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CHARACTERIZATION OF POTENT EXOPOLYSACCHARIDE PRODUCING BACTERIA ISOLATED FROM FRUIT PULP AND POTATO PEELS AND ENHANCEMENT IN THEIR EXOPOLYSACCHARIDE PRODUCTION POTENTIAL

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ABSTRACT

Exopolysaccharides (EPS) are environment friendly natural polymers secreted by microorganisms in the surrounding medium. Due to the presence of unique structural composition, EPS shows diverse applications such as in food formulations, pharmaceutical, cosmetics industry, etc. In the present investigation for the isolation of EPS producing bacteria, 30 samples comprising 14 fresh fruits, 14 spoiled fruits, 1fresh potato peels and 1spoiled potato peels were inoculated on 4 different solid media containing sucrose, glucose and galactose as carbohydrate substrate and MRS agar (MRSA). The bacteria producing higher exopolysaccharide were screened among bacteria isolated from fresh and spoiled fruits and potato peels. Total 105 EPS producing colonies were obtained, out of which 17 isolates, which showed viscosity more than 0.1060m.Pa.S^{-1} were selected. Based on 16S rRNA gene sequence analysis, 17 isolates, which represents 8 genera, out of this 40% isolates belong to genera *Bacillus*. Among the carbohydrate studied sucrose proved to be the choice of the isolates for EPS production as compared to other sugars. When SYE and EPS media were compared, EPS medium was found to be the best except for the isolate SR17. The selection of EPS medium as medium of choice, sucrose as source of sugar and its 5% concentration in the medium enhanced EPS production as high as 440% higher as compared to SYE medium.

Keywords: Exopolysaccharide, isolation, identification, fresh and spoiled fruits, potato peels, viscosity

INTRODUCTION

Exopolysaccharides (EPS) are key components of biofilm, which determines physico-chemical, and biological properties of biofilm formation. The exopolysaccharide play an important role in allowing microbes to live continuously at high cell densities in a stable mixed population of biofilm communities. Bacteria that produce exopolysaccharides have been identified from a variety of ecological niches and it is apparent that precise role played by exopolysaccharides is dependent on the natural environment, from which they are isolated. Ability to produce exopolysaccharides is a direct and logical response to selective pressures in that natural environment (**Weiner, 1997**).

EPS are used in food, textile, detergents, beverages, pharmaceutical (**Nwodo *et al*., 2012**), biotechnology, agricultural, paper, paint, cosmetic, medical and petroleum industries (**Quesada *et al*., 1993**) drug delivery (**Sosnik, 2014**), cancer therapy (**Zhang *et al*., 2013**) and in the formulation of the culture media due to their unique structure and physical properties. Some of these applications include their use as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents (**Sutherland, 1998**). Due to their bioactive role and their extensive range of applications increasing attention is being paid to the production of these biomolecules. A new approach to encounter EPS with novel properties might entail investigating different environments. Isolation of bacteria from fresh vegetables and fruits, ready to eat salads (**Trias *et al*., 2008a**), citrus fruits waste (**Marína *et al*., 2007**), vegetable salads (**Wright *et al*., 1976**), processed vegetables (**Franzetti *et al*., 2005**) dried fruits (**Askari *et al*., 2012**) have been done but with the importance of these bacteria in food spoilage (**Nguz *et al*., 2005**), pathogenicity (**Palacio *et al*., 2012**), antibacterial activity (**Askari *et al*., 2012**) and production of organic acids (**Aslim *et al*., 2005; Trias *et al*., 2008b; Mridul and Preethi, 2014**). To the best of our knowledge, no data are available for the isolation of EPS producers from potato peels and fruit pulp. Thus, in this context attempts were done to isolate EPS producing bacteria from potato peels and fruits. Work was also carried out to enhance their EPS production potential.

MATERIAL AND METHODS

Screening and isolation of exopolysaccharide producing bacteria

From the local market 14 types of fresh fruits and potato peels were procured and used for isolation of EPS producing bacteria. One gram pieces of fresh fruit and potato peels were homogenized in 10 ml sterile peptone broth and incubated at 28±2 °C for 24 h. After incubation, 10 folds serially diluted suspensions were prepared and 0.1 ml aliquots were spread on yeast extract (YE) agar plates containing glucose, galactose or sucrose as carbon source. Fruits were stored at 28±2 °C and were allowed to spoil and after every 15 days, isolation was performed up to two months as mentioned for fresh samples. Plates were incubated for 48 h at 28±2 °C. Mucoid colonies were screened and re-streaked on another agar plate with the same composition to obtain a pure culture. de Man, Rugosa and Sharp (MRS) medium **(Atlas and Parks, 1997)** was also used for isolation. Isolated cultures were characterized on the basis of colony morphology, microscopic observations and routine biochemical tests. The identification work was done according to the methods described in Bergey's Manual of Systematic Bacteriology **(Brenner *et al*., 2005; Vos *et al*., 2009)**. Selected cultures were further identified by 16S rRNA partial gene sequence analysis.

Ehancement in EPS production

EPS production was carried out in 250 ml Erlenmeyer flasks containing 100 ml of medium. The base medium contains (g/L): yeast extract 1.0; $MgSO_4$ 0.5; KH_2PO_4 1.0 and glucose/galactose/sucrose/mannitol 30.0 as a source of carbohydrate. The pH of all four media was adjusted to 7.0. All media constituents were sterilized at 121°C for 20 min except carbohydrates. All carbohydrates were filter sterilized and added to the sterile base medium under aseptic condition. All the test flasks were inoculated with actively growing 15 hours old 10% v/v culture, having cell count of 10^7 cells/ml. The flasks were incubated on a rotary shaker at 28±2 °C for 5 days. Viscosity and production were checked after every 24 h. A comparative study for production of EPS was also performed using EPS medium (g/L): caseine hydrolysate 15.0, sodium acetate 12.0, K_2HPO_4 10.0, yeast extract 5.0, sodium chloride 2.5, L-cystine 0.5

and sucrose/glucose/galactose 50.0, pH 7.2 **(Atlas and Parks, 1997)**.Influence of carbohydrate on EPS production was studied by supplementing medium with 30g/L glucose/galactose/sucrose/mannitol. Further concentration of sucrose in the medium was also optimized by providing 30,50 and 70g/L sucrose.

Recovery of EPS

Cells were harvested from the respective broth medium by centrifugation at 10,000 g for 10 min. After centrifugation, three volumes of chilled acetone was added into the supernatant and stored overnight at 4 °C. Precipitated material was collected by centrifugation (10 min at 10,000 g) and the pellets were dried at 65 °C till constant weight. Dry weight was measured from all the dried pellets **(Razack *et al*., 2013)**.

RESULTS AND DISCUSSION

All the colonies on 4 media were observed, based on difference in colony morphology 105 bacterial isolates were selected as EPS producers as they showed slimy gummy colony morphology (Table 1). Out of 105 isolates, 68 bacterial isolates were from fresh samples and 37 from spoiled samples. Medium with sucrose resulted in growth of nearly 50% of the total isolates. As compared to spoiled fruits or potato peels, fresh samples showed more variety of EPS producers except watermelon sample. In case of sucrose containing medium 6 types of colony reappeared in spoiled samples, whereas in glucose containing medium 3 samples showed reappearance of single isolate. In case of galactose and MRSA none of the samples showed the presence of isolates isolated from fresh sample. During the course of two months storage out of 68 isolates which were isolated from fresh samples 14% colony reappeared in spoiled samples. A number of bacterial isolates decreased during storage of samples, due to drying of samples as well as the antagonists effect of the survived organisms.

Table 1 Isolates and source of isolation

Source	Number of morphologically different colonies picked from yeast extract agar with different cabohydrates and MRS agar (MRSA)							
	Sucrose		Glucose		Galactose		MRSA	
	Fresh	Spoiled	Fresh	Spoiled	Fresh	Spoiled	Fresh	Spoiled
Potato peels	3(1)	1	3	1	2	1	-	-
Orange	4(2)	1	2	1	1	-	-	-
Apple	1	1	2(1)	1	2	-	-	-
Chikoo	3(1)	1	2	1	2	-	-	-
Pomegranate	2	-	3(1)	1	1	-	-	-
Strawberry	3	1	2	1	1	1	-	-
Grapes	2(1)	3	1	-	1	-	-	-
Pineapple	2	1	1	1	1	-	-	-
Sweet lime	2	-	1	-	1	-	-	-
Guava	1	1	1	-	-	2	-	-
Berry	1	1	1	1	1	-	-	-
Lichee	3	2	-	1	-	-	-	1
Custard apple	2(1)	3	1	1	-	-	-	1
Mango	3	-	2(1)	2	1	1	-	-
Watermelon	-	1	-	-	-	1	-	-
Total	**32**	**17**	**22**	**12**	**14**	**6**	**-**	**2**

( ) = reappearance of isolate after 60 days of storage.

Amongst the isolates, 87% isolates were gram positive and 13% were gram negative, 42% showed pigmentation and 44% colonies were opaque. The selected 17 cultures, which gave EPS production more than 0.9 g/L were represented by 83% gram positive bacteria and 17% gram negative bacteria. Biochemical test and sugar utilization pattern revealed that all the isolates showed casein hydrolysis, and oxidative test positive; where as, ammonia production, deaminase test, decarboxylase test and fermentative test were negative. Out of 17 isolates, 6 isolates were able to produce only acid in all 21 sugars. A similarity index of 17 isolates based on biochemical test and sugar utilization pattern is shown in Table 2.

Table 2 Similarity index based on biochemical characteristics and sugar utilization pattern.

	% Similarity																
	Isolates																
Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	1	0.55	0.57	0.64	0.37	0.71	0.6	0.6	0.62	0.37	0.6	0.55	0.62	0.46	0.6	0.46	0.57
		1	0.84	0.86	0.55	0.66	0.80	0.86	0.84	0.73	0.68	0.77	0.77	0.75	0.86	0.66	0.68
zz2																	
3			1	0.93	0.51	0.81	0.95	0.97	0.91	0.68	0.91	0.82	0.88	0.82	0.93	0.68	0.80
4				1	0.53	0.64	0.95	0.93	0.97	0.6	0.88	0.84	0.86	0.82	0.95	0.66	0.75
5					1	0.37	0.55	0.53	0.51	0.6	0.53	0.55	0.57	0.57	0.57	0.46	0.44
6						1	0.57	0.64	0.62	0.46	0.62	0.62	0.57	0.6	0.64	0.57	0.55
7							1	0.93	0.88	0.57	0.86	0.77	0.84	0.82	0.88	0.71	0.75
8								1	0.93	0.64	0.93	0.84	0.86	0.84	0.95	0.73	0.77
9									1	0.57	0.86	0.84	0.84	0.77	0.93	0.71	0.75
10										1	0.57	0.6	0.6	0.66	0.6	0.55	0.48
11											1	0.8	0.84	0.77	0.93	0.68	0.75
12												1	0.75	0.68	0.84	0.68	0.71
13													1	0.75	0.82	0.57	0.68
14														1	0.80	0.62	0.66
15															1	0.68	0.77
16																1	0.66
17																	1

Based on biochemical test and sugar utilization characteristics out of 17 cultures, none of the culture showed 100% similarity. The maximum similarity observed was 97% amongst culture 3 and 8 (C1A and SRA 8), 4 and 9 (SRA 1 and SRA 5), 12 cultures showed more than 80% similarity, out of these 6 cultures showed more than 90% similarity. The similarity index of the isolate (SRA 17) was 37% to 60%; whereas, that of isolate 10 (SRA 15) was 37% to 73%. This data clearly shows the wide diversity of EPS producers isolated from the selected samples.
Based on 16S rRNA partial gene sequence, of selected 17 isolates, *Bacillus subtilis* comprised 25% of the isolates and if genus *Bacillus* is considered, it comes out to be near 60% of the total selected isolates. Remaining 40% are represented by 7 different genus (Table 3).

Table 3 Identification of selected isolates based on 16S rRNA sequencing

Source	Isolate code	Isolate identified	GenBank accession number
Potato peels	SRA 18	*Escherichia coli*	KTG30840
Sweet lime	SRA 6	*Bacillus tequilensis*	KM406457
Chikoo	C1A	*Bacillus subtilis* subspecies *spizizani*	KM406418
Custard apple	SRA1	*Bacillus soneresis*	KM406421
Spoiled orange	SRA 17	*Xanthomonas campestris*	KTG30839
Spoiled custard apple	SRA 9	*Lactobacillus fermentum*	KM406420
Pomegranate	SRA 4	*Bacillus* species	KM406429
Sweet lime	SRA 8	*Bacillus methylotrophicus*	KM406458
Pomegranate (i)	SRA 5	*Bacillus licheniformis*	KM406430
Spoiled guava	SRA 15	*Bacillus subtilis*	KP178604
Chikoo 2	C2A	*Bacillus subtilis* species	KM406419
Spoiled lichee	SRA 3	*Leuconostoc pseudomestenteroids*	KM406428
Mango	SA 100	*Enterobacter cloacae*	
Orange	SRA 2	*Bacillus amyloliquefaciens*	KM406427
Spoiled mango	SRA 7	*Bacillus* species	KM406459
Potato peels 1	SRA 14	*Micrococcus* species	KP178617
Orange	SRD 1	*Panebacillus polymyxa*	KJ830755

Carbohydrates are the major component of the cytoskeleton and an important nutritional requirement for the growth and cell development. Production of EPS was studied using YE broth containing 3% sucrose, glucose, galactose and mannitol as a source of carbon. In this study, it was found that irrespective of selected isolates, production of EPS was more than at least 2 fold higher in sucrose containing media as compared to any other sugar used in the media except isolates SRA 3 and SRA 18 (Fig. 1). EPS dry weight production ranged between 0.9 to 5.4 g/L in sucrose containing medium; whereas, it was 0.06 to 3.79, 0.03 to 0.99, 0.02 to 0.6 g/L in glucose, galactose and mannitol containing medium respectively.

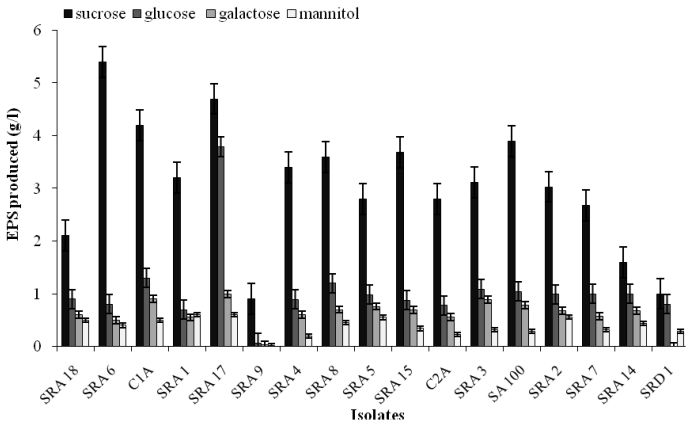

Figure 1 Influence of different sugars on EPS production.

Influence of sucrose concentration on EPS production is shown in Fig. 2. EPS production was 1.06 to 3.8 and 1.2 to 3.5 fold higher in the presence of 5% sucrose as compared to 3% and 7% sucrose in the medium respectively.Variation of EPS production by different isolates in types of sugar used and the concentration of sucrose added in the medium indicates the metabolic and physiological diversity of the isolates and their preference for the sugar as well as concentration of sucrose in the medium.

Comparative study of EPS production between YE broth containing 5% sucrose and EPS medium was also performed. It was observed that constituents of EPS medium gave a higher production of EPS in comparison to Yeast Extract broth except for isolate SRA17. The differences of EPS produced in the EPS medium as compared to SYE were as small as 1.03 and as high as 4.4 fold (Fig.3). The selection of EPS medium as a medium, sucrose as a source of substrate and 5% concentration of sucrose resulted in more than 4.4 fold increase in the EPS production. Amongst selected isolates under the experimental conditions, *Bacillus* species gave higher EPS production as compared to species of *Xanthomonas* and *Leuconostoc* obtained in this study, which are reported to be EPS producer.

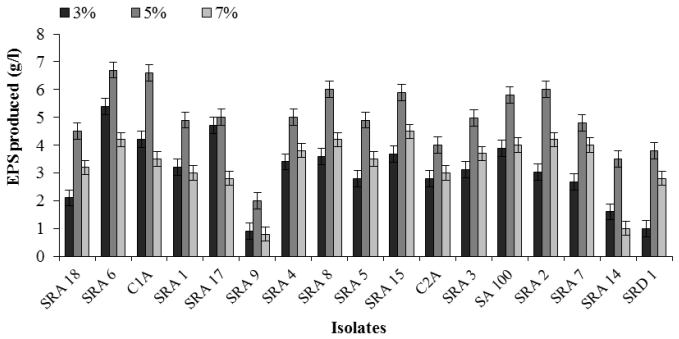

Figure 2 Influence of different sucrose concentration on EPS production.

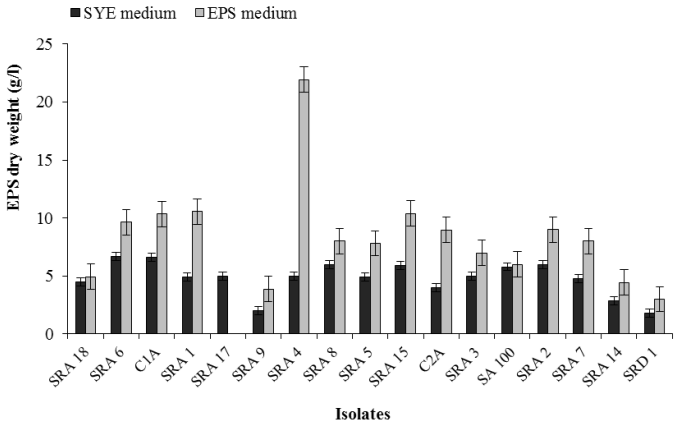

Figure 3 Influence of different medium on EPS production.

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