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SCREENING AND DNA IDENTIFICATION OF THREE NEWLY ISOLATED BACTERIAL STRAINS FROM VARIOUS ENVIRONMENTAL BIOTOPES IN ALGERIA, PRODUCING EXTRACELLULAR POLYSACCHARIDES (EPSs)

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ABSTRACT

Exopolysaccharides (EPSs) of microbial origin exhibit substantial advantages compared to their synthetic equivalents, due to their lower toxicity, higher selectivity, and better degradability. In this context, this study aimed to characterize three newly isolated strains producing extracellular polysaccharides, which are indigenous to different biotopes in Algeria. The EPSs-producing bacteria were preliminarily screened according to the red ruthenium coloration. The production of EPSs was evaluated by measured viscosities of the enriched biomass in HJL broth medium and by studying the effect of pH and temperature. Phylogenetic analysis of the three best performing strains, based on partial sequences of their PCR amplified 16S rRNA genes was used to distinguish the individual isolates. As a result, three genetically distinct strains designated LGM-TAM3, LGM-TAM4, and LGM-TAM5 were found to belong to the genera *Serratia* and *Aeromonas*. Specifically, these strains were found closely related to *Serratia marcescens subsp. Sakuensis*, *Aeromonas media*, and *Serratia nematodiphila* with16S rRNA similarities to type strains, of 86.20, 99.70, and 99.85%, respectively. Furthermore, the best EPSs production was recorded by the strain LGM-TAM5 at pH 7.5 and 30 °C with a viscosity value of 1289 cP. Based on FTIR spectroscopic analyses, functional groups of polysaccharides were identified in the crude EPSs of each strain, which mainly include a hydroxyl group (-OH stretching), (C-H) of aliphatic groups, and carboxylate groups (-COO-). These interesting results are encouraging as the study's first phase and will be supported by additional research to better understand the biological fonctions and the potential uses of the investigated EPSs.

Keywords: EPSs, *Serratia* sp., *Aeromonas* sp., FTIR, viscosity, rDNA 16S

INTRODUCTION

Polysaccharides are the most common organic compound on the planet. They can be found in nature, soil, water, animals, plants, and microorganisms **(Casillo *et al.*, 2018)**. Based on where they are found in the cell, microbial polysaccharides are classified into the following groups: intracellular polysaccharides, structural polysaccharides, and extracellular polysaccharides **(Asgher *et al., 2021*)**. These extracellular polysaccharides are known as "Exopolysaccharides" (EPSs), a phrase coined by Sutherland (1972) to describe such carbohydrate polymers with a high molecular weight produced by various microorganisms **(Asgher *et al.*, 2021)**. While plants, animals, fungi, algae, and bacteria are all common sources of industrial polysaccharides, microbial polysaccharides (fungi and bacteria) offer the particular benefit of great structural reproducibility due to the closely managed development conditions imposed during their growth **(Casillo *et al.*, 2018)**.
EPSs are classified into two types: homopolysaccharides (e.g. dextran) and heteropolysaccharides (e.g. xanthan and gellan). Homopolysaccharides are made up of only one type of monosaccharide, whereas heteropolysaccharides are made up of many monosaccharides synthesized inside the cell, resulting in complex structures. Heteropolysaccharides are the most common type of EPSs found in bacteria **(Barcelos *et al.*, 2019)**.
Different studies have reported the production of EPSs by several bacterial genera. **Thu *et al.* (2012)** and **Cervino *et al.* (2018)** described the production of alginate by *Azotobacter* spp. and *Pseudomonas* spp. Numerous studies have described the production of dextran by *Streptococcus* spp. and *Leuconostoc* spp. **(Bowen and Koo 2011; Takashima *et al.*, 2015; Senpuku *et al.*, 2017)**. Moreover, Gram-positive bacteria, Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria and *Acetobacter* spp. are reported to produce cellulose **(Augimeri *et al.*, 2015; Chunshom *et al.*, 2018; Kumar *et al.*, 2019a).**
Microbial EPSs have found different commercial applications, in food products, cosmetics, agriculture, medicine, the petroleum industry, and in research laboratories. For instance, xanthan ($C_{35}H_{49}O_{29}$) produced by *Xanthomonas campestris* was reported to have several applications in the food industry (as a texturizing agent), petroleum industry, and health care. Dextran ($C_{18}H_{32}O_{16}$) produced by *Leuconostoc mesenteroides* is used as food hydrocolloid, in wound care, and as a drug encapsulating agent. The well-known Hyaluronic acid ($C_{14}H_{21}NO_{11}$) n produced by *Streptococcus zooepidemicus* is used in human health, and cosmetics **(Rana and Upadhyay, 2020)**.

In this context, our study aimed to screen, identify (16S rRNA sequencing) and characterize EPSs-producing bacteria which have been isolated from different biotopes in Algeria. The chemical structure of their crude EPSs was characterized using FTIR analyses.

MATERIALS AND METHODS

Sampling

Samples of various biotopes were collected on February 2019 from the soil of a salt lake (Sabkha of Oran) (GPS coordinate 35°33'02.0"N 0°46'10.3"W), sediment water and wastewater of marine and domestic origin, respectively, and seawater (from the coastal waters) in Oran, Algeria. Another sample was collected from the thermal water at Boughrara Hammam station (GPS coordinate: 34°53'33.4"N 1°44'51.0"W) in Tlemcen, Algeria. All samples were collected in sterile flasks using sterile materials, then transported to the laboratory and stored at 4 °C.

Media composition

The HJL medium, which is used to isolate bacteria, is made up of the following ingredients (g/l): Tryptone (30), Beef extract (2), yeast extract (10), glucose (10), KH_2PO4 (5), distilled water (1L). The HJL agar medium was prepared by adding 15g/l of agar. The pH was adjusted to 6.5-6.8 using (5 M) NaOH and (6 N) HCl solutions. All the media were sterilized by autoclaving at 121 °C for 20 min.

Enrichment, isolation and screening of EPSs-producing bacteria

One gram of Sebkha soil or one millilitre aliquotes of different water samples were aseptically and separately decanted into test tubes containing 9 ml of HJL medium and incubated at 30 °C for 48-72h. Then, following the method described by **Chaida *et al.* (2021a)**, we used the decimal dilution technique for bacterial isolation. A series of decimal dilutions (10^{-1}-10^{-6}) were performed using 0.85% sodium chloride solutions, and the previously prepared bacterial cultures. Each dilution was plated on HJL agar Petri plates and incubated at 30 °C for 48-72h. After incubation, colonies that possessed a slight or strong mucoid aspect recovered and purified. For further studies, the pure isolates were stored at (-20 °C) on an HJL medium supplemented with glycerol (20%, v/v). The ability of bacteria

to produce EPSs was visualized using red ruthenium coloration (**Larouci *et al.*, 2017)**. In brief, after a 24-48h incubation period at 30 °C, EPSs-producing colonies were selected based on their viscous and white appearance on the medium's surface **(Larouci *et al.*, 2017)**.

Effect of pH and temperature on EPSs-producing bacteria

The apparent viscosity of EPSs produced was determined on cultures grown in HJL broth medium. Erlenmeyer flasks (500 ml) each containing 100 ml of HJL broth medium, were adjusted to three different pH values (pH 5.5, 7.5 and 9.5), and then were inoculated with 10% (v/v) of bacterial cultures (OD = 1 at 600 nm of absorbance) and incubated for 4 days at a constant temperature of 30 °C non-static conditions (150 rpm) **(Okoro *et al.*, 2021)**. To investigate the effect of temperature on EPS production, the pH of the medium was fixed to pH 7.5 and the incubation was carried out at various temperature (20, 30, and 40 °C). After incubation, the viscosity was measured at 26°C using a viscometer (Digital viscometer SNB-1) at a constant speed of 6 rpm. The viscosity is expressed in pascal seconds (1 Pa.s = 1000 cP). The abiotic control (uninoculated) was used under the same experimental conditions. Results are expressed as the mean of duplicate tests ± standard deviation.

Molecular characterization : 16S rRNA amplification and sequencing

Some tests were used based on phenotypic and biochemical characterization, such as catalase test, Gram stain and microscopic observation. In addition, based on molecular characterization, the 16S rDNA gene was amplified using colony PCR and sequenced for phylogenetic analysis to identify bacterial strains **(Bensalah *et al.*, 2009)**. For amplification of the 16S rDNA gene, the following PCR program was carried out using a TC3000 thermocycler: predenaturation at 95 °C for 15 min, denaturation at 94 °C for 1 min, hybridization at 60 °C for 1 min, and elongation at 72 °C for 1.5 min, followed by final elongation at 72 °C for 10 min. The reaction mixture included a pair of universal primers (0.5µl forward primer (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'), and 0.5 µl Reverse primer (1492R: 5'-TACGGGTACCTTGTTACGACTT-3') which targeted the 16 rRNA gene. In addition, the reaction mixture comprised 2.5 µl of the buffer solution, 2 µl DNTP, 0.25 µl Taq polymerase, 5 µl of the bacterial cells, and 14.25 µl of distilled water **(Chaida *et al.*, 2021b)**. Amplified PCR products of the correct size were visualized using ultraviolet light after migration on an electrophoresis gel made up of 1.2 g of agarose and 100 ml of Tris-Borate-EDTA (TBE) buffer with ethidium bromide as a DNA stain. Sequencing was performed according to the Sanger method, and the 16S rDNA sequences were imported into the BioEdit Sequence Alignment Editor ver. 7.2 **(Hall, 1999)**, then were aligned with other 16S rDNA sequences via BLAST (Basic Local Alignment Search Tool) using the NCBI (National Center for Biotechnology Information) database. For phylogenetic analysis, a phylogenetic tree was constructed using MEGA 11: Molecular Evolutionary Genetics Analysis **(Tamura *et al.*, 2021)**, and was generated using the bootstrap method with 1000 repetitions.

EPSs extraction

The EPSs were extracted using a modified **Evans and Linker (1973)** method that involves collecting all of the EPSs produced on an HJL agar medium, mixing these materials with 25 ml of distilled water and centrifuging them at 10.000 rpm for 30 minutes. The extraction of EPSs was then performed on the resultant pellet by adding a ratio (75:25) of cold (-20 °C) ethanol (95%) and distilled water and incubated overnight at 4 °C. After centrifugation at 10.000 rpm for 20 minutes, the supernatant was discarded and the crude EPSs were dried at 45 °C, and lyophilised for further analysis **(Buksa *et al.*, 2021)**.

EPSs characterization using FTIR analyses

For chemical structure characterization of the crude EPSs, a Bruker Alpha Fourier Transform Infrared spectrometer with a diamond ATR (Attenuated Transmission Resonance) module and computer control was used to generate infrared spectra **(Margaret *et al.*, 2019),** with a frequency range of 360-4000 cm^{-1} and a resolution of 2 cm^{-1}. The wavenumbers are expressed in cm^{-1} .

Statistical analyses

Viscosities measuring at different temperatures and pH ranges was performed at two replicates tests. The means and standard deviations of the viscosities were calculated using IBM SPSS 21 (Trial version).

RESULTS AND DISCUSSION

Isolation and screening of EPSs-producing-bacteria

Among 14 strains isolated on HJL medium from all samples, 6 strains designated LGM-TAM2, LGM-TAM3, LGM-TAM4, LGM-TAM5, LGM-SS4, and LGM-ET1 were further screened by the ruthenium red coloration method (Table 1). Various research studies have reported on this basic technique for detecting and screening bacterial EPSs production. For instance, according to Bouzar et al. (1996), the same procedure was used to select EPSs-producing *Lactobacillus delbruckii* subsp. *Bulgaricus*. Furthermore, **Larouci *et al.* (2017)** used this method as a preliminary screening tool for EPSs-producing *Leuconostoc* sp.

Table 1 Screened bacterial strains using the ruthenium red coloration method with some phenotypic characteristics

Bacteria code	Bacteria origin	Microscopic observation	Gram coloration	Catalase test	ruthenium red coloration
LGM-TAM3	Sediment water	Rods	-	-	+
LGM-TAM4	Waste water	Rods	-	+	+
LGM-TAM5	Sea water	Rods	-	+	+
LGM-TAM2		Cocci	-	+	+
LGM-SS4	The soil of the Sebkha	Cocci	+	+	+
LGM-ET1	Thermal water	Cocci	+	+	+

Legend : (+) – positive, (-) – negative

Effect of pH and temperature on EPSs-producing bacteria

The viscosity of the HJL broth medium was used to assess the effect of pH on EPSs production. The results showed that all strains produced the most EPSs at pH 7.5. Nevertheless, the strains LGM-TAM3 and LGM-TAM4 had higher viscosity values, equal to 1008 cP and 698 cP, respectively. Interestingly, the strain LGM-TAM5 produced the most EPSs, reaching the maximum viscosity value of 1289 cP (Figure 1). While for temperature, the results showed that all of the strains produced the most EPSs at 30 °C, and the strain LGM-TAM5 recorded the best EPSs production with a value of 1289 cP. As an exception, the strain LGM ET1 produced considerably more EPSs at 40 °C with a value of 1250 cP consistent with this strain's isolation from a thermal water source (Figure 2). Based on those results, the best three EPSs-producing strains (LGM-TAM3, LGM-TAM4, and LGM-TAM5) were selected for phylogenetic analyses (Figure 3)**.**

In this context, our findings are consistent with most literature-based studies. **Wilkinson (1958)** states that pH values close to neutrality are best for most EPSs-producing strains. However, after reviewing the literature, we found that the viscosity values of the various strains were influenced by several factors such as temperature, pH, and the composition of the medium that was utilized (i.e., carbon and nitrogen source and their ratio) **(Kumar *et al.*, 2019a)**, as well as the genus/species of the strains used. In our study, the strains LGM-TAM3, LGM-TAM4, and LGM-TAM5 recorded viscosity values that are particularly fascinating compared to counterparts from other genera. For instance, some works have shown that the viscosity of the fermented nmedium inoculated with *Enterobacter agglomerans* was 800 cP at neutral pH 7 and 30 °C, where the strain produces more EPSs. This author showed that the viscosity decreases when the pH is lower or higher than 7 **(Monique, 1991)**, while the viscosity of the fermented medium inoculated with *Halomonas maura* was 70 cP at pH 7 **(Arias *et al.*, 2003)**. Furthermore, based on the yield of crude EPSs (expressed in g/l) after extraction and drying, studies have also shown that, maximum dextran synthesis in *Leuconostoc mesenteroides* species is achieved at an initial pH of 6.8 **(Moosavi-nasab *et al.*, 2010)** or pH 7 **(Sarwat *et al.*, 2008)**, while, maximum EPSs production was recorded at pH 6 by the strain *Propionibacterium Acidipropionici* **(Monique, 1991)**.

It has been shown in various studies that temperature affects the generation of EPSs, as our research revealed. However, when the strain *Enterobacter agglomerans* was incubated at different temperature 20, 25 and 30 °C, the best production of EPSs was noticed at 25 °C at pH 6.5 and 8, with a viscosity value equal to 850 cP **(Monique, 1991)**. In addition, based on the yield of purified EPSs (expressed as mg of EPSs per g (dry weight) of cell material), strain CAM025 (a member of the *Pseudoalteromonas* genus) inoculated at -2 °C and 10 °C produced EPSs at a yield 30 times greater than the strain inoculated at 20 °C **(Nichols *et al.*, 2005)**. While, the *Aeribacillus pallidus* 418 strain produced more EPSs when the temperature was 55 °C **(Radchenkova *et al.*, 2013)**.

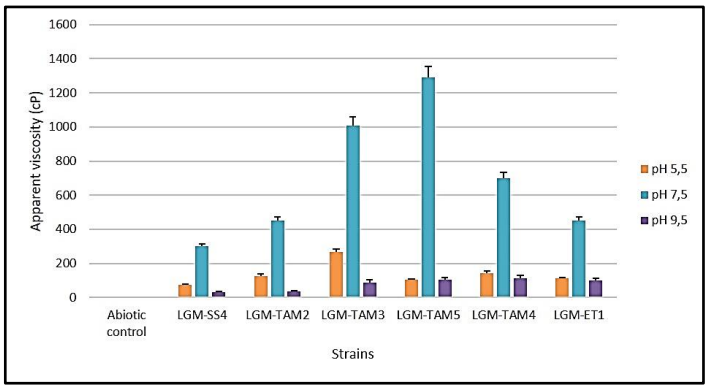

Figure 1 Effect of pH on the EPSs-producing bacteria after 4 days of incubation at 30 °C

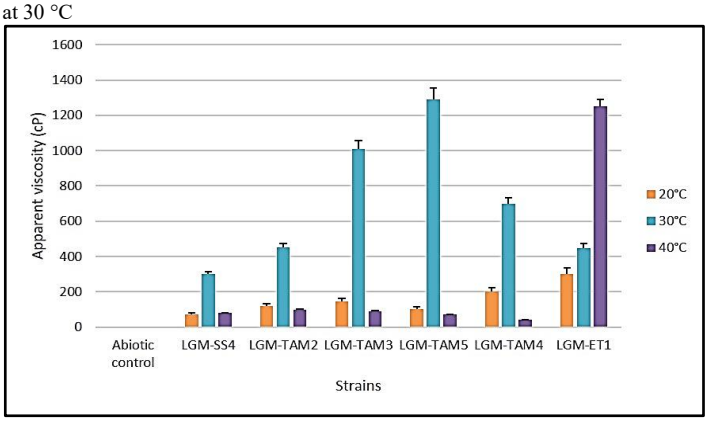

Figure 2 Effect of temperature on the EPSs-producing bacteria after 4 days of incubation at pH 7.5

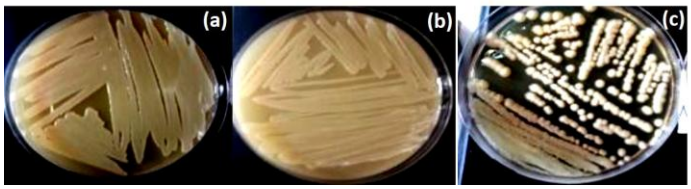

Figure 3 The EPSs production on the HJL agar medium of the strains: LGM-TAM3 **(a)**; LGM-TAM4 **(b)**; LGM-TAM5 **(c)**

Molecular characterization

The separation of the amplified 16S rRNA gene of the selected strains was performed in electrophoresis gel using a smart ladder (Figure 4). Phylogenetic analyses revealed that strains LGM-TAM3 and LGM-TAM5 were most closely related to members of the *Serratia* genus, particularly the species *Serratia marcescens subsp. sakuensis* and *Serratia nematodiphila* with a sequence 16S rDNA similarity percentage of 86.20 and 99.85%, respectively, to their type strains. Moreover, strain LGM-TAM4 was most closely related to members of the *Aeromonas* genus, particularly the species *Aeromonas media*, showing a sequence 16S rDNA similarity percentage of 99.70% to the type strain (Figure 5). The corresponding sequences were deposited in the GenBank/ENA/EMBL databases as *Serratia nematodiphila* strain LGM-TAM5 (GenBank Accession no.: OP218430), *Aeromonas media* strain LGM-TAM4 (GenBank Accession no.: OP218431), and *Serratia* sp. strain LGM-TAM3 (GenBank Accession no.: OP218432). Table 2 shows the percentage of 16S rDNA similarities of the three strains, and each is compared to the closest type strain.

The literature has provided descriptions of EPSs generation by *Serratia* genus of bacteria. For instance, **Badireddy *et al.* (2008)** documented *Serratia marcescens'* synthesis of EPSs and their characterization utilizing spectroscopic investigations. Additionally, *Serratia* sp. 1's generation of EPSs utilizing wastewater sludge as a raw material was reported by **Bezawada *et al.* (2013)**. Other studies have shown the *Serratia* sp. strain Gsm01 EPSs' potential as an antiviral agent against a yellow strain of the cucumber mosaic virus (CMV-Y) **(Ipper *et al.*, 2008)**. Likewise, numerous studies in the literature have reported the production of EPSs by *Aeromonas* spp. **(Pandey *et al.*, 2010; Castro *et al.*, 2014)**.

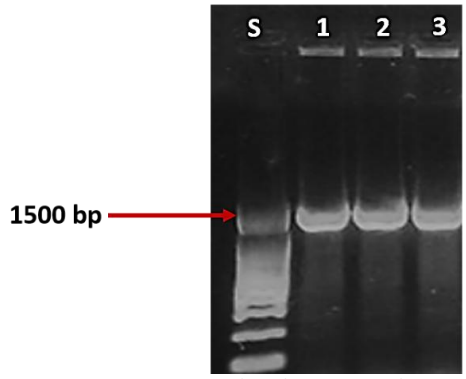

Figure 4 Electrophoresis profile of 16S rRNA of the three strains (1: LGM-TAM3; 2: LGM-TAM4; 3: LGM-TAM5; S: Smart Ladder)

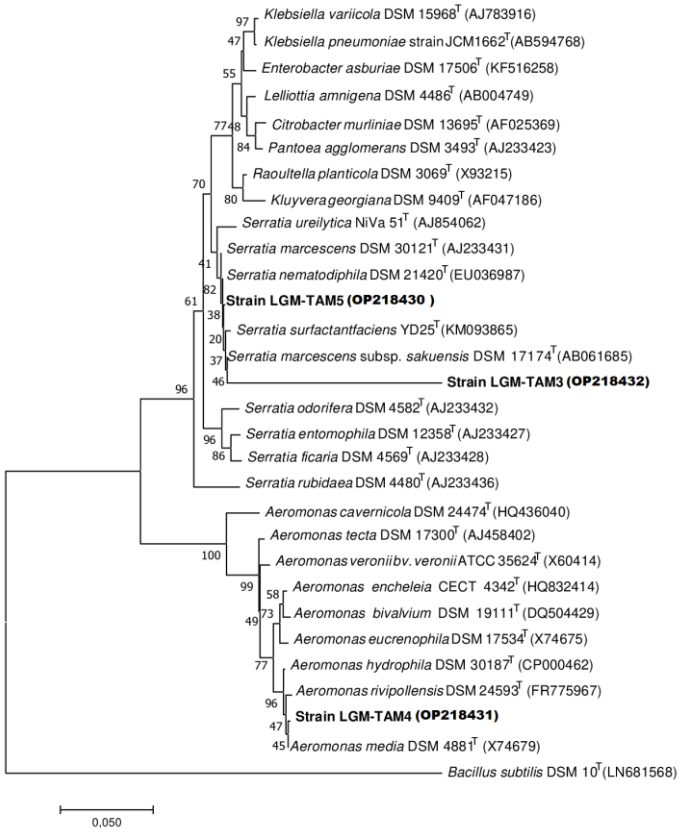

Figure 5 Phylogenetic tree by Neighbor-joining method based on 16S rRNA sequences of strains LGM-TAM5, LGM-TAM3, and LGM-TAM4 and related type strains species. The tree was generated with 1000 repetitions Bootstrap method, and the evolutionary history was inferred using the Tamura Nei model. The percentages (%) at the node represent the probability values of similarity robustness. Bar = 0.05 nucleotide substitution per site. *Bacillus subtilis* was used as an out group.

Table 2 Origin**,** percentage of 16S rRNA similarities, and the accession numbers of the strains LGM-TAM3, LGM-TAM4, and LGM-TAM5

Strains code	Origin	16S rRNA similarity percentage with type strain (%)	Accession numbers
LGM-TAM5	Sea water	*Serratia nematodiphila* DZ0503SBS1^T (EU036987) **99.85**	OP218430
LGM-TAM4	Waste water	*Aeromonas media* DSM 4881 (X60410) **99.70**	OP218431
LGM-TAM3	Sediment water	*Serratia marcescens subsp. sakuensis* (AB061685) **86.20**	OP218432

Crude EPSs characterization: FTIR analyses

The FTIR spectra of the crude EPS of the strains LGM-TAM5, LGM-TAM3, and LGM-TAM4 indicated the presence of functional groups belonging to polysaccharides (Figure 6, a, b, c). The elongation of the absorption peak appearing at 3200 cm^{-1} indicated the presence of a hydroxyl group (-OH stretching) which confirms the polysaccharidic nature of the EPSs investigated in this study. The band at 2900 cm^{-1} is assigned to the valence vibrations (C-H) of aliphatic groups. The absorption bands between 1607 and 1419 cm^{-1} are characteristic of carboxylate groups (-COO-) corresponding to uronic acids. A large region of absorption between 1200 and 1300 cm^{-1} is likely due to C-O and C-O-C vibrations indicating the presence of carbohydrates. The intense peak at 1003 cm^{-1} is characteristic of C-C bonds recognized to be an important component of polysaccharides.

In this study, FTIR analyses of EPSs produced by strains LGM-TAM5 and LGM-TAM3 are in agreement with the results reported by **Kumar *et al.* (2019b)**. These scientists used infrared spectroscopy to show the EPSs produced by *Serratia* sp. ISTD04 is polysaccharide-based. The polysaccharide fraction was described in the same study along with a modest protein fraction and lipids **(Kumar *et al.*, 2019b)**. Furthermore, the main functional groups in the FTIR spectrum of the EPSs produced by strain LGM-TAM4 confirmed the polysaccharide character of the substance, in line with earlier studies by **Pandey *et al.* (2010)** on the EPSs production by the pathogenic *Aeromonas hydrophila* strain An4.

More investigations will be carried out to quantify and characterize the eventual proteic and lipidic fractions of the EPSs produced by the three strains LGM-TAM3, LGM-TAM4, and LGM-TAM5. Additional methods, such as ^{1}H and ^{13}C NMR analyses for structural configuration, will be performed to thoroughly examine the chemical nature of EPSs.

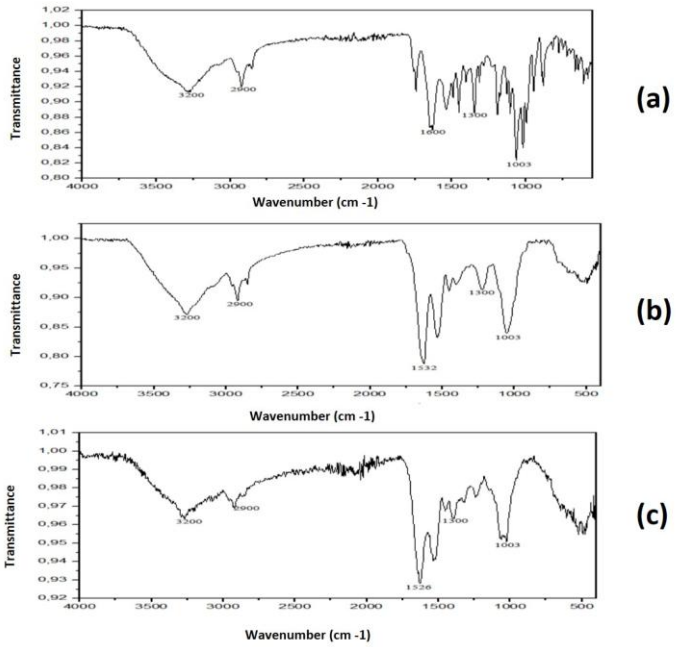

Figure 6 FTIR spectra of the crude EPSs of the strains : LGM-TAM5 **(a)** ; LGM-TAM4 **(b)** ; LGM-TAM3 **(c)**

CONCLUSION

In this study, three strains designated LGM-TAM3, LGM-TAM4, and LGM-TAM5 were screened in comparison to other isolates for their abilities to secrete particularly large amounts of EPSs in the HJL broth medium by measuring the viscosity. Molecular characterization by sequencing the 16S rRNA of each strain showed the affiliation of the strains at the *Serratia* and *Aeromonas* genera. Furthermore, the best EPSs production was recorded by the strain LGM-TAM5 at pH 7.5 and 30 °C with a viscosity value equal to 1289 cP. Their crude EPSs polysaccharide composition was validated by chemical characterization utilizing FTIR studies. The detailed chemical structure of each EPSs will be the subject of further research in the future. However, multiple carbon and nitrogen sources and renewable substrates will be evaluated at various concentrations, pH, and temperature ranges to optimize production parameters. In addition, various assays will be performed to determine the potential uses for each EPSs, like antimicrobial activity, sequestration of heavy metals and other.

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Conflicts of interest: The authors declare that there is no conflict of interest.

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