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Isolation and characterization of glycolipid biosurfactant produced by a *Pseudomonas otitidis* strain isolated from Chirimiri coal mines, India

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Abstract

Background: Biosurfactants (BSs) are amphipathic, surface active molecules produced by microorganisms and can reduce the surface tension and interfacial tension. The present study emphasizes the isolation and structural characterization of the BS produced by *Pseudomonas otitidis* P4.

Results: An efficient BS producing bacterial strain isolated from the unexplored coal mining site of Chirimiri, India was identified as *P. otitidis* P4 based on morphological, biochemical and 16S rRNA gene sequence analysis. The surface tension of the culture medium was reduced from 71.18 to 33.4 mN/m. The surface tension and emulsification activity of the BS remained stable over a wide range of temperature, pH and salt concentrations indicating its scope of application in bioremediation, food, cosmetics, and pharmaceutical industries. Structural attributes of BS were determined by biochemical tests, thin layer chromatography (TLC), Fourier transform infrared (FTIR) and nuclear magnetic resonance (^{1}H NMR) spectroscopy analyses, which confirmed the glycolipid nature of BS. Lipid and sugar fractions were the main constituents of the extracted BS. Thermogravimetric (TG) and Differential scanning calorimetry (DSC) analyses showed the thermostable nature of BS. As determined from TGA graph, the degradation temperature of biosurfactant was found to be 280 °C while complete weight loss was observed after 450 °C.

Conclusion: The BS isolated from *P. otitidis* P4 was identified as glycolipid and showed high emulsification activity and stability in a wide range of temperature, pH and salinity which makes it suitable for various industrial and environmental applications.

Keywords: *Pseudomonas otitidis*, Biosurfactant, Polycyclic aromatic hydrocarbons, Bioremediation

Background

Surfactants are one of the important group of chemical compounds having a wide range of industrial applications such as detergent, cosmetics, food, agricultural, pharmaceutical, paint, textile and paper (Vaz et al. 2012; Geys et al. 2014; Rebello et al. 2014). Majority of the currently used surfactants are chemically synthesized from petroleum-based resources. The use of these surfactants pose detrimental effects on the environment due to their partially biodegradable and ecotoxic nature (Vaz et al. 2012; Rebello et al. 2014). Environmental awareness have increased the demand for bio-based surfactants as they could reduce the level of synthetic surfactants prevalence in environment and also the toxicity associated with it (Harshada 2014). Biosurfactants (BSs) are amphiphilic molecules comprising of hydrophilic and hydrophobic moiety. The hydrophilic head is usually composed of carbohydrate (mono-/di-/poly-saccharides), amino acid, peptide anions or cations or phosphate group. The hydrophobic tail is generally consist of a long chain of saturated, unsaturated, linear or branched fatty acid (Banat et al. 2010; Smyth et al. 2010a, b). These

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Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 2 of 16

surface-active molecules can lower the surface and interfacial tension on liquid–liquid or liquid–solid phase boundaries (Pacwa-Plociniczak et al. 2011; Ramani et al. 2012). These compounds can be synthesized by a variety of microorganisms (bacteria, yeasts and filamentous fungi) (Sanchez et al. 2007; Díaz De Rienzo et al. 2016) utilizing both water-immiscible hydrocarbons i.e., plant/animal derived oil, n-alkanes, polycyclic aromatic hydrocarbons (PAHs) and/or water-soluble compounds i.e., glucose, fructose, sucrose, xylose, galactose, mannitol, glycerol, ethanol. (Díaz De Rienzo et al. 2016; Desai and Banat 1997; Mukherjee et al. 2006; Sajna et al. 2015).

BSs produced by microorganisms represent a heterogeneous group of secondary metabolites (Chandrasekaran and Bemiller 1980), play major roles in the survival of the producing microorganisms by increasing the bioavailability of hydrophobic substrates (facilitating nutrient transport), interfering in microbe–host interactions and quorum sensing mechanisms (bacterial pathogenesis), or by acting as antimicrobial, insecticidal, antibiofilm and anti-adhesive agents (Rodrigues et al. 2006; Marchant and Banat 2012; Inès and Dhouha 2015).

BSs can be categorized based on the following characteristics: ionic charges (anionic, cationic, non-ionic and neutral), molecular weight (high molecular and low molecular weight), and secretion type (intracellular, extracellular and adhered to microbial cells) (Inès and Dhouha 2015). On the basis of chemical structures, microbial derived BSs can be classified as glycolipids, polypeptides, lipoproteins, phospholipids, fatty acids, neutral lipids, particulate and polymeric surfactants (Geys et al. 2014; Gudiña et al. 2013; Healy et al. 1996). Among these groups, the most common BSs that have been studied till date, are the glycolipids and the lipopeptides produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* strains, respectively (Gudiña et al. 2015). Glycolipids, the most popular biosurfactants, are composed of a hydrophobic moiety (long chain fatty acid) in combination with a hydrophilic moiety of carbohydrate (Muller et al. 2011).

BSs offer several benefits over their chemically synthesized counterparts which include high biodegradability, low toxicity and can be produced from renewable substrates (Kiran et al. 2010). Furthermore, BSs are usually effective at extreme pH, temperatures and salt concentrations and have less impact on the environment than chemical surfactants (Gudiña et al. 2013). Due to these unique properties, BSs can be used in several industries including food, beverages, cosmetics, agrochemicals, petrochemicals, petroleum, metallurgy, mining and many more. Besides pharmaceutical and agricultural applications, some other interesting applications of BSs include bioremediation of pollutants, clean-up of oil containers and microbial enhanced oil recovery (Sajna et al. 2015;

Perfumo et al. 2009; Das et al. 2008; Gharaei-Fathabadp 2011; El-Sheshtawy et al. 2015).

In general, biosurfactant producing microorganisms improve the bioavailability of hydrocarbons by reducing the surface tension, interfacial tension (IFT) and critical micelle concentration (CMC) in both aqueous solutions and hydrocarbon mixtures (through emulsification by micelles formation) and thus helps in bioremediation of persistent organic pollutants including (Desai and Banat 1997; Das et al. 2008; Maier 2003). PAHs are a group of the petroleum hydrocarbons known for their mutagenic, carcinogenic, teratogenic, endocrine-disrupting properties (Samanta et al. 2002; Liu et al. 2012; Xia et al. 2012). The US Environmental Protection Agency (US EPA) has listed 16 PAHs as priority pollutants for remediation (Sayara et al. 2011). Pyrene (a four-ring PAHs) is one of the most abundant high-molecular weight PAHs present in the environment and due to its low bioavailability recalcitrant property, removal from the environment is a challenging task. Degradation of pyrene or other PAHs occurs mostly by microbial means and many bacterial species have been involved in this process (Zhong et al. 2011; Kumari et al. 2013; Ma et al. 2013; Ghosh et al. 2014).

The present work intends to explore the production, structural characterization and stability of BS by a pyrene degrading bacteria isolated from coal mining site of Chirimiri, Chhattisgarh, India. The effects of various carbon and nitrogen sources on BS production was also investigated. To the best of our knowledge this is the first report of production and characterization of glycolipid BS by a pyrene degrading bacterium *Pseudomonas otitidis* P4.

Methods

Enrichment and isolation of strains

To enrich selectively for pyrene degrading bacteria, 5.0 g of soil sample was added into an Erlenmeyer flask containing 50 mL carbon free mineral medium (CFMM) supplemented with 100 mg/L of pyrene was incubated at 30 ± 2 °C with shaking at 150 rpm for 7 days. CFMM contained the following (g/L); NH_4NO_3—3.0, KH_2PO_4—2.2, $Na_2HPO_4 \cdot 12H_2O$—0.8, $MgSO_4 \cdot 7H_2O$—0.1, $CaCl_2 \cdot 2H_2O$—0.05, $FeCl_3 \cdot 6H_2O$—0.05 at pH 7.0. For each subculture an aliquot of 5 mL enriched culture was transferred into another Erlenmeyer flask containing 45 mL fresh CFMM with increasing concentration of pyrene. CFMM flasks containing only pyrene served as negative controls. This step was repeated eight times to obtain a pyrene degrading enriched consortium. Positive growth was determined by an increase in the turbidity and change in colour of medium containing samples as compared to the control. From enriched culture, 0.1 mL

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 3 of 16

of 10-fold serially diluted culture broth was spread over a CFMM agar plate. The plate was sprayed with pyrene (1000 mg/L) for the isolation of pyrene-degrading bacteria. The bacterial colonies surrounded by a clear zone were scored positive (John et al. 2012). All the isolates were purified through subculturing, maintained on CFMM supplemented with pyrene and preserved as glycerol stocks (15 %, v/v).

Growth of the selected isolates was studied by inoculating each isolate in culture tube containing 20 mL of the CFMM supplemented with pyrene (1000 mg/L) and incubating at 30 ± 2 °C for 7 days. The ability of each isolate to utilize pyrene was indicated by increase in turbidity of the medium measured at 600 nm using a UV spectrophotometer (Shimadzu UV-1800).

Characterization and identification of isolate
Phenotypic characterization
The selected isolate P4 was preliminary characterized on the basis of colony morphology (color, shape, size, margin, texture, elevation) following their growth on nutrient agar medium (NAM) plates (HiMedia). Physiological and biochemical tests were performed as described in Bergey's manual of systematic bacteriology (Holt et al. 2000).

Phylogenetic analysis by 16S rRNA gene sequences
The identity of the selected isolate was confirmed based on 16S rRNA gene sequence analysis. Genomic DNA was isolated from the bacterial sample using Chromous bacterial genomic DNA isolation kit. The universal primers of 16S rDNA fragments, 27F and 1492R, were used to amplify the 16S rDNA. The sequences of primers were as follows: (27F) AGAGTTTGATCMTGGCTCAG and (1492R) TACGGYTACCTTGTTACGACTT (Chromous Biotech, Bangalore). A phylogenetic tree was constructed using partial 16S rRNA gene sequences of the isolate and the other sequences, closely related with the reference strain, obtained from NCBI database. Clustal Omega was used for multiple sequence alignment of sequences. Neighbor joining tree was constructed with complete deletion using bootstrapping at 10,000 bootstraps trials with Kimura-2 parameter using MEGA 6.0 software (Das and Tiwary 2013). The isolate P4 was finally identified as *P. otitidis*. The sequence of the 16S rRNA gene of the strain P4 is available in NCBI under the GenBank accession number KP877124.

Screening for BS production
The BS producing ability of the isolate P4 was studied using standard parameters, described earlier such as foaming test (Ferhat et al. 2011), drop collapsing test (Tugrul and Cansunar 2005), emulsification index (Deepak and Jayapradha 2015), BATH assay (Rosenberg 1984), cetyl trimethyl ammonium bromide (CTAB) agar test (Satpute et al. 2010). Sodium dodecyl sulphate (SDS) and distilled water were used as positive and negative controls, respectively.

Determination of surface tension
The surface tension of the cell-free medium was determined at different intervals of incubation of the strain P4 in CFMM with pyrene using stalagmometer at a constant temperature 30 ± 2 °C and calculated by the following formula:

$$\gamma 0 = \gamma \, (n/n0)$$

where $\gamma 0$ = surface tension of reference solvent (for water 71.18 ± 0.02 mN/m), γ = surface tension of the BS solution, $n0$ = drop numbers of the reference solvent, n = drop numbers of the BS solution.

All surface tension values were recorded in triplicates (Mishra et al. 2014).

Isolation and purification of BS
For extraction of BS, Erlenmeyer flasks containing 500 mL carbon-free mineral medium (CFMM) supplemented with 1000 mg/L pyrene was inoculated with 10 mL of freshly grown bacterial culture and shaken incubated at 150 rpm for 7 days at 30 ± 2 °C. After incubation the culture medium was centrifuged at 10,000 rpm at 4 °C for 15 min to remove bacterial cells. The supernatant was collected and pellet was discarded. The pH of the supernatant was adjusted to 2.0 with 6.0 N HCl before incubating it at 4 °C for overnight. The crude precipitate was collected by centrifuging the acidified solution at 4 °C and 10,000 rpm for 15 min. The pellet obtained was dissolved in 0.1 M $NaHCO_3$ and extracted by vortexing with an equal volume of chloroform and methanol (2:1, v/v). The lower organic phase containing BS was collected by separating funnel. This extraction process was repeated three times until honey color disappeared (Mishra et al. 2014). The crude BS was dialyzed against distilled water for 2 days at 4 °C in a dialysis membrane (molecular weight cutoff 7000 Da, HiMedia, India). The dialyzed sample was dried at 45 °C and the weight of BS was expressed in terms of g/L. Purified BS was stored at 4 °C to use for further physico-chemical characterization.

Effect of carbon and nitrogen sources on BS production
Effect of carbon sources on the yield of BS was evaluated by growing the isolate in different carbon sources viz., sodium acetate, glycerol, L-ribose, glucose, fructose, mannitol, sucrose, starch, diesel, phenanthrene and pyrene. The carbon source was added into medium

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 4 of 16

at a concentration of 2 % (w/v or v/v). After optimization of carbon source, most suitable carbon source was used for evaluation of nitrogen source on BS production. Different nitrogen sources such as ammonium nitrate, ammonium sulphate, sodium nitrate, urea, beef extract, peptone, yeast extract and amino acids (L-aspartic acid, L-asparagine, L-isoleucine, L-glutamine, tyrosine) were evaluated for the production of BS in three different concentrations (3, 0.3 and 0.03 % w/v). Effect of nitrogen starvation was also studied where no nitrogen salts were added to the mineral medium. Extraction of BS was done by the method as described above and the yield of BS and biomass were expressed in terms of g/L.

Study of BS stability

The effect of various environmental factors such as temperature, pH and salt concentrations on the stability of the BS was evaluated as described by Obayori et al. (2009). Thermal stability of the BS was determined by incubating the culture broth supernatant at a wide range of temperatures (0–100 °C) for 30 min. The effect of pH was determined by varying the pH (3.0–11.0) of the supernatant and determining the emulsification index at a constant temperature of 30 ± 2 °C. The effect of salinity on emulsification index was determined by adding various concentrations of NaCl (2.0–10.0 %, w/v) to the supernatant and determining the emulsification index after 30 min at optimum temperature.

Characterization of BS
Determination of ionic character

The ionic character of the BS was determined by agar double diffusion technique as described by Van Oss (1968). Agar plate of low hardness (1 % agar) with two regularly spaced rows of wells were made with the help of borer. The wells in one row were filled with the BS solution and those on the other side were filled with a pure compound of known ionic charge. Sodium dodecyl sulphate (SDS) and CTAB at a concentration of 25 mM were chosen as the anionic and cationic substances, respectively. The appearance of any precipitation line between the wells which is an indicative of the ionic character of BS, was monitored over 48 h of incubation period at room temperature.

Compositional analysis of BS

The total carbohydrate content of BS was assayed by the phenol sulfuric acid method (Dubois et al. 1956) using D-glucose as the standard. The lipid content was determined by the colorimetric method (Van Handel 1985) using oleic acid as the standard. Total protein content was measured by Bradford method (1976), using bovine serum albumin as the standard.

Thin layer chromatography (TLC)

The preliminary characterization of BS was performed by TLC analysis. BS was separated on a silica gel plate using chloroform:methanol:glacial acetic acid (65:15:4, v/v/v) as mobile phase. The resulted spots were visualized by spraying different colour developing reagents. Protein and carbohydrate spots were visualized by spraying ninhydrin reagent and Molish reagent (5 % 1-naphthol in alcohol), respectively, whereas iodine crystals were used to detect lipid fraction of BS. Plates were heated at 110 °C for 10 min after application of the spraying agents.

Fourier transform infrared spectroscopy (FTIR) analysis and ^{1}H Nuclear magnetic resonance (NMR) analysis

To identify various types of chemical bonds and functional groups and characterize the components of BS, FTIR spectroscopy in the range of 4000–400 cm^{-1} was performed at a resolution of 4 cm^{-1} using a FTIR spectrophotometer (Shimadzu IR affinity-I, Japan).

Further characterization of BS was done with NMR spectroscopy. The extracted BS was re-dissolved in deuterated chloroform (CDCl$_3$) and the respective ^{1}H NMR spectra was recorded at 25 °C using an NMR spectrophotometer (Bruker Avance III, 400 MHz).

Thermal gravimetric analysis (TGA) and differential scanning calorimetric (DSC) analyses

To determine the decomposition temperature of BS, TGA analysis was carried out using Perkin Elmer, Diamond TG/DTA system. Approximately, 5.0 mg of BS sample was loaded in the sample pan and heated over a temperature range of 50–700 °C at a constant temperature gradient of 10 °C/min under nitrogen atmosphere (injected at a flow rate of 100 mL/min). For DSC analysis, 6.8 mg of sample was precisely weighed and loaded in the sample pan of the DSC instrument (Mettler Toledo DSC 822e) and heated from −50 to 400 °C at a constant heating rate of 10 °C/min using alumina (Al$_2$O$_3$) as internal standard under nitrogen atmosphere.

Determination of pyrene degradation (quantitative estimation)

For degradation studies, desired concentration of pyrene (1000 mg/L) was taken in pre-sterilized conical flasks (capacity 100 mL). After acetone evaporation, 45 mL of CFMM broth was inoculated with 5 mL of enriched bacterial culture. A set of uninoculated flasks containing only pyrene served as the abiotic control to determine the PAH loss, if any. The autoclaved bacterial cultures raised in the same medium were used as the biotic controls. All flasks were kept for incubation at 30 ± 2 °C in the dark on a rotary shaker at 150 rpm. The residual amount of pyrene was extracted at the end of 10th day, with equal

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 5 of 16

amount of ethyl acetate and quantified spectrophotometrically by comparing the optical density (OD) of different samples at λ_{max} (λ_{max} for pyrene = 273 nm) with the standard curve of pyrene (Das and Mukherjee 2007). Degradation percentage was calculated by the following formula:

$$\% \text{ degradation} = \left(\frac{I_0 - I_f}{I_0} \right) - \left(\frac{I_0C - I_fC}{I_0C} \right) \times 100$$

I_0 = Initial concentration in test
I_f = final concentration in test
I_0C = Initial concentration in control
I_fC = final concentration in control.

Gas chromatography–mass spectrometry (GC–MS) for the identification of the pyrene degradation products

The residual amount of pyrene and degradation metabolites were extracted at the end of 10th day, with equal amount of ethyl acetate. The organic phases were combined and evaporated at 40–45 °C until the volume was reduced to approximately 1 mL. An aliquot of 1 mL of this solution was then diluted in 10 mL ethyl acetate for GC–MS analysis. The sample was derivatized with in BSA + TMCS (BSA + TMCS:Sample, 5:1) and heated up to 70 °C for 30 min. GCMS analysis was performed on Agilent 7890A, 6890 N, Thermo Trace GC Ultra (GC)-Thermo DSQ II (MS) equipped with a DB-5 MS column (30 mL × 0.25 mm ID × 0.25 μm film thickness, Phenomenex, Inc., Torrance, CA, USA). The split less sample was injected onto a capillary column (DB-5 MS). Helium was the carrier gas at a rate of 2 mL/min. The column temperature was started at 70 °C for 2 min, programmed to 250 °C at a rate of 3 °C/min, and held at 320 °C for 10 min. Injector and analyzer (detector) temperature were 270 and 280 °C, respectively. The mass spectrometer was operated in electron impact (EI) ionization mode with electron energy of 70 eV. Solvent cut-off was set from 0 to 5 min. The injection volumes, MS scan range were 1 μL and 30–600 m/z, respectively. GC–MS library (NIST 2011) search was used to confirm the metabolites of pyrene degradation.

Results and discussion

Enrichment, isolation and characterization of the isolates

Out of 10 bacterial strains isolated from coal mining site of Chhattisgarh, one most potent strain named as P4 (enriched up to 2000 mg/L of pyrene) was selected for further studies. The isolate P4 showed the rapid growth in CFMM supplemented with pyrene (OD_{600} = 1) after 24 h of incubation compared to other isolates. The strain was characterized on the basis of morphological and biochemical parameters and was found to be a Gram negative rod, endospore forming, cream/slimy, circular and

motile bacterium (Table 1). On the basis of partial 16S rRNA gene sequence analysis, the isolated strain P4 was further identified as a member of the genus *Pseudomonas* revealing 100 % identity to *P. otitidis*. Comparison with NCBI sequences after neighbor-joining analysis of different *pseudomonads* indicated that the closest relatives of this strain (P4) were *Pseudomonas guezennei* strain TIK669 (99 %), *P. aeruginosa* strain P60 (98 %) and *Pseudomonas resinovorans* strain AF22 (98 %). Figure 1 shows a relative position of strain P4 in the phylogenetic tree.

There is no report available in the literature on PAHs degradation and biosurfactant production capacity of *P. otitidis* till date. Nevertheless, other species/strains

Table 1 Phenoytypic and biochemical characteristics of the strain P4

Biochemical characteristics	Strain P4
Shape	Rod
Gram reaction	–
KOH test	+
Endospore formation	+
Acid fast staining	–
Motility	+
Catalase test	+
Oxidase test	+
Nitrate reduction test	+
Oxidation and fermentation of glucose	
Without paraffin oil	–
With paraffin oil	–
Determination of carbohydrate fermentation and hydrogen sulfide production	
Glucose	–
Sucrose	–
Lactose	–
H_2S production	–
Gas production	–
Indole test	–
Methyl red (MR)	–
Voges Proskauer (VP)	–
Citrate test	+
Growth of mannitol salt agar	+
Starch hydrolysis	+
Urea hydrolysis	–
Gelatin hydrolysis	+
Casein hydrolysis	+
Deamination of phenylalanine	–
Decarboxylation of	
Ornithine	–
Arginine	–
Lysine	+
Growth on different hydrocarbons (BTEX)	+

BTEX Benzene, Toluene, Ethyl benzene, Xylene

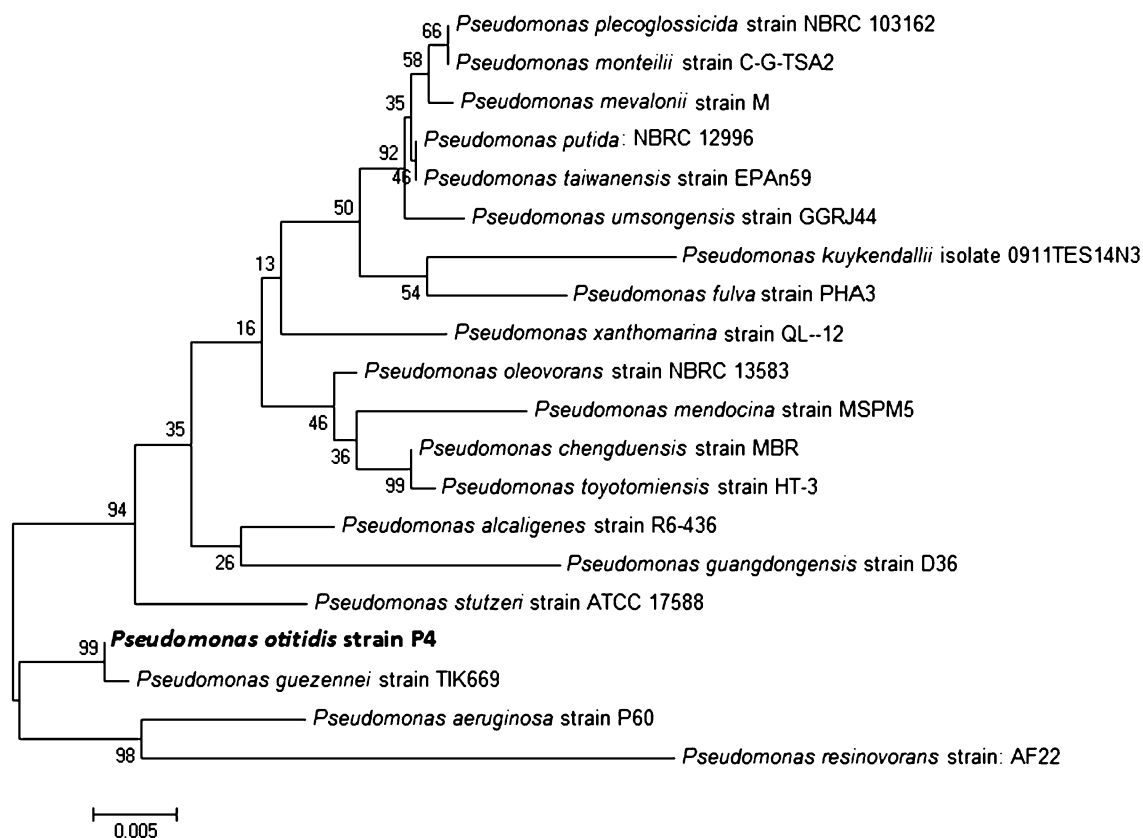Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 6 of 16

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequencing of strain P4 obtained using the neighbor-joining method, showing the position of isolated strain. *Scale bar* equals approximately 0.005 substitutions per nucleotide position

belonging to the genus *Pseudomonas* have been reported to degrade a wide range of recalcitrant xenobiotics (Mulet et al. 2011) including PAHs such as pyrene (Ma et al. 2013). *Pseudomonads* are also reported to produce a wide range of BS including glycoplipids (mainly rhamnolipids), lipopeptides and viscosins having diverse industrial applications, e.g., in the bioremediation, pharmaceutical, food-processing petroleum, cosmetic and industries (Gudiña et al. 2015; Ben Belgacem et al. 2015).

Screening for BS production

The strain P4 was screened by various methods including drop collapse assay, CTAB blue agar test, emulsification index assay and BATH assay. After shaking the surfactant solution, it showed a good foaming ability. Complete disappearance of foam in the solution was detected after 1.5 h. Emulsification of hydrocarbons is also used as an indirect method of testing the surface activity (Belcher et al. 2012). Emulsion formation is a stable interaction of the hydrophobic and the hydrophilic phase and is greatly depends on the solvents used (Deepak and Jayapradha 2015; Mahanty et al. 2006; Ron and Rosenberg 2002). For

the emulsification test vegetable oil, xylene and diesel were used as substrates in which vegetable oil served as the best substrate (60.7 %) followed by xylene (41.5 %) and diesel oil (13.6 %). Ferhat et al. (2011) have found that the BS produced by *Brevibacterium* 7G and *Ochrobactrum* 1C could not emulsify the kerosene and diesel oil, but emulsification were recorded for both BSs when motor oil (approx. 52 and 45 %) and sunflower oil (approx. 93 and 90 %) were used. In contrast to the report (Ferhat et al. 2011), Abouseoud et al. (2008) have reported diesel oil and kerosene (55 %) as the best substrates for emulsification and sunflower oil (45 %) as less efficient substrate for emulsification. Our study indicated that the biosurfactant produced by *P. otitidis* have the ability to emulsify different hydrocarbons and thus it could increase the availability of the recalcitrant hydrocarbons. This property demonstrates the applicability of this strain against diverse hydrocarbon contamination (Aparna et al. 2012). Drop collapse assay mainly depends on the destabilization of liquid droplets by surfactants. In this assay, water drop did not collapse and remained in the form of a bead, however, the positive control (SDS) and the BS solution
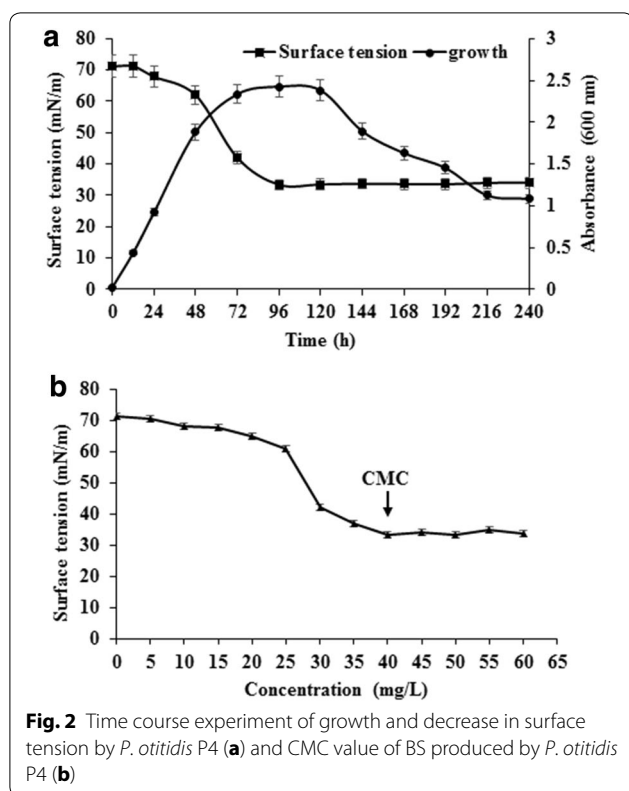
Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 7 of 16

Fig. 2 Time course experiment of growth and decrease in surface tension by *P. otitidis* P4 (**a**) and CMC value of BS produced by *P. otitidis* P4 (**b**)

collapsed (Additional file 1: Figure S1). These qualitative experiments suggested the surface and wetting activities of the BSs (Youssef et al. 2004). CTAB–methylene blue agar test is a simple method for the detection of anionic surfactants. The strain P4 showed a positive reaction for biosurfactant production on CTAB–methylene blue agar medium, as the presence of blue halos around the bacterial colonies were observed after 48 h of incubation at 37 °C. Relative hydrophobicity of the bacterial cell surface was examined by BATH assay which demonstrated that the strain possessed a higher value of cell surface hydrophobicity (69.3 %). The emulsification activity and cell surface hydrophobicity was directly proportional to the production of BS. All these screening methods confirmed the production of BS by our pyrene degrading bacteria. Several other authors (Ferhat et al. 2011; Abouseoud et al. 2008; Youssef et al. 2004) have advocated the use of these screening techniques for the identification of microorganisms with potential to produce BSs.

Production of BS and determination of surface tension
The surfactant production was established from the late exponential phase until the end of the stationary phase as the maximum reduction of surface tension occurred during this period of incubation. Ron and Rosenberg (2002) have also reported that surfactants are generally
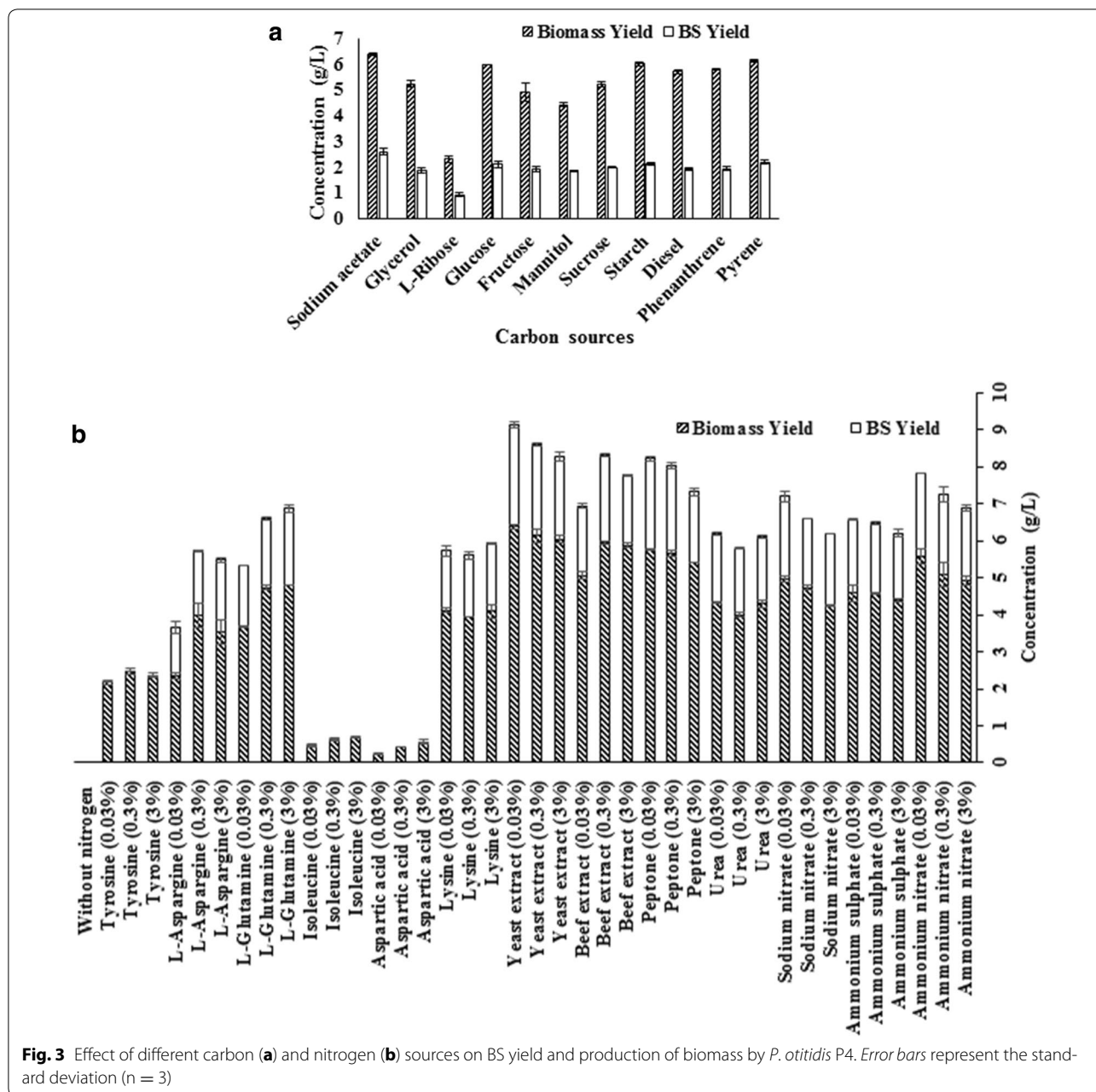
produced during the logarithmic and stationary phase of growth and the release of cell-bound surfactant in the growth medium results in the reduction in surface tension even after the stationary phase. The BS produced by the *P. otitidis* P4 showed excellent surface tension reducing capacity as it reduced the surface tension of the medium from 71.18 to 33.4 mN/m (Fig. 2a). The CMC of the BS was found to be approximately 40 mg/L (Fig. 2b). This result is comparable to the recent reports on BS production by *P. aeruginosa* #112 and PA1 (Gudiña et al. 2015; Mendes et al. 2015). According to the previous reports, the BSs produced by bacterial strains for instance *P. aeruginosa* are more effective in decreasing the surface tension of the medium. Most of the BSs produced by this bacterium have shown to reduce the surface tension to values around 27–28 mN/m (Gautam and Tyagi 2000).

Effect of carbon and nitrogen sources on BS production
Carbon and nitrogen are the most important factor in the BS production medium, as it is necessary for microbial growth and the synthesis of enzymes and proteins depends on it. Carbon and nitrogen sources used in production medium have a great impact on composition as well as on the yield of BSs (Desai and Banat 1997; Perfumo et al. 2009; Das et al. 2008; Tugrul and Cansunar 2005; Mendes et al. 2015).

In the present study, the isolate P4 was able to utilize all the tested carbon and nitrogen sources for growth but the production of BS was not seen in the amino acids (isoleucine, tyrosine and aspartic acid). Sodium acetate (2 %) gave the best biosurfactant yield while L-ribose gave the least yield among the carbon sources evaluated (Fig. 3a). Preference of carbon source for the production of BSs by microorganisms appears to be strain dependent, as different strains produce BSs in different carbon sources which could be either water-miscible or water-immiscible substrates (Díaz De Rienzo et al. 2016; Desai and Banat 1997; Sajna et al. 2015; Wu et al. 2008; Kumari et al. 2012).

The results for evaluation of nitrogen source revealed that yeast extract (0.03 %) served as the best nitrogen sources for BS production by *P. otitidis* P4 strain (Fig. 3b). It was also observed that the production of biosurfactant enhanced when grown in nitrogen limiting condition. No growth and BS production was observed in the medium without any nitrogen source (nitrogen starvation) in the present study. Zavala-Moreno et al. (Zavala-Moreno et al. 2014) have reported that the production of glycolipids enhanced when grown in nitrogen limiting or starving conditions. According to Maneerat (2005), production of surface active compounds increases when the concentration of nitrogen depleted in the medium which is due to reduction in the activity

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 8 of 16

Fig. 3 Effect of different carbon (**a**) and nitrogen (**b**) sources on BS yield and production of biomass by *P. otitidis* P4. *Error bars* represent the standard deviation (n = 3)

of isocitrate dehydrogenase. This enzyme is NAD and NADP dependent which catalyzes the oxidation of isocitrate to 2-oxoglutarate in the citric acid cycle. Isocitrate and citrate starts accumulating in the mitochondria due to decline in the activity of isocitrate dehydrogenase, and further transported to the cytosol. In the cytosol, citrate synthase converts citrate into oxaloacetate and acetyl-coA which is the processor of fatty acid synthesis and hence BS production increases.
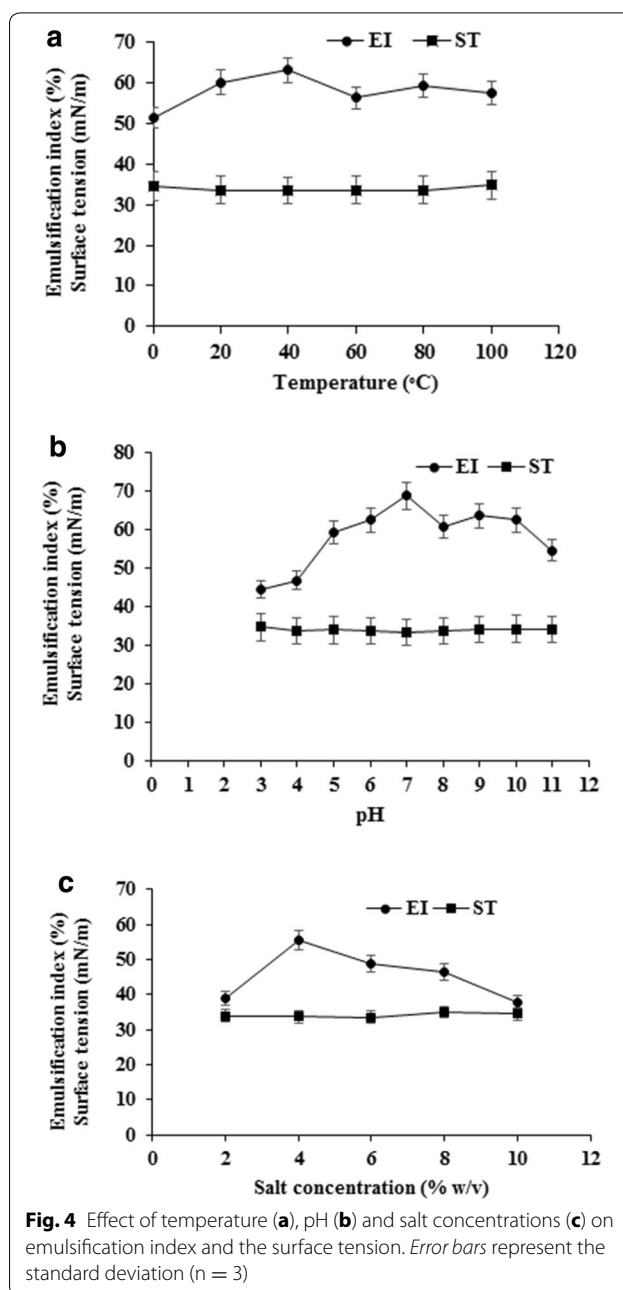
Isolation and purification of BS

The dried yield of BS and biomass in optimized conditions were found to be 2.75 ± 0.07 and 6.38 ± 0.05 g/L, respectively, after 7 days of incubation. The dried BS isolated from *P. otitidis* appeared as off white powder and was soluble in various organic solvents such as methanol, ethyl acetate, chloroform and hexane. Kumari et al. (2012) have reported the production of 0.05 and 0.01 g/L of BS by the two bacterial strains of *Pseudomonas* sp.

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 9 of 16

BP10 and *Rhodococcus* sp. NJ2 with high emulsification activity of 75 and 35 %, respectively. Ferhat et al. (2011) have also reported the production of 2.0 and 2.5 g/L of BS on the basis of dry weight for *Brevibacterium* 7G and *Ochrobactrum* 1C strains, respectively.

Study of BS stability

Application of BSs in different fields greatly depends on its stability under different environmental conditions such as temperature, pH and salinity. At different range of temperatures, no significant changes were observed in the emulsification index after incubation period of 30 min. The BS produced by *P. otitidis* P4 was found to be thermostable because heating at 80–100 °C caused no significant effect on surface tension and emulsification activity (Fig. 4a). Temperature is one of the most important parameter that significantly influence the growth of microorganism and thus the biosurfactant production. Any drop or rise in emulsification activity under extreme temperature could be due to some structural alteration in the BS molecule (Aparna et al. 2012). BS stability at extreme temperatures was reported by Aparna et al. (2012) and Kiran et al. (2010) for *P. aeruginosa* strain and *Brevibacterium aureum* MSA13, respectively. The results of thermostability of BS produced by *P. otitidis* P4 shows the potential application of the biosurfactant in various industries i.e., pharmaceutical, food, and cosmetics as well as in microbial enhanced oil recovery (MEOR) where heating step is very important (El-Sheshtawy et al. 2015; Abouseoud et al. 2008). The surface tension and the emulsification activity of the BS remained stable at a wide range of pH (3–11). The highest emulsification (68.7 %) was found at neutral pH (pH = 7) although a significant stable emulsification activities were also observed at acidic pH (pH = 3, 44.4 %) as well as alkaline pH (pH = 11, 54.5 %) (Fig. 4b). A drop in emulsification under extreme pH may be due to partial precipitation of the BS (Abouseoud et al. 2008). The surfactant was able to withstand a broad salt concentration range from 2.0 to 10.0 % and the maximum emulsification activity was observed at 4 % NaCl (Fig. 4c). The reason behind the stable surface tension at extreme salt concentration is well explained by Helvaci et al. (2004). They have described that the electrolytes present in the culture medium can directly affect the carboxylate groups of the glycolipids. At alkaline pH, the solution interface has a net negative charge due to the presence of ionized carboxylic acid groups. In the presence of NaCl, Na^+ ions shields this negative charge in an electrical double layer, causing the development of a close-packed monolayer and subsequently a reduction in surface tension values. Stability studies showed that the BS retained its activity at extreme temperature, pH and salt concentrations. Therefore, the

Fig. 4 Effect of temperature (**a**), pH (**b**) and salt concentrations (**c**) on emulsification index and the surface tension. *Error bars* represent the standard deviation (n = 3)

BS exhibited potential for various industrial applications. Several other reports on the stability of BSs at extreme conditions are listed in Tables 2, 3 and 4.

Characterization of BS

Determination of ionic character

Agar double diffusion tests revealed the appearance of precipitation line between the cationic compound selected (barium chloride) and the BS produced by *P. otitidis*, while no line was formed between the BS and

Table 2 Emulsification and surface tension reducing activity of the biosurfactant produced by different strains over a wide range of temperature

Strain /organism	Temp. (°C)	E_{24} (%)	Surface tension (mN/m)	References
P. otitidis P4	0	51.4	34.5	This study
	20	60	33.5	
	40	63	33.4	
	60	56.2	33.6	
	80	59.3	33.5	
	100	57.5	34.7	
Ochrobactrum 1C	20	95.23	32.0	Ferhat et al. (2011)
	50	72.72	32.0	
	70	85.10	31.0	
	100	94.0	30.0	
Pseudomonas sp. 2B	4	64.0	30.0	Aparna et al. (2012)
	30	83.0	28.0	
	50	81.0	28.0	
	75	80.0	28.0	
	100	78.0	28.0	
	121	75.0	28.0	
Bacillus siamensis RT10	0	49.0	39.0	Varadavenkatesan and Murty (2013)
	20	67.0	38.5	
	30	70.0	38.0	
	40	69.9	37.9	
	50	68.0	38.0	
	60	67.5	38.0	
	70	67.5	38.0	
	80	67.0	38.5	
	90	65.0	38.5	
	100	66.0	38.0	
	121	67.0	38.0	

Table 3 Emulsification and surface tension reducing activity of the biosurfactant produced by different strains over a wide range of pH

Strain/organism	pH	E_{24} (%)	Surface tension (mN/m)	References
P. otitidis P4	3	44.4	34.7	This study
	4	46.8	33.8	
	5	59.3	33.9	
	6	62.5	33.5	
	7	68.7	33.4	
	8	60.6	33.5	
	9	63.6	34.1	
	10	62.5	34.2	
	11	54.5	34.0	
Ochrobactrum 1C	4	70.58	31.0	Ferhat et al. (2011)
	7	93.0	31.0	
	9	95.0	30.5	
	11	95.0	30.5	
Pseudomonas sp. 2B	2	57.0	34.19	Aparna et al. (2012)
	4	67.0	31.48	
	6	73.0	30.39	
	7	84.0	28.39	
	8	80.0	30.0	
	10	75.0	34.0	
	12	69.0	35.0	
Bacillus siamensis RT10	2	56.0	39.0	Varadavenkatesan and Murty (2013)
	4	65.5	39.0	
	6	70.0	39.0	
	7	69.0	39.0	
	8	65.0	39.0	
	10	67.5	39.0	
	12	60.0	38.8	

the anionic compound (SDS) suggesting that the BS extracted from *P. otitidis* was anionic in nature.

Compositional analysis of BS

Biochemical analysis revealed that the BS produced by the strain P4 was mainly composed of lipid and carbohydrate. The carbohydrate content was found to be 386.25 μg/mL whereas the lipid content was 0.381 mg/g equivalent to oleic acid. A minor fraction of protein (0.83 μg/mL) detected in the purified sample was possibly due to the residual cell debris that might have coprecipitated with the BS during extraction process.

Thin layer chromatography (TLC)

TLC analysis of purified BS showed a single spot in replica plates with R_f value of 0.9. The BS fraction showed positive reaction with Molish reagent and iodine vapour,

indicating the presence of carbohydrate and lipid moieties. When sprayed with ninhydrin reagent, no spots were detected indicating the absence of free amino acids in the BS. The above result of TLC analysis demonstrated the glycolipid nature of BS. Similar reports of the production of glycolipids BS by *P. aeruginosa* (R_f value 0.85) and *P. cepacia* (R_f value 0.9) are then in the literature (Silva et al. 2010, 2014).

FTIR and ^1H NMR analyses

FTIR analysis (Fig. 5a) revealed the presence of aliphatic hydrocarbon chains (lipid) along with polysaccharide moiety which confirmed the glycolipid nature of BS. The absorption bands at 3464.15 and 3356.14 cm^{-1} indicated the presence of free –OH groups due to H-bonding of polysaccharides and –OH stretching of carboxylic acid groups, respectively. The absorption peak at 2920.23 and 2360.87 cm^{-1} suggests the stretching vibration of

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 11 of 16

Table 4 Emulsification and surface tension reducing activity of the biosurfactant produced by different strains over a wide range of salt concentrations

Strain/organism	Salt conc. (g/L)	E_{24} (%)	Surface tension (mN/m)	References
P. otitidis P4	2	39.1	34.0	This study
	4	55.5	33.72	
	6	48.8	33.6	
	8	46.6	35.0	
	10	37.7	34.5	
Ochrobactrum 1C	5	95	31.0	Ferhat et al. (2011)
	10	79	30.5	
	50	90	31.0	
	100	64	31.0	
Pseudomonas sp. 2B	0	82.0	28.5	Aparna et al. (2012)
	2	80.1	28.6	
	4	80.0	29.5	
	6	79.0	30.0	
	8	78.0	30.5	
	10	76.0	31.0	
	12	75.5	32.0	
	15	75.0	33.0	
	20	70.0	34.0	
Bacillus siamensis RT10	0	73.5	39.0	Varadavenkatesan and Murty (2013)
	2	70.0	39.5	
	4	67.5	40.0	
	6	66.5	40.1	
	8	68.0	40.2	
	10	66.0	40.3	
	12	65.0	40.8	
	15	67.0	41.0	
	20	64.0	42.5	

−CH bond of respective sugar moiety and the presence of phosphines (P-H_3), respectively. The absorption peaks between 1740.08 and 1708.89 cm^{-1} indicated the C = O stretching in ester groups of lipids and fatty acids while the deformation vibration of alkyl groups was confirmed by the presence of peaks at 1384.89 and 1361.74. The absorption peaks at around 1458.48 and 1180.14 cm^{-1} indicated the stretching bands of carbon atoms with hydroxyl groups in the structure of sugar moiety and bands at 1080.44 and 848.68 cm^{-1} was associated with the stretching vibrations of glycosidic linkage which confirmed the glycolipid nature of the BS. The sugar residue present in the structure of BS provides the hydrophilic characteristic whereas the lipid fraction was responsible for hydrophobic characteristics. The FTIR spectra of glycolipid was nearly the same as those reported for other glycolipid BS produced by *Enterococcus faecium* MRTL9

(Sharma et al. 2015) and *Strenotrophomonas maltophilia* NBS-11 (Hemlata et al. 2015).

The chemical shifts (δ) in ^{1}H NMR spectrum of BS (Fig. 5b) showed the presence of sugar and lipid moieties. The peaks at δ 0.88 and 1.6 confirmed the presence of long chains of aliphatic fatty acids of lipid fraction in the BS. Additional peaks at δ 1.25–1.3 indicated the methyl (−CH_3) group of constituent sugar moiety. Peaks at around δ 7.3 and 7.55 were also detected which corresponds to carboxylic acid and ester groups of fatty acids of lipids which assures the glycolipid nature of BS. Similarly, Hemlata et al. (2015) and Jain et al. (2012) and have reported the characteristic NMR spectra of BSs isolated from *E. faecium* and *Cronobacter sakazakii*, respectively.
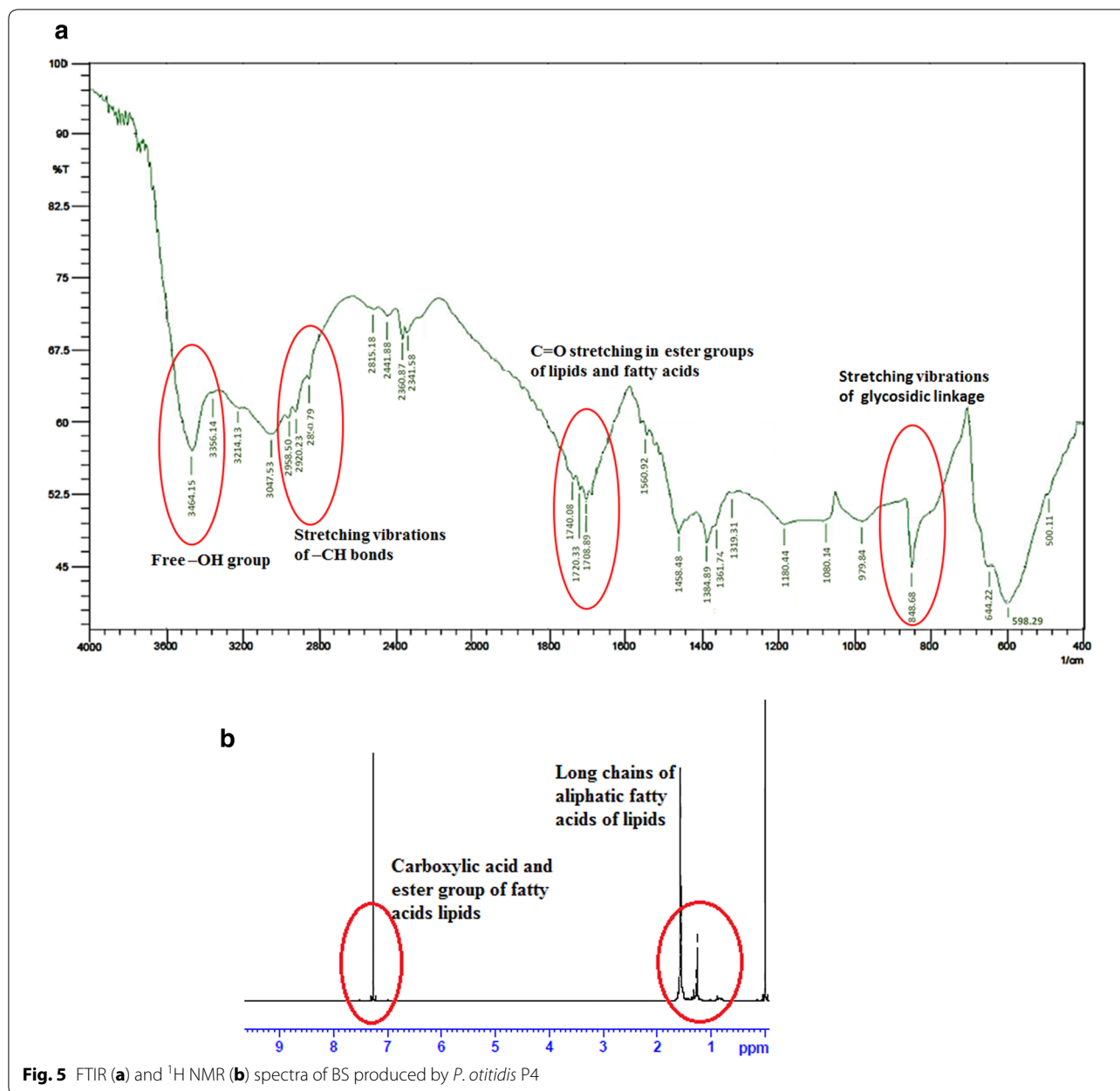
TGA and DSC analyses

The commercial application of any BS depends on its thermal stability at extreme temperatures. In the first step of TGA (Fig. 6a), the glycolipid lost its weight by 7.27 % during the temperature range 70.4–92.9 °C, possibly due to the loss of water and solvent molecules present in the sample. This step was followed by a rapid weight loss by 21.38 % at 149.9 °C which could be due to the degradation of thermolabile components such as lipid and sugar content of the BS. The non-labile component was degraded by only 8.88 % over the temperature range 280.5–450 °C. As determined from the TGA graph, the degradation temperature of BS was found to be 280 °C while complete weight loss was observed after 450 °C. A similar thermostable BS up to 250 °C produced by *E. faecium* has been characterized recently (Sharma et al. 2015). Nevertheless, our BS sample appeared more thermostable, even beyond 250 °C.

The phase transitions viz., glass transition (Tg), crystallization (Tc) and melting temperatures (Tm) in glycolipid BS were recorded with DSC analysis. In the beginning some peaks of weak transitions at 0–15 °C were observed which was possibly due to presence of moisture in the sample or the purge gas used. The DSC thermogram (Fig. 6b) of BS showed Tg at midpoint 92.5 °C (onset temperature 88.5 °C) and Tc at midpoint 112.83 °C (onset temperature 100.85 °C). The Tm1 and Tm2 peaks were observed at 143.63 °C (onset temperature 133.61 °C) and 283.5 °C (onset temperature 280.85 °C), respectively. BS produced by *P. otitidis* P4 was found to be thermostable and showed similar thermal degradation pattern as glycolipids.

Determination of pyrene degradation (quantitative estimation)

Pseudomonas strain P4 was able to utilize pyrene (1000 mg/L) as the sole source of carbon and energy. In

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 12 of 16

Fig. 5 FTIR (**a**) and ^{1}H NMR (**b**) spectra of BS produced by *P. otitidis* P4

the experimental controls, the PAH loss due to abiotic or biotic factors were negligible. The 4-ring compound pyrene was almost degraded after 10 days of incubation in the dark. The residual concentration of pyrene on 10th day was 75.87 mg/L (91.24 % degraded). Feng et al. (2014) have also reported 50 % degradation of pyrene (20 mg/L) within 2.5 days of incubation, by a *Pseudomonas* strain PAHAs-1. Recently, a fusant strain F14 reported by Lu et al. (2014) has been found to degrade 100 mg/L pyrene up to 48 % after 10 days of incubation.

GC–MS analysis for the identification of the pyrene degradation products

The GC–MS technique is of great interest to elucidate some structures obtained which may constitute intermediates of pyrene biodegradation. Many linear alkanes and four trimethylsilylated metabolites, which had retention times of 10.68, 13.19, 15.33 and 18.07 min were identified as 1, 2-benzenediol, benzoic acid, 1,2-benzenedicarboxylic acid and phthalic acid, respectively (Fig. 7). In the present study, we found the presence of phthalic acid

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 13 of 16
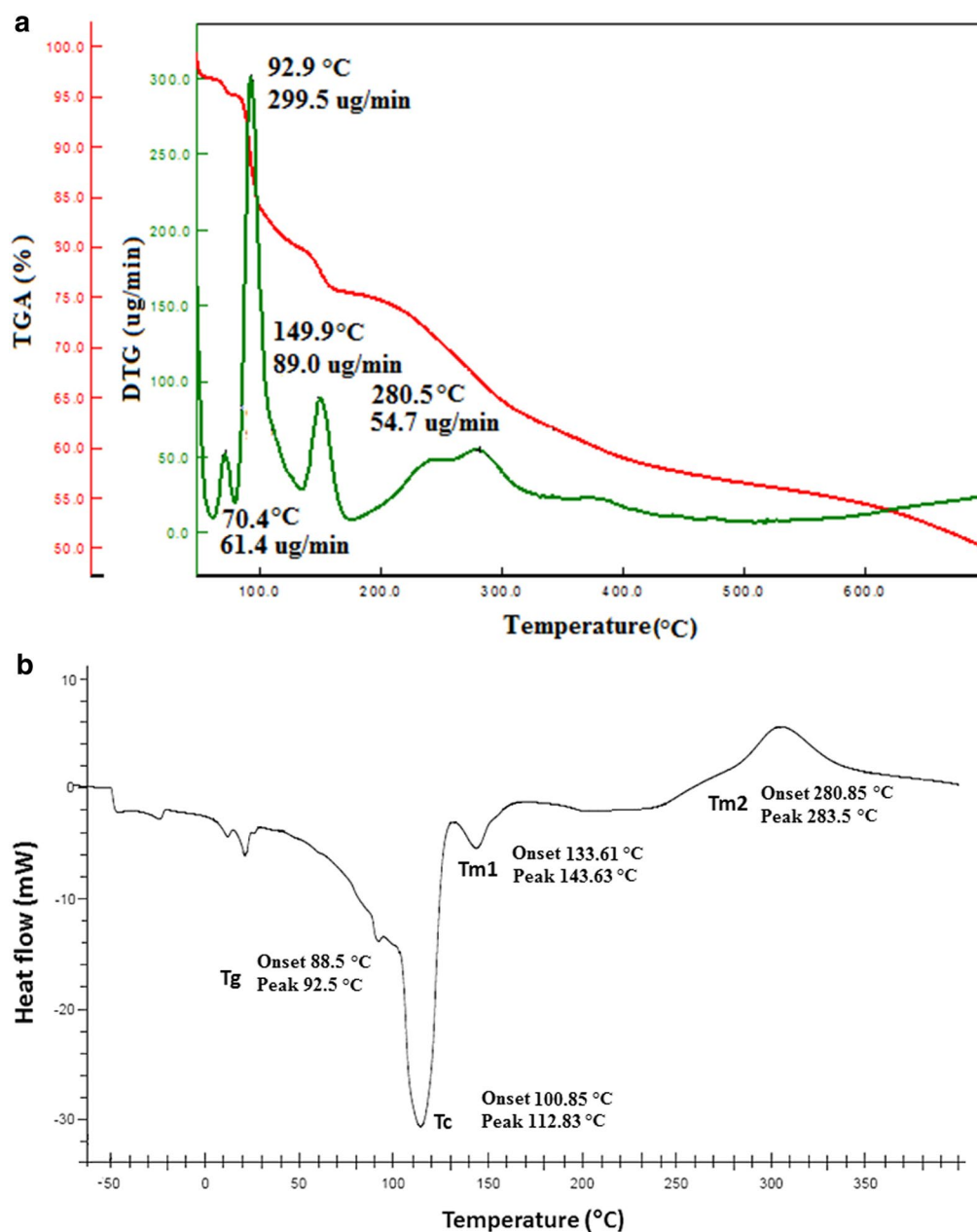
Fig. 6 TGA (**a**) and DSC (**b**) thermogram of biosurfactant obtained from *P. otitidis* P4

derivatives as the main detected intermediate of pyrene biodegradation pathway. These findings suggested that the pathway for the degradation of pyrene by *P. otitidis* P4 proceed via the phthalic acid pathway similar to the pathway reported for *Pseudomonas* sp. strain BP10 (Kumari et al. 2013) and Jpyr-1 (Ma et al. 2013). The present study suggested that *P. otitidis* P4 was a potent pyrene degrader and its biosurfactant production and emulsification abilities, were attributed for the bioavailability of pyrene.

Conclusions

The results obtained from the present investigation indicated that a promising pyrene degrading and BS producing strain has been isolated, characterized and identified as *P. otitidis* P4. Structural characterization by TLC, FTIR and ^{1}H NMR confirmed that the BS produced by *P. otitidis* P4 was glycolipid in nature. The BS exhibited high emulsification activity and stability in a wide range of temperature, pH and salinity which makes them suitable

Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 14 of 16

Fig. 7 GC-MS analysis of pyrene biodegradation metabolites; pyrene (**a**) and metabolites detected during degradation (**b**)
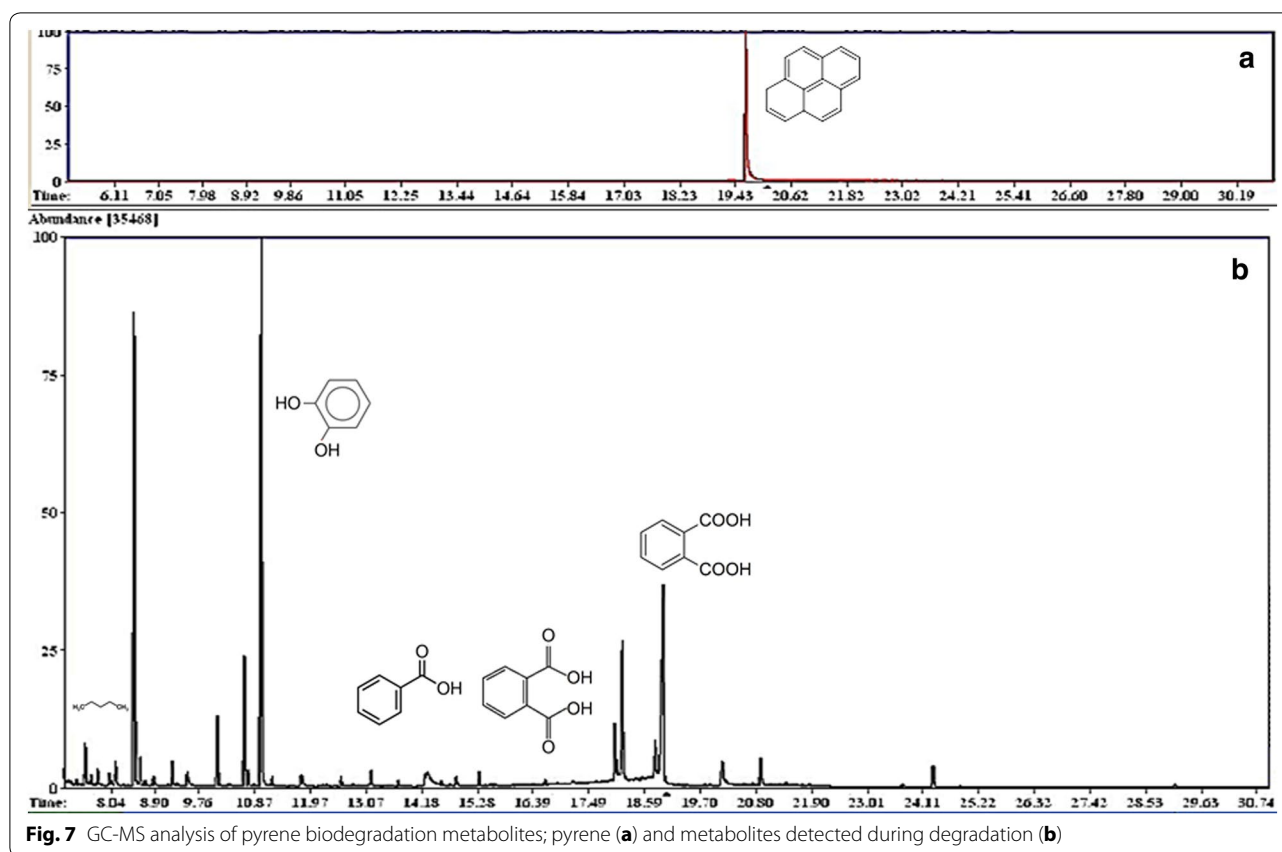
for various industrial applications such as food, pharmaceutical and cosmetics industries. This strain may potentially be used for bioremediation of PAHs contaminated soils as well as for the production of industrially useful BS. Further studies of the biological activity of glycolipids are in progress to unravel the novel and promising applications of BSs produced by *P. otitidis* P4 in different fields.

Additional file

Additional file 1. Figure S1. Screening for BS production (A) Drop collapse method (B) Modified drop collapse method (C) Emulsification assay.

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Singh and Tiwary *Bioresour. Bioprocess.* (2016) 3:42

Page 15 of 16

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Page 16 of 16

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