



Characterization of extracellular polymeric substance producing isolates from wastewaters and their antibacterial prospective

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ABSTRACT

Bacteria have the ability of biofilm formation, in which the cells attach to each other within a self-produced matrix of extracellular polymeric substance (EPS). The aim of the present research work was to isolate EPS-producing bacteria from wastewater. Total 21 bacterial isolates were screened for EPS production based on mucoid and slimy colonies. Out of 21 isolates, nine efficient isolates were selected for the production of EPS. These efficient bacterial strains were also checked for their antimicrobial potential against *Salmonella* sp., *Escherichia coli*, and *Klebsiella* sp. The isolates ASA3, H2E7, H2F8, and ASB4 inhibited the growth of *Salmonella* sp., *E. coli*, and *Klebsiella* sp, while isolate ASB5, H2C6, and H2E9 only showed inhibitory effects against *Salmonella* sp. The maximum concentration of EPS (i.e., 17.2 g/l) was produced by strain ASB4 within 3 days of incubation.

1. INTRODUCTION

Today, biopolymers are in great demand for their various applications and extracellular polymeric substance (EPS) production by microorganisms is of great concern now-a-days. [1]. EPSs are secondary metabolites secreted by bacteria and accumulate over the cell surface [2]. The main components of extracellular polymeric substance are polysaccharides and proteins but also include some other macro-molecules [3]. Many microbial EPSs have gum like properties [4]. These microbial extracellular polymeric substances are highly soluble in water but many EPSs are insoluble and not easily separated from the cells [5].

Due to physicochemical and rheological properties, the extracellular polymeric substance is industrially important as they are used in textiles, food, cosmetics, agriculture, packaging industries, and pharmaceutical industries [6,7]. The EPS-producing bacteria have a good capability to convert nutrient into EPS [8]. It mainly consists of two types of groups, i.e., homopolysaccharides and heteropolysaccharides [9]. Some of the microbial exopolysaccharides have commercial applications,

such as dextran, xanthan, pullulan, gellan, and hyaluronic acid [10]. The EPS also have been reported to be generally recognized as safe compounds [11]. It also has a great role in the field of bioremediation, among them, are sorption of heavy metals from wastewater [12,13], metabolizing and mineralizing of persistent organic pollutant [14] and improving activated sludge settleability [15]. In the medical field, the extracellular polymeric substance has a defensive role against desiccation, phagocytosis, antibiotics, helps to lower the cholesterol levels and prebiotic potential [16], used in oral, ophthalmic, and nasal drug formulations [10] and anticancer drugs [17]. There are many microorganisms reported earlier from different genera responsible for the production of EPS as a secondary metabolite. Among bacteria, *Lactobacillus plantarum* KX041 [7], *Bacillus* sp. MC3B-22, and *Microbacterium* sp. MC3B-10 [13], *Leuconostoc mesenteroides* [18], *Sphingomonas paucimobilis* [19], *Kocuria rosea* ZJUQH [8], *Alteromonas infernus* [20], and *Escherichia coli* [21]. Many fungi have also been reported for EPS production that include *Fusarium equiseti* ANP2 [22], *Schizophyllum commune* IBRC-M 30213 [23], *Fusarium solani* DO7 [24], *Cordyceps militaris* [25], etc. By keeping all the facts in mind and its vast application in various fields, the present research work was based on isolation and characterization of EPS-producing bacteria.

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2. MATERIALS AND METHODS

2.1. Bacterial Cultures and Media Used

All the microorganisms used in this study were isolated from biofilms formed over the slides dipped in wastewater, and *Salmonella*, *E. coli*, *Klebsiella* sp., *Pseudomonas* sp., *Staphylococcus aureus* were used as test microorganisms for antimicrobial activity test. Different types of growth media were used viz. Nutrient agar medium (Peptone, 5 g/l; Beef extract, 3 g/l; Sodium chloride, 5 g/l; pH: 7.2) and casein hydrolysate agar medium (Sodium nitrate, 2 g; Dipotassium hydrogen phosphate, 1 g/l; Magnesium sulfate, 0.5 g/l; Potassium chloride, 0.5 g/l; Carboxymethyl cellulose, 5 g/l; and Peptone, 2 g/l; pH: 7). Each medium was first adjusted to appropriate pH and the agar-agar was added @2% whenever the solid medium was required before being sterilized by autoclaving at 121°C for 15 minutes.

2.2. Isolation of Biofilm Forming Bacteria

Glass slides were sterilized with 1N HCl and washed with distilled water. The sterilized glass slides were dipped into wastewater at different sites in Rohtak, Haryana for 20 days. After completion of 20 days, slides were rinsed with distilled water to remove debris and loosely attached bacteria [26]. The surfaces of the slides were scraped and suspended in 10 ml of sterilized normal saline. Bacterial cultures were isolated using the serial dilution technique. The agar plates were incubated at 37°C for 72 hours. Bacterial colonies having different morphology were selected and purified on nutrient agar plates [27].

2.3. Screening of EPS-producing Bacteria

Screening of EPS-producing bacteria was carried out on a morphological basis and formation of strings [28]. The colony characteristics, viz., shape, color, and polysaccharide production were observed on agar medium.

2.4. Production and Extraction of EPS

The EPS-producing colonies of each isolate were inoculated in appropriate media for the highest production of EPS. The one loopful inoculum was inoculated in 100-ml broth media and then incubated at 37°C for proper growth and production of EPS. The EPS extraction was carried out using the modified protocol of Subramanian et al. [26]. The bacterial broth culture was centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was precipitated with 97% chilled ethanol. The Precipitated EPS was collected by centrifugation at 10,000 rpm for 30 minutes. The dry weight of the extracted EPS was measured.

2.5. Microscopic Studies and Biochemical Characterization of Isolates

The studies were carried out microscopically by different staining methods that involved simple staining and Gram staining. Biochemical tests were carried out as per the method given by Cappuccino and Sherman [29] with 24 hours old cultures. The various biochemical tests involved for the present study were starch hydrolysis, casein hydrolysis, cellulose hydrolysis, urease, Indole, Methyl Red, Voges-Proskauer and Citrate tests (IMVIC) citrate utilization test, etc.

2.6. Antimicrobial Activity of EPS-producing Isolates

The antimicrobial activity of isolates was studied by using agar well diffusion method. The test was performed according to a procedure developed by Rajoka et al. [30]. Twenty milliliters of the melted agar medium was poured in petriplate and 100 µl of 24 hours. Old broth culture of the test microorganism was spread on each plate. When the inoculums got absorbed in media, wells were cut on the surface using borer. The supernatant of isolates was poured into the well against different test microorganisms and incubated at 37°C for 24–48 hours. After incubation, the plates were observed for a clear zone of inhibition around the well and diameter was measured.

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Biofilm-forming Bacteria

Total of five samples of biofilms were collected from five different sites (Table 1). Bacteria from these samples were isolated on nutrient agar media amended with glucose (1.0%). In all, 50 representative bacteria were isolated, purified, and maintained as pure cultures. A similar method of isolation has also been used by Dhanya et al. [27] and isolated *Pseudoalteromonas* sp. from sea water. The EPS-producing bacteria are involved in the formation of biofilms, which in turn mediates close association of bacteria to abiotic and biotic surfaces [31]. According to another study by Gu et al. [8], EPS production is directly related to salt tolerance by *K. rosea* ZJUQH. Stress conditions are always preferable for EPS production [32].

3.2. Screening of EPS-producing Colonies

Out of 50 isolates, 21 isolates were screened for EPS production. The method used for the selection of EPS-producing microorganisms was based on their slimy colony and string formation by touching with inoculating loop on the growth medium (Table 1; Fig. 1). The EPS-producing colonies were mucoid in shape and produced a large amount of yellow pigmentation (Fig. 1C and 1D). The mucoid colonies have a glossy and slimy appearance on agar

Table 1: Sites of wastewater sample collection and the selected EPS producing isolates.

Sr. No.	Name of the sample	Wastewater sample collection sites	Bacterial isolates with mucoid and slimy appearance
1	H1	Hostel No. 1 (Boys), MD University, Rohtak, Haryana, India	H1A, H1B
2	H2	Hostel No. 1 (Boys), MD University, Rohtak, Haryana, India	H2A, H2B, H2C, H2D, H2E1, H2E2, H2F
3	AS	Sector 14, Rohtak, Haryana, India	ASA1, ASA2, ASA3, ASB4, ASB5, ASC6, ASD7
4	P1	Indira Colony, Rohtak, Haryana, India	P1A, P1B, P1C
5	P2	Wastewater treatment plant, Nehru colony, Rohtak, Haryana, India	P2A, P2B

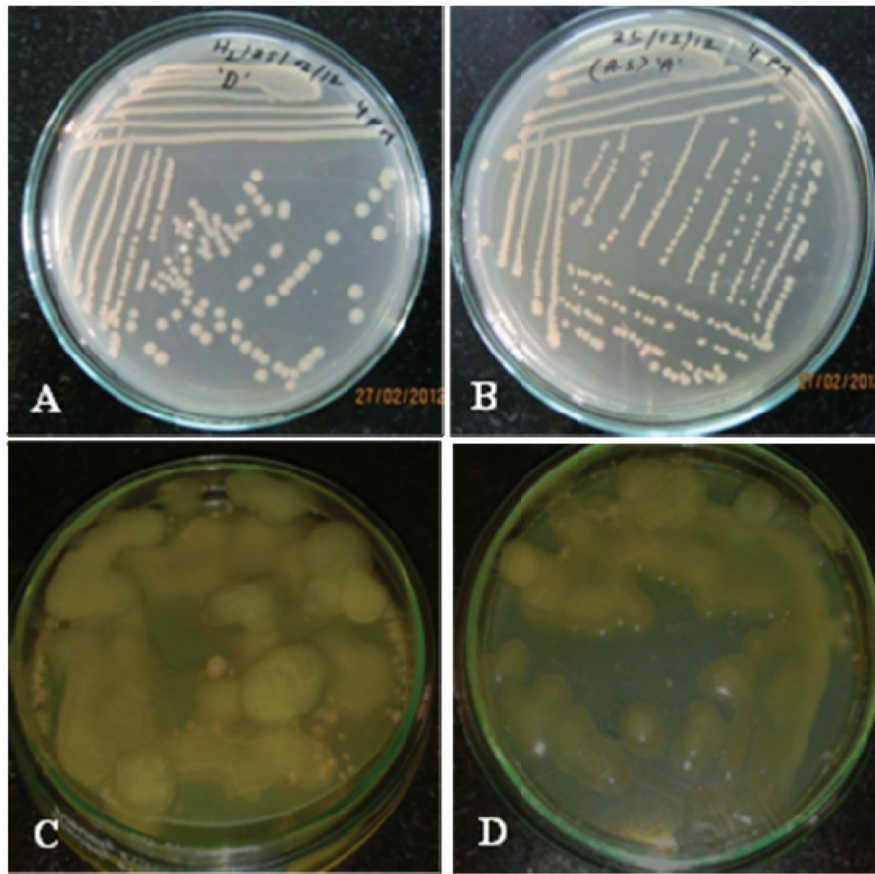


Figure 1: Bacterial isolates purified colonies (Plate A) H2D isolate and (Plate B) A.SA isolate. Plate (C) and (D) are the bacteria producing yellow pigmentation after overproduction of EPS during preservation.

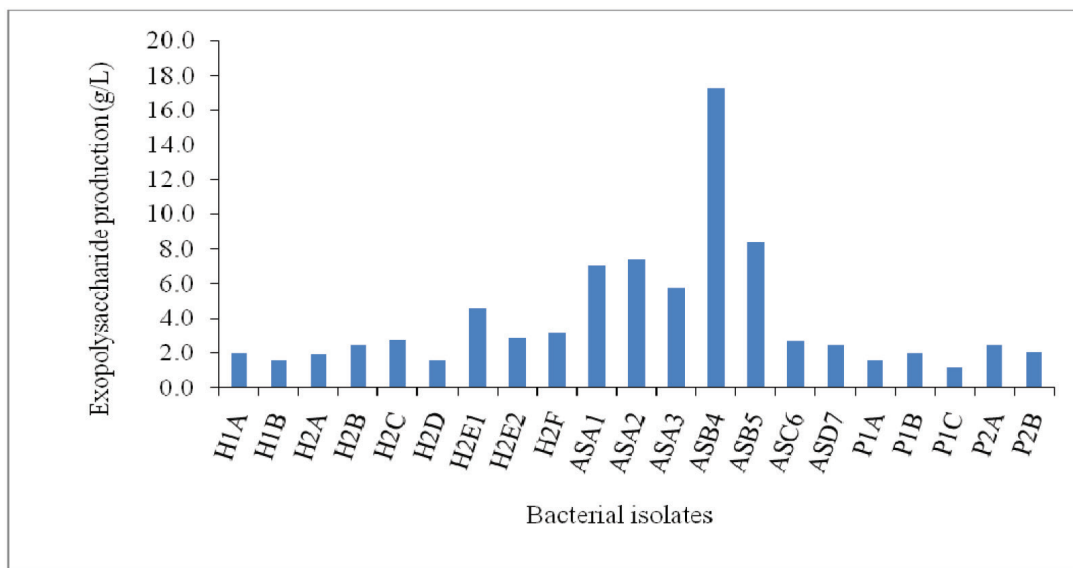


Figure 2: Production of EPS by different bacterial isolates.

media were the primary criteria for selection of EPS-producing bacteria [33]. Earlier, the similar property of string formation and the mucoid colony has been reported by Bacosa et al. [34] and Rühmann et al. [28].

3.3. Extraction of Exopolysaccharide (EPS)

All the 21 isolated microorganisms were used for EPS production and extraction using casein hydrolysate broth medium. On fourth day strain, ASB4 showed the highest EPS productivity, i.e., 17.2 g/l (Fig. 2). The concentration of EPS produced by 21 different bacterial strains varied from 1.2 to 17.2 g/l. Out of 21 isolates, nine were best EPS producers (ASA1, ASA2, ASA3, ASA4, ASA5, H2C6, H2E7, H2F8, and H2E9) and were further selected for EPS extraction. The strain ASB4 showed the highest productivity of 17.2 g/l, whereas the EPS produced by the isolates ASB5, ASA2, ASA1, and ASA3 was 8.4, 7.4, 7.0, and 5.8 g/l, respectively. The rest of the isolates showed average production (1.2–4.6 g/l) of EPS. In another study reported earlier, the production of exopolysaccharide was 4.65 mg/ml by isolate VS2B [35]. The comparative study revealed that isolate ASB4 was the highest producer of EPS. The EPS concentration from *Bacillus subtilis* was found in the range from 3.5 to 5.5 g/l [36].

3.4. Microscopic Studies of Isolates

Among nine isolates, six were rod-shaped and three were small rods. Five out of nine isolates were Gram-positive and four were Gram-negative (Table 2). The highest EPS-producing strain ASB4 was Gram-positive and rod-shaped. The bacterial isolate ASA1 was a small rod and Gram-positive, while the strain ASA2 was a small rod and Gram-negative. The strain ASB4 was found to be rod-shaped and gram-positive, whereas the isolate H2E9 was rod-shaped and Gram-negative. Earlier, it has been reported that most of the EPS-producing bacteria were Gram-positive and rod-shaped. Although, EPS producing bacteria has earlier been identified as Gram positive, Gram negative and maybe coccus or rods [37].

3.5. Biochemical Characterization of Isolates

Biochemical characterization showed that most of the bacterial isolates showed amylase positive except ASBB, H2E7, H2FB, and

H2E9 (Table 2), and all of the isolates showed cellulase, casein hydrolysis, and urease test as negative, while most of the isolates were V-P, indole, and citrate utilization test positive. Six of the nine isolates were methyl red negative and three were positive. In our study, the strains ASA1, ASA2, ASA3, ASB5, and H2C6 (Table 2) showed a positive test for starch hydrolysis, i.e., these strain produced amylase enzyme. Amylase is the enzyme that hydrolyzes starch and forms simple glucose. The strains H2C6, H2E7, and H2E9 showed methyl red positive, whereas ASA1, ASA2, ASA3, ASB4, ASB5, H2C6, and H2F8 showed Voges–Proskauer test positive (Table 2). The isolates ASA1, ASA2, ASA3, ASB4, and ASB5 showed indole production test positive. The strains ASA1, ASA2, ASA3, ASB4, and ASB5 utilized sodium citrate as a carbon source, while the isolates H2C6, H2E7, H2F8, and H2E9 showed negative results for citrate utilization test. The unknown bacterial sample can be identified using biochemical tests as each bacterium has its own metabolic pathway. The biochemical tests have earlier been carried out to analyze the ability of bacteria to use enzymes and degrade amino acids, lipids, carbohydrates, and proteins [38]. It has been reported that EPS-producing bacteria having oxidase, protease, and starch hydrolysis as positive but catalase negative [39,40].

3.6. Antimicrobial Activity by Bacterial Isolates

The test microorganisms, such as *Salmonella* sp., *E. coli*, *Klebsiella* sp., *Pseudomonas* sp., and *S. aureus*, were used in this study (Table 3; Fig. 3). The determination of the antimicrobial effect was carried out by the agar well diffusion method. In this study, strains ASA3, ASB5, H2C6, H2E7, H2F8, and H2E9 inhibited the growth of *Salmonella* sp. and the strain H2E7 inhibited the growth of *E. coli* and *Klebsiella* sp., whereas the strains ASA3 and H2F8 showed inhibition against *Klebsiella* sp. and *E. coli*, respectively (Table 3; Fig. 3). However, none of the isolates showed growth on *Pseudomonas* sp. and *S. aureus* plates. Antimicrobial activity reached the maximum after 48 hours of incubation of strains H2E7 and H2F8 against *Salmonella* sp. (Table 4). The highest antimicrobial activity shown by H2C6, H2E7, and H2E9 strains were 1.6, 1.4, and 1.4 cm against *Salmonella* sp. (Table 4), whereas the lowest antimicrobial activity was 1.0 and 1.1 cm by ASA3 strain against *Salmonella* sp. and *Klebsiella* sp. The strains H2C6, H2E7, and H2F8 showed the highest antimicrobial activity against

Table 2: Morphological, microscopic, and biochemical characterization of isolates.

Sr. No.	Name of the isolate	Microscopic Studies				Name of the tests					
		Morphology	Gram's staining	Starch hydrolysis	Cellulose hydrolysis	Casein hydrolysis	Urease	Methyl Red	Voges–Proskauer	Indole	Citrate test
1	ASA1	Small rod shaped	+ve	+	–	–	–	–	+	+	+
2	ASA2	Small rod shaped	–ve	+	–	–	–	–	+	+	+
3	ASA3	Rod shaped	+ve	+	–	–	–	–	+	+	+
4	ASB4	Rod shaped	+ve	–	–	–	–	–	+	+	+
5	H2B5	Rod shaped	+ve	+	–	–	–	–	+	+	+
6	H2C6	Rod shaped	–ve	+	–	–	–	+	+	–	–
7	H2E7	Rod shaped	–ve	–	–	–	–	+	–	–	–
8	H2F8	Small rod shaped	+ve	–	–	–	–	–	+	–	–
9	H2E9	Rod shaped	–ve	–	–	–	–	+	–	–	–

Table 3: Antimicrobial activity of EPS producing isolates.

Sr. No.	Name of the isolate	Name of the test microorganisms				
		<i>Salmonella</i> sp.	<i>E. coli</i>	<i>Klebsiella</i> sp.	<i>Pseudomonas</i> sp.	<i>Staphylococcus aureus</i>
1.	ASA1	-	-	-	-	-
2.	ASA2	-	-	-	-	-
3.	ASA3	+	-	+	-	-
4.	ASB4	-	-	-	-	-
5.	ASB5	+	-	-	-	-
6.	H2C6	++	-	-	-	-
7.	H2E7	+	+	+	-	-
8.	H2F8	+	+	-	-	-
9.	H2E9	+	-	-	-	-

(++) = Good, (+) = Moderate, (-) = Negative

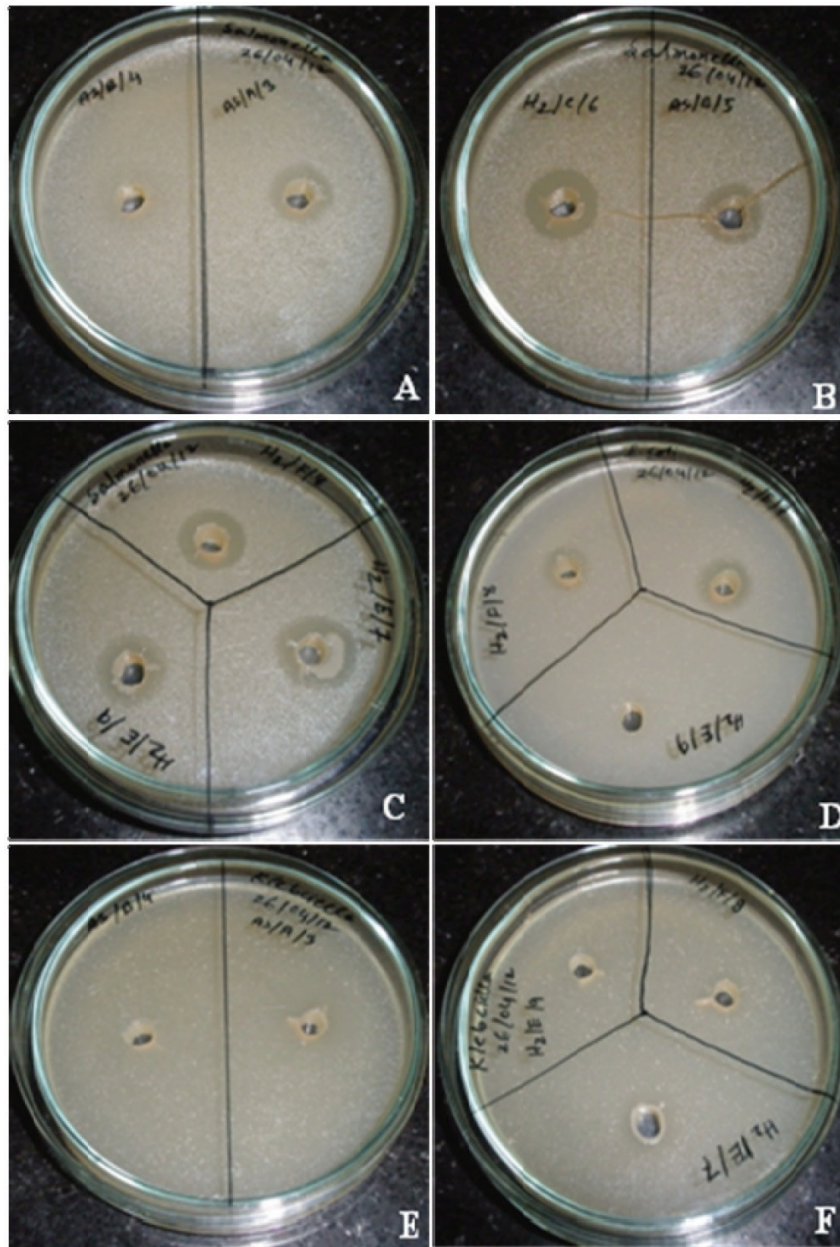


Figure 3: Plate (A) strain ASA3, plate (B) ASB5 and H2C6, and plate (C) H2E7, H2F8, and H2E9 showed antimicrobial activity against *Salmonella* sp. In plate (D), strain H2E7 and H2F8 showed antimicrobial activity against *E. coli*. In plate (E) and (F), strain A.SA3 and H2E7 showed antimicrobial activity against *Klebsiella* sp.

Table 4: Antimicrobial activity (inhibition zone diameter in centimeter) of the selected isolates against *Salmonella* sp., *E. coli*, and *Klebsiella* sp.

Bacterial isolates	<i>Salmonella</i> sp.			<i>E. coli</i>			<i>Klebsiella</i> sp.		
	12 hours	24 hours	48 hours	12 hours	24 hours	48 hours	12 hours	24 hours	48 hours
ASA3	NI	NI	1	NI	NI	NI	NI	NI	1.1
ASB5	NI	NI	1.1	NI	NI	NI	NI	NI	NI
H2C6	1.6	1.6	1.6	NI	NI	NI	NI	NI	NI
H2E7	1.1	1.2	1.4	NI	1	1.1	NI	NI	1.2
H2F8	1.3	1.4	1.4	NI	NI	1	NI	NI	NI
H2E9	NI	1	1.1	NI	NI	NI	NI	NI	NI

NI: No inhibition.

Salmonella sp. Similar study on antimicrobial activity against some microorganisms has earlier been carried out by researchers by using EPS-producing bacteria [41,42]. It was reported that most of the EPS-producing bacteria effective against the Gram-positive bacterium, especially *B. subtilis* and *M. tetragenus* and their effectiveness depends on the concentration of culture filtrate and incubation time [43]. But in the present study, we found that EPS-producing strains were effective against Gram-negative bacteria, especially *Salmonella* sp., *E. coli*, and *Klebsiella* sp. but not against Gram-positive bacteria. Various factors are responsible for the antimicrobial activity of exopolysaccharides, including molecular weight, composition, and the chelating activities [44]. In our study, the strains ASA3, ASB5, H2C6, H2E7, H2F8, and H2E9 showed antimicrobial activity against *Salmonella* sp. (Table 3; Fig. 3A–C). Two strains H2E7 and H2F8 showed antimicrobial activity against *E. coli* (Fig. 3D), and the strains ASA3 and H2E7 showed antimicrobial activity against *Klebsiella* sp. (Table 3; Fig. 3E and F).

4. CONCLUSION

In the present study, an attempt was made to isolate efficient EPS-producing bacteria from wastewater samples collected from different sites. A total of 21 bacterial isolates were used to EPS extraction and nine of them were efficient EPS producer. These isolates were checked for antimicrobial activity. On fourth day strain, ASB4 showed the highest EPS productivity, i.e., 17.2 g/l. Further studies are needed to study the application of produced exopolysaccharides at the industrial level.

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