissolved solids, total solids, total suspended solids, chemical oxygen demand and dissolved organic and inorganic matter. These values show that Lemna incubated test water sample has fewer amount of pollutants by comparing with the control sample. *Lemna minor* can be effectively used as a phytoremediation agent for the purification of drinking water.

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BIOACTIVE PEPTIDES IN GERMINATING SEEDS OF *Phaseolus aureus*

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ABSTRACT

Antimicrobial activity of bioactive peptides is of great significant in the present condition in which antibiotic resistant pathogens are a threat to the modern medicine. Antimicrobial peptides are superior to conventional plant derived antimicrobial agents due to their increased activity, lower accumulation capacity, and fewer side effects. In the present study an attempt has been made to identify the presence of antimicrobial peptides in germinating seeds of *Phaseolus aureus.* Seeds were collected from the local market, washed in distilled water and allowed to germinate for 7 days. The extracts of the germinating seeds were prepared for seven consecutive days in phosphate buffer and protein content was analysed. Antibacterial activity studies against five different bacterial species, *E.coli*, Staphylococcus sp, proteus sp, Klebsiella sp and Lactobacillus sp were carried out. It is found that a significant inhibitory effect was observed during the 4^{th} day of germination against the *E coli*, proteus and Staphylococcus sp. Protein content showed an increase during the 7^{th} day of germination. This study shows that *Phaseolus aureus* seeds contain bioactive peptides with antibacterial activity. Studies on the nature and purification of the peptides are in progress.

INTRODUCTION

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. They usually range from two to twenty amino acids in length. Bioactive peptides have different biological effects on human health, such as antiproliferative, antimicrobial properties, blood pressure-lowering effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, etc. These peptides may already be present in foods as natural components or may derive from hydrolysis by chemical or enzymatic treatments. Bioactive peptides and proteins are emerging as health foods because of their ability to arrest disease propagation. Mainly naturally occurring compounds from foods such as rice, peas, vegetables, fruits and so have been found to posses properties that help to

slow disease progression, inhibit pathological mechanism ,or suppress activities of pathogenic molecules. Proteins and peptides play significant role in such activities (Hartman & Miesel, 2007).Particularly rich source of bioactive peptides are milk and egg. But they are also found in meat of various kinds as well as many plants. These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. Depending upon the amino acid sequence, these peptides may exert a number of different activities *in-vivo* effecting cardiovascular, endocrine, immune and nervous systems. In addition to nutrient utilization, bioactive peptides are also produced from milk, by starters employed in the manufacture of fermented milk or cheese (Korhonen & Pihlanto, 2003). Cereals and legumes are key component of healthy and balanced diet. There were observed correlations between lower risk and occurrence of chronic diseases and the adherence to dietary patterns like the Mediterranean diet, in which cereal grains, legumes and derived products represents a stable food. In the research for a dietary approach to control or prevent chronic degenerative disease, protein derived bioactive peptides may represent one such source of health enhancing component. These peptides may already be present in foods as natural component or May derived from hydrolysis by chemical or enzymatic treatments. Many reports are present in literature regarding the bioactivity of peptides in-vitro and wide range of activities has been described, including anti-microbial properties, blood pressure lowering, cholesterol lowering, immuno modulatory effects and opioid like activities. However it is difficult to translate these observed effects to human. In fact, the active peptide may be degraded during digestion, or may not be observed at a concentration to necessary to exert its function.(Marco *et.al.*,2014)

In the present study an attempt has been made to identify the presence of antimicrobial peptides in germinating seeds of *Phaseolus aureus.*

MATERIALS AND METHODS

Collection of the sample

The plant *Phaseolus aureus* was selected for the present study. Seeds of *Phaseolus aureus* were collected from the local market.

Preparation of extract

Seeds were washed in distilled water and allowed to germinate for 7 days. The experiment was done with seven different petriplates in triplicates each containing 5 grams of seeds. The germinating seeds were collected in each day. Seeds were finely ground with pre cooled mortar and pestle using 20 ml of ice cold phosphate buffer of pH 7.4. The extract was centrifuged at 10,000rpm at 4^{o}C and supernatant was collected for the experiment.

Estimation of protein content

The protein content in the crude extract of the germinating seeds and ungerminating seeds, which served as the control was analysed using the method developed by Lawry *et al*. 1951.

Antimicrobial assay

Antibacterial activity studies against five different bacterial species, including gram positive and gram negative bacteria such as *E.coli*, Staphylococcus sp, proteus sp, Klebsiella sp and Lactobacillus sp were carried out. Well diffusion method (Gurinder and Daljit, 2009) as well as Kirby-Bauer method (1966) were employed for the study. Prior to the analysis, the protein content of the crude extract was determined. Antimicrobial study was carried out using extract with a concentration of 10ug protein. Inhibitory zones were compared with standard chloromphenicol inhibitory zone (30mg).

RESULT AND DISCUSSION

Seeds contain reserve food for the germinating plantlet. It is well protected within the seed coat. During the germination the enzymes in the seeds will get activated and cause the hydrolysis of the stored products within the seed to nourish the developing seedling (Miintz, 1996). These small peptides protect the germinating seedling from the surrounding microbial environment (Saad *et al*., 2010). Hence an attempt is made to explore the possible antimicrobial peptides generated in germinating seeds of *Phaseolus aureus*.

The antimicrobial activity studies showed that till the 3rd day of germination, seeds did not show any antimicrobial activity. On the fourth day of germination, all antimicrobial activity was noted towards all the organisms tried with a maximum inhibition to Proteus species (Table 1). Thereafter the activity was found to be decreasing gradually with respect to *E.coli*, *Proteus* and *lactobacillus* sp. Inhibitory zone of *Klebsiella* sp showed a different pattern with maximum inhibition on the 7th day of germination. In the case of *Staphylococcus* sp inhibition was maximum on the 5th and 6th day of germination. Anti microbial activity of peptides in

germinating seeds, against both gram positive and gram negative bacteria was reported earlier by Aliahamadi *et al* in 2011, in which more inhibition was towards gram positive bacteria. But in the present study such conclusion could not be made.

Table 1 The inhibitory zone of the extract under different stages of germination

Bacteria	**Inhibition zone diameter (cm)**			
	4th day	**5th day**	**6th day**	**7th day**
Gram negative Bacteria				
E.Coli	1.33	1.23	1.15	0.75
Klebsiella sp	0.5	1	1	1.3
Proteus sp	2.5	0.4	0.4	0
Gram positive bacteria				
Staphylococcus sp	1.05	1.5	1.5	0
Lactobacillus sp	0.6	0.2	0	0

The seeds are well protected with the pod and seed coat. The greater chance to microbial attack to seeds is the time of germination. During this period, the emerging seedling is exposed to the surrounding microbial environment. The exposure to the environment occurs during the third or fourth day of germination. The absence of significant inhibitory zone during the first days of germination may be explained by this fact. The 4th day of germination was the stage in which the first leaves were exposed from the cotyledons, which need maximum protection from the pathogens. This may be the reason for the observed hike in the antimicrobial activity during the 4th day. As the plantlets get older they acquire other means of protection from pathogens such as the synthesis of secondary metabolites and phenolic compounds, which are very abundant in

mature plants. Hence as the plantlet get older the quantity of antimicrobial peptides decreases as observed in the present study.

Fig 1 Zone of inhibition maximum observed by different organisms

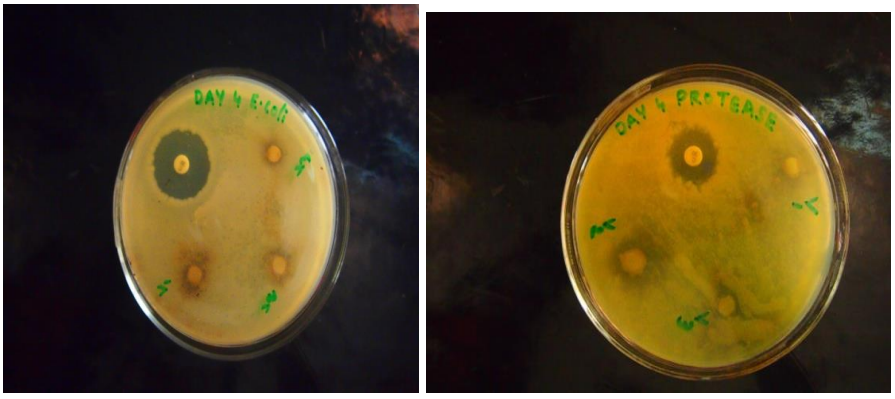
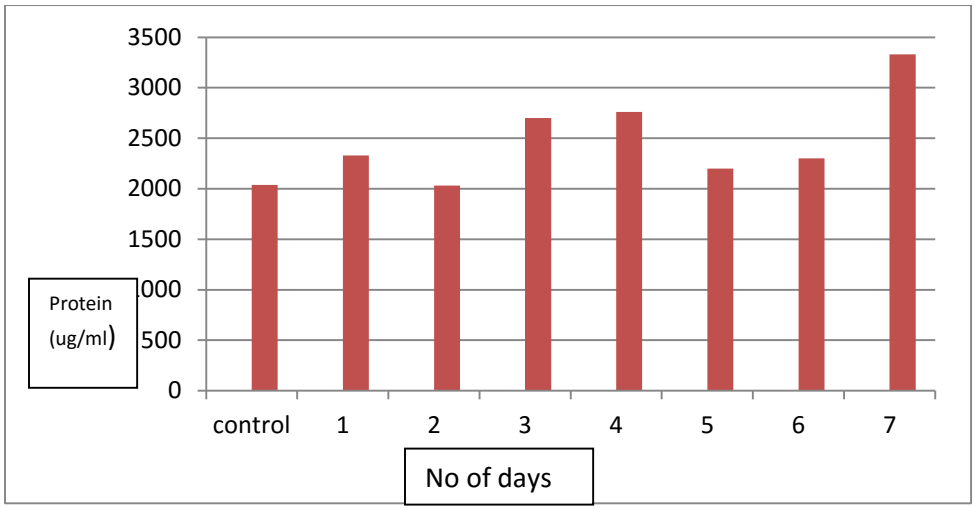


Fig 2 – comparison of inhibitory zone with chloromphenicol (30mg)

Along with the antimicrobial activity, the protein content of the seeds under different stages of germination was also analysed (Fig 3).

Fig 3 Protein content of the extract in ug/ml



A fluctuation in the levels of protein content was observed in the samples in different stages of germination. Protein is the main storage component in the seeds. During germination of the seeds, the reserve protein starts hydrolysis by various enzymes which get activated on germination. During this process, the developing seedling gets nourishment from the stored protein. This is a stage where new proteins are produced and some are disappearing from the seeds. This may be the reason for the observed fluctuation in the levels of protein content in the seeds during different stages of germination. Such changes in protein content during seed germination was reported by Dogra *et al* in 2013.

CONCLUSION

Present study reveals that the germinating seeds of *Phaseolus aureus* are potential sources of antimicrobial peptides against both gram positive and gram negative bacteria tested in the present study. But the activity of the peptides vary during the different stages of germination and the extent of inhibition of different microbes also vary. But noticeable change in the susceptibility of gram positive and gram negative bacteria could not be identified through the present study. Studies regarding the nature of the peptides involved in the observed antimicrobial activity is under progress. Isolation and purification of these peptides will provide a novel unconventional antimicrobial agent with least side effects and with poor accumulating property.

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IN-SILICO STRUCTURAL AND FUNCTIONAL ANALYSIS OF A CONSERVED HYPOTHETICAL PROTEIN FROM *P. Aeuroginosa* PA14

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ABSTRACT

The entry of genome sequencing era has provided a number of protein sequences being deposited in the databases. But only 50-60% of them were functionally as well as structurally annotated. Rest, being unknown of their function and need to be characterized. These are known as hypothetical proteins. *Pseudomonas aeruginosa* is an opportunistic pathogen behind many nosocomial infections. It can often be isolated from wounds, burns and urinary tract infections.

In this study hypothetical protein PA14_49650 has been annotated using a bioinformatics approach. NCBI CDD search showed that this hypothetical protein has a conserved domain of Cupin Super family. Homology modeling was performed to define three-dimensional structure of PA14_49650 using 1SQ4_A as the template, which shares 73% homology to the hypothetical protein. $(PS)^2$v2 server was used for model refinement. Model validation was done using PROCHECK Ramachandran plot. It indicated that the homology model is reliable. KEGG automatic annotation server (KAAS) was used to reveal the involvement of the hypothetical protein in metabolic pathways. Lab based validation technique will provide more information regarding its actual function in microbial pathways.

An extensive study is required to open a new way to aid molecular docking for therapeutic designing.

Keywords : *Pseudomonas aeruginosa* , Hypothetical protein, Homology modeling, Functional annotation

INTRODUCTION

Pseudomonas aeruginosa is an important soil bacterium, with a complex metabolism capable of degrading polycyclic aromatic hydrocarbons, and producing interesting, biologically active secondary metabolites. Production of these products is likely controlled by complex regulatory networks making *Pseudomonas aeruginosa* adaptable both to free-living and pathogenic lifestyles. The complexity of its metabolism is reflected in a relatively large genome size (about 6.5 Mb).

In addition to being able to colonize a wide variety of environments, *Pesudomonas aeruginosa* is also a pathogen with a wide host range. PA14, a clinical isolate from a human burn patient, has been demonstrated to cause pathogenesis in such diverse. PA14 is also an excellent model for the study of pathogenesis and biofilm formation. The ubiquitous bacterium *Pseudomonas aeruginosa*is the quint- essential opportunistic pathogen. Certain isolates infect a broad range of host organisms, from plants to humans. PA14 is a highly virulent strain that causes disease in a wide range of organisms.

The genome of *P.aeruginosa* comprises a large number of hypothetical proteins for which the function is unknown. Detection of hypothetical protein would be of benefit to genomics enabling the discovery of so far unknown or even predicted genes. Hypothetical protein is a protein that is predicted to be expressed from an open reading frame, but for which there is no experimental evidence of translation. Several in silico methods are available for descriptive predictions of proteins with unknown function.

METHODOLOGY

Sequence retrieval and subcellular localization prediction

The amino acid sequence of a *P.aeuroginosa* hypothetical protein PA14_49650 was retrieved from NCBI for study. The sequence ID was gi|115584293.For physicochemical characterization, theoretical isoelectric point (pI), amino acid composition, molecular weight,extinction coefficient,instability index, aliphatic index and grand average of hydropathycity (GRAVY) were computed using Expasy'sProtoparam server. Predicting subcellular localization is important in understanding protein function. It is carried out by CELLO v.2.5, which is a multiclass support vector machine classification system.

Homology Modeling of PA14_49650

Homology modeling was used to determine the 3D structure of PA14_49650. A BLASTP search against Protein Data Bank (PDB) was performed to find suitable template for homology modeling. PDB ID: 1SQ4_A was chosen as the best template showing sequence similarity between query and template protein sequence. Online server PS^2 (PS Square), a Protein Structure Prediction Server was used to construct the protein 3D model using integrated modeling package MODELLER.

Energy minimization, validation and visualization

The model constructed was refined using ModRefiner by a two-step atomic level energy minimization. Validity of the model was checked using PROCHECK Ramachandran plots. Root mean Squared Deviation (RMSD), superimposition of query and template structure, and visualization of generated models was performed using UCSF Chimera 1.10.2.

Functional annotation of PA_49650

Hypothetical protein PA14_49650 was analyzed for the presence of conserved domains using NCBI Conserved Domain Database (NCBI-CDD). STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), a database of known and predicted protein interactions was used to determine the protein interactions in which PA14_49650 has involved. To predict the disulphide bonding state of cysteins, online server DISULFIND was used.KEGG automatic annotation server (KAAS) was used to predict the involvement of PA14_49650 in various metabolic pathways. To predict the virulent nature of the hypothetical protein online tool VICMpred based on SVM method was used.

RESULT AND DISCUSSION

Expasy'sProtoparam tool predicted various physico-chemical properties of PA14_49650.The hypothetical protein PA14_49650 was predicted to be 278 amino acid, with a molecular weight of 31.6 KDa and an isoelectric point of 5.38. An isoelectric point below 7 indicates a negatively charged protein. The calculated pI value will be useful in developing buffer system for purification by isoelectric focusing method. Aliphatic Index (AI) is the relative volume of a protein occupied by the aliphatic side chains (A, V, L and I). Predicted AI for PA14_49650 was 71.51, which showed that thishypothetical protein is thermally flexible why because the range of the aliphatic indices for hypothetical proteins is from 65.36 to138.39. Low AI value indicates that the protein is thermally unstable. The GRAVY value of PA14_49650 was predicted to be -0.347, which is indicative of a hydrophilic and soluble protein. Lower GRAVY values indicate better interaction with water. Sub cellular localization of proteins is very important as cellular functions are always localized in specific compartments. The predicted sub cellular location of PA14_49650 is cell cytoplasm, i.e. it is a cytoplasmic protein.

Homology Modeling of PA14_49650

Homology or comparative modeling is one of the most common structure prediction methods in structural genomics and proteomics. A number of online servers are there for homology modeling. Comparative modeling always need a suitable template, which is identified from PDB through homology search using BLASTP. Hypothetical protein PA14_49650 showed maximum identity to 1sq4_A, which is an X-ray diffraction model of crystal structure of the putative Glyoxylate induced protein from *Pseudomonas aeruginosa*. The query sequence and template ID was then given as input to the $(PS)^2$ server for homology modeling using MODELLER.

Energy minimization, validation and visualization

Even though there is no steric clash, the generated model is subjected to energy minimization using ModRefiner. Structure assessment methods including RMSD, Ramachandran plot were used to check the quality and reliability of the predicted model. The RMSD value indicates the similarity between two 3D structures. Both query and template structures are need to be superimposed for the calculation of RMSD (Fig:2). The RMSD value obtained for PA14_49650 and 1sq4_A was found to be 0.457 Angstrom. Lower the RMSD value, the more similar the structures. To check the quality of the structures Ramachandran plot (Fig: 3) was obtained. It displayed 91% in the most favored regions with 8.5%, 0.4% residues in additionally allowed and disallowed regions respectively.A good quality model would be expected to have over 90% in the most favored regions. Both RMSD value and Ramachandran plot was confirmed the quality of the homology model of PA14_49650.

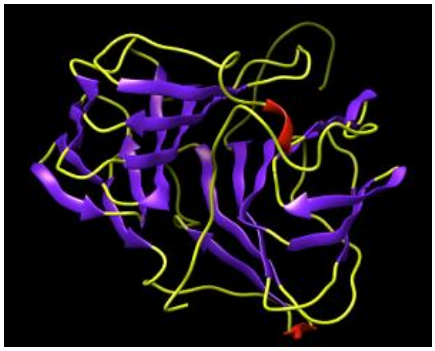

Fig:1-Homology model of PA14_49650

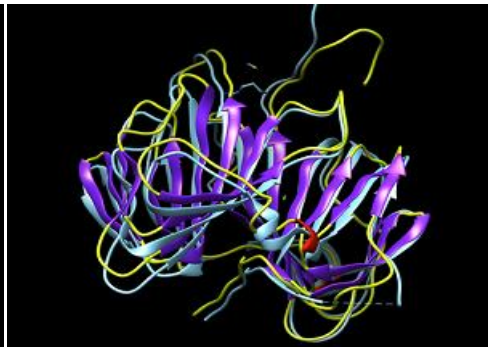

Fig:2-superimposed structure of PA14_49650 and template1SQ4_A

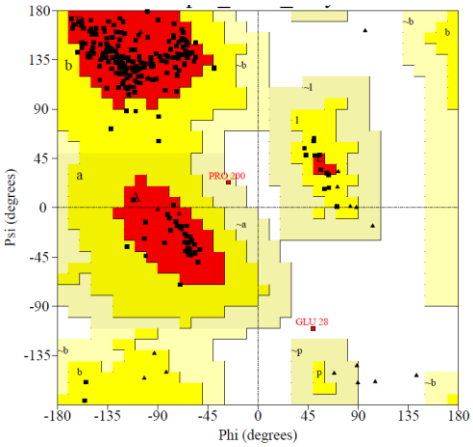

Fig: 3 Ramachandran plot of *PA_49650*

Functional annotation of PA_49650

Based on consensus predictions made by Pfam, NCBI-CDD and InterProScan , it is suggested that PA14_49650 is a multi domain protein of Cupin-2, cupin like superfamily. And this hypothetical protein is currently classified as putative allantoin-urate catabolism protein. DISULPHIND prediction revealed no disulphide bond in this hypothetical protein, showed that protein is thermally unstable. STRING tool was used to predict the interaction with other proteins. The result showed that PA14_49650 has interacted with various proteins like ureidoglycolatehydrolase, allantoicase,allantoateamidohydrolase etc. which are involved in allantoincatabolism. KEGG was used to identify the involvement of PA14_49650 in *P.aeroginosa* metabolic pathways. The search performed via KAAS (KEGG automated annotated server) showed that PA14_49650 was involved in purine metabolism precisely in allantoin degradation to ureidoglycolateII (ammonia producing). Finally VICMpred tool was predicted that there is no virulence factor present with this hypothetical protein.It is suggested that PA14_49650 may be involved in any cellular process.

CONCLUSION

This study has used homology-modeling approach to propose the three-dimensional structure and possible functions for the *P.aeuroginosa* hypothetical protein PA14_49650.It is expected that many of the hypothetical proteins may play important roles in many cellular processes. Extensive study required for extending their utility in molecular docking.

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BIOTECHNOLOGY : FUTURE LIES IN INFORMATICS, SHARING, COLLABORATION & CONNECTIVITY

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ABSTRACT

The Cancer phenomenon has plagued humanity for decades, and still stands as the greatest unsolved mystery of Biological Research. While certain breakthroughs have been had in the recent past, the incidents have been on the rise. A better understanding of the human genome facilitated by the Human Genome Project (HGP), furthered by Next-Generation Sequencing (NGS), and companies like Illumina, have helped in the cause, no doubt. The regional pattern followed by certain cancers is an indication to focus in-house regional research. These geographical regions have their own traditional forms of cure - Ayurveda in India being a good example. These "alternative" forms of treatment have had their fair share of success; however, there stands a need to fully standardize research and their methods . Collaboration with modern medicine – both in the respective regions and at the global cancer research levels – is set to open newer faster ways to handle cancer - to detect, to treat, also as means of palliative care. More integration with Information technology (IT) and a solid Internet backbone, will accelerate the overall process. Places like India can use their existing IT Infrastructure in this field. Bioinformatics can be diversified, spread and made ubiquitous to address the problems of analysis Exabytes of "Big Data" to be generated (greater than 2EB by 2025, as per certain studies). An individual should be able to choose his/her preferred/suited method of treatment after expert consultation –personalized medicine via genomics. Funding can come from the fast growing Pharma Industry. More data sharing between (Cancer) research institutes – both regional and international, can avoid rework, saving badly needed time – making way for faster research. As say at the MIT- the only cure for cancer is continual research.

Index Terms—Cancer, information technology, alternative medicine, collaboration, genomics, bioinformatics, human genome project,

INTRODUCTION

The focus on this paper is the informatics side of Biotechnology, Bioinformatics to deal with the unprecedented amounts of "big" biological data generated with the advancement of genome studies, and how collaboration and sharing at various levels - can assist in the most intensive & challenging genomic disease, Cancer.

Genomics, Big Data, and Medicine [GBDM] need to be further integrated, collaboration done regionally, the strength of the internet backbone fully utilized, and particularly in the Indian context, involve & integrate a standardized AYUSH as a complete "scientific" alternate 2.0 form of medicine. Post the Human Genome Project (HGP), and the Next-Generation Sequencing (NGS), the 3rd generation sequencing advanced by private companies like Illumina has brought down the sequencing time span, And with companies with the likes of 23&ME [1] sequencing of the human genome to $1000 and in 26 hours [2], we have genomics personalized!

Cancer & Multiple-levels of Collaboration:

Now cancer is a unique polymorph, following regional patterns with certain cancers like stomach cancer, is an indication to focus in-house regional research. See Fig1

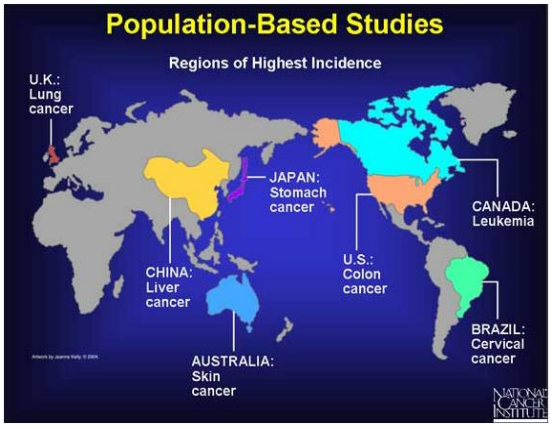

Fig1 . Regional Nature seen in Cancer

The figure suggests a demographic nature of cancer, with the changes in diet pattern can't be ruled out. [3]

Also, these geographical regions having their own traditional forms of cure with Ayurveda, now of the AYUSH methodology prevalent in India. These "alternative" forms of

treatment have had their fair share of success, albeit at earlier stages of cancer. And suggestive of the regional pattern, such "alternative" treatment forms may best suited for local inhabitants – giving them a personal choice of medicine, apart from the highly advanced genomic personalized medicine.

This strongly advocates the need for standardization, with their methods properly defined and documented inclusive of wider Clinical trials. Only a standardized alternate medicine be consider for any serious collaboration with modern allopathic medicine which is much advanced, especially on detection and diagnosis.

Regional collaboration : both in the respective regions and at the global cancer research levels – will set to open newer faster ways to handle cancer - to detect, to treat, also as means of palliative care. Why re-invest the wheel, and waste time?

More integration with Information technology (IT) and a solid Internet backbone, with compute and sharing in the cloud with accelerate the overall collaboration process. Countries like India can channel their existing IT prowess, advance it to address upcoming the technological IT challenges be it the sheer size of data (in Exabytes)

Bioinformatics can be diversified, spread and made ubiquitous to address the problems of analysis Exabytes of "Big Data" to be generated (greater than 2EB by 2025, as per certain studies). An individual should be able to choose his/her preferred/suited method of treatment after expert consultation –personalized medicine via genomics. Funding can come from the fast growing Pharma Industry. More data sharing between (Cancer) research institutes – both regional and international, can avoid rework, saving badly needed time – making way for faster research.

Indian IT & BT Perspective

The Pharma Industry is the 3rd largest in volume worldwide with the potential to topple the IT Industry in pay [4]

Additionally, local research may be able to facilitate citizens from neighboring Pakistan [5], Bangladesh & Sri Lanka providing a cheaper yet efficient alternative; medical tourism has already picking up – funding from this field can be siphoned. It's already a Cancer surgery destination [6]. Alternative forms must be standardized and thus instilling more confidence in both the local and foreigners patients alike.

It would be unfair not to touch upon what India boasts the most – it's Software Superpower status- here its IT expertise & resources would come in handy. Each Human being is equal to 3Billion nucleotide base with 25-30,000 protein-coding genes. A Cubic centimeter of a tumor mass has 10 trillion cells.

So all the (bio) Linux systems with NoSQL/Oracle/MySQL Databases in Exabytes (and more) and smart (bio)perl/python/R scripting program(mers) - would not be wasted a least "bit". Powerful Cluster and Cloud computing would still need to do the Individual Specific Codes and match them against the common drug and cancer sequence databases.

Indian Academia and Industry.

Only a hand-in-hand approach with campus inputs, industry feedbacks and practical technology updates, can keep the both parties abreast of the current trends in ever happening the Genomics world. **Closer collaboration is a must.** Here, a mention of the a better use of potential that India hold with more 40K [7] biotechnology students with more than 50% women, churned out each year (as of 2013)5 using the concept of a distributed yet highly connected, industry-trained and updated workforce is to be explored in the connected world we live in. India should not be left behind or out as it was in the Human Genome Project [Fig2]

Fig 2. Human Genome Project [HGP]: Countries in Collaboration

As say at the MIT- the only cure for cancer is continual research. We at the Genomics Central, say, it's through continual collaborative research.

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EFFECT OF PH AND INCUBATION PERIOD ON PROTEASE PRODUCTION BY *Aspergillus niger* USING FISH SCALE AS SUBSTRATE

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ABSTRACT

Proteases refer to a mixture of enzymes which include proteinases, peptidases and amidases. They are one of the largest group of industrial enzymes and their sources range from animals to microorganisms. In this study, *A. niger* was used for the production of protease by surface fermentation using fish scale as the substrate. The study was conducted with the aim of analysing effect of different parameters on production of protease. For this, *A. niger* was inoculated into Potato dextrose broth containing powdered fish scale with different pH; 4.5, 5 and 5.5. The culture was incubated for 2, 3, 5 and 7 days. Protease assay of the culture supernatant was carried out using casein as substrate and tyrosine as standard. The results showed that the different parameters have influenced the protease production by *A. niger*.

INTRODUCTION

Proteases are widely known for their applications in medicine, industry [1,2] and as a basic research tool. To name a few, they are used in detergents, food industry, leather industry, meat processing and dairy industry. Proteases are found in a wide diversity of sources[3], from micro organisms to plants and animals. Microbial sources-bacteria and fungi- are mainly utilised for the large scale production of the enzyme since microbial proteases are predominantly extracellular and therefore released into fermentation medium. This aids in an efficient and easier downstream processing. Another factor that contributed to the preference of microbial source is their fast growth rate and relatively easy way of culturing. Microorganisms account for a two-third share of commercial protease production. All the major type of proteases-acidic, neutral and alkaline-were identified and isolated from microbes. Different fermentation techniques[4,5,6] like solid state, submerged and surface fermentation have been studied for production of proteases. To enhance production of microbial enzyme, it is routine to include any compound that can serve as the substrate in the production media. The universal substrate for protease is casein and is widely used in its production. Studies on alternative compounds as enhancers or substrates for protease production have been reported. Wastes rich in protein, generated in different industries, can serve as a potential source of substrate for protease production [7,8]. Production of microbial

enzymes is influenced by substrate concentration as well as a number of other parameters including pH, temperature and culture period[4,9,10]. This study was aimed to utilize fish scales as substrate for protease production from *Aspergillus niger* through surface fermentation and to analyse the effects of pH and culture duration.

MATERIALS AND METHODS

Preparation and processing of fish scales

Fish scales of *Scomberomorus commerson* was collected from a local fish market. It was then thoroughly washed with distilled water and steamed for 15 minutes. Afterwards, the scales were properly dried and powdered.

Production media

Production of protease was carried out in sterile potato dextrose broth with 1 gram of powdered fish scale. Spores of *A. niger* was inoculated in to the media and cultured under various conditions.

Effect of pH and culture period on protease production

The effect of pH on protease production was determined by growing *A.niger* in production media with an initial pH of 4.5,5 and 5.5. The inoculated media with different pH was incubated for different time intervals (days), 2, 3, 5 and 7.

Protease assay

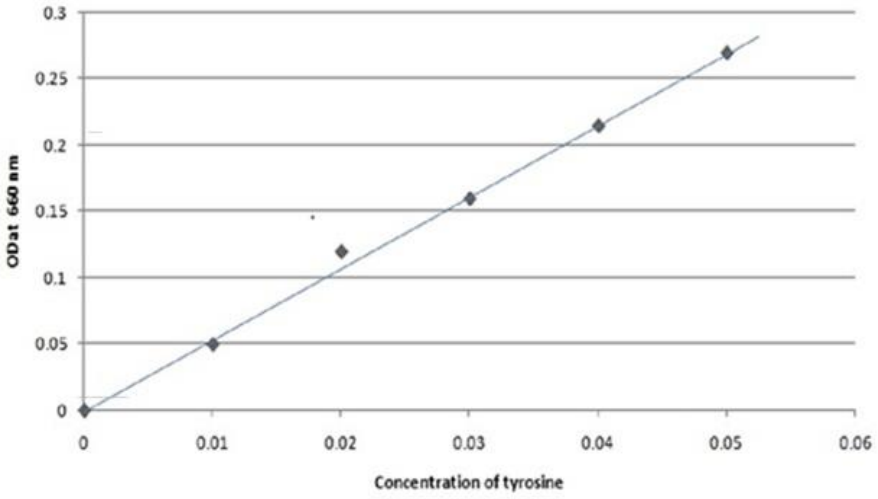
The culture was filtered and the filtrate was centrifuged for 10 minutes at 10,000 rpm. The supernatant was used for the assay. Protease activity was determined using casein as substrate. 1.0 ml of 0.1% casein solution was added to 1.0 ml of supernatant and incubated for 30 minutes at 37 ^{0}C. The reaction was stopped by adding 10% TCA. Mixture was kept in ice for 15 minutes and centrifuged at 10,000 rpm for 10 minutes. To 0.1 ml of the supernatant, 5 ml of 500mM Na_2CO_3 was added, followed by 1 ml of folin ciocalteu reagent. The mixture was incubated for 30 minutes at 37^{0}C. Absorbance was read at 660 nm and the amount of tyrosine released by the protease activity was calculated from a tyrosine standard graph. A substrate blank without casein and enzyme blank without supernatant served as controls. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μg of tyrosine/min under standard conditions.

RESULTS AND DISCUSSION

Microbial enzyme production is profoundly influenced by components of media and culture conditions like pH, temperature and incubation period. Optimized media and culture conditions increase efficiency of production. In the present study, potential of fish scale as substrate for protease production was analysed. Various parameters affecting protease production like pH and culture duration was also investigated. Protease assay was performed using casein as substrate and tyrosine as standard.

Powdered fish scale

Surface fermentation of *Aspergillus niger*



It was observed that as the culture period increased, production of protease decreased. Significant production could be seen in 2nd and 3rd day of culture. Protease is released by the microorganism into the medium for the breakdown of the protein rich substrates and to utilise

the peptides or amino acids for its growth. Since the organism (*A.niger*) might have reached stationary phase of its growth by 7th day, protease production is not significant.

pH also influenced production of protease. On 2nd day, protease production was highest in medium with pH 5 while there was no significant production in pH 4.5. On the contrary, on 3rd day, production of protease was highest in media of pH 4.5.

Incubation period	**pH**	**Enzyme activity (IU/litre)**
2nd day	5	146
2nd day	5.5	36.5
3rd day	4.5	118.5

CONCLUSION

Present study demonstrated that fish scale can be exploited as a substrate for protease production. Use of fish scales will in turn help in managing waste generated by fishing industry. It can also serve as a cheap alternative for the usual expensive media components. Further studies are planned for an in depth analysis of various factors needed for optimised protease production from *Aspergillus niger*.

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ANALYSIS OF MICROBIAL GENES FROM SOIL FOR RUBBER DEGRADATION

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ABSTRACT

The growing problem of environmental pollution caused by synthetic plastics has led to the search for alternative materials such as biodegradable plastics. Of the biopolymers presently under development, starch/natural rubber is one promising alternative. Several species of bacteria and fungi are capable of degrading natural rubber and many can degrade starch.This study indicated that this bacterium causes the biodegradation of the new biopolymer and natural rubber and confirms that this new biopolymer can be degraded in the environment and would be suitable as a 'green plastic' derived from natural sources. Starch was completely removed and polyisoprene chains were broken down to produce aldehyde and/or carbonyl groups. The degradation of natural rubber (NR), synthetic poly(cis-1,4- isoprene) (SR), and cross-linked NR (latex gloves) by Gram-positive and Gram-negative bacteria was analyzed by weight loss, gel permeation chromatography, and determination of the protein content.Weight losses of 11-18% and an increase in protein up to 850 microg/ml after incubation by the bacterial isolate with latex gloves as a carbon source indicated degradation of the polymer.

INTRODUCTION

Natural rubber (NR) is a biopolymer (polyisoprene) that is synthesized by many plants and some fungi. Commercially produced from *Hevea brasiliensis* trees. NR contains a minimum of 90% rubber hydrocarbon together with small amounts of proteins, resins, fatty acids, sugars, and minerals(Rose and Steinbuchel, 2005) .Organic impurities in the rubber can support microbial growth, and many reports on its biodegradability have been assumed that degradation of the rubber backbone is initiated by oxidative cleavage of the double bond (Tsuchii, 1995;

Jendrossek *et al.*, 1997; Linos and Steinbuchel, 1998; Rose and Steinbuchel,*et.al*, 2005) .Biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms .The microbial organisms transform the substance through metabolic or enzymatic processes. It is based on two processes: growth and cometabolism. In growth, an organic pollutant is used as sole source of carbon and energy. This

process results in a complete degradation (mineralization) of organic pollutants .The biodegradability of various rubbers and products plays an important role from the view of protecting rubber goods against biological damage and deterioration and of providing environmentally compatible solutions for the disposal and recycling of rubber waste. Microbial degradation is a natural process by which organic compounds including rubber polymers are converted by the action of bacteria to simpler compounds, mineralized and redistributed through the elemental cycles (Enoki *et al.,* 2003; Cui *et al*., 2005) .Bacteria, fungi and the mycelium-forming actinomycetes degrade vulcanised NR products but microbial rubber degradation alone can be a very slow process After usage of these natural rubber products the disposal of these products are the world wide solid waste problem. It became obvious that bacteria as well as fungi, are capable of degrading rubber and that rubber biodegradation is a slow process (Gallert, 2000; Jendrossek *et al*., 1997; Nette *et al.,* 1959; Ibrahim *et al*., 2006) One of the solutions to reduce this problem is to recycle the used waste rubber. But due to the chemical cross linking formed during vulcanization it is not possible to simply melt and reshape the products as in case of polythene. So other alternatives such as microbial degradation of the product should be developed.

The present study was taken to isolate the natural rubber degrading fungi /Bacteria from the soil so that it can be used to degrade the rubber waste and to study the enzymes responsible for degradation.Microbial degradation is mainly carried out by various microorganisms such as bacteria and fungi (Lions *et al*., 2000). The introduction of latex overlay agar plates, which consisted of a bottom agar layer of mineral salt medium and a layer of latex or latex agar on top, for isolation and cultivation of rubber-degrading microorganisms was an important achievement (Spence, D., and C. B. van Niel. 1936.). Formation of clear zones was inhibited by addition of glucose, indicating that there was regulation of the expression of rubber-degrading enzymes. One disadvantage of latex overlay agar plates is that not all rubber-degrading bacteria can be cultivated in this way, because many do not form halos on such plates and because too little polyisoprene is locally available to allow formation of visible colonies by these organisms. Rubber-degrading bacteria were therefore divided into two groups according to the growth type and other characteristics (Linos, A.,*et.al.* 2000.).With one exception, representatives of the first group belongs to the clear-zone-forming actinomycetes mentioned above and metabolizes the polyisoprene by secretion of one or several enzymes.

MATERALS AND METHODS:

The present study was carried out for the screening of rubber degrading microorganism from the soil. Soil samples were collected from rubber plantation premises of Calicut and Palakkad district, Kerala, India, from the depth of 6-10cm in the rhizosphere and non-rhizosphere regions.

Collection of Sample:

The present study was carried out for the screening of rubber degradation microorganism from the soil. Soil samples were collected from rubber plantation areas of Calicut and Palakkad district, Kerala, India, from the depth of 6-10cm in the rhizosphere and non-rhizosphere regions, from rubber plantations.

Isolation and Enumeration of *Actinomycetes* from the Soil

The soil sample was collected and transported to the laboratory. One gram of soil sample was weighed and diluted in 100ml of distilled water. 1gm of soil weighed and diluted in 100ml of sterile distilled water + 0.5 ml latex was also added to the soil sample and then the soil sample Serial dilution was performed and was plated in the mineral salt medium as it is selective media for the isolation of the rubber degrading microorganisms then incubated at 37°C for 5 days in the room temperature on for microbial growth.

Identification of the *Actinomycetes*

Colony Morphology

The colour and texture of the colonies of the isolated strains 1, 2, 3, 4, 5, 6, on the MSM media was observed.

Identification of the Culture by Slide Cultural Technique& Gram staining

It is very essential to observe *Actinomycetes* under undisturbed conditions to study their morphological features such as arrangement of spores, presence of sporangia as well as to distinguish between aerial and substrate mycelium. The cover slips from the slide culture were removed on to a clean glass slide with the mycelial growth on the upper surface. The Microscopic examination of the *Actinomycetes* was done by using gram staining procedure.

Screening of *Actinomycetes*

Screening of *Actinomycetes* In Mineral Salt Medium Agar (Lions and Steinbuchel, *et al.*, 1998).The *Actinomycetes* colonies are selected and streaked on to the Mineral Salt Medium

Agar incorporated with latex as sole carbon source. After sterilization media was mixed well and poured in to plates, then inoculated and incubated at 37ºC for 4 days. Then the plates are then incubated at 37°C for 4-5 days. The media is used as the screening of rubber degrading work.

Latex Coagulate Method:

Latex coagulate method was performed to know the organisms have the ability to decolourize the latex .Two methods were performed in latex coagulate method i.e. is with latex pure sample and latex and ammonia.

Latex Pure Sample

Latex sample was taken about 20ml in the centrifuge tube and it was centrifuged for 10,000rpm for 15 mints, after centrifugal the cream portion was transformed to the conical flask then the culture was inoculated and then incubated at room temperature for 37ºC 1 week.

Latex +Ammonia Sample

Ammonia latex sample was taken about 20ml in the centrifuge tube and it was centrifuged for 10,000rpm for 15 mints, after centrifugation the cream portion was transferred to the conical flask then the culture was inoculated and then incubated at room temperature for 37ºC for 1 week.

Confirmation of genomic DNA by agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal submarine electrophoresis unit. 30ml of 1 % Agarose gel was prepared with 1X TBE buffer (do not mix) and heated the content to get up to clear solution for casting Agarose gel. After cooling the solution, 7 µl of staining dye solution was added into the casting system.The gel was allowed to solidify, and then carefully disassembled from the casting system without disturbing the wells and placed in 1X TBE buffer filled electrophoresis tank (the buffer level should be above gel). 5 µl of genomic sample DNA mixed with 2 µl of gel loading dye and then loaded to gel.The power card terminals was connected at respective positions, run the gel at 50 V, till the gel loading dye migrate more than half the length of gel. Then switched off the unit and visualized the isolated DNA under UV Transilluminator.

Bruker MALDI Biotyper for the identification bacteria and fungi in the laboratory:

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI- TOF MS) is increasingly used for the identification of bacteria and fungi in the laboratory. By the mold database of Bruker Daltonik (Bremen, Germany), the Filamentous Fungi Library 1.0, Using the manufacturer recommended interpretation criteria,genus and species identification rates were 78.3% and 54.2%, respectively

MALDI-TOF MS analysis:

The acquisition and analysis of mass spectra was performed by a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0) with the Filamentous Fungi Library 1.0 (Bruker Daltonik) and default parameter settings as published previously . The Bruker bacterial test standard (Bruker Daltonik) was used for calibration according to the instructions of the manufacturer. For each strain two preparations of sample material were analyzed.

RESULTS

Isolation and Enumeration of Microbes from the Soil

The soil samples were collected, weighed from different microsites of various rubber plantation and serially diluted the dilutions upto 10^3 were prepared in sterilized distilled water. Samples were plated on MSM medium. The *Actinomycetes* colonies were isolated, purified and maintained on MSM medium.

Colony Morphology, Colony Characteristics of *Actinomycetes* Sp.

The 6 selected isolates showed difference in their culture character. The *actinomycetes* are identified on by the earthy smell, powdery texture mycelia. The 6 isolates on MSM media showed the difference in the *mycelial* character.

1. Fast growing with dull white coloured spores powdery colonies.
2. Greenish brown colored, powdery, substate mycelium well isolated colonies.
3. Grey powdery coloured colonies other three have similar nature

Identification of the culture by slide culture techniques:

Morphological, Physiological Characterization

Cell morphology	**Branched *Mycelium* rods with spiral spore chains**	**Branched *Mycelium* rods with straight chains**	**Branched with spiral spore chains**	**Branched *Mycelium* Long rods with branched *Mycelium***
Gram reaction	Positive	Positive	Positive	Positive

DNA Isolation:

DNA was isolated from LB medium checking the proper growth of the culture by checking OD value in spectrophotometer and then DNA isolation was done and with the isolated DNA gel running was performed.

Latex Coagulase Test

Latex pure:

The latex sample was inoculated with the culture and latex was not solidified it was in liquid stage only. Latex was completely degraded.

Latex + ammonia the preservative:

The latex sample was inoculated with the culture and the latex + ammonia sample was not solidified. it was in colourless liquid stage only according to our inference latex was completely degraded.

MALDI- TOF MS (Matrix assisted laser desorption ionization-time flight mass spectrometry):

Data interpretation:

The Biotyper software 1 compares each sample mass spectrum to the reference mass spectra in database, calculates an arbitrary unit score value between 0 and 3 reflecting the similarity between sample and reference spectrum, and displays the top 10 matching database records. Standard Bruker interpretative criteria were applied. Briefly, scores of ≥2.0 were accepted for species assignment, and scores of ≥1.7. But <2.0 for an identification to the genus level.

CONCLUSION

Rubber is a natural product obtained from rubber tree as rubber latex.Rubber latex is obtained through tapping and the collected rubber latex is processed by rubber processing factory, during the rubber processing they release waste effluents which cause pollution to the nearby areas. In the present work an effort has been made to screen and characterize the rubber degrading *Actinomycetes* from soil and study its activity. The certain filamentous fungi are capable of degrading it to the level of CO_2. *Actinomycetes* are filamentous bacteria which are found in soil. *Streptomyctes* are able to degrade to the highest rubber degrading ability than the other *Actinomycetes.*

- The basic idea behind the project is to isolate rubber degrading *Actinomycetes,* Characterize and study its activity

The colony Morphology, Cell Morphology and Gram reaction of these isolates were studied,and maldi-tof was performed for organism identification then its activity for biodegradation was also checked and came to conclusion that the organisms have the activity for biologically degrade rubber.Latex coagulase test was done to know the efficiency of the isolate to degrade the rubber latex and all the isolates showed their efficiency to decolorize the natural rubber latex and the latex was not solidified. Six isolates showed as positive and designated as RCA 1,2,3,4,5,6 these isolates were screened for their activity. Each isolates showed different colony character as white powdery colonies, greenish brown colonies, grey and brownish and grey mixed coloured colonies was found.

The rubber degradation of non filamentous bacteria is usually limited compared to filamentous bacteria and it has not been so extensively studied. An attempt to study the degradation by coagulase test was performed with the rubber latex and all the isolates showed positive result by showing decolourizing of the rubber latex within two weeks. The rubber latex is a natural product as its processing cause the waste effluent which causes pollution. The aim of the work was to biodegrade with the microbes to minimize pollution. The procedure was carried out which is more eco friendly by any other chemical process.The genus level identification was carried out by biochemical methods and comparing the result with bergeys manual of determinative bacteriology. The DNA isolation performed and gel running was done and the bands of all the samples came on the similar pattern. The isolates which confirm that it belongs to *Steptomyctes sp* by maldi-tof results.

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