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Research Article

BIOPROSPECTING OF TRADITIONAL SWEET MANUFACTURING EFFLUENT FOR EXOPOLYSACHHARIDE PRODUCING BACTERIA AND THEIR BIOTECHNOLOGICAL APPLICATIONS

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Abstract

This work was aimed to isolate, purify, characterize and to study the biological applications of extracellular polysaccharide (EPS) produced by *Agrobacterium fabrum* strain C 58 isolated from effluent of a traditional sweet manufacturing unit. The isolated bacterium *Agrobacterium fabrum* strain C 58 was found to produce 16.21 g/L crude EPS in terms dry weight at 28 ^{0}C when brown sugar was supplemented as the source of carbon. The polysachharide was further purified by anion exchange chromatography on a column of DEAE Cellulose -52 yielding one fraction which eluted at 0.2M NaCl. The monosachharide composition of EPS by TLC indicated it to be a hetero polysachharide composed of glucose, galactose, mannose and rhamnose. The FT-IR analysis proves the presence of biologically important functional groups and alpha glycosidic linkage between individual glycosyl residues. The biopolymer at a concentration of 1 % exhibited significant lipid emulsifying capacity against various vegetable oils. The effectiveness of polysaccharide in inhibiting free radicals evaluated by DPPH radical scavenging appeared to be significant. This is the first report about isolation of potent EPS producers from a traditional sweet manufacturing unit effluent which confirms that these samples can be used as a potential habitat for bioprospecting extracellular polymer producing bacteria. The diversity offered by microorganisms in these diverse habitats thus renders a hope for screening new habitats for isolating and developing new polysaccharides with properties superior to those of the existing polymers.

Keywords: Exopolysaccharide; Lyohilisation; Emulsification Index; Thin layer Chromatography; DPPH Assay.

Introduction

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides (EPS). The ability to produce extracellular polysaccharides is widely spread among bacteria. These secreted polymers are attached to the cell surface or released as extracellular slime in the surroundings of the cell (Knoshaug *et al.*, 2000). The unique physical and chemical characteristics of the secreted extracellular polysaccharides are determined by their individual sugar components and their mode of linkage. Thus EPS show great diversity and novelty in their structures and chemical properties. The rheological and physiological properties of bacterial extracellular polysaccharides are quite different from those of natural gums or synthetic polymers (Ahn *et al.*, 1998) and its physicochemical characteristics vary depending on the microbial source and culture conditions. Additionally, bacterial extracellular polysaccharides are non-toxic, biodegradable, and environmental friendly (Shoda & Sugano, 2005). These polysaccharides can be used as active ingredients in pharmaceutical products, cosmetics or as raw materials for industrial synthesis of aromas (Linton *et al.*, 1991; Cresenzi 1995), as thickeners, stabilizers, emulsifiers, gelling agents and water-binding agents in the food, cosmetics, bioplastics, pharmaceuticals, oil industries etc. (Sutherland, 2002). There are reports suggesting the presence of unique physiological activities as anti-tumour, anti-viral and anti-inflammatory agents as well as an inducer for interferon, platelet aggregation inhibition and colony stimulating factor synthesis in some polysaccharides (Wiley, 2003). Scientific developments in the recent years have opened new frontiers and enabled a better understanding of the polysaccharides secreted by various bacteria. The screening of bacterial polysaccharides is promising because of the enormous range of polysaccharides that have yet to be adequately explored. Many bacterial polysaccharides have been extensively characterized and developed for commercial applications.

Industrial effluents can be a rich source for microbial biocatalysts as microorganisms present in it are adapted for growth on various organic chemicals that are used in various manufacturing processes (Bramucci *et al.*, 2003). The intense competition for limited carbon resources in

wastewater may result in the evolution of novel genes and biochemical pathways in the specialized environments of industrial wastewater.

Agrobacterium is a Gram negative, rod shaped aerobic bacteria which thrives well on a media containing carbohydrates and releases large quantities of mucus. *Agrobacterium* species are reported to produce two types of extracellular polysaccharides namely water soluble acidic polysaccharide like succinoglycan (Hisamatsu *et al.*, 1980) and water-insoluble extracellular polysaccharides like curdlan (Lee and Lee, 2001).These polysaccharides were believed to promote nodule invasion by nitrogen-fixing microorganisms (Glazebrook *et al.*, 1990).

Materials and Methods

Isolation and Identification of Bacteria

Sweet shop effluents were collected from different regions of Udaipur in presterilized ampoules. The samples were serially diluted and plated on media suggested by Tallgren *et al.*, 1999 for the isolation of EPS producers. The polysaccharide producers were screened on the basis of colony morphology as mucoid and ropy (Vescovo *et al.*, 1989). An isolate with shiny morphology was isolated, purified maintained on agar slants at 4 °C slants for further use.

The selected bacterial isolate was identified on the basis of its microscopic morphology, biochemical characteristics and molecular identification. The isolate was further identified by 16S rDNA sequencing. Total genomic DNA was extracted from the isolate following the method of Sambrook *et al.*, 1989. Universal primers 8F and 1492R were used for amplification of *16S rRNA* gene by PCR. The sequence was compared with those available in the NCBI genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W.

Growth and EPS Production

Production of extracellular polysachharides by the isolate was determined during growth in medium containing (g/L) 40 gms brown sugar; 0.2 gms $MgSO_4 .7H_2 O$; 9 gms $K_2H PO_4$; 3 gms KH_2PO_4; 2 gms yeast extract; 2 gms peptone; 2 gms NaCl; 15 gms agar. Erlenmeyer flasks (250 mL) containing 50 mL media were inoculated with 18 h grown bacterial culture and agitated on a rotary shaker at 150 rpm at 28 °C for 10 days. Cell growth was determined by taking an aliquot of the medium (10 ml) by centrifuging it at 12,000 rpm at 4ºC for 20 min and drying the cell pellet at 80 ºC for 24 h. To determine the EPS yield, EPS from cell-free culture broth was precipitated by mixing the supernatant with cold ethanol (4 ºC, 1:2, v/v) and left overnight at 4ºC. The precipitate (EPS) was lyophilized and then dry weight of was determined. All the experiments were carried out in triplicates

Isolation of Extracellular Polysaccharide from Broth

The culture broth was diluted with sterile water and centrifuged at 15000g for 20 min to remove the bacterial cells. The pellet obtained after centrifugation was weighed to calculate the biomass of bacteria and discarded. To the clear supernatant fraction 5% trichloro acetic acid was added and left overnight at 4 ºC and centrifuged at 15000 rpm again. The supernatant fraction was collected again and three volumes of chilled ethanol was added for precipitating EPS and left overnight at 4 ºC (Ruas-Madiedo *et al.*, 2002). The precipitated EPS was weighed, dialyzed against deionized water for 2 days at 4 ºC and lyophilized to calculate the dry weight EPS produced by the selected isolates. The lyophilized EPS will be used for the further assays. The polysachharides secreted by the isolates was quantified according to phenol sulphuric acid method (Dubois *et al*., 1956) using glucose as the standard sugar.

Purification of EPS

The crude polysaccharide (10 mg in 5 ml water) was subjected to anion exchange chromatography on a column (1.0 x 25cm) of DEAE Cellulose-52, pre equilibrated using deionised water and eluted with a volume of deionised water followed by a continuous gradient of NaCl from 0.0 to 0.5 M in deionised water with a flow rate of 45 ml h^{-1}. Fractions (5 ml) were collected and an aliquot (0.1 ml) was tested for total carbohydrate by the phenol sulphuric acid method (Dubois *et al*., 1956). The respective polysaccharide fractions were pooled and dialyzed overnight against deionizied water and finally lyophilized.

Analysis of Monosaccharide Composition by TLC

One mg EPS were hydrolysed with one ml of 4 M trifluroacetic acid at 100 ºC for 2 hours. Sugar components were identified by thin layer chromatography using standard sugars for identification. TLC was developed with n- propanol: water (85:15) as solvent system. Sugars were visualized by spraying with freshly prepared aniline-diphenylamine-orthophosphoric acid reagent on plates and baking the plates for 15-20 min at 80-100 ºC (Dawson *et al*., 1986).

FT IR Analysis

The polysaccharides were characterized using a FT IR imaging system (Bruker 3000 Hyperion Microscope with Vertex 80, Germany). The dried polysaccharides were grounded with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 400-4000 cm-1

Biological Applications of Isolated Polysachharides

Lipid Emulsifying Test

Emulsifying effect of the isolated extra cellular polysaccharide was tested using the method of Kurane and Nohata (1991). Equal volumes of various vegetable oil and 1% biopolymer solution in distilled water were shaken for 10 min at 150 rpm on a rotary shaker to make a lipid

emulsion. The emulsion was centrifuged at 2000 *g* for 5 min and the height of the emulsified layer was measured. The lipid emulsifying activity was expressed as percentage of the height of emulsified layer by the height of whole layer. Xanthan, dextran, gum ghatti, gum acacia and guar gum were used as the standard. The emulsifying activity of the biopolymer and standard gum against vegetable oils like olive, sunflower, soyabean and mustard oil were tested.

DPPH Radical Scavenging Assay

The free radical-scavenging activity of EPS was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals using the method of Shimada *et al*. (1992). Freshly prepared DPPH at a concentration 0.1 mM in methanol were added to 1 mL of EPS solution of different concentrations (100µg-2000 µg) in water. After 30 min, absorbance was measured at 517 nm using a UV visible spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. Ascorbic acid was used as a positive control. The results were shown as the average of three independent experiments. Means and standard deviation were calculated for concentration. The ability to scavenge the DPPH radical was calculated using the following equation:

$$Scavenging\ effect\ (\%) = \frac{(Ac - As)}{Ac} \times 100$$

Where, A_C = the absorbance of the control reaction; A_S = the absorbance of the sample extract.

Results

Among the 15 strains with mucoid appearance isolated from traditional sweet shop effluents the strain MRL- 2A produced significant amount of extracellular polysaccharide that was capable of forming viscous solutions in water. On the basis of morphological, cultural and biochemical characters the bacterial isolate was tentatively identified up to generic level according to the key suggested in Bergey's manual(1957). Thus a combination of rapid biochemical tests and genotypic identification using *16S rRNA* gene sequencing enables the successful identification of the isolate. At the first stage conventional methods based on growth morphology and microscopic observations were used to classify the bacterial isolates.

Microscopically the isolate MRL 2A was Gram-negative, non-spore-forming rod (Fig. 1& 2). The isolate produced white to beige colour, convex, circular, smooth and glistening colonies. They were positive for catalase and oxidase, citrate reduction test, esculin hydrolysis and reduced nitrate to nitrite. The isolate produced acid from a variety of carbohydrates like sucrose, arabinose, arabitol, mannitol and maltose etc. On the basis of morphological, microscopical and biochemical characteristics the isolate showed its similarities to the genus *Agrobacterium.*

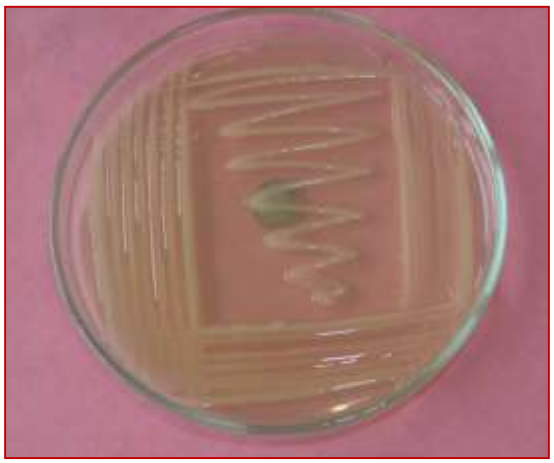

Fig.1: EPS from *Agrobacterium tumefaciens*

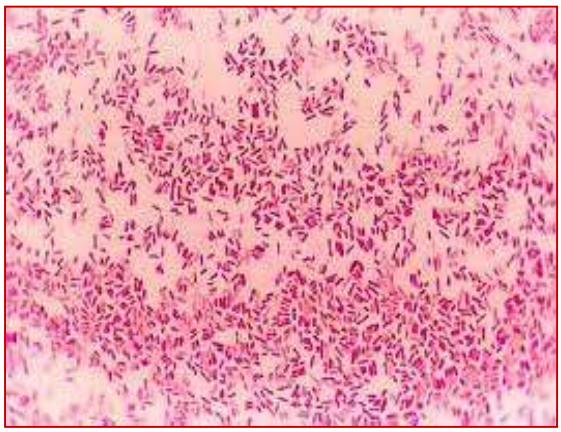

Fig.2: Gram Staining of Isolate

The identification of bacteria at the species level was confirmed by molecular methods and use of 16srRNA sequencing. Phylogenetic relationships based on *16S rRNA* gene sequences and phenotypic variations were used to assign isolates into distinct species. The *16S rRNA* gene sequences generated were aligned and compared with other *16S rRNA* gene sequences in the Gene Bank by using the NCBI BLAST program. To determine the closest type strain of the isolate a phylogenetic tree was constructed from automated BLAST searches. The *16S rRNA* gene sequences of the isolate had the highest matching similarities with *Agrobacterium fabrum* strain C 58 (99%). The gene sequence was submitted in Gene Bank and assigned with a Gene bank accession number NC003063.2 (Fig. 3).

The isolate on agar plate at 28°C developed translucent gelatinous colonies. It was also observed that the liquid culture medium became viscous during bacterial growth under aerobic conditions due to the formation of extracellular polysaccharide. The cell growth and EPS yield were monitored for a period of ten days. EPS production reached the maximum on the 6th day of incubation (16.21 g/L) thereafter it decreased (Fig. 4). Results of cell growth, however, showed a different pattern with increase till 5th day followed by a decline in growth.

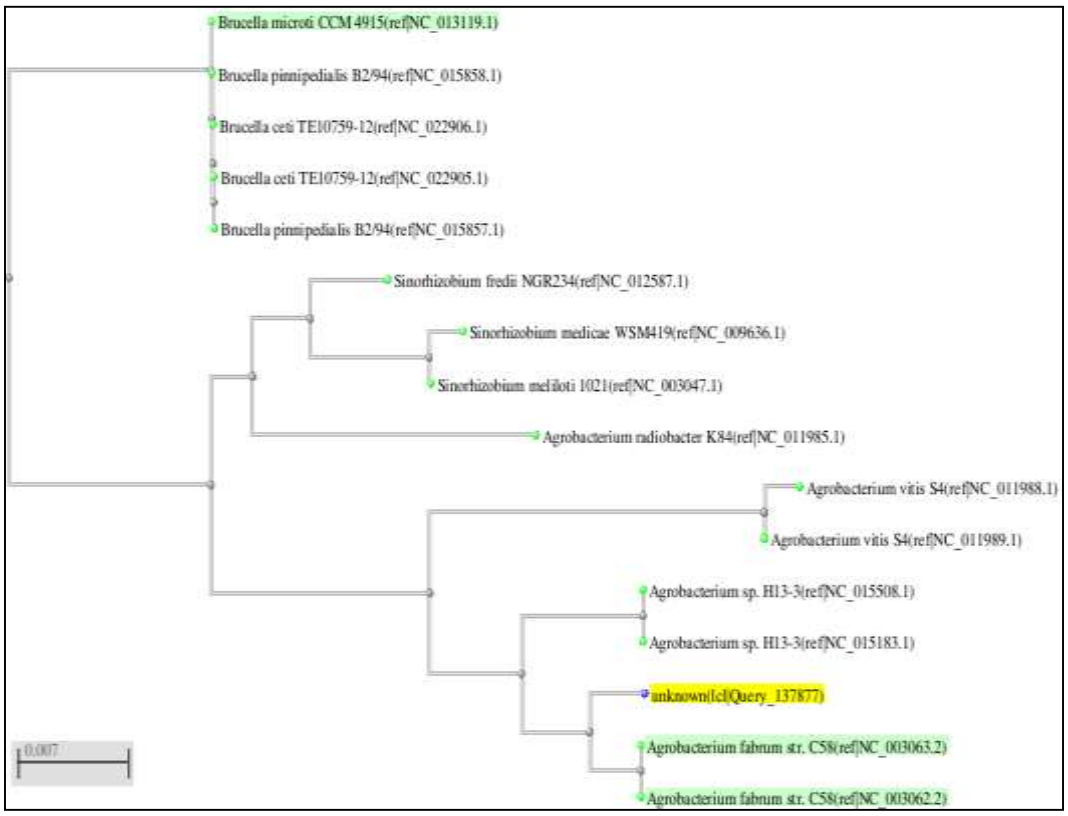

Fig 3: Phylogenetic analysis based on *16S rRNA* gene sequences

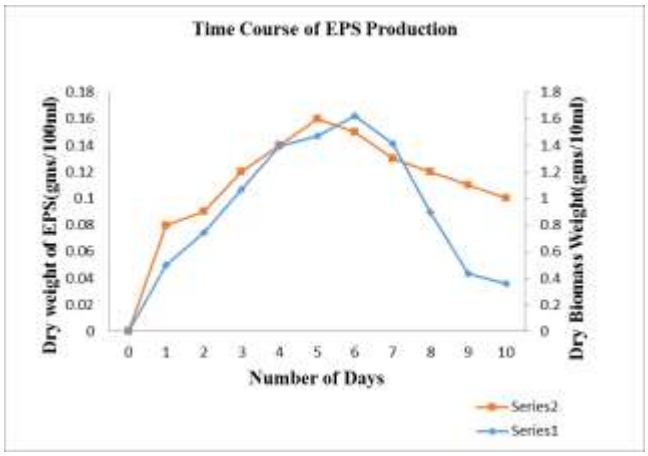

Fig. 4: Bacterial Growth and EPS production
[Data are the mean± SD (n = 3) from three independent experiments.]

Partial purification of crude EPS was carried out by repeated fractional precipitations with ethanol followed by dialysis. The dialyzed EPS loaded on the anion-exchange column Cellulose DEAE-52 showed only one elution peak which eluted at 0.2M NaCl as "The dialysed EPS gave only one elution peak which eluted at 0.2 M NaCl. The presence of one chromatographic peak attested to the fact that the polysaccharide from *Agrobacterium fabrum* strain C 58 contained only one species of polysaccharide. The eluted polysachharide was hence an anionic polymer as it adsorbed to anion-exchanger DEAE Cellulose 52 because of its negative charges. The eluted fraction showed no absorbance peaks at 260 and 280 nm indicating absence of contaminating nucleic acids and proteins.

The monomeric composition of EPS produced by *Agrobacterium fabrum* strain C 58 was analyzed by TLC after hydrolyzing with acid. On spraying with detection reagent the standard monosachharides gave yellow green colour for rhamnose, blue grey for mannose and dark blue colour for glucose and galactose respectively

The Rf value of the bands obtained by hydrolysate corresponded to glucose, galactose, mannose and rhamnose. This implied that the polysaccharide is a hetero-polysaccharide. (Fig. 5)

Fig. 5: TLC of acid hydrolysed EPS

Infrared spectroscopy allows the measurement of molecular vibrations of covalent bonds. In carbohydrate analysis using IR spectra, α and β conformers can be clearly distinguished within the anomeric region vibrational bands since the α and β-configuration are well separated in the 950 to750 cm^{-1} region, where 870–840 cm^{-1} corresponds to α conformers, while the β conformers lie around 890 cm^{-1} (Kacurakova *et al*., 2001; Yang *et al*., 2009) An absence of β configuration was indicated as there was no absorption peak at 890 cm^{-1}. Absorption peak at 861 cm^{-1} was indicative of α-glycosidic linkage between individual glycosyl residues in EPS (Kodali *et al*., 2009). In addition, a medium-broad C–O stretching was observed around 1056 $cm^{-1}$was attributed to the ether linkages present within oligomers (Wang *et al.*, 2010).The infrared spectra of extracted polysaccharides (Fig 2.) shows absorption peaks at 3444 cm^{-1}, 2924 cm^{-1} and 1635 cm^{-1} indicating O-H stretching, a C-H bond, and a C=O bond, respectively. The spectrum peak at 3444 cm^{-1}indicated the presence of OH group and NH_2 group in the molecule (Desoukyet *et al*., 2008) (Fig. 6).

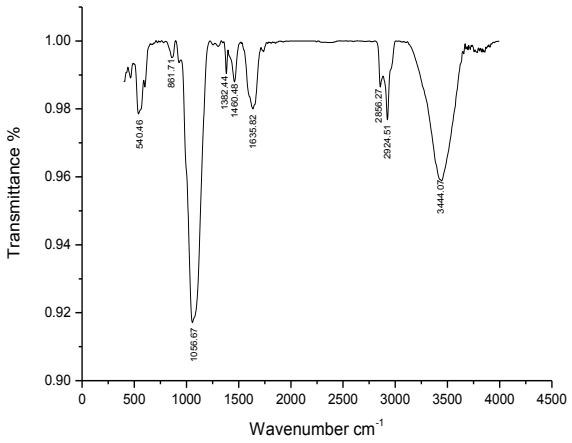

Fig. 6: FTIR analysis of exopolysachharides

Microbial and plant gums have been known to possess lipid emulsifying effects. Especially, xanthan gum of microbial origin is well known for its high emulsifying activity and thus has been widely used in the food industry (Cirigliano and Carman, 1985). The emulsifying effect of the crude polysaccharide examined against various vegetable oils like olive, sunflower, soyabean and mustard oil reveals that the biopolymer obtained from isolate showed emulsifying capacity. The emulsifying capacity of the isolated polysaccharide is listed in Fig. 7. The EPS efficiently emulsified sunflower and olive oil (76%) at a concentration of 1 % w/v and emulsion remained stable for 10 days. The emulsifying ability of the EPS was comparable to that of natural gums and those produced from various microorganism.

The DPPH free radical was a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant. So it has been widely accepted as a tool for evaluating the free radical scavenging activities of natural compounds. Antioxidant activities of various concentrations of polysaccharides evaluated by DPPH assay are presented in Fig 8. The scavenging activity of the exopolysaccharide was seen to increase with increase in concentration of polysaccharides. It was observed that at higher concentration ie, 2 mg/ml the polysaccharide exhibited very good scavenging activity with 80.74% hence it can be used as a promising antioxidant.

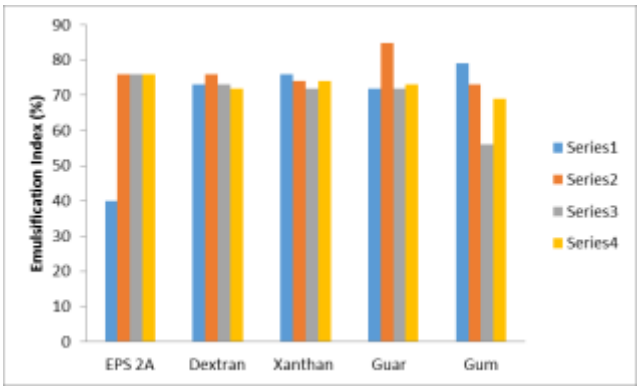

Fig. 7: Comparison of Lipid emulsifying capacity of isolated biopolymers with standard polymers.
[The results are represented as Mean± SD, *n* = 3.]

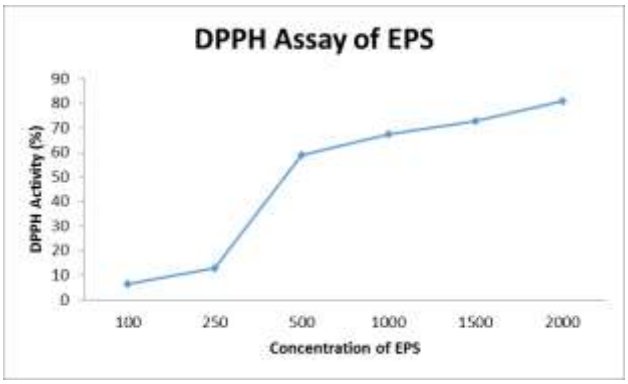

Fig. 8: DPPH Assay of EPS
[The results are represented as Mean± SD, *n* = 3]

The huge market potential of bio polymers and their high value added applications in various sectors suggests that research works should be intensified to explore promising samples that has to be out sourced for isolation of biopolymer producing bacteria and for their high value added applications in various sectors. The present work reports the isolation of a potent EPS producer identified as *Agrobacterium tumefaciens* from a traditional Indian sweet manufacturing unit effluent. The isolated biopolymer was viscous and possessed good emulsifying and antioxidative properties which is an indicative of its potential use in industrial applications.

Discussions

The complex biochemical composition of the products like milk, sugar, ghee etc used for manufacturing Indian sweets generate a nutrient rich effluent which can serve as an excellent place for the growth and multiplication of many bacteria. The purpose of this study was to obtain a potent

EPS producer from such an effluent. Review of literature revealed the reports of isolation of exopolysaccharides secreting bacteria from milk products, fermented food products, psycrophilic regions, deep sea hydrothermal vents and soil etc. (Nakajima *et al.*, 1992*;* Bukola *et al*., 2008; Guezennec 2002; Bueno and Cruz, 2006). This is the first study which reports the novelty of sample used for isolating significant EPS producers.

The identification of bacteria based on phenotypic, morphologic and biochemical tests is often misleading. Therefore identification of bacteria based on *16s rRNA* gene sequencing confirms the strain as *Agrobacterium fabrum* strain C 58. Extracellular polysaccharides produced by *Agrobacterium* species have been reported in the literature (Lee *et al*. 1997; Kim *et al.*, 1999; Kim *et al.*, 2003, Wu *et al.*, 2008). The polysachharide produced by *Agrobacterium* described in this work is water soluble and aqueous solutions of the polysaccharide were viscous. Hisamatsu *et al.* (1980) isolated water-soluble exocellular polysaccharides from nine strains of *Agrobacterium* containing succinic acid, pyruvic acid and acetic acid. Their structures, which were similar to succinoglycan, were composed of (1 - 3), (1 - 4), and (1 - 6)-linked D-glucosyl and (1 - 3) linked galactosyl residues. The analysis of monomer composition of the polymer suggests that it is a heteropolysachharide. Majority of EPS reported from *Agrobacterium* is of curdlan type which is a homopolysachharide made up of glucose units.

Functional nature of the isolated EPS confirmed absorption peaks at higher frequencies common characteristics of polysaccharides (Silverstein and Webster 1998; Stuart 2004). The presence of peak around 861 nm^{-1}in FTIR analysis shows the α-linkage of the isolated polysaccharides which seems to be different from other reports. York *et al.* (1980) reported that *Agrobacterium* species produce neutral β- (1, 2) D glucose polymer with 22 glucose residues per molecule. The glucose residues occurred equally in β- (1, 3,), β- (1, 4), β- (1, 6) linkages while galactose is linked by β- (1, 3) linkages. Hisamatsu *et al.* (1977) reported that the water soluble polysachharides of *Agrobacterium* are succinoglycans with succinate residue covalently linked to β glucan residues.

The commercial applicability of EPS largely depends on the viscometric behaviour under various environmental conditions (Marinho-Soriano and Bourret, 2005). The bioactivities of polysaccharides can be affected by many factors including chemical components, molecular weight, structure, conformation, even the extraction and isolation methods.

The emulsification activity of the exopolymer is determined by its strength in retaining the emulsion for a certain period of time. The presence of acetyl group imparts somewhat hydrophobicity to the EPS that might contribute to its emulsifying capacity and surface active property (Ashtaputre and Shah, 1995).The emulsifying ability of purified EPS was quite comparable to that of other commercially available gums. Therefore, it might be concluded that purified EPS would be a potential bioemulsifier with significant emulsifying activity. Sutherland (2001) reported several polysaccharides from Gram-negative bacteria that have been commercialized but currently only a limited number are excellent viscosifying or suspending agents with high stability under a range of pH and temperature conditions. Xanthan and pullulan produced by *Xanthomonas campestris* and *Aureobasidium pullulans* have been most widely used in the food and other industrial fields, because of their high gelling and emulsion stabilizing functions.

DPPH radicals have been widely used as a model system to study the scavenging activity of various natural compounds. The colour change of DPPH from purple to yellow at absorbance 517 nm decreases due to the formation of DPPH-H through donation of hydrogen by antioxidants. The polysaccharide exhibited good antioxidant activities and could be a potential source of antioxidants for food supplements or ingredient in pharmaceutical industry. The DPPH activity may be attributed to its hydroxyl group and other functional groups in the polysaccharide, such as –COOH, C=O and -O- which can donate electrons to reduce the radicals to a more stable form, or react with the free radicals to terminate the radical chain reaction (Sakanaka *et al*.2005)

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