Bioproduction of exopolysaccharide by *Agrobacterium tumefaciens* using date molasses and their potential applications

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Abstract

The present study aimed to optimize the production of exopolysaccharides (EPS) by Agrobacterium tumefaciens using dates molasses (DM) as a carbon source. Various culture conditions, seed culture, inoculum size and nitrogen supplements were tested to achieve maximum bacterial growth and EPS yield. After optimizing the media and cultural conditions, the EPS was purified by gas-liquid chromatography. The purified EPS was characterized for its functional properties including sugar composition, viscosity, emulsification and serological properties. The results indicated that DM has a high percentage of sugars (55.77%), with potassium as the main element (60.40%). A. tumefaciens produced a single EPS at 96 hours of fermentation under static conditions at 30 °C, 3 to 10% DM, 7 g/L KNO_3 and 36 h of seed culture.

The succinic acid in the basal medium and inducing agents such as ribose, glucose and sucrose stimulated the growth and EPS yield of the bacterium. D- glucose was observed to be the main component in the purified EPS 100% glucose. The viscosity property increased with EPS concentration and showed a reversible behavior as the temperature rose from 10 to 100 °C. An emulsification of 45% was obtained in the filtrate containing the EPS. The antisera against crude EPS showed activity in the double immunodiffusion test, indicating that the crude acetone-precipitated polymer was immunogenic. However, the pure polymer did not show any precipitation. Pure EPS is non-toxic, emulsifying and non-immunogenic, making it suitable for blood plasma expander or food additive.

Keywords: *Agrobacterium tumefaciens*, Exopolysaccharide, Date molasses, Bacterial polysaccharide, Emulsifier.

Introduction

Plant and algae based polysaccharides have been used for centuries[43]. Over the last two decades, technology has been developed to produce a highly diverse array of microbial exopolysaccharides (EPS)[4,7]. Stress stimulation also induces EPS production in bacteria, algae, fungi, mammals and plants[11]. EPS protects bacteria from environmental stresses and supports bacterial growth[51]. Although EPS varies in biological and biochemical composition, polysaccharides can be degraded easily and are safe for the environment and human consumption. Over the last decade, the world has become increasingly aware of the inherent utility potential of bio-polysaccharides derived from various biogenic sources[5,36].

The glycocalyx is a polysaccharide coating found on the outer surface of most bacterial cells. These polymers form a capsule when covalent bonds bind to cell surfaces[30]. Extremes in temperature, pH, salinity and other forms of environmental stress, are thought to prompt bacteria to produce EPS as a means of adaptation[57]. Molecular factors such as fucose linkage, sugar type, sulfate concentration and molecular weight contribute to the biological action of EPS. The unique properties of EPS allow their use as additives to enhance the texture and rheological properties of food products[10]. The compounds are also suitable for pharmaceutical applications and possess immunomodulatory, antitumor, antiviral, anti-inflammatory and antioxidant properties[7,37]. Also, bacterial polymers like dextran produced by several species like Streptococcus dextranicum, Streptococcus mutans and others are used for pharmaceutical purposes as blood expanders[2,7].

It has been reported that EPS (like cyclic-β-(1,2)-glucan, succinoglycan, cellulose and curdlan) serves crucial roles in surface adhesion and structural stability in *Agrobacterium tumefaciens* biofilms[32]. To a great extent, EPS from Agrobacterium is essential to cellular function[26,32]. It has been reported that some species of Agrobacterium produce EPS in both water-soluble and water-insoluble forms[28,45]. The aim of the present work is to study the fermentation parameters that favour a maximum polysaccharide production from bacteria grown on DM-containing media. In addition, characterized EPS structure and properties have many possible applications.

Material and Methods

Proximate composition of date molasses: The crude date molasses (DM) was obtained from the black strap left after the Shakra date was manufactured and was supplied by Saudi Arabia Dates Company, EI Kasseim-El-badaee. The DM was used as the sole carbon source and was analyzed to determine the chemical composition of moisture, ash, total titratable acidity, total sugars, individual sugars, total nitrogen, total lipids, trace elements and optical activity[13,14].

Seed culture preparation and cultivation: Fermentation was initiated by inoculating 2% (1x107 CFU) inoculum from a liquid culture grown for 48 hours at 30°C on a rotary

shaker. *Agrobacterium tumefaciens* MSI (National Collection of Industrial Bacteria, Scotland) was propagated in 100 ml Kole flasks with 100 ml of a basal medium made up of the following ingredients (g/L): Date molasses- 60 (equivalent to 3346 mg total reducing matter (55.77%), before adding to media, the muddy residue was removed by centrifuging the molasses for 20 minutes at 4000 rpm), NH_4NO_3 - 2.5, KH_2PO_4 - 0.5; $MgSO_4.7H_2O$ - 0.01, Peptone – 2, yeast extract- 2, $FeCl_2$ - 0.05, pH- 5.5[22]. A sterilized medium mixture was mixed with standard inocula and incubated (either shaken or static, as stated) at 30 °C for the required time. The results obtained during this study are the arithmetic mean of at least two experiments conducted in triplicate[25]. To assess the age of the seed culture, 50 ml of sterilized modified basal medium was dispensed in 250 ml Erlenmeyer flasks and mixed with standard inocula (2 ml of pure slants). The culture flasks were shacked at 30°C for different periods (6, 12, 24, 36 and 48 hrs). Two ml were transferred as inocula to the modified fermentation medium in Kole flasks from these aged cultures.

Cell dry weight determination: The bacterial cells were centrifuged for 20 minutes at 7000 rpm during each experimental treatment. Cells were washed twice with saline and dried at 60°C until they reached a constant weight

Recovery and determination of the polysaccharide: The cells were recovered by centrifugation and 5 volumes of cold ethanol were added to precipitate the polysaccharide[3]. After centrifugation, the precipitate was washed with alcohol, acetone, or ether and dried. Drying resulted in an off-white powder of polysaccharide.

Effect of molasses medium on the growth and EPS production by *A. tumefaciens*: In this experiment, the basal medium containing 60 g centrifuged DM/L was modified by adding different levels of centrifuged DM ranging from 20 to 100 g/L. The reaction of the fermentation medium was initially adjusted to pH 5.5 and the fermentation process was continued for 4 days under static conditions. Subsequently, the production of EPS was estimated at each DM level.

Effect of nitrogen source on the growth and EPS production by *A. tumefaciens*: To examine the effect of the nature of the nitrogen source on growth and EPS productivity by the experimental bacterium, the nitrogen source constituent of the basal medium was replaced by nitrogen equivalent amounts (0.87.5 g N/L) of one at a time $(NH_4)_2SO_4$, $NaNO_3$, NH_4C1, $(NH_4)_2HPO_4$ and KNO_3. Further, based on the obtained results, the nitrogen source that supported more growth of *A. tumefaciens*, was selected. Different concentrations of the selected compound (1-9 g/L) were used further to estimate EPS growth and production by *A. tumefaciens.*

Effect of seed culture age and size of inoculum on the growth and EPS production by *A. tumefaciens*: Portions (50 ml) of the modified basal medium were dispensed in 250 ml Erlenmeyer flasks, sterilized and mixed with standard inoculum of the tested organism (2 ml of pure slants). The culture flasks were shacked at 30°C for different periods (6, 12, 24, 36 and 48 hours). Two ml were transferred as inoculum to the modified fermentation medium in Kole flasks from these aged cultures. To estimate the effect of the inoculum size, the fermentation flasks (each containing 100 ml of modified basal medium) received different volumes (1-5 ml/ flask) of 12 hr old seed cultures, each at a time. The inoculated flasks were incubated for 4 days under static conditions at 30°C. Later the growth of the *A. tumefaciens* and the EPS production was estimated as described above.

Effect of natural supplements and inducing agents on the growth and EPS production by *A. tumefaciens*: The response of the *A. tumefaciens* to well-defined complex substances was investigated by adding one-at-a-time of some natural substances to the growth medium. Thus, the formulation of the modified basal medium was supplemented by separate addition of peptone, beef extract, casein hydrolysate, malt extract, corn steep liquor yeast extract, soybean and ground nuts at 2 g/L.

Also, the effect of inducing agents like monosaccharides (ribose, glucose, galactose), disaccharides (sucrose), 6-deoxy-sugars (rhamnose), alditol (mannitol) and organic acids or their sodium salts (succinic, malic, pyruvic, acetic, citric acid) was estimated[38]. Thus the formulation of the modified basal medium was supplemented with separate addition of each of the above mentioned agents at 0.5 g% level. The samples were incubated under static conditions at 37 °C for 4 days and the growth of *A. tumefaciens* and the EPS production was determined.

Purification and application of the recovered EPS: The Ninhydrin method was used to purify crude polysaccharides, as described[15]. The obtained polysaccharide was dialyzed against distilled water for 24 hours. Following reprecipitation with alcohol, the polysaccharide was washed, dried and used for further study[40].

Acid hydrolysis and chromatographic separation: Using a sealed tube at 100°C for 12 hours, 200 mg of the purified polysaccharide was heated with 10 ml 1N H_2SO_4. The purified polysaccharide (200mg) suspended in 10 ml of 1 N H_2SO_4 in a sealed tube was heated at 100°C for 12 hours. After filtering out the flocculent material, the filtrate was neutralized with 0.01 N $Ba(OH)_2$. The solution was evaporated to dryness at 80 °C under reduced pressure. The degraded sugar residue was extracted with boiling methyl alcohol until a brown residue of barium salt of the degraded sugars was formed.

A mixture of Amberlite resin IR-120 (H^+) and IRA-400(OH^-) was used to deionize the methyl alcohol extracts. The obtained syrup was examined by paper chromatography using

Whatmann no.1 filter paper. Butanol, distilled water and ethyl alcohol (50:40:10) were mixed and shacked in a separating funnel. In the chromatogram jar, the upper layer acted as a mobile phase while the lower layer acted as a saturating layer. This experiment used para-anisidine hydrochloride (1 % in butanol) as a spraying agent.

Methylation of the purified EPS: As part of the Hakomori method, 2 g of pure polysaccharides were dissolved in dimethyl sulfoxide for a few minutes. Then, they were stirred with methyl sulfinyl carbanion equivalent to the hydroxyl present in the polysaccharide under a nitrogen stream. A weighed amount of sodium hydride was dissolved in dimethyl sulfoxide according to the method of Chaykovsky and Corey[8]. Under a nitrogen stream, the mixture was stirred at room temperature for 10 minutes. then add excess methyl iodide and stir for another 20 minutes. After 1/10 dilution with water, the methylated products were extracted with chloroform, washed with water and evaporated in vacuum. To remove the traces of dimethyl sulfoxide, few ml of an ether-petroleum ether mixture were used to remove the residue and then washed with water[57].

Methanolysis and hydrolysis of the methylated product: In a sealed tube, 0.4 g of methylated product and 3% methanolic hydrogen chloride were heated at 100°C for 12 hours. Using cooled ethereal diazomethane, the mixture was neutralized and concentrated. In sealed tubes, the sample was boiled with 4% hydrochloric acid at 100°C for 7 hours. Silver chloride precipitated from the acid was neutralized with silver carbonate and then filtered. Hydrogen sulphide gas was used to treat the filtrate. Amberlite IR 120 (H+) and IRA-400 (OH-) ion exchange resins were used to deionize the filtrate and the syrup was analyzed by paper chromatography.

Gas-liquid chromatography: Method of quantitative estimation of alditol acetates using gas-liquid chromatography[42]. was used in the present study. Chromatographic analyses were performed on a Perkin Elmer Sigma gas chromatograph with a flame ionization detector. Columns packed with Gas-chrom-Q (80-100 mesh) coated with 5% DC-200 were used for acetylated and methylated sugar analyses (2 m x 3 mm). Chromatograms were carried out isothermally at 220°C and the injector and detector temperatures were 250°C. Nitrogen was used as a carrier gas at a flow rate of 30 to 45 ml per minute. There was a flow rate of 20 and 200 mu/min for hydrogen and oxygen respectively. A comparison of standard mixtures' retention times under the same conditions was used to identify the components.

Determination of viscosity property of EPS: The viscosity of the polysaccharide was measured using a conventional Ostwald viscometer at 30°C[25]. Apparent relative viscosity $\eta_{(app)}$, was determined as follows:

$$\eta(\text{app}) = \frac{ty}{to}$$

where t_y is the falling time of sample at 30 °C and t_o is that of water at 30 °C.

Determination of the lethal dose 50 (LD50): LD_{50} was determined using the Carlos Sevik method for the pure polysaccharides under investigation before their use as blood plasma exchangers or in food preparation[42]. Male albino mice (20-30 g) were divided into 5 groups of 6 animals. In preliminary experiments, the minimal dose that causes death for all animals (LD_{100}) (108 mg/30g mouse) and the maximum dose that does not cause death for any animal (7 mg/30g mouse), were determined (data not shown). Five doses at equal logarithmic intervals were chosen between these two doses (1.03, 1.23, 1.43, 1.63 and 1.83, d = 0.2) (logarithm of mg polysaccharide/g mouse). The polysaccharide was injected intraperitoneally into experimental animals in 0.9% saline.

The LD50 was calculated according to the formula mentioned:

$$m = X_k + \frac{1}{2}d - \frac{dr_1}{N}$$

where m = Log LD_{50}, x_k = Log dose causing 100% mortality = 2.03, d = Logarithmic interval of doses = 0.2, r_1 = Sum of number of animals dead at each of the individual doses = 21 and N = Number of animals on each of the dose levels = 6.

∴ m= 1.43 = Log LD_{50}
LD_{50} = 27 mg/ 30 g = 900 mg/kg

Serological properties of EPS

Antisera production: We prepared antisera specific to the tested polymer in two rabbits weighing 2-3 kg each (3 rabbits in each group). Multiple intraperitoneal injections with crude acetone precipitated polymer and pure polymer emulsified in incomplete Freund adjuvant were administered to each rabbit. The blood samples were collected after 4 weeks of experimentation and were allowed to stand for one hour at 37 °C to collect antisera. Clarified antisera were centrifuged at 35000 rpm for 20 minutes and stored at -15°C.

Determination of the immunogenic nature of EPS: The experiment was carried out using the Ouchterlony method[36]. Agarose (Sigma, type I) 0.5 g was dissolved in 0.1 M Tris/HCI (pH 8.6) containing 1% (v/v) Triton X-100. A total of 25 µl antisera were then added at a temperature of 55°C. The mixture of agarose and antisera was raised against the pure polymer or antisera was raised against the crude acetone preparation each at a time in separate plates. Different concentrations of antigens (purified and crude polymer) were placed in the wells. Each agarose gel plate was incubated at 37 °C for 24 hours.

Statistical analysis: Data in this study were analysed by using SPSS version 16. One-way ANOVA (Analysis of variance: F test) was applied to assess the significance of

variation in the production of dry weight, EPS and yield coefficient (Yp/c).

Results

Proximate chemical composition of date molasses: In the current study, the chemical composition of DM was estimated before being used as a base medium for the growth of *A. tumefaciens*. DM was perceived to have about 29% water and 55.77% of total sugars. The total protein and lipid contents were noticed to be 2 and >1% respectively. The ash content was obtained at 7.63%, with a total titratable acidity of 1.84. Among the elements, potassium was observed to be at a high amount at 60.40%, while manganese was at a negligible amount at 0.05%. Table 1 provides the approximate chemical composition of the DM.

Growth characteristics of *A. tumefaciens* in the presence of DM and EPS yield: To study the EPS production of *A. tumefaciens* in the presence of DM, different compositions of the medium and the production of EPS in different growth phases of the starter culture were performed. Growth yield regularly accelerated during the first 96 h of surface cultures with 400 mg/100 ml. A 12-fold increase in EPS production was recorded from the biomass when fermentation was allowed to proceed from 12 to 96 h. However, a decrease in growth yield was observed on further incubation, indicating the end of the stationary phase and the beginning of the decline phase. Under shaking conditions, the growth yield was lower when compared to the static condition by the end of 96 hrs of incubation. However, a 32-fold increase in biomass was noticed (Table 2).

Table 1
Proximate chemical composition of date molasses (DM)

Constituents (%)	g/100 DM fresh weight
Water content	29.00
Total sugars	55.77
Crude proteins	1.83
Total lipids	0.40
Ash content	7.63
Total titratable acidity	1.84
Elements (%)	
Potassium	60.40
Sodium	11.30
Calcium	7.90
Phosphorus	6.50
Iron	5.30
Copper	4.70
Magnesium	3.80
Zinc	0.41
Manganese	0.05

Table 2
Growth and EPS production by *A. tumefaciens* during static and shaking conditions at different growth periods

Mode of incubation	Incubation period (hours)	Final pH	Dry weight (D.wt) mg/100 ml	Extracellular polysaccharide (EPS) mg/100 ml	Yield coefficient (yp/c)
Surface (static) culture	12	5.5	33	80	0.06
	24	5.5	40	110	0.08
	36	5.5	89	130	0.10
	48	5.5	150	144	0.11
	60	5.0	243	165	0.12
	72	5.0	300	183	0.14
	84	5.0	375	210	0.16
	96	5.0	400	230	0.17
	108	5.0	390	211	0.16
F value		6.65**	158.3**	19.251**	56.516**
LSD 0.05		0.300	32.560	3.431	0.015
LSD 0.01		0.412	44.602	4.700	0.021
Shaked culture	12	5.5	10	10	0.01
	24	5.5	10	20	0.01
	36	5.5	50	67	0.05
	48	5.5	67	90	0.07
	60	5.0	131	120	0.09
	72	5.0	186	130	0.10
	84	5.0	275	149	0.11
	96	5.0	320	155	0.12
	108	5.0	298	140	0.10
F value		6.65**	53.430**	47.731**	115.002**
LSD 0.05		0.300	1.540	2.380	0.012

The EPS produced by cells was detected in the static and shaking condition after 12 h of incubation and up to 4th day of incubation. During the stationary phase, 2.9 and 15.5 times higher EPS yield with a maximum productivity of 230 and 155 mg/100 ml were observed in static and shaking conditions. Comparatively, the EPS yield was 1.5-fold lower in shaking than under static conditions.

Effect of date molasses concentration on the growth and production of EPS by *A. tumefaciens*: Different concentrations of processed DM (ranging from 20 to 100 g/L) were added to the fermentation medium to observe the effect of DM on the EPS yield. It was noticed that the bacterium produced considerable amounts of EPS in both centrifuged and H_2SO_4-treated DM samples (Table 3). Comparatively, the productivity, specific activity and yield coefficient were less in H_2SO_4-treated DM samples than in the centrifuged DM.

The dry mass and EPS yield at various concentrations of centrifuged DM are presented in table 4. The obtained results suggest that the sugar level exerted variable influences on the metabolic activities of the experimental bacterium. A low growth yield was observed at 2% DM followed by a regular increase in growth yield and EPS production as the DM level concentration increased. Thus, more than 5.7- and 5.3-fold increases in the cell mass and EPS production were recorded on raising DM levels from 3 to 10%. DM level of 10% showed maximal EPS production (580 mg/100 ml), Growth yield continued to increase with an 11% DM level, but EPS production declined. It should be noted that high levels of DM (6- 10%) supported a high yield coefficient value. Therefore, a DM level of 10% was selected as the optimal concentration for further experiments.

Effect of the nitrogen source and the nitrogen level on EPS production by *A. tumefaciens*: Fig. 1 shows the response of different nitrogen elements to the growth and EPS production by *A. tumefaciens*. The obtained data showed that KNO_3 supported the highest EPS outcome (600 mg/100 ml) and adequate growth yield (520mg/100 ml) with the highest coefficient value of 0.27. At the same time, the other tested nitrogen source gave a moderate growth yield and EPS production (Fig. 1A).

To assess further, KNO_3 was added to the basal medium at different concentrations of 1-9 g/L and the effect of the growth of *A. tumefaciens* and EPS production was monitored. It was perceived that the KNO_3-supplemented medium had a marked effect on the metabolic activities of *A. tumefaciens*. Good growth and high EPS biosynthesis were achieved at KNO_3 level 7 g/L. As the concentration of KNO_3 increased to 9g/L, the metabolic activities of the bacterium showed a decline (Fig. 1B).

Table 3
EPS production in *A. tumefaciens* in media supplemented with centrifuged and H_2SO_4-treated DM samples

Type of DM	Centrifuged DM (g/l)	H_2SO_4 - treated DM (g/l)
Cell mass (g/l)	2.8	1.5
EPS yield (g/l)	4.2	2.5
Productivity (g EPS/L/h)	0.044	0.026
Specific activity (g EPS/g cell/h)	0.012	0.017
Yield coefficient (Yp/c)	0.24	0.15

Table 4
Growth of *A. tumefaciens* and EPS production as influenced by different concentrations of DM.

DM level g%	Dry weight (mg/100 ml)	EPS (mg/100 ml)	Yield coefficient (Yp/c)
2% = 1.115 g TRS	16	50	0.11
3% = 1.1673 g TRS	79	110	0.16
4% = 2.230 g TRS	160	180	0.20
5% = 2.788 g TRS	230	250	0.22
6% = 3.346 g TRS	280	320	0.24
7% = 3.906 g TRS	300	380	0.24
8% = 4.461 g TRS	350	440	0.25
9% = 5.022 g TRS	390	500	0.25
10% = 5.577 g TRS	450	580	0.26
11% = 6.135 g TRS	530	440	0.18
F. Value	999.9**	999.9**	48.9**
LSD at 0.05	280.1	326.0	0.211
LSD at 0.01	280.9	326.8	0.214

TRS- total reducing sugars, pH of medium 5.5, incubation period was 4 days, * means the significant result, ** means highly significant result ($p<0.05$).

Effect of seed culture and size of inoculum: The results of this investigation are presented in table 5, indicating that *A. tumefaciens* increased as the culture grew and a maximum yield of EPS (560 mg/100 ml) was achieved at 36 hr age of seed culture. However, the yield of EPS was high at 12 hr old seed cultures (740 mg) with a yield coefficient of 0.33. The seed cultures of younger or older ages showed lower efficiencies in EPS production.

Further, the growth yield progressively increased with inoculum size, reaching its maximum value of 800 mg/100 ml with 5 ml of seed culture with a yield coefficient of 0.36. A decline in the EPS yield was noticed on increasing the inoculum size over 5 ml[56,57].

Response of natural additives and inducing agents on *A. tumefaciens* growth and EPS production: According to the results, basal medium supplemented with ground nuts, malt extract, casein hydrolysate, beef extract and soybean enhanced the growth of *A. tumefaciens*. However, a decline in the EPS yield was noticed by other additives. Notably, the biosynthesis of EPS was slightly retarded by adding these natural additives (Table 6).

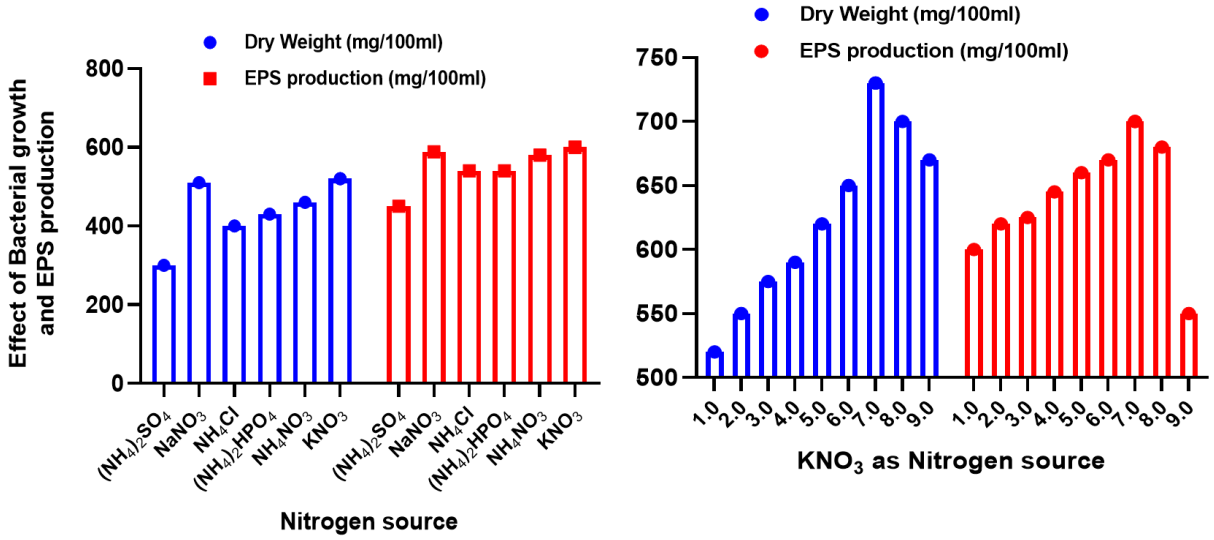

Fig. 1: Effect of the (A) nitrogen source and (B) the KNO_3 level on growth and EPS production by *A. tumefaciens*

Table 5
***A. tumefaciens* growth and EPS production as influenced by the age of seed culture and size of inoculum**

Age of seed culture (hrs)	Dry weight (mg/100 ml)	EPS (mg/100 ml)	Yield coefficient (Yp/c)
6	670	550	0.25
12	720	740	0.33
24	780	660	0.30
36	820	560	0.25
48	700	520	0.23
F. Value	139.9**	660.7**	68.4**
LSD at 0.05	741.9	609.4	0.278
LSD at 0.01	743.2	610.5	0.279
Size of inoculum ml/100ml			
1	540	420	0.19
2	720	740	0.33
3	750	760	0.34
4	825	796	0.36
5	820	800	0.36
6	816	750	0.34
F. Value	999.9**	999.9**	679.16**
LSD at 0.05	747.0	712.7	0.322
LSD at 0.01	747.6	713.2	0.323

Molasses level- 10%, pH of medium 7.0, KNO_3 level- 0.7 g%, age of seed culture 12 hr, the incubation period was 4 days, * means the significant result, ** means highly significant result ($p<0.05$).

Table 6
***A. tumefaciens* growth and EPS production as influenced by natural additives and inducing agents**

Natural additives (2 g/l)	Dry weight (mg/100 ml)	EPS (mg/100 ml)	Yield coefficient (Yp/c)
Control	1350	910	0.4
Ground nuts	1540	900	0.4
Malt extract	1500	900	0.4
Casein hydrolysate	1490	900	0.4
Beef extract	1480	900	0.4
Soybean	1390	860	0.39
Yeast extract	1300	830	0.37
Peptone	1312	830	0.37
Corn steep liquor	1260	830	0.37
F. Value	445.5**	62.7**	4.52**
LSD at 0.05	1405.5	875.7	0.393
LSD at 0.01	1406.4	876.5	0.394
Inducing agents (g/l)			
Control	1350	910	0.41
Ribose	1520	900	0.40
Glucose	1420	910	0.41
Galactose	1320	890	0.40
Rhamnose	1300	890	0.39
Sucrose	1390	890	0.40
Mannitol	1280	800	0.36
Succinic acid	1550	1100	0.49
Malic acid	1390	890	0.40
Sodium pyruvate	1370	910	0.41
Sodium acetate	1360	908	0.41
Sodium citrate	1330	810	0.36
F. Value	272.0**	5.8**	20.5**
LSD at 0.05	1383.5	907.4	0.404
LSD at 0.01	1384.2	909.0	0.406

Molasses level- 10%, pH of medium 7.0, KNO_3 level- 0.7 g%, age of seed culture 12 hr, size of inoculum 5%, the volume of culture medium- 200 ml, the incubation period was 4 days, * means the significant result, ** means highly significant result ($p<0.05$).

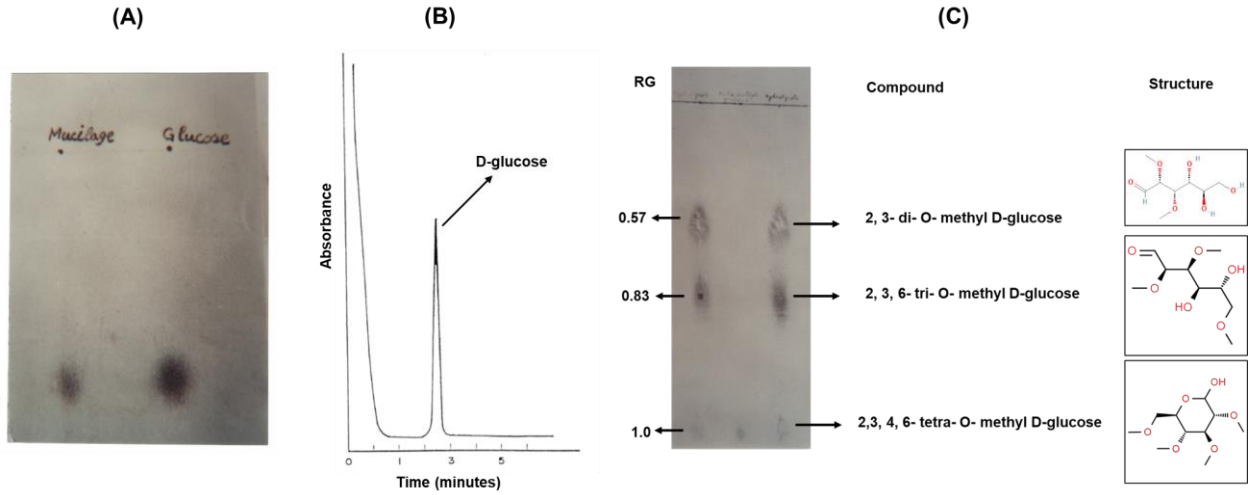

Fig. 2: Paper chromatogram of (A) the sugar components, (B) acid hydrolysate of purified EPS and (C) methylation of EPS produced by *A. tumefaciens*

On the other hand, the inducing agents like ribose, glucose, sucrose, succinic acid, sodium pyruvate, sodium acetate and sodium citrate led to appreciable stimulation of the growth yield of the bacterium whilst rhamnose and galactose retarded the growth of the organism. About EPS production, an increase in yield values was recorded upon adding succinic acid and ribose sugar, while the other tested inducing agents slightly retarded the EPS yield (Table 6).

Functional properties of purified EPS

Sugar components and methylation of the purified EPS: Analysis of the sugar composition and absolute configuration of EPS by paper chromatography has shown monosaccharide D-Glucose to be the primary component of the purified EPS. The EPS produced by *A. tumefaciens* showed only one spot on paper chromatography corresponding to D-glucose (Fig. 2A). The gas-liquid chromatography confirmed a single peak. The peak area was identified by comparing the retention time of the respective standard run under the same conditions.

It was identified a 100% D-glucose (Fig. 2B). The hydrolyzed methylated product of EPS revealed the presence of three different spots on the chromatographic sheet, corresponding to 2,3, di-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose and 2,3,4,6-tetramethyl-D-glucose with RG of 0.57, 0.83 and 1.0 respectively (Fig. 2C).

Viscosity properties: The relative viscosities of the EPS were estimated and it was perceived that the viscosity increased with increasing EPS concentration (Fig. 3A). The viscosity of a solution containing 3% polysaccharide showed a reversible behavior when the temperature was successively raised from 10 to 100°C (Fig. 3B).

Purified EPS as a plasma expander or in food preparation and as an emulsifier: The degree of toxicity before being recommended as a blood plasma expander was estimated by calculating LD50. The crude EPS's toxicity level was low, 900 mg/kg and proved to be non-toxic. Hence, the chance to use EPS in food preparations as a blood plasma expander is applicable. Also, the results showed that the filtrate containing the EPS exhibited an emulsification of 45%, indicating its possible use as an emulsifying agent.

Serological properties of EPS: We have performed immunochemical studies on EPS using rabbit antiserum obtained by immunizing animals with whole cells of *A. tumefaciens*. The titer of anti-EPS serum measured by the passive hemagglutination test was 1/2048 (Fig. 4A). This serum showed activity in double immunodiffusion test with purified EPS of *A. tumefaciens*, indicating that crude acetone precipitated polymer was immunogenic in nature (Fig. 4). In contrast, the purified polymer did not elicit a specific antibody response when injected into rabbits (Fig. 4B).

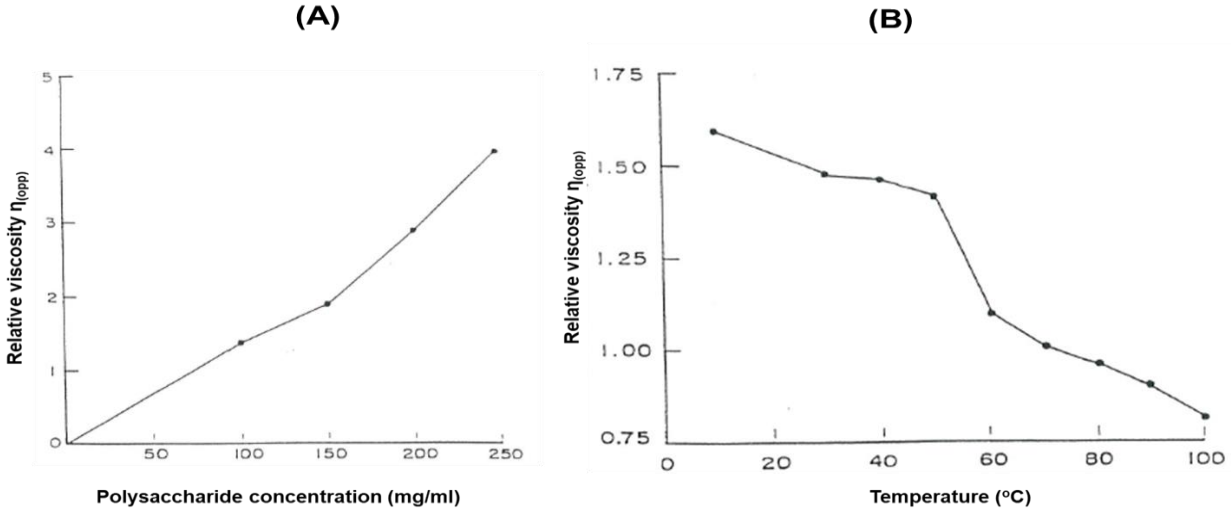

Fig. 3: (A) Estimation of relative viscosity (RV) and (B) effect of temperature on RV of the purified EPS

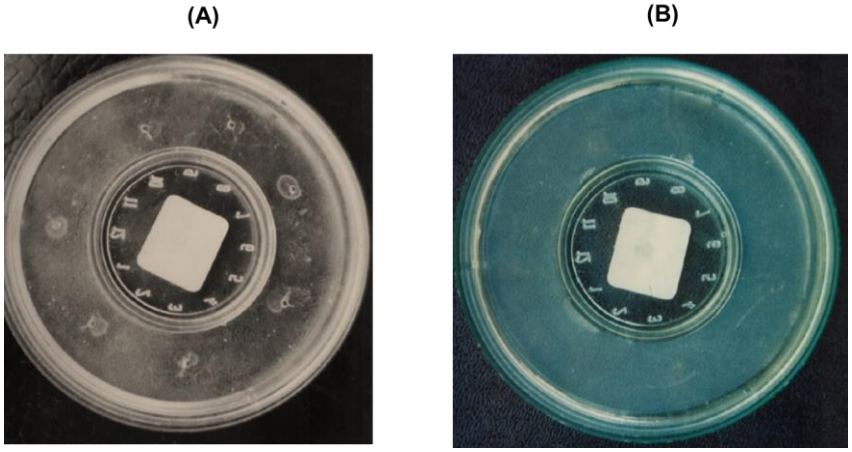

Fig. 4: Antigen-antibody reaction of the insoluble complex by Ouchterlony double diffusion, where A) shows the precipitation of polymer by acetone and B) shows no precipitation of the pure polymer.

Pure EPS from *A. tumefaciens* reacted with serum anti-A. tumefaciens, while pure EPS from *A. tumefaciens* did not show any precipitation lines. The immunoprecipitation test has shown that EPSs from *A. tumefaciens* isolated from rabbits gave higher reactivity patterns whereas EPS isolated from *A. tumefaciens* revealed much weaker reactivity with this antiserum. Perhaps the structural variations between these EPSs are reflected in their varying degrees of immunoreactivity.

Discussion

To a considerable extent, the synthesis of EPS is one of the mechanisms, microorganisms use to cope with environmental stress, such as high or low temperatures, acidity or salt[57]. Extracellular biopolymers are one of several self-protection mechanisms used by endemic microorganisms to ensure their survival in environments with exceptionally high salt and pH[7,44]. The medium growth of the current study and the response to EPS production varied. The maximum yield of cell biomass and EPS synthesis was obtained at pH 7.0, while pH 3.0 inhibited bacterial growth. Each species has a unique optimal pH for EPS production. However, many bacteria thrive under slightly neutral to alkaline conditions[24].

Increasing the aeration conditions in the present investigation improved the synthesis of EPS polymers. *A. tumefaciens* also produced more EPS after being subjected to more aeration and agitation. The ratio of cellular metabolite release is affected by aeration, nutritional availability and dissolved oxygen levels[38]. Interestingly, *A. tumefaciens* showed excellent ability to produce EPS during growth on DM, the most dominant raw feedstock material in Saudi Arabia. The production of EPS from *A. tumefaciens* using agroindustrial wastes such as rice bran and sugar cane molasses was reported by Razack et al[41]. Similarly, bacteria could produce levan (an EPS) using date syrup as primary material[34].

Many microorganisms are believed to use sugars in DM (mainly sucrose) as a carbon source for biopolymer synthesis[34,46]. It was postulated that the growth and yield of EPS are high due to the availability of carbon sources at low concentrations of molasses. In higher amounts of molasses, suppressing catabolite oxidative pathways will limit bacterial growth, producing lower levels of EPS[24,39]. However, in our study, with a DM concentration of 2%, a low growth yield was observed, followed by a steady increase in EPS and growth yield as the DM concentration increased. These results are consistent with the notion that EPS synthesis is stimulated by a sufficient carbon supply from molasses at low molasses concentrations while EPS production is inhibited at higher molasses concentrations[33,36]. A sugarcane molasses concentration of 1-3% was previously reported to be optimal to produce EPS from *Bacillus sphaericus* and *B. subtilis* EPS[33,39]. According to research by Razack et al[41], nitrogen is essential in the synthesis of microbial biomass and EPS and a higher carbon content is preferred to a lower nitrogen concentration. However, in our analysis, KNO_3 acted as a suitable nitrogen source. It improved the growth and production of EPS by *A. tumefaciens* at a concentration of 7 g / L. Increasing the sugar content and adding a yeast extract will increase the production of EPS[33]. Many authors have previously documented EPS production when yeast extract was present. Since yeast extract is rich in B vitamins and nitrogen, it is often used in nutritional supplements[35,39].

Carbon sources such as glucose and sucrose are widely used in the synthesis of bacterial EPS[12]. Agricultural waste, industrial waste and by-products are being studied as potential fermentation media alternatives, as they are 30% less expensive than traditional media[17]. However, there are drawbacks in using low-cost waste substrates, such as the possibility of unintended polymer or by-product synthesis as a result of impurities and potentially divergent metabolic pathways resulting from diverse nutritional compositions. In our study, growth and polysaccharide production improved when the concentration of DM increased to 10%. However, *A. tumefaciens biomass* increased at 11% DM, but EPS production was reduced by 31.8%.

12-hour microbial seed culture was found to be the most efficient in polymer formation. This might be due to the high metabolic activity of cells in this growth stage. In addition to that, using an inoculum of actively growing cells may rapidly increase sugar consumption and accelerate polymer formation. The size of the inoculum also showed a marked effect on the growth and formation of EPS by *A. tumefaciens*. A study by de Jesus Assis et al[10] showed that the production of cell and xanthan polymers was higher when the inoculum size increased from 1 to 4%.

EPS synthesis by various bacterial strains has been documented before. Prior studies agreed that the capacity of microbial cells to produce EPS is influenced by the concentrations of carbon and nitrogen in the culture medium[22]. According to research by Miqueleto et al[35], high C/N ratios are desirable for synthesising EPS by immobilising bacterial biomass. Although microbial biomass production increased with increasing N content in the medium, EPS synthesis exhibited the reverse behavior, with enhanced production occurring at lower N concentrations[43]. Bacteria, including *Xanthomonas*, *Pseudomonas* and *Rhizobium*, are known to have this behaviour, as described by Kimmel et al[28].

Carbohydrates such as glucose, sucrose, starch, maltose, sugar concentrates and lactose are commonly cited as carbon sources for producing microbial EPS. However, other sources have been identified[25], Elleuch et al[14] also found that lactose was used as a carbon source in their investigation. However, *Saccharophagus degradans* showed no signs of growth in this medium and EPS production did not increase when glycerol was introduced. Although yeast extracts

improved biomass and EPS production, ground nuts showed maximum yield and efficiency than yeast extract. Sayyed et al[43] suggested a high EPS yield from *Rhizobium* sp. and noticed that the ground nut powder served as a nitrogen source. There is much influence on the productivity of EPS and its composition based on the type of carbon source[54].

Sucrose, a disaccharide sugar, is believed to be a precursor to EPS in several bacteria[17,50]. EPS generated by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 was shown to be affected by sugar supply, with glucose producing three times more EPS than fructose[20]. In our study, ribose yielded the maximum bacterial biomass and EPS production, followed by glucose. Cerning et al [6] found that *Lactobacillus casei* CG11 produced EPS when administered a variety of sugars (glucose, galactose, lactose, sucrose, maltose and melibiose). However, glucose was the most efficient.

A regular increase in relative viscosity was observed when the polysaccharide concentration was increased from 50 to 250 mg/ml. A reversible effect on relative viscosity was increased from 10 to 100°C. For example, 1% guar gum solution has sufficient stability against shear stress and temperature and can maintain a viscosity of up to 10,000 mPas. Guar gum dominates the market for hydraulic fluid additives due to its excellent thickening effects and low price[54]. Chromatographic methods used for qualitative and quantitative measurements of the polysaccharide components require hydrolysis and neutralization of the polysaccharide. D-glucose was shown to be one of the EPS hydrolysate components of *A. tumefaciens* in our analysis.

According to gas-liquid chromatography, the primary components of the methylated derivatives of EPS hydrolysate are 2,3, di-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose and 2,3,4,6-tetramethyl-D-glucose. The pure EPS from *B. licheniformis* contains →6)-D-Fruf-(2→,→1,6)-D-Fruf-(2→and D-Fruf-(2→ in a molar ratio of 5:1:1. In the same study, the data as a whole were consistent with the polysaccharide being a levan, characterized by (2,6)-linked major chain and (1,2)-linked side branches.

The study of microbial polysaccharides has gained popularity and various types of polymers with a wide range of shapes and functionalities can be produced by various microbial strains[51]. Biopolymers such as xanthan and emulsan of *Xanthomonas campestris* and *Acinetobacter calcoaceticus* are commercialised due to their emulsifying properties[5]. The EPS obtained by *A. tumefaciens* showed a non-toxic nature with a 45% emulsification property. Pig fat is the primary source of natural emulsifiers, while palm and coconut oil are rarely used[50]. Thus, the activity of EPS as an emulsifier obtained from DM can be a good source and can be used for bioremediation. On the basis of the determination of the lethal dose of the polysaccharide, it was found to be nontoxic, indicating that it could be used in foods and as a blood plasma expander without any risk of adverse effects.

In a trial for the formation of anti- *A. tumefaciens* EPS through immunization and Ouchterlony double diffusion, the immune precipitation was not observed with the pure polymer. Increased scientific and medicinal interest in polysaccharides and polysaccharide-protein complexes as capsules is attributable to their immunomodulatory and anticancer properties[9]. When EPS is negatively charged, it may activate immune cells, whereas EPS without charge has the opposite effect. Therefore, neutral EPS could protect bacteria that produce EPS from host immune systems[21]. However, *Bifidobacterium breve* UCC2003 strains that produce surface-linked EPS, did not elicit a robust immunological response[16,58]. The determination of the lethal dose of the obtained EPS revealed its nontoxicity, indicating its possible safety use in food and blood plasma expanders.

Conclusion

It is reasonable to infer that date molasses include a wide variety of microorganisms that could be useful in biotechnological applications. *A. tumefaciens* stands out as an adaptable bacterium that can generate substantial amounts of EPS from a variety of sugars including ribose and glucose. The EPS obtained was nontoxic and can act as an excellent emulsifying agent and as a safe blood plasma expander with a nonimmunogenic property.

Consequently, nutritional restriction enhances EPS production in *A. tumefaciens*; therefore, statistical optimization is proposed to clarify the influence of nutrient limitation on biopolymer biosynthesis. A study is being conducted to determine the complete chemical structure of this EPS and its potential application *in vivo*.

References

1. Adlam C. et al, Purification, characterization and immunological properties of the capsular polysaccharide of Pasteurella haemolytica serotype T15: Its identity with the K62 (K2ab) capsular polysaccharide of Escherichia coli and the capsular polysaccharide of Neisseria meningitidis serogroup H, *J. Gen. Microbiol.*, **131**, 1963–1972 **(1985)**

2. Al-Farga A., Natural antioxidants and their relationship to health, *BAOJ Nutrition*, **2**, 1–2 **(2016)**

3. Antón J., Meseguer I. and Rodríguez-Valera F., Production of an extracellular polysaccharide by Haloferax mediterranei, *Appl Environ Microbiol*, **54**, 2381–2386 **(1988)**

4. Bhatia S.K., Kumar N. and Bhatia R.K., Stepwise bioprocess for exopolysaccharide production using potato starch as carbon source, *3 Biotech*, **5**, 735–739 **(2015)**

5. Caruso C. et al, Production and biotechnological potential of extracellular polymeric substances from sponge-associated Antarctic bacteria, *Appl Environ Microbiol*, **84**, e01624-17 **(2018)**

6. Cerning J. et al, Carbon source requirements for exopolysaccharide production by Lactobacillus casei CG11 and partial structure analysis of the polymer, *Appl Environ Microbiol*, **60**, 3914–3919 **(1994)**

7. Chambi D. et al, Exopolysaccharides production by cultivating a bacterial isolate from the hypersaline environment of Salar de Uyuni (Bolivia) in pretreatment liquids of steam-exploded quinoa stalks and enzymatic hydrolysates of Curupaú sawdust, *Fermentation*, **7**, 33 **(2021)**

8. Chaykovsky M. and Corey E.J., Some new reactions of methylsulfinyl and methylsulfonyl carbanion, *J Org Chem*, **28**, 254–255 **(1963)**

9. Darwati, Khoirun Nissa and Desi Harneti, Dulxanthone A from Ethyl Acetate Extract of Garcinia nigrolineata Stem Bark, *Res. J. Chem. Environ.*, **27(3)**, 54–57 **(2000)**

10. de Jesus Assis D. et al, A study of the effects of aeration and agitation on the properties and production of xanthan gum from crude glycerin derived from biodiesel using the response surface methodology, *Appl Biochem Biotechnol*, **172**, 2769–2785 **(2014)**

11. De Vuyst L. and De Vin F., Exopolysaccharides from lactic acid bacteria, Comprehensive Glycoscience, 477–519 **(2007)**

12. Donot F., Fontana A., Baccou J.C. and Schorr-Galindo S., Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction, *Carbohydr Polym*, **87**, 951–962 **(2012)**

13. Du R. et al, Purification, characterization and antioxidant activity of dextran produced by Leuconostoc pseudomesenteroides from homemade wine, *Carbohydr Polym*, **198**, 529–536 **(2018)**

14. Elleuch M. et al, Date flesh: Chemical composition and characteristics of the dietary fibre, *Food Chem*, **111**, 676–682 **(2008)**

15. Elschner T. et al, Modification of cellulose thin films with lysine moieties: A promising approach to achieve antifouling performance, *Cellulose*, **25**, 537–547 **(2017)**

16. Fanning S. et al, Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection, Proc Natl Acad Sci USA, **109**, 2108–2113 **(2012)**

17. Freitas F. et al, Fucose-containing exopolysaccharide produced by the newly isolated Enterobacter strain A47 DSM 23139, *Carbohydr Polym*, **83**, 159–165 **(2011)**

18. Ghosh S., Ghosh P., Saha P. and Maiti T.K., The extracellular polysaccharide produced by Rhizobium sp. isolated from the root nodules of Phaseolus mungo, *Symbiosis*, **53**, 75–81 **(2011)**

19. González-García Y. et al, Biosynthesis of extracellular polymeric substances by the marine bacterium Saccharophagus degradans under different nutritional conditions, *Int J Polym Sci*, **2015**, 1–7 **(2015)**

20. Grobben G.J. et al, Analysis of the exopolysaccharides produced by Lactobacillus delbrueckii subsp. bulgaricus NCFB 2772 grown in continuous culture on glucose and fructose, *Appl Microbiol Biotechnol*, **48**, 516–521 **(1997)**

21. Hakomori S., A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide, *J Biochem*, **55**, 205–208 **(1964)**

22. Haug A. et al, An extracellular polysaccharide from Beijerinckia indica, containing L-guluronic acid residues, *Acta Chem Scand*, **24**, 1855–1856 **(1970)**

23. Hidalgo-Cantabrana C. et al, Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria, *Probiotics Antimicrob Proteins*, **4**, 227–237 **(2012)**

24. Jailani A., Ahmed B., Lee J.H. and Lee J., Inhibition of Agrobacterium tumefaciens growth and biofilm formation by tannic acid, *Biomedicines*, **10**, 1619 **(2022)**

25. Kallesha N., Prasad Ningegowda, Mahadeva Swamy M. and Ramu Gnanendra Chikkamagalur, Oxidation of oxcarbazepine with Chloramine-B in HCl Medium: A kinetic and mechanistic approach, *Res. J. Chem. Environ.*, **28(2)**, 44–50 **(2024)**

26. Kalpana R. et al, Exopolysaccharide from Bacillus cereus VK1: Enhancement, characterization and its potential application in heavy metal removal, *Colloids Surf B Biointerfaces*, **171**, 327–334 **(2018)**

27. Kennedy J., Polysaccharides structural diversity and functional versatility, Dumitriu S., Ed., Marcel Dekker Inc., New York, USA, Carbohydr Polym, **40**, 89 **(1999)**

28. Kimmel S.A., Roberts R.F. and Ziegler G.R., Optimization of exopolysaccharide production by Lactobacillus delbrueckii subsp. bulgaricus RR grown in a semidefined medium, *Appl Environ Microbiol*, **64**, 659–664 **(1998)**

29. Kvittingen L. and Sjursnes B.J., Demonstrating basic properties and application of polarimetry using a self-constructed polarimeter, *J Chem Educ*, **97**, 2196–2202 **(2020)**

30. Lee J.H., Lee I.Y., Kim M.K. and Park Y.H., Optimal pH control of batch processes for production of curdlan by Agrobacterium species, *J Ind Microbiol Biotechnol*, **23**, 143–148 **(1999)**

31. Lopez-Santamarina A. et al, Potential use of marine seaweeds as prebiotics: A review, *Molecules*, **25**, 1004 **(2020)**

32. Lynch K.M., Zannini E., Coffey A. and Arendt E.K., Lactic acid bacteria exopolysaccharides in foods and beverages: Isolation, properties, characterization and health benefits, *Annu Rev Food Sci Technol*, **9**, 155–176 **(2018)**

33. Martínez-Cánovas M.J. et al, Salipiger mucescens gen. nov., sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium isolated from hypersaline soil, belonging to the α-Proteobacteria, *Int J Syst Evol Microbiol*, **54**, 1735–1740 **(2004)**

34. Matthysse A.G., Exopolysaccharides of Agrobacterium tumefaciens, *Curr Top Microbiol Immunol*, **418**, 111–141 **(2018)**

35. Miqueleto A.P. et al, Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment, *Bioresour Technol*, **101**, 1324–1330 **(2010)**

36. Moosavi-Nasab M., Farahnaky A., Askari H. and Bakhtiyari M., Production of succinoglycan biogum from date syrup by Agrobacterium radiobacter and investigation of its rheological

properties, *Gums and Stabilisers for the Food Industry*, **15**, 51–60 **(2009)**

37. Osemwegie O.O. et al, Exopolysaccharides from bacteria and fungi: Current status and perspectives in Africa, *Heliyon*, **6**, e04205 **(2020)**

38. Ouchterlony Ö., Antigen-antibody reactions in gels, *Acta Pathologica Microbiologica Scandinavica*, **32**, 231–240 **(1953)**

39. Prasanna P.H.P., Grandison A.S. and Charalampopoulos D., Bifidobacteria in milk products: An overview of physiological and biochemical properties, exopolysaccharide production, selection criteria of milk products and health benefits, *Food Res Int*, **55**, 247–262 **(2014)**

40. Radchenkova N. et al, Optimization of the aeration and agitation speed of Aeribacillus palidus 418 exopolysaccharide production and the emulsifying properties of the product, *Process Biochemistry*, **49**, 576–582 **(2014)**

41. Razack S.A., Velayutham V. and Thangavelu V., Medium optimization for the production of exopolysaccharide by Bacillus subtilis using synthetic sources and agro wastes, *Turkish Journal of Biology*, **37(3)**, 280-288 **(2013)**

42. Sawardeker J.S., Sloneker J.H. and Jeanes A., Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography, *Anal Chem*, **37**, 1602 **(1965)**

43. Sayyed R.Z., Jamadar D.D. and Patel P.R., Production of exo-polysaccharide by Rhizobium sp., *Indian J Microbiol*, **51**, 294–300 **(2011)**

44. Sevcik C., LD50 determination: Objections to the method of Beccari as modified by Molinengo, *Toxicon*, **25**, 779–783 **(1987)**

45. Shannon E. and Abu-Ghannam N., Seaweeds as nutraceuticals for health and nutrition, *Phycologia*, **58**, 563–577 **(2019)**

46. Sheng G.P., Yu H.Q. and Li X.Y., Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review, *Biotechnol Adv*, **28**, 882–894 **(2010)**

47. Shivakumar S. and Vijayendra S.V.N., Production of exopolysaccharides by Agrobacterium sp. CFR-24 using coconut water - a byproduct of food industry, *Lett Appl Microbiol*, **42**, 477–482 **(2006)**

48. Tang Z.X., Shi L.E. and Aleid S.M., Date and their processing byproducts as substrates for bioactive compounds production, *Brazilian Archives of Biology and Technology*, **57**, 706–713 **(2014)**

49. Tanaka A., Cho Y.T., Nabeshima S. and Fukui S., Production of polysaccharides from lower alcohols and glycols by nitrogen-fixing Pseudomonas sp., *J Ferment Technol*, **52**, 739–746 **(1974)**

50. Van Dyk J.S., Low Ah Kee N., Frost C.L. and Pletschke B.I., Extracellular polysaccharide production in Bacillus licheniformis SVD1 and its immunomodulatory effect, *Bioresources*, **7(4)**, 4976–4993 **(2012)**

51. Vargas-García M.C. et al, Influence of nutritional and environmental factors on polysaccharide production by Azotobacter vinelandii cultured on 4-hydroxybenzoic acid, *J Ind Microbiol Biotechnol*, **27**, 5–10 **(2001)**

52. Vidhyalakshmi R. et al, Production, characterization and emulsifying property of exopolysaccharide produced by a marine isolate of Pseudomonas fluorescens, *Biocatal Agric Biotechnol*, **16**, 320–325 **(2018)**

53. Weiner R., Langille S. and Quintero E., Structure, function and immunochemistry of bacterial exopolysaccharides, *J Ind Microbiol*, **15**, 339–346 **(1995)**

54. Xia S. et al, Application of polysaccharide biopolymer in petroleum recovery, *Polymers* (Basel), **12**, 1860 **(2020)**

55. Xiao J.H. et al, Optimization of submerged culture requirements for the production of mycelial growth and exopolysaccharide by Cordyceps jiangxiensis JXPJ 0109, *J Appl Microbiol*, **96**, 1105–1116 **(2004)**

56. Xu W., Liang L. and Zhu M., Determination of sugars in molasses by HPLC following solid-phase extraction, *Int J Food Prop*, **18**, 547–557 **(2014)**

57. Yilmaz M., Celik G.Y., Aslim B. and Onbasili D., Influence of carbon sources on the production and characterization of the exopolysaccharide (EPS) by Bacillus sphaericus 7055 strain, *J Polym Environ*, **20**, 152–156 **(2011)**

58. Yin W., Wang Y., Liu L. and He J., Biofilms: The microbial "protective clothing" in extreme environments, *Int J Mol Sci*, **20**, 3423 **(2019)**.

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