

Production and Partial Characterization of a Glycoprotein Bioemulsifier Produced by *Lactobacillus plantarum* subsp. *plantarum* PTCC 1896

N. Bakhshi¹, M. Sheikh-Zeinoddin¹, and S. Soleimanian-Zad^{1, 2*}

ABSTRACT

Given the growing interest in the production of new and low cost bioemulsifiers, the rice and wheat bran and straw were investigated in this study for the production of bioemulsifier by *Lactobacillus plantarum* subsp. *plantarum* PTCC 1896 (probiotic). The strain produced bioemulsifier only in the rice bran hydrolysate medium. The bioemulsifier amount reached around 0.7 g L⁻¹ for 72 hours of fermentation. The new biomolecule was extracted, purified, and its structural and thermal properties were evaluated. The functional groups and the structure of the molecule were revealed by GPC, FT-IR, ¹HNMR and ¹³CNMR techniques. The bioemulsifier was a water soluble extracellular high molecular weight (>10⁷ Da) α -glucan (81.74%) bound with protein (18.18%). Thermal behavior was studied using DSC and TG analysis. Thermal analysis showed the bioemulsifier broke down above 211.74°C, and the melting point was 182.0°C with the enthalpy value of 101.7 J g⁻¹. These results might provide incentives for the industrial production of the biodegradable and safe bioemulsifier introduced in this study, which seems to offer potential applications in the food and medical industries.

Keywords: Probiotic, Rice bran hydrolysate, Structural properties, Thermal analysis.

INTRODUCTION

Microbial Surface Active Compounds (MSACs) are amphiphilic compounds produced by different microorganisms which either adhere to cell surface or excrete extracellularly (Beltrani *et al.*, 2015). MSACs can be divided into the following two main classes: low-molecular-weight compounds called biosurfactants, such as lipopeptides, glycolipids and glycopeptides, and high-molecular-weight compounds known as bioemulsifiers, which are polymers of proteins, polysaccharides and lipid, such as glycoprotein, lipopolysaccharides and lipoproteins. The major criterion used for choosing an

effective biosurfactant is its ability to reduce surface tension. Cooper (1986) considered a culture as promising if it could reduce the surface tension of a liquid medium to 40 mN m⁻¹ or less. Willumsen and Karlson (1996) provided a similar definition: a good biosurfactant producer is defined as the one being able to reduce the surface tension of the growth medium by ≥ 20 mN m⁻¹, as compared with distilled water. Bioemulsifiers are more effective in forming and stabilizing water in oil or oil in water emulsions, but do not necessarily decrease surface or interfacial tension (Portilla-Rivera *et al.*, 2010; Beltrani *et al.*, 2015). Usually, different microbial genera produce structurally different MSACs, whereas

¹ Department of Food Science and Technology, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, Islamic Republic of Iran.

² Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, 84156-83111, Islamic Republic of Iran.

* Corresponding author; e-mail: soleiman@cc.iut.ac.ir



strains belonging to the same genera produce structurally similar MSACs. However, small differences in the molecular structures of MSACs can have major impact on their functions and potential industrial applications (Saimmai *et al.*, 2012).

MSACs are preferred to the synthetic ones due to environmental compatibility, biodegradability, lower toxicity, higher selectivity, and effectiveness at a wide range of temperature, pH, and salinity levels (Jain *et al.*, 2013). Due to their diverse functional properties including wetting, emulsification, foaming, antimicrobial and anti-adhesive activities, they find wide applications in many industrial areas (Markande *et al.*, 2013; Campos *et al.*, 2014).

MSACs obtained from microorganisms like lactobacilli, which are Generally Recognized As Safe (GRAS), offer a great promise for medical and food applications. In recent years, production of MSACs by lactobacilli has attracted much attention due to their antimicrobial and anti-adhesive activities (Gudina *et al.*, 2010).

In spite of numerous advantages of MSACs over synthetic chemical surfactants, their large scale and economical production is strongly associated with cost-effective bioprocesses and the use of renewable and low-priced raw materials. While it is advantageous, this process faces its own limitations when it comes to selecting proper wastes for use as culture media with the right balance of nutrients to allow cell growth and MSAC accumulation by lactobacilli. Moreover, the properties of the final product largely depend on the composition of the culture media and/or conditions. Therefore, designing a low-cost and at the same time efficient culture medium should be a priority for bioemulsifier production by lactobacilli (Portilla-Rivera *et al.*, 2010; Thavasi *et al.*, 2011).

Lignocellulosic materials represent an abundant renewable feedstock. Considering the large effect of the substrate cost on the economic feasibility of microbial production, efficient application of

lignocellulosic by-products is highly recommended (Li *et al.*, 2010; Portilla-Rivera *et al.*, 2010). Wheat and rice comprise a major portion of human food not only on a global scale, but more specifically in Asia, where they are widely grown and used (FAOSTATE, 2012). This large amount of wheat and rice could be beneficially exploited to produce significant amounts of by-products such as bran and straw as the potential sources of nutrients for microorganisms employed in generating value-added products (Li *et al.*, 2010).

The main objective of the present work was to investigate the efficacy of using low-cost agricultural by-products as sole nutrient sources in producing a biodegradable and safe bioemulsifier for application in food and pharmaceutical industries. For this purpose, rice and wheat bran and straw were screened as carbon sources for extracellular bioemulsifier production by *Lactobacillus plantarum* subsp. *plantarum* PTCC 1896. In the second stage, such properties as bioemulsifier amount, structure and thermal properties of bioemulsifier produced on the selected carbon source were investigated.

MATERIALS AND MEETHODS

Microorganism

Lactobacillus plantarum subsp. *plantarum* PTCC 1896 (*Lp* PTCC 1896) used in this study was previously isolated from Iranian infants' fecal flora in our laboratory (Mirlohi *et al.*, 2009). The strain has reportedly exhibited probiotic activity in MRS (de Man Rogosa and Sharpe, Merck, Germany) medium (Mirlohi *et al.*, 2009) and has been successfully used as a biocontrol agent in suppressing gray mold rot (*Botrytis* infections) in strawberries (Zamani-Zadeh *et al.*, 2013). The isolated strain was kept frozen at -80°C in MRS broth containing 30% (v v^{-1}) glycerol solution. For further experiments, the microbial culture was activated twice in MRS broth at 37°C for 18 hours under microaerobic conditions (10%

CO₂) in a CO₂ incubator (Binder TM, Germany).

Screening Agricultural Byproducts for Bioemulsifier Production

Preparation of Media

Agricultural byproducts, namely, Rice Bran (RB), Rice Straw (RS), Wheat Bran (WB), and Wheat Straw (WS) were used as the feedstock for fermentative bioemulsifier production. They were obtained locally (Isfahan, Iran) and milled using a ball-mill hammer and then they were passed over a 60-mesh sieve. Then, 100 g of each of the RB and WB powders and 50 g of each of the RS and WS powders were transferred into a 2-L flask containing 400 mL of 1.5% H₂SO₄. Hydrolysis process was carried out at 80°C for 20 hours at 220 rpm (IKA®KS4000 I control, Germany) (Li *et al.*, 2010). Hydrolysates produced from the agricultural byproducts were neutralized with powdered CaCO₃ to a final pH of 6.0 and the CaSO₄ (neutralizing product) was separated from the hydrolysates supernatant by centrifugation (7,500×g, 5 minutes at 25°C) (Sigma k-16, Germany). The clarified liquors were sterilized at 121°C for 15 minutes to be used as the fermentation media (Portilla-Rivera *et al.*, 2010). Total sugar, reducing sugar, and protein contents of each hydrolysate were determined using the methods suggested by Dubois *et al.* (1956), Miller (1959) and Bradford (1976), respectively.

Fermentation Process

Once the substrates had been prepared, fermentation was performed in 250 mL Erlenmeyer flasks containing 100 mL of each hydrolysate diluted with distilled water up to 20 g L⁻¹ of the reducing sugar. Each medium was inoculated with 1 mL of the 18 h inoculum of the strain grown in MRS broth (a starting cell count of 7.31±0.07 Log

Colony Forming Unit (CFU) mL⁻¹ in all flasks). The media were incubated in a shaking incubator (Jaltajhiz, Iran) at 37°C and at 120 rpm for 72 hours. The cells were precipitated by centrifugation (10,000×g, 5 minutes at 10°C) (Sigma k-16, Germany) and emulsification activities of cell-free culture supernatants were determined according to the procedure described by Paraszkiwicz *et al.* (2002). Briefly, canola oil was added to the cell-free culture supernatant (1:1) and vortexed for 2 min. Emulsification Index (EI) values were determined after 24 and 72 hours of Emulsion formation (EI₂₄ and EI₇₂, respectively) using the following formula:

$$EI = (HEL/HL) \times 100$$

Where, *HEL* is the height of the emulsified layer, and *HL* is the height of the total liquid. Emulsion Stability (ES, %) corresponds to the ratio of EI₇₂ to EI₂₄.

Time-course study of bioemulsifier production in rice bran hydrolysate medium was performed by determination of the bacterial cell dry weight and EI values (EI₂₄ and EI₇₂) of the cell-free culture supernatant at different time intervals. All fermentation experiments were carried out in triplicate.

Extraction and Purification of Bioemulsifier

The chloroform-methanol (2:1) was added to the cell-free culture supernatant and stored overnight at 4°C. The bioemulsifier was subsequently separated by centrifugation (7,500×g, 5 minutes, and 10°C) (Sigma k-16, Germany). The crude bioemulsifier was dialyzed against distilled water at 4°C in a Cellu-Sep® membrane (molecular weight cut-off 6,000–8,000 Da, Membrane Filtration Products, Seguin, USA). Then, the dialyzed solution of bioemulsifier was purified by ultrafiltration using Amicon Ultra-15 Centrifugal Filter Unit with Ultra-100 membrane (Merck Millipore, USA), freeze-dried (Christ alpha-1-4, Germany), weighted and stored at -20°C.



Estimation of Weight Average Molecular Weight (Mw)

The Mw of the freeze-dried bioemulsifier was determined by a Gel Permeation Chromatography (GPC) system (Shao *et al.*, 2014). A GPC system equipped with a PL Aquagel OH mixed-H 8 μm column (Agilent 1100 series, Santa Clara, USA) and a Refractive Index (RI) detector was used. Sample (1 mg mL⁻¹) was eluted with water with a flow rate of 1 mL min⁻¹ at 30°C and acquisition interval of 0.43 seconds.

Chemical Composition

Purified bioemulsifier was subjected to chemical analysis for estimation of total sugars (Dubois *et al.*, 1956) and protein (Bradford, 1976).

Proteinase K Treatment

To investigate the role of proteinous fraction in emulsification activity, supernatant was treated with Proteinase K. The cell-free culture supernatant was treated with Proteinase K (1 mg mL⁻¹; 30 U mL⁻¹, Roche TM, Germany) at 37°C for 2 hours and the emulsification activity was studied as described above (Markande *et al.*, 2013).

Monosaccharide Characterization

The monosaccharide composition of the freeze-dried bioemulsifier was estimated by hydrolyzing in 4 M HCl at 121°C for 4 hours followed by using NaOH to adjust the pH to neutral. The reducing sugar content was then estimated by High Performance Liquid Chromatography (HPLC) system (Navon-Venezia *et al.*, 1995). Hydrolysis products and standard sugars (glucose, xylose, galactose, arabinose and mannose) were applied to a HPLC (Jasco International Co., Tokyo, Japan) equipped with a Aminex HPX-87H column (300×7.8 mm, Bio-Rad, Richmond, CA,

USA). The injected quantity was 20 μL . The column was eluted with H₂SO₄ (0.005M) at a flow rate of 0.6 mL min⁻¹ and detected using a Refractive Index (RI) detector.

Fourier Transform Infrared (FT-IR) Analysis

The freeze-dried bioemulsifier was mixed with potassium bromide (1:100 w w⁻¹). The mixtures were then pressed to obtain translucent pellets. The infrared absorption spectrum of the bioemulsifier was recorded using a FT-IR spectrophotometer in the frequency range of 4,000–400 cm⁻¹ (Jasco International Co., Tokyo, Japan) to identify the functional groups.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The spectra of ¹H and ¹³C NMR were obtained to identify the freeze-dried bioemulsifier chemical structures (Shao *et al.*, 2014). The sample was recorded as solution in Deuterium Oxide (D₂O) at 60°C on an Ultrashield 400 MHz spectrometer (Bruker, Germany).

Differential Scanning Calorimetric (DSC) Analysis

DSC analysis was carried out to investigate the thermal properties of the bioemulsifier. DSC analysis of the bioemulsifier was carried out with a differential scanning calorimeter (Sanaf, Iran). About 10 mg of freeze-dried sample was loaded on a platinum pan and scanning was performed in the range of 30–300°C with a temperature gradient of 6.2 °C min⁻¹ under a nitrogen atmosphere (Miao *et al.*, 2015a).

Thermal Gravimetric (TG) Analysis

TG analysis was carried out to investigate the thermal degradation behavior of the

bioemulsifier. TG analysis of the bioemulsifier was carried out with STA 449F3 Jupiter system (Netzsch, Germany). About 8 mg of freeze-dried sample was loaded on a platinum pan and scanning was performed in the range of 30–500°C with a temperature gradient of 5 °C min⁻¹ under a nitrogen atmosphere. The Derivative ThermoGravimetry (DTG) trace was also obtained by taking the first derivative of the TG curve (Miao *et al.*, 2015a).

RESULTS AND DISCUSSION

Screening Agricultural Byproducts for Bioemulsifier Production

It is known that the functional properties and chemical structures of bioemulsifiers are strongly influenced by both the producing microorganism and certain parameters of the culture medium (Thavasi *et al.*, 2011). In this context, different agricultural byproducts (Table 1) were screened for bioemulsifier production by *Lp* PTCC 1896, based on the emulsification index of the culture supernatant. The established criterion for emulsion-stabilizing capacity is the ability of a bioemulsifier to preserve at least 50% of the original emulsion volume 24 hours after its formation (Beltrani *et al.*, 2015).

Among the hydrolysates used in this study, the rice bran hydrolysate was the only substrate which showed emulsification activity, probably because of its low C:N ratio (1.1:1) (Table 1), which is known to be a critical factor implicated in the production of bioemulsifiers (Toledo *et al.*, 2008).

Growth and Production of Bioemulsifier

The growth and bioemulsifier production of *Lp* PTCC 1896 grown in the rice bran hydrolysate medium are summarized in Figure 1. Bioemulsifier production occurred during an exponential growth phase and it was continued up to 72 hours (Figure 1-b). A good emulsifier is the one producing a stable emulsion; thus, for emulsion forming ability, the two factors introduced included emulsion index at 24 and 72 hours (EI₂₄ and EI₇₂, respectively) and Emulsion Stability (ES= EI₇₂/EI₂₄). The emulsion formation at the beginning of the fermentation was due to the presence of hydrolysate compounds and their emulsion was not stable because the EI₇₂ was 43.37±2.76%. However, during the fermentation, both emulsion index and emulsion stability were high due to the bioemulsifier production. Maximum EI₇₂ and ES observed at the end of fermentation were 64.70±0.71 and 97.73±1.02%, respectively (Figure 1-b). These results indicated that production of bioemulsifier was growth-associated. This phenomenon was common in exopolysaccharide by lactic acid bacteria of the genus *Lactobacillus*, *Leuconostoc* or *Streptococcus*, and the glucansucrase from Glycoside-Hydrolase family 70 was the key enzyme responsible for polymerization of high molecular weight α-glucan (Miao *et al.*, 2015a). A similar production kinetic has been reported for bioemulsifier producing *Acinetobacter radioresistens* KA53 (Navon-Venezia *et al.*, 1995). It is known that the media and culture conditions affect bioemulsifier yields and structures, including fermentation time, pH,

Table 1. Chemical composition of sterilized substrates used in this study.^a

Substrate	Total sugars (g L ⁻¹)	Reducing sugar (g L ⁻¹)	(g	Protein (g L ⁻¹)	C:N ratio
Rice bran hydrolysate	56.45±1.77	41.90±0.98		51.15±0.75	1.1:1
Rice straw hydrolysate	39.66±3.72	27.47±1.86		26.49±1.17	1.5:1
Wheat bran hydrolysate	144.99±1.96	78.90±3.65		59.40±1.60	2.4:1
Wheat straw hydrolysate	41.36±0.82	26.90±4.92		12.69±1.18	3.3:1

^a Data are presented as means±SD (n =3).



temperature, and nutrient media components. The optimization of culture conditions for bioemulsifier from *Lp* PTCC 1896 will be addressed in our future study.

Extraction and Quantification of Bioemulsifier

The use of chloroform-methanol (2:1) resulted in forming a yellowish water soluble bioemulsifier precipitated at the interface, which was separated and dialyzed. Almost 100% of the emulsification activity that was present in the culture supernatant was recovered in the precipitate. The dialyzed solution of the crude bioemulsifier was separated by GPC (Figure 2). One sharp (I) (80.11%) and two small peaks (II and III) (19.89%, supposed as contaminants) were

separated on the column. Thus, for further purification, the dialyzed sample was ultra-filtered (100 kDa) and freeze-dried. The total amount of the purified bioemulsifier extracted was $0.70 \pm 0.05 \text{ g L}^{-1}$, which was within the range described by other researchers (between 0.1 and 3.15 g L^{-1}) (Navon-Venezia *et al.*, 1995; Toledo *et al.*, 2008; Jain *et al.*, 2012; Beltrani *et al.*, 2015). Many studies have reported the production of biosurfactants/bioemulsifiers by lactobacilli under nutrient-rich media. In laboratory studies, yeast extract, peptone, and corn steep liquor, mainly as nitrogen sources, along with minerals, are used to supplement agricultural waste hydrolysates in media. Also, detoxification of acid hydrolysate was performed to further reduce toxic compounds. The supplementation and detoxification steps could lead to higher finished price of microbial products (Moldes *et al.*, 2007; Portilla-Rivera *et al.*, 2010). This study is the first report on the production of bioemulsifier from a *Lactobacillus* strain grown in a waste based medium, without any supplementation and detoxification. Thus, this new carbon source could be considered for the industrial production of bioemulsifier.

Chemical Composition of Bioemulsifier

The bioemulsifier exhibited high M_w exceeding the exclusion limit of the column (10^7 Da), as indicated by early elution at 3.81 minutes of retention time and confirmed by calculating the exclusion limit through the extrapolation of dextran standards retention times (Figure 2). The purified bioemulsifier consisted of $81.74 \pm 3.96\%$ polysaccharide and $18.18 \pm 1.26\%$ protein. No emulsification activity was observed after Proteinase K treatment of the bioemulsifier produced, indicating that the proteinous fraction played a major role in the emulsification activity. The acid hydrolysed bioemulsifier resulted in single sugar; the monomer was identified as glucose, showing that the bioemulsifier was composed of a glucan. Our present

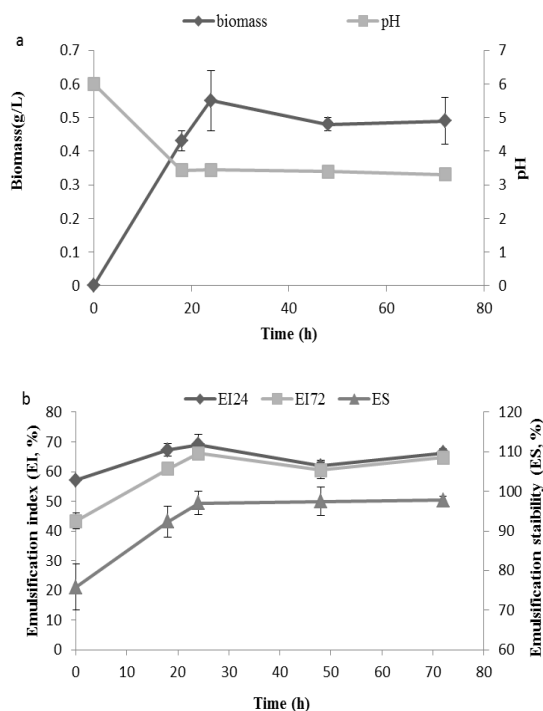


Figure 1. (a) Growth and (b) Bioemulsifier production by *Lp* PTCC 1896 cultivated in rice bran hydrolysate medium at 37°C and 120 rpm for 72 hours. Emulsification Index (EI) values at different time intervals were determined after 24 and 72 hours of emulsion formation (EI₂₄ and EI₇₂, respectively) using cell-free culture supernatant and canola oil. Data are presented as means \pm SD (n= 3).

work is the first report on the production and characterization of the extracellular polysaccharide-protein bioemulsifier from a *Lactobacillus* strain. Alasan, the extracellular polysaccharide and proteins containing emulsifier of *A. radioresistens* KA53, has been studied extensively. It consists of protein (25%) and heteropolysaccharide (75%) containing four major sugars, viz. glucosamine, galactosamine, glucose (minor), and galactose, with the molecular weight of approximately 10^6 Da (Navon-Venezia *et al.*, 1995). Liposan, an extracellular bioemulsifier produced by *Candida lipolytica*, is composed of 83% carbohydrates and 17% proteins (Cirigliano and Carman, 1985). *Curvularia lunata* IM2901 produced a bioemulsifier consisting of a complex of polysaccharide (48%) and protein (25%). It is also identified as a polymer of *D*-glucose (Paraszkievicz *et al.*, 2002). Bioemulsifiers produced from *Bacillus subtilis*, *Alcaligenes faecalis*, *Enterobacter sp.*, *Halomonas sp.*, and *Cronobacter sakazakii* are comprised of carbohydrate, protein, uronic acid and sulfate (Calvo *et al.*, 2002; Toledo *et al.*, 2008; Jain *et al.*, 2012). Proteins present in bioemulsifiers have been proved to play major roles in emulsification with

hydrocarbons and oils, both as enhancers in a polysaccharide-protein complex and as standalone emulsifiers (Navon-Venezia *et al.*, 1995; Yadav *et al.*, 2012; Markande *et al.*, 2013).

FT-IR Characterization

For the primary identification of the main functional groups present in the bioemulsifier, it was submitted to FT-IR analysis (Figure 3). The FT-IR spectrum of bioemulsifier exhibited a variety of typical absorption peaks of polysaccharides and proteins. The obvious absorption band at $3,420.14\text{ cm}^{-1}$ was due to the stretching frequency of the hydroxyl groups (O-H), which was typical of polysaccharides (Shao *et al.*, 2014; Beltrani *et al.*, 2015). The weak band at $2,925.48\text{ cm}^{-1}$ was attributed to C-H stretching, whilst the one at $1,458.89\text{ cm}^{-1}$ was ascribed to the C-H bending vibrations (Shao *et al.*, 2014). The absorption at $1,654.62$ and $1,542.77\text{ cm}^{-1}$ indicated the C=O stretching of Amids I and N-H bending vibration in Amides II, respectively (Dikit *et al.*, 2010). Each particular polysaccharide showed specific bands in the $1,200\text{--}1,000\text{ cm}^{-1}$ region, and the location and intensity of these bands

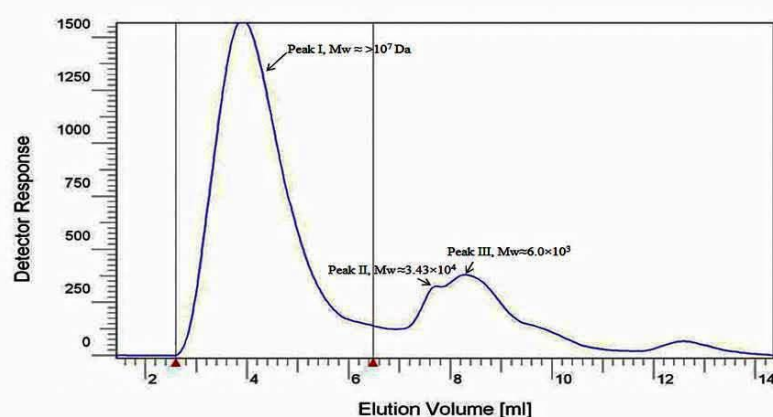


Figure 2. Gel Permeation Chromatography (GPC) analysis of the bioemulsifier synthesised by the strain *Lactobacillus plantarum* A. A GPC system equipped with a PL Aquagel OH mixed-H $8\ \mu\text{m}$ column (Agilent1100 series, Santa Clara, USA) and a Refractive Index (RI) detector was used. Sample (1 mg mL^{-1}) was eluted with water with a flow rate of 1 mL min^{-1} at 30°C and acquisition interval of 0.43 seconds.

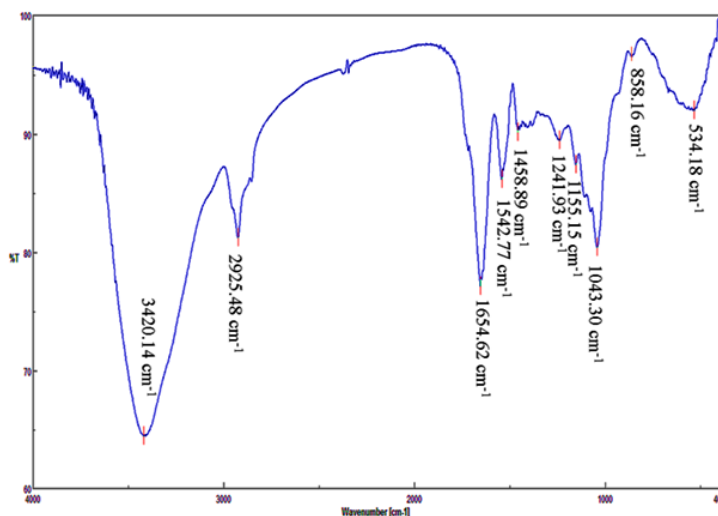


Figure 3. FT-IR spectrum of the bioemulsifier from *Lb* PTCC 1896.

were also specific for each polysaccharide. The intense band at $1,043.30\text{ cm}^{-1}$ was associated with the bioemulsifier polysaccharide fraction. The band at $1,155.15$ and $1,043.30\text{ cm}^{-1}$ corresponded to the C—OH stretching vibrations and the (C—O—C) glycosidic band vibration, thereby showing the pyranose configuration of the sugar residues. Also, the bands at 858.16 cm^{-1} were associated with the stretching of the α -isomeric carbon, which was further confirmed by the results of ^1H NMR characterization (Shao *et al.*, 2014).

NMR Characterizations

Figure 4 shows ^1H NMR and ^{13}C NMR spectra of the bioemulsifier dissolved in D_2O . In the ^1H NMR spectrum (Figure 4-a), chemical shifts of an anomeric region were observed at 5.14–5.55 ppm, corresponding to α -configuration evidenced by the FT-IR spectrum as well (Shao *et al.*, 2014). The chemical shifts at 5.55 and 5.14 ppm were attributed to the anomeric proton of the α -1, 4-linked *D*-glucopyranose and α -1, 6 *D*-glucopyranose, respectively. Moreover, the broad peak of α -1, 4 linkage was split into two overlapping peaks (5.55 and 5.54 ppm) which corresponded to the α -1, 4, 6-linked *D*-glucopyranose residues and non-reducing

terminal residue, respectively. The chemical shifts from 3.59 to 4.33 ppm were assigned to the protons of carbons C2–C6 of the sugar ring (Miao *et al.*, 2015b). The protein groups related to the glucan–protein structure seemed to be present, as indicated by the signal at 1.03–1.51 ppm (Dikit *et al.*, 2010; Jain *et al.*, 2013). These attributions were considered once the groups N—CH₃ and N—H were generally observed at 0.5–3.0 ppm, respectively (Gonzaga *et al.*, 2005).

In the ^{13}C NMR spectrum (Figure 4-b), the chemical shifts at around 99.74 ppm were identified as the C1 of α -1, 4-linked *D*-glucopyranose, α -1, 6-linked *D*-glucopyranose and α -1, 4, 6-linked *D*-glucopyranose, respectively. Whereas the signals at around 77.22 ppm corresponded to the C4 of α -1, 4-linked *D*-glucopyranose and α -1, 4, 6-linked *D*-glucopyranose, and those at 60.75 ppm were attributed to the C-6 of non-reducing terminal residues and α -1, 4-linked *D*-glucopyranose. Some major signals in the non-anomeric region (69.43–73.23 ppm) were attributed to the C2, C3, C4 and C5 substituted glucose residues (Miao *et al.*, 2015b). The presence of additional peaks at 19.89 ppm may suggest the consideration of the presence of glucan–protein structure (Gonzaga *et al.*, 2005). The data suggested that polysaccharide fraction of the

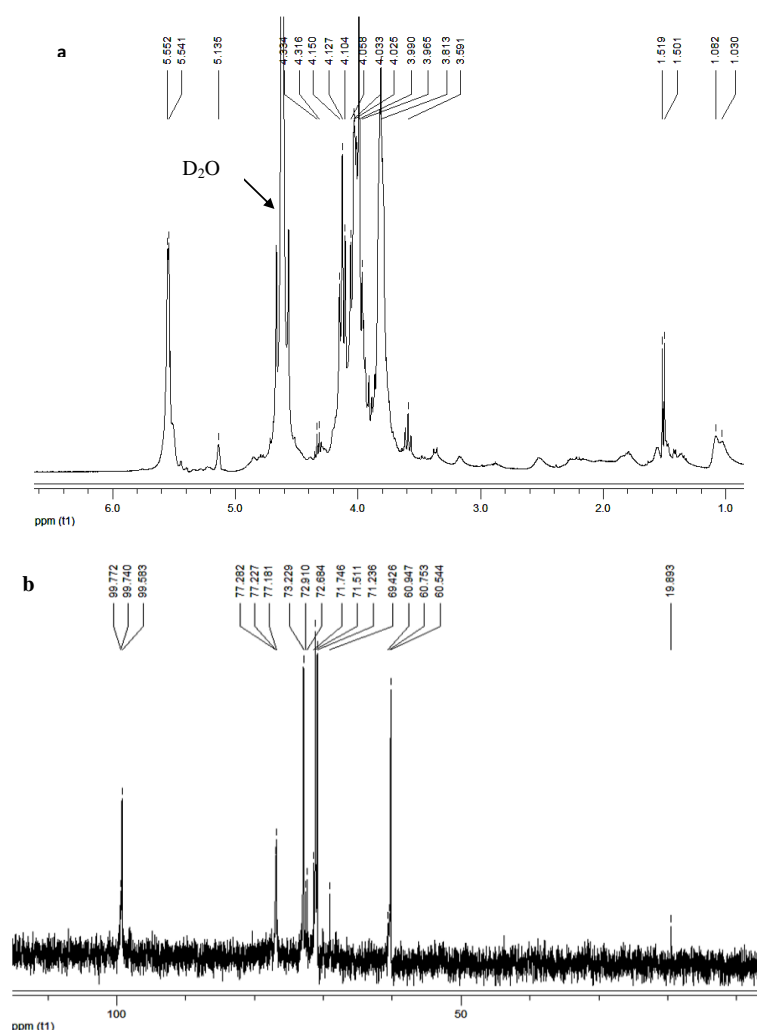


Figure 4. (a) ^1H NMR and (b) ^{13}C NMR spectra of the bioemulsifier from *Lp* PTCC 1896 in Deuterated water (D_2O) at 60°C .

bioemulsifier from *Lp* PTCC 1896 was a glucan that comprised mainly of α -1, 4 bonds, with fewer α -1, 6 linkages and α -1, 4, 6 branching points (Miao *et al.*, 2015b). Similar characteristic spectral peaks of NMR were also observed in the bioemulsifier (polysaccharide-protein complex) obtained from *Cronobacter sakazakii* (Jain *et al.*, 2012).

Thermal Properties (DSC and TG Analysis)

DSC analysis was conducted in order to study the melting point and changes in

enthalpy (H) values of the bioemulsifier (Figure 5-a). The first transition at 167.8°C was associated with the crystallization point, and the second one represented melting (182.0°C) with an enthalpy of transition of 101.7 J g^{-1} . The melting temperature and enthalpy of transition of the bioemulsifier isolated in this study were higher than those of the α -D-glucan from *L. reuteri* SK24.003 (147.7°C and 78.4 J g^{-1}) (Miao *et al.*, 2015a).

Thermal stability of bioemulsifier is an important characteristic determining its commercial utilization. Degradation of bioemulsifier took place by two well differentiated steps as observed in TG

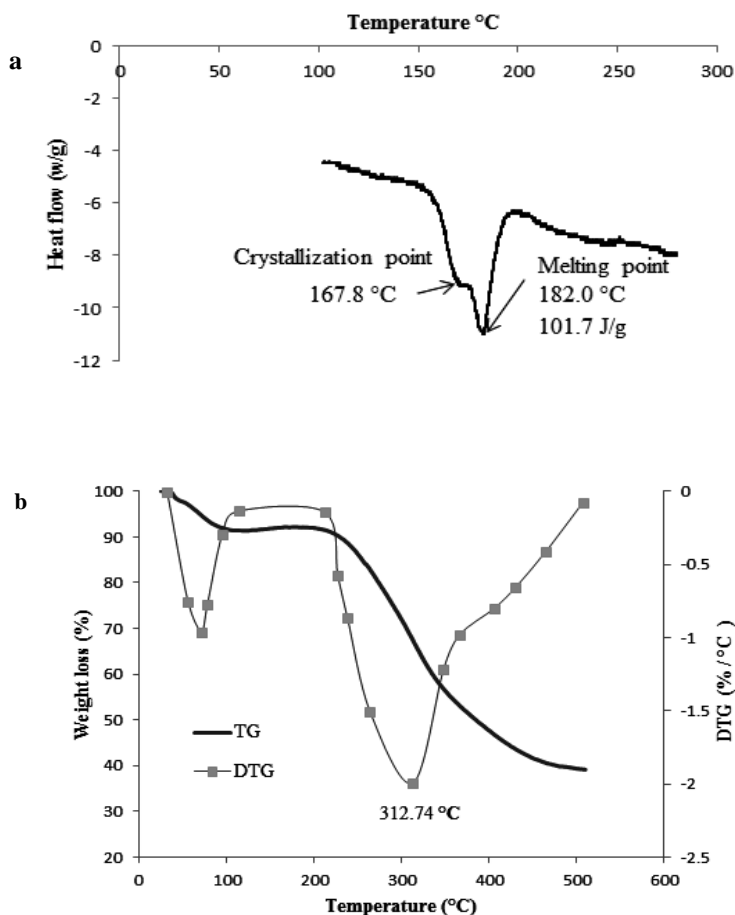


Figure 5. (a) DSC and (b) TG and DTG analysis of the bioemulsifier from *Lp* PTCC 1896. About 8-10 mg of the freeze-dried bioemulsifier was loaded on a platinum pan and scanning was performed under a nitrogen atmosphere. Temperature gradients of 6.2 and 5 °C min⁻¹, respectively, were used for the DSC and TG analysis.

analysis (Figure 5-b). An initial weight loss (9.60 %) was recorded from 30 to 113.74°C due to the loss of water molecules, which was followed by second phase degradation (211.74-500°C). The bioemulsifier was broken down above 211.74°C and the weight was dramatically lost (52.84%) for the second degradation process, with the maximum degradation at 312.74°C, which was due to the depolymerization of exopolysaccharide-protein. A similar degradation pattern was reported for other polysaccharide-protein complexes such as bioemulsifier produced by *Cronobacter sakazakii* and the natural hydrocolloids Arabic gum (Jain *et al.*, 2012; Miao *et al.*,

2015a). Due to the high degradation temperature of the bioemulsifier, it would be safe for use in food processing, where, in most processes, temperature rarely overpasses 150°C.

CONCLUSIONS

Probiotic lactobacilli culture would be a valuable alternative to produce bioemulsifiers for food applications if the lactobacilli could be grown in low-cost and safe culture media. The results of screening agricultural byproducts for bioemulsifier production by probiotic *Lp* PTCC 1896

offered excellent opportunities for the discovery of a new molecule with distinctive properties. The strain was found to produce a water soluble extracellular high molecular weight α -glucan bound with protein. The emulsification activity together with high thermo-stability (high melting point and high degradation temperature) could have promising prospects for food applications. The rice bran is inexpensive and easy to obtain, showing satisfactory results for the production of bioemulsifier. In view of its applications, the process optimization for its production, and the structure–function relationship in food systems should be further investigated.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Isfahan University of Technology for their financial support. The authors declare that no conflict of interest exists.

REFERENCES

1. Beltrani, T., Chiavarini, S., Cicero, D. O., Grimaldi, M., Ruggeri, C., Tamburini, E. and Cremisini, C. 2015. Chemical Characterization and Surface Properties of a New Bioemulsifier Produced by *Pedobacter* sp. strain MCC-Z. *Int. J. Biol. Macromol.*, **72**: 1090-1096.
2. Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.*, **72**: 248-254.
3. Calvo, C., Martínez-Checa, F., Toledo, F., Porcel, J. and Quesada, E. 2002. Characteristics of Bioemulsifiers Synthesized in Crude Oil Media by *Halomonas eurihalina* and Their Effectiveness in the Isolation of Bacteria Able to Grow in the Presence of Hydrocarbons. *Appl. Microbiol. Biotechnol.*, **60**: 347-351.
4. Campos, J. M., Stamford, T. L. M. and Sarubbo, L. A. 2014. Production of a Bioemulsifier with Potential Application in the Food Industry. *Appl. Biochem. Biotechnol.*, **172**: 3234-3252.
5. Cirigliano, M. C. and Carman, G. M. 1985. Purification and Characterization of Liposan, a Bioemulsifier from *Candida lipolytica*. *Appl. Environ. Microbiol.*, **50**: 846-850.
6. Cooper, D. 1986. Biosurfactants. *Microbiol. Sci.*, **3**: 145.
7. Dikit, P., Methacanon, P., Visessanguan, W., H-kittikun, A. and Maneerat, S. 2010. Characterization of an Unexpected Bioemulsifier from Spent Yeast Obtained from Thai Traditional Liquor Distillation. *Int. J. Biol. Macromol.*, **47**: 465-470.
8. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.*, **28**: 350-356.
9. FAOSTAT, 2012. p. <http://faostat.fao.org/site/339/default.aspx>.
10. Gonzaga, M. L. C., Ricardo, N. M. P. S., Heatley, F. and Soares, S. D. A. 2005. Isolation and Characterization of Polysaccharides from *Agaricus blazei* Murill. *Carbohydr. Polym.*, **60**: 43-49.
11. Gudina, E. J., Teixeira, J. A. and Rodrigues, L. R. 2010. Isolation and Functional Characterization of a Biosurfactant Produced by *Lactobacillus paracasei*. *Colloids Surf B Biointerfaces*, **76**: 298-304.
12. Jain, R. M., Mody, K., Joshi, N., Mishra, A. and Jha, B. 2013. Effect of Unconventional Carbon Sources on Biosurfactant Production and Its Application in Bioremediation. *Int. J. Biol. Macromol.*, **62**: 52-58.
13. Jain, R. M., Mody, K., Mishra, A. and Jha, B. 2012. Isolation and Structural Characterization of Biosurfactant Produced by an Alkaliphilic Bacterium *Cronobacter sakazakii* Isolated from Oil Contaminated Wastewater. *Carbohydr. Polym.*, **87**: 2320-2326.
14. Li, Z., Han, L., Ji, Y., Wang, X. and Tan, T. 2010. Fermentative Production of L-Lactic Acid from Hydrolysate of Wheat Bran by *Lactobacillus rhamnosus*. *Biochem. Eng. J.*, **49**: 138-142.
15. Markande, A. R., Acharya, S. R. and Nerurkar, A. S. 2013. Physicochemical Characterization of a Thermostable Glycoprotein Bioemulsifier from



- Solibacillus silvestris* AM1. *Process Biochem.*, **48**: 1800-1808.
16. Miao, M., Ma, Y., Huang, C., Jiang, B., Cui, S. W. and Zhang, T. 2015a. Physicochemical Properties of a Water Soluble Extracellular Homopolysaccharide from *Lactobacillus reuteri* SK24.003. *Carbohydr. Polym.*, **131**, 377-383.
 17. Miao, M., Ma, Y., Jiang, B., Cui, S.W., Wu, S. and Zhang, T. 2015b. Structural Elucidation and *In Vitro* Fermentation of Extracellular α -D-Glucan from *Lactobacillus reuteri* SK24.003. *Bioact. Carbohydr. Diet. Fibre*, **6**: 109-116.
 18. Miller, G. L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.*, **31**: 426-428.
 19. Mirlohi, M., Soleimani-Zad, S., Dokhani, S., Sheikh-Zeinodin, M. and Abghary, A. 2009. Investigation of Acid and Bile Tolerance of Native Lactobacilli Isolated from Fecal Samples and Commercial Probiotics by Growth and Survival Studies. *Iran. J. Biotech.*, **7**: 233-240.
 20. Moldes, A. B., Torrado, A. M., Barral, M. T. and Domínguez, J. M. 2007. Evaluation of Biosurfactant Production from Various Agricultural Residues by *Lactobacillus pentosus*. *J. Agric. Food Chem.*, **55**: 4481-4486.
 21. Navon-Venezia, S., Zosim, Z., Gottlieb, A., Legmann, R., Carmeli, S., Ron, E. Z. and Rosenberg, E. 1995. Alasan, a New Bioemulsifier from *Acinetobacter radioresistens*. *Appl. Environ. Microbiol.*, **61**: 3240-3244.
 22. Paraszkiwicz, K., Kanwal, A. and Długoński, J. 2002. Emulsifier Production by Steroid Transforming Filamentous Fungus *Curvularia lunata*. Growth and Product Characterization. *J. Biotechnol.*, **92**: 287-294.
 23. Portilla-Rivera, O., Torrado, A., Domínguez, J. M. and Moldes, A. B. 2010. Stabilization of Kerosene/Water Emulsions Using Bioemulsifiers Obtained by Fermentation of Hemicellulosic Sugars with *Lactobacillus pentosus*. *J. Agric. Food Chem.*, **58**: 10162-10168.
 24. Saimmai, A., Rukadee, O., Onlamool, T., Sobhon, V. and Maneerat, S. 2012. Characterization and Phylogenetic Analysis of Microbial Surface Active Compound-Producing Bacteria. *Appl. Biochem. Biotech.*, **168**: 1003-1018.
 25. Shao, L., Wu, Z., Zhang, H., Chen, W., Ai, L. and Guo, B. 2014. Partial Characterization and Immunostimulatory Activity of Exopolysaccharides from *Lactobacillus rhamnosus* KF5. *Carbohydr. Polym.*, **107**: 51-56.
 26. Thavasi, R., Jayalakshmi, S. and Banat, I. M. 2011. Application of Biosurfactant Produced from Peanut Oil Cake by *Lactobacillus delbrueckii* in Biodegradation of Crude Oil. *Bioresour. Technol.*, **102**: 3366-3372.
 27. Toledo, F. L., Gonzalez-Lopez, J. and Calvo, C. 2008. Production of Bioemulsifier by *Bacillus subtilis*, *Alcaligenes faecalis* and *Enterobacter* Species in Liquid Culture. *Bioresour. Technol.*, **99**: 8470-8475.
 28. Willumsen, P. A. and Karlson, U., 1996. Screening of Bacteria, Isolated from PAH-Contaminated Soils, for Production of Biosurfactants and Bioemulsifiers. *Biodegradation*, **7**: 415-423.
 29. Yadav, K. K., Mandal, A. K., Sen, I. K., Chakraborti, S., Islam, S. S. and Chakraborty, R. 2012. Flocculating Property of Extracellular Polymeric Substances Produced by a Biofilm-Forming Bacterium *Acinetobacter junii* BB1A. *Appl. Biochem. Biotechnol.*, **168**: 1621-1634.
 30. Zamani-Zadeh, M., Soleimani-Zad, S. and Sheikh-Zeinoddin, M. 2013. Biocontrol of Gray Mold Disease on Strawberry Fruit by Integration of *Lactobacillus plantarum* A₇ with Ajwain and Cinnamon Essential Oils. *J. Food Sci.*, **78**: M1582-M1588.

تولید و شناسایی جزئی بیوامولسیفایر گلیکوپروتئینی تولیدی توسط لاکتوباسیلوس
پلاتناروم زیر گونه پلاتناروم PTCC 1896

ن. بخشی، م. شیخ زین الدین، و س. سلیمانان زاد

چکیده

با توجه به علاقه روزافزون به تولید بیوامولسیفایرهای جدید و ارزان قیمت، در این مطالعه سبوس و ساقه برنج و گندم برای تولید بیوامولسیفایر توسط لاکتوباسیلوس پلاتناروم زیر گونه پلاتناروم PTCC 1896 (پروبیوتیک) ارزیابی شدند. این سویه فقط در محیط عصاره هیدرولیزی سبوس برنج بیوامولسیفایر تولید کرد. بازده تولید بیوامولسیفایر در حدود 0.7 g L^{-1} بعد از ۷۲ ساعت تخمیر به دست آمد. مولکول زیستی جدید، استخراج، خالص سازی و خصوصیات ساختمانی و حرارتی آن بررسی شد. گروه های عاملی و ساختار مولکول با استفاده از روش های GPC، FTIR، $^1\text{H NMR}$ و $^{13}\text{C NMR}$ تعیین گردید. بیوامولسیفایر یک α -گلوکان (۸۱/۷۴٪) متصل به پروتئین (۱۸/۱۸٪) محلول در آب و با وزن مولکولی بالا (10^6 Da) بود. رفتار حرارتی با استفاده از DSC و TG بررسی شد. آنالیز حرارتی نشان داد که بیوامولسیفایر بالاتر از $211/74^\circ\text{C}$ تجزیه می شود و نقطه ذوب آن $182/0^\circ\text{C}$ با آنتالپی ذوب $101/7 \text{ J g}^{-1}$ بود. این مطالعه اطلاعات اولیه ای برای تولید صنعتی بیوامولسیفایر مقرون به صرفه و ایمن فراهم می کند که به نظر می رسد می تواند در مصارف غذایی و دارویی مورد استفاده قرار بگیرد.