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ISOLATION AND CHARACTERIZATION OF EXOPOLYSACCHARIDE PRODUCED BY TRIBUTYLTIN CHLORIDE DEGRADING MARINE BACTERIUM *ALCALIGENES* SP. 2-6 STRAIN S3 FROM GOA, INDIA

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Isolation and Characterization of Exopolysaccharide Produced by Tributyltin Chloride Degrading Marine Bacterium *Alcaligenes* sp. 2-6 Strain S3 from Goa, India

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Abstract – The aim of the study was to isolate and characterize the Exopolysaccharide (EPS) produced by Tributyltin Chloride (TBTC) degrading marine bacterial isolate under TBTC stress from Goa, India. A highly potent TBTC degrading marine bacterial isolate was isolated and identified to be Alcaligenes sp. 2-6 strain S3 using morphological, biochemical and molecular characteristics (16S rDNA Sequence & taxonomic phenogram). The isolate grew and produced 98.3µg/mL EPS in Zobell marine broth (ZMB) and 46.8µg/mL in Mineral salts medium (MSM) supplemented with 5mM TBTC. The exopolysaccharide was extracted and chemically analysed following standard procedures, which revealed its acidic nature and the presence of neutral sugars, proteins, uronic acids and methyl pentoses. The Fourier transformed infrared (FTIR) spectroscopic scan revealed the presence of functional groups as hydroxyl, carboxylic and amides, corresponding to acidic hetero-polymeric polysaccharide, possessing good emulsification activity. A highly potent TBTC degrading marine bacterium was isolated and identified as Alcaligenes sp. 2-6 strain S3, which produced more EPS in ZMB than in MSM under TBTC stress. The EPS was chemically analysed and the FTIR spectrum revealed it to be an acidic heteropolysaccharide, with good emusifying activity. This is the first report of EPS production and characterization by TBTC degrading marine bacterium Alcaligenes sp. 2-6 strain S3, which significantly contributes towards an understanding of the chemical composition and applications of the EPS in environmental bioremediation of TBTC contaminated sites of Goa, in India.

Key Words: Alcaligenes sp. 2-6, Exopolysaccharides, Emulsification, Bioremediation, Hetero-Polysaccharide.

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INTRODUCATION

Exopolysaccharides are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeated units within the polymer. Bacterial exopolymers are important in the interaction between bacteria and their environment and are chemically diverse. The major organic fractions of the EPS are carbohydrates, proteins and humic substances (Nielsen and Jahn 1999). A wide range of chemical structures of homopolymeric or heteropolymeric type, made up of sugar and non-sugar components, is possible and the range of monosaccharide combinations, together with non-carbohydrate constituents and varied linkage types, makes the exopolymer an excellent emulsifying agent and attributes diversity in bacteria (Keene and Lindberg 1983). The bacterial exopolymers are usually acidic heteropolysaccharides possessing the functional groups (e.g. hydroxyl, carboxyl and phosphoric acid) associated with EPS, which exhibits high affinity towards certain metal ions (Mittleman and Geesey 1985). Many interesting physical and chemical properties of microbial exopolysacharides, have found a wide range of applications in the field e.g., stabilizing, suspending, thickening, gelling, coagulating, film-forming, and water retention capability, e.g., in detergents, textiles, adhesives, paper, paint, food and beverage industries, oil recovery, mining industry and petroleum industries (**Sutherland 1996; 1998).** A wide range of bacteria from various environmental habitats are known to produce complex and diverse exopolysaccharides (EPS) occurring as capsular polysaccharides intensively associated with the cell surface or as slime polysaccharides, loosely associated with the cell (Uhlinger and White 1983). In recent years there has been a growing interest in the isolation and characterization of microbial EPS owing to their importance in adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds, and protection against osmotic shock

(Decho 1990; Hoagland et al. 1993). Despite their importance, very few studies have been carried out on chemical characterization of EPS by marine fouling bacteria (Rodrigues and Bhosle 1991; Majumdar, D'Souza, and Bhosle, 1999; Muralidharan and Jayachandran, 2003). Keeping in view of the commercial importance of the exopolysaccharides, the present investigation deals with the isolation, chemical and physical characterization of extracellular polysaccharides produced from the highly potent TBTC resistant marine bacterium *Alcaligenes* sp. 2-6 strain S3.

MATERIALS AND METHODS

Isolation and Culturing of bacteria for EPS production

TBTC resistant marine bacterium was isolated from the sediments of Western India Shipyard Ltd., (WISL) in Murmugoa port trust (MPT) harbor, Vasco-da-gama, in Goa. The bacterial isolate was obtained by serial dilution plating on ZMA with TBTC incubated at 28±0^{o}C for 24 h. A total of 45 distinct colonies were isolated and the exopolymer producing bacterial strains were screened for their ability to produce exopolymer based on colony morphology (mucoid phenotypes). One among the screened isolates grew well at 5mM TBTC, formed a viscous exopolymer after the ice cold ethanol precipitation was identified as *Alcaligenes* sp. 2-6 strain S3 according to *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984). and 16S rDNA sequence [Courtesy: Prof. Satoru Suzuki and Dr. Kitamura from CMES, Ehime University, Matsuyama, Japan]. *Alcaligenes* sp. 2-6 strain S3 was grown in Zobell marine broth and optimized mineral salts medium supplemented with NaCl to a final concentration of 1.0 % (w/v) and 1mM, 2mM and 5mM TBTC in 500 mL Erlenmeyer flasks on a rotary shaker at 28±2 ^{o}C for 48hrs and pH adjusted to 7.0 for EPS production. 250 mL of the medium was dispensed in 500 mL Erlenmeyer flasks and inoculated with 5 % (v/v) of an overnight culture at room temperature (28±2 °C) on a rotary shaker at 160 rpm. The sub samples of 5 mL were drawn at regular intervals for measuring bacterial growth (A_{600} nm) and EPS production.

Extraction and purification of Exopolysaccharide

The 200 mL of bacterial culture was centrifuged at 12,000 rpm for 20 min at 4 ^{o}C. The cell pellets were freeze-dried and weighed. The supernatants were pressure-filtered through cellulose nitrate filters with the following pore sizes: 0.8, 0.45 and 0.25 µm (Millipore filters). EPS were precipitated from the final filtrate after the addition of three volumes of cold ethanol and the solution was chilled to 4 ^{o}C overnight. The resulting precipitate was recovered by vacuum filtration through a scintered glass apparatus. An additional 100 mL cold ethanol was added to the filtrate and the solution was placed at –20 ^{o}C overnight. The precipitate was recovered, washed with 70–100 % ethanol–water mixtures. After washing with ethanol, the EPS was pooled and dried in a desiccator and stored at room temperature. To remove excess salts, the EPS was redissolved in distilled water and dialyzed at 4^{o}C for 24 hrs against distilled water. Excess water was removed under vacuum before lyophilization. Exopolysaccharide extracted was lyophilized using a Labonco lyophilizer (Kansas City, MO, U.S.A.) at 3000 psi. The lyophilized exopolysaccharide was stored at room temperature for chemical and physical analysis.

Quantitative & Chemical analysis, Emulsifying activity and FTIR scan of EPS

The lyophilized exopolysaccharide was hydrolyzed with 2 N HCl for 2 hrs at 100 °C in ampoules flushed with N_2 before sealing. After hydrolysis, the solution was evaporated under reduced pressure at 40^{o}C (Read and Costerton 1987). Wet weight and dry weight of the exopolysaccharide was determined. The lyophilized sample was used for further chemical and physical characterization. The lyophilized exopolymer was dissolved in ultrapure milli-Q water (0.1 mg/mL) for chemical analysis. Exopolymers were assayed for total carbohydrate content using the phenol sulphuric acid assay with glucose as standard (Dubois et al.1956). Uronic acids were assayed using the method described by Dische (1962) with glucuronic acid as standard. Methyl pentoses were assayed using Cysteine Hydrogen chloride method with arabinose as standard (Dische and Shettles 1948). Sulphated sugars were determined by measuring sulphates according to the method of Terho and Hartiala (1971) after hydrolysis of the polymer, K_2SO_4 was used as standard. The protein content of the EPS was determined according to Lowry *et al*. (1951), BSA was taken as standard (Table 1.).

The emulsifying activity of EPS was assayed by modifying the method described by Rosenberg *et al.* (1979). Lyophilized EPS (0·5 mg) was dissolved in 0·5 mL deionized water by heating at 100 °C for about 15–20 min and allowed to cool to room temperature (28±2 °C). The volume was then made up to 2 mL using phosphate-buffered saline (PBS). The sample was vortexed for 1 min after the addition of 0·5 mL hexadecane. The absorbance was read at 540 nm immediately before and after vortexing (A_0). The fall in absorbance was recorded after incubation at room temperature for 30 and 60 min (A_t). A control was run simultaneously with 2 mL phosphate buffer saline (PBS) and 0·5 mL hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for time *t:* A_t/A_0 x100.

The major structural groups of the purified EPS was detected using Fourier transformed infrared (FTIR) spectroscopy (Abu et al. 1991). Pellets for infrared analysis was obtained by grinding a mixture of 2 mg exopolysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16-mm diameter mold. The Fourier transformed-infrared (FTIR) spectra was

recorded on a Bruker Vector 22 instrument (Bruker SA, Wissembourg, France in the region of 4000–400 cm^{-1}) region and spectra traced with a Hewlett Packard plotter.

RESULTS

The bacterial isolate was identified to be *Alcaligenes* sp.2-6 Strain S3 (Gen Bank Accn. No. EU 428755) by standard morphological, physiological, biochemical tests and molecular characteristics (16S rDNA Sequence & taxonomic phenogram) (Fig.1). *Alcaligenes* sp.2-6 Strain S3 showed variation in growth pattern and EPS production when grown in ZMB and MSM supplemented with 1 mM, 2 mM, and 5 mM TBTC respectively. The isolate showed better growth in nutrient rich medium i.e., ZMB, than in MSM supplemented with TBTC (Fig. 2). In ZMB it produced large quantity of EPS than in MSM (Fig. 3). *Alcaligenes* sp.2-6 showed the highest exopolymer production with reference to yield (µg/mL) at 5mM of TBTC (Table-1). It produced large quantity of EPS in ZMB 98.3µg/mL than in MSM 46.8µg/mL (Fig. 3). The chemical analysis of the exopolymer revealed its acidic nature and the presence of functional groups such as neutral sugars, proteins, uronic acids and methyl pentoses (Table 1). The FTIR spectrum of the EPS produced by *Alcaligenes* sp.2-6 Strain S3 in the presence of 5mM TBTC showed two additional peak than control without TBTC (Fig 4) corresponding to an assymetrical weak C–H stretching band of methyl group at 2925 cm^{-1} and a weak peak at 1404 cm-1 revealing the presence of C=O stretching symmetrical of COO^- and C-O bend COO^- (Fig. 5). The emulsifying activity of the exopolymer determined by its strength in retaining the emulsion breaks rapidly within an initial incubation of 30 min. The absorbance reading after 30 and 60 mins gave good indication of the stability of the emulsion (Rosenberg et al. 1999). The dialyzed fraction of the exopolymer retained 42.48 % and 36.08 % of the emulsification activity, while the non-dialyzed fraction of the exopolymer produced 34.32 % and 11.0 % of emulsification activity at 30 mins and 60 mins respectively (Table 1).

DISCUSSION

The bacterial isolate identified as *Alcaligenes* sp.2-6 Strain S3 was Gram negative rod, facultative anaerobe, motile, oxidase, catalase, and casein positive, gelatinase and starch negative, produced mucoid colonies on ZMA. The isolate failed to utilize trehalose, adonitol, galactose, lactose, salilcin, raffinose, maltose, arabinose and sucrose, but utilized rhamnose, xylose, cellubiose, melibiose, saccharose and mannose. It was negative to lysine and ornithine decarboxylase. The isolate produced large quantity of EPS in ZMB than in MSM, as ZMB is a nutrient rich medium for all marine isolates. The EPS also showed good emulsifying activity and stability after 30 and 60 mins for both dialyzed lyophilized and non-dialyzed.

The FTIR spectrum of the EPS produced by *Alcaligenes* sp.2-6 Strain S3 in ZMB (Control) (Fig. 4) and ZMB with 5mM TBTC (Fig. 5) reveals similar stretching at 3400 cm^{-1} (control) and 3416 cm^{-1} (TBTC) corresponding to hydroxyl groups, a broad assymetrical stretching of P=O noticed at 1095 cm-1(Control) and 1078 cm^{-1} (TBTC) corresponded to the presence of Phosphodiesters (PO_2), a intense symmetrical peak (Control) noticed at 909 cm-1 and a weak assymetrical peak (TBTC) at 871 cm^{-1} corresponded to the Glycosidic linkage especially at the "Anomeric region" and a similar weak peak at 549 cm^{-1} (Control) and at 539 cm^{-1} (TBTC) corresponded to fingerprint region. The stretching at 1404 cm^{-1} could be assigned due to stretching of S=O sulphate groups.

Most of the EPS-producing marine bacteria, isolated from various TBTC contaminated sites are Gram-negative rods belonging to the genus *Pseudomonas, Alteromonas sp*. *Alteromonas atlantica, Alcaligenes sp. Vibrio*, *Flavobacterium,* and *Alteromonas colwelliana* are known to produce acidic polysaccharides (Geesy et al. 1992). The Exopolysaccharide produced by *Alcaligenes* sp.2-6 Strain S3 exhibited good solubility in distilled water. Most exopolysaccharides are produced both in the exponential and stationary phases, with the exception of non-marine pseudomonads, which produces exopolysaccharide only in the stationary phase (Uhlinger and White 1983). Though several methods for extraction of EPS from microbial cultures are reported (Decho 1990; Bhosle et al. 1995). We found fairly effective extraction of exopolysacchardie by cold ethanol precipitation with the incubation at 4 ^oC for 24 h. The frequency and type of functional groups present in the EPS affect the structure and overall physicochemical characteristics of the polymer in the surrounding aqueous environment (Decho 1990). Exopolymers are highly hydrated molecules (up to 99% water) possessing hydroxyl and carboxyl groups, which can have a hydrophilic character in aqueous solutions (Decho 1990; Sutherland 2001). Up to 20% to 50% of EPS produced by marine bacteria may be uronic acids (pKa 3.2–3.4). A comparison of functional groups presents that EPS having a higher number of variable functional groups was more complex than the other exopolysaccharides previously reported. The presence of acidic sugars in the EPS may be important, considering the heavy-metal-binding properties of this polymer. The EPS excreted by *Alcaligenes* sp. 2-6 Strain S3 is highly surface active, which is probably due to an uronic-acid containing polymer. Grobe *et al*. (1995) have analyzed the chemical composition of EPS excreted by *Pseudomonas aeruginosa.* The presence of neutral sugars, proteins, uronic acids and methyl pentoses suggested that the exopolymer is a heteropolysachharide indicating the acidic nature of the exopolymer. The heteropolysachharide containing multiple sugars have been reported in different Gram negative bacteria, such as *Psuedomonas flourescens* strain III 3, which was found to contain glucose,

glucosamine, rhamnose, fucose, arabinose and acetate, the plant pathogen *Psuedomonas andropogonis* which produces an acidic exopolymer containing glucose, glucouronic acid, mannose, rhamnose and galactose, the exopolymer of *Psuedomonas mendocina* P_2d was found to contain rhamnose, fucose, glucose, ribose, arabinose and mannose. Upon biochemical examination of the EPS produced by Strain S3 several prominent functional groups typical for hetero-polysaccharides such as hydroxyl, carboxylic and amides were found (Fishman et al. 1997). The EPS also possessed good emulsification activity. High level of EPS production in *Alcaligenes* sp. 2-6 Strain S3 may possibly be involved in biofilm formation under TBTC stress. Similar report is available in *Pseudomonas aeruginosa* USS 25W where TBTC stress are involved in development of biofilms (Upal 2003)**.** Implications for the role of these TBTC resistant bacterial polysaccharides in the marine environment requires further characterization using NMR and mass spectroscopy to elucidate the complete molecular structure of EPS produced by *Alcaligenes sp*. 2-6 strain S3. In addition to this we intend to study the emulsification activity of EPS using petroleum hydrocarbons such as crude oil, lubricants, diesel and petrol, beside n-hexadecane. Biotechnological uses for microbially produced EPS include environmental bioremediation of toxic heavy metals, polyaromatic hydrocarbons, biological recovery of crude oils and several other industrial applications, as reviewed by Gutnick and Bach (2000) and (Sutherland 1998; 2001). Though biotechnological potential of the biopolymer produced by *Alcaligenes* sp. 2-6 strain S3 from marine environment of Goa, in India remains largely untapped. The current study will enhance our knowledge on the ecological significance and provide insight into the biotechnological potential of the biopolymer.

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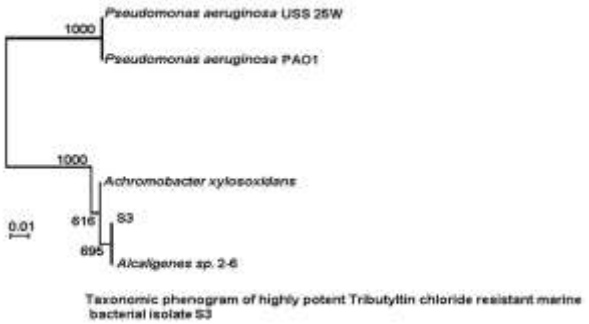
Table-1. Chemical Characterization and Emulsifying activity of Exopolymer (µg/mg) produced by *Alcaligenes* sp. 2-6 strain S3.

ND= Not detected, -ve= Below detection limits

Growth media	Wet weight	Dry weight	Total carbohydrates	Proteins	Uronic acid	Sulphates	Methyl pentose	Incubation time (Min)	Sample OD at λ 540 nm	% Emulsifying activity
ZMB (Control)	48.0 mg/mL	4.3 mg/mL	38.0	156.0	114.6	-ve	174.0			
C+ 1mM TBTC	66.2 mg/mL	6.5 mg/mL	46.3	158.0	117.4	-ve	196.0			
C+ 2mM TBTC	81.8 mg/mL	7.6 mg/mL	61.8	171.0	122.8	-ve	243.0			
C+ 5mM TBTC	128.3mg/mL	13.0 mg/mL	98.3	208.0	136.7	-ve	274.0			
MSM (Control)	ND	ND	ND	ND	ND	ND	ND			
C+1mM TBTC	34 mg/mL	2.9 mg/mL	21.8	78.0	84.4	-ve	96.0			
C+2mM TBTC	48.6 mg/mL	4.2 mg/mL	32.1	96.0	88.1	-ve	137.0			
C+5mM TBTC	68 mg/mL	7.4 mg/mL	46.8	164.0	114.0	-ve	186.0			
EPS Non-dialyzed								0	0.22	100%
								30	0.08	34.32
								60	0.02	11.0%
EPS Dialyzed and Lyophilized								0	0.47	100%
								30	0.19	42.48
								60	0.16	36.08



Taxonomic phenogram of highly potent Tributyltin chloride resistant marine bacterial isolate S3

Figure 1

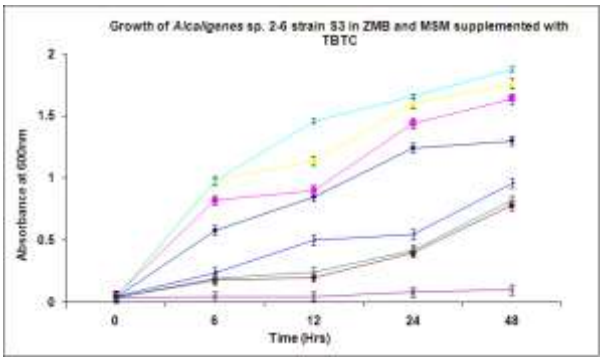


Figure 2

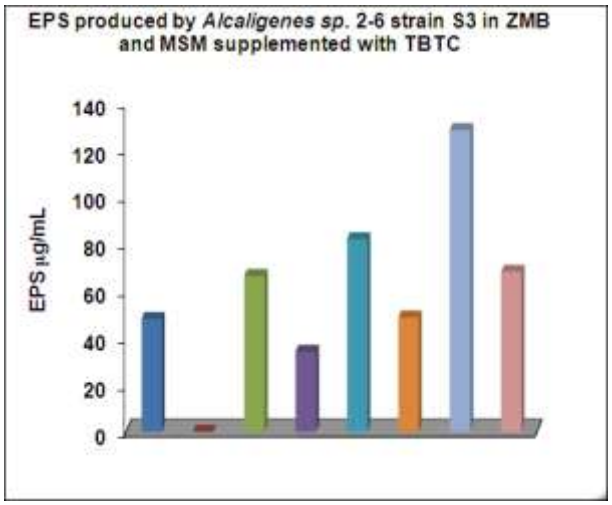


Figure. 3

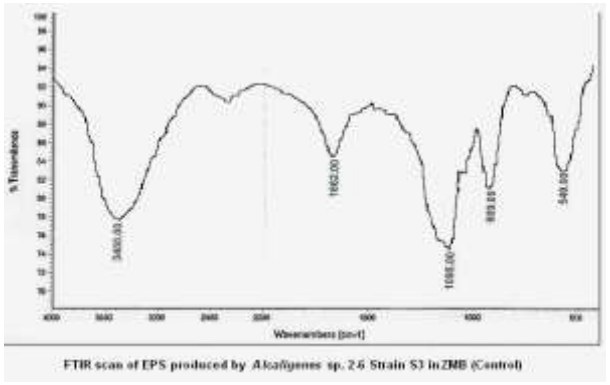


Figure. 4

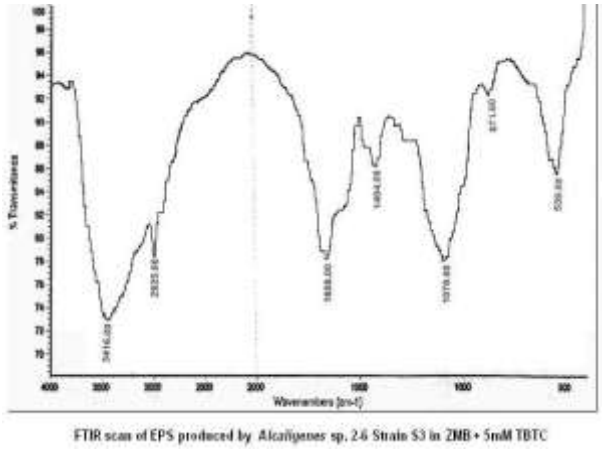


Figure. 5

Legends to figures

Figure-2. Growth of *Alcaligenes sp.* 2-6 strain S3 in ZMB and MSM supplemented with TBTC

Figure-3. EPS produced by ***Alcaligenes sp.*** **2-6 strain S3 in ZMB and MSM supplemented with TBTC**

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