

**“BIOPROSPECTING MARINE MICROORGANISMS THROUGH
CULTURE DEPENDANT METHODS”**

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Executive Summary

Bioprospecting Marine Organisms through Culture Dependant Methods

The study was conducted with the aim of bioprospecting microbes from marine samples and developing a strategy for discovering useful bioactive agents from microorganisms. For this initially marine water samples were collected from three sampling locations viz. Sangumugham Vizhinjam and Veli coast, Thiruvananthapuram and were characterized for their physico-chemical properties. As actinomycetes were known to possess excellent activities, first part of the work focuses on actinomycetes cultures. A total of 20 actinomycetes strains were isolated from the selected marine sample. They were characterised morphologically and biochemically. The isolated strains were screened for cellulase, protease, lipase, amylase, chitinase and L-asparaginase activity. Effect of incubation period, pH and temperature on enzyme activity was determined. The second part of the study focuses on the isolation and characterization of bacterial strains from selected samples. A total of six strains were isolated and they were checked for their potential for selected enzyme activity, production of IAA, Biosurfactant, EPS and also their pollution degradation potential such as dye degradation. The final part of the study focused on the Isolation and characterization of Fungal strains from the selected samples. The isolated fungal strains were screened for their enzyme activity and their potential to produce EPS. The results of the study are summarised hereunder:

- The physico- chemical characters of the marine samples indicate that highest temperature was observed in Veli coast followed by Vizhinjam. The pH values showed great fluctuation among the sampling sites. Veli coast showed acidic waters compared to Sangumugham and Vizhinam.
- Compared to other two samples, Veli coast showed maximum conductivity, salinity and TDS which may be resulted from the effluent discharge from TTP.
- Sangumugham showed maximum DO value followed by Vizhinam and minimum DO value was in the Veli coast which may be due to the industrial discharge.
- A total of 20 actinomycetes strains were isolated from the marine samples. A total of 20 actinomycetes strains were isolated from the selected marine samples. 10 (A1, A2, A3, A4, A5, A6, A7, A8, A9 and A10) from Sangumugham. 8 (B1, B2, B3, B4, B5, B6, B7 and B8) from Vizhinjam and 3(T1, T2 and T3) from Veli, coast.
- The morphological characters of the strains were identified using cover slip method

- All the isolated strains were primary screened for cellulase, protease, lipase, amylase and L-asparaginase by spot inoculation method.
- The strains which showed positive enzyme production were selected for further enzymatic assay using shake flask method.
- In order to find out the effect of incubation period on enzyme production each of the selected enzyme activity was checked for 3rd, 6th, 9th ad 12th days of incubation. The various parameters like pH (4.0 to 9.0) and temperature (20°C to 50°C) were also determined.
- In the case of cellulase enzyme activity all the strains showed a similar trend of enzyme production from 3rd to 9th day of incubation i.e. the enzyme production increased gradually from 3rd to 6th day of incubation and then decreased from 9th to 12th day of incubation. Almost all the strains showed maximum enzyme production on the 6th day of incubation. Of all the strains, strain A3 showed maximum enzyme production followed by strain T2. Minimum enzyme production was shown by strain B7.
- The effect of pH on the enzyme activity was studied. It was observed that all the isolates showed maximum enzyme activity at a pH range of 7 and 8. Except strain A5, all the other strains showed optimum activity level at pH 7. A5 showed maximum enzyme activity of at pH 8. The enzyme activity of the isolates showed significant reduction at pH 4, 5 ad 6.
- The effect of temperature on cellulase enzyme production indicated that a temperature of 30-40°C is found to be favourable for the enzyme activity for all the strains. Strains A3, A4, B6 and T2 showed maximum enzyme activity at 40°C. All the other strains except B5 and B7 showed maximum activity at 30°C. B5 ad B7 showed same activity at both temperatures. The cellulase enzyme activity found to get decreased at 20 and 50 °C.
- In the case of lipase activity out of the 14 strains, strain B1, B2, B6, T1 ad T3 showed maximum activity on 6th day of incubation where as all the other strains showed maximum enzyme production on 9th day. Maximum enzyme production was shown by strain A3 on 9th day of incubation .All the other strains showed almost equal range of enzyme production.
- The influence of pH on lipase production a revealed that all the selected strains in the preset study showed maximum enzyme activity at pH 7. The enzyme activity gradually increased from increasing the pH from 4to 8, reached its peak at pH 7 and then occurred a sudden decrease from 8 to 9. The result indicated that all the isolates showed a good range of lipase activity at pH 6, 7 and 8

- The effect of temperature on lipase production the enzyme assay of the isolated strains indicate that the isolated strains showed maximum lipase production at 40 °C. At 20 °C the enzyme activity seems to be reduced.
- In the case of protease production, except strains A1 and A4, all the other strains showed maximum enzyme production on the 6th day of incubation where as A1 and A4 showed maximum enzyme production on the 9th day. Of all the strains strain B6 showed maximum protease activity followed by T1 and T2. All the other strains showed almost similar range of enzyme production
- The influence of pH on the protease activity revealed that almost all the strains were able to produce a good protease activity both at pH 7 and 8 with maximum enzyme production at pH 7. Except strain A9 almost all the strains showed moderate range of activity at pH 6. A9 showed least enzyme activity at pH 4. The enzyme production capacity of all the strains seems to get reduced at pH 4, 5 and 9.
- The influence of temperature on the protease activity indicate that out of the 13 strains which showed protease activity, except strains A1, A4, A8 and B4 all the other 9 strains showed greatest enzyme activity at 40°C. Whereas A1, A4, A8 and B4 showed maximum activity at 30°C. The results also revealed that except strain B2 and T3 almost all the strains showed moderate range of enzyme activity at 20 and 50°C. Strain B2 showed least activity at 20°C. No protease activity was observed in strains B2 and T3 at 50 °C.
- The effect of incubation period for the amylase production was showed that all the strains showed good amylase activity at the 3rd and 4th day of incubation with maximum activity at 3rd day. Among the isolated strains A10 showed maximum activity followed by A9 and A1. Least activity was shown by the strain A5.
- The influence of pH on the amylase production revealed that the activity of all the strains increased with increasing the pH from 5 to 7 and thereafter a decline in activity was observed from 8-9. So in the present study pH 6-7 was considered as the favourable pH for the selected strains. Strain A9 showed maximum activity at pH 6 and 7. Except A9, B6 and T1 all the other strains showed maximum activity at pH 7 where as these three strains showed maximum activity at pH 6.
- Regarding the influence of temperature on amylase production the results indicate that the isolated strains showed maximum activity at 40 and 50°C. Out of the 14 strains possessing amylase activity, 5 strains (A1, A5, A7, A10 and T2) showed maximum activity at 40 °C where as the rest of the strains showed

- maximum activity at 50⁰ C. At 20 and 30⁰ C almost all the strains showed reduced amylase activity
- Influence of incubation period on the L-asparaginase activity exposed that all the isolates showed maximum activity on the 6th day of incubation and thereafter from 9th to 12th day of incubation, the enzyme activity was found to be decreased. Maximum enzyme activity was shown by Strain A3. The other strains showed almost equal range of asparaginase activity with slight variations.
 - The effect of pH on L-asparaginase production indicated that except strain A7 all the strains showed good enzyme activity at both pH 8 and 7 with maximum enzyme activities at pH 8. Strain A3 showed maximum enzyme activity compared to other strains. At pH 6 and 9 all the strains showed moderate enzyme activity. Strain A7 showed maximum enzyme activity at pH 4.
 - The effect of temperature on the enzyme activity specifies that except strain A2 all the isolates showed good enzyme activity at 30 and 40⁰ C. All the strains showed maximum activity at 40⁰ C except strain A3 which showed maximum activity at 30⁰ C. When compared to other strains least asparaginase activity was shown by strain A2. At 20 and 50⁰ C the selected isolates showed moderate rate of enzyme production.
 - The present study points out that the isolated strains were enzymatically active and the enzyme production is temperature, pH and time dependant.
 - In this study almost all the strains possess more number of enzyme activities. The secondary screening of the isolated strains showed that strain A3 showed superior activity in the case of cellulase, lipase and production.
 - The isolates showed a potential to produce a wide range of enzymes which may be due to the result of natural selection of microorganisms in order to survive in the competing environment
 - The enzyme activity of the 20 actinomycetes varied from isolate to isolate depending upon the growth and physical condition of the isolates which may be due to the nature of the sample from which they were isolated.
 - A total of 12 bacterial strains were isolated from the marine samples. 4 (Ab1, Ab2, Ab3 and A4) from Sangumugham., 4 (Bb1, Bb2, Bb3 and Bb4) from Vizhinjam and 3 (Tb1, Tb2 Tb3 and Tb4) from Veli, coast.
 - Biochemical characters of the selected strains were done based on standard procedures. Further studies on the molecular characterization of the isolates are in progress.
 - All the selected strains were screened for their potential to produce biosurfactants. Out of 12 strains 8 (Ab3, Ab4, Bb1, Bb3, Bb4, Tb1, Tb2, Tb3 and Tb4) strains showed growth in hydrocarbon added medium.

- Four (Ab4, Bb4, Tb1 and Tb3) strains which showed maximum growth were selected for further screening tests such as emulsification activity and blue agar plate method. Strain Tb1 and Tb3 showed > 30 % emulsification activity and these strains were considered as positive strains for biosurfactant production.
- None of the strains showed positive result for blue agar plate which indicates that the strains are not potent for glycolipid production.
- Six bacterial strains showed positive amylase activity.
- Almost all the isolates showed a dip in amylase production at neutral pH after a good level of activity at acidic pH.
- The highest amylase producer is Bb1 and the organism produced the least is Bb3.
- In the case of Tb3 and Bb2, though the production was low in acidic as well as neutral pH, the production increased manifold in alkaline pH.
- It can be seen that all bacterial strains invariably showed the greatest activity at pH 9, that is alkaline condition.
- Except Bb1 and Bb2, other strains showed maximum activity at 37°C.
- Ab2 showed maximum activity (2.45 Units/mL) at 37°C. This was followed by Bb3 (2.15 Units/mL) and Ab1 (1.98 Units/mL). A notable finding is that the strain Ab2, the maximum amylase producer, produced the least amount at 20°C.
- All the strains Except Bb1 showed the highest production of amylase at the third day of incubation.
- Ab2 produces the maximum amount of enzyme (2.45 Units/mL).
- Except in Bb2 which produced highest secretion in pH 9, all the others showed minimum activity at this pH.
- Bb2 was found to show maximum amylase activity also at this alkaline pH, which indicates its potential to grow and metabolize best at alkaline conditions.
- In the case of temperature of incubation, except Bb2, all other isolates showed a highest activity at 37°C.
- Bb4 showed maximum protease activity at 37°C which comes about 742 Unites/mL.
- Ab2, showed higher production of enzymes at the third day of incubation.
- Of all the isolates, exceptionally good activity was shown by Bb4 and then Tb4.
- Tb3 which showed a heightened production at neutral pH.
- The isolate Tb3 showed a good activity at 37°C.
- Tb4 also shows a similar profile although the other cultures showed almost similar production at 20°C as well as 50 degrees and temperature did not cause much change in the production of cellulase.
- Most of the isolates showed maximum activity at day 3.

- Except for Tb1 and Tb2 in which the day 6 also witnessed considerable cellulase production, the other strains had almost comparable activities in all the temperature.
- In Lipase production, except in Ab1, all other isolates produced lipase at neutral pH.
- In the case of Ab1, the microbe shows acidophilic nature and makes its optimum pH for secretion of lipase is 4.0.
- Two of the microbial isolates (Tb1 and Tb2) showed considerable production of lipase at 37°C.
- Ab2 showed a small increase in the temperature of 37°C.
- Isolate Ab1 showed highest lipase activity on the first day of incubation.
- Bacterial strains Tb1, Tb2, Ab2 and Ab3 showed a better activity on the third day of incubation.
- Bb4 showed same lipase activity on first, third and sixth day of incubation.
- None of the strains showed L-asparaginase activity
- Out of 12 strains, 5 strains exhibit IAA production. The IAA producing strains are Bb1, Bb2, Tb2, Tb3, and Tb4.
- All the positive strains showed almost equal range of IAA production except in the case of strain Tb3 which showed maximum production of IAA ($14.35\mu\text{g mL}^{-1}$). The strain Bb2 showed minimum IAA ($6.05\mu\text{g mL}^{-1}$) production whereas all the other strains showed almost equal range of production.
- When grown at different temperatures all the strains showed maximum production at 37°C except the strain Tb2 which showed maximum production at 20°C.
- The IAA production by all the strains seem to get reduced at high temperature except in the case of strain Tb4 which showed comparatively high amount of IAA at 50°C than at 20°C (8.11 and $3.82\mu\text{g mL}^{-1}$ respectively)
- Of the isolates, Tb2 showed better production at 20°C and the isolate Tb4, at 50°C.
- Strains Bb1 and Tb3 showed equal range of production both at acidic and alkaline pH (3.52 , 5 and 3.23 and $5.6\mu\text{g mL}^{-1}$) respectively.
- Both the strains showed maximum production at neutral pH.
- In the isolates Bb2 and Tb3, the production decreased at low pH when compared to pH 9. Strain Tb3 showed maximum production of IAA at pH 7 ($14.35\mu\text{g mL}^{-1}$). At pH 4, it produces $10.76\mu\text{g mL}^{-1}$ of IAA and in the case of alkaline pH IAA production seems to get reduced ($7.52\mu\text{g mL}^{-1}$).

- The isolate Tb2 made maximum secretion of IAA (10.94 µg/mL) at 24 hours of incubation. The production of IAA by this isolate was greatest at 20°C and at neutral pH.
- Isolate Tb4 showed IAA secretion which was greatest at 72 hours (14.35µg/mL) of incubation which decreased slightly after that at 120 hours of incubation.
- Out of 12 strains, 5 strains exhibit EPS production and the strains are Ab4, Tb1, Tb2, and Tb3.
- All the strains produced proteins in EPS at highest quantities at 20°C.
- Except in Tb2, after a dip in the production at about 37°C, most of the bacterial isolates increased the production of proteins at 50°C.
- Except Bb4 and Tb1, all others showed a slight increase in the production of protein content in EPS. Of these, Tb3 showed a sharp increase in the secretion of proteins in the EPS at 120 hours.
- In case of incubation time, except Bb4 and Tb1, all others showed a slight increase in the production of protein content in EPS.
- Tb3 showed a sharp increase in the secretion of proteins in the EPS at 120 hours.
- The concentration of carbohydrates in EPS produced in the selected isolates were quantified and is found that it is almost same at both higher and lower temperatures and was found to be minimum at room temperature.
- Except Tb1 the EPS in terms of carbohydrates was found to be highest in alkaline pH.
- The content was much higher than at acidic as well as neutral pH.
- As far as incubation time is concerned, isolates Ab4 and Tb3 increased the secretion of carbohydrates upon passing of time whereas Bb4 and Tb2 decreased the secretion as incubation time increased.
- Phosphate solubilization activity was screened for only two of the 12 strains isolated, Tb1 and Tb3.
- All the isolated strains were primary screened for their tolerance to selected dyes congo red and methyl red in marine Zobell broth added with 0.1 % of respective dye.
- The tolerance test was confirmed with the Plate assay by streaking the isolates in Basal Salt Medium added with 1 % of selected dye.
- The bacterial strains which showed maximum tolerance to the selected dyes were selected for further decolourisation study.
- Among the bacterial strains, Strain Ab1, Ab2, Bb2, Bb3, Tb2 and Tb3 showed maximum tolerance to congo red and these strains were further selected for decolourization assay of congo red.

- The strains Ab1, AB3, Bb1, Bb3, Tb2 and Tb3 which showed maximum tolerance to congo red were selected for further decolourisation assay.
- Strain Tb2 showed maximum decolourisation and the strain Bb1 showed comparatively low congo red decolourisation with the other strains.
- In the case of methyl red decolourisation assay strain Tb2 showed maximum potential whereas least efficiency was shown by Bb2.
- The optimum decolourisation of the selected dyes by bacterial strains was studied at different parameters like pH (4, 7 and 9), temperature (20, 30 and 50°C), azo dye concentration (10, 50, 100, 150 and 200 ppm), co-substrate (starch, glucose and mannitol), and incubation period (24,48,72 and 96 hrs).
- Selected strains were able to decolourize congo red over a wide range of pH. Strain Tb2 showed maximum decolourisation at pH 7 followed by Tb3 and Ab3.
- The growth and decolourisation followed a similar trend in all strains. But there occurred a controversy that Strain Bb3 and Tb3 showed a good growth rate but its percentage decolourisation was found to be minimum at pH 4 it was noticed in the case Bb3 at 7 pH also. The growth rate of Bb3 was found to be very low but it showed high decolourisation.
- All the strains showed a moderate rate of decolourisation and growth at 20 and 37 ° C. There after there occurred a sudden decline in decolourisation was observed as the temperature increases from 37 to 50 ° C.
- The influence of congo red concentrations on decolourisation revealed that all the isolates showed almost equal range of decolourisation at 100 and 150 ppm of dye. Strain Bb1 was found to tolerate all the tested concentrations
- All the strains showed significant decolourisation rate and growth when added with different co-substrates. All the strains showed their maximum decolourisation potential when added with starch when compared to glucose and mannitol with slight variation which is insignificant
- Regarding the influence of incubation period on congo red decolourisation for almost all the strains 48 hours of incubation was found to be the optimum time period for growth and incubation period except the strains Bb1 and Bb3 which showed maximum growth and decolourisation at 72 hrs of incubation.
- In the case of methyl red Ab1, Bb2 and Tb3 prefer pH 7 for maximum decolourisation. whereas the strains Bb3 and Tb2 showed maximum decolourisation at pH 4. At the same time strain Ab3 showed maximum decolourisation and growth at 9 pH.
- All the strains showed maximum methyl red decolourisation at 37°C.

- As the methyl red concentration increases from 10 to 100 there occurred an increase in growth and decolourisation was noticed and there after a sudden drop was found as the concentration increased from 100 to 200 ppm.
- Except Ab1 all the other strains showed maximum decolourisation and growth when added with mannitol. Ab1 showed maximum reduction with glucose.
- Almost all the strains showed maximum decolourisation of methyl red at 48 hrs of incubation after that decolourisation range seems to get stable.
- Bioremediation potential of the efficient bacterial strain was evaluated by seed germination study with green gram seeds. Green gram showed good germination in the presence of treated dye compared to untreated dye.
- In the case of bacterial strains soaked in congo red added bacterial metabolites maximum germination was shown by the seeds soaked in Bb3 metabolite and minimum was found with Tb3.
- In the case of methyl red added bacterial effluent seeds soaked in Tb2 metabolite showed maximum decolourisation followed by Tb3, Ab1, Ab3 and Bb2 .
- A total of three (VF1, VF2, VF3) fungal strains were isolated from marine sample from Veli coast.
- Based on standard procedures biochemical characters of the selected strains were done.
- Three fungal strain showed positive amylase activity.VF3 showed highest amylase production and VF1 showed least production activity.
- Almost all the strains showed good level of amylase production in alkaline pH
- VF1 strain showed least production of amylase enzyme in acidic pH
- In the case of temperature VF2 and VF3 showed maximum amylase production at 37 ° C. Both the strain showed great level of enzyme activity at different temperature.
- VF1 showed least production at 20°C.VF2 and VF3 showed equal range of enzyme production.
- In lipase expect VF1 all other showed great level of lipase production at neutral pH. Of that VF3 strain showed highest level of lipase production
- With regard to temperature, VF2 and VF3 strains has equal range of production. At 50°C VF3 showed least production of enzyme.
- In the case of incubation period on lipase production, VF2 showed maximum production on 3rd day.VF3 also showed good range of lipase production on 3rd day of incubation
- In the case of L- Asparaginase activity, VF1 and VF2 showed considerable range of enzyme production. All the stain showed good level of production at neutral pH.

- At 37°C VF1 and VF2 showed highest range of enzyme production .In the case of incubation period, at 12th day all the strains showed maximum L-Asparaginase production. VF2 showed highest range of production.VF2 and VF3 showed equal range of production.
- In the case of cellulase VF2 and VF3 showed highest cellulose production at neutral pH.
- All the strains showed considerable range of cellulase production. All the strains showed maximum cellulose production on 12th day.
- In the case of protease VF1showed highest production .Mmost of the strain showed highest production in pH 7.
- VF2 and VF3 showed highest protease production in 37°C.
- VF3 has highest protease production on 12th day and VF3 showed least production of protease on 9th day
- In the present study EPS production by fungal isolates depends on the culture parameters
- VF2 strain showed maximum EPS production at different pH, temperature and incubation period.
- In both the strains low pH, 30 ° C temperature and 15 th day of incubation period was found to be favourable for the EPS production.

Based on the present study, it may be concluded that the actinomycetes that live in marine environments can be used as very important tools in environmental biotechnology. It is clearly indicated that marine water system can be considered as an important resource for screening useful enzyme producing actinomycetes. The selected actinomycetes in the present study showed excellent activity of cellulase, protease, amylase, L-asparaginase and lipase enzymes. These isolates may be effectively used in large scale production for commercial, industrial and pharmaceutical applications in the coming future. However, it's through testing at industrial conditions is advised. the marine bacterial strains can be considered as a significant tools in environmental biotechnology. Biosurfactant producing bacterial strains in the present study needs much more screening tests for confirmation. The selected bacterial strains in the present study showed excellent activity of cellulase, protease, amylase and lipase enzymes. These isolates may be effectively used in large scale production for commercial, industrial and pharmaceutical applications in the coming future. In the present study the selected bacterial strains isolated from the marine water samples have a high efficiency to tolerate congo red and methyl red. This has increased the scope for using these marine for the decolourisation harmful dyes. These strains can be positively used for further decolourisation studies. Further characterizations would help to commercially exploit these organisms for biodegradation of textile azodyes. Moreover, a lot

of future work is needed to isolate new microorganisms capable of effectively degrading wide range of textile dyes and to create an environment free from textile dye pollution. Further investigations are required to make use of the full potential of these organisms for enzyme production by modern protein engineering technology.

Significance of the study

The study is intended to isolate marine microbes with potent enzymatic and antibiotic activity. Oceans once considered being 'biological deserts' has shifted to the status of 'rain forests' owing to the isolation of many novel microbes and associated bioactive compounds. This relatively untapped recourse is now being studied and potentials unveiled. This study will help in the isolation of a number of different microorganisms coming under bacteria and fungi with varying degrees of potential activity which can be further screened for diverse activities and production of bioactive metabolites. Some of the activities are anticipated to be identified in this work and the rest can be subsequently studied on proper storage of the organisms. But it forms only a glimpse of what actually present hence the knowledge can later be extrapolated through a metagenomic approach.

Its potential contribution to knowledge in the field of social relevance or national importance

The outcome of the work may reveal the presence of many industrially as well as medically important molecules which on further purification, characterization and optimization can be used in respective fields. Since the molecules are of biological origin, they would be safe and less harmful than corresponding synthetic compounds. They are thought to cause minimum environmental problems. Since the products with antibiotic properties are natural the chemical structure will be complex and the development of resistance will be very slow. Their mass production does not involve the use of strong chemicals. Handling and cultivation of microbes are also relatively easy.