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Antibacterial Activity of Polysaccharide *Bacillus Subtilis* and *Leuconostoc Mesenteroides* under Submerged Fermentation

Engy Elia Baheeg[1*], Mona Mansour Oraby[1], Khadiga A. Abou-Taleb[1*], Shimaa Abdel Raouf Amin[1*]

[1]*Agricutural Microbiology Department, Faculty of Agriculture, Ain Shams University, 11241, Cairo, Egypt*

Abstract

This study aimed to synthesize exopolysaccharides from bacterial isolates from the Egyptian ecology and apply them to combat foodborne bacteria. In the investigation, 66 bacterial isolates were isolated from various sources (soil, cane juice,and spoilage cucumber). Among the 66 bacterial isolates tested, 40 exhibited the capacity to produce exopolysaccharides (29 isolates produced levan, and 11 isolates produced dextran), giving the slimy colonies and mucoid viscosity appearance in the broth medium. The 40 polymer bacterial isolates evaluated for antibacterial efficacy against foodborne pathogenic bacteria strains (*Escherichia coli* O157H7*, Salmonella typhimirium* As3*, Staphylococcus aureus* As4*, Bacillus cereus* DSMZ345*, Pseudomonas aeruginosa* ATOC27853and *Listeria monocytogenes* As1). Six polymers-producing isolates out of 40 demonstrated the highest significant antibacterial activity, with minimum inhibitory concentration values ranging from 1000 to 12.5 mg/mL. The chosen polymers exhibited bacteriostatic properties. The most efficient isolates were phenotypically identified as *Leuconostoc mesenteroides* for the dextran-producing isolates with codes J1, J2, SCs1, SCs3, and SCg2, and *Bacillus subtilis* for the levan-producing isolate with code B. Among the six isolates, *L. mesenteroides* isolate code SCg2 produced the most dextran polymer with a dry weight of 6.36 g/L, productivity of 0.088 g/L/h, and a polymer yield coefficient relative to biomass of 1.55 g/g/L.Further nuclear magnetic resonance research demonstrated that the dextran polymer was composed of glucose with α-(1→6) links, with a 1H NMR spectrum ranging from δ 3.44 to 3.92 ppm. Dextran polymer produced from *L. mesenteroides* isolate code SCg2 was therefore selected for consumption during food preservation as a film coating to increase the product's shelf life.

***Keywords*:** Antibacterial activity; Bacterial isolation; Dextran; Levan; Phenotypic identification; Submerged fermentation

1. Introduction

A class of naturally occurring polysaccharides A class of naturally occurring polysaccharides called microorganism-derived polysaccharides can be found in nature during any metabolic process. Elevated molecular weight polymers called microbial polysaccharides share a significant portion of the cellular carbohydrates within and outside of the microbial cells. A diverse range of bacteria produces an assortment of polysaccharides, and the majority are water-soluble gums with distinctive physical features **[1].** Due to the variety of their chemical structures and special qualities, microbial polysaccharides are widely used in the food, pharmaceutical, biomedical, and bioremediation industries. High viscosity in aqueous solutions makes polysaccharides ideal for gelling agents, thickening, and stabilizing **[2- 3].**

Exopolysaccharides (EPS) can be divided based on repeating units' composition into two groups: (i) homopolysaccharides (HoPS) and (ii)heteropolysaccharides (HePS). The former (HoPs) present only one type of monosaccharide (D-glucose or D fructose), and the main ones are α-glucans, β-glucans, or fructans. They can be further classified based on the glycosyl type, linkage variety, and the position of carbon involved in the bond. HePS contains three to eight repeating units of two or more monosaccharides (e.g., rhamnose, fructose, galactose, or glucose) **[4].** Despite being assembled from comparable building blocks (the carbohydrate ring structure of pyranose and furanose), the vast variability in their structural and functional features

*Corresponding authors e-mail: Engyelia@agr.asu.edu.eg.(E. E. Baheeg);Khadija_aboutaleb@agr.asu.edu.eg (Kh. A.Abou-Taleb)

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accounts for their broad spectrum of utility. Rod-shaped molecules such as schizophyllan and scleroglucan from the fungi *Schizophyllan commune* and *Sclerotium rolfsii*, respectively, linear random coil-type structures such as pullulan from the fungus *Aureobasidium pullulans*, and intermediate forms such as alginates from the bacteria *Azotobacter vinelandii* can all be found. It is possible to have neutral polysaccharides like dextran from the bacteria *Leuconostoc mesenteroides* or extremely electronegative or "polyanionic" compounds like xanthan released by *Xanthomonas campestris*. **[5].**

EPS-producing bacteria are classified into several phylogenetic groups that involve Gram-negative bacteria from the following classes: the Betaproteobacteria class, containing the genera *Alcaligenes* and *Achromobacter*; the Gammaproteobacteria class, which includes the genera *Azotobacter*, *Pseudomonas*, *Enterobacter*, *Alteromonas*, *Pseudoalteromonas*, *Xanthomonas*, *Halomonas*, *Erwinia*, *Vibrio,* and *Klebsiella* genera. Additionally, they contain Gram-positive bacteria from the following classes: Actinomycetia, which consists of the *Bifidobacterium* and *Rhodococcus* genera; Bacilli, which contains *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Leuconostoc,* and *Streptococcus* genera; and Clostridia, which includes *Sarcina* sp. **[6,7**].

Microbial infections bring on many foodborne ailments after consuming tainted food. Various preservation techniques have been created to guarantee the microbiological food safety, dietary benefits, and sensory qualities of food. However, as consumers become more worried about their health, the need for natural antimicrobial agents is rising. Furthermore, the development of multidrug-resistant microbes due to the overuse of antibiotics has shifted researchers' and the food industry's attention to natural antimicrobials. We discuss naturally occurring antimicrobial substances derived from bacteria, viruses, algae, plants, animals, and mushrooms **[8].** Mammalian cell surfaces are richly coated with glycoconjugates or glycocalyx. Pathogens typically use glycoconjugates in microbial infections to detect and bind to host cells **[9].** The alterations in glycans throughout time and space reflect methods infections employ on the host surface to avoid detection **[10].** Antibiotic resistance and chronic infections threaten public health and food safety. Hence, the ability of LAB-derived EPS to prevent biofilm formation may be utilized to reduce the incidence of infectious diseases caused by pathogenic bacteria that produce biofilms. It may also offer a viable substitute for conventional antibiotics in light of the growing evidence demonstrating pathogen resistance to these drugs **[4, 11]**.

As antibacterial agents and biomaterials, polysaccharides and their derivatives have been used in numerous experiments and applications. Different polysaccharides have been investigated for antimicrobial applications, including dextran, hyaluronic acid, chitosan, cellulose, and other plant/animal-derived polysaccharides and their derivatives **[12].** Polysaccharides are a class of natural polymers worth investigating to develop new antibacterial uses. Creating various cytokines (such as interleukin, interferon, etc.), stimulating macrophages and lymphocytes, and stimulating antibody formation to improve organismal immunity are crucial physiological roles that polysaccharides play **[13].** Additionally, polysaccharides have some anti-inflammatory, antiviral, and antibacterial properties, mostly via preventing viral and bacterial proliferation **[14, 15].** Moreover,polysaccharides, macromolecules of more than 10 glycoside-bonded monosaccharides, are naturally abundant, secure, and residue-free. They are necessary for life. The considerable bioactivities of polysaccharides, such as their hypoglycemic, hypolipidemic, anticancer, antioxidant, immunity-boosting, antibacterial, and other bioactivities, have also drawn much interest **[16].** Pathogenic bacteria were impacted by the antibacterial mechanisms of polysaccharides, which influenced the creation of biofilms, nucleic acids, mycoproteins, intracellular metabolic pathways, cell walls, and cell membranes **[17].** Due to their accessibility, biocompatibility, and ease of modification, polysaccharides—one of the main biopolymers- have been studied for their potential use in antimicrobial applications.

According to a study by Souza *et al*. **[18]**, there has been a 17% increase in the use and application of these natural materials in recent years (2017–2021). This growth translates into a potential global market worth up to $10 billion USD, and it is anticipated to reach more than 22 billion USD by the end of this decade (2030). In order to extract new polysaccharides, there is interest in experimenting with alternative sources (such as residues) and polysaccharide extraction methods that yield higher yields or purer extractions.

The present study is designed to take advantage of certain natural materials of microbial origin, such as polysaccharides, as antimicrobial activity toward foodborne pathogens.

2. Materials and methods

2.1. Samples collection

Three samples (soil, spoilage cucumber, and sugarcane juice) were gathered from several locations of Shoubra El Khema and Al harm from Qalyubia and Giza Governorates, Egypt. At a depth of 10 cm,

soil samples were taken from the cultivated lands' rhizosphere. Samples were placed in sterile plastic bags, transported to the lab in an ice tank, and stored there for additional research.

2.2. Pathogenic bacterial strains used

Six pathogenic bacterial strains were obtained from the Microbiological Resource Centre (MIRCEN), Cairo, Egypt. These bacteria included *Escherichia coli* O157H7, *Salmonella typhimirium* As3, *Staphylococcus aureus* As4, *Bacillus cereus* DSMZ345, *Pseudomonas aeruginosa* ATOC27853and *Listeria monocytogenes* As1. The antibacterial activity of each of these strains was examined. The tested strains were maintained on a nutrient agar medium slant **[19]**. It was composed as follows (g/L): beef extract, 3; peptone, 5; agar, 20; distilled water up to 1000 ml and adjusted pH to 7 at 37°C, while the inoculum preparation process employed the same medium without agar.

2.3. Isolation and screening of polymers producing bacterial isolates

One gram of each sample was added to 9 ml of sterilized water, mixed thoroughly, and serial dilutions ($10^{-1}$to 10^{-6}) were done, and then 1 ml from each dilution was taken and cultured on the agar plate. The medium used for the isolation of levan-producing isolates was sucrose mineral salt agar **[20],** which was composited (g/L) of sucrose, 20; yeast extract, 3; $MgSO_4$, 0.2; K_2HPO_4, 5; distilled water up to 1000 ml and adjusted pH to 7 at 37°C. The medium used for the isolation of dextran-producing isolates was glucose mineral salt agar, which was the same medium as sucrose mineral salt agar as mentioned above,in which sucrose was replaced by glucose and McCleskey agar medium **[21]**, it was composited (g/L) of sucrose, 100; peptone, 10; yeast extract, 5; $CaCO_3$, 7; distilled water up to 1000 ml and adjusted pH to 7 at 37°C.

The agar medium plates were incubated at 30°C for 24-48 h. The colonies given viscous slimy growth were picked and purified several times to obtain pure culture-produced polymers and then maintained on nutrient and McCleskey agar media slants and stored at 4°C for the next studies.

2.4. Maintenance of cultures

Stock culture slants were maintained at 4°C on a preservation medium after an incubation period at 30°C ±2 for 24-48 h.

2.5. Inoculum preparation and fermentation process

To prepare a standard inoculum, the tested isolates were cultured in nutrient and McCleskey broth individually at 30°C for 24 h (in which a mean viable count of 2.1 - 2.7 $\times 10^7$ colony forming unit (CFU)/ml culture broth was obtained). This served as the production medium's inoculum. To carry out the fermentation process, 100 ml of sterile production medium for levan manufacture was placed inside each of the 250 ml plugged Erlenmeyer flasks **[22]**; it was composition (g/L) of sucrose 20; yeast extract 5; $CaCl_2$, 0.05; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 5.2; KH_2PO_4, 3.18; $MgSO_4$, 0.3 and adjusted pH to 7. For dextran production on dextran broth medium **[23]**, it was composed (g/L) of sucrose, 150; bacto-peptone, 5; yeast extract, 5; K_2HPO_4, 15; $MnCl_2.H_2O$, 0.01; NaCl, 0.01; $CaCl_2$, 0.05 and adjusted pH to 7. The productive mediums were inoculated with 1% standard inoculum and incubated at 30°C on a rotary shaker (150 rpm) for 48- 72 h. The fermentation medium was centrifuged at 8496 xg for 20 min. The pellets were used to assay the biomass (cell dry weight) by being washed with distilled water about three times and dried in an oven (Heraeus T5050N) at 80°C until constant weight. The supernatant (cell-free extract) was used to determine the polymer as described below.

2.6. Polymers precipitation

The supernatant (cell-free extract) was used for levan or dextran precipitation using absolute chilled ethanol (99.9%) **[24]**. An equal amount of absolute chilled ethanol (99.9%) was added to 10 ml cell-free extract (supernatant) and incubated in the refrigerator at 4°C for 30 min. The precipitated dextran or levan were centrifuged at 8496 xg for 20 min, and the supernatants were discarded. Following a buffer phosphate wash, the precipitated pellets were extracted again by centrifugation at 8496 xg for 10 min. The pellets obtained were dried in an oven at 60 - 70°C until constant weight. The dry weight of polymers was measured.

2.7. Identification of the most efficient polymer (dextran) using ^{1}H nuclear magnetic resonance (^{1}H-NMR) assay

^{1}H-NMR analysis was carried out to characterize the polymer's structure further. Thirty milligram purified EPS was dissolved and H/D exchange was conducted three times with 99.96% deuterated water D_2O (Sigma Aldrich). ^{1}H-NMR spectra were carried out on Bruker 400 MHz (Bruker Corp., Billerica, MA, USA) spectrophotometer, Faculty of Pharmacy, Cairo University, Cairo, Egypt.Tetramethylsilane (TMS) was used as an internal standard and chemical shifts were recorded in ppmon δ scale and coupling constants (J) were given in Hz.^{13}C-NMR spectra were carried out on Bruker 100 MHz spectrophotometer, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

2.8. Application of the selected polymers as antibacterial activity

2.8.1. Evaluation of polymers' antibacterial activity

This experiment used dextran and levan polymers to study their effect as antimicrobial agents. The antibacterial efficacy of the above polymers toward pathogenic bacterial strains was evaluated by the agar well diffusion method as recommended by Choksi *et al.* **[25]** on a nutrient agar medium. The tested pathogenic bacterial strains were grown separately in a broth medium and incubated at 30°C for 24 h. One milliliter of each bacterial suspension was placed into sterile Petri plates, then 10 ml of melted nutrient agar medium was poured and left to solidify. Each Petri plate was divided into two equal quarters using a marker pen, and wells of 7 mm in diameter were made in each quadrat of the plate using a sterile cork borer. The wells were loaded with an acceptable quantity of polymer agents (100 µl). The plates could stand until polymer agents were completely absorbed and then incubated at 30°C for 24 h. After 24 hours of incubation, the growth inhibition was obvious with the unaided eye, and the diameter of the inhibition zone (IZD) was measured using a ruler. The growth inhibition zone around the wells was measured to gauge the antibacterial activity.

2.8.2. Determination of minimum inhibitory concentration (MIC) using well agar diffusion

The MIC assay was carried out in accordance with the guidelines established by the Clinical and Laboratory Standard Institute **[26]**. Two-fold serial dilutions (1, 1/2, 1/4, 1/8, and 1/16) of the tested suspension fermented of bacterial isolate polymers were conducted by beginning to transfer 1 ml from stock to the first tube containing 1 ml of distilled water, then transferring 1 ml to corresponding tubing, respectively. These dilutions were put into inoculation plate wells, as mentioned above. The naked eye examined the tested agent at its lowest concentration (highest dilution) preventing the appearance of growth, and was described as MIC.

2.8.3. Determination of minimum bactericidal concentration (MBC) The MIC assay-tested plates that showed no growth were subcultured into nutrient agar and incubated for 24 h at 30°C in order to calculate the minimal bactericidal concentration (MBC). The lowest concentration on the nutrient medium that showed no growth was identified as the MBC value **[27]**.

2.9. Phenotypic identification of the most efficient bacterial isolates

The most efficient bacterial isolates producing polymers were completely identified up to species based on their cultural, morphological, and physiological features based on the key of Bergey's Manual**[28]** for identifying dextran isolates andfor identifying levan isolate **[29, 30]**.

2.10. Polymer parameter calculations

The polymer parameter was calculated as follows equations:

Polymer productivity (g/L/h) [31].

= Polymer dry weight (g/L)/ Fermentation time (h) **Eq. 1**

Polymer yield coefficient relative to biomass ($Y_{p/x}$) (g/g/L)[32].

= Polymer dry weight (g/L) / Biomass dry weight (g/L) **Eq. 2**

Polymer Yield (%) [33].

= (Polymer dry weight (g/L) / Original sugar (g/L)) x 100 **Eq. 3**

2.11. Statistical analysis

The collected data were subjected to statistical analysis using IBM® SPSS® Statistics **[34]**.

3. Results and Discussion

3.1. Isolation and screening of polymers producing bacterial isolates

The results presented in **Table 1** showed that sixty-six bacterial isolates were obtained from different sources on both glucose and sucrose mineral salt agar media at 30°C for 48 hours of incubation periods.

Forty-three bacterial isolates with a percentage distribution of 65.15% were isolated from different plant rhizosphere soils of lettuce, basil, tomato, and cabbage with 19, 9, 10, and 5 isolates, respectively. Six bacterial isolates were obtained from sugarcane juice, and 17 bacterial isolates were taken from spoilage cucumber, with percentage distribution of 9.09% and 25.76%, respectively.

Furthermore, Abou-Taleb*et al*. **[35]** isolated 37 bacterial isolates from rhizosphere soils of different plants, including *Abelmoschus esculentus* (4 isolates with 10.8 %), *Pisum sativum* (5 isolates with 13.5 %), *Zea mays* (9 isolates with 24.3%) and *Vicea faba* (19 isolates with 51.4%). Moreover, Rawal *et al.* **[36]** obtained 11 mucoid colonies from various soil samples and spoilage fruits**.** In addition**,** Abdalrahim *et al.* **[37]** gathered 55 bacterial isolates from fermented fruits. Moselhy *et al.***[38]** exhibited that 165 bacterial isolates in all, divided into 40 (24.2%), 103 (62.4%), and 22 (13.3%) isolates, were obtained from the soil rhizosphere.

As a preliminary study for selecting the polymer producers (levan and dextran) on production medium,

40 isolates out of 66 bacterial isolates showed growth. They demonstrated positive results on plate agar, which gave the slimy colonies and the broth medium mucoid viscosity appearance (**Fig. 1 A, B**).

Table 1: Survey of bacterial isolates on different agar media

Source of isolates		No. of isolates				No. of total isolates	%
		Medium 1		Medium 2			
		No.	%	No.	%		
Soil	▪ Lettuce	6	31.58	13	68.42	19	
	▪ Basil	3	33.33	6	66.67	9	
	▪ Tomato	3	30	7	70	10	
	▪ Cabbage	2	40	3	60	5	
Total		14	32.56	29	67.44	43	65.15
Cane juice		6	100	-	-	6	9.09
Spoilage Cucumber		12	70.6	5	29.4	17	25.76
Total isolates		32		34		66	

Medium 1, Glucose mineral salt agar medium; Medium 2, Sucrose mineral salt agar medium; No., Number

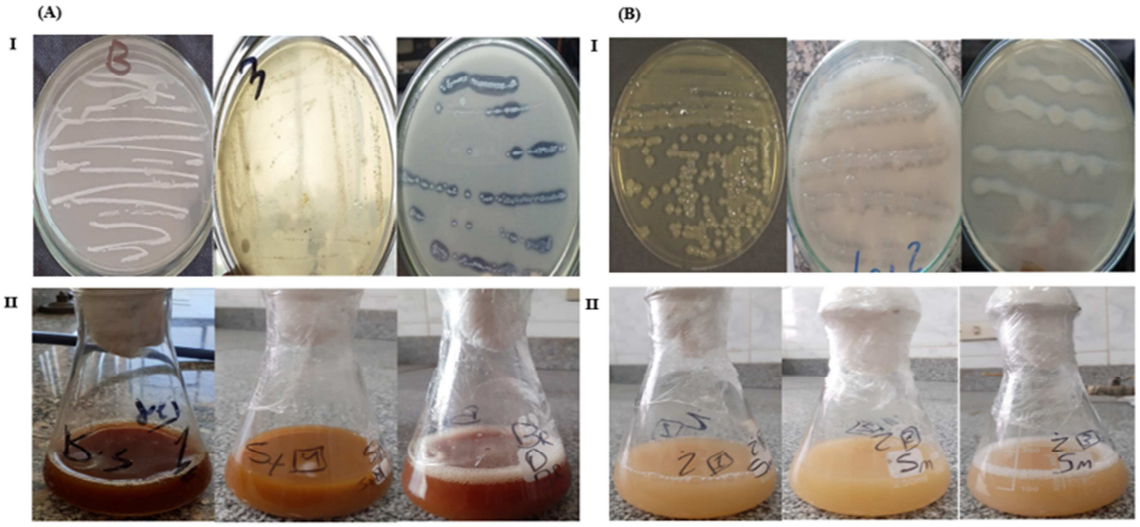

Figure 1: (A) Bacterial isolates producing levan on (I) plate agar and (II) broth medium. (B) bacterial isolates producing dextran on (I) plate agar and (II) broth medium

Results in **Table 2** exhibited that 29 isolates (67.44 % of total isolates) out of 40 bacterial isolates produced levan polymer isolated from plant rhizosphere soils of lettuce, basil, tomato, and cabbage had 17, 5, 5, and 2 isolates producing a polymer with a percentage of the distribution of 58.62, 17.24, 17.24, and 6.90 %, respectively. As well as 11 isolates (47.80 % of total isolates) among 40 bacterial isolates that produced dextran polymer were isolated from sugarcane juice (4 isolates with 66.70 %) and spoilage cucumber (7 isolates with 41.20 %)**.** Twenty-six isolates (with a percentage distribution of 39.40) among 66 bacterial isolates showed no sign of polymer production.

Specifically, Abou-Taleb *et al.***[35]** stated that all 37 bacterial isolates revealed signs of growth on the agar plate with sucrose. The colonies had a mucoid, slimy look that indicated the formation of the polysaccharide. Hassan *et al.* **[39]** found that *Bacillus subtilis* SH1 produced polymers. Moreover, two B14 and Y8 bacteria were isolated from a compost sample, and their ability to create polysaccharides on a fermentation medium was examined **[40]**. More than 100 bacteria species have been shown to produce levan **[41].** The most studied levan producers among Gram-positive bacteria are the species of the class Bacilli including *Bacillus subtilis*, *Bacillus licheniformis*, *Paenibacillus polymyxa*, *Lactobacillus reuteri*, *Leuconostoc citreum* and others **[42, 43].**

Both isolates produced mucous, smooth, and irregularly edged colonies when cultivated on a solid sucrose medium. In addition, Ramya *et al.***[44]** found that *Bacillus* sp. isolated from rhizosphere soil

produced unclear but highly viscous polysaccharides. As well as Davidović *et al.***[45]** reported that *Leuconostoc mesenteroides* T3 obtained dextran. Some of the most commonly used EPSs are xanthan from the genus *Xanthomonas*, and dextran from the *Leuconostoc*, *Streptococcus*, and *Lactobacillus* genera **[46].**

Table 2: Number and percentage distribution of bacterial isolates producing polysaccharides on production media at 30°C for 48-72 h using shake flasks at 150 rpm

Polymers produced	Source of isolates		No. of isolates and % of the distribution				No. of total isolates
			Producing polymer		Non-producing polymer		
			No.	%	No.	%	
Levan polymer	Soil	▪ Lettuce	17	58.62	2	14.30	19
		▪ Basil	5	17.24	4	28.60	9
		▪ Tomato	5	17.24	5	35.70	10
		▪ Cabbage	2	6.90	3	21.40	5
	Total		29	67.44	14	32.56	43
Dextran polymer	Cane juice		4	66.70	2	33.30	6
	Spoilage Cucumber		7	41.20	10	58.80	17
	Total		11	47.80	12	52.20	23
No. of total isolates			40	60.60	26	39.40	66

No., Number.

3.2. Application of the selected polymers as antibacterial activity

3.2.1. Evaluation of polymers' antibacterial activity

Antibacterial activity against *Escherichia coli* O157H7*,Salmonella typhimirium* As3 *and Pseudomonas aeruginosa* Atoc27853(as Gram^{-ve}), and *Staphylococcus aureus* As4*, Bacillus cereus* Dsmz345*, and Listeria monocytogenes* As1 (as Gram^{+ve}) are shown in **Fig. 2 and Table 3.** The results showed that six polymer-producing isolates out of 40 bacterial isolates had antibacterial activity, inhibiting the growth of the tested pathogenic bacterial strains and causing a halo-zone (no growth) around the bacterial growth on agar plates, which was observed with necked eyes and expressed with a + sign **(Fig. 2 and Table 3).** Whereas the isolates didn't have antibacterial activity, their growth on agar plates was marked with a - sign **(Table 3).**

Data in **Fig. 2 and Table 3** indicated that the polymer produced by isolates SCg2, SCs1, SCs3 inhibited all six tested pathogenic Gram-positive and -negative bacterial strains. In contrast, polymers J1 and J2 inhibited only 4 tested pathogenic strains of *L. monocytogenes* As1, *E. coli* O157H7, *S. typhimirium* As3, and *P. aeruginosa* Atoc27853. Meanwhile, polymer B inhibited only two tested Gram-negative pathogenic strains of *E. coli* O157H7 and *P. aeruginosa* Atoc27853. These positively charged polymer molecules may have been adsorbed on the negatively charged microbial cell surface, diffused through the cell wall, and interacted with the cytoplasmic membrane, causing irreversible damage to the integrity of the cell membrane and, ultimately cell death. This may explain why these polymers are more effective against Gram-negative pathogenic bacteria. This method of action is thought to be less susceptible to acquiring resistance **[47, 48]**, inhibited Gram-negative pathogenic bacteria by interacting with the cell membrane protein, such as *Escherichia coli* **[49]**, and the extracellular polysaccharides could stop the growth of *E. coli* ATCC35218 by lowering the cell membrane's hydrophobicity % **[50]**.

The results emerged from the work recorded the antibacterial activity of the EPS from *B. subtilis* SH1 (2.8, 4, and 4.6 AU/ml) was registered against *Pseudomonas aeruginosa* at 50, 100, and 200 mg/ml, respectively **[39]**.EPS from *Lactobacillus reuteri* SHA101 and *Lactobacillus vaginalis* SHA110 have been demonstrated to exhibit inhibitory zones against *Salmonella typhimurium* CMCC, *Staph.s petrasii* subsp. *pragensis* KY 196,531 and *E. coli* ATCC 25,922 of (14, 15 mm), (12.2, 9.5) and (13.5 and 10.6), respectively**[4, 51]**. Ağçeli *et al.* **[52]** reported that at 1000 µg/mL levan, the highest zone of inhibition was observed against *E. coli* produced from *Pseudomonas mandelii* about 16 mm. With inhibitory zone diameters that fall between 10 and 20 mm, 12 and 16 mm, and 10 and 16 mm, respectively, probiotic cultures of lactic acid bacteria as an antibacterial agent demonstrated more significant antibacterial action against *E. coli* O15H7, *S. typhimirium* As3, and *S. shigae* As2 **[53]**.

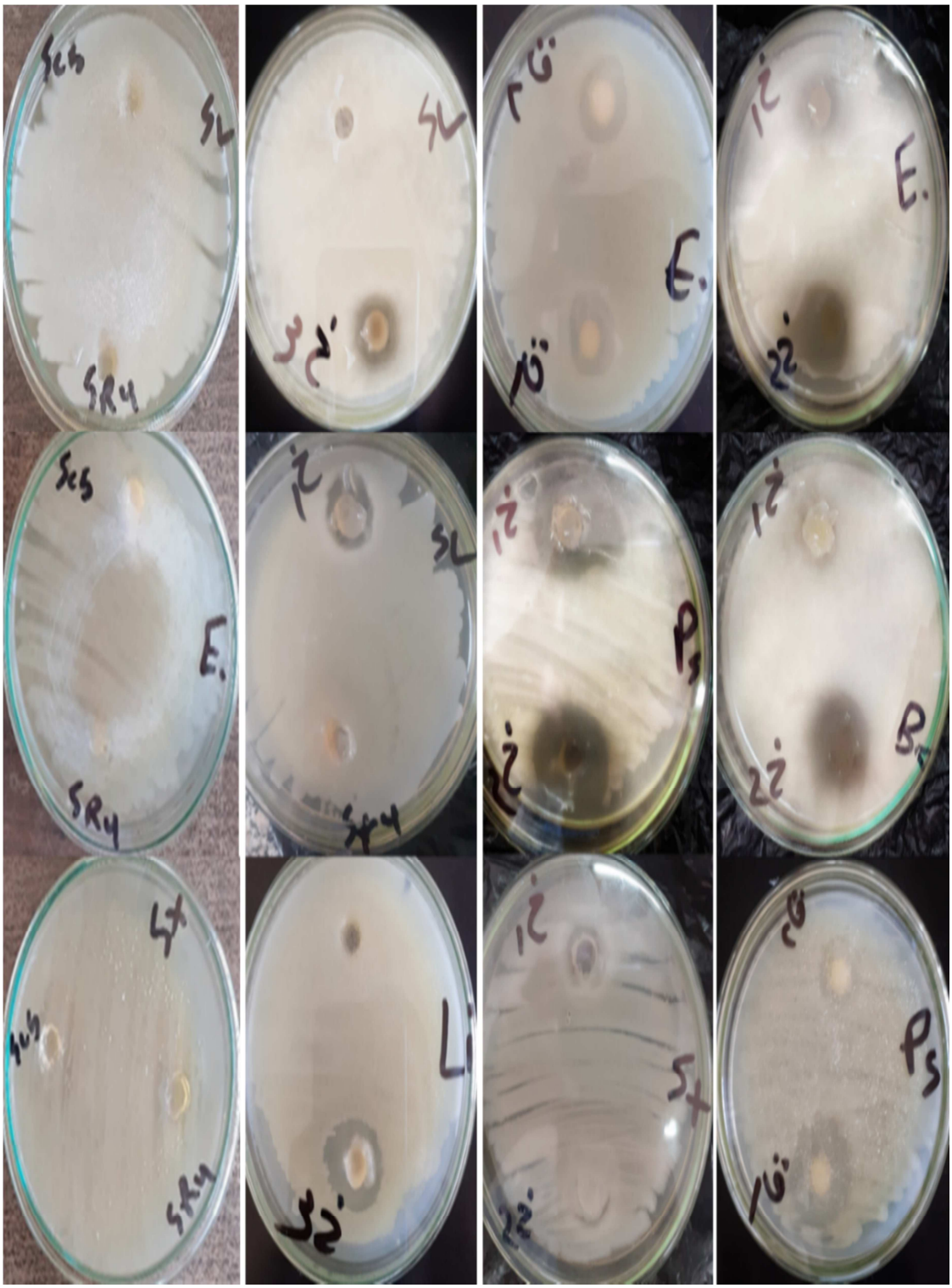

Figure 2: Antibacterial activity of polymers produced by the selected bacterial isolates against pathogenic bacterial strains on agar plates

Table 3: Detection of polysaccharides produced by the different isolates as antibacterial activity against Gram^{+ve} andGram^{-ve} bacteria

Polymer-producing isolate codes	Antibacterial activity against					
	Gr $^{+ve}$ bacteria			Gr $^{-ve}$ bacteria		
	Staphylococcus aureus As4	*Bacillus cereus* Dsmz345	*Listeria monocytogenes* As1	*Escherichia coli* O157H7	*Salmonella typhimirium* As3	*Pseudomonas aeruginosa* Atoc27853
Lg1	-	-	-	-	-	-
Lg2	-	-	-	-	-	-
Lg3	-	-	-	-	-	-
Lg4	-	-	-	-	-	-
Lg5	-	-	-	-	-	-
Lg6	-	-	-	-	-	-
Ls1	-	-	-	-	-	-
Ls2	-	-	-	-	-	-
Ls3	-	-	-	-	-	-
Ls4	-	-	-	-	-	-
Ls5	-	-	-	-	-	-
Ls6	-	-	-	-	-	-
Ls7	-	-	-	-	-	-
Ls8	-	-	-	-	-	-
Ls9	-	-	-	-	-	-
Ls10	-	-	-	-	-	-
Ls11	-	-	-	-	-	-
Bg3	-	-	-	-	-	-
Bs1	-	-	-	-	-	-
Bs3	-	-	-	-	-	-
Bs4	-	-	-	-	-	-
Bs6	-	-	-	-	-	-
Tg3	-	-	-	-	-	-
Tg4	-	-	-	-	-	-
Tg6	-	-	-	-	-	-
Ts1	-	-	-	-	-	-
Ts3	-	-	-	-	-	-
Cbg	-	-	-	-	-	-
J3	-	-	-	-	-	-
J4	-	-	-	-	-	-
B	-	-	-	+	-	+
J1	-	-	+	+	+	+
J2	-	-	+	+	+	+
SCg1	-	-	-	-	-	-
SCg2	+	+	+	+	+	+
SCg3	-	-	-	-	-	-
SCs1	+	+	+	+	+	+
SCs2	-	-	-	-	-	-
SCs3	+	+	+	+	+	+
SCs7	-	-	-	-	-	-

(-), growth; (+), no growth. Lg and Ls, polymer isolated from Lettuce soil; Bs and B, polymer isolated from Basil soil; Bg, Tg and Cbg, polymer isolated from Tomato soil; Ts, polymer isolated from Cabbage soil; J, polymer isolated from Cane juice; SCg and SCs, polymer isolated from Spoilage cucumber

The six selected polymer-producing bacterial isolates that had antibacterial activity were measured by the inhibition zone diameter on agar plates and the data illustrated in **Fig. 3**. Results revealed that the strain E. *coli* O157H7 had more significant (p<0.05) sensitivity to the polymer produced from isolates SCg2, SCs3, and J1 with not significant (p<0.05) mean diameter zone inhibition of 15.7, 14.3, and 13.0 mm, respectively. Polymers J2 and SCs1 inhibited this strain with 11.3 and 9.0 mm of means diameter zone inhibition. Meanwhile, this strain appeared less sensitive to polymer B, which means diameter zone inhibition reached 3.0 mm.

Moreover, the strain *P. aeruginosa* Atoc27853 had a highly significant sensitivity to polymer SCg2 (27.0 mm of means diameter zone inhibition), followed by SCs3, and J1 with no significant (p<0.05) means diameter zone inhibition of 21.3 and 20.0 mm, respectively. The lowest mean diameter zone inhibition was recorded by polymer B (4.0 mm).

Whereas, strains *L. monocytogenes* As1 and *S. typhimirium* As3 were more sensitive to the tested polymers in the following order SCg2 > SCs3 > J1> SCs1 > J2 with means diameter zone inhibition of 17 mm > 14.3 mm > 12.7 mm > 10.3 mm >10.0 mm and SCg2 > SCs1 > SCs3 > J2 > J1 with means diameter zone inhibition of 16.0 mm > 12.0 mm > 10.0 mm > 9.3 mm > 9.0 mm, respectively. At the same time, both strains were resistant to polymer B.

The Gram-positive bacterial strains *B. cereus* Dsmz345 and *Staph. aureus* As4 were susceptible to polymers SCg2 (19.0 and 13.0 mm of means

diameter zone inhibition), SCs3 (8.0 and 10.0 mm of means diameter zone inhibition), and SCs1 (8.0 and 8.7 mm of means diameter zone inhibition) while they were resistant to polymers J1, J2, and B.

Besides that, it could be summarized data that the polymer produced by the SCg2 isolate was given the highest significant inhibition against all the tested pathogenic bacteria.

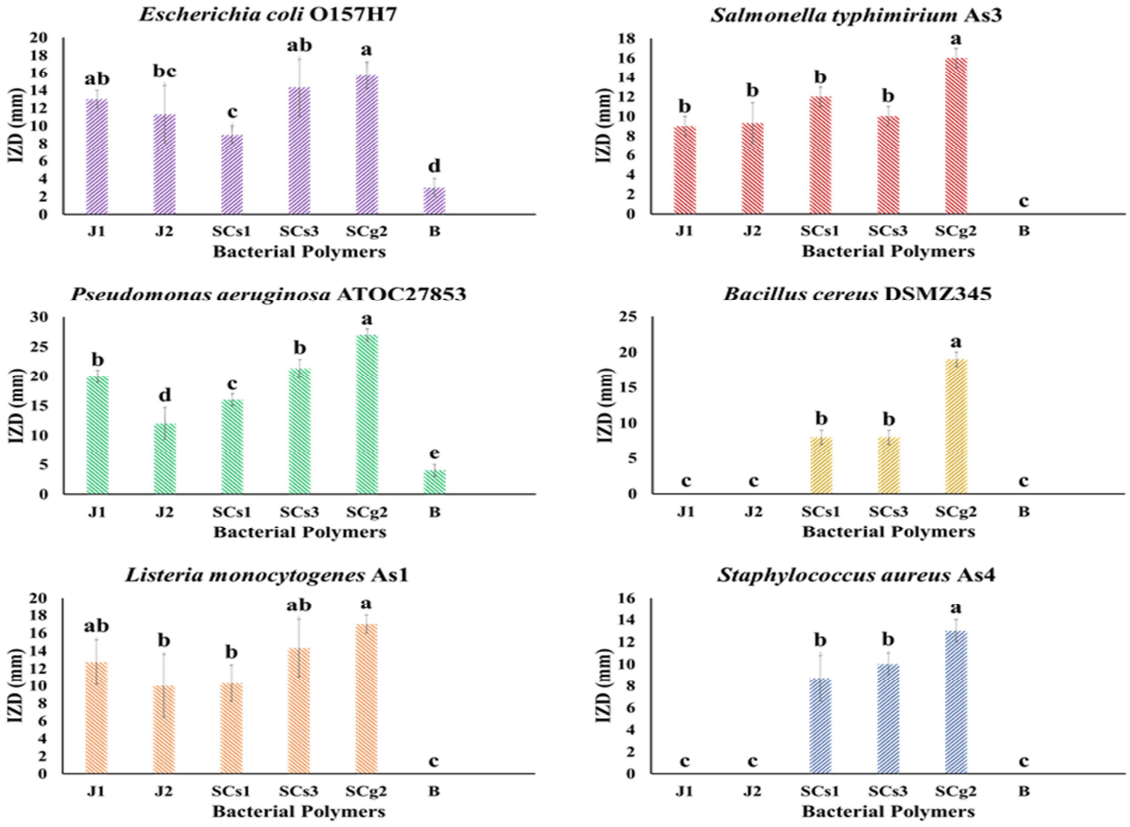

Figure 3: Diameter zone inhibition (mm) of polymers produced by the selected isolates against pathogenic bacterial strains.
IZD, Inhibition zone diameter.All tested polymers were dextran except B polymer was levan. B, polymer isolated from Basil soil; J, polymer isolated from Cane juice; SCg and SCs, polymer isolated from Spoilage cucumber
[a,b] values in the above column with the same letter do not differ significantly according to Duncan's at the 5% level. Bar indicated to ± standard division

3.2.2. Determination of minimum inhibitory concentration (MIC) using well agar diffusion and minimum bactericidal concentration (MBC)

After 24 hours of incubation, the MIC values of the tested polymers produced from the selected isolates were used against six tested pathogenic bacterial strains, as shown in **Table 4**. The results indicated that the tested polymers were diluted in a two-fold dilution ranging up to 1/16 of the polymers with final concentrations of 400, 200, 100, 50, and 25 mg/mL for polymers J1 and J2 isolates, 300, 150, 75, 37.5, and 18.75 mg/mL for polymers SCs1 and SCs3, and 200, 100, 50, 25, and 12.5 mg/mL for polymers SCg2. The results of the MIC values demonstrated that the polymer produced by SCg2 isolate exhibited activity at dilution ranging from 1 to 1/8 (from 200 to 25 mg/mL concentrations) against the six tested pathogenic bacteria, except in *Staph. aureus* As4exhibited activity at dilution ranging from 1 to ¼ (from 200 to 50 mg/mL concentrations). The polymers produced by J1 and J2 isolates showed activity at dilution 1 and ½ (400 and 200 mg/mL concentrations) against *L. monocytogenes* As1, *E. coli* O157H7, *S.typhimirium* As3, and *P. aeruginosa* Atoc27853. The polymers produced by SCs1 and Scs3 exhibited activity at dilution ranging from 1 to ¼ (from 300 to 75 mg/mL concentrations) against the tested pathogenic microorganisms, except in *Staph.s aureus*exhibited activity at dilution 1 and ½ (300 and 150 mg/mL concentrations). The polymer produced

by B isolate exhibited activity at dilution 1 and ½ (1000 and 500 mg/mL concentrations) against *E. coli* O157H7 and *P. aeruginosa* Atoc27853, as seen in Table 4.

The results of the MBC assay demonstrated that polymers produced by J1, J2, SCs1, SCs3, SCg2, and B isolates had a bacteriostatic effect on all the tested pathogenic microorganisms (data not shown).

According to **[54]**, the tested extracts had an effective MIC of 0.32 mg/mL against the strains of *Staph. aureus* St3, *E. coli* Ec3, and *S. typhi* Sa1. On the other hand, the *P. aeruginosa* Ps9 strain had intermediate susceptibility to grape extract with a MIC of 0.16 mg/mL and low susceptibility to mulberry extracts with a MIC of 0.08 mg/mL. The exopolysaccharides (EPS) of *Lacticaseibacillus paracasei* AS20(1) showed inhibitory effects on the growth of *L. monocytogenes* (MIC of 0.935 mg/ml, MBC of 0.935 mg/ml), *Yersinia enterocolitica* (MIC of 12.5 mg/ml, MBC of 50 mg /ml) and *B. cereus* (MIC of 6.25 mg /ml, MBC of 12.5 mg/ml) **[4, 55]**. Moreover, the minimum inhibitory concentrations of bacteriocin against *L. monocytogenes* PTCC 1302 (p<0.05), *S. aureus* PTCC 1113(p<0.05), *L. inovani* PTCC1303 (p<0.05), and *S. enterica* PTCC 1709 (p<0.05) were 6.25, 25, 6.25, and 12.5 µg/L, respectively **[56].**

Table (4): Determination of the minimum inhibitory concentration of the tested polysaccharides polymers against pathogenic bacterial strains

Bacterial polymer codes	Polymer concentrations (mg/mL)	Polymer dilutions	Pathogenic bacterial strains					
			Staph. aureus As4	*B. cereus* Dsmz345	*L. monocytogenes* As1	*E. coli* O157H7	*S. typhimirium* As3	*P. aeruginosa* Atoc27853
J1	400	1	-	-	+	+	+	+
	200	½	-	-	+	+	+	+
	100	¼	-	-	-	-	-	-
	50	1/8	-	-	-	-	-	-
	25	1/16	-	-	-	-	-	-
J2	400	1	-	-	+	+	+	+
	200	½	-	-	+	+	+	+
	100	¼	-	-	-	-	-	-
	50	1/8	-	-	-	-	-	-
	25	1/16	-	-	-	-	-	-
SCs1	300	1	+	+	+	+	+	+
	150	½	+	+	+	+	+	+
	75	¼	-	+	+	+	+	+
	37.5	1/8	-	-	-	-	-	-
	18.75	1/16	-	-	-	-	-	-
SCs3	300	1	+	+	+	+	+	+
	150	½	+	+	+	+	+	+
	75	¼	-	+	+	+	+	+
	37.5	1/8	-	-	-	-	-	-
	18.75	1/16	-	-	-	-	-	-
SCg2	200	1	+	+	+	+	+	+
	100	½	+	+	+	+	+	+
	50	¼	+	+	+	+	+	+
	25	1/8	-	+	+	+	+	+
	12.5	1/16	-	-	-	-	-	-
B	1000	1	-	-	-	+	-	+
	500	½	-	-	-	+	-	+
	250	¼	-	-	-	-	-	-

(-), growth; (+), no growth. B, polymer isolated from Basil soil; J, polymer isolated from Cane juice; SCg and SCs, polymer isolated from Spoilage cucumber. ***Staph., Staphylococcus; B., Bacillus; E., Escherichia; S., Salmonella; P., Pseudomonas.***

3.3. Phenotypic identification of the most efficient polymers producing isolates

The most efficient polymers-producing isolates were completely phenotypically identified (**Fig. 4**) up to species according to phenotypic (cultural, morphology, and physicochemical) characteristics according to the key of Bergey's Manual**[28]** for identifying dextran isolates andfor identifying levan isolate **[29, 30]**. Results in **Fig. 4** (**AI, II**) indicated that the J1, J2, SCs1, SCs3, and SCg2 isolates

producing dextran polymers were classified as *Leuconostoc mesenteroides* which investigated to be organized in pairs or chains, smooth, shiny, greyish white, positive for Gram stain, negative for catalase, nonmotile, and non-spore-forming. Growth happens between 5 and 30 °C, with 20 and 30 °C being the optimum range for growth. Growth occurs at pH 6.5 but not at pH 4.8 or when 10% (v/v) ethanol is present.

Moreover, the EPS-producing strain isolated from soybean paste was identified as *Leuconostoc mesenteroides* SN-8 **[57]**. As well as the classification of strain number 3 (LM03) biochemically as *L.mesenteroides var. mesenteroides* to demonstrate that the polymer produced was dextran **[58]**.

The B isolate producing levan polymer was classified as *Bacillus subtilis*(**Fig. 4 BI, II**),which showed as motile, aerobic, Gram-positive rods that produce cylindrical spores and are centrally located in unswollen sporangia. Cells typically exist alone or in pairs; chains are quite uncommon. Colonies have a moderately rounded to irregular shape, dull surfaces that might wrinkle, and margins that range from undulate to fimbriate; they also become opaque. The hue is white and could eventually turn creamy (Fig. 4). 28–30 °C is the optimum growth temperature, with a minimum of 5–20 °C and a maximum of 45–55 °C.Though no limits have been identified, growth occurs between pH values of 5.5 and 8.5 with up to 7% NaCl present. Oxidase variable, catalase positive. Starch and casein are hydrolyzed. Specifically, *B. subtilis* Natto CCT7712 was demonstrated to be an efficient levan producer **[59]**. Phenomy identification was used in order to recognize the high-efficiency levan-producing isolate V8 as *B. lentus* V8 **[35]**.

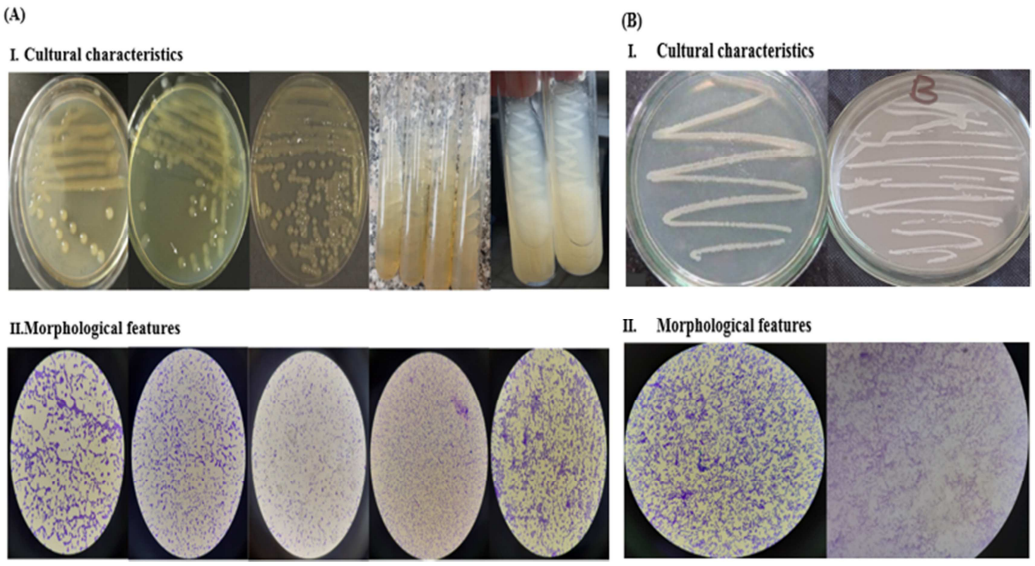

Figure 4: The most efficient isolates for dextran (A) and levan (B) polymers production.
I. Cultural characteristics.; II. Morphological features under light microscope (1000 X).

3.4. Polymer production using submerged fermentation

Under submerged fermentation, the dextran and levan polymers produced by the selected isolates (*Leuconostoc mesenteroides* codes J1,J2, SCs1, SCs3, SCg2, and *Bacillus subtilis* code B) that had antibacterial activity Results in **Fig. 5** showed the cell dry weight, polymer dry weight, polymer yield (%), $Y_{p/x}$ (g/g/L), and productivity (g/L/h) for *L. mesenteroides* codes J1, J2, SCs1, SCs3, SCg2, and *B. subtilis* code B isolates during propagation on productive medium supplemented with 150 g/L sucrose for dextran production and with 20 g/L sucrose for levan production at 30°C for 48-72 h.

Results showed that the cell dry weight on medium enhanced by 150 g/L sucrose after 48 h for *L. mesenteroides* codes J1 andJ2 being 3.56 and 3.63 g/L, and after 72 h for *L. mesenteroides* codes SCs1, SCs3, and SCg2 being 3.06, 4.05, and 4.11 g/L, respectively. While the cell dry weight on medium enhanced by 20 g/L sucrose after 48 h for *B. subtilis* code B isolate being 2.05 g/L.

The polymer production for *L. mesenteroides* codes J1, J2, SCs1, SCs3, and SCg2, and *B. subtilis* code B isolates being 3.78, 3.89, 4.11, 5.60, 6.36, and 2.77 g/L of polymer dry weight, respectively.

The polymer yield (%) on medium supplemented with 150 g/L sucrose after 48 h for J1

and J2 isolates was 2.52% and 2.59%, and after 72 h for *L. mesenteroides* codes SCs1,SCs3, and SCg2 being 2.74%, 3.73%, and 4.24%, respectively. Meanwhile, the polymer yield (%) on medium supplemented with 20 g/L sucrose after 48 h for *B. subtilis* code B isolate was 13.85%. The amount of polymer productivity was attained after 48 h of fermentation for *L. mesenteroides* codes J1 and J2, and *B. subtilis* code B isolates being 0.079, 0.081, and 0.058 g/L/h, respectively. While the amount of polymer productivity was achieved after 72 h of fermentation for *L. mesenteroides* codes SCs1, SCs3, and SCg2 being 0.057, 0.078. and 0.088 g/L/h, respectively. The polymer yield coefficient relative to biomass ($Y_{p/x}$) for *L.mesenteroides* isolate codes J1, J2, SCs1, SCs3 and SCg2, and *B. subtilis* code B being 1.06, 1.07, 1.34, 1.38, 1.55, and 1.35 g/g/L, respectively. The exopolysaccharide production by *Bacillus subtilis is in a* medium with sucrose (2.98 g EPS/L) **[24]**. Also, levan production from production media (40% sucrose) by local isolation of *B. subtilis* subsp. *subtilis* w36 was 4.9 g / 100 ml **[60]**. The lactic acid bacterial genus Leuconostoc B and C bacterial isolates could produce EPSs (14 g/L for isolate B and 32 g/L for isolate C) after 24 h **[61]**. The strain *L. mesenteroides var. mesenteroides* produced the highest concentration of dextran (26.87 g/L) in 76 h of incubation **[58]**.

Furthermore, *L. mesenteroides* code SCg2 was the most efficient polymer-producing isolate, producing the greatest dextran polymer with the highest productivity and yield. As a result, the dextran *L. mesenteroides* code SCg2 polymer was chosen for the next study.

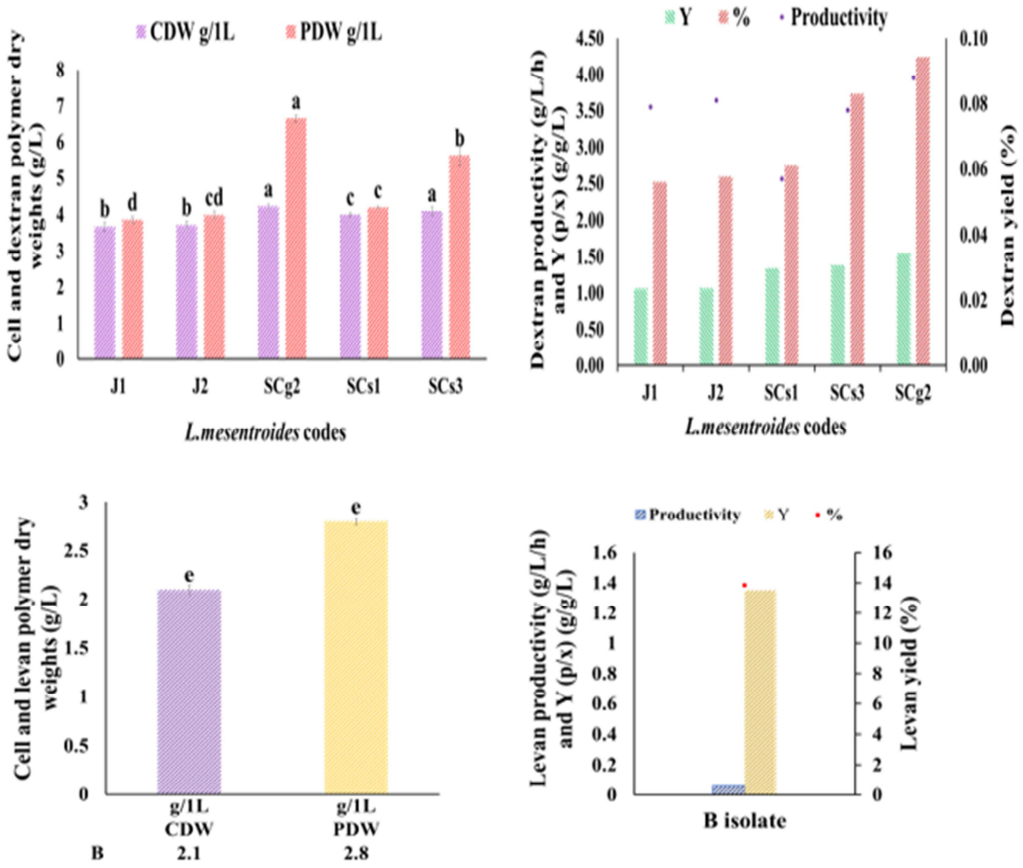

Figure 5: Biomass and polymers (dextran and levan) dry weight from the selected bacterial isolates using shake flasks (150 rpm) at 30°C for 48-72 h and kinetics parameters calculated. (A) Bacterial-producing dextran and (B) Bacterial-producing levan.

Y(p/x)= polymer yield coefficient relative to biomass; polymer yield (%)= initial sugar for dextran production was 150 g/L, and for the levan show was 20 g/L. B, polymer isolated from Basil soil; J, polymer isolated from Cane juice; SCg and SCs, polymer isolated from Spoilage cucumber.

- **[a, b] Values in the above column with the same letter do not differ significantly from each other according to Duncan's at 5% level. Bar indicated to ± standard division.**

3.5. Identification of the most efficient polymer (dextran) using ^{1}H-NMR

To learn more about the structural details of the dextran polymer produced by L. mesenteroides, NMR investigation was carried out. Two sections may be identified in the EPS 1H NMR spectrum: the ring proton region (δ3.1–4.5 ppm) and the anomeric region (δ4.5–5.5 ppm). **[62]**, The dextran polymer's 1H nuclear magnetic resonance (NMR) spectrum **(Fig. 6 and Table 5)** revealed chemical shifts that were consistent with the glycosyl residues serving as the biopolymer's repeating unit. The dextran polymer's H-2 to H-5 signals were located at δ3.51, δ3.64, δ3.44, and δ3.85, whereas the H-6 signals were located at δ3.92. The lone anomeric proton signal detected at 4.90–4.91 ppm corresponded toH-1 of the α-(1–6)-linked D-glucosyl residues comprising the main chain.

A characteristic signal of an α-(1→6) glycosidic chain was identified in the 1H NMR spectrum of the EPS, where a doublet was found in the anomeric region (H-1) at δ 4.98 ppm. Due to the C2-C6 connected protons, there was a ring proton area at δ 3.53-3.96 ppm. Double-doublet (dd) signals were detected at δ 3.57 and 3.75 ppm for H-2 and H-3, triplet (t) signals for (H-4) glucose carbonyl hydrogens were observed at 3.53 ppm, and double dubletos (dl) were observed at δ 3.92 and 3.96 ppm for H-5 and H-6. **[63].**

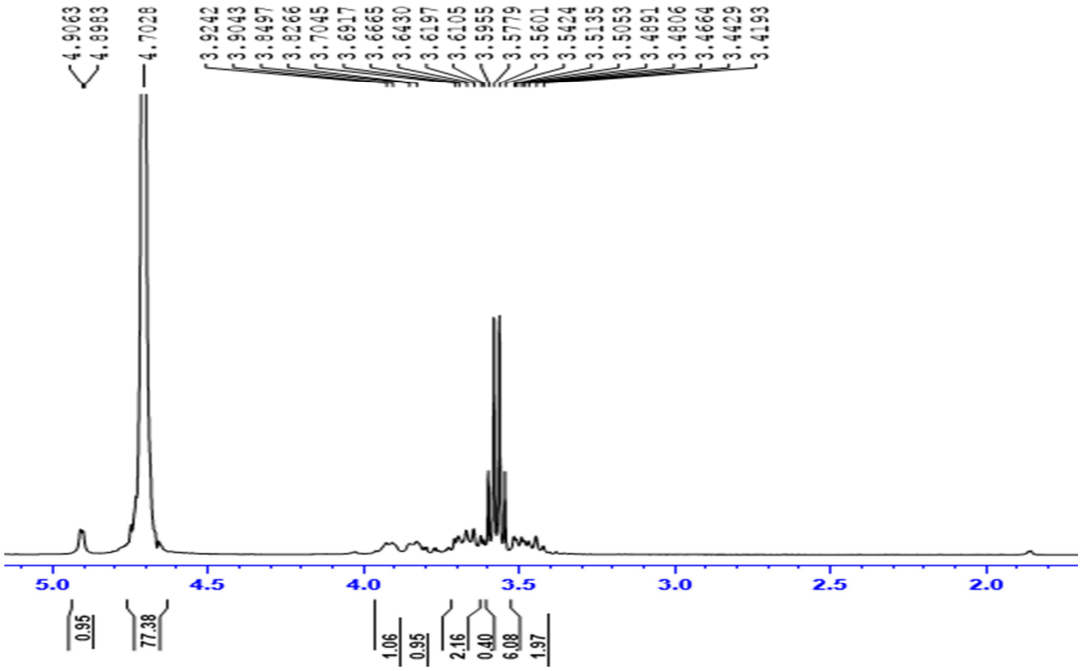

Figure 6: ^{1}H NMR spectrum (δ 3.44–3.92 ppm) of polymer produced *Leuconostoc mesenteroides*isolate

Table (5): Chemical shifts of proton in glucose skeleton of the produced bacterial dextran polymer

Proton atom ^{1}H NMR	Chemical shift (ppm) of dextran polymer	
	assignment result cited from Wang *et al.* [64]	The produced bacterial polymer
H_1	4.98	4.90- 4.91
H_2	3.52	3.51
H_3	3.63	3.64
H_4	3.45	3.44
H_5	3.84	3.85
H_6	3.92	4.92

1H NMR, 1H nuclear magnetic resonance; ppm, part per million

4. Conclusion

Among the 66 bacterial isolates used in this study, *L. mesenteroides* code SCg2 produced dextran polymer, which had antibacterial efficiency against Gram-positive and negative pathogenic bacteria with MIC values ranging from 200 to 25 mg/mL. This bacteria's polymer dry weight, yield, and productivity were 6.36 g/L, 4.24%, and 0.088 g/L/h, respectively.The polymer produced by the L. mesenteroides code SCg2 isolate was identified by NMR analysis as a dextran with a linear backbone made up of consecutive D-glucopyranose units joined by α-(1→6) linkages. As a result, this polymer will be used in food manufacturing as antibacterial substances, food additives, and food storage.

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