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In Vitro enzymatic screening and assessment of the Lichen Associated Bacteria and its evaluation of antifouling studies

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Abstract

Parmeloid Lichen was collected from the Yercaud hills, Tamil Nadu. Lichen-associated bacteria of fifty cultures were isolated by using the pour plate technique. The total colonies observed in surfactant samples obtained after 5 minutes of washing was 0.89x10^2 CFU/g which showed a considerable increase to 2.9 x10^2 CFU/g after 90 minutes of washing. From that, sixteen Lichen-associated Bacteria were purified and characterized. Gram Staining was done for the identification of the Lichen-associated Bacteria. Herein, five potential bacterial isolates were screened from the sixteen Lichen-associated Bacteria by using the qualitative enzyme activity. Lichen-associated,12,13, and 14 were screened and subjected to the quantitative enzymatic assay. Isolate 4 showed a higher Protease specific enzymatic activity of 80 Units/mg, higher specific enzymatic activity of 30.7 Units/mg, lipase enzymatic activity of 14.69 units/ml and a higher cellulase specific enzymatic activity of 31.76 Units/mg.

Key words: *Parmeloid, Bacterial isolates, specific enzyme activity, protease, lipase*

Introduction

Marine biofouling is a complex process of colonization that becomes the major issue, where aquatic species, microbial slimes and macrophytes eventually colonize the ship hulls in a short span. Bioaccumulation leads to a rise in irregularity on the surface, which leads to an increment in hydrodynamic wear and drag. This, in turn, leads to increased fuel consumption, removal of paints and repainting over the surface, and all other associated environmental compliance measures all contribute to repair costs of biofouling [6]. It is one strategy to prevent biofouling by hydrolyzing adhesives of fouling organisms using enzymes [15]. Compared to

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organometallic antifoulants, enzymes show more advantages including high efficiency, biodegradability, and environmental friendliness. Except for the hydrolyzing function, enzymes also influence the attachment of marine fouling organisms like producing biocides. Enzyme-based fouling control technologies have attracted worldwide attention and have been investigated continuously over the past 20 years. Fouling does not appear immediately on a submerged material. It involves microbe invasion, bio-adhesive deposition, and subsequent growth. As a result, the development of ecologically safe coatings is highly essential and quickly expanding [16]. Most of the approaches to novel control methods are based on the search for new bioactive compounds that might repel or inhibit the adhesion of fouling organisms or acts as a good biocide [3]. Lichen-associated bacteria are an integral component of lichen thalli and thus the classical view of lichens should be widened to include bacteria. The lichen-associated bacteria (LAB) often possess high diversity and abundance as well as some possible roles in lichen symbiosis [10]. Most lichen-associated bacteria are found to be non-photosynthetic bacteria [4]. Recent research on lichen-associated bacteria revealed that lichen-associated microbes are a fundamental component of lichen thalli [2]. Bacterial species obtained from four different lichen species at multiple locations harbored a distinct community of bacteria and all the communities were dominated by *Alphaproteobacteria* [8]. Still, most of this symbiotic association remains unexplored. These lichen-associated bacteria may possess some un-investigated potentials which have to be inquired about and experimented with in the future. Industrial enzymes are important for various biotechnological applications. Proteases are the key antifouling active components in enzyme-based antifouling coatings. Several enzymes used as antifouling active substances include protease, cellulase, amylase, wood poly enzyme, and lipase. Enzyme-based antifouling technology has a broad developing space in the fouling control field. Many enzymes such as protease and amylases have been proposed as a substitute for traditional antifouling compounds. Enzymes may affect the settlement and adhesion of biofoulants occurs in diverse ways. Initially, they may outbreak the adhesive of settling organisms, thus averting the settlement event. Furthermore, enzymes may damage the polymers in the biofilm matrix formed by proliferating settled organisms [11]. In this paper, we will discuss the various enzymes derived from lichen-associated bacteria and their utilization in the antifouling process.

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Materials and Methods

Collection and identification of lichen samples from Yercaud hills

Lichen samples were collected at four different altitudes from Yercaud hills, Salem, and Tamil Nadu. It is situated at an altitude of 1515 meters (4970 ft) above sea level. No specific permissions were required for sampling at the specified location. Lichen samples were collected at each location from at least five arbitrarily selected trees or rocks. On each sampling, at least 10 lichen thalli were collected from each location to analyze single or bulk lichen thalli samples. Specimens appearing healthy were carefully removed from tree bark or rocks using sterile gloves and stored in individual plastic bags. Thalli samples were transported and stored under refrigeration until processing within 24 h after sampling [1]. Identification of lichen samples was performed by K-Test, C-Test, KC-test, and PD test followed by Thin layer Chromatography [10]. Microchemical tests are to be done to determine the order, genus, and Species level lichen taxonomy [19].

Symbionts isolation from macerated lichen samples and gram staining

1g Fresh lichen thalli were washed with running tap water followed by Tween20 and 1minute treatment with Mercuric chloride and washed with sterile distilled water. The surface-sterilized lichen thalli were macerated with sterile distilled water in the mortar and pestle. The macerated sample was then serially diluted (10^{-3}, 10^{-4}, $10^{-5}$and 10^{-6}) and pour plated in Zobell marine agar medium for the isolation of bacterial symbionts. The plates were incubated for 24 hours at 37ºC. Gram staining was performed for the observed symbionts. Morphological identification of gram-positive actinobacteria with the developed vegetative and aerial mycelium was viewed under optical microscopy achieved by gram staining technique [5].

Qualitative Screening of potential Lichen associated Bacteria

The sixteen isolates isolated from the lichen were suspected to various enzymatic screening to identify the potential isolate that is to be accompanied in the production of anti-fouling compounds.

Protease enzymatic screening

Protease enzymatic production was carried out by inoculating the LAB in minimal media agar with milk powder as a source and incubated overnight, production of protease enzyme is confirmed by a clear zone around the culture streak [18].

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Polyphenol oxidase production

L-DOPA agar medium was prepared, autoclaved, and sterilized. It was then poured into sterile Petri plates and then allowed to solidify. A single streak inoculum of each organism was made on the center of appropriately labeled plates. The inoculated plates were then incubated for 7 days. The presence or absence of a color change in the colonies to black color was observed [7].

Amylase enzymatic screening

Amylase enzymatic production was carried out by inoculating the LAB in minimal media with starch as a source and incubated for 48 hours, production of starch enzyme is confirmed by the clear zone obtained after iodine stain. Iodine stain was prepared by dissolving 0.34g of iodine and 0.66g of potassium iodide in 100 ml of water [13].

Lipase enzymatic screening

Lipase enzymatic production was carried out by inoculating the LAB in phenol red agar media with olive oil as a source and incubated for 48-72 hours, production of protease enzyme is confirmed by the color change of the media from red to yellow. Lipase enzymatic screening was performed to identify the lipid cell wall cleaving ability of the lichen-associated bacteria [9].

Cellulase enzymatic screening

Cellulase enzymatic production was carried out by inoculating the LAB in minimal media with carboxymethylcellulose as a source and incubated for 48 hours, production of cellulase enzyme was confirmed by the clear zone obtained after staining with 01% congo red for 15 minutes and 1M NaOH for 15 minutes [12]. The isolates passed in the maximum number of enzymatic tests were subjected to the quantitative estimation of that particular enzyme.

Quantitative protease enzyme

Protease enzymatic production was carried out by inoculating the LAB in minimal media with 1% milk powder as a source and incubated for 48 hours. 100µl of supernatant from the broth was exposed to the protease assay by using L-Tyrosine as a standard. 0.1ml supernatant was taken in the fresh test tube, makeup to 1ml with dis.H_2O, 5ml of 0.65% casein dissolved in 1N NaOH were added and incubated for 10minutes at 40ºC, 5ml of 110mM TCA

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reagent was then added and incubated for 30 minutes at 37ºC. 2ml of the above mixture was then added to the fresh test tube to which 5ml of 500mM Na_2CO_3 and 1ml of 0.5M folin's cioculteu reagent were added and incubated for 30 minutes in dark. In standard 0.1, 0.2, 0.3, 0.4, and 0.5 ml of L-Tyrosine was added and makeup to 1ml, to which 1.2 ml of TCA was added and incubated for 30 minutes at 37ºC. In which 5 ml of Na_2CO_3 and 1ml folin's ciocalteu reagent were added and incubated for 30 minutes in dark. Absorbance was taken at 660nm [18]. The enzymatic activity was calculated using the formula,

$$\text{Enzymatic activity: } \frac{\text{Moles of tyrosine equivalent} \times \text{total vol. of reaction mixture}}{\text{Total vol. of supernatant} \times \text{Reaction time} \times 2}$$

Specific enzymatic activity: Enzymatic activity/ Protein concentration

Quantitative amylase enzyme

Amylase enzymatic production was carried out by inoculating the LAB in minimal media with 1% starch as a source and incubated for 48hours. 100µl of supernatant from the broth was exposed to the amylase assay by using glucose as a standard. 0.1ml of supernatant was added to fresh test tubes and makeup to 1ml with the distilled water to this 1ml of 1% CMC dissolved in 0.05M sodium citrate buffer of pH 4.8 was added and incubated at 40ºC for 15 minutes. In to which 1ml of DNS was added and incubated at 100ºC for 5minutes and cooled to room temperature [13]. Absorbance was recorded at 540nm. The enzymatic activity was calculated using the formula,

$$\text{Enzymatic activity: } \frac{\text{Moles of glucose equivalent} \times \text{total vol. of reaction mixture}}{\text{Total vol. of supernatant} \times \text{Reaction time} \times 2}$$

Specific enzymatic activity: Enzymatic activity/ Protein concentration

Quantitative cellulase enzyme

Cellulose enzymatic production was carried out by inoculating the LAB in minimal media with 1% CMC as a source and incubated for 48 hours. 100µl of supernatant from the broth was exposed to the cellulase assay by using glucose as a standard. 0.1ml of supernatant was added to fresh test tubes and makeup to 1ml with the distilled water this 1ml of 1% starch dissolved in the acetic acid buffer of pH 4.9 was added and incubated at 40ºC for 15 minutes. In to which 1ml of DNS was added and incubated at 100ºC for 5minutes and cooled to room

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temperature. Absorbance was recorded at 540 nm [12]. The enzymatic activity was calculated by,

$$\text{Enzymatic activity: } \frac{\text{Moles of glucose equivalent} \times \text{total vol. of reaction mixture}}{\text{Total vol. of supernatant} \times \text{Reaction time} \times 2}$$

Specific enzymatic activity: Enzymatic activity/ Protein concentration

Quantitative lipase enzyme

Lipase enzymatic production was carried out by inoculating the LAB in phenol red media with 2% olive oil as a source and incubated for 72 hours. 100µl of supernatant from the broth was exposed to the lipase assay and 1ml para nitrophenyl acetate was used as a substrate and incubated for 10 minutes at 37 ºC Absorbance was recorded at 410 nm [9].

$$\text{Enzymatic activity: } \frac{\text{Absorbance value} \times \text{Final volume}}{\text{Co-efficient value} \times \text{Initial value}} \times 10$$

Specific enzymatic activity: Enzymatic activity/ Protein concentration

Antimicrobial activity for the lichen associated Bacteria

The antimicrobial screening of the starch, casein and cellulose broth was performed to identify which source has resulted in highest amount of antimicrobial agents by the agar well diffusion assay. The antimicrobial activity against *E.coli* and *Proteus vulgaris* in muller hinton agar was carried out for culture broth, pellet suspended with phosphate buffer of pH 7, lyophilized ethanolic extract of the culture supernatant and incubated for 24 hours. The clear zone of diameter around the well were calculated [14, 17].

RESULT AND DISCUSSION:

ISOLATION AND STAINING OF LICHEN ASSOCIATED BACTERIA

Lichen samples collected from yercaud hills, Salem Districts of Tamil Nadu were identified by National Botanical Research Institute (NBRI), Lucknow as *Parmelinella wallichiana* and *Parmotrema tinctorum.* Isolation of bacteria from Lichen sample was done by pour plate technique in Nutrient Agar plates. A substantial number of colonies was found even in higher dilutions. An increase in the washing period of the lichen thalli has increased the recovery of ectolichenic bacteria. The total colonies observed in surfactant samples obtained after 5 minutes of washing was 0.89×10^2 CFU/g which showed a considerable increase to 2.9×10^2

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CFU/g after 90 minutes of washing, the increase in colony count was due to the slow discharge of bacterial colonies on the surface of the lichen into the surfactant solution. The result obtained was in correlation with [4] were increased in thalli washing time has increased the recovery of the total number of ectolichenic bacteria where the total colony-forming units attained after 5minutes of washing was 1.4 x10^2 CFU/g which has increased to 3.3 x10^2 CFU/g after 90 minutes of washing period.

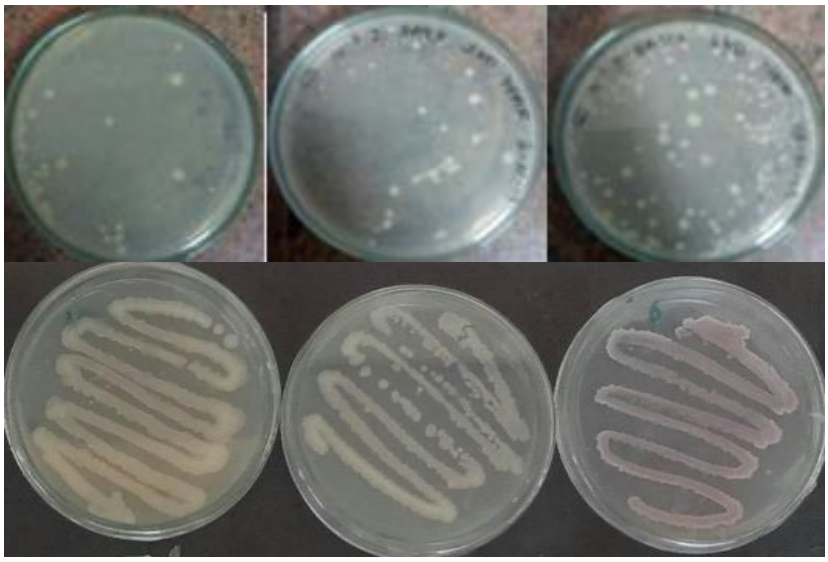

Figure 1. Isolation and streaking of Lichen-associated Bacteria by using pour plate techniques.

Table 1. Morphological appearance of the isolated Lichen associated bacteria

Organism	**Gram staining**
1	Gram positive, diplobacillus
2	Gram-positive, rod
3	Gram-negative, bacillus chains
4	Gram-positive, cocci
5	Gram negative, diplobacillus
6	Gram-negative, rods
7	Gram-negative, cocci
8	Gram-positive, cocci
9	Gram-positive, cocci
10	Gram-positive, cocci
11	Gram positive, bacillus
12	Gram-negative, rods
13	Gram positive, bacillus
14	Gram positive, bacillus
15	Gram positive, bacillus
16	Gram-positive, rod

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The absolute number of endolichenic bacterial colonies obtained was approximately equal to 0.7 $x10^2$ CFU/g which was parallel to [4] where the total number of restored colonies was $1x10^3$ CFU/g. An overall number of 50 colonies were selected and isolated from the plates and subcultured on slants. Among that 16 bacterial isolates of morphologically different colonies were selected, 10 were ectolichenic bacteria and the other 6 were endolichenic bacteria. Subsequent quadrant streaking was done for the isolates in order to improvise the purity of the strains. Gram staining of the isolates was done to ensure the purity as well as to evaluate the morphological aspect of the strains. Gram staining results of the isolates were shown in Table 1.

Quantitative protease enzyme

A quantitative enzymatic assay of protease enzymatic production was carried out for the above five isolates. The isolate 4 showed higher specific enzymatic activity of 80 Units/mg when compared with the isolate 13 with a specific enzymatic activity of 25.6 Units/mg whereas the isolate 12 produced 31.7 Units/mg as a moderate performance and the result was in accordance with [18], he produced protease enzyme for seven isolates with the specific enzymatic activity of 76 Units/mg and the result was recorded in Table 3.

Quantitative amylase enzyme

A quantitative enzymatic assay of amylase enzymatic production was carried out for the five isolates. The isolate 4 showed a higher specific enzymatic activity of 30.7 Units/mg compared with isolate 12 with a specific enzymatic activity of 16 whereas the isolate 12 produced 26.9 Units/mg showing the moderate performance and the result was in accordance with [13], produce the specific amylase activity of 27 Units/mg and the result was recorded in Table 3.

Quantitative lipase enzyme

A quantitative enzymatic assay of lipase enzymatic production was carried out for the five isolates. The isolate 4 showed higher enzymatic activity of 14.69 units/ml when compared with isolate 2 with an enzymatic activity of 14.41 and the result was following [9], who produced the 13 units/ml of lipase enzyme from their isolate and result was recorded in Table 3.

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Quantitative cellulase enzyme

A quantitative enzymatic assay of cellulase enzymatic production was carried out for the five isolates. The isolate 4 showed a higher specific enzymatic activity of 31.76 Units/mg when compared with the isolates 14 and 13 with the specific enzymatic activity of 19.98 Units/mg whereas the isolate 12 with a specific enzymatic activity of 26.7 Units/mg and the result was by [12], who produced the 28 Units/mg of cellulose enzyme in his research and the result was recorded in Table 3.

Table 2. Qualitative enzymatic screening of lichen isolates

Isolates	Amylase	Polyphenol oxidase	Protease	Lipase	Cellulase
1	+	+	+	+	+
2	+	+	+	-	+
3	-	+	-	+	-
4	+	+	+	+	+
5	+	+	+	-	+
6	+	+	+	+	-
7	+	+	-	-	-
8	-	+	-	-	-
9	+	+	+	-	+
10	+	+	+	-	+
11	-	+	-	-	-
12	+	+	+	-	+
13	+	-	+	+	+
14	+	+	+	+	+
15	+	-	-	+	-
16	-	+	+	+	-

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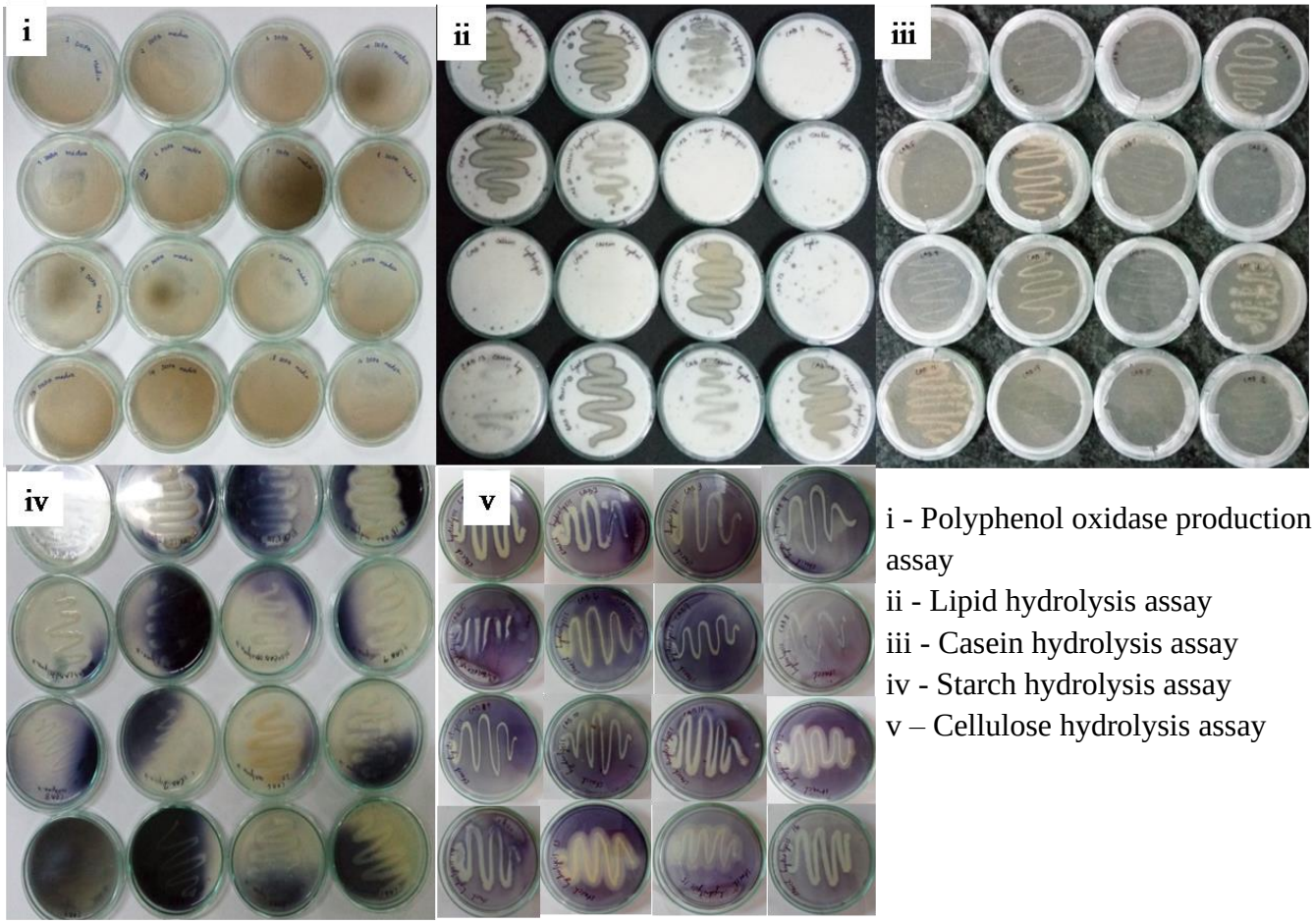

Figure 2. Potentiality of the lichen isolates to produce various kinds of enzymes

Table 3. Quantitative enzymatic screening of lichen isolates

Isolate	Protease Assay		Cellulase Assay		Amylase Assay		Lipase Assay
	Enzymatic activity (units/ml)	Specific enzymatic activity (Units/mg)	Enzymatic activity (units/ml)	Specific enzymatic activity (Units/mg)	Enzymatic activity (units/ml)	Specific enzymatic activity (Units/mg)	Enzymatic activity (units/ml)
2	5.96	29	15.2	27.6	16.9	21.4	14.41
4	6.6	80	15.8	31.76	13.6	30.7	14.69
12	8.56	31.9	19.2	26.9	15.16	29.98	14.36
13	7.7	25.6	20.0	16	12.9	19.98	17
14	7	27	18.82	17	14.7	19.98	16.4

Antimicrobial activity of the lichen-associated bacterial isolate

The ethanolic extract of the quantitative broths lipase, protease, amylase, cellulose and the metabolites was mixed with the Dimethyl sulfoxide and tested for the antimicrobial activity

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against the *E.Coli* and *Protease vulgaris*, and the results were tabulated in table 4.6. Casein metabolites shown the clear zone of 1.8 cm against *P.vulgaris* and cellulose metabolites shown the clear zone of 1.8cm against *E.coli,* and the standard tetracycline disc recorded 2.5 and 2.7 cm for *E.coli* and *P.vulgaris* respectively. The supernatant of the mass production sample was shown the inhibition of 1.6 and 2 cm for *E.coli* and *P.vulgaris* respectively.

Table 4. Antimicrobial activity of the metabolites from quantitative enzymatic broth

Source of LAB metabolites and Std disc.	Anti-microbial Activity Zone diameter (cm)	
	E. coli	*P. vulgaris*
Starch	1.5	1.7
Casein	1.7	1.8
Cellulase	1.8	2
Tetracycline Disc	2.5	2.7

Conclusion

. Lichens are small group of curious plants recognized as a rich source of secondary metabolites. Parmeloid Lichen such as *Parmelinella wallichiana* and *Parmotrema tinctorum* was collected from the Yercaud hills, Salem Districts of Tamil Nadu. Lichen associated bacteria of fifty cultures were isolated by using the pour plate technique. 5 bacterial isolates were selected based upon the enzymatic activity of the bacterial isolates. The specific enzymatic activity of all the lichen isolate have showed the better production in accordance with the other organisms, thus once again the result reveals the unique potential of the lichen associated symbionts, it could be the best source for the production of antifouling compounds. The anti-microbial activity of the ethanolic extract of the casein, cellulose, protease and lipase have shown the antimicrobial activity of 180 mm for the *E.coli* and *P.vulgaris* thus, it proved the efficiency. Hence, in the quantitative enzymatic screening, isolate 4 produced the extremely higher enzyme production in all the four assays, and it was subjected to the antifouling study. Thus, from this study it is evident that, enzyme from lichen associated bacteria would be the ideal to combat biofoulings, in the further research it can be formulated and can used against the marine foulers.

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