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ISOLATION, IDENTIFICATION, PRODUCTION AND EXTRACTION OF EXOPOLYSACCHARIDE (EPS) FROM SEA WATER OF TITHAL BEACH, VALSAD

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ABSTRACT

Exopolysaccharides (EPS) are high molecular weight polymers which are long chain sugar residues and secreted by microorganisms into the surrounding environment. EPS shows diverse applications such as in food formulations and pharmaceutical industry, etc. Five different bacteria were isolated on Zobell marine agar medium from sea water of Valsad district, Gujarat, India and screened by using YEM (Yeast Extract Mannitol) congo red agar medium. Bacteria were identified by their morphological and biochemical characterization. Analytical method such as phenol sulfuric acid method, biuret assay, specific viscosity and molecular weight of EPS producing bacteria were carried out. The EPS was characterized by thin layer chromatography and result shown the presents of L-rhamnose, L- GlcNAC, D-ribose and D-xylose combination. Isolates of EPS producing were also studied for biofilm production in test tubes and found the potential biofilm former bacteria. Among 5 isolates studied, EPS production was highiest in colony 2.

KEY WORDS: *Exopolysaccharide, Emulsifying activity, Viscosity, TLC, Biofilm*

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INTRODUCTION

In recent years, the interest in environmental friendly natural polysaccharide has increased due to their huge potential in varied sectors, possessing several advantages over synthetic or chemical polymers. The term exopolysaccharide (EPS) was proposed by Sutherland (1972) to describe high molecular weight carbohydrate polymers produced by many bacteria.[1] The matrix of exopolysaccharide can be defined as those materials which can be removed from microorganisms without disrupting the cells and without which the microorganism is still viable. The microbial EPS are having unique rheological properties due to their pseudoplastic behavior and capacity of forming viscous solutions at low concentrations.[2] EPS can be classified into two group based on their monomeric composition. Homopolysaccharide (e.g. pullulan, dextran and cellulose) are repetitive structure of single sugar, majorly consisting of pentose and hexose moiety. Heteropolysaccharide (xanthan, alginate and succinoglycans) consists of two or more sugar residues in repeating units.[3] Microbial EPS protects microbial cell in the natural environment against adverse condition such as a desiccation, antibiotic, osmotic stress or toxic compounds (e.g. toxic metal ions and ethanol), predation by protozoans, phagocytosis and phage attack.[4] Biosynthesis of EPS depends on environmental conditions and organisms used for production. Bacterial exopolysaccharide is to be synthesized intracellularly and exported to extracellular environment. The steps involved in biosynthesis of EPS include sugar transport into the cytoplasm, synthesis of sugar-1-phosphates, activation of and coupling of sugars and export of EPS.[5] Three general mechanisms for the production of EPS in bacteria are Wzx/Wzy-dependent pathway, ATP-binding cassette (ABC) transporter-dependent pathway and the synthase-dependent pathway.[6] EPSs have been important as stabilizer, emulsifier, antibacterial, antifungal, antiviral, anticarcinogenic and antioxidant agents.

MATERIAL AND METHODS

Sample collection

Sea water was collected from various sites of Tithal Beach, Valsad, Gujarat, India. Sea water was collected in a sterile plastic bottle and bought to the laboratory and stored in the refrigerator for further study. The physico-chemical properties of collected water sample were analyzed.

Isolation of EPS producing bacteria

Water samples were inoculated into sterile Zobell Marine broths and enrichment at 20˚C on shaker for 48 hours. From enrichment broth, serial dilution method was performed and 0.1 ml dilution taken on sterile Zobell agar plate for spreading. All plates were incubated at room temperature for 72 hours.[7]

Screening of EPS producing bacteria

YEM (Yeast Extract Mannitol) congo red agar plates were used for further confirmation and screening of EPS producing bacteria. Selected isolated colonies were streaked on YEM congo red agar plates and incubated at room temperature for 72 hours. Black or pink color mucoid colonies were selected and stored at 4˚C on Zobell agar slant.

Morphological and biochemical characterization

Morphological characterization of selected isolates was done by Gram staining and capsule staining method. Motility of the selected isolates was checked by hanging drop method. Biochemical characterization included Catalase test, Oxidase test, Indole test, Methyl Red test, Voges Proskauer test, Citrate Utilization test, Hydrogen sulphide Production test, Urea Hydrolysis test and carbohydrate fermentation of various sugars such as glucose, fructose and lactose.

EPS Production and Extraction

The isolates were inoculated into the sterile Zobell marine broth use as growth medium and incubated at 27-28˚C on shaker at 90 rpm for 72 hours in BOD incubator. For the production of EPS, growth medium was transferred into the production medium consists of Glucose (5g), Peptone (5g), Yeast extract (3g), KH_2PO_4 (3g), $MgSO_4$ (0.2g) and incubated at 27-28˚C on shaker at 90 rpm for 72 hours.[8] After incubation, broth was centrifuged at 10,000 rpm for 15 minutes. Supernatant was collected in sterile test tube and added equal volume of ethanol into it and kept at 4˚C for overnight. On the next day, precipitated material was collected by centrifugation at 10,000 rpm for 15 minutes. Supernatant was discarded and the pellets were dried in hot air oven.

Analytical method

The amount of carbohydrate was measured by phenol sulphuric acid method. To 1 ml of sample, 1 ml 5% (w/v) phenol was added followed by 5 ml concentrated sulphuric acid. The sample tubes were kept in ice while adding sulphuric acid. The mixture was incubated at RT for 20 min and the absorbance read at 490 nm.[9] The amount of protein present in sample was determined by Biuret assay. To 0.5 ml of sample, 1 ml of biuret reagent was added and mixture was incubated at RT for 20 min and the absorbance read at 540 nm.[10] The specific viscosity of broth was measured with Ostwald viscometer.[11] On the basis of above results the molecular weight of EPS was calculated using the following formula:

$$M_w = 0.9\ (\eta_{sp})\ \text{x}\ 10^6$$

Where, M_w =the molecular weight of EPS, η_{sp} = specific viscosity of EPS solution.[9]

Emulsifying activity

The emulsifying activity of EPS was described by Nielsen and Jahn.[12] 0.05 gm EPS was dissolved in 0.5 ml deionized water. The samples were heated at 100 ˚C for 20 min and allowed to cool at room temperature. The total volume 2ml was adjusted with phosphate buffer saline having pH -7.0. The sample mixtures were vortexed for 1 min after the addition of Tween 80 (hydrophobic substrate). The samples were incubated at 37˚C with shaking speed of 90 rpm. Before and after incubation vortexing the samples (A_o) and read the absorbance at 545 nm. The decrease in absorbance was recorded after the incubation at 37˚C with shaking for 30 and 60 min (A_t). The control was run without EPS and with 2 ml PBS. The emulsification activity was expressed as percentage retention of emulsion during incubation for time *t:* A_t/A_0 x 100.[13]

Screening method for biofilm producing isolates

Tube method was used for screening of biofilm. Nutrient broth medium with 1% glucose was prepared and inoculated with isolates. The inoculated tubes were incubated for 48 hrs at 37˚C on shaker. After 48 hrs, tubes were decanted and washed with PSB (phosphate saline buffer) and allowed it to dry. The dried tubes were stained with 0.5 % crystal violet solution and excess stain was removed by using distilled water. The tubes were dried in an inverted position and observed for biofilm formation.[8]

Statistical analysis of data

The data obtained were analyzed by using Microsoft Office Excel (Version 8.1). The data were presented as mean ± standard deviation (SD). Probability value (P) of less than 0.05 was considered statistically significant.

RESULTS

Many different organisms are present in marine water. But only few organisms have the capacity to produce exopolysaccharide (EPS). Sea water samples were collected from diferent sites of Tithal beach, Valsad, Gujarat, India. Table no 1 indicates the physiochemical characteristics of sea water. Bacteria were isolated by using standard plate count method. Serially diluted samples were spread on sterile zobell agar plates. These plates were incubated up to 72 hours at room temperature. Among all different colonies mucoid colonies were selected and further screened through YEM congo red agar plates. The isolates were initially differentiated on the basis of cultural and cellular morphological characteristics shown in table no 2.

Table 1
Physiochemical analysis of water samples

Sample No.	pH	Temperature	TSS	TDS	Hardness
1	7.0±0.2	33±2.0˚C	4500±300 mg/L	12,900± 100 mg/L	1433±33 mg/L
2	7.2±0.1	30±2.0˚C	4000± 100 mg/L	13100± 100 mg/L	1370±30 mg/L
3	7.0±0.1	30±1.0˚C	4250± 100 mg/L	12500± 100 mg/L	1420± 20 mg/L
4	7.4±0.2	32±1.0˚C	5150±50 mg/L	13250± 50 mg/L	1443± 10 mg/L
5	7.2±0.2	28±4.0˚C	4850± 150 mg/L	12500± 50 mg/L	1390± 10 mg/L

Note: 'n' value= 3.0 and 'p' value=≤ 0.05

Table 2
Morphological Characters

Characters	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5
Size	Large	Small	Large	Small	Large
Shape	Round	Round	Irregular	Irregular	Irregular
Surface	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Edge	Entire	Entire	Undulate	Undulate	Undulate
Elevation	Convex	Convex	Convex	Flat	Convex
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	Pink	Pink	Pink	Black	Black

Microscopic and biochemical characteristics of isolates were shown in table 3, 4 and 5.

Table 3
Microscopic Characters

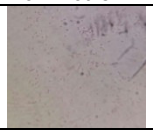
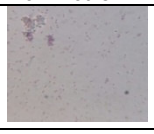
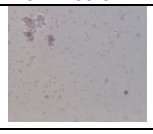
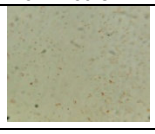
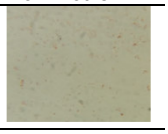
Characters	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5
Gram staining	Negative	Negative	Negative	Negative	Negative
Capsule staining	Capsulated	Capsulated	Capsulated	Capsulated	Capsulated
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Observation					

Table 4
Microscopic observation

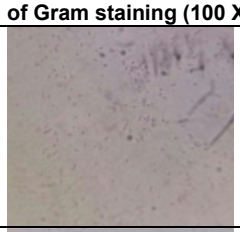
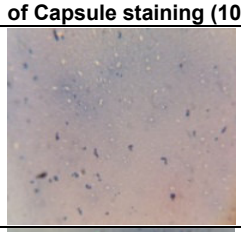
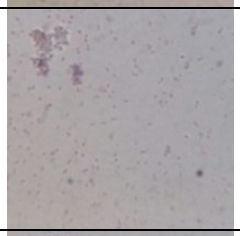
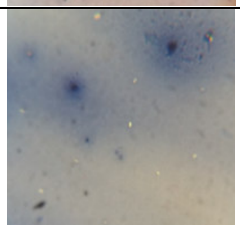
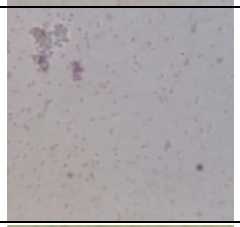
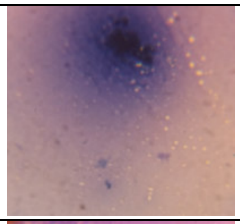
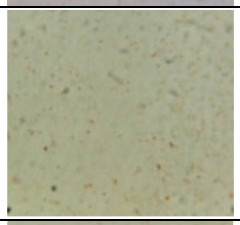
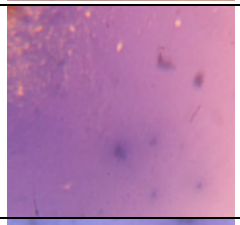
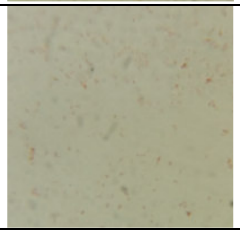
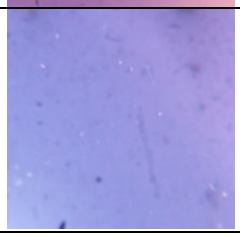
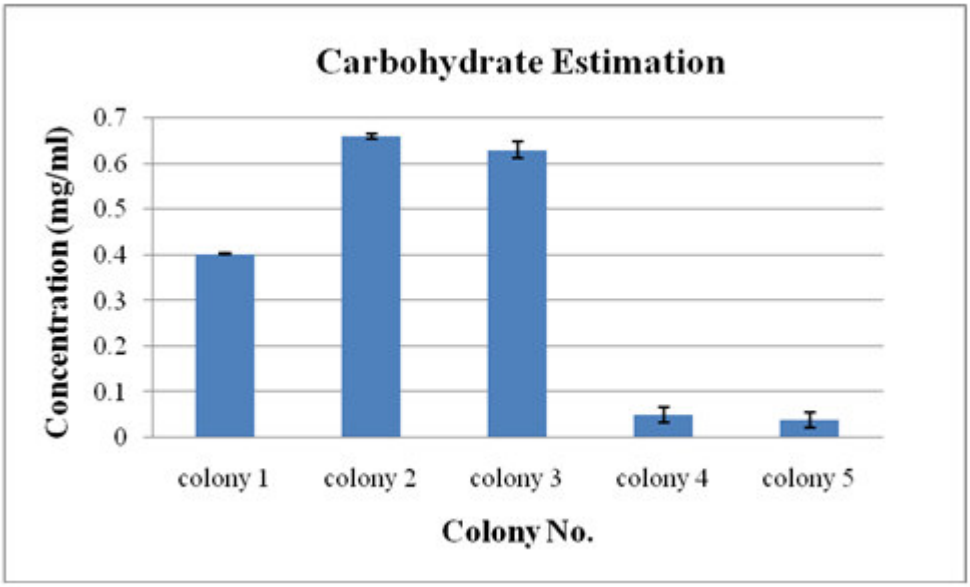
Colony No.	Microscopic observation of Gram staining (100 X)	Microscopic observation of Capsule staining (100 X)
1		
2		
3		
4		
5		

Table 5
Biochemical Characterization

Tests	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5
Indole test	Negative	Negative	Negative	Negative	Negative
Methyl Red test	Negative	Negative	Negative	Negative	Negative
Voges Proskauer test	Positive	Positive	Positive	Positive	Positive
Citrate Utilization test	Positive	Positive	Positive	Positive	Negative
H_2S Production test	Positive	Negative	Negative	Negative	Negative
Urea Hydrolysis test	Negative	Negative	Negative	Negative	Negative
Carbohydrate Fermentation:					
a. Lactose	Positive	Positive	Positive	Negative	Negative
b. Glucose	Positive	Positive	Positive	Negative	Negative
c. Fructose	Positive	Positive	Positive	Negative	Negative

Carbohydrate estimation

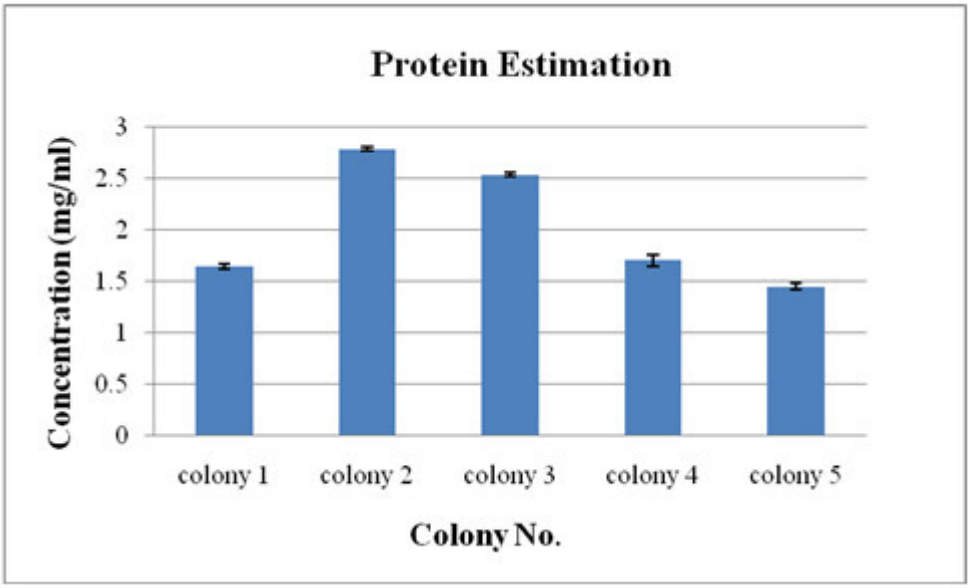
Graph 1
Phenol sulphuric acid analysis for total carbohydrate estimation



Carbohydrate content in all colonies were different. Among the 5 isolates, colony 2 represents high amount of carbohydrate.
In above, graph 1 shows that colony 2 consists of high amount of carbohydrate i.e.0.66±0.01 mg/ ml compare to colony 3 and colony 1. Colony 4 and colony 5 represent low amount of carbohydrate which was 0.05±0.03 and 0.04±0.03 mg/ml, respectively.

Protein estimation

Graph 2
Protein estimation by Biuret assay



The same way, graph 2 also shows that colony 2 (2.78±0.03 mg/ml) consists of high amount of protein content compare to colony 3 (2.53±0.03 mg/ml). Colony 1 and colony 4 consist of 1.64±0.04 and 1.7±0.1 mg/ ml protein, respectively and the lowest amount protein presents in colony 5 (1.45±0.05 mg/ml).

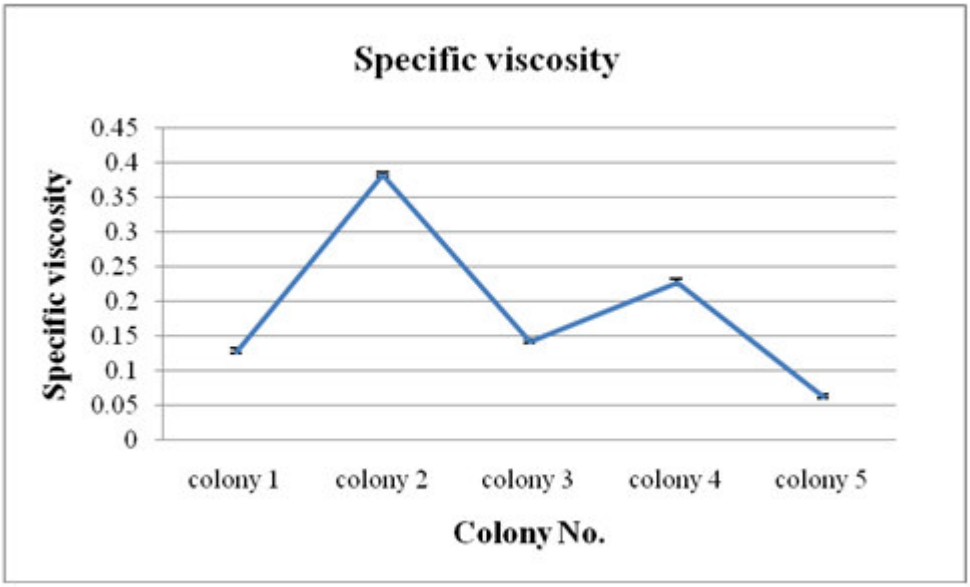
Graph 2
Represents that colony 2 contains high amount of protein content compare to other isolates.

Viscosity measurement and molecular weight analysis

The molecular weight of the polymer is measured by using viscometer. Graph 3 and 4 indicated that as molecular weight of EPS increased, the specific viscosity also increased.

Graph 3

Specific viscosity of EPS



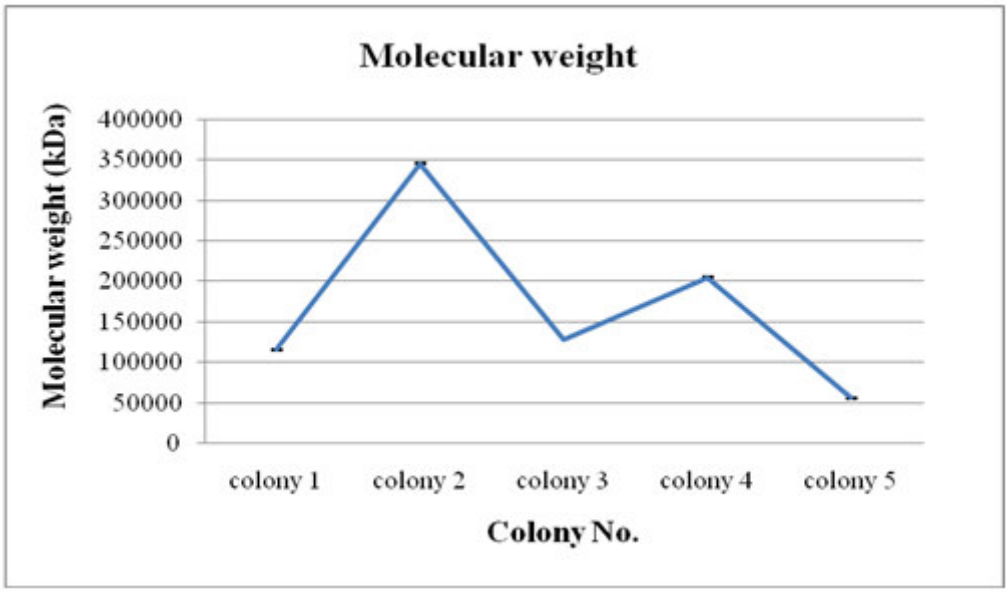
Graph 3 represents that colony 2 shows high specific viscosity compare to other isolates, while colony 5 shows the lowest amount of specific viscosity.

Graph 3

Specific viscosity of 5 isolates

Graph 4

Molecular weight of EPS



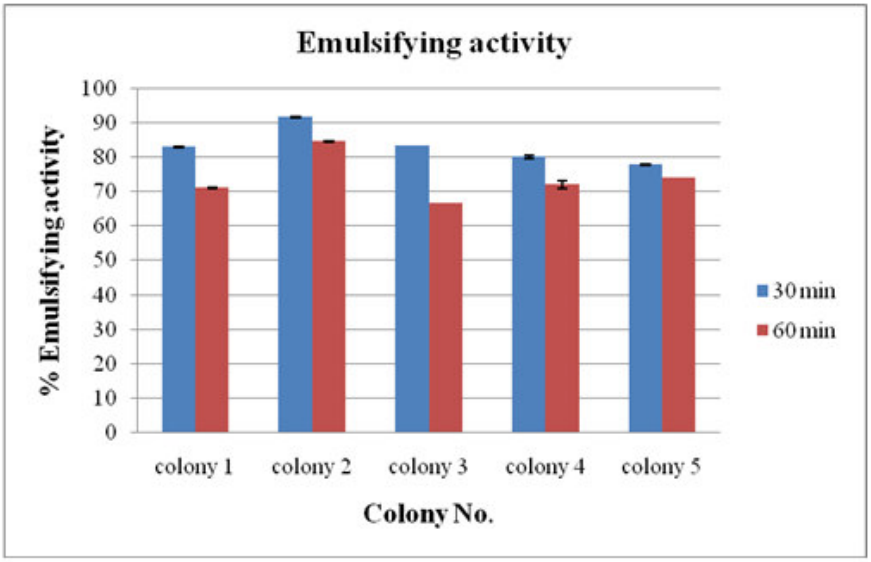
Graph 4 represents that colony 2 shows high molecular weight i.e 345600 kDa compare to other isolates, while colony 5 shows low molecular weight i.e 55800 kDa.

Graph 4

Molecular weight of EPS of isolates

Emulsifying activity

Graph 5
Analysis of Emulsifying Activity



Note: 'n' value= 3.0 and 'p' value=≤ 0.05

Graph 5
Represents that colony 2 shows the highest emulsifying activity compare to other isolates.

The emulsification activity of the EPS is determined by its strength in retaining the emulsion breaks rapidly within an initial incubation of 30 min. The absorbance reading after 30 and 60 min gave a fairly good indication of stability of EPS emulsion. Among the 5 isolates, colony 2 exhibited highest emulsifying activity as represented in in graph 5.The tube method is more suitable for detecting biofilm producers. Biofilm formation was considered positive when violet color appears in the tube. This biofilm line was stained with 0.1% crystal violet solution. All 5 isolates were capable to produce biofilm that shown in figure 1.

Figure 1
Screening of biofilm producing bacteria by tube method

DISCUSSION

Exopolysaccharides are produced as the normal metabolic products in bacteria. EPS generated within the cells helps in adhesion to substratum, maintaining the cellular structure, playing a protective role and preventing from dessication. EPS are used commercially as food additives, stabilizers, binders, suspending agents, cosmetics and in drug delivery systems. It was observed that viscosity of the culture broth increased as the growth of cells increased and remained stagnant after an extent. Also, molecular weight increased with increase in viscosity.[7] The functional groups in the molecular chain of the polymer are considered as important determinants of emulsifying activity. In our study, we used only Tween 80 as an

emulsifying agent because it is a nonionic surfactants, viscous and water soluble. Screening method for biofilm production was carried out, where 0.1 % crystal violet solution was used for possible biofilm detection. In our study, we found that all 5 isolates were capable to produce biofilm. Similar work has been done in recent past where two isolates had been identified as strong producers of biofilm.[8]

CONCLUSION

Five different isolates from marine water sites were screened for EPS producing activity. Out of five isolates, isolate No-2 produced higher amount of EPS. The isolate were studied and identified by morphological and biochemical test. The extracted EPS have functional properties like good emulsification activity and viscosity. Our research data showed that EPS producing bacteria also able to form biofilms. Further studies are needed to evaluate the potential of the biofilm exopolysaccharides for industrial application.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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