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ORIGINAL ARTICLE

Characterization of a novel bioemulsifier from *Pseudomonas stutzeri*

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Abstract This study describes a novel and efficient alasan-like bioemulsifier produced by *Pseudomonas stutzeri* NJtech 11-1, which was isolated from the Shengli Oilfield. The strain was found to produce a new and interesting emulsion stabilizer. The crude bioemulsifier showed super stability with 50% salinity and broad pH 3–10. The emulsion index (EI_{24}) was increased to 100% after heating from 45 to 95 °C and the emulsion could be stable for at least 30 days. The yield of Ps-bioemulsifier (pure bioemulsifier) was 0.68 ± 0.05 mg mL^{-1}. The Ps-bioemulsifier was composed of carbohydrates ($80 \pm 2.6\%$) and proteins ($9.5 \pm 0.5\%$). A low concentration (0.2 mg mL^{-1}) of the Ps-bioemulsifier was obtained maximum emulsifying activity at pH 7.1 and its emulsifying activity strengthened by suitable salinity. Furthermore, Ps-bioemulsifier could also emulsify cyclohexane, hexadecane, kerosene, xylene hydrocarbons efficiently. Therefore, the Ps-bioemulsifier showed emulsifying characteristics which make it a good candidate for potential applications in bioremediation and microbial enhanced oil recovery.

Keywords *Pseudomonas stutzeri* · Bioemulsifier · Emulsion index · Emulsifying activity · Microbial enhanced oil recovery

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Introduction

Several kinds of bioemulsifiers are synthesized by varieties of microorganisms from different genus. Bioemulsifiers are broadly classified as low-molecular-mass and high-molecular-mass emulsifiers according to their molecular weight (Rosenberg and Ron 1999; Camargo-De-Morais et al. 2003; Bonilla et al. 2005; Marchant and Banat 2012). Low-molecular-mass bioemulsifiers generally belongs to lipopeptides and glycolipids such as surfactins (Sandrin et al. 1990; Kim et al. 1997; Nitschke and Pastore 2006) and rhamnolipids (Rendell et al. 1990; Maier and Soberón-Chávez 2000); while high-molecular-mass bioemulsifier including emulsans (Rosenberg et al. 1979a, b; Zosim et al. 1987; Kaplan and Rosenberg 1983) and alasan (Navon-Venezia et al. 1995, 1998), emulsifier YM-1(Zheng et al. 2012), emulsifier AM1 (Markande et al. 2013) and others unnamed bioemulsifiers (Amaral et al. 2006; Luna-Velasco et al. 2007), they are usually consist of hetero-polysaccharides, lipopolysaccharides, lipoproteins and polysaccharide–protein. On one hand, high-molecular-mass bioemulsifiers are quite similar to low-molecular-mass bioemulsifiers, in that they can solubilize hydrocarbons or other hydrophobic substrates in water, developing stable emulsion layers. On the other hand, they are different, because high-molecular-weight bioemulsifiers can effectively emulsify hydrophobic substances at low concentrations but are less effective for surface tension reduction (Ron and Rosenberg 2010).

Bioemulsifiers have some advantages over synthetic emulsifying agents, such as higher biodegradability, lower toxicity, better environmental compatibility, higher selectivity and activity over a wide range of temperatures, pH, and salt content, as well as the possibility of production from renewable resources (Desai and Banat 1997). These characteristics enable wide applications of

bioemulsifiers in fields such as bioremediation (Juwarkar et al. 2007) and microbial enhanced oil recovery (MEOR) (Ron and Rosenberg 2002). Currently, three major bioemulsans are widely reported in the literature: RAG-1 emulsan, BD4 emulsan and alasan. RAG-1 emulsan (Sar and Rosenberg 1983), which is produced by *Acinetobacter calcoaceticus* RAG-1, is the most commonly used bioemulsifier. RAG-1 emulsan is a complex of anionic hetero-polysaccharides and proteins, the fatty acids of which is associated with the polysaccharide backbone via *O*-ester and *N*-acyl linkages appear to play a very important part in the polymer (Sar and Rosenberg 1983; Belsky et al. 1979). BD4 emulsan, produced by a strain of *A. calcoaceticus* BD4, is a surface-active extracellular polysaccharide-protein complex (Kaplan and Rosenberg 1983). Alasan is produced by *Acinetobacter radioresistens*, and is a complex composed a polysaccharide (apo-alasan), together with covalently bound alanine and proteins (Navon-Venezia et al. 1995, 1998; Toren et al. 2001).

To our best knowledge, *Pseudomonas* always used for fermentation of glycolipid bioemulsifer or applied to decompose organic substances, while seldom used for producing high-molecular-weight bioemulsifier. In this paper, we present a new and interesting emulsion stabilizer produced by a newly isolated *Pseudomonas* strain. One of its most remarkable features is its heat-activated emulsification activity. Our goal was to estimate the impact of temperature, pH, and salt content on the activity of the bioemulsifier and to investigate the effects of various chemical and physical conditions on its physicochemical and functional properties. We thus provide a detailed characterization of this bioemulsifier, which will hopefully guide its industrial applications in the future.

Materials and methods

Medium compositions and growth conditions

The basal salt medium (BSM) was employed for bacteria isolation and bioemulsifier production, which comprising 2.5 g L^{-1} $NaNO_3$, 2.0 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 2.0 g L^{-1} KH_2PO_4, 0.02 g L^{-1} $CaCl_2$ and 0.1 g L^{-1} $MgSO_4{\cdot}7H_2O$ (pH 7.3 ± 0.02). Crude oil (5.0 g L^{-1}, Shengli Oilfield, extracted in 2016) or glucose (30 g L^{-1}, w/v) was added to BSM as the sole carbon source. Inoculation fluid was cultivated with Luria-Bertani (LB) medium including 5.0 g L^{-1} yeast extract, 10.0 g L^{-1} tryptone, 10.0 g L^{-1} NaCl, to reach an OD600 of 1.0. The LB medium was solidified with 2.0% agar to achieve LB plates. Crude oil and contaminated soil samples were collected from Shengli Oilfield, China.

Isolation and identification of strain

The bioemulsifier producer was isolated according to a published enrichment culture technique (Kumar et al. 2006), which is added 0.5-g crude oil contaminated soil sample to 100 mL BSM was incubated for 4 days used as initial inoculums, and then a 1 mL inoculums was transferred to a fresh BSM with 5.0 g L^{-1} crude oil and incubated for another 4 days. Finally, the enrichment culture was diluted and plated onto LB agar plates. The pure microbial colonies were obtained by repetitive streaking the morphologically different colonies onto LB agar plates for several times, and the strains with the ability to emulsify diesel oil were selected for further study. The strains were identified according to partial 16S rDNA sequencing. Amplification of the 16S rDNA gene and nucleotide sequencing was performed by the Bact16S service from Bio Molecular Research (GENEWIZ, China). A complete 16S rDNA gene sequence was determined by PCR amplification using the Primers 27F: 5′-AGAGTT TGATCCTGGCTCAG-3′ and 1492R: 5′-CGGTTACCT TGTTACGACTT-3′, and the obtained sequences were compared with those in GenBank database (National Center for Biotechnology Information) using nucleotide–nucleotide blast (Blastn) network service.

Preparation of crude bioemulsifier

Pseudomonas stutzeri NJtech 11-1 was incubated in 50 mL BSM with glucose as sole carbon source in a 250-mL flask at 37 °C and 200 rev min^{-1} for 72 h. After that, the fermentation broth was centrifuged (8000*g* at 4 °C for 25 min) and the supernatant was used as the crude bioemulsifier.

Emulsion index determination

A 1.5 mL aliquot of each crude bioemulsifier (used as water layer) and 1.5 mL of diesel was mixed in cylindrical glass vials. The mixtures were then emulsified by maximum vortexing (QT-2, Qite, Shanghai, China) for 2 min and placed at room temperature for the indicated number of hours (Cooper and Goldenberg 1987). The emulsion index (EI_x) was determined according to the emulsion layer height and the total mixed liquid height, via the following equation:

$$EI_x = \frac{\text{Height of the emulsifier layer (cm)}}{\text{Total height of the mixture (cm)}} \times 100\%$$

whereby x is the time after initial emulsification (h).

Preparation of purified Ps-bioemulsifier

The crude bioemulsifier was precipitated with three volumes of cold acetone at 4 °C overnight (Freitas et al. 2009). The precipitate was re-dissolved in distilled water, dialyzed (7000 Da, biosharp, USA) against 1000 mL of distilled water at 25 °C for 48 h, lyophilized, and finally weighed. The resulting pure bioemulsifier named Ps-bioemulsifier was directly used for further study.

Chemical composition of Ps-bioemulsifier

Protein concentrations were measured using the Bradford assay (Bradford 1976). The standard protein curve was determined with bovine serum albumin (BSA). The total carbohydrate concentration of the Ps-bioemulsifier was measured using the anthrone-sulfuric acid assay (Hodge and Hofreiter 1962). Analysis of lipid components was carried out using ammonium molybdate-perchloric acid method (Lou and Clausen 1967).

Protease K and trypsin treatment

A stock solution comprising 1 mg mL^{-1} of deproteinized Ps-bioemulsifier was prepared by treating the Ps-bioemulsifier with proteinase K (GENEWIZ, China) at 10 μg mL^{-1} final concentration in 20 mL TM buffer (pH 7.8), or with 50 U mL^{-1} trypsin (Sinopharm Chemical Reagent Co., Ltd, China) in 20 mL TM buffer (pH 8.0) for 60 min at 55 and 37 °C, respectively. After dialyzing against 1000 mL of distilled water for 24 h (fresh water was replenished three times), the deproteinized solution was lyophilized and stored at 20 °C for further experiments.

Viscosity measurement

The Ps-bioemulsifier was dissolved in TM buffer (pH 7.5 ± 0.02) including 20 mM Tris–HCl and 10 mM $MgSO_4$. Viscosity of the Ps-bioemulsifier solution (0.2 mg mL^{-1}) was measured by an Ubbelohde viscometer (Huabo, Shanghai, China).

Emulsifying activity and emulsion stability assays

The standard emulsifying activity assay with some modifies based on the method of Rosenberg et al. (1979b) reported was applied to measure the emulsifying activity. 100–260 mg of diesel oil or other hydrocarbon substrate was added to a final volume of 20 mL of TM buffer, and either 0.10 or 0.25 mg mL^{-1} of Ps-bioemulsifier. The samples were incubated at 30 °C with reciprocal shaking at 200 strokes min^{-1} for 60 min. A WGZ-200 turbidimeter (XINRUI, Shanghai, China) was used to determine the turbidity of the resulting emulsions in the range of 10–200 NTU. One unit was defined as one NTU. Emulsifying activity was defined as the number of NTU units per Ps-bioemulsifier weight (NTU mg^{-1}), and quantified using the following formula:

$$\mathrm{EA} = X \times \frac{20\ \mathrm{mL}}{1\ \mathrm{mL}} \times \frac{1}{Y\ \mathrm{mL}} \times \frac{1}{Z\ \mathrm{mg\ mL^{-1}}} \left[\mathrm{NTU\ mg^{-1}}\right]$$

whereby X is the turbidity measured by the turbidimeter, Y is the volume of the tested solution (mL) and Z is the concentration of the Ps-bioemulsifier (mg mL^{-1}).

After this, the resulting emulsion layers were left to passively stand at room temperature with no shaking for 24 h and the turbidity determined again. The emulsion stability was calculated as the percent of the remaining turbidity of the emulsion after 24 h.

Emulsifying activity toward different oils and hydrocarbons

0.2 mg mL^{-1} of Ps-bioemulsifier solutions were used to study the effect of different oils (diesel and kerosene) and hydrocarbons (cyclohexane, hexane, 1-octane, hexadecane, liquid paraffin, toluene, xylene and styrene) on the emulsifying activity of bioemulsifier.

Results

Isolation and identification of the bioemulsifier producer

A total of 14 bacterial strains were isolated from different Shengli Oilfield samples. The pure cultures were grown to stationary phase in glucose medium, harvested by centrifugation, and the extracellular fluids screened for emulsifying activity by the standard emulsification assay. The strain that yielded the highest emulsification value (EI_{24} = 57 ± 0.14%), referred to as NJtech 11-1.

The species identity of strain 11-1 was determined via its partial 16S rDNA gene sequence, and a 1407 bp 16S rDNA gene corresponding to strain11-1 was found in GenBank under the accession number KX580703. The 1407 bp sequence was searched against the NCBI database, and showed the highest similarity (99%) with different strains of *P. stutzeri* (Fig. 1). Based on these results, the isolated strain was named *P. stutzeri* NJtech 11-1. The strain has been preserved in the China Center for Type Culture Collection (CCTCC) under the reference number M 2015742.

Strain 11-1 was also identified by physiological characterization. It grew on glucose, lactose, sucrose, sodium acetate, ethanol, citrate, glutamate, serine, tryptophan and

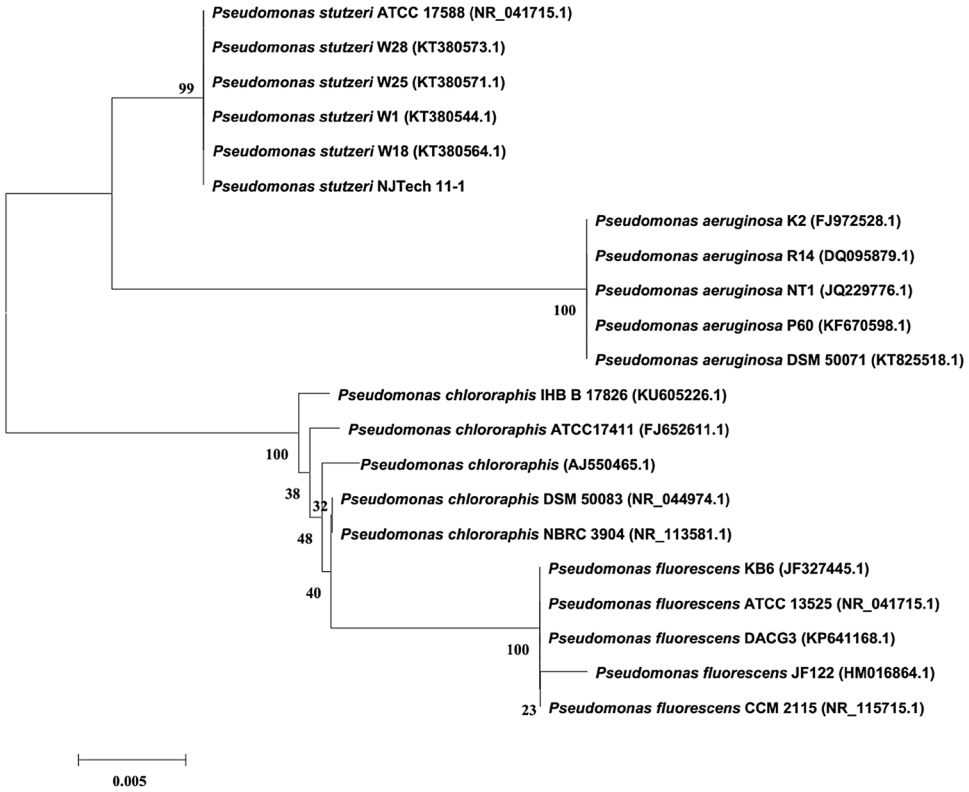

Fig. 1 Phylogenetic neighbor-joining tree based on the 16S rDNA sequence of strain NJtech 11-1

L-phenylalanine as sole carbon sources, but failed to grow on aspartate. It did not hydrolyze gelatin. It is Gram-negative, non-spore-forming, rod shaped, and the colonies have a light yellow, moist and translucent appearance.

Effects of salinity and pH on emulsion index of crude bioemulsifier

The effects of salinity, temperature and pH on emulsion index of crude bioemulsifier are shown in Fig. 2. The emulsion stability of bioemulsifier was poorly affected by salinity (Fig. 2a, b). At concentration of 50% NaCl, $MgCl_2$ and $CaCl_2$, the EI_{24} was 45, 48 and 40%, respectively, while the original value (57%) was obtained without any salt (Fig. 2a). After still standing for 30 days, the crude bioemulsifier with salt showed better emulsion stability than crude bioemulsifier without salt (Fig. 2b). The highest EI_{24} was obtained at pH 8 and 9 and the emulsion index of 30 days was still higher than 50% at pH 3–9 (Fig. 2c).

Emulsifying properties of heat-treated crude bioemulsifier

The emulsification ability of the resulting solutions was determined by measuring the EI_{24}, as shown in Fig. 3a. Heat treatment greatly enhanced the emulsifying activity of supernatant fluid toward diesel at temperatures ranging from 45 to 95 °C for 60 min. After being heated at 85 °C, the supernatant fluid exhibited the highest activity; the EI_{24} of a ten-fold dilution reached 50% toward diesel. Moreover, all of the heated supernatant dilutions formed emulsions with diesel that were stable for at least 30 days (Fig. 3b).

Production and characterization of Ps-bioemulsifier

With glucose as sole carbon source, the production of the Ps-bioemulsifier (pure bioemulsifier) was 0.68 ± 0.05 mg mL^{-1} after 72 h of fermentation in shake flasks. As shown in Table 1, no lipids were detected in the Ps-bioemulsifier via thin layer chromatography; it however contained approximately $80 \pm 2.6\%$ of polysaccharides and $9.5 \pm 0.5\%$ of proteins. The Ps-bioemulsifier was therefore putatively classified as a glycoprotein. When the concentration of Ps-bioemulsifier was 1 mg mL^{-1}, the surface tension of the solution was 53.5 ± 0.2 mN m^{-1} and EI_{24} reached $66.5 \pm 0.05\%$ toward diesel oil. Therefore, the Ps-bioemulsifier apparently possesses only emulsifying activity but no significant surface activity.

Proteinase K and trypsin treatment

Deproteinization of Ps-bioemulsifier with proteinase K and trypsin led to a large loss of emulsifying activity (Table 2), indicating that the proteins component of

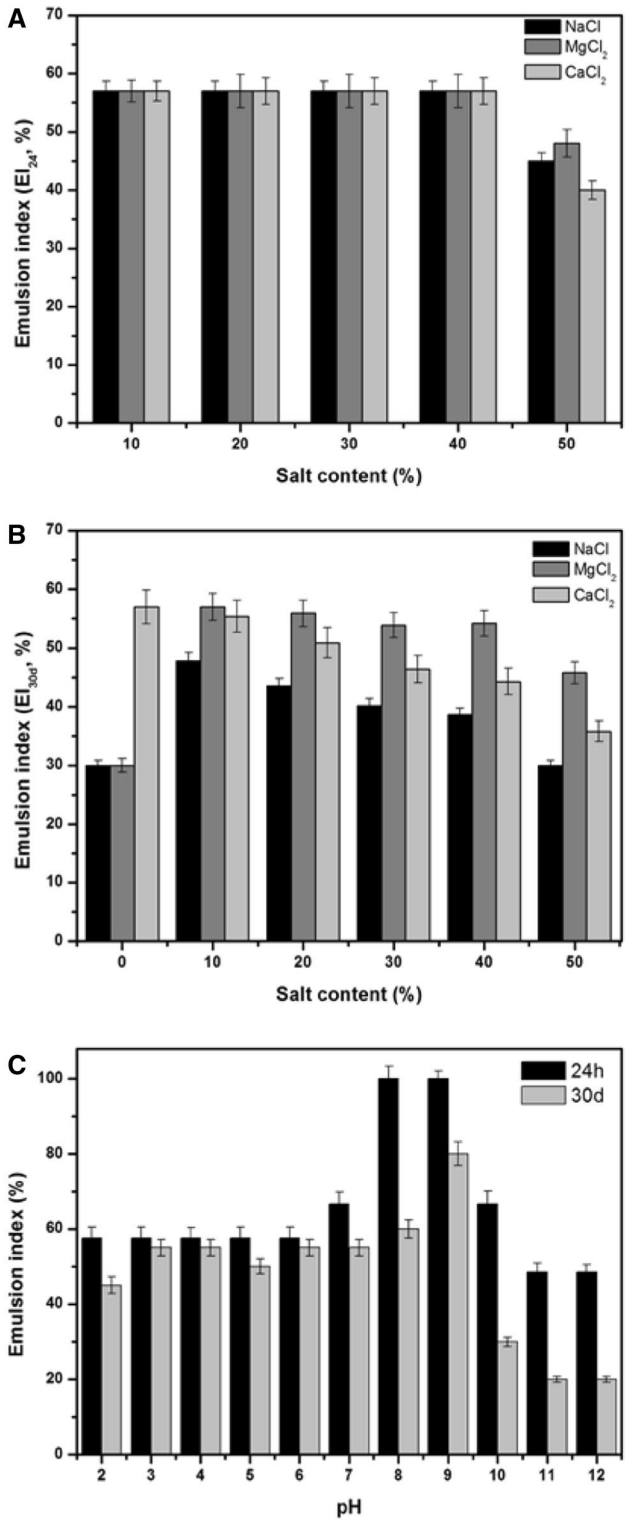

Fig. 2 Effects of salt content (**a** EI_{24} and **b** EI_{30d}) and pH (**c**) on the emulsion index of crude bioemulsifier. The broth was centrifuged at 8000*g* for 20 min. The pH was adjusted to 2–12 using NaOH or HCl. EI_{24}: emulsion index after 24 h; EI_{30d}: emulsion index after 30 days; Data points represent the average of three replicate tests; *error bars* represent standard deviation

Ps-bioemulsifier play a major role in the emulsifying activity of the compound.

Comparison of Ps-bioemulsifier and chemical emulsifier

Diesel-in-water emulsions containing different concentrations of Ps-bioemulsifier or Tween 60 were prepared by reciprocal shaking for 1 h. The results were showed in Fig. 4. Although the turbidity and emulsion stability of Ps-bioemulsifier was inferior to Tween 60 at a concentration between 0.05 and 0.3 mg mL^{-1}, the emulsions formed by Ps-bioemulsifier and diesel oil were quite stable after 24 h still-standing, and the EI_{24} of each sample remained above 50%. The turbidity of diesel emulsions, and therefore the emulsion stability, was even greater, and remained above 70%. Furthermore, the working concentration of the Ps-bioemulsifier was very low (0.05 mg mL^{-1}), which means that the Ps-bioemulsifier possesses exceptionally good emulsifying activity which may make it economically viable in the future.

Effects of salt content and pH on emulsifying activity and stability of Ps-bioemulsifier

Ps-bioemulsifier was able to emulsify diesel in the presence of 0.1–1.1 M NaCl (Fig. 5a). NaCl at a concentration of <0.7 M enhanced the emulsifying activity, and the highest value was observed with 0.1 M NaCl, reaching 515.0 ± 20.7 NTU mg^{-1}. However, the activity was inhibited in the presence of >0.9 M NaCl. Although the emulsion stability decreased slightly when the concentration of NaCl was increased above 0.9 M, it still remained above 50%. In fact, the emulsion containing 1.1 M NaCl was quite stable and the emulsion index was greater than 50% after 30 days. $CaCl_2$ weakened the emulsifying activity and stability of Ps-bioemulsifier (Fig. 5b). The highest emulsifying activity was only 300.5 ± 15.0 NTU mg^{-1} with 0.1 M of $CaCl_2$, barely higher than the control (262.0 ± 13.1 NTU mg^{-1}). Interestingly, $MgCl_2$ (0.05–0.3 M) enhanced the emulsifying activity, especially at the concentration of 0.05 M $MgCl_2$, whereby the specific activity reached the maximum value of 475.5 ± 23.7 NTU mg^{-1} (Fig. 5c). The emulsion stability decreased as the content of $MgCl_2$ increased. It is however important to note that the emulsion stability of each sample containing calcium and magnesium salts maintained above 50%, which means that the emulsion layer was still stable.

The emulsifying activity was studied in the pH range of 3.0–10.6, and the maximum values were observed at pH 7.1. The emulsion stability decreased markedly at pH 3–4 and pH 8–10.6 (Fig. 5d). However, the emulsion index (EI_{24}) of each sample within this broad pH range remained above 50%.

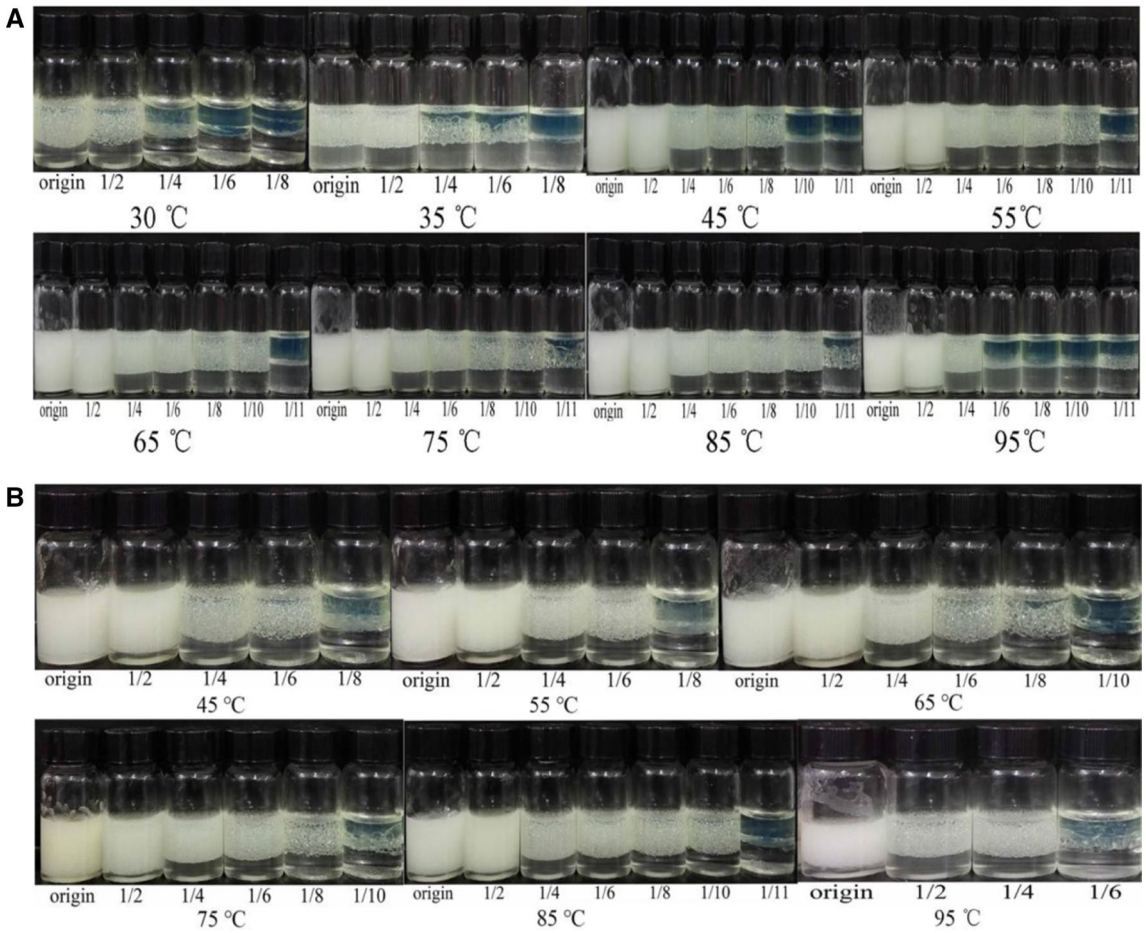

Fig. 3 Influence of temperature on the emulsion index of crude bioemulsifier after 24 h (**a**) and 30 days (**b**). The crude bioemulsifier was subjected to heat treatment between 30 and 95 °C for 60 min, cooled to room temperature and diluted with distilled water. Each assay was repeated at least twice

Table 1 Characterization of Ps-bioemulsifier

Ps-bioemulsifier (mg mL^{-1})	Protein (%)	Polysaccharide (%)	Lipid (%)	Surface tension (mN m^{-1})	Emulsion index (EI_{24}%)
1.0	9.5 ± 0.5	80 ± 2.6	0	53.5 ± 0.2	66.5 ± 0.05

The surface tension of the Ps-bioemulsifier solutions (1.0 mg mL^{-1}) was measured by a BZY-3B tension meter (CNSHP, Shanghai, China) using the Wilhelmy Plate method (Lin et al. 1994). Each assay was done at least three times and the standard deviations (SD) were shown

Taken together, an emulsion containing 0.2 mg mL^{-1} of Ps-bioemulsifier was stable after being exposed to a wide range of NaCl (0.1–1.1 M), $CaCl_2$ (0.1–0.6 M) and $MgCl_2$ (0.05–0.3 M) concentrations, as well as in a wide pH range (3–10.6).

Effect of heat treatment on viscosity and emulsifying activity of Ps-bioemulsifier

Great changes occurred in the viscosity and emulsifying activity of the compound after heating at 30–95 °C (Fig. 6). The emulsifying activity of Ps-bioemulsifier complex increased significantly with the heat treatments at increasing temperatures. The maximum emulsifying activity was observed after heating at 85 °C for 60 min, and was about 2.3-fold of the original (emulsifying activity at 30 °C). However, heat treatment at 95 °C resulted in a reduced activity compared to 85 °C. The maximum reduced viscosity was observed at 45 °C. Heat treatment from 30 to 45 °C, the reduced viscosity of the Ps-bioemulsifier solution improved 1.3-fold over original (the reduced viscosity at 30 °C) and decreased sharply from 55 to 95 °C. All of the emulsions were quite stable with values consistently above 70%.

Table 2 Deproteinization of Ps-bioemulsifier

Ps-bioemulsifier (mg mL^{-1})	Protein (%)	Treatment	Emulsifying activity (NTU mg^{-1})	Emulsion stability (%)
0.2	9.5 ± 0.5	None	262.0 ± 13.1	73.3 ± 3.6
	<0.1	ProK	7.3 ± 0.5	0
	<0.1	Trypsin	8.1 ± 0.5	0

Each assay was done at least three times and the standard deviations (SD) were shown

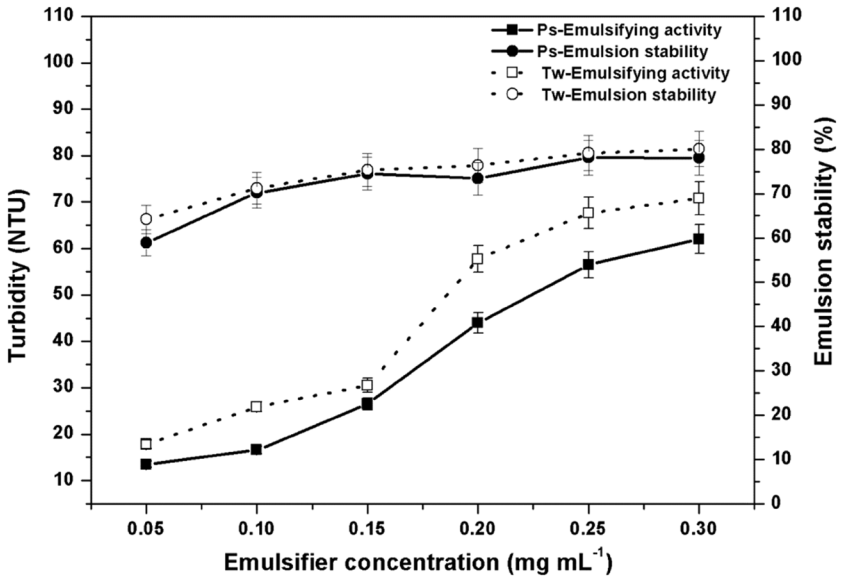

Fig. 4 Comparison of bioemulsifier and chemical emulsifier. 260 mg of diesel was added to a Ps-bioemulsifier solution (0.05–0.30 mg mL^{-1}, dissolved in 20 mL of TM buffer, pH 7.5). The solutions were treated at 30 °C with reciprocal shaking (200 strokes min^{-1}) for 60 min. *Ps* Ps-bioemulsifier, *Tw* Tween 60. Each assay was done at least three times; the *error bars* represent standard deviation

Emulsifying activity towards different oils and hydrocarbons

Data on the ability of Ps-bioemulsifier to stabilize highly dispersed oil-in-water emulsions are summarized in Table 3. The emulsifying activity toward different oils and hydrocarbons varied significantly. Among aliphatic hydrocarbons, the short-chain species including cyclohexane (7.6 ± 1.2 NTU mg^{-1}), hexane (8.5 ± 1.7 NTU mg^{-1}) and 1-octane (71.3 ± 3.6 NTU mg^{-1}) were emulsified poorly or not at all, whereas the long-chained ones such as hexadecane (136.3 ± 3.2 NTU mg^{-1}), liquid paraffin (351.1 ± 8.7 NTU mg^{-1}), diesel (262.0 ± 13.1 NTU mg^{-1}) and kerosene (185.1 ± 6.5 NTU mg^{-1}) were emulsified very well. Maximum turbidity was obtained with toluene (858.0 ± 15.4 NTU mg^{-1}). Other aromatic hydrocarbons such as xylene (798.0 ± 14.5 NTU mg^{-1}) and styrene (256.0 ± 12.1 NTU mg^{-1}) were also emulsified effectively. Thus, the Ps-bioemulsifier possesses a promising emulsion-stabilizing capacity for hexadecane, diesel, kerosene, toluene, styrene and xylene, as shown by emulsion stability values higher than 50%, while lower emulsion stability was achieved with cyclohexane, hexane, octane and liquid paraffin.

Discussion

A novel bioemulsifier produced by *P. stutzeri* NJtech 11-1 is efficient over a wide range of salinity, temperature and pH. The study in this paper showed that crude bioemulsifier could tolerate 50% NaCl, $MgCl_2$ and $CaCl_2$ with only 29.8% of loss and the crude bioemulsifier with salt showed better emulsion stability than that without salt after still standing for 30 days, while the EI_{24} of the bioemulsifier from *Pseudomonas* sp. strain LP1 reported by Obayori et al. (2009) reduced 84% at 10% NaCl. It revealed that this bioemulsifier was suitable for the reservoir of high salt concentration. The highest EI_{24} was obtained at pH 8 and 9 and the emulsion index of 30 days was higher than 50% at pH 3–9. Therefore, the novel bioemulsifier from *P. stutzeri* have potential applications in environments such as oil wells and some industrial applications which require extreme temperature.

The purified Ps-bioemulsifier precipitated with cold acetone from crude bioemulsifier, is composed of polysaccharides (80 ± 2.6%) and proteins (9.5 ± 0.5%) and this is in common with emulsan and alasan (Bach et al. 2003). The Ps-bioemulsifier was found to lose its emulsifying activity after enzymatic deproteinization with protease K and

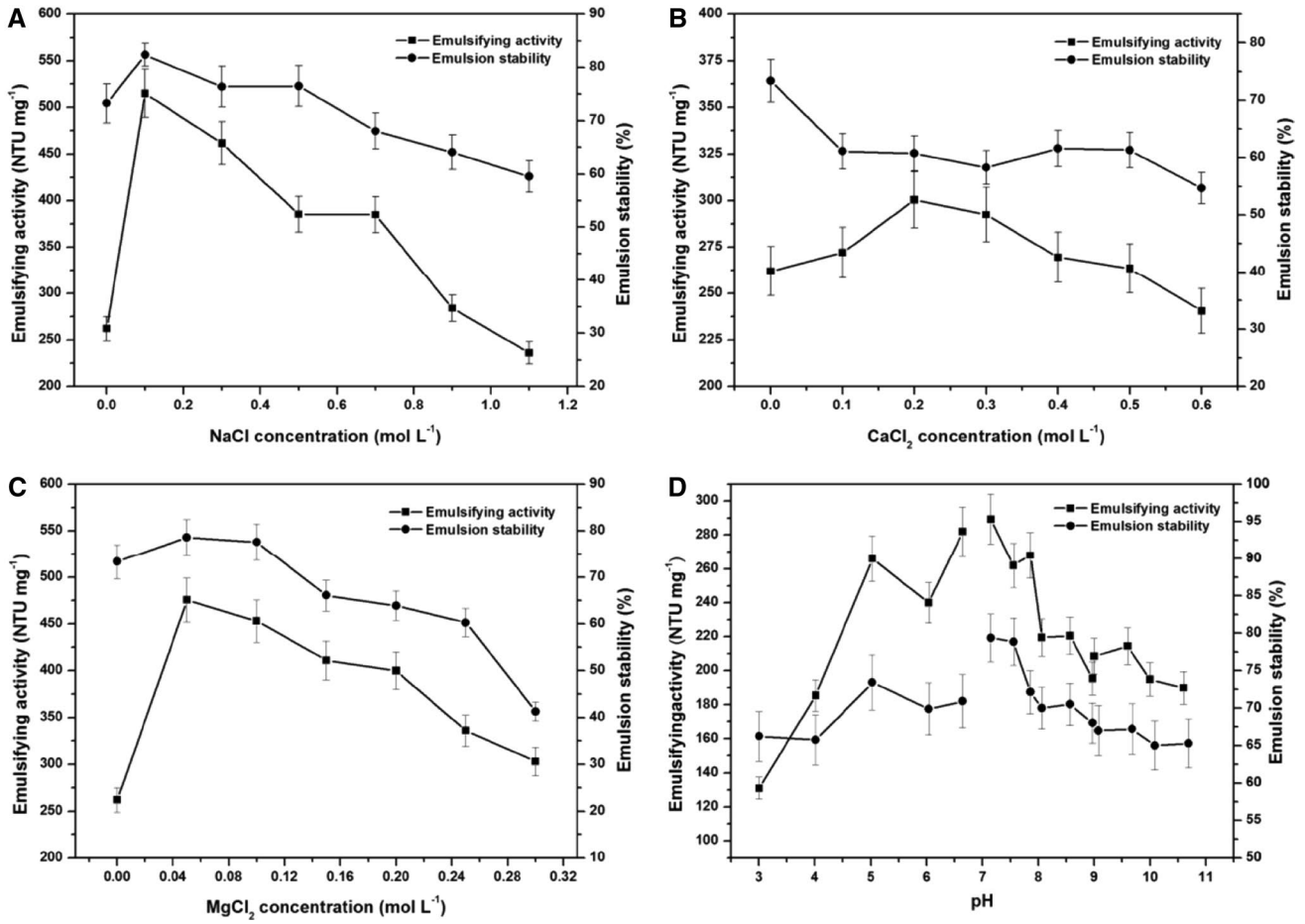

Fig. 5 Influence of NaCl (**a**), $CaCl_2$ (**b**), $MgCl_2$ (**c**) and pH (**d**) on emulsifying activity and emulsion stability of Ps-bioemulsifier. The Ps-bioemulsifier solution was 0.2 mg mL^{-1}. The emulsification experiment was carried out with 20 mM citrate buffer for the pH range 3.0–6.6, 20 mM Tris–HCl buffer for the pH range 7.1–8.9 and 20 mM glycine buffer for the pH range 9.0–10.6. Every assay was done at least three times; the *error bars* represent standard deviation (SD)

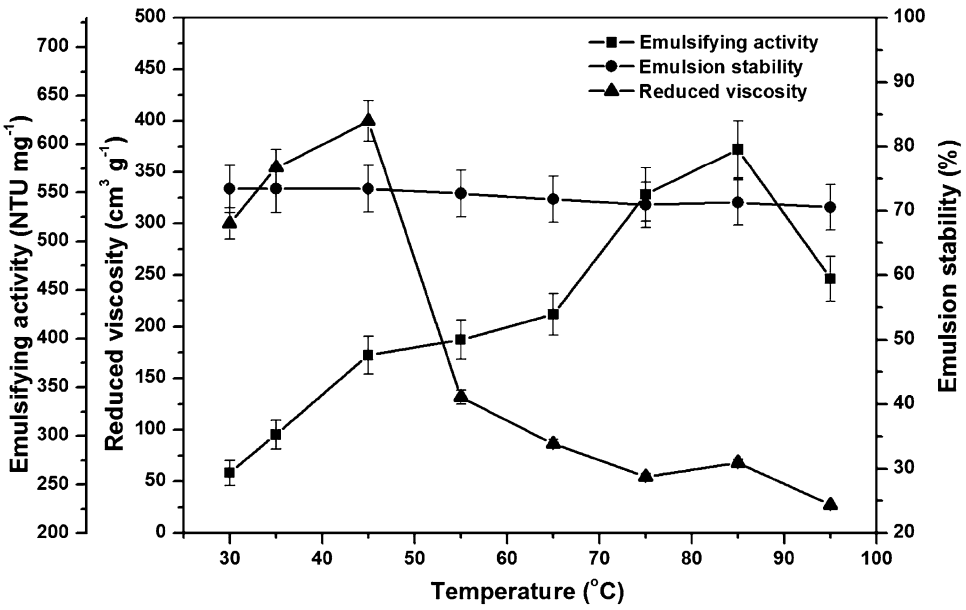

Fig. 6 Effect of temperature on viscosity, emulsifying activity and emulsion stability of Ps-bioemulsifier. Solutions of 0.2 mg mL^{-1} Ps-bioemulsifier (dissolved in TM buffer, pH 7.5) were heated to the indicated temperatures in a thermo-block and subsequently incubated for 30 min. After that, samples were cooled to 30 °C and the emulsifying activity, reduced viscosity and emulsion stability were determined at 30 °C. Each assay was done at least three times; the *error bars* represent standard deviations

Table 3 Hydrocarbon substrate specificity of Ps-bioemulsifier

Hydrocarbon substrate	Emulsifying activity (NTU mg^{-1})	Emulsion stability (%)
Alkanes		
Cyclohexane	7.6 ± 1.2	26.7 ± 0.9
n-Hexane	8.5 ± 1.7	25.6 ± 0.5
1-Octane	71.3 ± 3.6	46.7 ± 1.0
Hexadecane	136.3 ± 3.2	66.7 ± 1.3
Liquid paraffin	351.1 ± 8.7	41.2 ± 0.8
Diesel	262.0 ± 13.1	73.3 ± 3.6
Kerosene	185.1 ± 6.5	76.9 ± 2.7
Aromatics		
Toluene	858.0 ± 15.4	86.7 ± 1.5
Styrene	256.0 ± 12.1	90.0 ± 0.9
Xylene	798.0 ± 14.5	88.5 ± 1.2

The Ps-bioemulsifier was used to emulsify various hydrocarbon substrates. Each assay was done at least three times and the standard deviations (SD) were shown

trypsin, which means that the main emulsifying activity of the compound is mediated by its protein components. This is also in good agreement with observations on emulsan and alasan, in which the functional proteins are esterase (Bach et al. 2003) and ompA-like proteins (Toren et al. 2002; Walzer et al. 2006), respectively.

The emulsifying properties of bioemulsifiers are quite different from species to species. RAG-1 emulsan is an efficient bioemulsifier that work at low concentrations (0.01–0.001%), however, RAG-1 emulsan is not able to emulsify pure cyclic, aromatic, or aliphatic hydrocarbon substances (Ron and Rosenberg 2010; Rosenberg et al. 1979a). Ps-bioemulsifier showed the highest emulsifying activity and the highest stability (85–90% of the original values after still standing for 24 h) towards aromatic hydrocarbons. These differences can be understood in terms of the emulsion forming and stabilizing capacity of polysaccharides as reported by Freitas et al. (2009), which is specific for particular hydrophobic complexes. The highest emulsifying activity of emulsan was obtained in the presence of divalent cations (2–10 mM Mg^{2+}) at pH values of 5.0–7.5. Alasan can stabilize oil-in-water emulsions of *n*-alkanes with chain lengths of ten or higher, liquid paraffin, alkyl-aromatics, crude oil and other oils, and its emulsifying activity was stable over a broad pH range (3.3–9.2), with a maximum at pH 5.0 (Belsky et al. 1979). In this work, the Ps-bioemulsifier was able to emulsify diesel over an even broader pH range (3.0–10.6) with a clear maximum activity at pH 7.1.

Similar with alasan (Rosenberg and Ron 2002), the distinguishing feature of Ps-bioemulsifier was that its emulsifying activity was greatly enhanced by heat treatment. After heating at 45–95 °C, the EI_{24} of the crude bioemulsifier (reached 100%) increased remarkably. This means that the effective working concentration of heated samples was much lower than the unheated samples. In other words, the heat treatment strategy could potentially reduce the production cost and is therefore especially promising for applications in MEOR.

Based on the results from this study, the newly isolated *P. stutzeri* NJtech 11-1 could produce an effective bioemulsifier using glucose as sole carbon source and the compounds exhibited great stability and activity over a range of challenging environmental conditions such as high salinity, high temperature and wide pH range, implying its significant potential for applications in MEOR.

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