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Morphological and Biochemical Characterization of Exopolysaccharide Producing Bacteria Isolated from Dairy Effluent

Indira M[1], Venkateswarulu T.C[1], Chakravarthy K[1], Ranganadha Reddy A[1], John Babu D[1], Vidya P Kodali[2*]

[1]*Department of Biotechnology, Vignan University, Vadlamudi, Guntur-522213, India.*
[2]*Department of Biotechnology, Vikrama Simhapuri University, Nellore-524001, India.*

Abstract
Microbial Exopolysaccharides are widely used as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents in food, pharmaceutical industries. Many bacteria possess an ability to synthesize and excrete Exopolysaccharides. The work was aimed to isolate and characterize the EPS producing bacteria from dairy effluent. Eight different strains are isolated by serial dilution and are screened for EPS activity. Among the isolates, 3 and 5 are found to be potential for EPS production. The morphological and biochemical characteristics of isolates 3 and 5 were studied.

Key words: Exopolysaccharides, Biofilms,Lubricants, suspending agents

INTRODUCTION

A biofilm is an assemblage of microbial cells that is irreversibly associated with surface and enclosed in a matrix of primarily polysaccharide material (EPS) (Rodney M. Donlan 2002). EPS molecules are regarded as the major factor influencing the microbial biofilm formation process (K. Czaczyk and K. Myszka, 2007). These exopolysaccharides are high molecular weight polymers composed of sugar residues secreted by microorganisms into the surrounding medium.

Biofilms are found in every type of environment, both natural and anthropogenic origin. Their development is conditioned by the presence of water, nutrients and oxygen (for aerobic bacteria). Pathogenic bacteria often form the biofilm in the human body, for example in the lungs, urinary or genital tract. In recent year's research have been focused on antibiofilm compounds particularly in medicine and health care. Anti-adhesion compounds have gained a lot of attention in controlling biofilm formation includes milk constituents, plant derived products, low molecular weight chitosan, polymers, dietary constituents, and furanone compounds (Essawi T et.al 200). Various chemicals have been tested for their antibiofilm activities. Unfortunately, those chemicals cannot be used as drug molecules to treat the diseases associated with the biofilm. The alternative to the chemical antibiofilm agents is natural source. Plant derived molecules have been found potential applications in pharmaceutical industry (Abraham et.al 2012).

EPS may account for 50% to 90% of the total organic carbon of biofilms and can be considered as the primary matrix material of the biofilm. The difference in the quantity of the biofilm EPS is due to its vary in chemical and physical properties, but it is primarily composed of polysaccharides (Fleming et.al 2000). These polysaccharides include intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confers the anionic property (Sutherland 2001).

Owing to the wide diversity in composition, exopolysaccharides have found multiple applications in various food and pharmaceutical industries. Polysaccharides from microbial origin also have been reported to have potential therapeutic applications (Vincent et al., 2000). Recently, a lot of attention is being paid on the microbial exopolysaccharides (EPSs) due to their health benefits and hence, they have been treated as highly potential molecules (Patricia Ruas-Madiedo et al, 2002, De Vuyst et al 1999).

The exopolysaccharides of some strains of lactic acid bacteria, e.g., *Lactococcus lactis* subsp. *cremoris*, contribute a gelatinous texture to fermented milk products (e.g., Viili), and these polysaccharides are also digestible. Capsular exopolysaccharides can protect pathogenic bacteria and contribute to their pathogenic. Animal pathogenic bacteria such as *Streptococcus pneumoniae, Streptococcus agalactiae*, plant pathogenic bacteria such as *Xanthomonas compestris, Sphingomonas elodea* (Sutherland, 1999) and strains of generally regarded as safe (GRAS), probiotic bacteria (intestinal microbes) and food grade bacteria such as lactic acid bacteria (LAB), dairy propionibacteria, bifidobacteria (Cerning, 1995, De Vuyst et al., 1999) and various Bacillus strains for eg. *B. licheniformis* (Larpin et al., 2002), *B. thermoantarcticus* (Manca et al., 1999) produce EPS in substantial quantities that can fulfill the market needs. EPSs have been proved to show important health benefits like antioxidant (Vidya Prabhakar & Sen, 2008) cholesterol lowering (Welman & Maddox, 2003), antitumor (Hosono et al, 1997), antiviral and immunomodulatory activities (Arena et al., 2006) . EPSs from probiotic bacteria has also showed antiulcer activity, Nagaoka et al., (1994)

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade procured from Strata gene, SRL and Sigma. Media components were purchased from Hi Media Laboratories. All glassware used was of borosil.

Sample collection & Isolation of pure cultures

Water sample was collected from the diary effluent of Sangam Diary located in the Vadlamudi region of Guntur (Dt), Andhra Pradesh. 1 ml of diary effluent was added to 10 ml of sterile distilled water. Mix vigorously and take 1 ml from this tube, add to another tube containing 9 ml of sterile water to get the dilution of $10^{-1.}$ From this tube 1ml was taken and added to another tube containing 9 ml of sterile water to get the dilution of 10^{-2}. This serial dilution procedure was repeated up to 10^{-9} dilutions. From each dilution 0.1 ml was placed on nutrient agar medium by spread plate method and the plates were incubated at 37°C for 24h to allow microbial growth. Colonies with different morphology were selected, pure cultures were developed on nutrient agar plates and stored at 4°C

Screening Method for Biofilm production

The procedures of (Christensen et al., 1982) and (Sujana et al 2013) were used for screening biofilm by tube method. Nutrient broth medium with 1% glucose was prepared and inoculated with isolated colonies. The inoculated tubes were incubated for 24h on shaker at 37ºC for growth of the organism. Next the tubes were decanted and washed with phosphate saline buffer (PSB) to remove planktonic bacteria and were allowed to dry. The dried tubes were stained with 0.1% crystal violet solution and excess stain was removed using distilled water. The tubes were dried in an inverted position and observed for biofilm production.

Morphological & Biochemical Characterization

The isolated strains were identified by morphological and biochemical characterization according to Bergey's Manual of Determinative Bacteriology (Krieg and Holt 1984).

Morphological features were identified by growing the isolated cultures on EPS medium and Gram staining was performed for each isolate. Different Biochemical tests were carried out includes Indole, Voges Proskauer, Citrate utilization tests, catalase test, starch hydrolysis, H_2S production test, Urease test and Carbohydrate fermentation of various sugars (Cappucino G and Natalie Sherman 1998).

Growth Curve for Isolated Strains

The medium was prepared for the two strains and sterilized by autoclaving at 121°C for 15 min. After cooling, 5% inoculum was added to the broth and incubated at 37ºC for 48 h. For every 2hrs sample was collected and optical density was measured at 600 nm. Microbial growth was measured up to 48 h and the values are tabulated to plot the growth curve.

EPS Production

The EPS production medium consists of Glucose – 5g, Peptone – 5g, Yeast extract – 3g, $KH_{2P}PO_4$- 3g, $MgSO_4$ - 0.2g, distilled water -1000ml. The isolated colonies were inoculated into this medium and incubated for 24-48 hours at a speed of 120rpm for the production of exopolysaccharide. After incubation the cell free supernatant was collected by centrifugation at 10,000 rpm for 10 min. EPS is precipitated by adding cold ethanol to the supernatant in the ratio of 1:3. After addition of cold ethanol a dense mass of EPS was settled at the bottom of the flask.

EPS Estimation

The EPS was estimated for the carbohydrate content using phenol sulfuric acid method. In this method standard sugar solution (glucose) 1µg/ml concentration was prepared for estimating the samples in test sample. From this the varying volumes such as 10µl, 20µl, 30µl… 100µl was taken in different test tubes. Next the samples containing EPS was prepared by mixing with distilled water and then specific volumes were taken into different test tubes. Each tube volume was made up to 1000µl using distilled water. To each of the standard and test sample tube 1ml of 80% phenol was added followed by 5 ml of concentrated sulfuric acid and allowed to stand at room temperature for 10 min. Then tubes were kept in water bath at 30ºC for 20 min and were cooled to room temperature and observed OD at 490 nm (Dubois et al., 1956)

RESULTS AND DISCUSSION

Screening for Biofilm production by Tube method

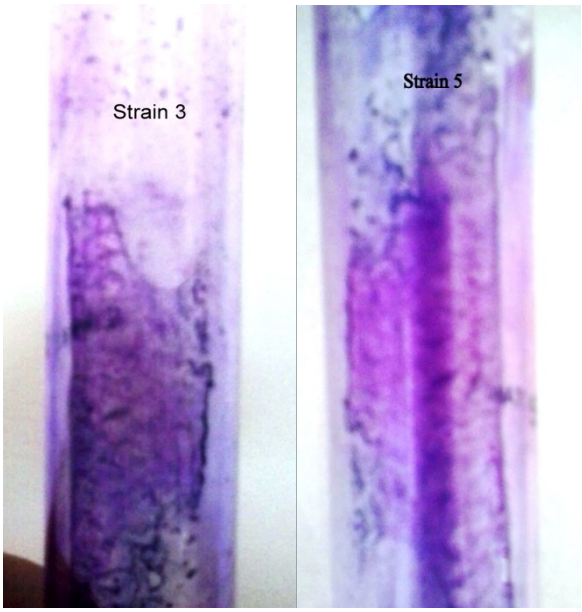

Figure 1: Biofilm production for strains 3 and 5 by tube method.

From serial dilution procedure 8 colonies with different morphology were selected. These colonies were screened for biofilm production. The tube method is more suitable for detecting biofilm producers. Biofilm formation was considered positive when a visible biofilm lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. This biofilm line was stained with 0.1% crystal violet solution. In this study the isolates were completely stained with 0.1% crystal violet solution, among those two isolates are strong producers of biofilm.

Morphological & Biochemical Characterization

Morphological features were observed for the isolates grown on Nutrient agar medium. Strain 3 and 5 were bacilli in shape and are Gram positive. The strain 3 is white, mucous in nature where as strain 5 is creamy yellow in color. These are represented in Table 1.

Strains 3, 5 showed positive for Catalase test and negative for Indole test, Citrate utilization test, and Hydrogen sulfide test. Vogues Proskaeur test, Urease test were positive for strain 3 whereas negative for strain 5. Oxidase test is negative for strain 3 and positive for strain 5. The results were represented in the Table 1.

In Carbohydrate fermentation Strains 3, 5 showed positive result for Fructose and Glucose where as negative for Lactose and Dextrose. The results were represented in the Table 1.

Table 1: Morphological and Biochemical characterization

S. No		Strain 3	Strain 5
Morphological features			
1	Gram staining	Gram positive	Gram positive
2	Shape	Bacilli	Bacilli
3	Color	White mucous	Creamy Yellow
Biochemical tests			
1.	Catalase Test	Positive	Positive
2.	Indole Test	Negative	Negative
3.	Citrate Utilization Test	Negative	Negative
4.	Vogues Proskaeur Test	Positive	Negative
5.	Starch Hydrolysis	Negative	Positive
6.	Hydrogen Sulfide Test	Negative	Negative
7.	Urease Test	Positive	Negative
8.	Oxidase Test	Negative	Positive
9.	Carbohydrate Fermentation a. Lactose Fermentation b. Fructose Fermentation c. Dextrose Fermentation d. Glucose Fermentation	 Negative Positive Negative Positive	 Negative Positive Negative Positive

Ethanol Precipitation

The supernatant containing exopolysaccharides were precipitated by adding 3 volumes of cold absolute ethanol and stored overnight at 4ºC. Finally, precipitate was observed which shows the presence of EPS. A dense mass of EPS is settled at the bottom.

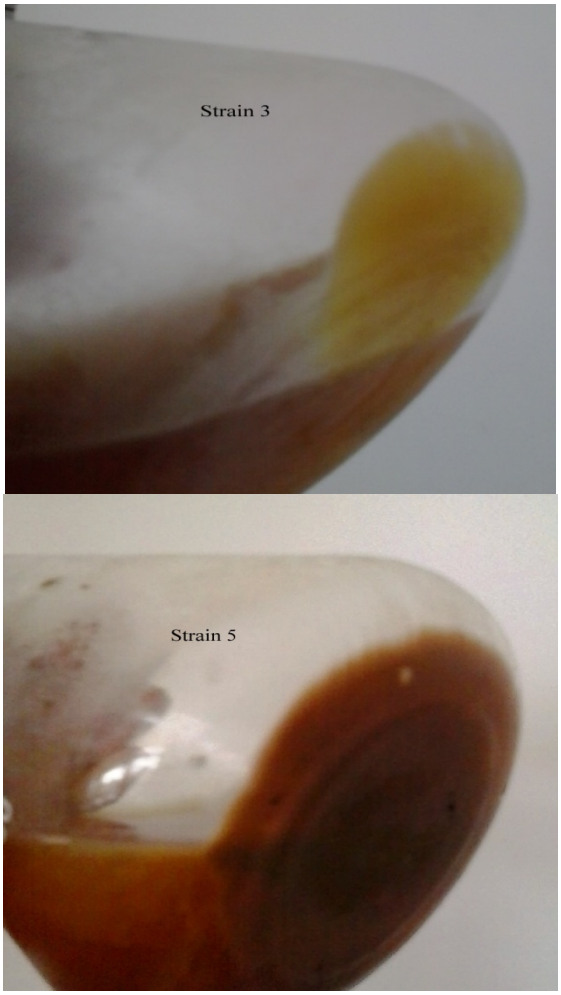

Figure 2: Images showing dense mass of EPS from Strain 3 & 5 settled at the bottom

CONCLUSIONS:

EPS producing bacteria were isolated from diary effluent and two isolates showed the positive response for exopolysacharide production. The maximum concentration of EPS by isolate 5 was found to be 132µg/ml at 24 hrs. The isolates were identified by morphological and biochemical tests.

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