Incidence of laccase and biosurfactant producing bacteria from mesophilic to extreme environments to harness potential contribution in dye degradation and antimicrobial activity

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Abstract

Three separate bacterial populations were isolated and screened from diverse locations, classified as mesophiles, halophiles and thermophiles. Halophiles and thermophiles exhibited greater laccase activity than mesophiles and enhanced biosurfactant property noted in thermophiles. Based on phenotypic and biochemical characteristics, T17 was identified as Pseudomonas sp. and H27 as Bacillus sp. Bacillus sp. (laccase positive, best grown at 5% NaCl) was assessed for its ability to degrade Gentian violet, Safranine and Bromothymol blue at 24, 48 and 72 h of incubation across various dye concentrations (1 to 10%). The most effective dye degradation occurred with safranine (24.12%), followed by bromothymol blue (22.85%) and gentian violet, which exhibited over 20.16% degradation at 72 h. Thermophilic Pseudomonas sp. was utilized to assess the antimicrobial activity of its partially purified biosurfactant.

It was particularly effective against Fusarium sp., Rhizoctonia sp. and Helminthosporium sp. at concentrations of 250 and 300 µg/ml, but it did not inhibit Alternaria sp. Similarly, the antibacterial activity of partially purified biosurfactant from Pseudomonas sp. was effective against all bacterial pathogens across a broad spectrum of concentrations ranging from 100 to 300 µg/ml. The findings underscore the extensive potential applications of laccase and biosurfactants sourced from halophiles and thermophiles.

Keywords: Laccase, biosurfactant, dye degradation, antimicrobial activity, extremophiles.

Introduction

The earth acquires tremendous types of bacteria in soil and water. Based on the nature of their habitats, those bacteria surviving at moderate temperatures and salinity conditions are regarded as mesophiles. Salt-loving bacteria are commonly known as halophiles[19] and those that tend to grow at higher temperatures (>40°C) are known as thermophiles[22]. Extremophilic bacteria have numerous applications in bioremediation processes, industries and pharmaceutical industries because they can thrive in extreme living conditions. Several mechanisms are involved in the protection and higher metabolic activities of extremophiles in comparison to the mesophilic bacteria.

Mesophiles and extremophiles have been reported to synthesize several industrially important enzymes and biomolecules with multiple biotechnological research applications due to their strong catalytic activities and higher stability[20]. Among many enzymes, a microbe may harbor laccase. It is a metalloenzyme containing four copper atoms in itself for the catalysis of oxidation and reduction of substrates[2]. The broad substrate specificity and catalytic efficiency for aromatic and non-aromatic compounds make laccases an important enzyme in bioremediation processes at industrial scales[7]. Laccase has been reported for stabilization of beverages[15], wood treatment[12], biofuel production[13], bioremediation of wastewater containing dyes[33,34] and polyphenols[24], pesticides and pharmaceuticals[16,23] etc. They are used as biosensors[8,36], for denim[29] and paper bio bleaching[4] etc.

Other than the laccase, extremophiles have also been reported for the production of secondary metabolites such as biosurfactants[5]. Biosurfactants are surface activated amphipathic molecules produced in exopolysaccharide membranes of microbes. Biosurfactants have several distinguishing properties, such as they act on surfaces of liquid (oil, water) or interfaces (oil-water)[30]. At the surface or interface of two immiscible liquids, a surface tension is applied. Water has a surface tension of 72 N/cm^2 which could drastically decline to 30N/cm^2 by the action of biosurfactant[31]. Such behavior of the biosurfactant makes it a suitable candidate for making emulsions of two immiscible liquids, harnessing dirt removal as detergent action[11,27], microbially enhanced oil recovery (MEOR)[3] and antimicrobial activity[14] etc.

Keeping in mind the attributes of bacterial laccases and biosurfactants from mesophiles, halophiles and thermophiles, the present investigation deals with the isolation of the three categories of bacteria and screening them for laccase and biosurfactant production. Afterwards, potential applications of laccase and biosurfactants were

made in terms of dye degradation and antimicrobial activity by partially purified biosurfactants.

Material and Methods

Sample collection: The work was carried out at the Centre for Molecular Biology, Central University of Jammu, India. Halophilic soil samples were collected from Drang salt mines and thermophilic samples were collected from hot springs, Manikaran, Himachal Pradesh. Mesophilic samples were collected from the campus of Central University of Jammu from different plant parts including mango leaves, mango bark, neem leaves, neem bark, curry leaves and curry bark phylloplane.

Isolation of mesophilic, halophilic and thermophilic bacteria: Plant parts were cut into dimensions of 1 cm^2 area and placed in nutrient broth for enrichment of mesophiles for 24h at 37°C at 180 rpm. Colonies were purified through quadrangular streaking and stored for further screening. Halophiles and thermophiles were isolated using serial dilution pour plate method in nutrient agar medium supplemented with 5% NaCl incubated at 37°C and nutrient agar incubated at 45°C respectively.

Screening for laccase production: Primary laccase screening was performed by supplementing nutrient agar with 0.2mM copper sulphate (inducer) and 2mM guaiacol (substrate) to which bacteria were inoculated and incubated under their said growth conditions. Appearance of brick red to brown colour of the colony ensured laccase positive bacteria.

Determination of laccase activity: Laccase activity was determined for all the positive bacterial isolates during primary screening. Isolates were grown at their ambient growth conditions and crude laccase was harvested from 24h grown old bacterial isolates through centrifugation (10000 rpm, 4°C for 10 minutes). Extracellular laccase was drawn in supernatant and used for laccase activity. All experiments were carried out in duplicate. Laccase activity was determined using sodium phosphate buffer (100mM, pH 7.5, 600 µL), guaiacol (100mM, 200 µL) and crude laccase (200 µL) using optimised reaction conditions (30°C for 10 minutes at 700rpm). Immediately after reaction, absorbance for the test and control samples was observed at 470 nm.

Laccase activity was calculated as per the formula:

$$E.A\ (U/ml) = (A_{470} \times V_t)/ (t \times V_e \times \varepsilon) \times D$$

where E.A is laccase activity (U/ml), A_{470} is absorbance at 470 nm, Vt is total volume of reaction mixture (ml), Ve is laccase volume in reaction, ε is guaiacol extinction coefficient (0.674 µM/cm) and D is dilution factor.

Screening for biosurfactant activity: The biosurfactant activity of the isolate was determined using two different tests as drop collapse assay (qualitative) and emulsification activity (quantitative).

Drop collapse assay: A calibrated microscopic slide was left at room temperature for an hour before oil was applied. After adding 20µl of cell-free supernatant to the oil drop on the slide, it was observed for one minute to see if it collapsed. The test was deemed negative unless the culture supernatant that caused the oil drop to collapse, revealed a positive drop collapse test. Distilled water with uninoculated oil drop was used as negative control[25].

Emulsification assay: In a cotton-plugged tube, 2 ml of culture was combined with 2 ml of mustard and olive oils separately and the mixture was vortexed at a high speed for two minutes to carry out the emulsification activity. After 0 h, 24 h, 48 h, 72 h and 96 h, the emulsion stability was assessed. To ensure that the emulsion formed was stable, emulsification was generated as E_0, E_{24}, E_{48}, E_{72} and E_{96} at 0, 24, 48, 72 and 96 h[17].

The emulsification indices, stability and volume were calculated by the formulae provided:

Emulsification activity (%) = (Emulsified height (cm))/(total height of liquid (cm)) × 100

Cultural, morphological and biochemical identification of screened isolates: Cultural identification was done based on the cultural characteristics of the bacteria on nutrient agar medium such as colony color, margin, elevation etc. Morphological identification of the bacteria was done by Gram staining and endospore staining to know whether the bacteria is Gram positive or Gram negative. Selected biochemical assays were done to know more about the physiology of the bacteria such as the IMViC assay, sugar fermentation etc.

Dye degradation ability of halophilic-laccase producing *Bacillus* sp.: Degradation of synthetic dyes namely gentian violet, safranine and bromothymol blue was performed using submerged shake flask method where 100 ml broth was inoculated with 2% v/v of 18h old grown culture. Different concentrations of dyes (1 to 10%) at varying time intervals (24 to 72h) were observed along with the treatment and media control for degradation. Initial and final absorbance of different dye concentration at different time interval (h) were observed using spectrophotometer at λ_{max} of each dye to calculate the percent degradation. λ_{max} of gentian violet is 590 nm in water, λ_{max} of safranine is 530nm and of bromothymol blue is 430 nm[10].

Degradation of dyes is calculated by the following formula:

Degradation of dyes (%) = (Initial absorbance at time,0 h-final absorbance at time, t (h)) / (Initial absorbance at time,0 (h)) ×100

Batch mode submerged production and solvent extraction of biosurfactant from thermophilic *Pseudomonas* sp.: 100 ml Bushnell Haas broth in 250 ml

capacity Erlenmeyer flasks was used for biosurfactant production where potassium dihydrogen phosphate (1g/L), dipotassium hydrogen phosphate (1g/L), ammonium nitrate (1g/L), ferric chloride (0.05g/L), magnesium sulphate heptahydrate (0.2g/L) and calcium chloride (0.02g/L) were dissolved in water. The pH of the broth was maintained at 7.2 and broth was supplemented with 2% mustard oil as sole carbon source, finally seeded with 2% bacterial inoculum. Flasks were incubated at 35˚C for 10-15 days at 180 rpm. Biosurfactants were harvested as crude by centrifugation at 10,000 rpm for 30 minutes. Chloroform-methanol extraction method was used for obtaining partially purified biosurfactant (PPBS).

The pH of the crude biosurfactant was dropped to 2 using concentrated HCl and allowed to rest for 10h in refrigerator at 4˚C followed by addition chloroform-methanol (2:1). The mixture was centrifuged at 10,000 rpm for 15 minutes. The pellet (Solvent extracted partially purified biosurfactant/PPBS) was decanted and the residual solvents were evaporated at 40˚C using a water bath[6].

Antimicrobial activity of partially purified biosurfactant (PPBS) of thermophilic *Pseudomonas* sp.: The antimicrobial activity of the produced biosurfactant was evaluated by agar diffusion method. Solvent extracted biosurfactant fraction was prepared as 50 µl, 100 µl, 150 µl, 200 µl, 250 µl and 300 µl per ml DMSO solution in vials to be used for antimicrobial test. Selected bacterial test strain was cultured in nutrient broth at 37℃ and incubated overnight. The grown culture was swab inoculated onto nutrient agar plate. Using well borer, 3 to 4 wells of diameter of 8 mm were made in the plate to which 100 µl of the solvent extracted biosurfactant was filled and to the other wells, same amount of DMSO (dimethyl sulphoxide) as a treatment control was used. The plates were incubated at 37˚C for 18 -24 h. The zone of inhibition was measured in mm[28].

For antifungal activity, spore suspension of the particular fungi was prepared in potato dextrose agar and poured (at 45°C) immediately to overlay the bottom agar of the same medium. Wells were punched and each well was filled with different concentrations of biosurfactant. Plates were incubated at 25°C for 2-3 days and the zone of inhibition was measured in mm.

Results and Discussion

Incidence, distribution of laccase and biosurfactant producing bacteria from mesophilic and extreme environments: High bacterial load from each sample was recorded. Among 100 halophilic bacterial isolates, 28 were laccase positive and 20 were biosurfactant producing. Among 40 samples of mesophiles, a total of 203 isolates were screened from a bacterial load of 7658 x 10^5 cfu/ml. Out of 203 bacterial mesophiles, 100 were laccase positive while 53 were biosurfactant producing. Similarly, from a bacterial load of 3872 x 10^5 cfu/ml, 40 thermophiles were screened and out of that 10 were laccase positive and 15 were biosurfactant producing (Table 1). Higher halophilic bacterial distribution was found inside salt mine (n=20) than outside the mine (n=8) including laccase. Equal numbers of biosurfactant producing strains from inside and outside were found (n=10,10 respectively).

A very high bacterial load was found from the phylloplane of the plant parts. Among all, curry bark showed highest distribution of laccase (n=91) and biosurfactant (n=20) producing strains. Thermophiles were obtained from samples of the Manikaran hot spring situated in Himachal Pradesh. Almost equal distribution of laccase-positive thermophiles was obtained from soil and water samples, while higher biosurfactant-producing strains were recovered from soil samples (10) (Table 2).

In accordance with the present investigation, a substantial amount of research has been conducted to demonstrate the prevalence of mesophiles, halophiles and thermophiles in natural environments in India and other countries. In a study conducted by Rafiee et al[26] thermophilic bacterial strains were isolated and identified from hot springs of Iran. These bacteria had potential antimicrobial activity towards *E. coli* and *Staphylococcus aureus*. The identified bacterial strains were thermophile growing at 45 to 75°C as well as they were alkaliphile growing well at more saline pH 9.

Table 1
Incidence of laccase and biosurfactant producing bacteria

Sample size	Total bacterial load on samples (cfu/ml)	Attributes	Isolates picked up for screening	Laccase		Biosurfactant	
				Number of positive isolates	Number of negative isolates	Number of positive isolates	Number of negative isolates
25	5576 x 10^5	Halophiles	100	28	72	20	80
40	7654 x 10^5	Mesophiles	203	100	103	53	150
15	3876 x 10^5	Thermophiles	40	10	30	15	25

Table 2
Distribution of halophilic, mesophilic and thermophilic -laccase and biosurfactant producing bacteria

Attributes	Site description	Sampling location/objects	Total isolates (N)	Screened (n)	Number of positive isolates	Number of negative isolates	Number of positive isolates	Number of negative isolates
					Laccase		Biosurfactant	
Halophiles	Salt mine, HP	Inside mine	100	75	20	55	10	65
	Salt mine, HP	Outside mine		25	8	17	10	15
Mesophiles	Plant phyloplane	Neem leaf	203	22	20	2	3	19
		Neem bark		27	24	3	5	22
		Mango leaves		17	7	10	10	7
		Mango bark		25	5	20	6	19
		Curry leaves		21	15	6	9	12
		Curry bark		91	29	62	20	71
Thermophiles	Hot spring, Manikaran, HP	water	40	20	5	15	10	10
		soil		20	6	14	5	15

It has been discussed by Kruglikov et al[18] that amino acid sequences in mesophiles and thermophiles differ in several terms. They classified the orthologs of amino acid sequences into weak polyampholytes/polyelectrolytes, boundary proteins, strong polyampholytes, negative strong polyelectrolytes and positive strong polyelectrolytes based on charges on the amino acid sequences. Thermophilic weak polyampholytes/polyelectrolytes have more disorders than in mesophiles. This explains higher compactness at higher temperatures in protein folding. In case of halophilic bacterial proteins, they thrive in lower levels of available water and higher salt concentrations.

Halophilic adaptation includes exclusion of cations for management of unfolded proteins stabilization[1]. Thus, it is known that both thermophiles and halophiles have better adaptation to external environments that make them dwell in extreme conditions. Their protein structure, conformation and folding are altered to maintain their functionality whereas mesophiles lack these special arrangements by the cell proteins.

Laccase activity of mesophiles, halophiles and thermophiles: All the bacterial isolates were subjected for laccase screening by plate assay. From the plate, strong brown colour producing strains by oxidation of guaiacol were selected for further study. The potent isolates screened five from each category were selected for quantitative laccase activity determination. From the five strains of mesophiles, highest laccase showing bacteria was M150, from halophiles highest laccase activity was shown by H27 and H70 while among thermophiles, highest laccase activity showing bacteria were T17 and T32 (Fig. 2).

Laccases are multi copper oxidases that catalyse huge range of substrates. The active site of laccase includes copper atoms arranged in fashion like T1 (type 1) and T2/T3 (Type 2). T1 fetches electron from substrate and transfers to type 2 copper centre which reduces molecular oxygen and releases water molecule. Specifically talking about laccases from halophiles have few adaptations such as it may lack halide binding sites that could alter enzyme activity, thereby it results in high salt tolerance. The second proposed mechanism is salt activated laccase which is still not very clear[21].

Biosurfactant activity of potent isolates: Emulsification indices of mustard and olive oil were generated by selected isolates (Fig. 3). A peculiar trend of emulsification indices was seen among all the isolates tested, where, 100% emulsion was obtained at 0h which gradually decreased over the incubation time period from 24h to 96h. Among halophilic strains H27, H33 and H90 had very high emulsification indices at 24h of incubation as 78%, 78% and 84% respectively, while H70 and H83 had 66% and 38% emulsification index for mustard oil. For olive oil, H33 and H83 had E_{24} more than 50% among the rest of halophilic strains. From the five strains of mesophiles all had high emulsification in case of mustard oil while in case of olive oil only M1 had 50% E_{24}.

All the thermophilic bacterial strains were recorded to produce high emulsification with mustard oil namely T2, T12, T17, T29 and except T32 as 82%,100%, 100%, 86% and 38% respectively but in case of olive oil only T2 and T17 showed above 50% emulsification indices. Emulsification index of mustard oil and olive oil for different isolates was calculated. Most of the isolates in case of mustard oil showed high E_{24} (≥50%) but in case of olive oil, only two isolates i.e. H33, H83, M1, T2 and T17 showed E_{24} ≥50% (Table 3).

Cultural, morphological and biochemical identification of T17 (biosurfactant producing thermophile) and H27

(Laccase producing halophile) bacteria: Two potent bacterial isolates, one from thermophile and other from halophile, were selected for further study. T17 was selected as thermophilic biosurfactant producing bacteria from Manikaran, Himachal Pradesh and H27 was selected as halophilic laccase producing bacteria from Drang salt mines, Himachal Pradesh, India. Based on cultural, morphological and biochemical identification; T17 was identified as *Pseudomonas* sp. while H27 was identified as *Bacillus* sp. Typical characteristics of the two bacteria have been summarized in table 4.

The current research study has included mesophilic and extremophilic bacterial isolation from different sites to compare their capability for laccase and biosurfactant properties.

Table 3
Biosurfactant attributes of selected isolates

Attributes	Isolate designation	Drop collapse-1% SDS	E_{24} (%) of Mustard oil	Drop collapse - Mustard oil	E_{24} (%) of Olive oil	Drop collapse - Olive oil
Halophiles	H 27	++	78±2.83	+	40±0.00	-
	H 33	++	78±2.83	+	52±5.66	-
	H 70	+	66±2.83	+	36±5.66	+
	H 83	+	38±2.83	+	56±0	-
	H 90	++	84±5.66	+	46±2.83	-
Mesophiles	M1	++	90±2.83	+	50±2.83	-
	M 13	++	78±2.83	+	34±8.49	-
	M 88	+	74±2.83	-	38±2.83	-
	M 102	+	90±2.83	-	44±5.66	-
	M 150	++	94±2.83	+	36±5.66	-
Thermophiles	T 2	++	82±2.83	+	36±5.66	-
	T 12	++	100±0	+	100±0	-
	T 17	++	100±0	+	100±0	-
	T 29	++	86±2.83	+	46±2.83	+
	T 32	++	38±2.83	+	40±0	-

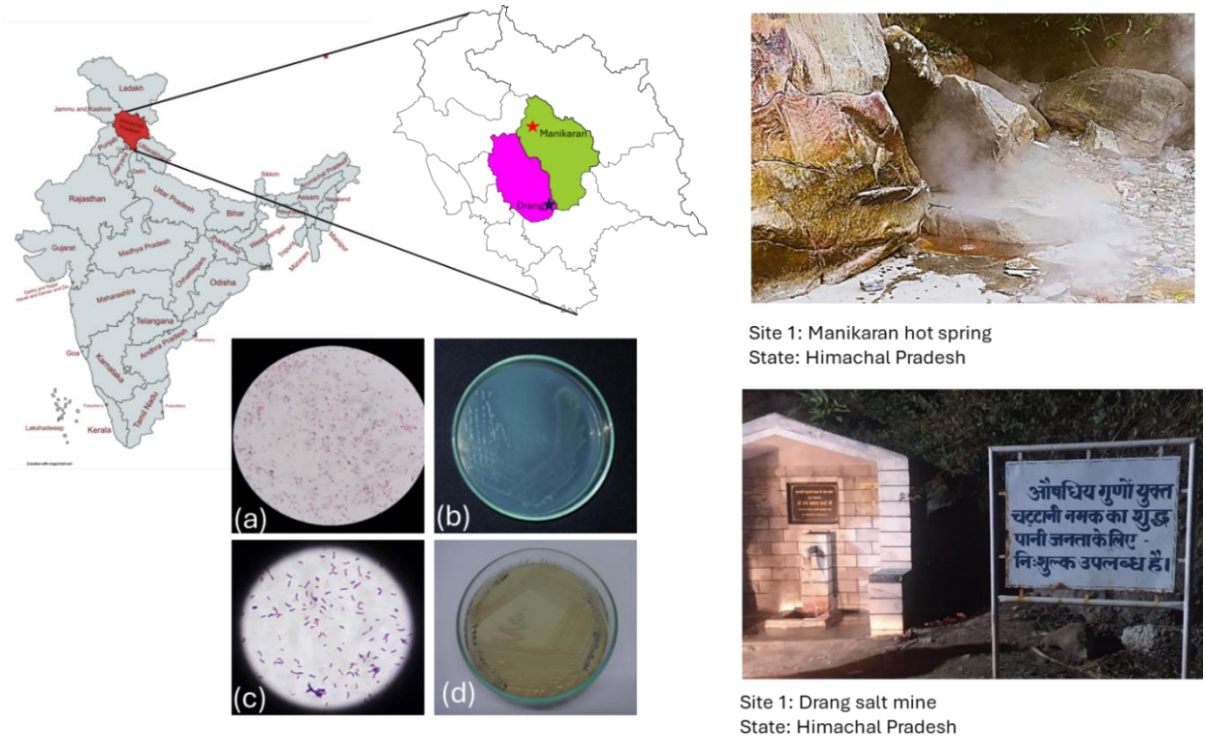

Fig. 1: (a) Gram staining of *Pseudomonas* sp. (b) Pure culture of *Pseudomonas* sp. on Nutrient agar medium (c) Gram's staining of *Bacillus* sp. (100X magnification, light microscope), (d) Pure culture of *Bacillus* sp. on Nutrient agar medium. *Pseudomonas* sp. is a biosurfacatnt producing thermophile from Manikaran, HP and *Bacillus* sp. is laccase producing halophile from Drang salt mine, Mandi, HP

Table 4
Cultural, morphological and biochemical identification of T17 and H27 isolates

Identifying characteristics of T17 and H27 bacterial isolates			
Characteristics	**Assay**	***Pseudomonas* sp. (T17)**	***Bacillus* sp. (H27)**
Cultural characteristics	Colony colour	Greyish	Cream white
	Margin	Entire	Undulate
	Elevation	Convex	Flat
	Shape of colony	Irregular large	Irregular large
	Opacity	Translucent	Opaque
	Pigmentation	Blue	No
	Consistency	Mucoid	Mucoid
Morphological characteristics	Gram stain reaction	Gram negative	Gram positive
	Cell shape	cylindrical rods	rods
	Cell arrangement	single	single
	Endospore	absent	present
	Endospore position	NA	subterminal
Biochemical characteristics	Oxidase test	positive	negative
	Catalase test	positive	positive
	MR	negative	negative
	VP	negative	negative
	Indole test	negative	negative
	Citrate utilization test	positive	positive
	Starch hydrolysis test	negative	positive
	Nitrate reduction test	positive	negative
	Urease	negative	positive
	Gelatin liquefaction	positive	positive
	TSI	B(AL) S(AL)	B(A) S(A)
	Motility	positive	positive
	Oxidation / fermentation	Oxidative	Oxidative + Fermentative
	Arginine hydrolysis	positive	negative
Carbohydrate fermentation assay	Glucose	A^+G^-	A^+G^+
	Sucrose	A^-G^-	A^-G^-
	Mannitol	A^+G^-	A^+G^+
	Lactose	A^-G^-	A^-G^-
	Trehalose	A^-G^-	A^-G^-
	D-galactose	A^-G^-	A^-G^-
	Xylose	A^-G^-	A^-G^-
	Sorbitol	A^-G^-	A^-G^-
	Rhamnose	A^-G^-	A^-G^-
	Fructose	A^+G^-	A^-G^-
	Arabinose	A^-G^-	A^-G^-

B(AL)S(AL) relates to Butt alkaline and slant alkaline; B(A)S(A): Butt acid and slant acid; signs in parenthesis to AG (+/_) refer to A^+G^-: acid positive gas negative

The mesophilic bacterial isolates were obtained from plant parts (leaves and bark) of neem, curry and mango; halophiles were recovered from Drang salt mines, Himachal Pradesh, while thermophiles were isolated from soil and water samples collected from Manikaran hot spring, Himachal Pradesh (Fig. 1).

Five bacterial isolates from each group were chosen for comparative analysis. Although mesophiles had a greater prevalence of laccase and biosurfactant producing strains, halophiles and thermophiles had similar numbers of laccase and biosurfactant positive strains. It was observed that laccase activity was higher in halophilic and thermophilic bacteria rather than mesophilic bacteria. Among the three categories of bacterial isolates, all of them showed good emulsification with mustard oil at 24h of incubation but a declining trend was observed with increase in incubation time, the emulsion was reduced showing the stability of the emulsion formed by the bacterial biosurfactant. For olive oil, fewer strains had higher biosurfactant activity at 24h of incubation from each category of halophile and thermophile. Two bacterial strains were analysed for dye decolorization assay and antimicrobial activity through biosurfactant was H27 (laccase producing halophile) biochemically identified

as *Bacillus* sp. and T17 (biosurfactant producing thermophile) as *Pseudomonas* sp. respectively.

Dye degradation by laccase producing halophilic *Bacillus* sp.: Three different dyes were analysed for degradation by halophilic laccase producing *Bacillus* sp. (H27-halophile) viz. Gentian violet, Safranine and Bromothymol blue. As shown in fig. 3, halophilic laccase positive *Bacillus* sp. was able to degrade all the three dyes at various concentrations (1 to 10%). It was observed that all the three dyes were highly degraded at their lowest concentrations and had declining trend towards higher concentrations of the dyes (from 1 to 10%). It was also seen that over the cumulative time period, degradation of the dyes enhanced at the 3rd day of incubation (72h).

Gentian violet was degraded up to 10.29±2.49% which increased to 17.57±5.41 at 48h and finally reached to 20.16±0.48% at 72h (Fig. 4a). In case of safranine, initial degradation at 24h was 16.82±1.81 followed by 22.14±0.23 at 48 h, finally reaching to 24.12±3.05 at 72h of incubation (Fig. 4b). Bromothymol blue dye was showing degradation of 18.56±0.54% at 24h that escalated to 21.97±2.14 at 48h and reached to 22.85±2.32%a at 72h (Fig. 4c).

Laccase producing bacteria are involved in dye degradation processes. The present investigation is in agreement to the research conducted by Sheela and Sadasivam[32] who used laccase producing bacteria for degradation of dyes. Dyes contain difficult degrading chromophore groups but they are easily degraded by enzymatic approach[8]. The present study showed the dye decolourization by *Bacillus* sp. that effectively degraded the gentian violet, safranine and bromo thymol blue at varying concentrations.

Antimicrobial activity of biosurfactant producing-thermophilic *Pseudomonas* sp.: Antimicrobial activity of partially purified biosurfactant from thermophilic *Pseudomonas* sp. (T17) was observed against fungal and bacterial pathogens. Antifungal activity was determined against *Fusarium* sp., *Rhizoctonia* sp., *Alternaria* sp. and *Helminthosporium* sp. It was seen that none of the concentration of partially purified biosurfactant was able to inhibit *Alternaria* sp.

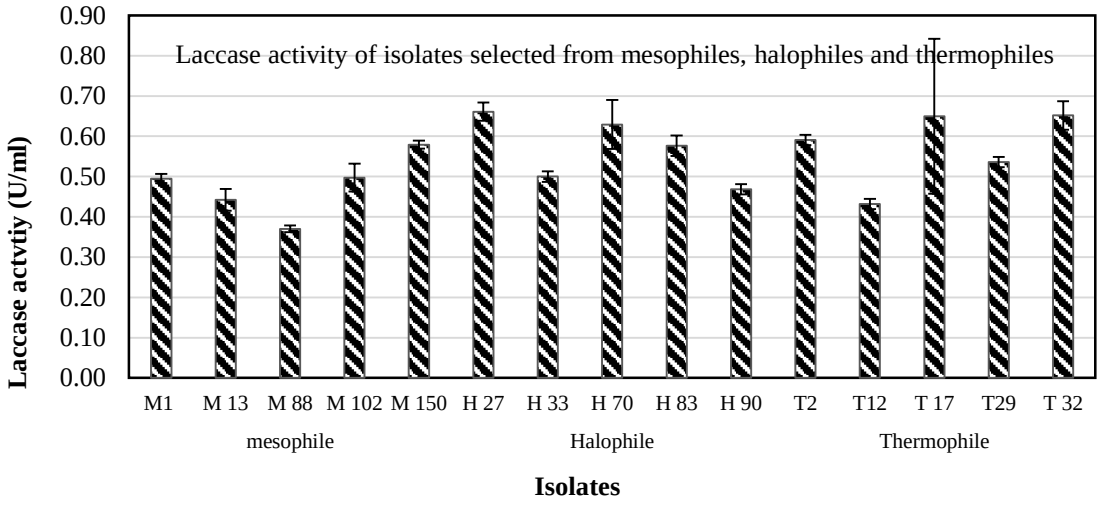

Fig. 2: Laccase activity of isolates selected from mesophiles, halophiles and thermophiles

Table 5
Antifungal activity of partially purified biosurfactant from *Pseudomonas* sp.
(Laccase and biosurfactant producing isolate)

Conc. of PPBS (µl/ml)	Zone of inhibition (mm)			
	***Fusarium* sp.**	***Rhizoctonia* sp.**	***Alternaria* sp.**	***Helminthosporium* sp.**
50	1.66±0.10c	0.67±0.11de	0.00	1.0±0.14d
100	2.03±0.01c	1.67±0.25cd	0.00	1.30±0.14d
150	2.32±0.27c	1.60±0.14cd	0.00	1.65±0.35d
200	2.33±0.31c	2.60±0.14c	0.00	2.60±0.14c
250	4.34±0.70b	**10±0.71a**	0.00	3.70±0.28b
300	**6.33±0.34a**	8.65±0.64b	0.00	**5.30±0.14a**
DMSO	0±0.00d	0±0.00e	0.00	0.00±0.00e
F_{cal}	23.84	126.7	0.00	40.93
F_{tab}	1.36	2.20	0.00	4.33
CD (0.05%)	1.26	1.08	0.00	0.855
F_{test}	S	S		S
Sed(±)	2.04	4.01	0.00	1.80

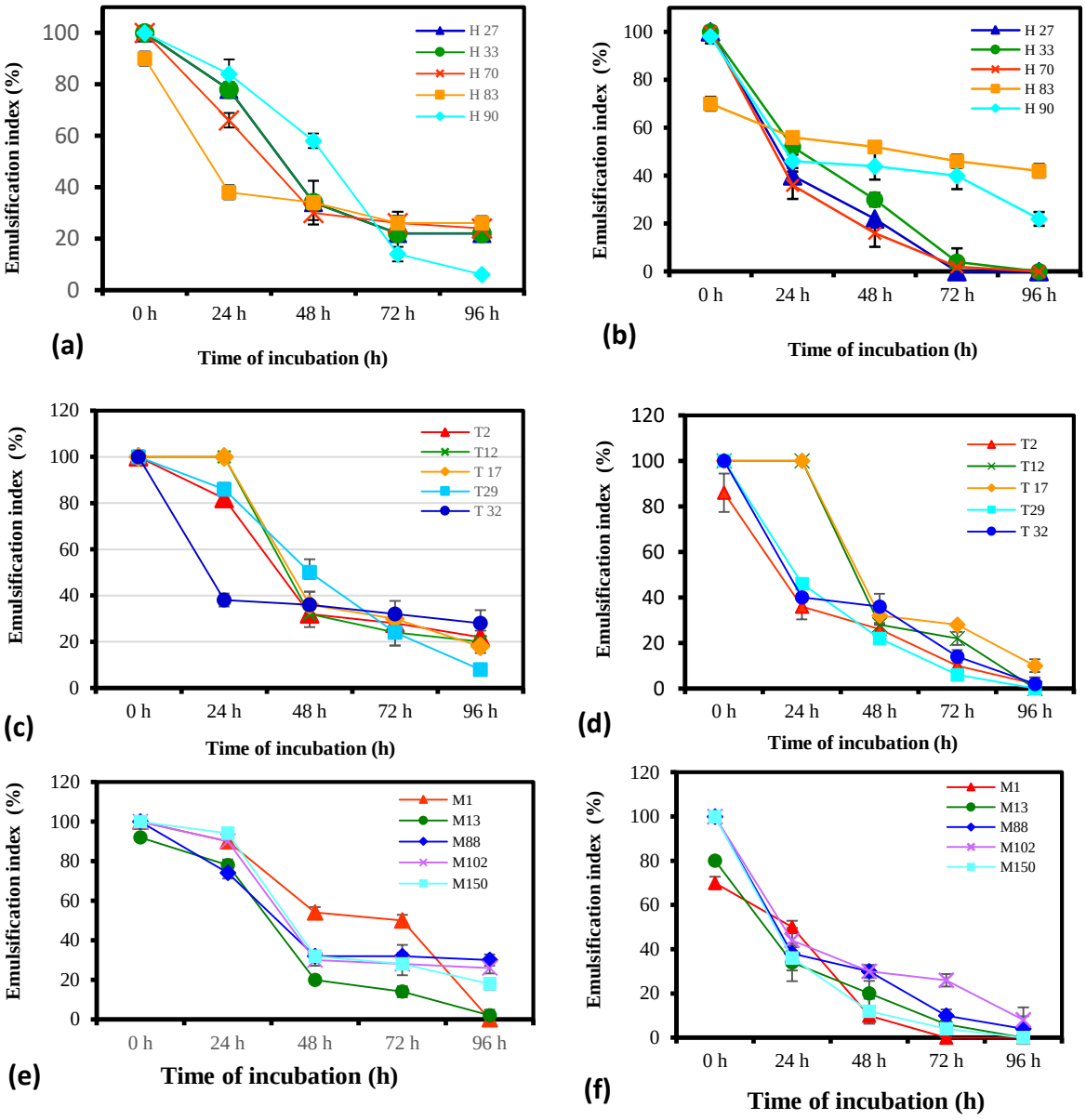

Fig. 3: Emulsification indices (%) of Mustard and olive oil by halophilic, thermophilic and mesophilic bacterial isolates at varying time intervals (0 to 96h) (a) Emulsification index of halophiles on Mustard oil; (b) Emulsification index of halophiles on Olive oil; (c) Emulsification index of thermophiles on Mustard oil; (d) Emulsification index of thermophiles on Olive oil; (e) Emulsification index of mesophiles on Mustard oil; and (f) Emulsification index of mesophiles on olive oil

The rest of the fungal pathogens were inhibited having higher zone of inhibition by higher concentration of biosurfactant. Highest zone of inhibition was recorded by 300 µl/ml PPBS against *Fusarium* sp. (6.33±0.34 mm) and *Helminthosporium* sp. (5.30±0.14 mm) while 250 µl/ml PPBS showed higher inhibition against *Rhizoctonia* sp. (10±0.71 mm).

The highest value is statistically analysed using One way ANOVA followed by Tukey's post hoc analysis. The lower-case letter represents the sequence of significantly different values and rank (Table 5). 250 and 300 µl/ml PPBS are statistically significantly inhibitory concentrations of biosurfactant for antifungal activity. In the same fashion, antibacterial activity of PPBS of *Pseudomonas* sp. was studied against five bacterial pathogenic strains namely, *Klebsiella* sp., *Staphylococcus aureus*, *Proteus* sp., *Salmonella* sp. and *E. coli*. The highest inhibitory conc. of PPBS of *Pseudomonas* sp. to *Klebsiella* sp. was 250 µl/ml having zone of inhibition 0.5±0.05 mm in comparison to other concentrations.

In case of *Staphylococcus aureus*, highest inhibitory conc. was 300 µl/ml (1.4±0.28mm) and lowest was 50 µl/ml (0.6±0.04 mm). *Proteus* sp. was highly inhibited at 100 µl/ml of biosurfactant and lower inhibition was recorded at 50, 150 and 300 µl/ml. *E. coli* was inhibited in the same fashion as *Proteus* sp.

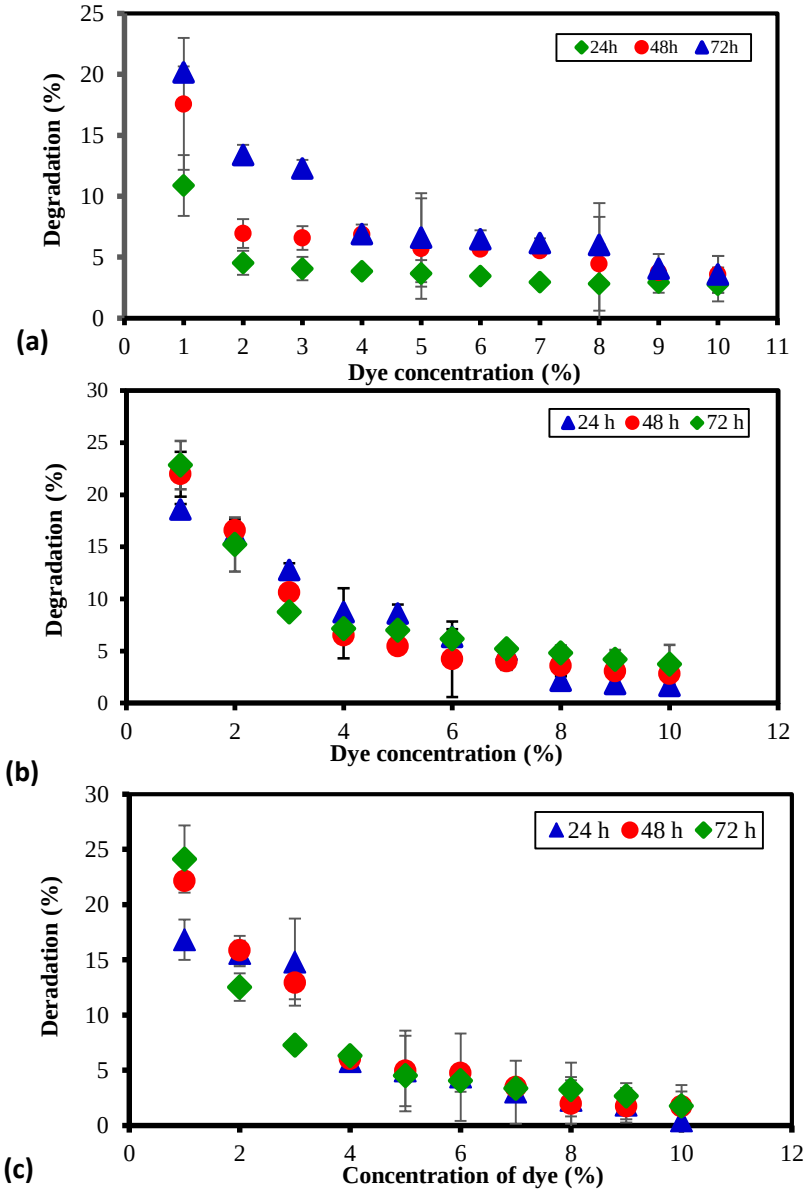

Fig. 4: Degradation of synthetic dyes by *Bacillus sp* sp. at different time intervals (24, 48 and 72h) (a) Gentian violet, (b) Bromo thymol Blue, (c) Safranine

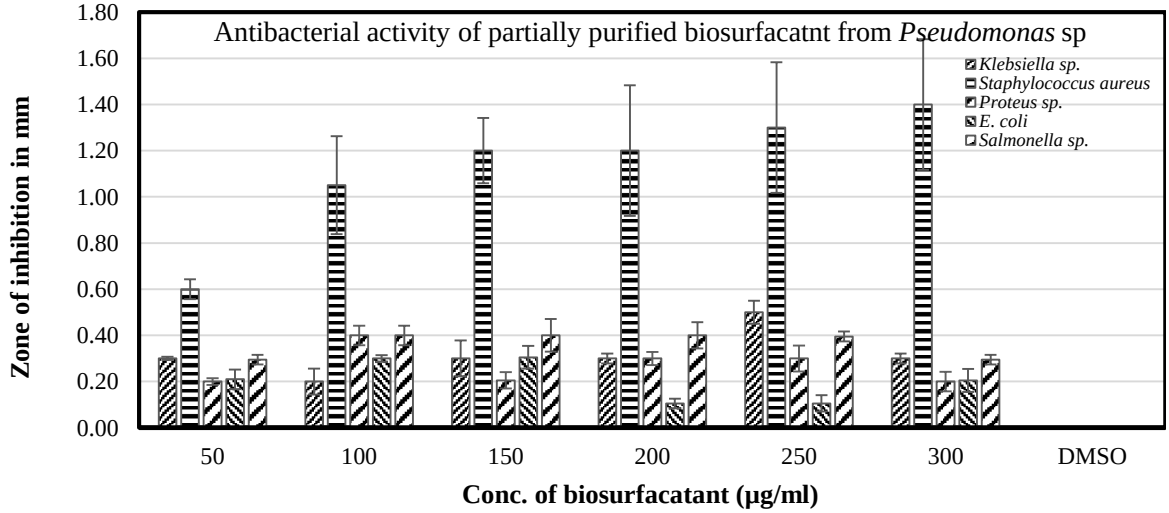

Fig. 5: Antibacterial activity of *Pseudomonas* sp. against selected bacterial pathogens

It was highly inhibited by 100 and 150 µl/ml showing inhibition zone of 0.30±0.01 and 0.31±0.05 mm. *Salmonella* sp. was inhibited by most of the concentrations of partially purified biosurfactant from 50 to 300 µl/ml as 0.30±0.02, 0.4±0.04, 0.4±0.07, 0.4±0.06, 0.4±0.02, 0.3±0.02mm respectively (Fig. 5). PPBS of *Pseudomonas* sp. was most effective against *Salmonella* sp. *Staphylococcus* followed by *Klebsiella*, *E. coli* and *Proteus* sp.

Thermophilic *Pseudomonas* sp. had efficient antimicrobial activity against fungal and bacterial pathogens. Similar kind of antimicrobial activity of biosurfactants has been studied by many researchers. Yamasaki et al[35] had studied the role of surfactin and rhamnolipids in inhibition of biofilm forming bacteria. Biosurfactants have structural similarity to chemical surfactants and they could penetrate the membrane system of the pathogens leading to leakage of the cellular contents which might be a reason for the antimicrobial action. The difference in the inhibition pattern of biosurfactant concentration in different species may be due to interaction of biosurfactant with the external cell wall components of the bacteria.

Conclusion

In conclusion, the present study explores the potential of halophilic bacteria *Bacillus* sp. and thermophilic bacteria *Pseudomonas* sp. as laccase and biosurfactant producers. *Bacillus* sp. efficiently degraded synthetic dyes, with safranine being highly susceptible. *Pseudomonas* sp., with its best growing temperature at 45°C, was effective against bacterial and fungal pathogens at a higher concentration of 250-300µl/ml. The study highlights the diverse applications of extremophiles in dye degradation and antimicrobial potential, making them useful biotechnological tools for industrial purposes and bioremediation therapeutics.

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