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Isolation and Screening Of Exopolysaccharide Producing Marine Bacteria And Evaluation Of Antibacterial Activity Against Human Pathogens

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Abstract:
Exopolysaccharides are long-chain polysaccharides containing branched, repeating units of sugars or sugar derivatives such as glucose, fructose, mannose and galactose etc, which are secreted into their surrounding environment during the bacterial growth .A total 160 bacteria were isolated on Zubell marine agar. The microorganisms were screened for their optimal EPS production. Out of 160 isolates,34%were screened on the basis of mucoidal colonies releasing gummy substances while growing on marine agar supplemented with glucose (3%). The exopolysaccharide was extracted from screened bacteria by ethanol precipitation method. Among them three isolates viz.AB4,MZ10 ,N8 gave more exopolysaccharide(9.2,7.7 and 9.4mg/l)was selected for further study. The bioactive potential of exopolysaccharide produced from AB4,MZ10,N8 was studied against MTCC pathogen viz. *S.aureus* ,*P.vulgariaus* ,*Ps.aeuroginosa*, *B.subtilis* ,*E.coli*.,*E.fecalis*, *S.typhi* and further study has been carried out by observing antimicrobial activity of bioactive EPS against human pathogens . Among them EPS extracted from AB4 ,MZ10 show 85.71 % inhibition against tested organisms. Outcome of this study will definitely contribute in the elucidation of antimicrobial components of (AB4) and further used as wound dressings.
Keywords :EPS ,Antimicrobial ,Ethanol, Precipitation .

INTRODUCTION :

Exopolysaccharides (EPSs) are high molecular weight, biodegradable polymers biosynthesized by a wide range of bacteria.(Vijayabaskar et al., 2011) EPS are metabolic products that accumulates on the cell surface of bacteria (Morgan et al.,1990).Marine bacterial exoploplysaccharide are fascinating industrial uses and bioactive compounds. Exoploysaccharides are generally composed of monosaccharide and some non carbohydrate substituent, like acetate, succinate, These EPS possess regular structures and thus have unique rheological properties and these molecules are highly pure.EPS could be acidic or basic in nature. Exopolysaccharide may be either homopolysaccharide or heteropolysaccharides. Of these some of the homopolysaccharides possess regular structures, but the dextrans and levans don't. With exception of bacterial alginate ,the heteropolysaccharide are composed of regular repeat units of 2-8 monosaccharides (Mayer et al .,1999).

In the natural environment, Exopolysaccharide (EPSs) is generally heteropolymeric (made of different monomeric units), non-sugar components like uronic acid, methyl esters, sulphates, pyruvates, proteins, nucleic acids and lipids. EPS also contain divalent metal cations that act as ionic bridges linking adjacent polysaccharide chain.(Shankar et al .,2014)Many bacterial cultures produce different types of EPS during its lifecycle. For example, most bacteria produce capsular form of EPS during the exponential growth phase and slime type EPS during the stationary growth phase (Wingender et al., 1999).

Biopolymers could either be intracellular or extracellular .The intracellular biopolymers are few and have very limited use; however ,the range of extracellular biopolymers are vast and may be grouped into four major

classes ;polysaccharide ,Inorganic polyanhydrides such as polyphosphate, polyesterand polyamides and have collectively termed as extracellular polymeric substances (Cerning .,1995)

In recent years, the increased demand for natural polymers or biopolymers for various industrial and biotechnological applications has led to a renewed interest in exopolysaccharides (EPS) production by microorganisms as soluble or insoluble polymers. Different types of polysaccharides produced by plants (cellulose, pectin and starch), algae (agar, alginate and carrageenan) and bacteria (alginate, dextran, gellan, pullulan and xanthan gum) are commonly used as food additives for their gelling, stabilizing or thickening properties. (Sutherland 1998).EPS produced by both prokaryotes (eubacteria and archaebacteria) and eukaryotes (phytoplankton, fungi, and algae).has a great deal of research interest (kumar et al .,2007).

The aim of this study was to screen high yield EPS producing marine bacteria and analyze the EPS Production and evaluate its antimicrobial activity.

MTERIALS AND METHODS

Collection of Marine Samples

Marine water and sediment samples were collected from twelve different coastal areas of India, name as Devka Beach Daman,Calangute beach Goa, Jhu chaupathy Mumbai, Gopalpur Orissa, Nagoba Beach, Alibagh beach ,Alibagh Amalapuram beach,Girgao chaupati Mumbai, Marud Zinzira Beach, Thirumullavaram beach Kerala, Elliot's beach Chennai, Angellar beach. Samples were collected from 515 cm depth and stored in dark during transport to laboratory for further study.

Enrichment and Isolation of Marine bacteria

For the enrichment of microorganisms, one gram/ml of each of twelve marine water and their sediment were inoculated in zubell marine broth aseptically and incubated at 30°C for 3 days in rotary shaker incubator. One gm/ml from each of the respective samples was mixed with 9 ml sterile Saline solution (0.85% NaCl), and the suspension was then serially diluted up to 10^{-8} dilution. From the diluted suspension 0.1ml from 10^{-4} onward was transfer onto the sterile sea salt medium plates by performing four quadrants method. This plate then incubated at 30±2°C for 2 days . After incubation the plates were observed for the presence of colonies. Isolated colonies were restreaked on same media to get pure culture.

Screening of exopolysaccharide producing marine bacteria

Primary Screening: based on mucoidal nature

Out of 160 isolates, potential EPS producing Bacteria were isolated on modified sea salt medium supplemented with 3% glucose and were selected to obtain pure cultures. Primary screening is carried out based on mucoidal colonies secreting gummy substances and glistening appearance which was confirmed by string test and Congo red agar plate method.

a.String test : Exopolysaccharide production was confirmed by the string test (Fang et al.,2004) by formation of a string (>5 mm) upon lifting of the loop indicates positive result.

b. Congo red agar plate method: The isolates were streaked onto Zobell marine agar medium supplement with 0.08% Congo red. The positive result determined by black, smooth, humid with mucoid type colony (Freeman et al., 1989). Selected screened isolates were maintained at 4° C and subculture periodically for consequent experiments.

Secondary screening :based on EPS Yield

The secondary screening was done on the basis of yield of exopolysaccharide by ethanol precipitation method. The Bacterial EPS quantification done by following method. After 72 hours of incubation, yeast extract glucose medium were centrifuged at 10000 rpm for 20 min. The EPS was then precipitated from the

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supernatant by addition of two volume amount of ethanol. The mixture were agitated with addition of ethanol to prevent local high concentration of the precipitate and left night at 4° C and next day it was centrifuged at 10000 rpm for 20 mins.

After centrifugation the precipitate was collected and dried at 60 °C.

a. **Qualitative test of extracted EPS :** EPS was confirmed by Anthrone Test.

b. **Quantitative test of extracted EPS:** on the basis of Yield of EPS which later on confirmed by Phenol sulfuric acid method (Dubios method).

Tertiary screening: based on antibacterial activity of EPS:

Tertiary screening was done by evaluating antibacterial potential of secondary screened isolates. Preliminary antibacterial easy was performed using *Escherichia. coli s.aureus ,,salmonella typhi* and *staphylococcus aureus, proteus vulgarius*, grown overnight in MH broth before bioassay. The antibacterial assay was carried out on 2.5% Mullter Hilton(MH) agar(Himedia).Agar plates were prepared by pouring 20ml of MH medium into each sterile Petriplate. At the time of bioassay,each test organism was suspended in 0.85%Nacl and turbidity adjusted to 10^{-7}-10^{-8} CFU ml, corresponding to 0.5 mcfarland standards according to NCCLS 1997 guidelines (now CLSi).Each MH plates were poured with soft agar(1%) containing test bacteria aseptically. Well was punched aseptically with cork borar and than Crude EPS (supernatant)after 3 days of incubation was used as a sample to determine the antibacterial activity.The plates were left for about 20 min in freeze for diffusion purpose, and thereafter incubated at 37 °C for 24 h.The diameters of inhibitions were measured in mm. All the experiments were performed in duplicate.

Identification of selected isolates: The morphological and biochemical test (according to Bergey's manual) were used to identify the potential bacterial isolates i.e AB4 MZ10,N8. Standard protocol was used for performing each test. Results of morphological analysis of selected three isolate are shown in the table No. two and three respectively.

Result and discussion

Sample collection ,enrichment and isolation of marine bacteria

NO.	MARINE SAMPLES Sampling site	NO.OF ISOLATES		EXOPOLYSACCHARIDE ISOLATES
		Water	Sediment	
1.	Amlapuram Beach	07	-	03
2.	Girgao chaupati, Mumbai	11	-	02
3.	Devka beach Daman	08	05	02
4.	Calangute beach, Goa	12	-	02
5.	Thirumullavaram beach, Kerala	11	09	05
6.	Jhu chaupti,Mumbai	12	-	03
7.	Gpalpur, Orrisa	06	-	02
8.	Nagoba beach	15	13	10
9.	Alibaug beach Alibag	10	04	01
10.	Elliots Beach, Chennai	07	-	02
11.	Anjerle beach	08	-	02
12.	Marud Zinzira beach	18	04	05
	Total	125	35	39

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Twelve marine water samples and its sediment were used for isolation of marine bacteria.Total 160 bacteria were found in these samples.Details of sampling site,sample type (water and sediment) and no of isolates obtained were given in table number one.

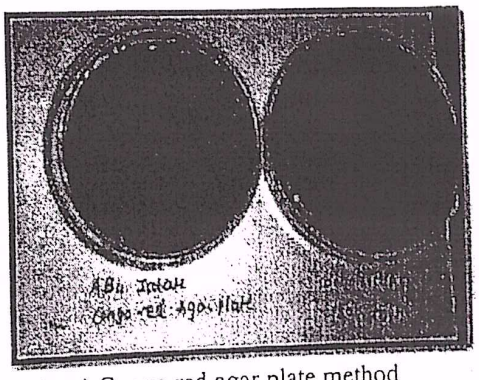
Table1: Results of Isolation of marine bacteria and primary screening for exopolysaccharide production

Primary Screening :

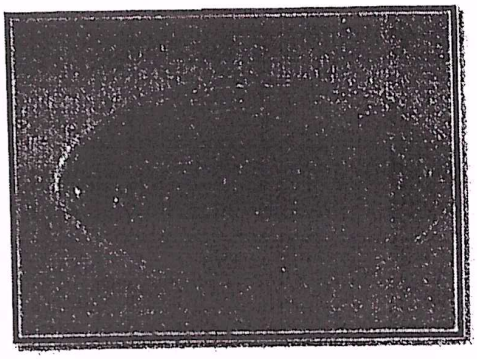
Amongst isolated 160 marine bacteria only 26% isolates had shown mucoidal nature .All the selected isolates were screened for exoplysaccharide production by string test and congo red agar plate .Among 26%isoltes AB4,MZ10 N8 show both the test positive .All the 03 isolate shows mucoid colonial characteristics. The mucoid colonies characteristics serve as the selection criteria for exopolysachharide producing bacteria (Fusconi and Godinho, 2002). Among these 03 isolates, AB4 show mucoidal and glistening type characteristics by lifting a colony by wire loop, it forms a string of length more than >5 mm. The results are in accordance with the method developed by past researchers for selection of exopolysaccharide producing bacteria (Vescovo *et al*, 1989). Isolate AB4 shows mucoid, glistening type, smooth, black colour growth on congo red gar plate indicative of positive test for exopolysaccharide production. The results are in accordance with Freeman *et al*., 1989. The results of primary screening represented in table no.2 and fig no.1

Table 2: Results of screening for exopolysaccharide production for AB4 ,MZ10,N8

Primary screening	Bacterial isolates		
	AB4	MZ10	N8
Colony characteristics	Mucoid	Mucoid	Mucoid
String test (mm)	>5mm	5mm	5mm
Congo red agar plate test	+++	++	+



a) Congo red agar plate method



b) Mucoid colony

Fig.1

Secondary Screening :

Qualitative

EPS production was confirmed qualitatively by Anthrone test showing bluishgreen colour (fig no. 2)

Quantitative

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EPS yield ranged from as low as 1.2mg/ml to as high as 10mg/ml .From isolate tested eleven isolates gave EPS Yield less than 3mg,ml,Fourteen and eleven isolates gave EPS yield between 3-6 mg/ml and 6-9 mg/ml respectively while 5 isolates gave EPS Yield above 9 mg/l.

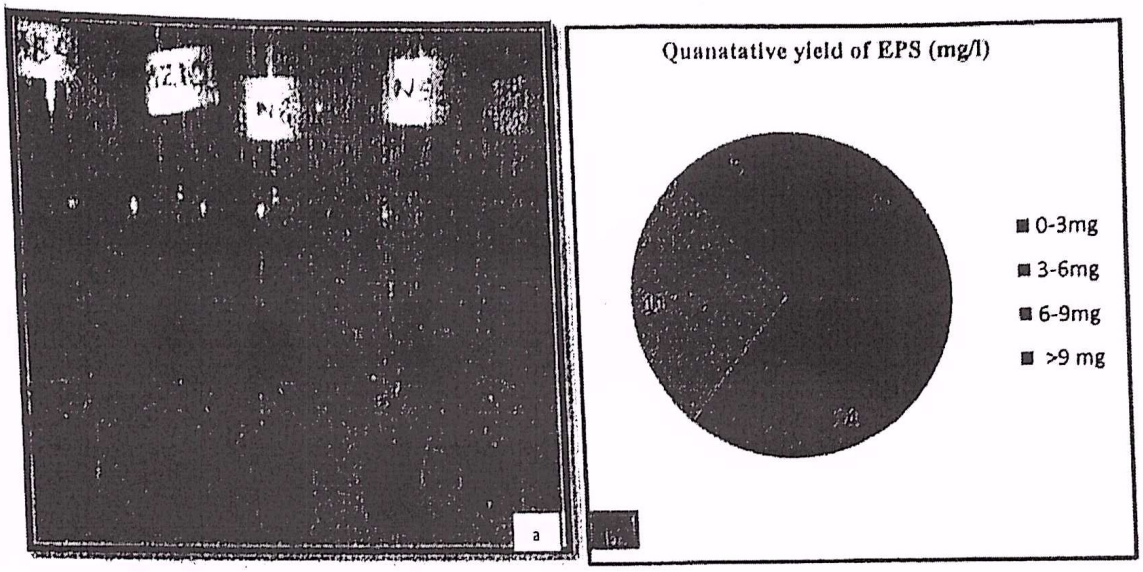

Figure 2 Secondary Screening of Exopolysaccharide (a) Anthrone Test, (b) Quantitative Yield of EPS

Tertiary Screening

The strains of sp. AB4 and MZ10 has shown 100% antibacterial activity. The highest spectrum of inhibition (80%) was observed towards species S.aureus . The data is presented in table number 3 and figure number 3. Similarly Nisha.P et al., (2013) has reported that isolated strain of is exopolysacharide in nature as it has shown antibacterial activity towards *Escherichia coli, Klebsiella spp., Salmonellatyphi* and *Staphylococcus sp.*

Table No.3. Antibacterial activity for Screening of Exopolysaccharides

	Zone of Inhibition (mm)								
Test organism	*AB4*	*MZ10*	*N8*	*NS3*	*KE3*	*MZ7*	*AL2*	*J7*	*%Inhibition*
E.coli	12	7	-	-	-	-	-	7	25
S.aureus	13	4	4	4	9	7	5	4	100
P.vulgarius	14	4	10	-	5	3	-	4	75
S.typhi	09	2	4	2	-	-	2	-	62.5
B.subtilis	05	3	13	-	-	-	-	-	37.5
E.fecalius	10	6	-	-	-	-	-	-	25
Ps.aeroginosa	-	-	14	5	-	-	-	-	25
%Inhibition	85.71	85.71	71.42	42.85	28.57	28.57	28.57	42.85	

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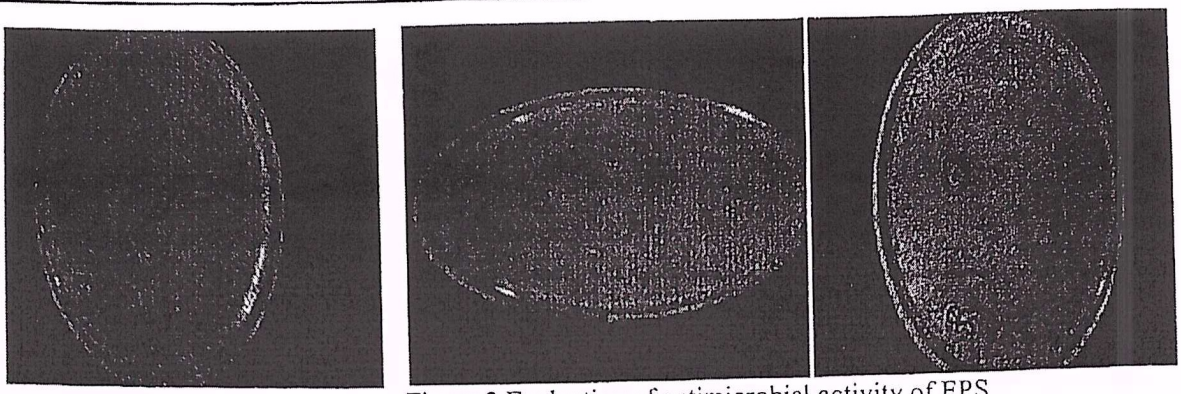
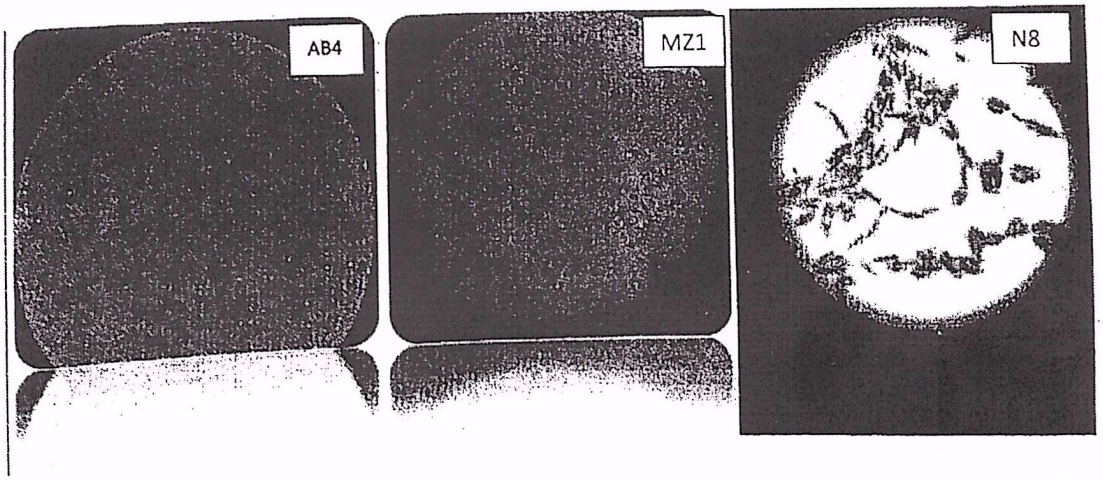


Figure 3 Evaluation of antimicrobial activity of EPS (AB4,MZ10,N8)against,*P.vulgariaus* , *S.aureus*, ,*E.fecalis*.

Identification of Marine isolates : Morphological characterization helps in partial identification of microorganism. Morphological characteristics of AB4,MZ10,N8 isolates were shown as Table 4.

Table No. 4 Morphological characterization of Isolates

Sr.No.	Colony characters	Observations		
		AB4	MZ10	N8
01.	Size	5mm	7mm	4mm
02.	Shape	Round	Round	Round
03.	Color	Blackish green	Offwhite	Offwhite
04.	Margin	Entire	Entire	Entire
05.	Surface	Smooth	Smooth	Smooth
06.	Elevation	Convex	Concave	Concave
07.	Consistency	Mucoid	Mucoid	Mucoid
08.	Opacity	Opeque	Opeque	Opeque
09.	Grams Nature	Gram Negative	Gram Negative	Gram positive
10.	Morphology	Short rods	Long Rods	Rods in chain
11.	Motility	Motile	Motile	Non motile



CONCLUSION:

A potential Haloalkalotolerent AB4 marine bacterium was selected having ability to produce 9.2 mg /l exopolysaccharide by ethanol precipitation method and showing better antimicrobial spectrum .Screened strain of AB4 will be further optimized to produce high yield of exopolysaccharide and its application will be studied.

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