Production and characterization of bioemulsifiers from *Acinetobacter* strains isolated from lipid-rich wastewater

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Abstract

In this study, two indigenous bacterial strains (Ab9-ES and Ab33-ES) isolated from lipid-rich wastewater showed potential to produce bioemulsifier in the presence of 2% (v/v) olive oil as a carbon source. These bacterial strains were identified as *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES by polymerase chain reaction and analysis of 16S rRNA gene sequences. Bioemulsifier production by these strains was found to be growth-linked. Maximum emulsifying activities (83.8% and 80.8%) were recorded from strains Ab9-ES and Ab33-ES, respectively. Bioemulsifier yields of 4.52 g/L and 4.31 g/L were obtained from strains Ab9-ES (XB9) and Ab33-ES (YB33), respectively. Fourier-transform infrared spectroscopic analysis revealed the glycoprotein nature of the bioemulsifiers. The bioemulsifiers formed stable emulsions only in the presence of edible oils. Maximum emulsifying activities of 79.6% (XB9) and 67.9% (YB33) were recorded in the presence of sunflower oil. The bioemulsifiers were found to be stable at a broad range of temperature (4–121 °C), moderate pH (5.0–10.0) and salinity (1–6%). In addition, bioemulsifier XB9 showed maximum emulsifying activities (77.3%, 74.5%, and 74.9%) at optimum temperature (50 °C), pH (7.0), and NaCl concentration (3%), respectively. On the contrary, YB33 demonstrated highest activities (73.6%, 72%, and 61.2%) at optimum conditions of 70 °C, pH 7.0, and NaCl concentration of 5%, respectively. Findings from this study suggest the potential biotechnological applications of the bioemulsifiers, especially in the remediation of oil-polluted sites.

Keywords *Acinetobacter* sp. · Bioemulsifier · Emulsifying activity · Lipid-rich wastewater · Remediation

Introduction

Lipid-rich wastewater is defined as wastewater that consists of lipids as well as a broad spectrum of organic and/or inorganic substances in large quantities (Adetunji and Olaniran 2018a). The production and discharge of raw and inadequately treated lipid-rich wastewater increase every year due to rapid urbanization and industrial growth. This poses serious threats to terrestrial and aquatic ecosystems (Rupani et al. 2010). Among the most effective treatment approaches, is the addition of microbial surface-active compounds (SACs) to the wastewater. This disperses the lipids and other pollutants in the wastewater and accelerates their

mineralization through increased bioavailability and subsequent uptake by direct cell contact (Franzetti et al. 2012).

Surface-active compounds are amphiphilic molecules, which form micelles and partition at the interface between fluid phases with varying degrees of polarity (Santos et al. 2016). They are classified into two groups: low-molecular weight and high-molecular weight SACs. The low-molecular weight compounds, also known as biosurfactants form micelles at the interface of immiscible liquids by reducing surface and interfacial tension as well as blocking hydrogen bonding and hydrophilic/hydrophobic interactions (Luna et al. 2012). They consist of glycolipids, fatty acids, phospholipids, neutral lipids, amongst others and have a molecular mass ranging between 500 and 1500 Da (Banat et al. 2010). Conversely, bioemulsifiers are high-molecular weight compounds that emulsify two immiscible liquids even at low concentrations without reducing surface or interfacial tension (Uzoigwe et al. 2015). They consist of polysaccharides, lipopolysaccharides, proteins, glycoproteins or lipoproteins, which

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confer upon them better emulsifying potential and ability to stabilize emulsions (Rosenberg and Ron 1999; Uzoigwe et al. 2015).

Bioemulsifiers are produced by a wide variety of microorganisms including bacteria, yeasts, and fungi using different substrates such as carbohydrates, hydrocarbons, vegetable oils and glycerol for cell growth (Kitamoto et al. 2002; Sarubbo et al. 2007). However, bioemulsifier production has been reported to be a common phenomenon among members of the genus *Acinetobacter* (Rosenberg 1986). Examples include emulsan, an extracellular polyanionic bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1 (Rosenberg and Ron 1999). Others include alasan from *Acinetobacter radiorensistens* (Navon-Venezia et al. 1995), biodispersan from *Acinetobacter calcoaceticus* A2 (Rosenberg et al. 1988). Many strains of *Acinetobacter* isolated from contaminated soil, mud, marine water, fresh water, and human skin have been reported to be bioemulsifier producers (Sar and Rosenberg 1983; Foght et al. 1989; Patil and Chopade 2001; Phetrong et al. 2008). However, few studies have been carried out on bioemulsifier-producing *Acinetobacter* sp. from lipid-rich wastewater (Saisa-ard et al. 2013).

In contrast to synthetic surfactants, bioemulsifiers are eco-friendly, biocompatible, less toxic with higher biodegradability and specifically active at extreme temperature, pH, and salinity. In addition, bioemulsifier can be produced from renewable inexpensive substrates (Pansiripat et al. 2010). Due to diverse functional properties such as emulsification, wetting, foaming, cleansing, phase separation, surface activity and reduction in hydrocarbon viscosity, bioemulsifiers are best employed in bioremediation, enhanced oil recovery, clean-up of oil-contaminated pipes and vessels, amongst others (Dastgheib et al. 2008).

In spite of their immense applications, large-scale production of bioemulsifier has been limited due to low yields and high production costs (Banat et al. 2014). Alternative approaches to ameliorate the aforementioned challenges include the selection of efficient strains for optimum bioemulsifier production (Marchant et al. 2014). Exploration of indigenous strains could be of great significance for bioemulsifier production since these strains can be assumed to perform better in their native environments than exotic strains. To the best of our knowledge, this is the first report on bioemulsifier production from *Acinetobacter* sp. isolated from lipid-rich wastewater in South Africa. This study, therefore, characterized glycoprotein bioemulsifier produced by two indigenous *Acinetobacter* sp. using different hydrophobic substrates. Thereafter, the stability of the bioemulsifiers (at extreme temperature, pH and salinity) was also determined to ascertain their potential industrial and environmental applications.

Materials and methods

Sample collection

Lipid-rich wastewater samples were collected from four different sites namely, wastewater treatment plant (WWTP), slaughterhouse (SH); edible oil mill (EOM) and soap (SP) industries, all in the KwaZulu-Natal (KZN) province, South Africa into sterile 500 mL Schott bottles and transported immediately to the laboratory for further analysis.

Bacterial isolation procedures

Lipid-rich wastewater samples (10 mL) were transferred to 250 mL Erlenmeyer flasks containing 100 mL mineral salt medium (MSM) and then incubated at 37 °C (120 rpm) for 48 h. The MSM composed of (in g/L): 1.0 KH_2PO_4 , 1.0 K_2HPO_4 , 1.0 NH_4NO_3 , 0.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.05 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ supplemented with 2% (v/v) filter-sterilized (0.2 μm) olive oil. Thereafter, bacterial cultures (5 mL) were transferred into another flask and cultivated under similar conditions. This process was repeated four times. The last enrichment cultures (0.1 mL) were serially diluted, plated onto nutrient agar (NA) and incubated at 37 °C for 24 h (Adetunji and Olaniran 2018b). Pure cultures obtained by sub-culturing on fresh NA plates were preserved at –80 °C in a storage medium (500 μL of 80% sterile glycerol and 500 μL culture suspension).

Screening of bacteria for bioemulsifier or biosurfactant production

Mineral salt medium supplemented with 2% (w/v or v/v) glucose, diesel, olive oil, sunflower oil, canola oil, castor oil or rice bran oil as sole carbon source was used. The pH of the medium was adjusted to 7.0 using 1 N NaOH or 1 N HCl. Homogenous bacterial culture [1% (v/v) of optical density (OD)_{600 nm} = 1] was inoculated into the MSM in 250 mL Erlenmeyer flasks and cultivated at 37 °C and 120 rpm for 7 d. A control flask for each carbon source without inoculum was subjected to similar incubation conditions. Bacterial growth was determined by measuring the OD at 600 nm using a UV–Vis spectrophotometer (UVmini-1240, Shimadzu, Australia). Cultures with an increase in OD when compared to control flask were scored positive (+) while those exhibiting no difference in turbidity were taken as no growth (–). Subsequently, the culture broth was centrifuged at 8000 rpm for 20 min at 25 °C. The obtained culture supernatants from respective test carbon sources were analyzed for bioemulsifier or biosurfactant production using oil-spreading test (Morikawa et al. 2000), emulsification assay

(Cooper and Goldenberg 1987), and surface tension (ST) measurement (Gudiña et al. 2015). Effect of the different carbon sources on bioemulsifier or biosurfactant production was studied. All experiments were done in triplicate.

Molecular identification of bioemulsifier-producing bacteria and phylogenetic analysis

Genomic DNA was isolated from bacterial cultures according to the boiling method of Akinbowale et al. (2007) with modifications. Amplification of the 16S rRNA gene was carried out using the universal bacterial primers F-5'-CAG GCCTAACACATGCAAGTC-3' and R-5'-GGGCGGTGT GTACAAGGC-3' (Marchesi et al. 1998). The polymerase chain reaction (PCR) mixture consisted of 1 × buffer, 1 mM MgCl₂, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), 0.4 μM of each primer, 2 U of *Taq* polymerase and 2 μL template DNA in a total volume of 25 μL. The mixture was subjected to amplification using a thermocycler (Bio-Rad T100, Singapore) under the conditions of 95 °C for 5 min (initial denaturation) followed by 30 cycles of; 95 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 1 min, 30 s (extension) and 72 °C for 5 min (final extension). Agarose gel electrophoresis was used for the observation of amplification products at 100 V for 45 min in a 1% *Tris*-acetate-ethylenediaminetetraacetic acid (TAE) buffer. The amplification products were viewed by UV illumination (Syngene, UK) after staining in 1 mg/mL ethidium bromide for 15 min and then sequenced (Inqaba Biotech, Pretoria, South Africa). The obtained sequences were compared with sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI) using the Nucleotide Basic Local Alignment Search Tool (BLAST N) program (<http://www.ncbi.nlm.nih.gov/BLAST>). The sequences were aligned using the multiple sequences alignment tool (Clustal W). Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software was used for the construction of a phylogenetic tree (Tamura et al. 2013).

Kinetics of growth and bioemulsifier production

Acinetobacter sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES were cultivated separately in a MSM supplemented with 2% (v/v) filter-sterilized (0.2 μm) olive oil. The pH of the medium was adjusted to 7.0 using 1 N NaOH or 1 N HCl. Bacterial culture [1% (v/v) of OD_{600 nm} = 1] was inoculated into the medium in 250 mL Erlenmeyer flasks and then incubated at 37 °C for 168 h at 120 rpm. A flask without inoculum was taken as a control. Samples were taken at 24-h intervals and analyzed for bacterial growth, emulsifying activity, and ST. Bacterial growth was measured at 600 nm using a UV–Vis spectrophotometer (UVmini-1240, Shimadzu, Australia). Thereafter, samples were centrifuged

at 8000 rpm (25 °C) for 20 min. ST and emulsification index (EI₂₄, %) of the obtained culture supernatants were determined, as described previously. All experiments were carried out in triplicate.

Extraction and partial purification of bioemulsifier

Bioemulsifier was precipitated from the bacterial culture supernatant using cold ethanol (1:3) followed by incubation overnight at −20 °C. Thereafter, the precipitated bioemulsifier was re-dissolved in distilled water (1%, w/v), dialyzed in cellulose tube membrane (cut-off: 12.4 kDa) (Sigma-Aldrich, USA) for 24 h against distilled water and then lyophilized. The obtained product was then weighed (Joshi et al. 2008).

Chemical composition of bioemulsifier

Standards methods were used for determination of chemical composition of the bioemulsifier. Carbohydrate content was determined by the phenol–sulfuric acid method of Dubois et al. (1956) using D-glucose as a standard. Protein content was measured as described by Lowry et al. (1951) using bovine serum albumin as a standard. Lipid content was estimated using the Folch extraction method (Folch et al. 1957). All experiments were done in triplicate.

Fourier-transform infrared (FT-IR) spectroscopic analysis

Functional groups of the bioemulsifier samples were elucidated by FT-IR spectroscopic analysis. The FT-IR spectra were generated in the wave number range of 3500–500 cm^{−1} using an ALPHA P platinum spectrometer (Bruker). The analysis of IR spectra was carried out using OPUS 6.5 (Bruker Optics) software. All measurements were recorded at room temperature.

Effect of different hydrophobic substrates on bioemulsifier activity

The emulsifying activity of the bioemulsifier was determined in the presence of different hydrophobic substrates including edible oils (sunflower oil, canola oil, castor oil, and rice bran oil), aromatic hydrocarbons (benzene, xylene, and toluene), aliphatic hydrocarbons (heptane, dodecane, and hexane) as well as hydrocarbon mixtures (motor oil, kerosene, and diesel). This was done by vortexing 2 mL of the bioemulsifier solution with the same amount of respective test hydrocarbons for 2 min. The mixture was then allowed to stand for 24 h. Emulsification index (%) was calculated, as previously described. The results were compared with those

obtained in the presence of sodium dodecyl sulfate (SDS). All experiments were carried out in triplicate.

Bioemulsifier stability studies

Effect of temperature, pH, and salinity on bioemulsifier activity

The influence of temperature, pH, and salinity on bioemulsifier activity was investigated by pretreating the bioemulsifier solution at varying temperatures, pH and NaCl concentrations. Bioemulsifier solution was exposed to different temperatures (4, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 121 °C) for 20 min and then cooled to room temperature. The effect of pH was assessed by adjusting the pH of the bioemulsifier solution to different values (2.0–13.0) using 1N HCl or 1N NaOH. Influence of salinity was investigated by supplementing bioemulsifier solution with different NaCl concentrations (1–10%, w/v). Emulsification index (%) was measured, as described previously in the presence of sunflower oil (substrate that gave highest emulsification index). SDS was used as a positive control. All experiments were carried out in triplicate.

Results and discussion

Isolation, screening, and identification of bioemulsifier-producing bacteria

A wide variety of industries produces lipid-containing waste effluent, which when improperly discharged without treatment, causes severe environmental and health hazards (Slavov 2017). To isolate indigenous bacteria capable of degrading the lipids through the production of extracellular SACs, lipid-rich wastewater samples were collected from different sites including WWTP, SH; EOM, and SP industries, all in the KZN province of South Africa. Twenty morphologically distinct bacteria including five from WWTP samples, seven from SH samples, five from EOM samples, and three from SP samples were isolated. These bacterial isolates were screened for the ability to produce SACs by cultivation in MSM supplemented with filter-sterilized glucose, diesel, olive oil, sunflower oil, canola oil, castor oil or rice bran oil as a carbon source. Bacterial growth was determined with respect to each carbon source. The obtained culture supernatants were tested for bioemulsifier or biosurfactant production using oil-spreading test and emulsification assay. Eighteen bacterial isolates showed positive results for both tests and were selected as bioemulsifier or biosurfactant producers (Table 1). All these bacterial isolates utilized olive oil as a carbon source. However, among the 18 bacterial isolates, five tested positive in the presence of glucose,

four in the presence of sunflower oil, and two each in the presence of canola oil, castor oil, and rice bran oil. Despite the growth of five bacterial isolates in MSM supplemented with diesel, bioemulsifier or biosurfactant production was not observed in the presence of this carbon source. Olive oil (2%, v/v) was chosen as a carbon source for further assays since this carbon source was mostly utilized for growth and bioemulsifier or biosurfactant production by all the selected bacterial isolates. Haba et al. (2000) reported the production of rhamnolipid from *Pseudomonas* sp. in the presence of olive oil as a carbon source. The ST of the selected 18 bacterial culture supernatants was measured to distinguish between biosurfactant and bioemulsifier producers. The ability of an organism to reduce ST below a threshold limit of 40 mN/m is an indication of its surface activity (Cooper 1986). In this study, among the selected bacterial isolates, 16 decreased the ST of the culture supernatants below 40 mN/m while 2 (Ab9-ES and Ab33-ES) produced ST above 40 mN/m (48.4 ± 1.9 mN/m and 46.4 ± 2.2 mN/m, respectively) (Table 2). This suggests that the bacterial isolates Ab9-ES and Ab33-ES are bioemulsifier producers and not biosurfactant producers (Patowary et al. 2017). Low-molecular weight biosurfactant reduced ST below 40 mN/m (Mulligan 2005) while high-molecular weight bioemulsifier formed and stabilized emulsions without remarkable ST reduction (Batista et al. 2006). Therefore, Ab9-ES and Ab33-ES were classified as bioemulsifier producers. Identification of strains Ab9-ES and Ab33-ES based on 16S rRNA gene sequences showed that they exhibit close homology (99% and 98%, respectively) to *Acinetobacter soli* KSM2 (KP297393.1). These study strains were therefore designated as *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES. The relatedness of these strains to other bioemulsifier-producing *Acinetobacter* sp. is illustrated in the constructed phylogenetic tree (Fig. 1).

Time course profile of growth and bioemulsifier production

Acinetobacter sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES were cultivated in MSM supplemented with 2% (v/v) olive oil as a carbon source for the production of bioemulsifier. The growth of these strains was monitored at 24-h intervals for 168 h to establish the exact phase at which optimum bioemulsifier production was achieved. The growth, ST and emulsifying activity profiles during cell cultivation are presented in Fig. 2. In both *Acinetobacter* sp., bioemulsifier production was found to be growth-linked, as a parallel relationship was observed between cell growth and emulsifying activity. Maximum cell growth ($OD_{600\text{ nm}} = 2.46 \pm 0.13$) at 144 h was attained by *Acinetobacter* sp. Ab9-ES. On the contrary, *Acinetobacter* sp. Ab33-ES recorded highest growth ($OD_{600\text{ nm}} = 2.28 \pm 0.1$)

Table 1 Growth and bioemulsifier or biosurfactant production by bacterial isolates cultivated in MSM supplemented with different carbon sources

Isolate	Source	Glucose		Diesel		Olive oil		Sunflower oil		Canola oil		Castor oil		Rice bran oil	
		G	B	G	B	G	B	G	B	G	B	G	B	G	B
SM5	EOM	+	–	–	–	+	+	+	–	+	–	+	–	+	–
SM7	EOM	+	–	–	–	+	+	+	–	+	–	–	–	+	–
SM19	EOM	+	–	–	–	+	+	+	–	+	–	–	–	+	–
SM20	EOM	+	–	–	–	+	+	+	–	+	–	–	–	+	–
SM23	EOM	+	–	–	–	+	+	+	–	+	–	–	–	+	–
Ab9-ES	SH	+	+	+	–	+	+	+	+	+	+	+	+	+	+
Ab22-ES	SH	+	+	+	–	+	+	+	–	+	–	+	–	+	–
Ab27-ES	SH	+	+	+	–	+	+	+	+	–	–	–	–	–	–
Ab33-ES	SH	+	+	+	–	+	+	+	+	+	+	+	+	+	+
Ab35-ES	SH	+	–	–	–	+	+	+	–	–	–	–	–	–	–
Ab38-ES	SH	+	+	–	–	+	+	+	+	+	–	–	–	+	–
Ab42-ES	SH	+	–	–	–	+	+	+	–	–	–	–	–	–	–
As5	WWTP	+	–	–	–	+	+	+	–	–	–	–	–	–	–
As33	WWTP	+	–	–	–	+	+	+	–	–	–	+	–	–	–
As53	WWTP	+	–	–	–	+	+	–	–	+	–	–	–	–	–
As72	WWTP	+	–	–	–	–	–	+	–	–	–	–	–	–	–
Ow9	WWTP	+	–	+	–	–	–	+	–	–	–	–	–	–	–
SE9	SP	+	–	–	–	+	+	+	–	–	–	–	–	–	–
SE13	SP	+	–	–	–	+	+	–	–	+	–	–	–	–	–
SE26	SP	+	–	–	–	+	+	+	–	+	–	–	–	–	–

2% (w/v, v/v); G growth, B bioemulsifier or biosurfactant production, + positive, – negative, EOM edible oil mill industry, SH slaughterhouse, WWTP wastewater treatment plant, SP soap industry

at 120 h. Optimum bioemulsifier production was recorded at different phases of growth in both studied *Acinetobacter* sp. In *Acinetobacter* sp. Ab9-ES, the EI₂₄ reached the highest value of $83.8 \pm 0.17\%$ at 168 h during stationary growth phase (Fig. 2a). This is in agreement with the findings of Pornsunthorntawee et al. (2008), where maximum biosurfactant production by *Bacillus subtilis* PT2 was observed during stationary phase. However, *Acinetobacter* sp. Ab33-ES attained highest emulsifying activity ($80.8 \pm 0.29\%$) at 120 h during late exponential phase (Fig. 2b). Maximum emulsifying activity ($60 \pm 1\%$) of *Aeribacillus pallidus* YM-1 has been reported during exponential phase (Zheng et al. 2012). Thereafter, the emulsifying activity decreased probably due to the degradation of the bioemulsifier. A slight reduction in ST from 70.9 ± 0.85 to 48.4 ± 1.9 mN/m and 70.0 ± 2.5 to 46.4 ± 2.2 mN/m was observed in the culture supernatants of *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively, as the incubation period increased from 24 to 168 h (Fig. 2). Ethanol precipitation of culture supernatants gave rise to the formation of dried whitish bioemulsifier powder, denoted as bioemulsifier XB9 and YB33 with yields of 4.52 ± 0.03 g/L and 4.31 ± 0.04 g/L

by *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively.

Composition of bioemulsifier

Preliminary studies on the chemical composition of the bioemulsifiers XB9 and YB33 were conducted. Results showed that XB9 consisted predominantly of carbohydrates (65.5%) and proteins (31%) while YB33 composed of carbohydrates (67.8%) and proteins (25%). This was further confirmed by FT-IR spectroscopic analysis (Fig. 3). The spectra obtained in both bioemulsifiers showed some similarities, especially broad absorption bands at 3275.86 cm^{-1} (XB9) and 3287.13 cm^{-1} (YB33), which revealed the presence of hydroxyl and amine groups typical of carbohydrates and peptides, respectively. Absorption peaks at higher frequencies have been attributed to polysaccharides (Stuart 2004). The presence of weak C–H stretching bands at 2926.78 cm^{-1} and 2978.14 cm^{-1} in YB33 characterized symmetric stretch ($\gamma\text{C-H}$) of CH_2 and CH_3 groups of aliphatic chains and O–H content of polysaccharides. In addition, the distinct absorption band at 1646.44 cm^{-1} in YB33 indicates stretching mode of CO–N bond of acetamido groups of *N*-acetylated

Table 2 Surface tension of bacterial culture grown in MSM supplemented with 2% (v/v) olive oil as a carbon source

Isolate	Source	ST (mN/m)
SM5	EOM	34.7 ± 1.5
SM7	EOM	39.1 ± 1.3
SM19	EOM	33.0 ± 2.1
SM20	EOM	35.6 ± 1.5
SM23	EOM	38.5 ± 1.2
Ab9-ES	SH	48.4 ± 1.9
Ab22-ES	SH	39.5 ± 1.9
Ab27-ES	SH	31.8 ± 2.2
Ab33-ES	SH	46.4 ± 2.2
Ab35-ES	SH	30.9 ± 1.4
Ab38-ES	SH	32.4 ± 2.5
Ab42-ES	SH	31.2 ± 1.5
As5	WWTP	30.9 ± 1.3
As33	WWTP	30.9 ± 1.2
As53	WWTP	37.4 ± 1.9
As72	WWTP	ND ^b
Ow9	WWTP	ND ^b
SE9	SP	30.2 ± 1.8
SE13	SP	33.7 ± 1.3
SE26	SP	32.8 ± 0.8

ND^b The value was not determined because no growth was observed in the production medium

sugars found mostly in microbial polysaccharides (Christensen 1989) as well as N–H bend of primary amines. Similarly, a band at 1634 cm⁻¹ in XB9 also corresponds to N–H bend. Weak absorption bands at 1459.62 cm⁻¹ and

1405.40 cm⁻¹ in XB9 correspond to C–H stretch of aliphatic amines. Furthermore, absorption bands at 1059.54 cm⁻¹, 978.69 cm⁻¹, 861.74 cm⁻¹, and 724.11 cm⁻¹ indicate C–N, C–H, N–H, and C–H groups, respectively, of aliphatic amines. Similarly, in the case of YB33, absorption bands stretching between 877.52 and 1453.07 cm⁻¹ are typical of C–H and C–N functional groups of amino sugars within the polysaccharides. The FT-IR spectra obtained in this study suggest that the bioemulsifiers XB9 and YB33 composed predominantly of polysaccharides and proteins. Based on the above results, both bioemulsifiers were tentatively identified as glycoproteins. Similar infrared spectra have been reported for bioemulsifier produced by *Ochrobactrum pseudintermedium* strain C1 (Bhattacharya et al. 2014) and *Solibacillus silvestris* AM1 (Markande et al. 2013).

Effect of hydrophobic substrates on emulsifying activity

The ability of the bioemulsifiers to form stable emulsions in the presence of wide range of hydrophobic substrates including edible oils (sunflower oil, canola oil, castor oil, and rice bran oil), aromatic hydrocarbons (benzene, xylene, and toluene), aliphatic hydrocarbons (heptane, dodecane, and hexane), and mixed hydrocarbons (motor oil, kerosene, and diesel) was investigated; results are presented in Fig. 4. The EI₂₄ values were compared to those of a known surfactant, SDS. The bioemulsifiers XB9 and YB33 exhibited higher emulsification activity in the presence of sunflower oil, canola oil, castor oil, and rice bran oil. However, less-stable emulsions were formed

Fig. 1 Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences of *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES showing their relatedness to other bioemulsifier-producing bacterial strains

Fig. 2 Time course profile of growth (OD), surface tension (ST), and emulsifying activity (EI₂₄) of (A) *Acinetobacter* sp. Ab9-ES and (B) *Acinetobacter* sp. Ab33-ES grown in MSM supplemented with 2% olive oil as a carbon source. Values indicate the average of triplicate values while the error bars represent the standard deviation

with other hydrocarbons, especially kerosene and diesel. This implied that the bioemulsifiers were able to emulsify edible oils efficiently. Among the edible oils, higher EI₂₄ values (79.6% and 67.9%) were recorded by bioemulsifiers XB9 and YB33, respectively, in the presence of sunflower oil. Biosurfactant produced by a novel *Pseudomonas* sp. 2B was reported to demonstrate maximum emulsifying activity (84%) in the presence of sunflower oil (Aparna et al. 2012). The emulsion-stabilizing capacity of an emulsifier is the ability to maintain at least 50% of the original volume of the emulsion for 24 h (Freitas et al. 2009). The emulsifying activities of the bioemulsifiers XB9 and YB33 were substrate-dependent. Most microbial surface-active compounds are substrate-specific and possess the ability to emulsify different hydrocarbons at varying rates (Luna-Velasco et al. 2007). Other researchers have reported similar results on the hydrocarbon substrate specificity of bioemulsifier (Sarubbo et al. 2001). The Bioemulsifiers XB9 and YB33 tested in this study were able to stabilize emulsions in the presence of edible oils, suggesting their potential applications as cleaning and emulsifying agents in the food industry as well as in the treatment of lipid-rich wastewater.

Effect of temperature, pH, and salinity on emulsifying activity

The effectiveness of bioemulsifier is dependent on emulsifying activity at a broad range of temperature, pH, and salinity (Markande et al. 2013). The influence of temperature, pH, and NaCl concentrations on the emulsifying activity of the bioemulsifiers is shown in Fig. 5. Both bioemulsifiers (XB9 and YB33) maintained stable emulsions at a broad range of temperature (4–121 °C). However, at 121 °C, YB33 recorded a lower EI₂₄ value (43.6%). The emulsifying activity increased from 4 to 50 °C (XB9) and 4 to 70 °C (YB33) (Fig. 5a). A slight decrease in EI₂₄ values was observed beyond these temperatures. This may be due to the denaturation of the protein component of the bioemulsifier during heat treatment. Highest emulsifying activity (77.3 ± 1.45%) was recorded at 50 °C by XB9 whereas; YB33 attained maximum activity (73.6 ± 0.46%) at 70 °C. Optimum temperature of 50 °C has been reported for high emulsifying activity of microbactan produced by *Microbacterium* sp. MC3B-10 (Camacho-Chab et al. 2013). Heat stability of liposan produced by *Candida lipolytica* has also been reported at temperature up to 70 °C (Cirigliano and Carman 1984). It is remarkable that bioemulsifier XB9 retained good emulsifying activity (52.6%) even at 121 °C. Such stability at extreme temperature has been reported for a lipopeptide biosurfactant produced by *Brevibacterium aureum* MSA 13 (Kiran et al. 2010). Hence, it could be concluded that bioemulsifiers are thermostable in nature.

The stability of the bioemulsifiers XB9 and YB33 was investigated at broad pH range (2.0–13.0) and emulsifying activity was measured (Fig. 5b). The bioemulsifiers XB9 and YB33 demonstrated emulsion-stabilizing capacity at pH ranges of 5.0–10.0 and 5.0–9.0, respectively. Maximum emulsifying activities (74.5% and 72%) were attained by XB9 and YB33, respectively, at pH 7.0 and these were found to be higher than those exhibited by synthetic surfactant, SDS. Pronounced reduction in activity was recorded at pH values above 7.0. This may probably be due to structural alterations of the bioemulsifiers under extreme pH conditions. Bioemulsifier from *Bacillus subtilis* has been reported to be active at pH 7.0 with a significant reduction in activity at pH values above 7.0 (Cooper and Goldenberg 1987). Optimum emulsifying activity (84%) of biosurfactant produced from a novel *Pseudomonas* sp. 2B was also observed at pH 7.0 (Aparna et al. 2012). The SDS maintained stable emulsion at a wide spectrum of temperature and pH with little differences in emulsifying activity.

Effect of salinity on the emulsifying activity of the bioemulsifiers was investigated at different NaCl concentrations (1–10%, w/v). The emulsifying activity increased as the NaCl concentration increases from 1 to 3% (XB9)

Fig. 3 FT-IR spectra of bioemulsifiers (a) XB9 and (b) YB33 produced by *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively

and 1–5% (YB33) (Fig. 5c). Maximum emulsifying activity (74.9%) was observed at NaCl concentration of 3% by XB9. On the contrary, YB33 demonstrated highest activity (61.2%) at NaCl concentration of 5%. Beyond these optimum salinity levels, the emulsifying activity declined drastically. Stability of these bioemulsifiers at NaCl concentrations up to 6% warrant intensified bioprospection in the remediation of polluted intertidal zone.

Conclusions

In the present study, glycoprotein bioemulsifier production from two indigenous *Acinetobacter* sp. isolated from lipid-rich wastewater was reported. The bioemulsifiers (XB9 and YB33) demonstrated higher emulsifying activity in the presence of edible oils including sunflower oil, canola oil, castor oil, and rice bran oil as well as stability

Fig. 4 Emulsifying activity of bioemulsifiers XB9 and YB33 in the presence of various hydrophobic substrates. Values indicate the average of triplicate values while the error bars represent the standard deviation

Fig. 5 Emulsifying activity of bioemulsifiers XB9 and YB33 in the presence of sunflower oil at different: (a) temperatures (b) pH and (c) NaCl concentrations. Values indicate the average of triplicate values while the error bars represent the standard deviation

at extreme temperature, moderate salinity, and pH. Hence, these bioemulsifiers represent a good candidate for various industrial and environmental applications, especially in the remediation of oil-contaminated wastewater.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication of this paper.

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