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Isolation of Biotechnologically Important Exopolysaccharide Producing Bacteria from Different Environments

Pijush Kumar Das, Konok Kanti Das, Tania Hossain[1] and Mihir Lal Saha*

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

Exopolysaccharides (EPSs) are carbohydrate polymers that occur naturally and are excreted by some microorganisms. The microbial EPS has a number of biotechnological uses in paints, textiles, cosmetics, medicine, etc. Interest in searching for novel EPS has increased due to the broad range of applications of EPS. Therefore, this study focused on isolating, screening, and identifying the most potent EPS-producing bacteria from different habitats. A total of 10 different samples were collected from in and around Dhaka University Campus. After initial screening, 88 distinct bacterial colonies were considered to be producing exopolysaccharides on the basis of thick mucoid colonies on nutrient agar medium. The findings showed that EPS-producing bacteria are widely dispersed throughout various habitats. Out of 88 isolates, 20 better isolates were finally selected for extensive study. The isolated Gram-positive bacteria belonged to the genera *Micrococcus*, *Streptococcus*, and *Bacillus*. While Gram-negative bacteria were identified as *Proteus myxofaciens*, *Chryseobacterium* sp., *Klebsiella pneumoniae* subsp. *Ozaenae*, *Edwardsiellaictaluri* and *Acinetobacter* sp. The EPS production ranged between 0.55 ± 0.04 and 2.21 ± 0.06 g/l. *Bacillus subtilis* (P11) was identified as the most promising isolate for EPS production, yielding the highest amount at 2.21 ± 0.06 g/l. The current study's findings showed that these local isolates could produce important exopolysaccharides and might be used in various biotechnological aspects.

Introduction

Microbial polysaccharides called exopolysaccharides (EPSs) are produced by microorganisms and released as either insoluble or soluble polymers. EPSs are crucial for intercellular communication, microbial cell attachment to solid surfaces and cell defense (Escárcega-González et al. 2018). For many years, plants and seaweeds have been used to produce polysaccharides; however, over the past two decades, microbial polysaccharides, particularly exopolysaccharides (EPS) produced by bacteria, have gained industrial significance and attracted growing attention (Sanalibaba et al. 2016).

*Author for correspondence: <sahaml@du.ac.bd>. [1]Centre for Advanced Research in Sciences, University of Dhaka, Dhaka-1000, Bangladesh.

EPSs are primarily classified into two types, such as heteropolysaccharides and homopolysaccharides. At the same time, EPSs are complex molecules, branched or unbranched and consist of long chains of different sugars (Mende et al. 2016).

Microbial exopolysaccharides are important biomaterials with a wide range of applications in industries such as food additives, textiles, brewing, pharmaceuticals, cosmetics, detergents, dredging and adhesives due to their diverse physicochemical properties and varied functions (Ahmad et al. 2015). Over the past few decades, interest in microbial EPS production has grown significantly, owing to their remarkable properties and the relatively simple process for extracting and purifying them from the culture medium (Singha 2012). The most widely employed source of EPS is bacteria because they multiply quickly, produce loosely linked mucoid layers that are easily isolated from cells using any EPS isolation techniques, and are nontoxic and environmental friendly (Angelin and Kavitha 2020).

Extracellular polysaccharide-producing microbes can be isolated from various ecological niches, including wastewater, organic waste, compost and marine environments, which serve as significant sources (Singha 2012). Nowadays, many studies have been focused on isolating novel EPS-producing bacteria from diverse environments. Numerous bacteria from various genera that produce EPS have been previously identified, *viz*., *Bacillus* sp., *Agrobacterium* sp., *Lactobacillus*, *Pseudomonas*, *Micrococcus luteus*, *Xanthomonas campestris*, *Klebsiella pneumoniae*, etc. (Dwivedi et al. 2018, Wang et al. 2017). These microbial biopolymers have a wide range of applications across various biotechnology sectors, including pharmaceuticals, textiles, cosmetics, food and other industries (Alsharabasy et al. 2016).

Considering these facts and their wide range of applications, the current study focuses on the isolation, screening, characterization, and identification of indigenous bacteria that produce EPS from different sources of Dhaka University campus and its nearby areas.

Materials and Methods

Various types of samples, such as garden soil, compost and cocopeat (made from coconut husk fibers), dumpsite soil and organic waste, wastewater, canteen basin biofilm and yogurt were collected from different locations of Dhaka University and adjacent areas. Soil samples were taken from the topmost layer of the garden, whereas wastewater samples were collected from 10 cm below the water surface. Sterile plastic polybags and bottles were used to collect samples. Samples were properly labeled after collection and transported into the lab, where a pH meter (HANNA HI 8424, Romania) was used to determine the samples' pH. All samples were stored at 4°C until further study.

Following the serial dilution plate technique, bacterial isolates have been grown on Luria Bertani Agar (LBA) and Nutrient Agar (NA) media. The plates were incubated for 72 hrs at 37°C. Isolates that formed dense mucoid colonies were chosen and purified

using the streaking method on newly made nutritional agar media to obtain different colonies (Nwosu et al. 2019). Exopolysaccharide-producing bacteria were selected mostly based on the glistening, slimy appearance of mucoid colonies on nutrient agar plates. Further EPS-producing bacteria was confirmed by observing the formation of a string longer than 5 mm when lifted with a loop, indicating a positive result (Fang et al. 2004). Various staining techniques, such as Gram staining and simple staining techniques were used for characterization. Important biochemical tests were conducted. Gram-negative bacteria were identified using the WHO Manual (Krieg and Holt 1984) and Bergey's Manual of Systematic Bacteriology, Vol. 1 (Krieg and Holt 1984), while Gram-positive bacteria were identified based on the guidelines provided in Bergey's Manual of Systematic Bacteriology, Vol. 2 (Sneath et al. 1986).

The EPS extraction was carried out according to Gangalla et al. (2021) with some modifications. To do this, selected bacterial isolates were cultured in 50 ml broth medium as inoculum preparation, comprising the following ingredients: 0.5% peptone, 0.3% beef extract and 2% sucrose at pH 7.0. In that 50 ml broth, one loopful of bacterial inoculum was inoculated and kept on an orbital shaker at 37°C (New Brunswick Excella E25 Incubator Shaker, USA) at 100 rpm for 24 hrs. For EPS production, 250 ml Erlenmeyer flasks were used with 50 ml of production media modified with 2% sucrose (Liu et al. 2010). The medium consists of the following components (g/l): beef extract 1.00, yeast extract 0.6, K_2HPO_4 3.00, NaCl 1.00, KH_2PO_4 3.00, $FeSO_4.7H_2O$ 0.001 and $MgSO_4.7H_2O$ 0.20. One ml of the inoculum was aseptically transferred to a conical flask that contained the production medium. The flasks were incubated for 72 hrs at 100 rpm at 37°C in an orbital shaker (New Brunswick Excella E25 Incubator Shaker, USA).

To extract EPS, the culture broth was subjected to centrifugation at 9,000 rpm for 10 min at 4°C using a centrifuge (HERMLE Labortechnik GmbH, Germany). After collecting the supernatant, it was mixed with two volumes of chilled ethanol and incubated for 24 hrs at 4°C. After that, the frozen solution was centrifuged for 15 min at 4°C at 9,000 rpm. The collected pellet was dissolved in distilled water and mixed with an equal amount of chilled ethanol. The final pellet was obtained by centrifuging the solution once again for 15 min at 4°C and 9,000 rpm. The collected pellets were dried for 2 hrs at 100°C in a dry heat oven (EYELA NDS-450D, Japan). After drying, the pellets were weighed in an electronic balance (Electronic Precision Balance, model: EK 600i-600), and the obtained results were recorded carefully. The amount of EPS produced by the bacterial isolates was estimated in triplicate.

Results and Discussion

The pH of the collected samples ranged between 4.68 and 7.76 (Table 1). It was found that the yogurt sample had the lowest pH (4.68), whereas effluent from Nazira Bazaar had the highest pH (7.76). Plates showed numerous bacterial counts, and the total bacterial load ranged from 3.8×10^5 to 5.0×10^7 CFU/g or CFU/ml on NA medium 3.2×10^5 to 4.3×10^7 CFU/g or CFU/ml on LBA medium, respectively (Table 1). The maximum

bacterial count (5.0×10^7 CFU/g) was observed on the NA medium from the dumpsite of Jagannath Hall and the minimum (3.2×10^5 CFU/g) bacterial load was found on the LBA medium from the yogurt sample. During this study, the highest number of EPS-producing bacteria (n = 5) was detected in the soil from the Jagannath Hall dumpsite. The findings showed that EPS-producing bacteria are widely dispersed throughout various soil samples, waste habitats, wastewater and dairy products due to the presence of various nutritional substances. According to Santal et al. (2019) and Talbi et al. (2023), soil, dairy products and wastewater were good sources of EPS-producing bacteria.

Table 1. Sampling sites, sample types, pH and bacterial load of the collected samples.

Sl. No.	Sampling site	Samples	pH	Bacterial load (CFU/g or CFU ml) of sample	
				NA	LBA
1	DU Arboriculture center	Compost	7.38	3.8×10^5	4.8×10^5
2	DU Arboriculture center	Cocopeat	5.72	5.2×10^5	7.8×10^5
3	DU Botanical Garden	Soil	6.06	8.1×10^5	7.8×10^5
4	Fazlul Huq Muslim Hall	Soil	6.52	1.52×10^6	1.23×10^6
5	Jagannath Hall Garden	Soil	6.70	1.54×10^6	1.1×10^6
6	Jagannath Hall dumpsite	Soil	7.51	5.0×10^7	4.3×10^7
7	Jagannath Hall dumpsite	Rotten veg.	5.78	6.7×10^5	4.2×10^5
8	INFS canteen	Basin biofilm	7.49	2.76×10^6	2.40×10^6
9	Jagannath Hall shop	Yogurt	4.68	4.8×10^5	3.2×10^5
10	Nazira Bazar	Wastewater	7.76	8.5×10^5	7.5×10^5

DU= University of Dhaka, INFS= Institute of Nutrition and Food Science, veg.= Vegetable, NA= Nutrient Agar, LBA= Luria Bertani Agar.

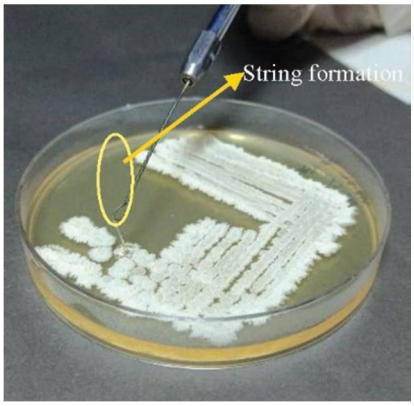

Fig. 1. String test showing positive result of the isolate P11.

Twenty distinct bacterial colonies were identified as exopolysaccharide-producing bacteria. All the isolates were analyzed for further confirmation of EPS producers by the string test (Fig. 1). The results showed that their string length ranged from 6 to 18 mm (Table 2). In a study, Bacosa et al. (2018) found a similar correlation between the development of mucoid colonies and string, suggesting the presence of potent EPS-

producing bacteria. Shukla and Dave (2018) also reported the same type of results. The Fig. 2 showed the results of the string length of the selected isolates. The isolate (P31 and P32) showed the highest string length (18 mm).

Table 2. String test result of the selected bacterial isolates.

Isolates	String length (mm)	Isolates	String length (mm)
P5	15.0	P56	10.0
P6	7.0	P60	8.0
P11	16.0	P61	8.0
P16	14.0	P72	6.0
P20	8.0	P74	7.0
P24	6.0	P76	8.0
P31	6.0	P80	18.0
P38	9.0	P83	18.0
P44	8.0	P85	12.0
P52	6.0	P88	10.0

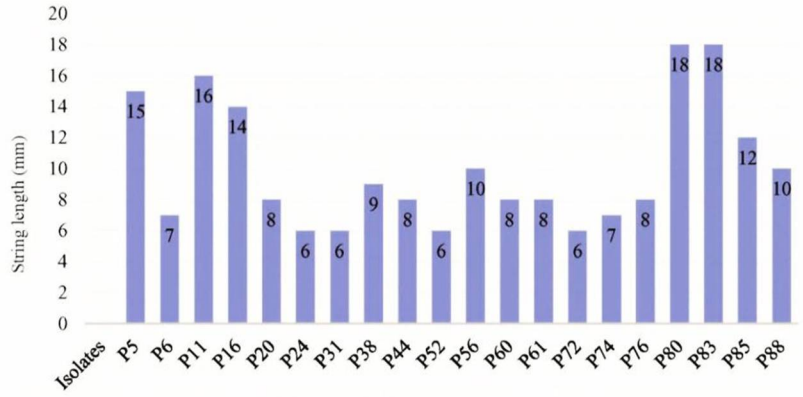

Fig. 2. String length of the selected isolates.

Among 20 isolates, 16 were rod-shaped and 4 were coccus. Ten isolates were Gram-positive, while the remaining bacteria were Gram-negative. All the Gram positive bacteria belonged to the genera *Micrococcus*, *Streptococcus*, and *Bacillus*, while the Gram negative bacteria were members of the genera *Klebsiella*, *Chryseobacterium*, and *Proteus*. Santal et al. (2019) and Afrin et al. (2022) showed that EPS-producing bacteria could be both Gram-positive and Gram-negative and may be rods and round in shape.

Out of 10 Gram positive isolates, there were seven different species under the genus *Bacillus*, including *B*. cereus, *B. subtilis*, *B. alvei*, *B. firmus*, *B. polymyxa*, *B. schlegelii*, and *B. pumilus*, and the other three Gram-positive bacterial isolates were identified as *Streptococcus pyogenes* (2) and *Micrococcus luteus*. Among the Gram-negative bacteria, *Proteus myxofaciens* (1), *Chryseobacterium* sp. (4), *Klebsiella pneumoniae* subsp. *Ozaenae* (3), *Edwardsiellaictaluri* (1), and *Acinetobacter* sp. (1) have been identified. The biochemical characteristics and provisional identification are presented in Table 3. Dwivedi et al.

(2018) and Afrin et al. (2022) mentioned that the majority of EPS-producing bacteria were members of the genus *Bacillus*, which was found to be very similar to the present investigation. *Klebsiella pneumoniae*, *Chryseobacterium*sp., and *Micrococcus luteus* were also found to be EPS producers in accordance with Asker et al. (2014) and Al-Hamdoni (2018).

Table 3. Morphological, biochemical characteristics and provisional identification of the isolated bacteria.

Isolates	Gram's staining	Biochemical tests																Provisional Identification
		Catalase	Oxidase	VP	MR	Hydrolysis of Starch	Hydrolysis of Casein	Pectinase	Citrate	Propionate	Indole	Lecithinase	Protease	Lipase	Urease	Lactose fermentation: Acid	Lactose fermentation: Gas	
P5	-	+	+	+	+	-	-	-	-	+	+	-	+	+	+	-	-	*P. myxofaciens*
P6	+	+	+	+	+	-	+	-	+	+	-	+	+	+	-	-	-	*B. cereus*
P11	+	+	+	+	-	+	+	-	+	-	-	-	+	+	-	-	-	*B. subtilis*
P16	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	*B. alvei*
P20	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	-	*Chryseobacterium*sp.
P24	-	+	-	-	+	-	-	-	+	+	-	+	-	-	-	-	-	*K. pneumoniae* subsp.*ozaenae*
P31	-	+	+	-	-	+	+	+	-	-	+	+	+	-	-	-	-	*Chryseobacterium*sp.
P38	-	+	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	*Chryseobacterium*sp.
P44	+	+	+	-	+	-	-	-	+	+	-	+	-	-	-	-	-	*S. pyogenes*
P52	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	*E. ictaluri*
P56	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	*Acinetobacter* sp.
P60	-	+	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	*K. pneumoniae* subsp. *ozaenae*
P61	-	+	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	*K. pneumoniae* subsp. *ozaenae*
P72	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	*M. luteus*
P74	+	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	*S. pyogenes*
P76	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	-	*Chryseobacterium*sp.
P80	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	*B. firmus*
P83	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	-	*B.polymyxa*
P85	+	+	+	+	+	+	-	+	+	-	-	-	+	-	+	-	-	*B. schlegelii*
P88	+	+	+	+	+	+	+	-	+	-	-	-	+	+	-	-	-	*B. pumilus*

"+" =Positive, **"-"** =Negative. VP = Voges-Proskeur, MR= Methyl Red.

In this investigation, an EPS production test was carried out using a carbon source consisting of a nutritional medium containing 2% sucrose. Nwosu et al. (2019) found that a nutrient medium supplemented with sucrose was an appropriate culture medium for the synthesis of exopolysaccharides. In the present investigation, EPS production by various bacterial isolates using sucrose as a carbon source varied from 0.55 ± 0.04 to 2.21 ± 0.06 g/l (Table 4).

Table 4. EPS production by the isolated indigenous bacteria.

Isolates	Bacteria	EPS production (g/l)	Isolates	Bacteria	EPS production (g/l)
P5	*P. myxofaciens*	1.67 ± 0.04	P56	*Acinetobacter* sp.	0.67 ± 0.04
P6	*B. cereus*	0.57 ± 0.05	P60	*K. pneumoniae* subsp.*ozaenae*	0.82 ± 0.05
P11	*B. subtilis*	2.21 ± 0.06	P61	*K. pneumoniae* subsp.*ozaenae*	0.76 ± 0.02
P16	*B. alvei*	1.74 ± 0.08	P72	*M. luteus*	0.73 ± 0.04
P20	*Chryseobacterium*sp.	0.96 ± 0.06	P74	*S. pyogenes*	0.91 ± 0.03
P24	*K. pneumoniae* subsp.*ozaenae*	0.73 ± 0.03	P76	*Chryseobacterium*sp.	1.14 ± 0.09
P31	*Chryseobacterium*sp.	0.81 ± 0.05	P80	*B. firmus*	1.15 ± 0.05
P38	*Chryseobacterium*sp.	0.80 ± 0.05	P83	*B. polymyxa*	1.12 ± 0.01
P44	*S. pyogenes*	0.97 ± 0.06	P85	*B. schlegelii*	1.48 ± 0.03
P52	*E. ictaluri*	0.55 ± 0.04	P88	*B. pumilus*	1.07 ± 0.06

Among the 20 isolates, many *Bacillus* species were found to be better EPS producers. *Bacillus subtilis* (P11) was identified as the most promising isolate for EPS production, yielding the highest amount at 2.21 ± 0.06 g/l. Razack et al. (2013) mentioned that EPS produced by different bacterial isolates ranged between 0.65 g/l (*Bacillus* spp.) and 3.69 g/l (*Chryseobacterium* sp.). In another study, Hu et al. (2022) mentioned that *Chryseobacterium* sp. could produce 3.24 g/l of EPS. In the present study, the isolated *Chryseobacterium* sp. could produce 0.80 to 1.14 g/l of EPS. In the present study, EPS produced by *Klebsiella pneumoniae* was recorded at 0.82 ± 0.05 g/l. Likewise, Sivakumar et al. (2016) observed that in their investigation, with a yield of 0.879 ± 0.014 g/l, which was found to be similar to the present study. In a different work, Asker et al. (2014) observed that EPS produced by *Micrococcus luteus* was 8.14 g/l, but the present investigation showed only 0.73 ± 0.04 g/l EPS produced by this isolate. During this study it was possible to extract crude EPS and the Fig. 3 clearly demonstrated crude EPS produced by *Klebsiella pneumoniae* subsp. *Ozaenae* (P61).

Fig. 3. Extracted crude EPS from the isolate *Klebsiella pneumoniae* subsp. *Ozaenae* (P61).

The purpose of this study was to isolate better EPS-producing isolates. The quantification of EPS obtained from different isolates showed that *Bacillus subtilis* was the most potent candidate for EPS production and can be applied in various biotechnological fields. EPSs are being utilized in several industries, including pharmaceuticals, medicine, food, cosmetics and textiles. More research using techniques like FTIR, Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (^{1}H NMR) is needed to obtain more structural information on the EPS, which is necessary for studying the application of produced exopolysaccharides in various potential industrial applications in the future.

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