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Rheological, emulsifying and thermostability properties of two exopolysaccharides produced by *Bacillus amyloliquefaciens* LPL061

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ABSTRACT

The rheological, emulsification, thermostability and certain physicochemical properties of two purified exopolysaccharides from *Bacillus amyloliquefaciens* LPL061 were studied. EPS1 showed entangled spider mesh structure that composed of dense rope with homogeneous hexagonal particles under scanning electron microscopy. EPS2 had a porous sponge structure with uniform cylindrical particles. The two exopolysaccharides showed higher intrinsic viscosity and better emulsification activity with sunflower seed oil, rice oil, olive oil and peanut oil compared to guar gum. EPS1 is the most promising one for applications in the industry, as it had high intrinsic viscosity, apparent viscosity and thermostablity in aqueous solution, dense entangled structure and good emulsification activity.

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1. Introduction

Exopolysaccharides (EPSs) produced by microorganisms usually occur in the form of slime when these are loosely attached to the cell surface or completely excreted to the environment. Microbial EPSs are generally recognized as safe and they can be widely applied in the industry as food additives, bioemulsifiers, pharmaceuticals, bioflocculants and chemical products. In addition, bacterial EPSs have been shown to be effective immunomodulatory, immunostimulatory, anti-tumor, anti-viral, anti-inflammatory and antioxidant agents. There is high demand for EPSs from different bacteria species as emerging industrial biopolymers (Gutiérrez, Leo, Walker, & Green, 2009; Kanmani et al., 2011, Liu et al., 2010, Wang, Ahmed, Feng, Li, & Song, 2008).

Bacterial polysaccharides have some advantages over plant or animal hydrocolloids in industry (Karim & Bhat, 2008; Saija, Welman, & Bennett, 2010). Most bacterial polysaccharides show higher water solubility compared to plant gums, and also exhibit better viscosifying, thickening, stabilizing, gelling and emulsifying activities than some other commercially used polymers such as guar gum, locust bean gum and gum arabic (Freitas et al., 2009; Jindal, Singh, & Khattar, 2011; Wang et al., 2008). Furthermore, EPSs produced by different bacterial species have been shown to have stable rheological and emulsifying properties over wide ranges of temperature, pH and ionic strength, which ensure their applications in various food products under different conditions (Kumar & Mody, 2009). Currently, the common EPSs produced by microorganisms used in market include xanthan from *Xanthomonas campestris*, gellan from *Sphingomonas paucimobilis*, bacterial alginates secreted by *Pseudomonas* species and *Azotobacter chrococcum*, bacterial cellulose from *Acetobacter xylinium*, hyaluronic acid from *Streptococcus equii* and succinoglycan from *Rhizobium* (Kumar Ganesh, Joo, Choi, Koo, & Chang, 2004).

Most EPS-producing bacteria demand better growth conditions, which greatly limits the further application of EPSs. *Bacillus* is an important group which has several advantages over other bacteria, in that they are easy to cultivate, preserve and conducive to industrial production. A number of *Bacillus* strains are demonstrated to be safe over many years as non-pathogenic species. What is more, their expolysaccharides (EPSs) also have a wide range of biological activity and technological characteristics. However, so far little information is available on the physicochemical and industrial

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properties of purified EPSs from *Bacillius* strains (Kumar Ganesh et al., 2004). In a previous study, we isolated a high EPS-producing strain named *Bacillus amyloliquefaciens* LPL061 (Zhang, Li, Fan, Liu, & Li, 2012). In addition, we optimized the conditions to produce EPSs from that strain and purified two fractions of EPSs which were named EPS1 and EPS2. Our preliminary data showed that the crude EPS had potent immunological acitivity (Han, Liu, Li, Liu, & Li, 2014; Li et al., 2013). In order to explore the possibility of applying our EPSs to industry, in this paper we described certain physicochemical, thermostability, rheological and emulsifying properties of two EPSs produced by *B. amyloliquefaciens* LPL061 and evaluated their potential value in industrial applications.

2. Materials and methods

2.1. Microorganisms and culture conditions

B. amyloliquefaciens LPL061 (GenBank: KJ432586) with high EPS yield isolated from carnation was used as the EPS-producing strain throughout this study (Zhang et al., 2012). The strain was cultivated in previously optimized EPS-producing medium containing 22 g L^{-1} sucrose and 18.4 g L^{-1} yeast extract at initial pH 7.0, 28 °C, 220 rpm for 24 h. (Li et al., 2013). The strain was maintained at −80 °C in 15% (v/v) glycerol.

2.2. Isolation and purification of EPSs

The EPSs were isolated and purified by using our previous method with slight modifications (Xu, Shen, Ding, Gao, & Li, 2011). In brief, the bacterial cells were spinned down by centrifugation at 10,000*g* for 10 min at 4°C. The cell-free supernatant was collected and precipitated by three times volume of 100% (v/v) cold ethanol overnight at 4 °C. Thereafter, the crude EPS was collected by centrifugation at 10,000*g* for 10 min and dissolved in deionized water (50%, w/v). The solution was treated with Sevag agent (chloroform: n-butanol = 4:1) to remove protein, and then dialyzed using the dialysis bag (Mw cut-off 8000–14,000 Da) for 48 h at 4 °C with twice replacement of deionized water per day. Crude EPS was lyophilized after dialyzed.

The crude EPS was fractionated using anion-exchange chromatography on a column (2.6 × 20 cm^2) of DEAE-Sepharose at a flow rate of 4 mL min^{-1} followed by a linear gradient of NaCl concentration (0–1 M). The carbohydrate content of the fractions was determined by phenol–sulfuric acid method using glucose as a standard. Two fractions were collected separately according to the carbohydrate elution profile and dialyzed against deionized water for 48 h, respectively. Then, the main fraction was confirmed by gel-filtration chromatography on a column of Sepharose CL-6B (1.6 × 80 cm^2) eluted with 50 mM NaCl (30 mL h^{-1}). Here, the two EPSs were selected for the emulsifying, rheological and physicochemical properties tests.

2.3. Physicochemical characterization of EPSs

2.3.1. EPSs concentration, molecular mass and monosaccharide composition

The carbohydrate content of EPSs was determined by the phenol–sulfuric acid method, using glucose as standard (Dubois, Gilles, Hamilton, Robers, & Smith, 1956). The protein content in the fraction was measured according to Bradford assay using bovine serum albumin (BSA) as the standard. The moisture content of lyophilised EPSs samples was determined as described earlier (Vijayendra, Palanivel, Mahadevamma, & Tharanathan, 2008).

Number and weight average molecular weights (*M*n and *M*w, respectively), as well as the polydispersity index (*M*w/*M*n) were obtained by gel permeation chromatography multi-angle-laser-light scattering (GPC-MALLS) with DAWN HELEOS MALLS II detector (Wyatt Technology, Santa Barbara, CA). The monosaccharide composition of two EPSs was determined using the method described by Xu et al. (2011). In brief, hydrolysis of the EPSs was carried out with 12 M H_2SO_4 at 100 °C for 2.5 h, the hydrolyzates were obtained by filtration and centrifugation at 4000*g* for 10 min after neutralizing the pH to 6.0 by adding $BaCO_3$. The supernatant was diluted 20-fold and subsequently fractionated by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex system. The DIONEX-2500 ion chromatography was equipped with a CarboPac PA20 sugar column. The column was eluted with a mixture of water and 200 mM NaOH in the volume ratio of 91:9 at a flow rate of 1.0 mL min^{-1}. Standard glucose, galactose, rhamnose, mannose, fructose and arabinose (Sigma, ≥99%) were prepared for comparison.

2.4. Determination of intrinsic viscosity

The intrinsic viscosity (η) of EPSs and guar gum was determined as described by Prasanna, Bell, and Grandison (2012). Diluted solutions of EPSs, guar gum (Sigma) (0.01–0.1%, w/v) were prepared with deionized water. Viscosity measurements were carried out using an Ubbelohde capillary viscometer (1619/03-C, Rheotek, UK) at 25 °C.

2.5. Rheological properties in aqueous solution

The rheological behavior of EPSs solutions, guar gum was studied with a Ex1500 rotary rheometer (TA Instrument, USA), with a parallel plate geometry (PP40; 40-mm diameter and 1-mm gap setting). For this, the lyophilised EPSs or gum (1%, w/v) was dissolved in deionized water. All measurements were carried out at 20 °C. A shear ramp was carried out by measuring the shear stress as a function of shear rate from 0.1 to 100 s^{-1}.

2.6. Scanning electron microscopic (SEM) analysis of EPSs

The lyophilized samples of EPSs were fixed to the SEM stubs with double sided tape. The samples were then coated with a layer of gold, ~10 nm thick. The samples were observed under a scanning electron microscope (S-3400N, Hitachi, Japan); accelerated voltage was operated at 15.0 kV.

2.7. Particle size distribution

EPSs sample 0.5% (w/v) was taken for measurement of particle size distribution by nano particle size analyzer (NicoMP380, Zeta Bekman coulter, USA) at 25 °C. The molecular size distribution changes of original EPSs and treated EPSs after boiling 30 min were measured.

2.8. Differential scanning calorimetric (DSC) analyses

DSC analyses of lyophilized EPSs were carried out with differential scanning calorimeter (TA-60WS, Shimadu, Japan). About 5–8 mg of lyophilized sample was loaded in a platinum pan and its energy level was scanned in the ranges of 20–400 °C under a nitrogen atmosphere, with a temperature gradient of 10 °C min^{-1}.

2.9. Emulsifying activity

2.9.1. Preparation of emulsions

The emulsifying activity of two purified EPSs of *B. amyloliquefaciens* were compared with guar gum (plant origin) using the method of Prasanna et al. (2012). Briefly, oil (3 mL) was added to

2 mL of EPSs or gum solution (1%, w/v) in a screw cap glass tube (100 mm × 13 mm) and stirred in a vortex for 2 min at 40 Hz (QL-901, China Aomen). After 24 h, 168 h and 360 h, the emulsion index (E_{24}, E_{168} and E_{360}) was determined as given below depending on the assay: E_{24}, E_{168} or $E_{360} = (h_e/h_t) \times 100$, where h_e is the height of the emulsion layer and h_t is the overall height of the mixture. All samples were stored at 25 °C.

2.9.2. Emulsifying activity of EPSs with different oils

Different emulsions were prepared as described in Section 2.9.1 using EPSs with different oils (sunflower seed oil, olive oil, peanut oil and rice oil) as a hydrocarbon (control) to determine the best oil which yields a stable emulsion based on E_{24}, E_{168} and E_{360}. All chemicals used in the emulsion study were obtained from Sigma-Aldrich (UK). The oils' storage time after production were less than six months.

2.9.3. Effect of concentration of EPSs on emulsion stability

Sunflower seed oil was selected as the best oil resulting in stable emulsions for both EPSs as indicated by E_{24}, E_{168} and E_{360}, and hence was used for subsequent assays. Therefore, the emulsions prepared with sunflower seed oil were studied with respect to different concentrations of EPSs (0.25–1.5%) using the emulsion index E_{168}.

2.9.4. Light microscopy and particle size distribution of emulsions

The particle size distribution of emulsions (1% EPSs or gum w/v) was measured using a modification of the method of Prasanna et al. (2012). Briefly, a light microscope (XSZ-HS3, Zhongyi Ltd, Beijing) was used to examine and photograph the emulsion after 168 h storage at 25 °C through a 10× objective lens. A volume of 60 μL of the emulsion was added to a cavity microscope slide and kept for 5 min to settle down, followed by observation under the light microscope.

2.10. Statistical analysis

Data were expressed as mean ± SD and statistically analyzed using the one-way ANOVA procedure of SPSS software (version 15.0). The differences among means were detected by the Tukey's post hoc test at 95% significance level.

3. Results and discussion

3.1. Physicochemical characteristics of EPSs

As shown in Table 1, the two exopolysaccharides (EPS1 and EPS2) from *B. amyloliquefaciens* LPL061 had different molecular mass and composition. On a weight basis, EPS1 and EPS2 accounted for 10.31% and 89.9%, respectively, of the total carbohydrate fraction. The molecular weights measured by different methods were different. In recent years, GPC-MALLs are considered to be the best method to determine the molecular weight and distribution of polysaccharides. The molecular weight of EPS1 (23.29×10^4 g mol^{-1}) was 2.37 times higher than that of EPS2 (9.81×10^4 g mol^{-1}). *M*w/*M*n ratio of EPS1 (1.32) is lower than EPS2 (2.17), indicating that the molecular weight distribution of EPS1 was more homogeneous than that of EPS2.

EPS1 and EPS2 are both heteropolysaccharides containing mannose and glucose in a relative molar proportion (mannose/glucose) of 96.9/3.1 in EPS1 and 65.3/34.7 in EPS2, respectively. This result was similar to the findings of Maric et al. (1996) in which *Bacillus thermoantarcticus* also produces two extracellular polysaccharides (EPS1 and EPS2) that were mainly composed of mannose. On a weight basis, EPS1 and EPS2 represented about 27% and 71% of the total carbohydrate fraction. The molecular weights of two EPSs were approximately 3.0×10^5 Da. EPS1 is a heteropolysaccharide

Table 1
Molecular weight, composition and intrinsic viscosity of the two EPS and guar gum.

Samples	Molecular weights				Monosaccharide ratio (%)		Composition (%)			Intrinsic viscosity (dL g^{-1})
	Mn/(10^4 g/mol)	*Mw*/(10^4 g/mol)	*Mz*/(10^4 g/mol)	*Mw*/*Mn*	Mannose	Glucose	Carbohydrate	Protein	Water	
EPS 1	17.6	23.29	31.59	1.32	96.9	3.1	93.33	ND	6.67	35.6
EPS 2	4.52	9.81	2.33	2.17	65.3	34.7	92.19	1.9	5.91	27.8
Guar gum	–	–	–	–	–	–	–	–	–	11.9

ND, not detected; –, not determined in this study; *M*w, weight-average molecular weight; *M*n, number-average molecular weight; *M*z, relative molecular mass; *M*w/*M*n, polydispersity index.

containing mannose and glucose in a relative molar proportion of 1.0 and 0.7, respectively. EPS2 is a sulfate homopolysaccharide containing mannose as the major component molar proportion of 1.0 and 0.7, respectively.

Protein was not detected in EPS1 and only 1.9% of protein was present in EPS2. The low quantity of protein in the EPSs indicated that our extraction and purification procedures adopted in this study were quite effective to yield high quality EPSs. In contrast, higher protein content in EPSs has been reported in some studies. In one study, the EPSs purified from *Bifidobacterium animalis subsp. lactis* IPLA-R1 had a protein content of 3.9% (Leivers et al., 2011). Furthermore, 4.2% protein content was reported for the EPSs isolated from *B. animalis* RH (Xu et al., 2011). In addition, higher protein content has been reported with some other bacterial species; including 8% of protein content for the EPS of *Bacillus sp.* 450 (Kumar Ganesh et al., 2004) and 10% of protein content for the EPS of *Pseudomonas oleovorans* (Freitas et al., 2009). These differences may be due to the different techniques used in the isolation and purification of EPSs. Interestingly, our lyophilised EPS1 and EPS2 were found to contain around 6.67% and 5.91% moisture, respectively, although they were freeze dried. This likely reflects trapped water being present in lyophilised EPSs.

3.2. Intrinsic viscosity

The intrinsic viscosity (η) is a measure of the hydrodynamic volume occupied by a given polymer in a given solvent (Freitas et al., 2011). In addition, it is a measurement of a polymer's contribution to the viscosity of a solution. The intrinsic viscosity of EPS1 (35.6 dL g^{-1}) was higher than that of EPS2 (27.8 dL g^{-1}) and approximately three times higher than that of guar gum (11.9 dL g^{-1}) (Table 1). The low intrinsic viscosity of EPS2 suggests that it has a poor thickening ability and higher concentration should be used if high viscosity is desired. On the other hand, due to the longer chain length and higher molar mass, EPS1 has higher thickening ability than EPS2. In addition, the chain rigidity of the polymer can affect the intrinsic viscosity (Freitas et al., 2011). There is no reliable information regarding the intrinsic viscosity of EPSs produced by strains of *Bacillus*. However, information on the intrinsic viscosity of EPSs from other bacterial strains such as *Lactococcus lactis subsp. cremoris* and *Bifidobacterium longum subsp. infantis* has been reported. The intrinsic viscosity of EPSs of *L. lactis subsp. cremoris* was 19.6 dL g^{-1} (Yang, Huttunen, Staaf, Widmalm, & Heikki, 1999), *L. lactis subsp.cremoris* B40 (32.0 dL g^{-1}) (Tuinier, Zoon, Stuart, Fleer, & de Kruif, 1999), *B. longum subsp. infantis* CCUG 52486 (3.2 dL g^{-1}) and *Bifidobacterium infant*is NCIMB 702205 (47.4 dL g^{-1}) (Prasanna et al, 2012)

3.3. Rheological properties of purified EPSs solution

As shown in Fig. 1, the two EPSs clearly showed a pseudoplastic or shear thinning property in aqueous solutions. The higher viscosities and the prominent shear thinning properties were observed for EPS1. Similarly, bioemulsifer of *Halomonas eurihalina* strain V2-7 exhibited pseudoplastic performance and viscosity decreased with increasing sheer rate (Martinez-Checa, Toledo, Mabrouki, Quesada, & Calvo, 2007). This property is important for various processes involved in food processing, such as mixing, pouring and pumping where different operative shear rates are applied.

In addition, a relationship was observed between the molecular weight of the polysaccharide and the viscosity in which the higher molecular weight of the polymer leads to higher viscosity. Therefore, a higher viscosity was observed with EPS1 (Mw 2.33×10^5 g mol^{-1}) than EPS2 (Mw 9.81×10^4 g mol^{-1}). These results are in agreement with the observations of Muralidharan and Jayachandran (2003) who showed that the viscosity of the EPSs solution is highly dependent on the average molecular weight.

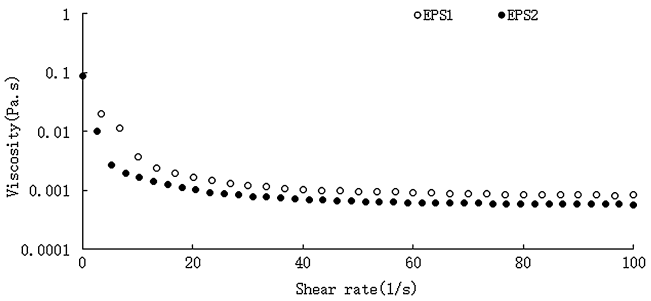

Fig. 1. Viscosity as a function of shear rate of aqueous solution of the two EPSs at concentrations of 1%(w/v).

3.4. Scanning electron microscopic analysis of EPSs

Scanning electron microscopy is considered to be a powerful tool to study the surface morphology of macromolecules and could be used to elucidate their physical properties (Wang et al., 2010). As shown in Fig. 2, a three dimensional spider mesh structure composed of dense rope with homogeneous hexagonal particles was observed in EPS1. Whereas the EPS2 had a porous sponge structure with uniform cylindrical particles (Fig. 2). At higher magnification (Fig. 2c and f), additional details of the microstructure of the two EPSs were visible. Both EPSs had smoother uniform hexagonal and cylindrical crystal particles rather than a sheet-like structure or filaments (Prasanna et al., 2012; Majumder & Goyal, 2009; Kumar Ganesh et al., 2004). There are no available SEM micrographs of EPSs from bacteria strains to compare with our results. In addition, the highly branched web nature of the EPS1 could contribute to its viscosity solution which was reflected in our viscosity assay. Moreover, the porous or web like structure of the EPSs could result in a higher water holding capacity, which is a desirable characteristic for a texturing agent in the food industry.

3.5. Thermostability

Thermal stability of EPSs is an important characteristic for its commercial utilization. This study selected particle size analyzer combined with differential scanning calorimetric to analyse the thermostability of EPSs.

3.5.1. The changes of particle size distribution before and after heating

Laser light scattering particle size analyzer was used to analyze the granularity of macromolecular diameter size. This study adopts particle size analyzer to determine the solution behavior of EPSs. The particle size distribution changes of EPSs aqueous solution (0.5% w/v) before and after heating were shown in Fig. 3. EPS1 aqueous solution changed slightly after heating as both samples showed one peak. The effective diameter slightly reduced from 324.8 nm to 305.5 nm, and polydispersity slightly reduced from 0.335 to 0.301 after boiling for 30 min. The particle size distribution of EPS2 changed significantly after heating, EPS2 aqueous solution showed a singe peak curve before heating and two peaks after boiling 30 min. Particle size increased from 288.45 nm to 519.0, polydispersity reduced from 0.363 to 0.246. This phenomenon suggests that EPS2 after heating can produce irreversible aggregation. These results also indicate that EPS1 particle size is larger than in the solution EPS2, which is consistent with the results of molecular mass. Collectively, EPS1 had a better heat quality and could be a good candidate for biotechnological and industrial applications.

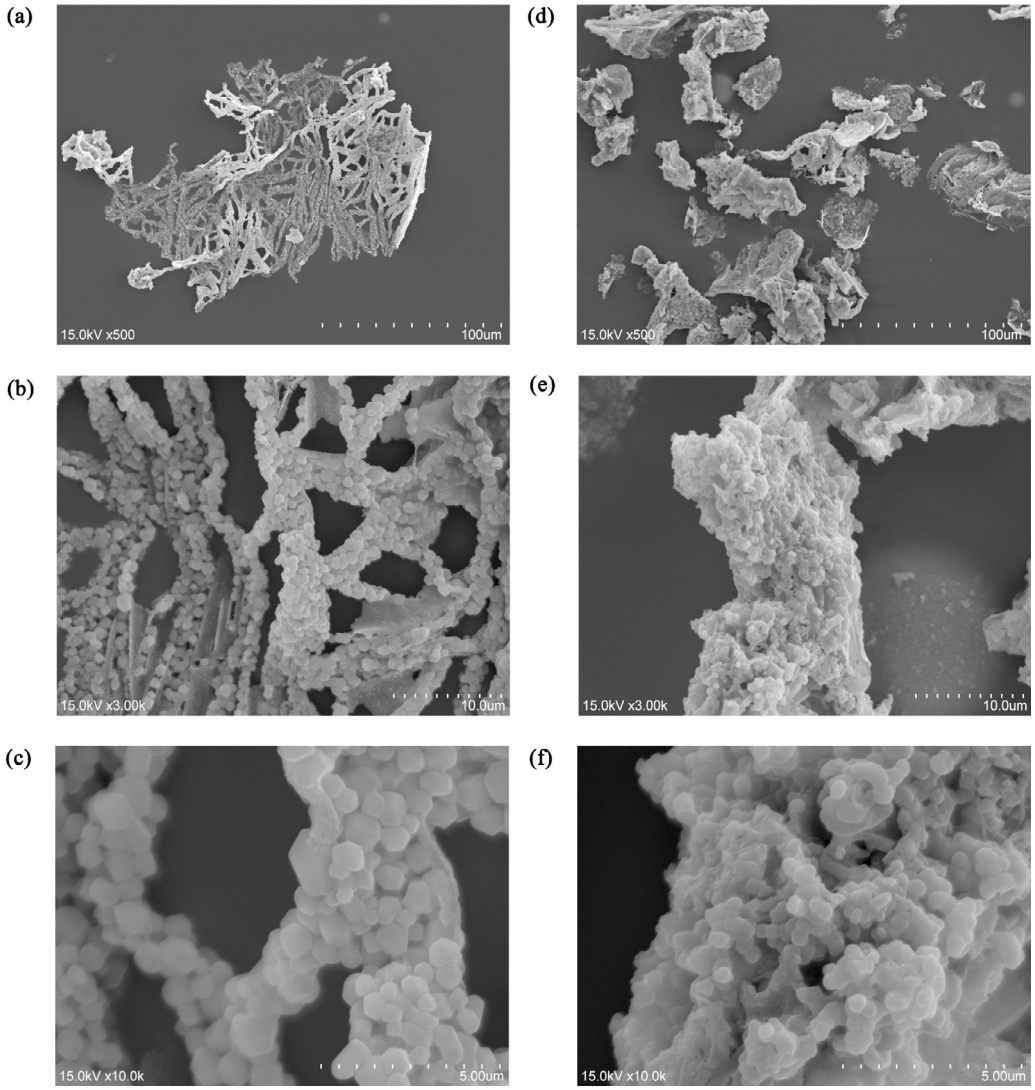

Fig. 2. Scanning electron micrograph showing the microstructure and surface morphology of purified EPS1 (a: 500×; b: 3000×; c: 10,000×) and EPS2 (d: 500×; e: 3000×; f: 10,000×).

3.5.2. *Differential scanning calorimetric (DSC) analyses*

The DSC thermogram (Fig. 4) showed exothermic peak of EPS1 with crystallization temperature (*T*c) of 123.79 °C (onset temperature 109.83 °C) accompanied with 37.88 J g^{-1} latent energy and the melting peaks were found at 224.09 °C(onset temperature 189.32 °C) with 80.43 J g^{-1} latent energy for Tm_1 and 301.09 °C(onset temperature 226.06 °C) with latent energy 29.80 J g^{-1} for Tm_2. The DSC thermogram showed exothermic peak of EPS2 with crystallization temperature (*T*c) of 125.82 °C (onset temperature 114.72 °C) accompanied with 48.49 J g^{-1} latent energy. The melting peaks were found at 295.21 °C (onset temperature 263.01 °C) with 69.87 J g^{-1} latent energy for *T*m. The melting peaks(*T*m) of guar gum were detected at 253 °C, 296 °C and 317 °C (Mudgil, Barak, & Khatkar, 2012). There is no reported literature about the calorimetric analyses of the EPSs produced by *Bacillus* species. However, similar observations were reported with biosurfactant produced by *Cronobacter sakazakii*, the biosurfactant with *T*c (117.11 °C), Tm_1 (178.11) and Tm_2 (242.77) (Rakeshkumar, Kalpana,

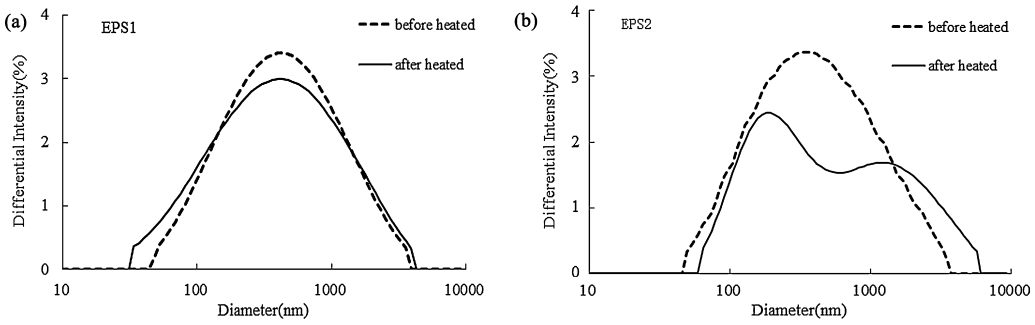

Fig. 3. Paricle size distribution before and after heated of EPS1(A) and EPS2(B).

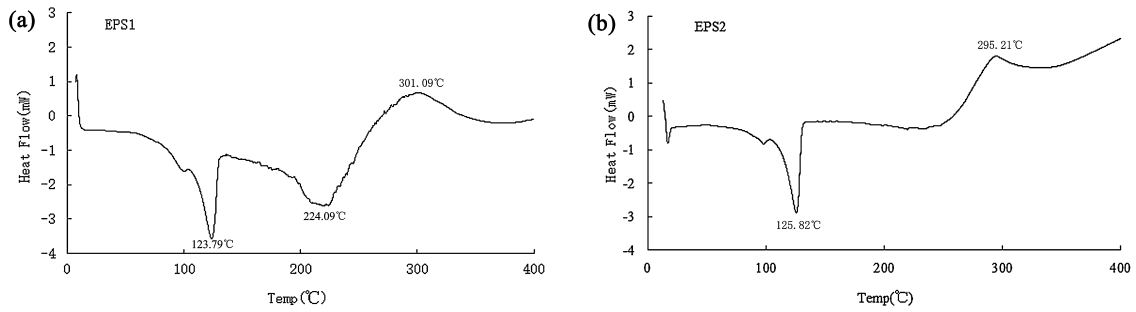

Fig. 4. DSC thermograms of two purified EPS obtained from *B.amyloliquefaciens* LPL061(A: EPS1; B: EPS2).

Table 2
Emulsification acivity of EPS1, EPS2 and guar gum (1 mg mL^{-1}) with different edible oils.

Oil	Type of polysaccharide								
	Guar gum			EPS1			EPS2		
	E_{24}	E_{168}	E_{360}	E_{24}	E_{168}	E_{360}	E_{24}	E_{168}	E_{360}
Sunflower seed oil	65.89 ± 2.47^{a}	61.32 ± 0.79^{a}	60.29 ± 0.77^{a}	68.26 ± 1.16^{a}	64.06 ± 1.37^{a}	63.61 ± 2.11^{a}	69.67 ± 1.15^{a}	64.87 ± 1.56^{a}	62.98 ± 2.18^{a}
Olive oil	58.61 ± 1.38^{b}	53.85 ± 1.15^{b}	53.42 ± 0.84^{b}	58.58 ± 0.74^{c}	53.28 ± 2.05^{b}	52.71 ± 1.34^{b}	59.54 ± 0.93^{c}	52.25 ± 0.73^{b}	51.89 ± 1.03^{b}
Peanut oil	60.36 ± 1.45^{b}	47.51 ± 0.56^{c}	46.50 ± 0.98^{c}	60.99 ± 0.82^{b}	55.69 ± 1.48^{b}	53.99 ± 1.27^{b}	65.78 ± 0.74^{b}	46.53 ± 0.63^{c}	46.03 ± 0.51^{c}
Rice oil	58.55 ± 1.56^{b}	55.55 ± 2.56^{b}	54.21 ± 1.23^{b}	59.91 ± 1.05^{c}	54.26 ± 2.55^{b}	54.26 ± 2.55^{b}	58.63 ± 1.43^{c}	56.71 ± 1.39^{b}	54.16 ± 2.09^{b}

Mean values (±standard deviation) within the same column not sharing a common superscript differ significantly ($p < 0.05$). E_{24}, emulsification index after 24 h; E_{168}, emulsification index after 168 h; E_{360}, emulsification index after 360 h.

Avinash, & Bhavanath, 2012). Our two EPSs were shown to have higher melting peaks, therefore have higher thermostability.

3.6. Emulsifying activity

3.6.1. Emulsifying stability of EPSs from LPL061with different oils

Emulsions prepared with the two EPSs, guar gum at a concentration of 1 mg mL^{-1} using different oils were compared, and the results are given in Table 2. Both EPSs showed higher emulsification activity with sunflower seed oil, olive oil, peanut oil and rice oil compared to guar gum. There is no reported literature about the emulsification activity of the EPSs produced by *B. amyloliquefaciens*. However, similar observations were reported with biopolymers produced by other bacterial species. More specifically, an emulsion prepared using hexadecane and the EPS of *Streptococcus phocae* PI80 was shown to have a higher emulsification activity (81.8) than guar gum (68.8) at 0.5 mg mL^{-1} concentration (Kanmani et al., 2011). In another study, the EPS of *Lactobacillus kefiranofaciens* ZW3 was shown to have a higher emulsifying activity (88.0) with hexadecane compared to guar gum (37.4) at similar concentrations (Wang et al., 2008).

The two EPSs of *B. amyloliquefaciens* LPL061 resulted in significantly higher emulsification stability with sunflower seed oil compared to the other edible oils. Furthermore, these emulsions prepared with sunflower seed oil and EPSs were stable for 7 days or 15 days at 25 °C. Similarly, higher emulsification stability with sunflower seed oil by some other bacterial EPSs has been reported. More specifically, it was reported that the EPS of *Bacillus coagulans* RK-02 showed a significantly higher emulsifying activity with sunflower seed oil (70) than mustard oil (62), soybean oil (62), castor oil (50) and rice oil (41) (Kodali, Das, & Sen, 2009). According to Freitas et al. (2009), emulsification forming and stabilizing capacity of EPSs is specific for certain hydrophobic compounds. This may be the reason for the differences observed in this study.

3.6.2. Effect of EPSs concentration on emulsion stability

In order to evaluate the possible effects of the EPSs concentration on the emulsion stability, a number of studies were carried out using emulsions prepared with different concentrations of EPSs and sunflower seed oil, and the emulsification indexes (E_{168}) were determined. Significant ($p < 0.05$) differences in the emulsification index were observed for the EPSs when the emulsion was prepared with EPSs concentrations ranging from 0 to 1.5 mg mL^{-1} (Fig. 5). However, no significant differences ($p > 0.05$) in the emulsification index were observed when the concentration of EPSs ranged from 1 to 1.5 mg mL^{-1}, although there was a small increase of the emulsion activity at higher concentrations. Therefore, 1 mg mL^{-1} EPSs is the optimum concentration since there is no significant improvement of the emulsification activity at higher concentrations. Similar results have been obtained with the EPSs of other bacterial species. Ashtaputre and Shah (1995) reported a similar trend in the emulsification index of the emulsions prepared with different concentrations of EPSs (0.25–3 mg mL^{-1}) derived from *Sphingomonas paucimobilis* and xylene; they concluded that 1 mg mL^{-1} was the best concentration for the emulsion. In another study, it was reported that 1 mg mL^{-1} of EPSs of *Enterobacter cloaceae* could effectively produce an emulsion with ground nut oil (Iyer, Mody, & Jha, 2006).

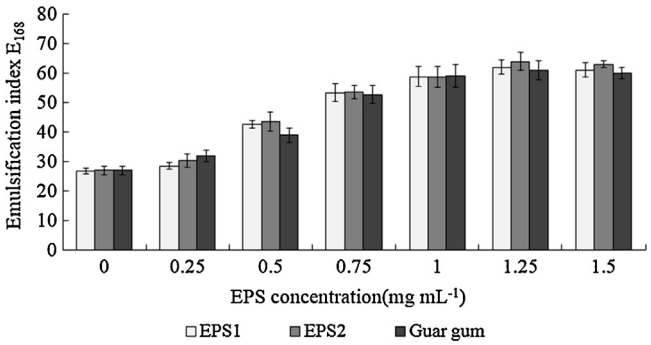

Fig. 5. Effect of different concentrations of EPS1, EPS2 and guar gum on the emulsification index of the emulsions prepared with sunflower seed oil. Vertical lines represent standard deviations. E_{168}: emulsification index after 168 h.

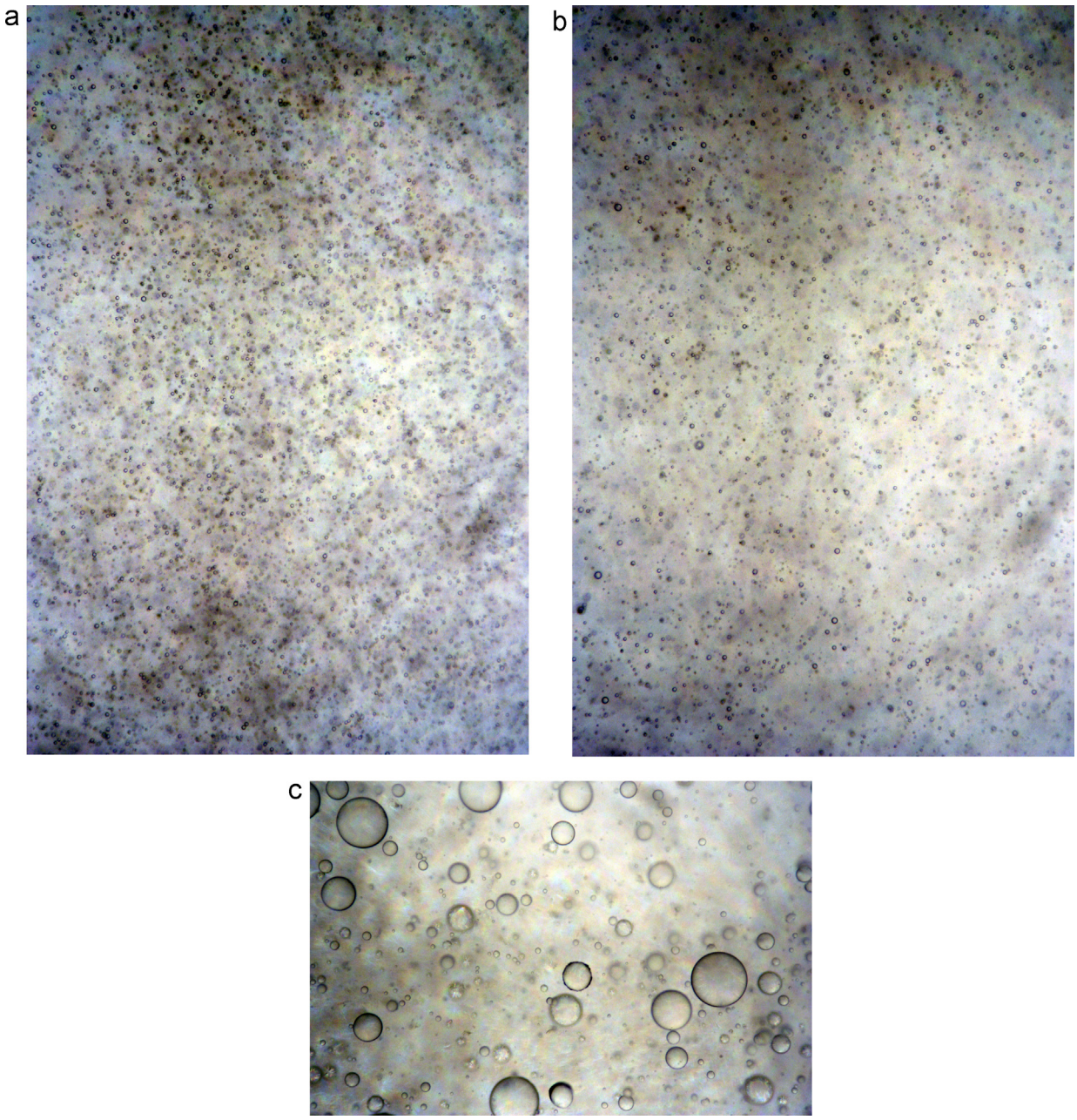

Fig. 6. Photomicrographs of emulsions prepared with 1%(w/v) of (a) EPS1, (b) EPS2 and (c) guar gum(100 ×)with sunflower seed oil.

3.6.3. Light micrographs and particle size distribution of emulsions

The emulsion droplet size is considered to be a very important parameter that determines the physical stability, such as flocculation and creaming rate of different emulsions (Kim, Morr, & Schenz, 1996). The emulsion prepared with the EPS1 and EPS2 were significantly different from that with guar gum (Fig. 6). Smaller, more densely packed and evenly distributed droplets were observed in the emulsion prepared with EPS1 and EPS2. Furthermore, large size droplets and flocculation of some droplets were observed in the emulsions prepared with guar gum. The emulsion prepared with the EPS1 and EPS2 had significantly smaller droplet size, where more than 80% of particles were smaller than 7 μm and the emulsion droplets ranged from 1 to 15 μm, whereas guar gum ranged from 1 to 58 μm. There is no recorded data of the emulsions prepared with the EPSs of *Bacillus* species to compare with these results. However, a droplet range of 10–40 μm was reported for an emulsion prepared with extracellular emulsifiers from *Lactobacillus pentosus* (Portilla-Rivera, Torrado, Dominguez, & Moldes, 2010). In addition, Ashtaputre and Shah (1995) reported that more than 80% of the droplets of an emulsion prepared with 1% of EPS of *Sphingomonas paucimobilis* were less than 5 μm. Furthermore, it is reported that smaller droplets can result in more stable emulsions (Horozov, Binks, & Gottschalk-Gaudig, 2007). Therefore, these results reveal that the two EPSs produced by *B. amyloliquefaciens* LPL061 have great potential to be used as emulsifiers in the food industry.

4. Conclusions

B. amyloliquefaciens produced two different EPSs. The EPS1 had a higher intrinsic viscosity value compared to EPS2 or guar gum. Both polymers showed non-Newtonian pseudoplastic behaviour. The SEM images revealed that EPS1 had a three dimensional spider

mesh structure composed of dense rope with homogeneous hexagonal particles, whereas the EPS2 had a porous sponge structure with uniform cylindrical particles. The two EPSs of *B. amyloliquefaciens* stabilized the emulsions more effectively with different edible oils, compared to guar gum. Stable emulsions were produced by both EPSs at an optimum concentration of 1 mg mL^{-1} with sunflower seed oil. Between these two EPSs, the most promising one from an application point of view in the food industry is EPS1, as it had a high intrinsic viscosity and apparent viscosity in aqueous solution, and a dense entangled mesh structure with higher emulsification activity, despite the fact that the EPSs had a slightly higher emulsification activity and smaller emulsion droplets.

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