JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 399-405

Micro Fibril and Capsular Exopolysaccharides Produced by *Alcaligenes CMG634*

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(Received: 11 January 2015; accepted: 20 March 2015)

An attached green seaweed bacteria CMG634 was isolated from Gaddani beach of Karachi coast (around 25.5°N, 66.7°E). It produced translucent and gelatinous colonies on agar plates. Strain CMG634 (AY596795) was identified as *Alcaligenes sp*. by 16S ribosomal RNA. Exopolysaccharide was analyzed by extracting the samples of broth at different intervals. When the isolated bacterium was grown at 30°C for a few days (1-5) on 0.5M NaCl nutrient broth. Partial purification of the extracted polysaccharide was done by dissolving of the precipitates in water and extracted by ethanol. The partially purified polysaccharide was subjected to acid hydrolysis and neutral fractions were analyzed by thin layer chromatography. Extracted polymer was found to be Glycoprotein in nature. Hygroscopic analysis of extracted EPS showed that maximum water was absorbed by EPS of 5th day. Scanning and transmission electron micrographs showed both capsular and slime in form of micro fibril.

Key words: Exopolysaccharides, 16S ribosomal RNA gene, thin layer chromatography, bioabsorbant, micro fibril,transmission electron micrographs.

Bacterial polysaccharides are usually associated with the outer surface of the bacterium. In crude terms these molecules can be divided into two groups. Either they may form an amorphous layer of extracellular polysaccharide surrounding the cell which may be organized into a distinct structure termed a capsule, or alternatively, the polysaccharide molecule may be more intimately associated with the cell surface either through linkage to a lipid-A moiety, as in the case of the lipopolysaccharide (LPS) molecules in Gram-negative bacteria, or linked to cell wall techoic acids as in Gram-positive bacteria [6,7].

Polysaccharides are highly hydrated polymers composed of repeating single units (monosaccharides) joined by glycosidic linkages. They can be homo- or hetero polymers and may be substituted with both organic and inorganic molecules[2]. Polysaccharides are an incredibly diverse range of molecules by virtue of not only the different possible monosaccharide units but also how these units are joined together[13]. The presence of a number of hydroxyl groups that may be involved in the formation of a glycosidic bond means that any two monosaccharides may be joined in a number of ways. Additional structural complexity may be achieved by the introduction of branches into the polysaccharide chain and the substitution with both organic and inorganic molecules. There fore, polysaccharide represent a rich source of structurally diverse molecules, many of which may have unique chemical and physical properties which have been exploited by man in a number of industrial, biomedical and food processes[13]. In natural and man made environment,

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the EPS (exopolysaccharide), play important role in biofilms which is normal habitat of many microbial communities in which varying number of microorganisms grow while attached to solid-liquid interfaces[10]. EPS can also function variously as enzyme stabilizer or concentrators as storage or nutrient reserves dispersants (emulsions) to release bacteria from nutrient spent surface[11]. Bioabsorbant polysaccharide of Alcaligenes latus B-16 is expected to be used in a wide range of applications including in absorbent materials such as sanitry products and diapers and even humectants used during irrigation of seedlings for promoting greenings of deserts[12].

MATERIALS AND METHODS

Isolation, Purification and Ribotyping

A sample of green seaweed and water from Gaddani beach of Karachi coast (around 25.5°N, 66.7°E). Bacterial strain was isolated purified and coded as CMG634 while it was preserved in 20% glycerol at –70°C. Strain CMG634 was maintained at 30°C in artificial seawater (ASW). The selected strain was identified by using 16S ribosomal RNA gene sequence homology primers used for amplification was 16S-3' – CCCGGGAACGTATTCACCG- and 16S-5'-GCYTAAYACATGCAAGTCGA-[3]. Sequence data obtained were analyzed by using BLAST algorithm. (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Culture condition, Production and Extraction of Exopolysaccharide

Fermentation of broth was performed in two stages. In stage I, CMG634 (AY596795) was grown in 500 mL conical flask containing 250 mL of 0.5M NaCl nutrient broth for 24 h at 100 rpm in room shaker, these broths were used as precultures. Stage II culture was prepared in 20 L. 0.5M NaCl nutrient broth by inoculating stage I cultures, so as to get 0.05OD (Optical density at 600 nm). Broths were incubated in room shaker at 100 rpm, at 30°C for 5days and same inoculation pattern were used for ASW supplemented with glucose.

Extraction procedure

The viscous culture broth of CMG634 from flask was centrifuged at 10,000 rpm for 30 minutes, and hard and compact cell pellets were separated. 10% NH_4Cl was added drop by drop to cell-free supernatant to precipitate[12] the extracellular protein fractions. The protein fractions were collected by centrifugation at 10,000 rpm for 30 minutes. The clear supernatant was added to equal volume of absolute ethanol, and the white columns of EPS precipitates floated on surface of ethanol. The EPS precipitates were hung on glass rod and transferred into sterile beaker. The EPS precipitates were then washed with two volume of absolute ethanol. The resulting precipitates were collected by glass rod. Sample was dried at 50° C until constant weight was reached and weighed the precipitates of EPS.

Chemical analysis of Exopolysaccharide (EPS)

Dry bioabsorbant about 200mg (W_1) was placed in a weighed small plastic cup and 2ml water was added to it, the plastic cup was placed at room temp for 24 h and next day the excessive water was removed and get dried by filter paper and the precipitates of EPS were again weighed (W_2) and then the weight of the plastic cup, filter paper, and dry bioabsorbent (W_1) was subtracted from the W_2 the resultant was the regain of water in grams (g) i.e. W_3.

Acid hydrolysis of extract

Nature of the biopolymer was analyzed by acid hydrolysis. Acid hydrolysis was done by adding10mg of dry ethanolic extract to 10ml of 20%HCl and 10ml of water was also added, place the reaction mixture at 110°C for 10h in heating mental. After hydrolysis, water was evaporated under reduce pressure and mixture was neutralization with Ag_2CO_3.

Thin layer chromatography hydrolyzate

Hydrolyzed sample was analyzed by running TLC card (Cellulose with fluorescent indicator 254 nm on aluminum cards layer thickness 0.1 mm) system used for the development of TLC cards was 80:20:1(Ethanol: H_2O : NH_3) while spray used was ninhydrin A (0.3g ninhydrin +100mL 1-butanol + 3mL glacial acetic acid) and was compared with standard amino acids. For the detection of sugars from the hydrolysed compounds TLC cards were developed with n-butanol: ethyl acetate: isopropanol: acitic acid: H_2O (7 : 20 : 12 :: 7 : 6) and spray used for the detection of sugars was anisaldehyde followed with 20% H_2SO_4 (0.5mL anisaldhyde in 50mL glacial acetic acid). Chromatogram was heated to 100-150°C until maximal visualization of the spots.

Quantitative analysis of carbohydrate and protein

Extracted and partially purified EPS was analyzed for total carbohydrate by Anthrone reagent (Sigma)[9]. The samples and reagents were cooled to 0°C before mixing and heated in a boiling water bath. The color was measured at 625nm against the blank. The quantitative estimation of protein was determined by Bradford procedure[1]. For the estimation of proteins present in extracted extracellular polysaccharide 100 ¼L of freeze dry samples were used. The absorbance was read at 595nm in 1ml plastic cuvettes.

Scanning and Transmission electron microscopy for the analysis of polysaccharide

Scanning electron microscopy (SEM) was performed of the selected strains. For this study cells were grown in nutrient broth and artificial sea water for 48 hr. at 30°C. Cultures broths were centrifuged. Cell pallets were fixed in 2 % (v/v water) triple distilled glutaraldehyde in ASW pH 7.0 for 2h at room temperature, and then washed four times with ASW (15 min / wash). Dehydration was through a 25-100 % (by vol.) ascending series of ethanol in distilled water, samples being left for an hour at each stage. Three transfers were made in absolute ethanol. The samples were coated for 5min using a Polaron E5100 series II 'cool' sputter coater fitted with Au/Pd target. The samples were examined at a voltage of 15-25 kV using a JEOL JSM-35 SEM.Centrifuged cells were directly mixed to equal volume of 5% glutaraldehyde in 0.1M phosphate buffer, pH 7.8. After 1h at 4°C, the cells were removed by low speed centrifugation for 5min.The pellet was washed carefully three times with 5ml phosphate buffer, with 10min between 5min centrifugation. The cells were resuspended in buffer and stained directly in 2.5mL 1% OsO_4 (in phosphate buffer). After staining over night samples were washed in buffer and dehydrated (15min intervals, except the last two washes, which were done twice) in water containing 25, 50, 75 ,90% and 100% acetone, followed by incubation in 100% propylene oxide 2x30mins and then 24hr in a 1:1(vol/vol) mixture of propylene oxide and Durcupan resin on a rotary wheel (4 r.p.m.) and 24hr in 100% Durcupan resin on a rotary wheel (4 r.p.m.) then pellets were transferred to flat embedding moulds with fresh resin and placed in a 60 degree centigrade oven for 24hr sections were cut with a Reichert-Jung Ultracut E microtome and collected on pioloform-coated copper grids. After staining with uranyl acetate and lead citrate, the sections were examined using a JEOL-1200EX transmission electron microscope.

RESULTS

Table 1. Identification of amino acids from hydrolysed sample

No.	Standard Amino acid	Color after spray	R_f Value	Spot No. Hydrolysate	from Color after spray	R_f Value	Spot identified
1	Lysine	Purple	0.024	1	Purple	0.024	Lysine
2	Arginine	Purple	0.0275	2	Purple	0.0275	Arginine
3	Proline	Yellow	0.275	3	Yellow	0.275	Proline
4	Histidine	Brownish yellow	0.310	-	-	-	-
5	Glycine	Purple	0.344	-	-	-	-
6	Serine	Purple	0.386	-	-	-	-
7	Asparagine	Purple	0.448	-	-	-	-
8	Alanine	Purple	0.462	4	Purple	0.462	Alanine
9	Cystine	Purple	0.482	-	-	-	-
10	Threnine	Purple	0.565	-	-	-	-
11	Glutamic Acid	Purple	0.586	5	Purple	0.586	Glutamic Acid
12	Valine	Purple	0.662	-	-	-	-
13	Lucine	Purple	0.758	6	Purple	0.758	Lucine
14	Methionine	Purple	0.779	-	Purple	0.779	Methionine
15	Tryptophane	Purple	0.793	7	Purple	0.793	Tryptophane
16	Tyrosine	Brown	0.8	8	Brown	0.8	Tyrosine
17	Phenyalanine	Purple	0.81	9	Purple	0.81	Lysine

Total number of spots observed in hydrolyzed sample after spray = 9

Study of Genetic Markers

CMG634 *was* isolated from green seaweed (Gadani, Pakistan), a G-ve rod, it was identified by 16SrRNA identified as *Alcaligenes* sp.and Genbank Accession number is AY596795 for 16S ribosomal RNA gene.

Time course of Exopolysaccharide production

Exopolysaccharide production was followed in 1L batch culture fermentation of nutrient broth, without pH control for 5 days at 30°C while aeration was provided by shaking 150rpm.Exponential growth continued for 21h. Exopolysaccharide production continued for 5day during the stationary phase (Fig. 1). The rate of EPS production increased at the end of exponential phase, viscosity of the culture started increasing and the pH get decreased. The fermentation pattern is typical of secondary metabolite formation. Highest EPS production was observed at 5day, while the amount, which was produced, was $2gL^{-1}$.

Hygroscopic activity and quantitative analysis of EPS

Water absorbance capacity was measured by observing the water loss during the drying process of the precipitates (wet to dry) as described in Fig. 2. Maximum exopolymer production was observed at 5day growth, extracted polymer also showed highest water regaining ability.

Quantitative analysis of Exopolysaccharide production under different nutrient conditions

The highest yields of bacteria and polysaccharide were obtained using the nutrient broth supplemented with 0.5M NaCl in which the doubling time of the bacterium was 110 to 120 min. When the medium was replaced to artificial seawater (glucose as carbon source), the total amount of exopolysaccharide decreased from 523to 350ug mL^{-1}. As function of time pH dropped from 7.0 to 5.0 after 72h growth. When extracted exopolymer were analyzed for carbohydrate analysis it was observed that amount of carbohydrate was significantly high than that of protein constituents under defined and undefined media while production of carbohydrates was more in the defined media. Amount of carbohydrate was $590ugmL^{-1}$ under undefined media while under minimal condition it was decrease to $370ugL^{-1}$.

Chemical analysis of Exopolysaccahride

In hydrolyzed sample of exopolysaccharide amino acids were detected (Table 1) while hydrolysis of carbohydrate were partially done due to which only Rhamnose sugar was detected and other sugars were not no successfully detected. Amino acids were identified through the Rf (Retention factor) value compared to the pure amino acids**,** which are applied simultaneously on the same plate (Table 1).

Scanning and Transmission electron microscope

Under scanning microscope polysaccharides were observed in different forms. Slime sheath over the cells were observed when the magnification was very low while at high

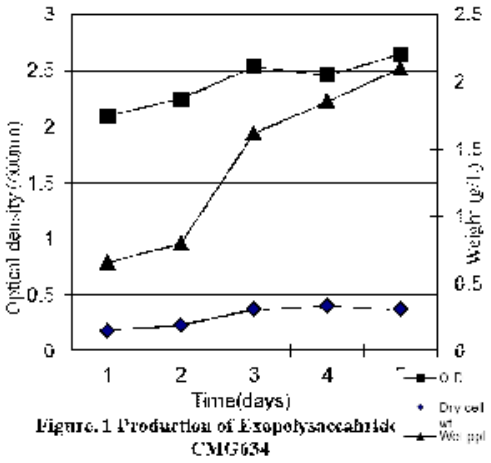

Figure. 1 Production of Exopolysaccahride CMG634

Fig. 1. Production of Exopolysaccahride by CMG634

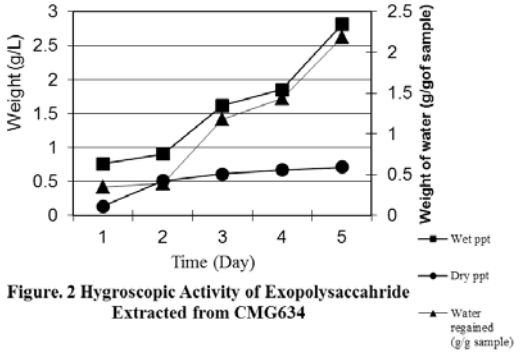

Figure. 2 Hygroscopic Activity of Exopolysaccahride Extracted from CMG634

Fig. 2. Hygroscopic Activity of Extracted Exopolysaccahride from CMG 634

magnification thread like extracellular polysaccharide were prominent in the field (Fig. 3 A and B) While under transmission electron microscopy different layers of slime were very clearly observed (Figure. 4 A and B).

DISCUSSION

Sea normally contains a versatile bacterial flora. Moderately halophilic bacteria are those which grow better in media containing 0.5M to 2.5M (w/v) of NaCl in the media. These constitute a very heterogeneous group and play an important ecological role due their abundance in hyper saline environments [5]. They are very interesting for biotechnological purposes since they produce secondary metabolites including substantial quantities of exopolysaccharide which could be of commercial interest. Keeping in view that bacterial extracellular polysaccharides (EPS) are polymers widely used in food and other industries as gelling, texturizing, suspending, bioabsorbing and encapsulating agents, the bacterial strain CMG634 was isolated from green seaweed. It was selected because it produced viscous broth when supplemented with nutrient broth with 0.5M NaCl while in minimal media (ASW with glucose) it also produced viscous and yellowish broths, while it produced translucent and gelatinous colonies on agar plates. This strain was identified as *Alcaligenes sp*. IS-18and gene sequences were deposited under the accession no AY596795.

The strain CMG634 was selected for

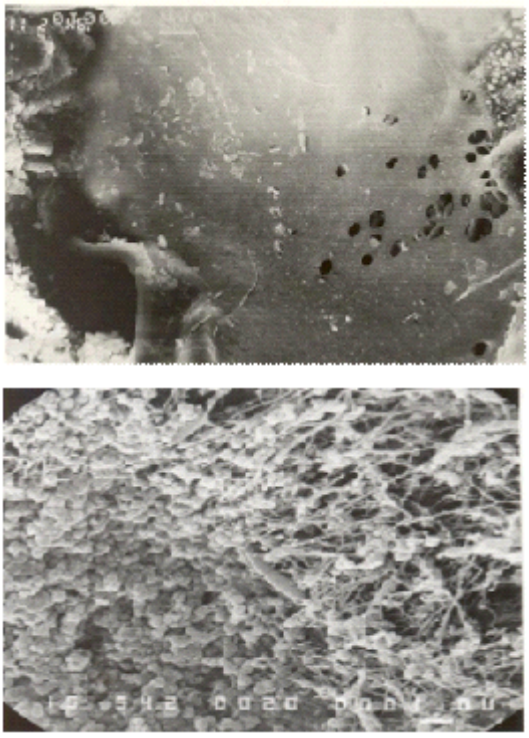

Fig. 3. Scanning electron micrographs of CMG634, A. Slime sheath over the cells, B extracellular ultra fine fibrils like polymeric structures of polysaccharide.

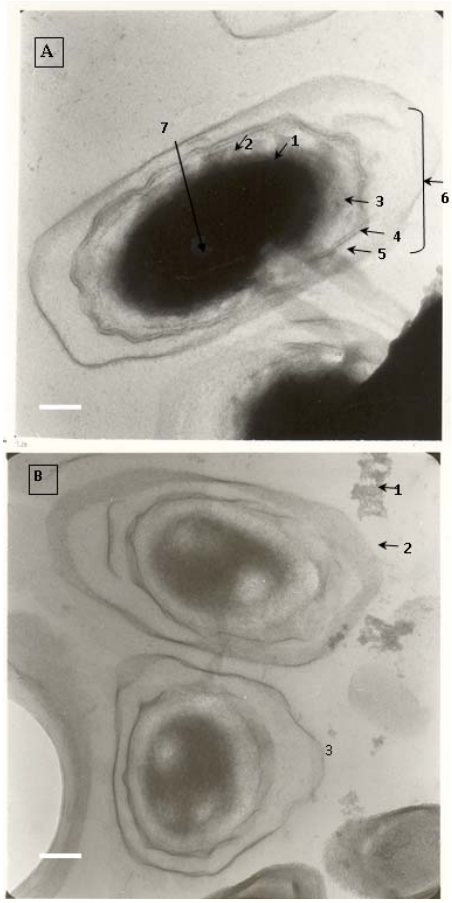

Fig. 4. Polysaccharide producing strain CMG634 under transmission electron microscope. A. Longitudinal section of bacterial cell producing polysaccharide (multi layer) in presence of nutrient broth.1. cell wall, 2. primary polysaccharide encase the cell wall, 3. primary polysaccharide 4. dense line appears to evolve secondary polysaccharide or slime layer, 5. Capsule, 6. slime lipopolysaccharide(secondary polysaccharide), when it released from cell into the environment as micro fibrils. 7. Electron dense cytoplasm.B.1. dark precipitates typical of polysaccharide, 2. longitudinal and 3. cross section of cells. ▬▬: 1.0µm

exopolymer extraction. Exopolysaccharide was analyzed by extracting the samples of broth at different intervals. When the isolated bacterium was grown at 30°C for a few days (1-5) on 0.5M NaCl nutrient broth. The liquid culture medium becames highly viscous with the bacterial growth under aerobic conditions due to formation of extracellular polysaccharide. Therefore cell growth and course of exopolysaccharide production analyzed by batch culture fermentation. Results showed that the exopolysaccharide produced in the late stationary phase. After 24h the amount produced was $0.5gL^{-1}$, after 48h to 120h EPS yield reached $2gL^{-1}$with increase in viscosity and constant cell mass after 48 to 120h (5th day). The viscosity of culture fluid reached maximum after 48h. It was observed that bacterial mass remained constant while the production of exopolysaccharide were increased (Fig 1). Maximum production of exopolysaccharide appeared at fifth day of incubation, which is typical of exopolysaccharide production[4]. When the incubations were prolonged i.e. above 10-day it was observed that the viscosity decreased which could be due to the degradation of the product, using EPS as sole carbon source[8].

Partial purification of the extracted polysaccharide was done by dissolving the precipitates in water and extracted by ethanol, polysaccharides were insoluble in ethanol. Proteins were removed by adding NH_4Cl. When extracted and partially purified compounds were analyzed for total carbohydrate and protein, it was revealed that under nutrient rich conditions total carbohydrates were~580 ug mL^{-1}whereas protein was 100ug mL^{-1}while in presence of artificial sea water in presence of glucose as sole carbon source it produced 289ug mL^{-1}, so it was concluded that the exopolysaccharide was substrate dependent and time dependent[4, 8]. When the partially purified polysaccharide was subjected to acid hydrolysis and neutral fractions were analyzed by thin layer chromatography. Amino acids were detected by comparing Rf values to the standers. A total of nine spots were detected by spray and identified on Rf values of standers. While sugar analysis of the hydrolyzate showed it has that one detectable sugar in it and which was Rhamnose, this may be due to two reasons either hydrolysis was incomplete with respect to sugars detection or the major component of the compound was Rhamnose. Extracted polymer was found to be Glycoprotein in nature.

Hygroscopic analysis of extracted EPS showed that maximum water was absorbed by EPS of 5th day (Fig. 2) the water which was absorbed was 2.73g while the weight of dry EPS was 0.57g and the water which was absorbed was 2.18g ~ 4.3 times more than its own weight Such type of bacterial exopolysaccharide had also been reported by Ryuichiro *et al*.[8] which were produced by A*lcaligene latus*.. This EPS is bioabsorbent, which has Rhmanose and nine amino acid as constituent components, it is biodegradable as shown by late phases of growth rates and perhaps it was used as a carbon source. It could be exploited for use in industrial products, thus being much safer for the soil, water and human beings. In fact is possible that it will be environmentally safe for the applications, including desertification prevention.

The growth of the bacteria is often accompanied by the production of polysaccharides, which are found out side the cell wall. These exopolysaccharide may be found as a capsule attached to the bacteria or the may be released to the environment as slime or remain attach to the cell as capsule[6]. Polysaccharide may be important to the bacterium for it adhesion, protection and infection, but it may have commercial and biomedical values as well[11]. As under study organism isolated from seaweed so polysaccharide used for the adhesion purpose Scanning and transmission electron micrographs showed both capsular and slime in form of micro fibril (Fig. 3B). SEM examination of the samples showed a polysaccharide slime layer forming an irregular but continuous sheet stretched across the field (Fig. 3A). If we combine the results of both SEM and TEM it was clear that two different morphologies of polysaccharide were present: a beaded, globular layer overlaid by a smooth, sheath like layer (Fig. 3 A) and second was web-like (Fig. 3B) morphology of polysaccharide slime produced in form of micro fibrils[6]. Electron dense precipitates of in TEM micrographs correlated to the SEM micro fibrils and the sheath stretched over the field is the capsular polysaccharide attached to the bacterial cell. So EPS can be released from the cell into the environment as a slime or remain attached to the cell to form capsule. Primary and secondary

polysaccharides were clearly distinct under TEM micrographs[4]. Bacterial strain produces produce EPS in two distinct forms: ropy EPS orloose slime that is excreted into the surroundings and capsularEPS that remain adhered to the cell surface creating a discretecovering[2].

The extracted exopolysaccharide from CMG634 have multipurpose commercial exploitation as the rheological characteristics of understudied hydroabsorbent biopolymer could be used for the stabilization of soil and to prevent soil erosion and high rate of evaporation in hot seasons. Hence this hydroabsorbent act as soil stabilizer and water reservoir may be helpful to prevent the, soil erosion, desertification, to plantation and also could be used for sanitary purpose as baby diapers and tissue papers.

CONCLUSION

We have isolated a bacterial strain of marine origin producing a high level ofexopolysaccharide (EPS) and identified the bacteria as *Alcaligenes* sp CMG634. The EPS exhibited a highlevel of viscosity and it was proposed that the EPS produced by CMG634 is highly viscous and contains a high level of water holding capacities.

ACKNOWLEDGEMENTS

This research article is the part of Dr. Nazia's PhD thesis.

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