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Exopolysaccharide production by *Brevundimonas diminuta* isolated from Marchica lagoon ecosystem in Morocco

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ABSTRACT

Polysaccharides are the most diverse families of biopolymers. *Brevundimonas diminuta*, a member of the Class *Bacilli*, has the ability to synthesize and secrete polysaccharides. The strain isolated from Marchica lagoon in Morocco produced extracellular polysaccharides (EPS), mainly during its exponential growth phase but also to a lesser extent during the stationary phase. The optimum pH and temperature for growth and exopolysaccharides (EPSs) production were 8 and 37°C, respectively. The dry weight of the polysaccharides products and biomass was found to be 306.85 ± 4.45 mg/100 mL and 77.75 ± 2.47 mg/100 ml, respectively. *B. diminuta* did not produce EPS with galactose as the carbon source and the addition of peptone improved EPS production. There is no production of EPS in the absence of mineral salts. Screening for emulsifying activities showed emulsion for hexane and toluene.

INTRODUCTION

In recent years, increasing attention is being paid to microbial exopolysaccharides (EPS) mainly because of their bioactive role and extensive range of potential applications in modern biotechnology especially in pharmaceuticals as antiangiogenic or antiproliferative agents or even in case of targeted drug delivery and environment (Di Gu et al., 2017; Dah Dossounon et al., 2017). The advantages of microbial EPS over plant and marine micro-algal biopolymers are due to their novel functionality, easily reproducible chemical and physical properties, and stable cost and supply. EPS generally consist of monosaccharides and some non-carbohydrate substituents (such as protein, nucleic acids, lipids, acetate, pyruvate, succinate and phosphate) (Aguilera et al., 2008; Gong et al., 2008; Bryan et al., 1986; Donot et al., 2012). Several researchers on the EPS production by the bacteria and their biotechnological applications are available in literature (Chen et al., 2017 ; Morris et al., 2012). However, it is important to study the optimal culture conditions for a good production of EPS, because factors such as the culture medium, pH, temperature and agitation influence the production of EPS and their composition (Mozzi et al., 1994).

Brevundimonas diminuta is an ubiquitous Gram bacilli. *B. diminuta* strain isolated from Marchica lagoon (Soil and water) in Morocco *was* reported as an EPS producer microorganism for the first time (Dah Dossounon et al., 2017). The EPS produced by this strain of *B. diminuta* had shown 20% antiproliferativeness against myeloid cancer and flocculating activity of 66% (Dah Dossounon et al., 2017). In view of these interesting activities of the EPS produced by *B. diminuta*, this work aims to establish the yield of EPS in the same strain of *B. diminuta* isolated from Marchica lagoon in Morocco. The influence of the incubation temperature, initial pH and different culture media on the production of EPS and the emulsifying activities of EPS were determined for the first time.

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Table 1. Formulae of six different fermentation culture media.

Media (Biokar)	Formula
M1 (PM)	10 g/l peptone, 5 g/l NaCl, 9 g/l Na_2HPO_4,1.5 g/l KH $_2PO_4$ 0.6 g/l $(NH4)_2S0_4$,1.36 g/l K_2HP0_4, 0.1 g/lMg$S0_4$, 0.02 g/l $CaCl_2$, 0.2 mg/lZn$S0_4$, 0.2 mg/lCu$S0_4$, 1.1 mg/lMn$S0_4$, 0.14 mg/l Fe$S0_4$; pH 8
M2 (YM)	O.5 g/l Yeast extract, 0.6 g/l $(NH4)_2S0_4$,1.36 g/l K_2HP0_4, 0.1 g/lMg$S0_4$, 0.02 g/l $CaCl_2$, 0.2 mg/lZn$S0_4$, 0.2 mg/lCu$S0_4$, 1.1 mg/lMn$S0_4$, 0.14 mg/l Fe$S0_4$; pH 8
M3 (P)	10 g/l peptone, 5 g/l NaCl, 9 g/l Na_2HPO_4,1.5 g/l KH_2PO_4;pH 8
M4 (Y)	O.5 g/l Yeast extract; pH 8
M5 (TSB)	17 g/l tryptone, 3 g/l papaic digest of soybean meal, 2.5 g/l glucose, 2.5 g/l K_2HP0_4 , 5g/l NaCl; pH 8
M6 (BCC)	17.5 g/l Beef heart extract, 10 g/l pancreatic peptone, 5 g/l NaCl, 2.5 g/l Na_2HPO_4, 2 g/l glucose; pH 8

MATERIAL AND METHODS

Bacterial strain

B.diminuta(NR_040805.1) was isolated from the lagoon Marchica (Soil and water) in Morocco. This bacterium was isolated in the contextof the project of the 7th PCRD research ULIXES «Unraveling and exploiting Mediterranean Sea microbial diversity and ecology for the xenobiotics and pollutants cleanup» in Morocco. Bacterial identification was done by sequence method using GenElute™ Bacterial Genomic DNA Kit and ABI 3130xl Genetic Analyzer. The identification of isolated strain wasperformed by direct sequencing of PCR amplified 16S rDNA gene fragments. The bacterium have been purified and maintained in glycerol at -20°C. This strain showed good performance for cell growth and EPS formation in culture environment rich in nitrogen source.

Growth media

For the evaluation of kineticsand the influenceof pHand temperature on the growth and EPS production, the bacterial strain was cultivated inyeast-peptone-glucose-mineral (YPMG) salts medium. Composition per liter: Glucose: 20 g; Peptone: 5g; yeast extract: 5g; $(NH_4)_2S0_4$:0.6 g; KH_2PO_4:3.18g; K_2HPO_4:5.2 g; $MgSO_4$:0.3 g; $CaCl_2$:0.05 g; $ZnSO_4$:0.2 mg; $CuSO_4$: 0.2 mg; $MnSO_4$:0.2 mg; $FeSO_4$:0.6 mg).

To determine the effect ofthe sources of carboninthe growth and the EPS production by *B. diminuta*, different sourcesofcarbon2% of[glucose, maltose, sucrose, galactose andlactose (all of Sigma)] were added separately to the minimal medium [0.6 g/l (NH4)2$S0_4$,1.36 g/lK_2HP0_4, 0.1 g/lMg$S0_4$, 0.02 g/l $CaCl_2$, 0.2 mg/lZn$S0_4$, 0.2 mg/lCu$S0_4$, 1.1 mg/lMn$S0_4$, 0.14 mg/l Fe$S0_4$).

The following media were used to determine the effect of different culture media on EPS production by *B. diminuta.* Formulae of six different fermentation culture media are shown in Table 1.

Culturing procedures

To establish the kinetics of EPS production, 200mL of the YPMGmedium was placed in 500ml Erlenmeyer flasks and 1mL of 16 h bacterial pre-culture was inoculated. The culture was maintained at a temperature of 37°C, pH 7 for 6 days. Some samples were taken during culture to monitor microbial growth and EPS production. 20 ml of culture was taken each time for EPS extraction in 50ml centrifuge tubes and the cell was dried and weighed.

We assayed the following variables to establish the influence of physicochemical parameters: incubation temperature (30, 37 and 40°C); initial pH (6, 7, 8, 9 and 10) andincubation eitherin a rotating shaker (100 rpm) for 4 days.All experiments were done using500 ml flaskseachcontaining 100ml of each medium. 1mL of 16 h bacterial pre-culture was inoculated. The test was done in triplicate.

Analytical determinations

B. diminuta growth was measured based on the dry weight per 100 ml of the culture. The cell dry weight (CDW) was determined by centrifugation (8600 × g, 4°C, 30 min) followed by drying to a constant weight in an oven at 100°C overnight.

EPS was quantified by dry weight determinations by the method of Castellane et al. (2014) modified. Briefly, the culture was centrifuged (8600 × g, 4°C) and the supernatant was filtrated on filter paper and the medium is treated with trichloroacetic acid (3%) for protein precipitation. The medium was recentrifuged at 8600 × g for protein removal. The polysaccharides contained in the supernatant were precipitated with cold 96% ethanol at a 1:3(v/v) supernatant: ethanol ratio. The mixture was refrigerated at 4°C overnight. After this period, the samples were centrifuged once again (8000 × g, 4°C) to separate the precipitate from the solvent. The precipitated product was dried at 37°C. For the purification, The EPS was dissolved in the solution of NaCl (1 M and 0.5 M) respectively. It was re-precipitated

with two volumes of absolute ethanol and centrifuged (8000g, 4°C, 20 min). This operation was carried out four times. The solvent precipitation achieved a partial purification of the polymer by eliminating the soluble components of the culture media. The precipitated product was dried at 37°C until a constant weight was observed, and a precision balance used to verify the quantity of EPS obtained (Castellane et al., 2014). The weight of the EPS is expressed in milligrams per 100 ml of culture (mg/100ml). The values shown for EPS were calculated by subtracting the amount of background interference in uninoculated medium (approximately 10mg of carbohydrate/100ml) from the amount in fermented broth. This test was triplicated.

Emulsifying activity

The capacity of the EPS to stabilize emulsions with several hydrophobic compounds was tested as described by Llamas et al. (2010). Briefly, we mixed equal volumes (5ml) of different EPS solutions (0.5%, w/v) in distilled water and various hydrophobic substrates with the vortex. The emulsification index after 24h (E_{24}) was determined, using the following equation:

$$E_{24}=(\frac{h_e}{h_t}) \times 100$$

Where h_e (cm) is the height of the emulsion layer and h_t (cm) is the overall height of the mixture. All tests were performed in duplicate. The tested compounds were mineral oil, Vaseline oil (commercial brands), hexane, diethylether, toluene, gasoline (all from Sigma). The test was also carried out for sodium alginate (Sigma) ant Tween 80, with the same concentration in deionized water.

All data are presented as means ± standard deviation (SD). All experiments were repeated three times.

RESULTS

We reported in this study, for the first time, the quantification of EPS produced by *B. diminuta* according to the initial pH, temperature of incubation anddifferent culture media. The 16S rDNA gene sequence of the strain was carefully studied by refereeing to the GenBank database using a BLAST search and was revealed to be identical to *B. diminuta* (GenBank Accession N° NR_ 040805.1).

Growth and EPS Production

To study the synthesis of the EPS as a function of the growth phase, *B. diminuta* was grown in YPMG medium. Cell dry weight and EPS production are determined during fermentation for six days at 37°C, 100 rpm and pH 7 without control over pH, as shown in Figure 1. The bacterium has a latency period of6 h and then enters to exponential phase.

The stationary phase starts from 96 h. The kinetics of the EPS production by *B. diminuta* showed that the EPS is produced during the exponential phase increasingly and reached a maximum value at 96 h. During the stationary phase, the quantity of EPS in the culture decreased after 96 h of incubation.

We studied the influence of different cultural parameters in order to be able to improve EPS production by *B. diminuta* strain. The dry biomass and isolated EPS were weighed and the values obtained are presented in Tables 2 and 3.

To evaluate the influence of pH, we grew *B. diminuta* in YPMG at 37°C and initial pH 6, 7, 8, 9 and 10. The amount of EPS varies considerably in different culture settings. The best production was at pH 8 (306.85 ± 4.45 mg/100 ml) (Table 2) under the conditions used in this study. There was production of EPS in the different pH tested and low EPS production was at pH 10.

We grew *B. diminuta* in YPMG at pH 7 at temperatures of 30, 37 and 40°C to evaluate the influence of temperature. The best production was obtained at 37°C (202.5 ± 3.53 mg/100 ml) (Table 3). Low EPS production for *B. diminuta* strain is at the temperature of 30°C.

The value ofcellular biomass at pH 8 (77.75 ± 2.47 mg/100 ml) is higher than that of other pH (Table 2). For the temperatures tested at pH 7, the value of the biomass at 30°C is higher than that of 37 and 40°C (Table 3).

We evaluated the specific yield of EPS production. This yield is given by the ratio of the total EPS to the cellular biomass. The best yield was obtained at pH 6 (6.12 ± 0.38) followed by pH 8 (3.94 ± 0.17), pH 7 (2.96 ± 0.09), pH 9 (0.71 ± 0) and pH 10 (0.26 ± 0.07). 37°C (3.17 ± 0.091) is the temperature at which there is a higher production of EPS followed by 40°C (1.21 ± 0.007) and 30°C (0.3 ± 0).

Effect of different carbon sources on growth andEPS production

B. diminuta was cultivated on minimal medium supplemented with 2% of glucose, maltose, sucrose, lactose and galactose, separately, at 37°C, pH 8. The results showed that the amount of growth and EPS produced by *B. diminuta* was comparatively different with different carbon sources. The Figure 2 shows that *B. diminuta* produces EPS with glucose, maltose, lactose and sucrose but no production with the galactose under the conditions used in this study (Figure 2B). Yield is maximal with maltose as a carbon source. *B. diminuta*

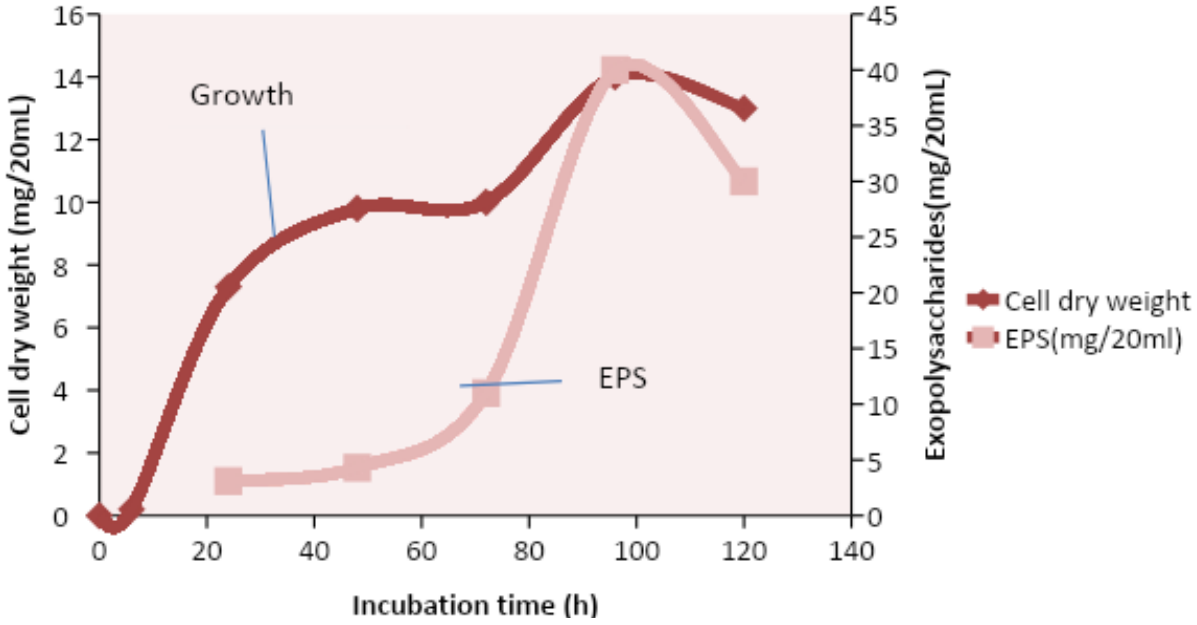

Figure 1. Profile of EPS production and cell dry weight by *B. diminuta* in YPMG medium (pH 7, 37°C).

Table 2. Evaluation of the influence of initial pH in the EPS production and growth in *B. diminuta* strain isolated in Morocco.

Strain	pH	EPS	Growth (mg/100 ml)	EPS/Growth
B. diminuta	6	128.55±0.63	21±1.41	6.12±0.38
	7	202.5±3.53	68.35±0.91	2.96±0.09
	8	306.85±4.45	77.75±2.47	3.94±0.17
	9	24.6±0.56	34.45±0.77	0.71±0
	10	13.3±4.66	49.95±2.75	0.26±0.07

Mean values (± standard deviation); YPMG medium, 37°C, 100 rpm, 4 days.

Table 3. Evaluation of the influence of the temperature in the EPS production and growth in *B. diminuta* strain isolated in Morocco.

Strain	Temp °C	EPS	Growth (mg/100 ml)	EPS/Growth
B. diminuta	30	22.68±0	73.85±0.21	0.3±0
	37	202.5±3.53	63.85±0.91	3.17±0.091
	40	55.45±2.05	45.5±1.55	1.21±0.007

Mean values (± standard deviation) culture conditions; YPMG medium, pH 7, 100 rpm, 4 days.

grew with all the carbon source tested as shown in Figure 2A with a maximum yield of sucrose.

Screening of the fermentation culture media

B. diminuta was cultivated on PM, YM, P, Y, TSB and BCC media. PM was richer in salts than the other media (YM, P, Y, TSB and BCC). There isno saltin medium Y. Peptone and yeast extract were used in PM and YM respectively. The medium TSB and BCC were rich in nitrogen source and contained glucose as carbon source unlike the others media. Based on cell biomass and EPS formation variations presented in Figure 3, it was found

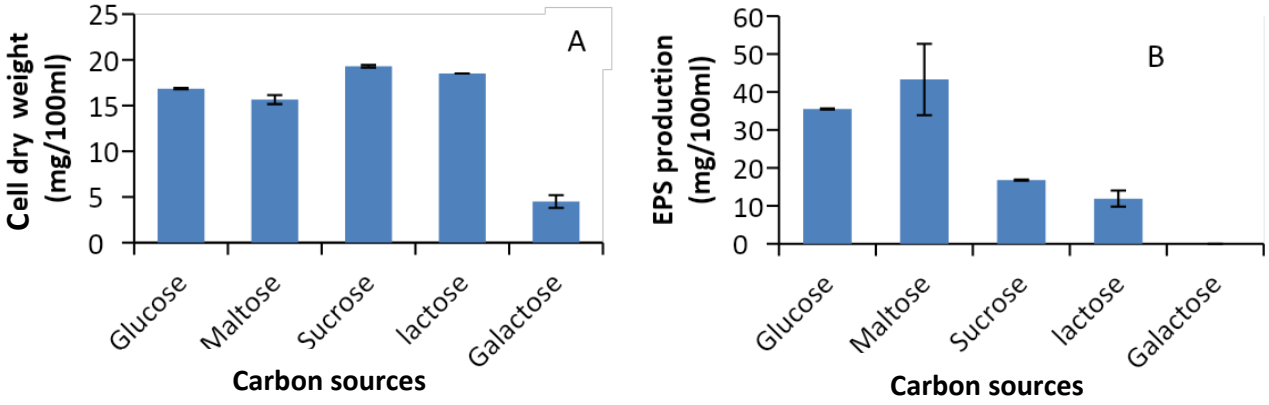

Figure 2. Effect of different carbon sources on the growth (A) and EPS production (B) by *B. diminuta.*

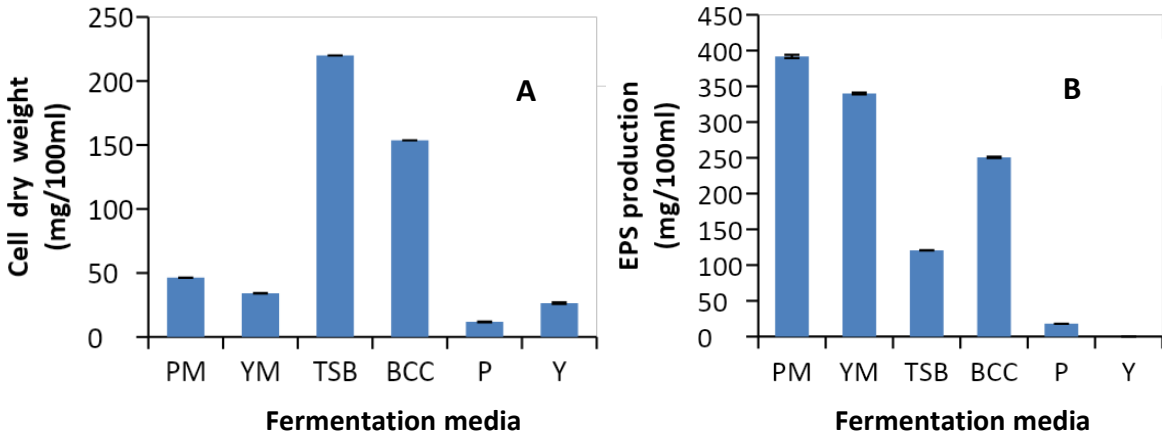

Figure 3. Screening of the fermentation culture media on the growth (A) and EPS production (B) by *B. diminuta.*

that the culture medium PM was considered to be the optimal fermentation culture for EPS production by *B. diminuta*. Although fermentation medium TSB is better for cell accumulation, it showed little beneficial effect on EPS production. There is no EPS production with medium Y, that medium contained only the yeast extract. As compared with these culture media compositions, it is clear that a high salinity and nitrogen source in the fermentation medium are useful for EPS production by *B. diminuta*.

Emulsion forming and stabilizing capacity

Several hydrophobic compounds, namely, mineral oil, Vaseline oil and hydrocarbons, were assayed (Table 4). For comparison, the same test procedure was performed with Tween 80 and sodium alginate. *B. diminuta* EPS produced in YPMG medium and yeast-peptone-mineral (YPM) salts medium (the previous medium without glucose) was tested. The EPS of *B. diminuta* in YPM medium has proven to possess emulsion stabilizing capacity for the toluene and hexane with emulsification indexes of 52 and 50%, respectively unlike the EPS of *B. diminuta* produced in YPMG medium, demonstrated to have a lower emulsion forming for mineral oil (30%) and diethylether (6%). Compared with alginate andtween 80, the EPS producedby*B. diminuta* in the medium YPM has shown an elevatedemulsion with toluene and hexane.

DISCUSSION

EPSs are polymers produced by many microbes, including bacteria (Amjres et al., 2015). EPSs are widely used in the pharmaceutical industry, the food industry,

Table 4. Emulsification index (E_{24}) for the EPS produced by *B. diminuta* (B.D) in YPMG and YPM media against several hydrophobic compounds in comparison with commercially available emulsion forming and stabilizing. All emulsions were prepared by mixing a 0.5%, w/v aqueous solution with each of the hydrophobic compound and left at room temperature for 24 h to determine E_{24}.

E24(%)	EPS-YPMG (*B.D*)	EPS-YPM (*B.D*)	Alginate	Tween 80
Mineral oil	30±7.07	0±0	0±0	67.5±3.53
vaseline oil	0±0	0±0	0±0	60±0
toluene	0±0	52±2.82	0±0	40±14.14
Gasoline	0±0	0±0	0±0	30±0
hexane	0±0	50±5.65	25±7.07	30±7.07
Diethylether	6±2.82	0±0	0±0	0±0

and the field of agriculture (Yu et al., 2017) because of their ecological, physiological and physicochemical properties (Palaniraj and Jayaraman, 2011). The bacterial strain studied in this work was *B. diminuta* which was isolated from Marchica lagoon in Morocco and was reported as an EPS producer microorganism for the first time by our research group (Dah Dossounon et al., 2017). The EPS produced by this strain of *B. diminuta* had shown 20% antiproliferative against myeloid cancer and flocculating activity of 66% (Dah Dossounon et al., 2017). On the basis of existing studies, the properties of polysaccharides are influenced by the location, composition, molecular mass and conformation or other criteria. We report here for the first time the influence oftheincubationtemperature, pH and different culture mediaon the growth andtheEPS production by *B. diminuta.* In this work, this bacterial strain showedthe fermentation profile characterizedbyanincrease in the EPS production duringtheexponential phase and a decrease duringthe stationary phase. The EPS production by *B. diminuta* strain exhibited a fermentation kinetic similar to that of Dah Dossounon et al. (2016) and Llamas et al. (2010). Under optimal growth conditions, the production of EPS starts during the exponential phase and increased concomitantly with the rise in number of viable cells. The decrease of amount ofpolysaccharides duringthe stationary phase of growth could be due to the activation of a glycohydrolase, degrading the polymers as suggested by Pham et al.(2000). In the present study, the pH and the temperature influence the production ofEPS. Indeed, the physicochemical parameters act on the bacterial growth, which directly influences theEPS production. These results are in agreement with those found by Mozzi et al. (1995) and Vijayabaskar et al. (2011).

The results showedthatthe amount of EPSproduced by *B. diminuta* was comparatively different with different carbon sources. The maximumEPS production was with maltose, maltoseis composed of glucose. This result could be explained by the fact that glucose is easily assimilated by the bacterium. Other studies have showed, in general that glucose provides the highest yield of EPS (Mozzi et al., 1995; Cerning et al., 1994). The galactosebasic medium supported growth of *B. diminuta* but not EPS production (Figure 3). These results are similar to those obtained by Mudoi et al. (2013) who found that there is a very low production of xanthan by *Xanthomonas campestris* with galactose as a carbon source. It has also been reported that the production of EPS is a response to the nutrient composition of the growth medium (Wrangstadh et al., 1990). In our study, high level of EPS production was achieved by *B. diminuta* in PM medium. In fact, the PM medium is a rich medium containing only the peptone and mineral salts. The yield of EPS could be strongly associated with the nitrogen source and mineral salts in the culture medium (Di gu et al., 2017). From our experiments, it follows that the peptone supply has a pronounced effect on the yield of EPS. Similar effects have been reported for *Aeromonas salmonicida* strain A450EPS production (Bonet et al., 1993). However there was no production of EPS in the medium Y. The minerals salts were present in all other media except the medium Y. This shows that salts such as NaCl and KH_2PO_4 are required for the production of EPS by *B. diminuta.* Lowering the amount of phosphate in the medium did not enhance polysaccharide (EPS or CPS) production, because it reduced the buffering capacity (Bonet et al., 1993). Similar conclusion was recorded by Mudoi et al. (2013) and Bonet et al. (1993) who found that at lower KH_2P0_4 concentrations, a decrease in *A. salmonicida* EPS yield was observed. The EPS production depends mainly on the type of producing microorganisms and the culture media. As shown in Table 4, the EPS of *B. diminuta* demonstrated to have emulsification indexes than alginate and Tween 80 for hexane and toluene. One important feature of the emulsions produced by the EPS described here is that they are very stable and are composed of small, uniform droplets, resulting in a fine, smooth consistency. Other known polysaccharides, such as xanthan, are capable of producing stable emulsions but they tend to be thicker and more viscous, which is not very desirable for some of

the uses for which emulsifiers such as these are intended (Llamas et al., 2010). Most of the commercially available products, such as Tween 80, are synthetic, generally toxic to the environment and not easily biodegradable. In view of this, natural polymers have emerged as advantageous alternatives because they are biodegradable, less toxic, have improved functionality and have activity under a wider variety of conditions (Banat et al., 2000).

Conclusion

In summary, the pH, temperature and carbon sources influence the EPS production by *Brevundimonas diminuta* isolated from Marchica lagoon in Morocco. Under optimum growth conditions, *B. diminuta* has the ability to produce significant quantities of EPS. This hypothesis is supported by the results of the EPS quantification. This bacterium is capable of transforming hexane and toluene. This bacterium may prove to be an excellent model species for the development of biotechnology products. We are currently studying the characterization of the composition of each EPS fraction depending on growth culture. Other monosaccharide such as galactose, mannose, osamine and uronic acid will be quantified.

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