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# ANTIMICROBIAL AND ANTIOXIDANT CHARACTERISTICS OF EXOPOLYSACCHARIDES PRODUCED BY *AGROBACTERIUM TUMEFACIENS* CS5 AND T1

**Mohamed A. Moselhy *, Fatma Shams Lotfy, Hussein E. Makboul, Abdallah S. Ali**

*Microbiology Department, Faculty of Agriculture, Cairo University, 12613 Giza, Egypt.*

**ABSTRACT**

This work was conducted to isolate and screen for bacteria that producing higher quantities of exopolysaccharides (EPSs) from the Egyptian soils. Forty soil samples were collected from different regions of three Governorates (Beheira, Giza, and Qalyubia). A total of 165 bacterial isolates were obtained from the soil rhizosphere. Thirty-five of them (21.2%) had ability to produce stable ropy colonies. After screening, two isolates Cs5 and T1 were selected for further study according to their capacity to produce a high quantity of EPS (7.1 and 5.7 g/l, respectively). The selected bacterial isolates were identified as non-similar strains of *Agrobacterium tumefaciens* based on their morphological, biochemical characteristics as well as 16S rRNA gene sequencing. To obtain maximum EPS production of both strains, nutritional and cultivation parameters were optimized using the one-factor-at-a-time method. Thus, the maximum EPS yield of 21.63 and 19.57 g/l were achieved from the strains Cs5 and T1, respectively. When molasses and corn steep liquor were substituted the synthetic carbon and nitrogen sources as low cost-substrates, EPS yields were improved to be 28.73 and 24.5 g/l for Cs5 and T1, respectively. The infrared (FT-IR) spectra for both extracts revealed the typical patterns of polysaccharide absorption. Moreover, HPLC analysis demonstrated that EPSs were heteropolysaccharide composed of glucosamine, glucose, and fructose. The produced EPS showed antioxidant capacity as well as strong antibacterial activity against *Staphylococcus aureus* (methicillin-resistant) and *Bacillus cereus* as hazardous human pathogens, suggesting their application in the food industry. While the produced EPS did not affect cancer cells.

Keywords: *Agrobacterium tumefaciens*, exopolysaccharides, optimization, antibacterial, antioxidant, and soil.

## 1. INTRODUCTION

Exopolysaccharides (EPSs) are long chains of carbohydrate polymers with high molecular weight. They are naturally derived from eukaryotic (*e.g*. plants, phytoplankton, algae, and fungi) as well as prokaryotic producers (*e.g*. eubacteria and archaebacteria) [1]. Microbes can produce EPS into two forms; either in capsule or in a slimy layer [2]. EPS-producing microorganisms are found in numerous habitats especially that provide a high level of organic compounds (high carbon/nitrogen ratio) such as effluents from sugar, paper or food industries as well as other wastewater of factories [3]. EPSs provide the microbial cells of some important functions such as protection from the environmental stresses (*e.g*. osmotic pressure, temperature, pH, desiccation, heavy metals, oxidants and UV light), cell-aggregation during cell adherence to the surfaces and biofilm formation [1, 4, 5].

In general, the chemical composition of EPSs is consisted of monosaccharides and non-carbohydrate substances (*e.g*. acetate, phosphate, pyruvate, and succinate). Moreover, EPSs are chemically assorted into two sets either homopolysaccharides or heteropolysaccharides. Homopolysaccharides (*e.g*. cellulose, dextran, and glycogen) are made up of the same sugar subunit in their repeating unit structure with different linkages. While, heteropolysaccharides *e.g*. xanthan and

*Corresponding author e-mail: mohamed.moselhy@agr.cu.edu.eg *Tel: +20 1008025400, Fax: +202 35717355.*
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hyaluronic acid are composed of repeating units of two or more types of sugar subunits [6,7].

Microbial EPS gained particular attention over the other natural sources of EPS due to their shorter production periods, the easier extraction methods and the higher production yields. Such microbial EPS are known as natural biopolymers, biodegradable, biocompatible, stable and low toxic in comparison to the synthetic polymers [8]. Also, microbial polysaccharides have more variability of structures and physicochemical characteristics that enabling their applicability in multiple areas in biotechnology [4]. These bio-applications include; medical and pharmaceutical uses (*e.g*. antioxidants, antimicrobial, antiviral, antifungal, antitumor, heavy metal removal and immune modulation). EPS-applications are more extended to food industries (*e.g*. stabilizers, thickeners, gelling agents, bio-adhesives, probiotic, and emulsifier), cosmetics, textile, oil recovery, metal mining and metal recovery [9–13]. As well, the EPS-producing microbes found in the rhizosphere could be an essential agent for maintaining soil productivity through the improving of the soil structure via aggregation of soil components [14].

Among EPS-producing bacteria, *Agrobacterium* sp. was identified as the second strong EPS producer (76 g/l) after *Bacillus* sp. (86.3 g/l), followed by *Alcaligenes faecalis*, *Xanthomonas campestris* and *Zymonas mobilis* (72, 53 and 50 g/l, respectively) as reported by Donot *et al*. [15]. Also, curdlan is the main biopolymer formed by *Agrobacterium* sp. For the economic production of the microbial EPS, the optimizations of nutritional and ecological parameters are principal requirements. Therefore, the essential strategy for the EPS optimization is a single factor such optimization approach, to change one variable at a time keeping the other constant [16]. Also, agro-industrial wastes are utilized as substrates (*e.g*. glycerol rich product from the biodiesel industry, cheese whey, molasses, sago starch, and lignocellulosic materials), suggesting another strategy for the cost-effective production of microbial EPS [17,18].

Recently, the research has been directed to discover novel bioactive compounds for the side effects of the clinically used anticancer drugs as well as the emergence of the microbial resistance to the commercial antimicrobial drugs [19]. Therefore, the objectives of the present study were to isolate bacteria from the soil rhizosphere that able to produce reasonable amounts of EPS. Then, screening of the obtained isolates to define the most promising isolate (s), which produced a significant quantity of EPS. Identification of the potent isolate (s) through biochemical and molecular methods. Optimization of growth parameters and utilization of the low-cost substrates for the maximum EPS production. Characterizations of the EPS structure, as well as their biological activities (antioxidants, antimicrobial and antitumor), were also examined.

## 2. MATERIAL AND METHODS

### 2.1. Soil Samples

Forty soil samples were collected from different sites in Beheira (4), Giza (30) and Qalyubia (6) Governorates, Egypt. Soil samples were obtained from the rhizosphere area of cultivated lands at a depth of 10-20 cm. Samples were put in sterile plastic bags which transferred to the laboratory in an ice tank and kept in the fridge for further studies.

### 2.2. Culture media for EPS production and optimization

Four different culture media were tested for EPS production and optimization: ATCC no.14 medium, Basal medium (BM), Fermentation medium (FM) and plate count agar (PCA) [14, 20]. The ATCC no.14 medium was composed of (g/l): $KH_2PO_4$ (0.2); $K_2HPO_4$ (0.8); $MgSO_4.7H_2O$ (0.2); $CaSO_4.2H_2O$ (0.1); $FeCl_3$ (0.002); $Na_2MoO_4.2H_2O$ (trace); yeast extract (0.5); sucrose (20.0); pH 7.2. The BM medium was composed of (g/l): $K_2HPO_4$ (5.0); yeast extract (3.0); $MgSO_4$ (0.2); glucose (20.0) pH 7.0. The FM medium was consisted of (g/l): dextrose (20.0); beef extract (15.0); NaCl (5.0); $K_2HPO_4$ (8.0); $KH_2PO_4$ (2.0); $MgSO_4$ (0.5); pH 7.0. The PCA medium without agar was comprised of (g/l): tryptone (5.0); yeast extract (5.0); glucose (20.0); pH 7.0.

### 2.3. Isolation and purification of exopolysaccharide producing bacteria

Soil samples were serially diluted from $10^{-1}$ to $10^{-6}$ in a sterile saline solution (0.85%). Then, one ml of each dilution was aseptically transferred to inoculate nutrient agar medium in triplicate using the pour plate technique. Nutrient agar medium was supplemented with glucose or sucrose (1%). The inoculated plates were incubated for 24-72 h at 30°C. During the incubation period, plates were observed to isolate colonies that produce mucous. The mucoid colonies were further purified by streaking four times to obtain pure cultures, that consequently stored frozen at –20°C in a nutrient broth medium containing glycerol 25%.

### 2.4. EPS production and extraction

Bacterial isolates were screened for production of polysaccharide by inoculation of nutrient broth medium (50 ml working volume in 250-ml Erlenmeyer flasks) supplemented with 1% glucose and incubated on a rotary shaker (100 rpm) at ambient temperature for 72 h. Then, the broth was centrifuged at 5000 rpm for 20 minutes. The supernatant was collected in a sterile test tube and three-volume of chilled ethanol were added and stored overnight at 4°C. The precipitated EPS was centrifuged for 20 minutes at 5000 rpm and the supernatants were discarded. The obtained pellet was dried in an oven at 60°C until constant weight and the EPS dry weight was recorded to know which organism showed high production of exopolysaccharides [21].

2.5. Identification of the potent EPS-producing bacteria

2.5.1. Cultural, morphological and biochemical characterization

The isolates with the highest EPS production (Cs5 and T1) were identified according to Bergey's manual of determinative bacteriology 9$^{th}$ edition [22]. The morphological characterization included Gram staining, motility, colony size, colony color, and colony consistency. Biochemical identification comprised the catalase, oxidase, urease, indole, and citrate utilization tests; gelatin, casein and starch hydrolysis; growth on McConkey`s agar medium and carbohydrate fermentation of various sugars such as sucrose, glucose, fructose, xylose, and lactose.

2.5.2. Molecular characterization of the selected bacterial cultures

The genomic DNA from two selected bacterial isolates was extracted [23]. The extracted DNA was subjected to 16S rRNA gene amplification using the universal primers F-27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1494 (5′-*CTACGGYTACCTTGTTACGAC*-3′). Polymerase chain reaction (PCR) was performed using a 16S rRNA bacterial identification PCR kit [The Min Elute PCR Purification Kit 50 (cat. nos. 28004 and 28006), Italy] to prepare reaction mixtures. PCR was done on a Gene Amplification PCR System using the following condition: 95°C for 4 min (initial denaturation 1 cycle), 95°C for 40 s (denaturation), 50°C for 30 s (annealing), 72°C for 1 min (elongation), 72°C for 10 min (final elongation) with 35 cycles by using PCR machine (Bio-rad T100 thermal cycler, Italy). The PCR products were checked via agarose gel electrophoresis. The electrophoresis was carried out in 1.5% agarose and the bands were observed and photographed using the gel documentation system. All operations were applied according to the manufacturer's instructions.

Based on the sequenced data, the obtained nucleotide sequences were used for sequence similarity analysis through BLAST (http://www.ncbi.nlm.nih.gov/blast). The sequences were deposited in the GenBank database and the accession numbers were obtained. The phylogenetic tree was constructed using MEGA 5.05 (http://megasoftware.net/) [24], for aligning the sequences by Neighbor-joining method [25] and maximum composite likelihood method [26]

2.6. Optimization of EPS production and preparing of the bacterial growth curve

The impacts of media type, medium components and cultivation conditions on EPS production by *Agrobacterium tumefaciens* Cs5 and T1 were optimized using the classical one-factor-at-a-time method [27]. For that purpose, four types of media (ATCC no. 14, BM, FM and PCA), five carbon sources (glucose, sucrose, lactose, fructose, maltose), different sucrose concentrations (0.5, 1, 1.5, 2, 2.5 and 3%) and nine nitrogen sources (organic and inorganic) were investigated. Growth conditions such as incubation temperatures (25, 30, 37, 45 and 50°C), initial pH (5, 5.5, 6, 6.5, 7, 7.5 and 8), agitation speed (zero, 50, 100 and 150 rpm), inoculum size (3, 7, 10, 12 and 15%) and incubation period (1-7 days) were also tested. All experiments were conducted in triplicate. The produced EPS were extracted, dried, and weighed as stated above.

To follow the bacterial growth, each strain was grown in triplicate flasks of ATCC no. 14 broth medium which inoculated with an inoculum size giving an initial count of $\approx$ 2.5 $\log_{10}$ CFU/ml. Inoculated flasks were incubated under optimum growth conditions for each strain. Over the time-course of incubation ranging from 3 to 168 h, samples were aseptically withdrawn from the flasks and their bacterial load was monitored by counting the grown colonies on ATCC no. 14 agar medium using the dilution plate method. Simultaneously, the produced EPSs were also determined.

2.7. EPS production from low-cost substrates

Sugar cane molasses (70.4 % sugar content) was obtained from the Hawamdiya sugar factory, Sugar and Integrated Industries Company, Hawamdiya, Giza that used as a raw carbon substrate. Molasses was clarified by mixing with distilled water comprising sulfuric acid (2 g/l) at a ratio of 1:1 [28]. The mixture was autoclaved at 121°C for 30 min and left overnight to settle. The clarified molasses was used at 2% concentration in

ATCC no. 14 broth medium for sucrose replacement as previously described by Abdul Razack *et al.* [21].

Corn steep liquor (CSL) was obtained from Starch and Glucose Factory, Mostorod, Cairo, Egypt. It is a by-product of the wet-milling process of the maize-starch industry. It is a viscous liquid consists of nitrogen (7.8 %) vitamins and minerals which used at a concentration of 20 ml/l of ATCC no. 14 broth medium for substitution of yeast extract. Then the EPS production was performed in the modified ATCC no. 14 liquid medium under the optimized conditions.

2.8. Characterization of EPS

2.8.1. Fourier transform infrared spectroscopy (FT-IR) analysis

The major functional groups in the produced EPS were detected by using a Fourier transform infrared spectrometer (Nicolet 380 FT-IR, Thermo Scientific, USA). The dried EPS was grounded with potassium bromide powder and pressed into a disc of 10 mm diameter. The FT-IR readings were between the frequency range of 4000 $cm^{-1}$ to 500 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ and 2 scans [16].

2.8.2. HPLC analysis for EPS monosaccharide composition

Partly purified EPS (20 mg) was hydrolyzed with 3 ml of 2 M trifluoroacetic acid (TFA) at 120°C for 8 h, afterward analyzed by HPLC (Agilent 1260 Infinity HPLC series, Agilent, USA), equipped with a quaternary pump, the used column was Phenomenex Rezex® RCM-monosaccharide, 300 mm × 7.8 mm operated at 80°C. The separation was completed using isocratic elution by HPLC grade water with a flow rate of 0.6 ml/min, the injected volume was 20 µl. The used detector was Refractive Index (RI detector) that operated at 40°C.

2.9. Bio-applications of the produced EPS

2.9.1. Antioxidant activity (free radical-scavenging activity)

The antioxidant activities of the EPS were estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [19], to determine free radical-scavenging potential of each EPS sample from two selected bacterial strains. The reaction mixture contained 2 ml of the EPS at different concentrations (40, 80, 100 and 120 µg/ml) and 2 ml of DPPH (0.2 mM in ethanol) was strongly shaken and kept in darkness at the ambient temperature for 30 minutes. Then the absorbance was measured at 517 nm by using UV-VIS Shimadzu spectrophotometer 2401PC (Shimadzu, Japan). The mean values were calculated from triplicate experiments. Antioxidant activity (radical scavenging activity) of EPS was expressed as a percentage of inactivated DPPH reagents from the following equation:

%DPPH "RSA" = [Abs. 517 of control – Abs. 517 of sample / Abs. 517 of control] ×100.

The effective concentration ($EC_{50}$) was considered as the EPS concentration (µg/ml) at which the DPPH absorbance was reduced by 50%.

2.9.2. Antimicrobial activity

The antimicrobial characteristics of the produced EPS were evaluated according to Bauer *et al.* [29] by using the disc-diffusion assay. The sterile paper discs (5 mm diameter) were saturated with 10 µl of the EPS samples (100 mg/ml solubilized in DMSO) and placed on the inoculated agar medium. The antimicrobial potency of the EPS was tested against a wide set of microorganisms including Gram-positive bacteria (*Bacillus cereus* ATCC 33018, *Staphylococcus aureus* MRSA ATCC 43300 and *Listeria monocytogenes* ATCC 7644), Gram-negative bacteria (*Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9072 and *Escherichia coli* ATCC 25922) on Mueller-Hinton agar medium at 30 - 37°C for 24 - 48 h. and fungal strains (*Macrophomina phaseoli* nrrl 62743 and *Candida albicans* ATCC 10231) on Sabouraud dextrose agar medium at 25°C for 24 - 72 h. Subsequently, the antimicrobial activity was evaluated by measuring the inhibition zone diameter (mm) around each of the EPS saturated discs. Discs loaded with penicillin G (10 µg), ampicillin (10 µg) and nystatin (100 units) were served as positive standard antimicrobials for Gram-positive bacteria, Gram-negative bacteria, and fungi; respectively.

2.9.3. Antitumor activity (cytotoxicity)

The EPS influence on cell viability was assessed by neutral red (NR) uptake assay [30]. The used cell cultures were human lung cancer (A-549) and human lung normal (WI-38) cell lines. These cell lines were obtained from the Tissue Culture Unit, Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt. Cells were cultivated in individual 96-well plates containing Dulbecco's Modified Eagle's medium (DMEM) complemented with 10% (v/v) fetal bovine serum (FBS) at a density of $10^5$ cells/well. After incubation at 37°C for 48 h, adhered cells were treated with 50 µl of the EPS solution at different concentrations (50, 200, 300 and 500 µg/ml in DMSO) for 48 h. DMEM was used as a negative control, while doxorubicin (100 µg/ml) was used as a positive control (giving 100% inhibition). The supernatant of each well was

replaced by 100 µl of fresh medium (without FBS) containing 20 µl NR solution (0.33%) at separate wells. The optical density (OD) of the well contents was read at 540 and 570 nm with an ELISA plate reader (BioTek Instrument, ELx808, USA). The experiments were recurring by using intact cells as the control and the assays were repeated in triplicates. Growth inhibition rates were calculated using the following formula: Growth inhibition rate (%) = [A-B/A] x 100.

Where: A and B are the absorbance of the supernatant of untreated and treated cell cultures, respectively. Then, the half-maximum inhibitory concentration ($IC_{50}$) was estimated from the plotted graph.

2.10. Statistical analysis

All the obtained experimental data were denoted as means ± standard deviation (SD) in triplicate. Statistics were tested by one-way analysis ANOVA (using XLSTAT 2019.1.2.56963 - ANOVA - Microsoft Excel 15.04420 software) to assess the variances among the means of parameters. Statistical significance was defined as $P < 0.05$.

## 3. RESULTS AND DISCUSSION

3.1. Isolation, screening, and identification of EPS-producing bacteria

Exopolysaccharides (EPSs) are natural polymers of high mass delivered by microbes into their environment. EPS can protect bacterial cells against various environmental stresses as well as engulfment with the predatory protozoa and white blood cells. Also, they can act as natural adhesives to stable surfaces. Furthermore, EPSs have significant bio-applications in the medical, pharmaceutical and food industries [31]. Therefore, the first part of this study was concerned with isolation and screening for the most forceful EPS-producing bacteria from Egyptian soil samples. The results showed that a total of 165 bacterial isolates were obtained from the soil rhizosphere, distributed as 40 (24.2%), 103 (62.4%) and 22 (13.3%) isolates on the sampling Governorates; Beheira, Giza, and Qalyubia, respectively. Thirty-five of the isolates (21.2%) could produce stable ropy colonies. After screening of these isolates for EPS production, two isolates (Cs5 and T1) were selected for further study according to their capability to produce a high quantity of EPS (7.1 and 5.7 g/l; Fig. 1). Comparable amounts of EPS were produced by *Bacillus velezensis* KY471306 (6.0 g/l) and *Rhodobacter johrii* CDR-SL 7Cii (6.2 g/l) as reported before by Moghannem *et al.* [32] and Sran *et al.* [33]. Mohamed *et al.* [19] obtained a higher quantity of EPS (10.3 g/l) by *Bacillus altitudinis* MSH2014. However, other bacterial isolates of this study produced amounts of EPS ranged between 0.82 and 2.76 g/l (Fig.1).

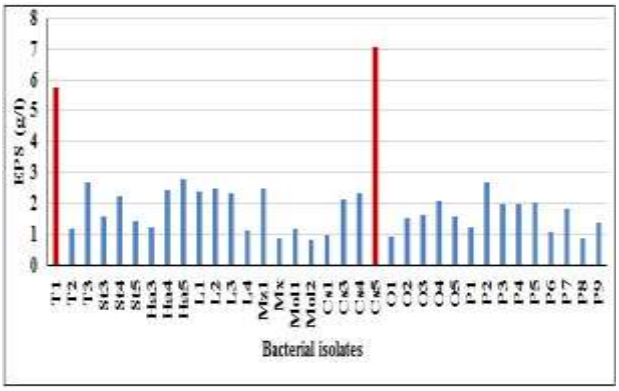


**Fig. 1: Screening of bacterial isolates for EPS production.**

Although the two isolates (Cs5 and T1) were obtained from different sampling sites, they revealed similar phenotypic characteristics. Their colonies were circular, convex, smooth, opalescent, with beige color and showed highly mucoid appearance on agar medium. The cells were Gram-negative, rod-shaped, motile and non-spore forming. Positive reactions were recorded for the following testes: catalase, oxidase, urease, citrate utilization, growth on McConkey`s agar medium and carbohydrate fermentation (sucrose, glucose, fructose, xylose, and lactose). Negative results were found for gelatin, casein and starch hydrolysis as well as indole test. So, the two isolates were identified as *Agrobacterium* spp. according to Bergey's manual of determinative bacteriology 9$^{th}$ edition [22].

The molecular identification using the partially sequenced 16S rRNA genes of both isolates (Cs5 and T1) showed that they belonged to the genus *Agrobacterium* with 100% identity to the species *A. tumefaciens*. Interestingly, despite the two strains were identified as the same species, they had different fingerprint profiles confirmed with 99.83% similarity between the 16S rRNA sequences, suggesting the variation between the two strains. This may lead to different EPS production capabilities between both isolates. A phylogenetic tree (Fig. 2) derived from16S rRNA gene sequences was performed by the neighbor-joining method to illustrate the relative positions of such strains (Cs5 and T1) and other *Agrobacterium* species. Moreover, the sequences were deposited in the GenBank database (NCBI) under accession numbers MN508443 and MN508444 for Cs5 and T1, correspondingly.

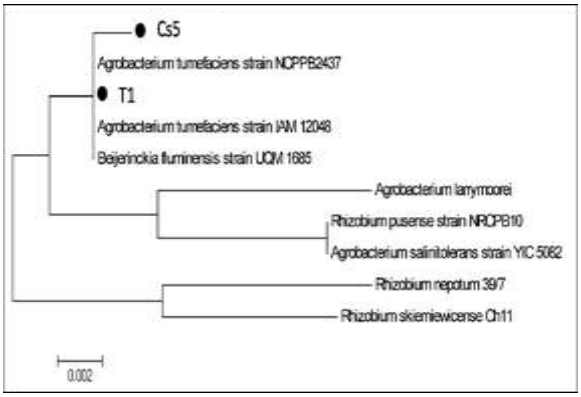


**Fig. 2: Phylogenetic tree indicating the estimated relationships between the *Agrobacterium tumefaciens* strains (Cs5 and T1) and their closest hits from the NCBI GenBank.**

3.2. Optimal parameters for EPS production by *Agrobacterium* strains (Cs5 and T1)

The nutritional requirements and the cultivation parameters for production of EPS by Cs5 and T1 are shown in Figs. 3 and 4, respectively. In this work, four broth media (ATCC no.14, BM, FM and PCA) were tested for the EPS production by the selected two strains. The results emerged that significant amounts of the EPS ($P < 0.05$) produced by Cs5 and T1 in the ATCC no.14 medium (averaged of 7.44 g/l; Fig. 3A). This finding may be referred to that medium ATCC no.14 included sucrose as a carbon source which is preferable by the selected strains for EPS production than glucose in the other three media. This result was supported by the findings of Mu'minah *et al.* [14] and Harahap *et al.* [34] that found the ATCC no.14 medium is the suitable medium for the production of EPS by soil microorganisms.

During the microbial production of EPS, the carbon source serves as a nutrient and energy source to support the cell growth for EPS synthesis [35]. Among the tested carbon sources, sucrose significantly supported ($P < 0.05$) the production of EPS by the two *Agrobacterium* strains followed by glucose whereas; lactose, fructose, and maltose gave the minimum yields (Fig. 3B). Sucrose and glucose are the most frequently carbon sources used by different microbes for EPS production [18]. Along with the type of the used carbon source, its concentration is also an important factor for EPS-producers. The *Agrobacterium* strain Cs5 favored sucrose concentrations of 2-3% ($P < 0.05$), with maximum EPS yield at 2.5% (7.4 g/l) while, the strain T1 got its maximum EPS production (6.6 g/l) at 2% sucrose (Fig. 3C). This finding is consistent with that of Triveni *et al.* [36] where the maximum EPS production by *Agrobacterium radiobacter* was obtained at a sucrose concentration of 2.62%. However, Shih *et al.* [35] recommended the sucrose concentration of 14% was needed for maximum curdlan production by *Agrobacterium* sp. Recently, Krishnamurthy *et al.* [13] proposed that sucrose concentration of 5% stimulated maximum EPS production (3.31 g/l) by *Bacillus cereus* KMS3-1. In the present study, sucrose concentrations beyond 2% for the strain T1 and 3% for the Cs5 led to a decrease in the EPS production. This mostly explained by increasing the osmotic pressure in the medium which caused plasmolysis, leading to cell death [21].

In the current work different organic and inorganic nitrogen sources were tested. Among the organic nitrogen sources, beef extract gave considerable amounts ($P < 0.05$) of EPS by both *Agrobacterium* strains (averaged of 7.2 g/l; Fig. 3D). While sodium nitrate as an inorganic nitrogen source greatly supported ($P < 0.05$) the maximum EPS production of both *Agrobacterium* strains (averaged of 7.7 g/l; Fig. 3D). Generally, the availability of carbon was accompanied by the limitation of nitrogen which is commonly reported as the better conditions for EPS production by different microbes [18]. Yeast extract was suggested before as an organic nitrogen source to encourage the productivity of the EPS [13,16,21]. Whereas, other researchers recommended different sources of inorganic nitrogen such as urea [35], ammonium phosphate [37], ammonium sulphate [38] and ammonium nitrate [39] to support the production of higher amounts of EPS.

The initial pH value of the growth medium is an essential factor because it affects cell growth, nutrient uptake and EPS production [40]. In this work, it was observed that the neutral pH significantly favored ($P < 0.05$) the production of EPS by the strain T1 (pH 7; 6.93 g/l) and strain Cs5 (pH 7.5; 7.13 g/l) as shown in Fig. 4A. This result accords with various reports showed that higher EPS production was obtained at neutral pH [13,16,39,40]. While the pH at 6.24 was optimum for EPS production from *Agrobacterium radiobacter* [36]. Shih *et al.* [35] recommended that controlling of pH between 5-7 led to the maximum curdlan production by *Agrobacterium* sp. On the contrary, Almansoory *et al.* [38] noticed that the production of the EPS by the bacterial consortium culture was increased at pH 8.

The temperature of incubation is also considered as a critical factor that can influence microbial growth and their enzymatic activities. The temperature range from 25 to 37°C was noticed to be appropriate for the growth and the production of EPS by the *Agrobacterium* stains, beyond this temperature range the EPS production was sharply declined.

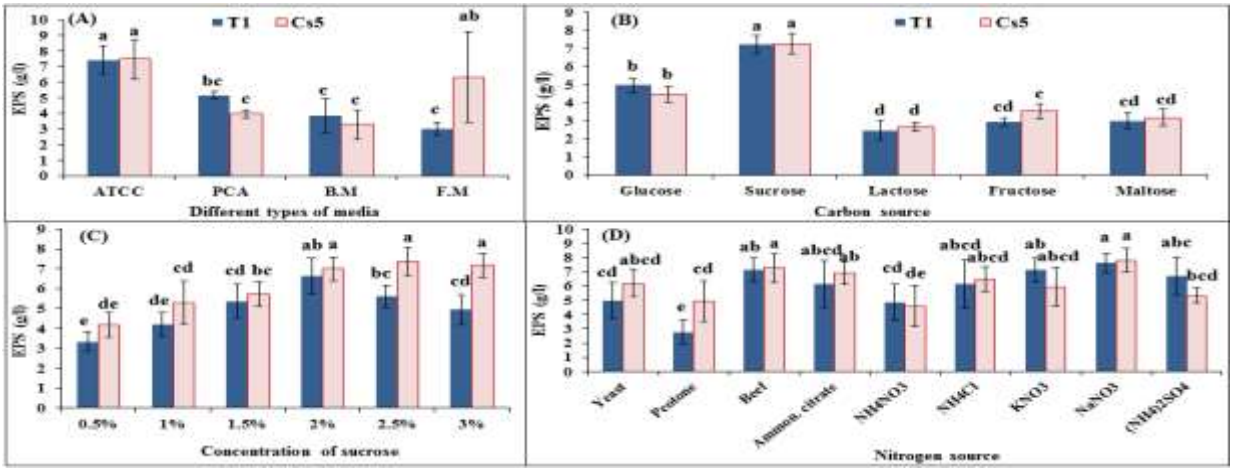


**Fig. 3: Effect of nutritional requirements; media type (A), carbon sources (B), sucrose concentrations (C) and nitrogen sources (D) on EPS production by *Agrobacterium tumefaciens* strains (Cs5 and T1); a-e = columns with the same letter are not significantly different (P < 0.05).**

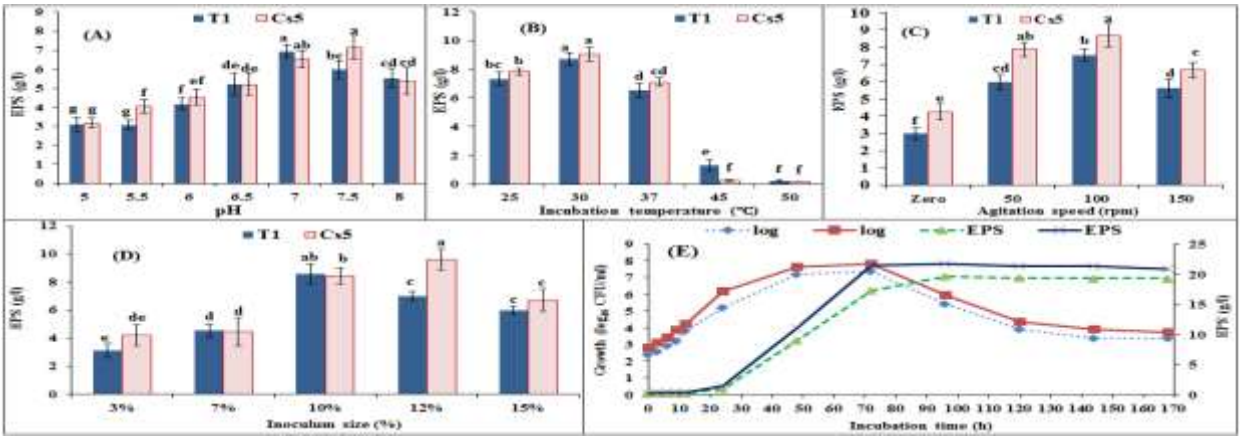


**Fig. 4: Effect of cultivation parameters; pH (A), incubation temperature (B), agitation speed (C), inoculum size (D) and incubation time (E) on EPS production by *Agrobacterium tumefaciens* strains (Cs5 and T1); a-f = columns with the same letter are not significantly different (P < 0.05).**

Within this temperature range, other researchers had better EPS production such as Oleksy-Sobczak *et al.* [5] at 25°C, McKellar *et al.* [42] at 28°C, Manivasagan *et al.* [40] and Lakra *et al.* [39] at 35°C. Definitely, at 30°C the highest EPS production was obtained by both *Agrobacterium* strains (averaged of 8.9 g/l; Fig. 4B). Several researchers agreed with these results despite the type of the used microbes; *Agrobacterium* sp. [35], *Aureobasidium pullulans* [43], *Bacillus velezensis* [32] and *Bacillus* sp. [44]. On the contrary, *Geobacillus stearothermophilus* could not produce considerable amounts of EPS at 55°C, nonetheless, it has been reported [45] to produce thermostable extracellular glycosidases which involved in EPS synthesis. The reason for that is the optimal growth temperature (55°C) of this microorganism may not match the optimal temperature for the activity of EPS producing enzymes.

Mixing and aeration are also relevant factors since they could impact the availability of oxygen and nutrients for microorganisms. It was found that the agitation speed of 100 rpm significantly stimulated (P < 0.05) the production of the EPS by the two different *Agrobacterium* strains (averaged of 8.12 g/l; Fig. 4C) followed by 50 rpm (averaged of 6.93 g/l); whereas, static flasks (zero rpm) produced the lowest amount of EPS (3.62 g/l). High agitation speed has been reported to give a high production rate of EPS by some microbes [33,35]. However, vigorous agitation rates may result in injury to the microbial cells or changing the characteristics of the produced EPS [10]. Contrariwise, the low agitation speed may not deliver enough amounts of nutrients and oxygen to the microbial cells leading to low productivity of the EPS.

As shown in Fig. (4D), the suitable inoculum size for the maximum production of EPS was varied between the two *Agrobacterium* stains. Strain T1 produced the highest EPS amount (8.6 g/l) at 10% inoculum size whereas, at 12% inoculum size, the strain Cs5 reached its maximum EPS production (9.6 g/l). Inoculum sizes above 12% or less than 10% significantly reduced EPS production. By the present results, previous reports have demonstrated that 10-11% inoculum size had a great effect on the production of the maximum amount of EPS [46,47]. In contrast, other studies recommended lower percentages of inoculum (1-5%) for the highest EPS production [33,36,43].

Time course for bacterial growth ($log_{10}$ CFU/ml) and the EPS production (g/l) were monitored over 168 h of fermentation. The presented data (Fig. 4E) revealed that the growth of both strains of *Agrobacterium* (Cs5 and T1) followed similar patterns with the superiority of strain Cs5 in the cell counts. The exponential growth phase lasted during a period of 48 h of fermentation, with no lag phase, while a short stationary growth phase was observed after only 24 h. Later on, the bacterial growth was sharply declined after getting its maximum value at 72 h of fermentation (Fig. 4E), then steadily decreased after 120 h till the end of the fermentation period. A similar growth pattern was presented by Malick *et al.* [45] for the EPS-producing bacterium *Bacillus amyloliquefaciens* 23350. A parallel increase of the EPS concentrations was noticed with bacterial growth through the fermentation period, especially after 24 h to 96 h (Fig. 4E). The productivity of EPS by *Agrobacterium* Cs5 and T1 strains was 0.23 and 0.20 g/ (l.h), respectively. This result supports evidence from previous observations [33, 45] who found that EPS production was growth-dependent since its concentration decreased after reaching the highest values at the end of the growth phase or shortly prolonged time. Hence, the produced EPS by both strains might be considered as primary metabolites. A slight decrease was observed in EPS production during the early decline phase. This could be referred to as the enzymatic degradation of polysaccharides or the alteration in physical and chemical parameters of culture media as earlier reported [33,45]. As summarized in Table 1, the cultivation of the *Agrobacterium* strains under the optimum cultural parameters achieved the maximum EPS concentrations of 21.63 and 19.57 g/l for the Cs5 and T1 strains, respectively, representing significant increases of 204.7% and 234.3%, in that order, as compared with the primary obtained results for both strains (Fig. 1). Comparable amounts of the EPS 22.6 and 21.4 g/l were produced by *Agrobacterium radiobacter* and *Agrobacterium* HX1126, respectively when cultured under their optimum cultivation conditions [36,48].

**Table 1. Outline of the optimum cultural parameters for the *Agrobacterium tumefaciens* strains (Cs5 and T1).**

| Parameters \ Bacterial strains | Cs5 | T1 |
|---|---|---|
| Media type | ATCC no. 14 | ATCC no. 14 |
| Carbon source | Sucrose | Sucrose |
| Sucrose concentration (%) | 2.5% | 2.0% |
| Nitrogen source | $NaNO_3$ | $NaNO_3$ |
| pH | 7.5 | 7.0 |
| Incubation temperature (°C) | 30°C | 30°C |
| Agitation speed (rpm) | 100 rpm | 100 rpm |
| Inoculum size (%) | 12.0% | 10.0% |
| Fermentation time (h) | 96 h | 96 h |
| EPS production (g/l) | 21.63 g/l | 19.57 g/l |

### 3.3. Efficacy of the low-cost substrates for EPS production

The commercial production of EPS requires higher production rates as well as a reduction of the fermentation expenses. Moreover, it is known that nearly 30% of the total costs of microbial fermentation processes are spent on culture media [18]. For that purpose, molasses and corn steep liquor (CSL) were used as low-cost carbon and nitrogen sources, respectively. The data illustrated in Fig. 5 indicated that the use of molasses and CSL instead of the synthetic carbon and nitrogen sources in the medium (ATCC no.14) under the optimum cultivation parameters greatly improved EPS production rates to be 0.29 and 0.26 g/(l.h) for Cs5 and T1, respectively. Therefore, the produced amounts of EPS were 28.73 and 24.5 g/l, representing an increase in the productivity by 33 and 25% for both strains, respectively. Such increase in the productivity could be attributed to molasses has high contents of minerals and vitamins besides to the readily fermented sugars [21].

Likewise, CSL comprises 40% protein, 21% lactic acid and 16% nitrogen-free extract as well as amino acids, vitamins and other growth factors that support microbial growth and productivity [49,50]. These results were previously supported by Abdul Razack *et al.* [21] and Moghannem *et al.* [32] who produced high amounts of EPS (4.86 and 4.2 g/l, respectively) by *Bacillus* sp. when molasses was used as carbon source. While Sharma *et al.* [49] obtained significant quantity (89 g/l) of pullulan from

*Aureobasidium pullulans* when utilized CSL as a fermentative substrate.

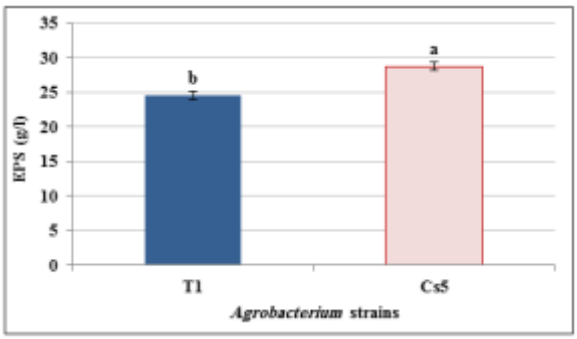


**Fig. 5: EPS productivity by *Agrobacterium tumefaciens* strains cultivated in medium containing molasses and corn steep liquor as low-cost substrates; a and b = columns with the same letter are not significantly different (P < 0.05).**

3.4. Characterization of the EPS produced by *A. tumefaciens* strains (Cs5 and T1)

3.4.1. FT-IR characterization of the EPS

FT-IR spectra of the produced EPS from *Agrobacterium* strains (Cs5 and T1) are displayed in Fig. (6). The IR spectra of the polymers have shown a broad intense band at 4000 $cm^{-1}$ to 400 $cm^{-1}$ with resolution of 4 $cm^{-1}$. Obviously, there were no major differences between the extracts of both strains regarding the functional groups. The spectrum of broadband at 3660 $cm^{-1}$ and 3650 $cm^{-1}$ for T1 and Cs5 strongly suggested the presence of broad stretching (O-H groups) which represents the extending vibration of the hydroxyl groups that characterize the carbohydrates, in addition to shifting of the hydroxyl group bands indicated the higher wave numbers in the spectrum of the exopolysaccharide isolated from Cs5 as well as water adsorption. This is the typical absorption band of exopolysaccharides and thus responsible for the hydrophilic nature of the EPS [16].

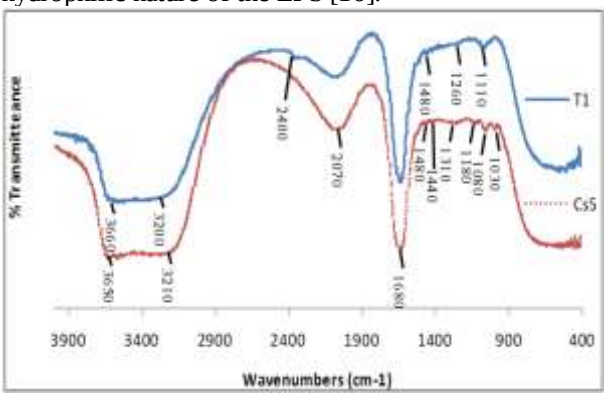


**Fig. 6: FT-IR analysis of EPS produced by *Agrobacterium tumefaciens* strains (Cs5 and T1).**

The peaks at 2088.96 $cm^{-1}$ and 2070 $cm^{-1}$ for Cs5 and T1, respectively were corresponding with stretching vibration of C≡C groups (terminal alkenes). The absorptions at 1660 $cm^{-1}$ and 1680 $cm^{-1}$ for both strains indicated the existence stretching vibration of C═O bond which found in carboxylic acids, anhydrides, ketones, esters and amides that are the typical property of the EPS [39,51]. The presence of a carboxyl group may serve as a binding site for metal ions which has been suggested as a potential mechanism for the antimicrobial activity [52]. Also, the occurrence of carboxylic group confirming that the sample contains aminosugars [53]. These findings are in one line with the sugar compositional analysis. Especially, the bands at 1480-1260 $cm^{-1}$ are attributed to the glycosidic units in the exopolysaccharides in the T1, whereas this band were shifted to 1440 $cm^{-1}$ is due to the vibration of methyl group (-$CH_3$). The specific backbone bands of the exopolysaccharides were present at stretching vibration of the -C-O- at 1310 $cm^{-1}$. Also, the peaks at 1030 to 1180 $cm^{-1}$ in Cs5 and T1 referred to the glycosidic linkages in the exopolysaccharides. Finally, the absorption from 1480 to 1000 $cm^{-1}$ are clearly corresponding to the reported fingerprint region used to characterize polysaccharides [54].

3.4.2. HPLC analysis of the EPS

Monosaccharide composition of EPS produced by *Agrobacterium* strains (Cs5 and T1) is shown in Fig. (7). The obtained results of two chromatograms revealed that the two extracts had similar independent peaks. Retention time and percentage of the major components of EPS for both strains were correspondingly; O-glucosamine (averaged; 7 min, 80%), glucose (averaged; 11 min, 10%) and fructose (averaged; 14.5 min, 8%), suggesting the heteropolysaccharide polymers. Unexpected peaks of sucrose appeared at 9.5 min with about 2% concentration for both extracts. This finding might be explained as incomplete hydrolysis due to the formation of stable dimers between mono-sugars within the EPS exhibiting strong glycosidic bonds, which are not easily broken under standard hydrolysis conditions [55]. Therefore, the hydrolysis conditions such as acid concentration, incubation temperature and time could be adjusted to obtain complete hydrolysis of polymers. It is noteworthy; *Agrobacterium tumefaciens* produces different types of EPS. Five of them have been extensively characterized; these are cyclic-β-(1, 2)-glucan, cellulose, curdlan, succinoglycan, and the unipolar polysaccharide (UPP). The latter is more likely produced in the current study because it exclusively includes N-acetyl glucosamine in its composition [56]. However, the EPS from *Agrobacterium*

*radiobacter* contained glucose, galactose, and rhamnose [36]. Recently, Liu *et al.* [48] reported that the monosaccharide of *Agrobacterium* HX1126 composed of glucose (97.2%), galactose (1.5%) and aminogalactose (1.3%).

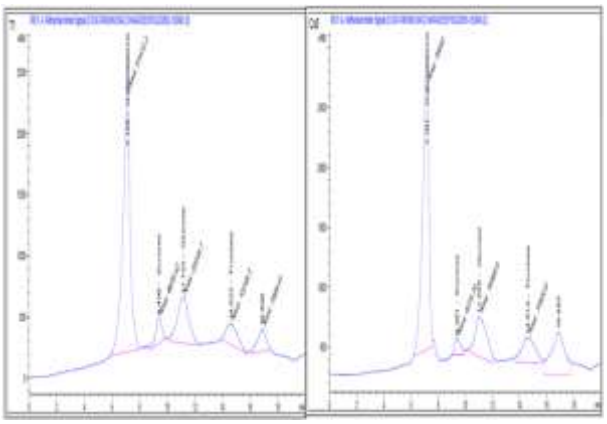

**Fig. 7: HPLC analysis of EPS produced by *Agrobacterium tumefaciens* strains (Cs5 and T1).**

3.5. Biological activities of EPS produced by *Agrobacterium* strains (Cs5 and T1)

3.5.1. Antioxidant activity

Free radicals are unstable molecules that are toxic and hazardous to the living organisms by causing harmful damages in the cells. Therefore, neutralization of such compounds is important to protect the cell viability. EPS are the most effective compounds in this regard due to the inhibiting activity of their oxidative reactions as well as their low toxicity [57]. In the present study, the EPS produced by the two strains of *Agrobacterium* showed concentration-dependent DPPH scavenging ability ranged from 40 to 120 µg/ml (Fig. 8). A reasonable antioxidant activity from 51 to 52.3% with $EC_{50}$ 39.2 µg/ml was recorded for the strain T1; however, strain Cs5 had an activity of 50.3 to 51.8% with $EC_{50}$ 39.7 µg/ml. Comparable antioxidant activities of 59.3 and 56.7% were produced by *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. lactis BB12, respectively, but at higher EPS concentration of 2 mg/ml [31]. Furthermore, the obtained antioxidant values were higher than *L. helveticus* MB2-1 (40%) but lower than *Neopestalotiopsis* sp. SKE15 (78.5%) as previously reported [11,58]. Two possible mechanisms were proposed for neutralizing unstable molecules by EPS; by providing electrons or hydrogen atoms to DPPH and/or interacting with its radical [31].

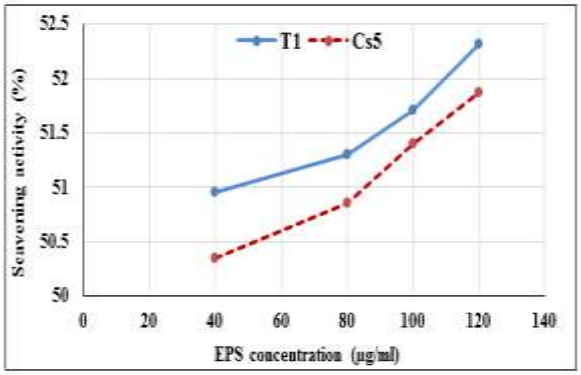


**Fig. 8: Antioxidant scavenging activity of EPS produced by *Agrobacterium tumefaciens* strains (Cs5 and T1).**

3.5.2. Antimicrobial activity

The results of the disc-diffusion assay (Table 2) demonstrated that EPS produced by the *Agrobacterium* strains (Cs5 and T1) had the highest significant ($P < 0.05$) antibacterial activity against *Staphylococcus aureus* the methicillin-resistant strain (MRSA) followed by *B. cereus* among the tested microorganisms. Also, it was noticed that the effect of the produced EPS on both tested bacteria surpassed the effect of the used standard antimicrobial agents, with the superiority of EPS from the strain Cs5. Whereas, *Salmonella typhimurium* was the only affected bacterium from Gram-negative group with lower susceptibility (Table 2). Unfortunately, the produced EPS from both *Agrobacterium* strains showed no antifungal activity. The results of this study are much interesting; because this special strain of *S. aureus* is a serious public health threat worldwide [11,59]. As well, *B. cereus* is another harmful organism to humans that causes foodborne illness. Consistent with these results, EPS produced from *Lactococcus lactis* F-mou presented higher antibacterial activity against Gram-positive bacteria, especially *B. cereus* [51]. The EPS inhibiting activity against Gram-positive bacteria could be referred to the interactions between EPS and murein as the effective and substantial compound of the cell wall in Gram-positive bacteria. Also, EPS may influence peptidoglycan synthesis enzymes which results in preventing the cell wall build-up leading to cell death [11]. On the contrary, Gram-negative bacteria were more resistant to the produced EPS. This result may be explained by the fact that Gram-negative bacteria outer membrane is a vital barrier that protects cells against toxic compounds, particularly hydrophilic antibacterial agents such as EPS [60]. Unlike these results, a study conducted by Mohamed *et al.* [19] reported that EPS from *B. altitudinis* MSH201 showed a wide spectrum effect against all tested microbes (bacteria and fungi).

**Table 2. Antimicrobial activity of EPS producedby *Agrobacterium tumefaciens* Cs5 and T1.**

| Tested microorganisms | Diameter of inhibition zone (mm) | | |
|---|---|---|---|
| | Cs5 | T1 | Standard positive control* |
| Gram positive bacteria (G+): | | | |
| *Bacillus cereus* (ATCC 33018) | 25 ± 0.0[b] | 20.5 ± 0.5[d] | 20 |
| *Staphylococcus aureus* MRSA (ATCC 43300) | 28.3 ± 0.0[a] | 24.3 ± 0.25[c] | 12 |
| *Listeria monocytogenes* (ATCC 7644) | NI | NI | 19 |
| Gram negative bacteria (G-): | | | |
| *Salmonella typhimurium* (ATCC 14028) | 9.1 ± 0.21[e] | 8.7 ± 0.17[e] | 20 |
| *Pseudomonas aeruginosa* (ATCC 9072) | NI | NI | 15 |
| *Escherichia coli* (ATCC 25922) | NI | NI | 18 |
| Fungi: | | | |
| *Macrophomina phaseoli* (nrrl 62743) | NI | NI | 15 |
| *Candida albicans* (ATCC 10231) | NI | NI | 15 |

*: penicillin G (10 µg) for G+ bacteria, ampicillin (10 µg) for G- bacteria and nystatin (100 units) for fungi; NI: No inhibition; Values are the mean ± SD; Different letters (a-e) represent significant differences between the data ($P < 0.05$).

3.5.3. Cytotoxic activity

The cytotoxic activity of the produced EPS from *Agrobacterium* strains against human lung cancer (A-549) and human lung normal cell lines (WI-38) was *in vitro* studied. The obtained results revealed that different tested concentrations (50, 200, 300 and 500 µg/ml) of EPS from both *Agrobacterium* strains (Cs5 and T1) had no cytotoxic effect on neither human lung cancer cells nor human lung normal cells (data not shown). Similar results were reported by EL Awady *et al*. [61] in which found that EPS from *Streptomyces hirsutus* NRC2018 had an anticancer effect on human colorectal adenocarcinoma cell line (CaCo-2), without any effect on the other cell lines; human hepatocellular carcinoma (HepG2), human breast adenocarcinoma (MCF-7), and human lung adenocarcinoma (A-549). In this context, many reports have noted that polysaccharides had anticancer activity and the mechanisms of inhibition could be summarized as follows: i) stimulation and enhancement of immune system; ii) induction of apoptosis; iii) inhibition of angiogenesis; and iv) cell cycle arrest [62,63].

## 4. CONCLUSIONS

In this study, two bacterial strains (Cs5 and T1) were selected from 35 isolates for further studies based on their potential production of EPS. Both strains were biochemically and genetically identified as non-identical *Agrobacterium tumefaciens* strains. The effects of nutritional requirements and cultivation conditions on EPS production by both strains were optimized using the one-factor-at-a-time method. Under the optimal cultivation parameters, EPS production was increased by more than two folds. Molasses and CSL could be cost-effective alternatives for higher EPS production for replacing synthetic carbon and nitrogen sources. The functional groups analysis revealed that both extracts showed similar major profiles which characterizing the EPS. Chromatographic analysis showed that EPSs were heteropolysaccharide consisted of glucosamine, glucose, and fructose. The produced EPS moderately exhibited the antioxidant as well as antibacterial activity against the tested Gram-positive pathogens. However, no antitumor activity was observed.

## 5. CONFLICTS OF INTEREST

"There are no conflicts to declare".

## 6. ACKNOWLEDGEMENT

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**الملخص العربي**

## الخصائص المضادة للميكروبات والمضادة للأكسدة للسكريات العديدة الخارجية المنتجة من *Agrobacterium tumefaciens* Cs5, T1

**محمد عبدالله مصيلحي[١]، فاطمة شمس لطفي[١]، حسين إمام مقبول[١]، عبدالله صبحى على[١]**

١- قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة – الجيزة – مصر

استهدفت الدراسة الحالية عزل وفحص البكتيريا التي تنتج كميات عاليه من السكريات العديده الخارجية (EPSs) من التربة المصرية. تم جمع أربعون عينة من التربة من مناطق مختلفة في ثلاث محافظات (البحيرة والجيزة والقليوبية). فقد تم الحصول على ١٦٥ عزلة بكتيرية من منطقة الريزوسفير بالتربة. منهم خمسة وثلاثون عزلة ( ٢١.٢%) لديهم القدرة على إنتاج مستعمرات لزجه. و بعد الفحص الأولى، تم اختيار عزلتين هما Cs5 و T1 للمزيد من الدراسة وفقًا لقدرتهما على إنتاج كميات كبيرة من السكريات العديده الخارجية (٧,١ و ٥.٧ جم / لتر على التوالي). ثم تم تعريف و تصنيف العزلات البكتيرية المنتخبة على أنها سلالات غير متشابهة من النوع *Agrobacterium tumefaciens* و ذلك بناءً على خصائصها المورفولوجية والكيميائية الحيويه بالإضافة إلى تسلسل الجينات S ١٦rRNA. و للحصول على أقصى إنتاج من السكريات العديده الخارجية لكل من السلالاتين، فقد تم دراسه تأثير التغذية و كذلك ظروف التنميه بإستخدام طريقه عامل واحد في كل مرة. و عليه فقد تم الحصول علي أعلي إنتاجية من السكريات العديده الخارجية قدرت بحوالي ٢١.٦٣ و ١٩.٥٧ جم / لتر من السلالاتين Cs5 و T1 على التوالي. و عندما تم استبدال مصدرى الكربون و النيتروجين الصناعيين فى البيئة الغذائية بالمولاس وناتج منقوع الذرة كمواد خام منخفضة التكلفة ، فقد تحسنت إنتاجيه السكريات العديده الخارجية إلى ٢٨.٧٣ و ٢٤.٥ جم / لتر لـ Cs5 و T1 على التوالي. كذلك تم الكشف عن المجاميع الوظيفية النشطة بواسطة جهاز FT-IR لكلا المستخلصين، وقد اظهرت النتائج أن الإمتصاص للأشعة تحت الحمراء متماشيا مع الأنماط النموذجية لامتصاص السكريات العديده الخارجية. علاوة على ذلك، فقد أشار التحليل الكروماتجرافى السائل (HPLC) إلى أن السكريات العديده الخارجية تكونت من أنواع مختلفة من السكريات الأحادية هى الجلوكوزأمين، الجلوكوز و الفركتوز. كذلك أظهرت السكريات العديده الخارجية نتائج جيدة كمضادات للأكسدة بالإضافة إلى تأثير قاتل قوى لبكتريا المكورات العنقودية (*Staphylococcus aureus*) من النوع المقاوم للمضاد الحيوى methicillin وكذلك البكتريا العصوية من النوع *Bacillus cereus* و هى من الأنواع البكتيرية الخطيرة على صحة الإنسان، مما يشير إلى امكانية تطبيقها كمضافات فى الصناعات الغذائية للوقاية منهما. بينما لم يكن للسكريات العديده الخارجية الناتجة أى تأثير على الخلايا السرطانية.