Bioresource Technology 101 (2010) 6118–6123

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Isolation and characterization of two new lipopeptide biosurfactants produced by *Pseudomonas fluorescens* BD5 isolated from water from the Arctic Archipelago of Svalbard

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ARTICLE INFO

Article history:
Received 15 December 2009
Received in revised form 19 February 2010
Accepted 26 February 2010
Available online 19 March 2010

Keywords:
Pseudomonas fluorescens BD5
Lipopeptide
Pseudofactins
Biosurfactant

ABSTRACT

The arctic freshwater bacterium *Pseudomonas fluorescens* BD5 produces biosurfactants when grown on 2% glucose. Crude biosurfactants were extracted from a cell-free culture supernatant with ethyl acetate and purified by preparative reversed phase high performance liquid chromatography (RP-HPLC). The chemical structure of the purified biosurfactants, pseudofactin I and II, was analyzed by matrix assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry and tandem mass spectrometry (MS/MS). Both compounds are novel cyclic lipopeptides with a palmitic acid connected to the terminal amino group of eighth amino acid in peptide moiety. The *C*-terminal carboxylic group of the last amino acid (Val or Leu) forms a lactone with the hydroxyl of Thr3. Pseudofactin II reduced the surface tension of water from 72 mN/m to 31.5 mN/m at a concentration of 72 mg/l. Its emulsification activity and stability was greater than that of the synthetic surfactants Tween 20 and Triton X-100; pseudofactins thus have a great potential for application in industrial fields such as bioremediation or biomedicine.

1. Introduction

Biosurfactants are biological surface-active compounds with both hydrophilic and hydrophobic moieties produced by diverse microorganisms such as bacteria and fungi (Fiechter, 1992; Lang, 2002). Microbial surfactants are in most cases low-molecular mass compounds such as lipopeptides, glycolipids, and phospholipids or high-molecular mass lipoproteins, lipopolysaccharides, proteins, polysaccharides and biopolymer complexes (Rosenberg and Ron, 1999; Ron and Rosenberg, 2001). The most commonly isolated biosurfactants are lipopeptides and glycolipids, predominantly produced by genera *Bacillus, Pseudomonas, Streptomyces,* and *Arthrobacter* (Morikawa et al., 1993; Yakimov et al., 1995; Kim et al., 1997; Richter et al., 1998; Steller and Vater, 2000; Grugurina et al., 2002; Roongsawang et al., 2002).

Biosurfactants have many advantages over synthetic surfactants. They are less toxic, more biodegradable and thus more environmentally friendly, and do not lose physico-chemical properties at different temperatures and pH levels (Mulligan, 2005). Due to their special properties they have found application in the food, pharmaceutical, cosmetics and agriculture industries, and in bioremediation (Banat et al., 2000; Noudeh et al., 2005). In recent years microbial surfactants have been found to possess several properties of therapeutic and biomedical importance (Singh and Cameotra, 2004; Rodrigues et al., 2006). They exert also antiadhesive action against several pathogenic microorganisms (Heinemann et al., 2000).

In general, lipopeptides lower the surface tension more than glycolipids. The lipopeptides have a hydrophilic part that contains polar amino acids, while the hydrophobic part contains lipophilic fatty acids and non-polar amino acids. *Pseudomonas* strains produce many types of bioactive compounds which have great potential for biotechnological and biomedical applications (Raaijmakers et al., 2006; Sinnaeve et al., 2009). A well-known group of compounds includes lipopeptides such as viscosin (Laycock et al., 1991), putisolvan (Kuiper et al., 2004), and massetolide (De Souza et al., 2003), which have environmental application and antagonistic activity against various pathogenic fungi.

In this report, we describe a strain isolated from the water of the Arctic Archipelago of Svalbard which was identified as *Pseudomonas fluorescens* BD5. This strain produced structurally novel cyclic lipopeptides with very good emulsification activity for aromatic and aliphatic hydrocarbons, and several plants oils. The preliminary structure of two new biosurfactants, named pseudofactin I and pseudofactin II, isolated by RP-HPLC, was determined by mass spectrometry (MALDI TOF).

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doi:10.1016/j.biortech.2010.02.109

2. Methods

2.1. Isolation of bacterial strain, culture conditions and emulsification assay of supernatant

Strain BD5 was obtained after spreading 100 μL of freshwater from the Arctic Archipelago of Svalbard on LB agar and incubation at 28 °C for 1 day. Single colonies were inoculated into 5 ml of LB medium containing 5 g/l yeast extract, 10 g/l bacto-tryptone and 10 g/l NaCl, incubated at 28 °C for 48 h and stored at −70 °C in 15% glycerol.

The strain was grown in 300 ml flasks containing 200 ml mineral salt medium (MSM) with the following composition: 7 g/l K_2HPO_4, 2 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 0.5 g/l sodium citrate $2H_2O$, and 0.1 g/l $MgSO_4 \times 7H_2O$ (pH 7.0). Glucose was added to MSM to a final concentration of 20 g/l. The flasks were inoculated with 5 ml overnight pre-culture of the strain and incubated at 10, 17, 23, 28 and 37 °C for 7 days without agitation. Growth of the strain BD5 with glucose as the sole carbon source was determined by measuring the absorbance of the culture at 600 nm.

Emulsification assays of the biosurfactants were performed using a previously described method (Cooper and Goldenberg, 1987). The emulsification activity of the supernatant was measured by adding 6 ml petroleum ether to 4 ml of aqueous sample in a test tube, vortexing for 2 min, and then leaving it to settle for 24 h. The emulsification index (E_{24}) was estimated as the height of the emulsion layer, divided by the total height, multiplied by 100.

2.2. Identification of bacterial strain

Strain BD5 was identified with the API 20E test for Enterobacteriacae (BioMérieux, Marcy l'Etoile, France) and by sequencing of its 16S rRNA gene. Genomic DNA was isolated from a one-day liquid culture using a GeneMATRIX Bacterial and Yeast Genomic DNA Purification kit 50 (EURx, Gdansk, Poland) following the manufacturer's standard protocol, and used as a target for polymerase chain reaction (PCR) amplification using primers 27F (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). PCR reactions were performed in the following mixture: 200 ng extracted chromosomal DNA, 10 pmol of each primer, 5 μl of 10*x* PCR buffer, 0.2 mmol of each dNTP (Biotools, Madrid, Spain), 1 U Yellow *Taq* polymerase (EURx, Gdansk, Poland) and ddH_2O up to a final volume of 50 μl. PCR conditions were as follows: 94 °C for 10 min; 30 cycles, 94 °C for 60 s, 58.5 °C for 60 s and 72 °C for 105 s; final synthesis at 72 °C for 5 min. The PCR products were subjected to agarose gel electrophoresis, purified using a GeneMATRIX PCR/DNA Clean-up Purification kits (EURx, Gdansk, Poland), and their nucleotide sequence was determined with an automated DNA sequencer. The 968-bp sequences obtained with 1492R primer was compared to rDNA sequences obtained from the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Purification of biosurfactants by RP-HPLC

Cell-free supernatant was obtained after centrifugation of the culture at 4700*g* at 4 °C for 15 min, extracted three times with ethyl acetate and evaporated under vacuum. The crude biosurfactants were dissolved in 5 ml of methanol, filtered through a 0.2 μm syringe filter and separated by RP-HPLC. HPLC was performed using a Waters 600 HPLC system (Waters, USA) equipped with an Xterra Prep RP_{18} OBD column (Waters, USA; 5 μm, 18 × 100 mm) held at 40 °C. The solvent system consisted of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The compounds were eluted at a flow rate of 4 ml/min with a linear gradient from the mixture A:B (70:30, vol/vol) to A:B (0:100, vol/vol) in 45 min. The absorbance of the eluate was measured at 210 nm. All the collected fractions were dried and stored at −20 °C for further studies.

2.4. Thin-layer chromatography (TLC) and lipopeptide detection

A volume of 10 μl of purified biosurfactants in methanol was spotted on a silica gel chromatography TLC plate (Silica gel 60; Merck, Darmstadt, Germany). The compounds were separated using a mobile phase of chloroform/methanol/water in the 65:25:4 ratio (vol/vol/vol). For the detection of peptides, the dry plates were sprayed with a solution of 0.25% ninhydrin in acetone and kept at 105 °C for 5 min. For the detection of lipids, the plates were treated with 0.1% bromothymol blue in 10% aqueous ethanol and iodine.

2.5. Mass spectrometry

All fractions collected after preparative HPLC were analyzed on a MDS SCIEX 4800 MALDI-TOF/TOF Analyzer (Applied Biosystem, Foster, CA, USA). The fractions were mixed with an equal volume of matrix solution, i.e. 0.1% α-cyano-4-hydroxycinnamic acid in acetonitrile–water-TFA (50:50:0.01, vol/vol/vol). The masses in the range 600–4000 Da were measured.

2.6. Acid hydrolysis and fatty acid and amino acid analysis

Purified fractions (100 μg) were hydrolyzed with 6 mol/l HCl at 100 °C for 24 h. The solution was cooled, and the fatty acids (FAs) were extracted at least three times with ether. The FAs were esterified with 3 mol/l HCl in methanol at 100 °C for 1 h. After cooling, the FAMEs (fatty acid methyl esters) were extracted with hexane and concentrated. The FAMEs were analyzed on a GC–MS apparatus (Thermo Scientific, Austin, TX, USA). The sample was injected into a capillary column HP-88 (100 m × 0.25 mm, J&W Scientific, Folsom, CA, USA). The temperature of the injector was 250 °C and the nitrogen carrier gas flow rate 1 ml/min. The column temperature was programmed from 50 to 250 °C at 5 °C/min. The aqueous fraction containing the free amino acids was subjected to TLC analysis with the solvent system *n*-butanol/acetic acid/water (4:1:1, vol/vol/vol). For detection of amino acids, the dry plates were sprayed with a solution of 0.25% ninhydrin in acetone and kept at 105 °C for 5 min.

2.7. Physico-chemical properties of the purified lipopeptide

The surface tension was determined by a Tensiometer Kruss K100 (Kruss GmbH, Hamburg, Germany) at 23 °C, according to the du Nouy's ring method (see, e.g. Huh and Mason, 1975). Surface tension measurements were performed on 50 ml samples of aqueous solution of purified pseudofactin II in the concentration range of 15–440 mg/l.

Emulsification activity of pseudofactin II was tested on different organic compounds: petroleum ether, benzene, *n*-hexane, cyclohexane, *n*-hexadecane, xylene, toluene, sunflower, olive and rapeseed oil. The purified lipopeptide dissolved in 5.0 ml distilled water (0.5% w/v) was mixed with 5 ml of each hydrophobic compound, and then vortexed at high speed for 2 min, after which the mixture was kept at 25 °C for 24 h. Synthetic surfactants Tween 20 and Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) in the same concentration were used for comparison. The emulsification index value (E_{24}) was then computed using the formula:

$$E_{24} = (\text{height of the emulsion layer}/\text{height of the total mixture}) \times 100.$$

3. Results and discussion

3.1. Identification of P. fluorescens BD5

An effective biosurfactant – producing strain, BD5, was isolated from the water around Wroclaw University scientific base in the Arctic Archipelago of Svalbard. This strain was isolated among 130 morphologically distinct bacteria based on the lowest surface tension of its culture supernatant. Cells of the isolated strain were rod-shaped, gram-negative and aerobic. The optimal temperature for growth of strain BD5 in MSM with 2% glucose was 28 °C. The strain BD5 was arginine dihydrolase-, citrate utilization-, gelatinase-, and cytochrome oxidase-positive and β-galactosidase-, lysine decarboxylase-, ornithine decarboxylase-, urease-, tryptophane deaminase-, indole-, and acetoin production-negative. These biochemical results classified it as belonging to *Pseudomonas* genus. Comparison of a 968-bp 16S rRNA nucleotide sequence from strain BD5 with sequences in the GenBank database revealed 99% identity with the corresponding sequences from *P. fluorescens* Pf0–1 and *P. fluorescens* Pf-5.

3.2. Purification, characterization and TLC analysis of biosurfactants from P. fluorescens BD5

The emulsification index of the culture filtrate increased from 2% to 67%, stabilizing after 92 h (Fig. 1). The biosurfactants were partially purified from the culture filtrate by extraction with ethyl acetate. The extracted biosurfactants were then subjected to preparative RP-HPLC. With a linear gradient of acetonitrile and water, the crude biosurfactants were resolved into five fractions, see Fig. 2. The collected HPLC fractions were tested for surface activity by the drop-collapsing assay (data not shown), and only two fractions corresponding to the peak retention times of 34.38 and 35.57 min appeared to be active. The peak with retention time 35.57 min showed the highest surface tension reduction activity. Isolated biosurfactants were used for further identification. Both active fractions were applied on TLC to characterize their homogeneity. The TLC showed single spots with iodine as the staining reagent. Both compounds have an identical R_f value of 0.74 and hence are very likely to have a very similar structure. This finding (see also the very similar retention times for the peaks from RP-HPLC) was further supported by the fact that both fractions showed positive reactions with the two spray reagents, i.e. ninhydrin and bromothymol blue, indicating the presence of peptide and lipid moieties in the molecule.

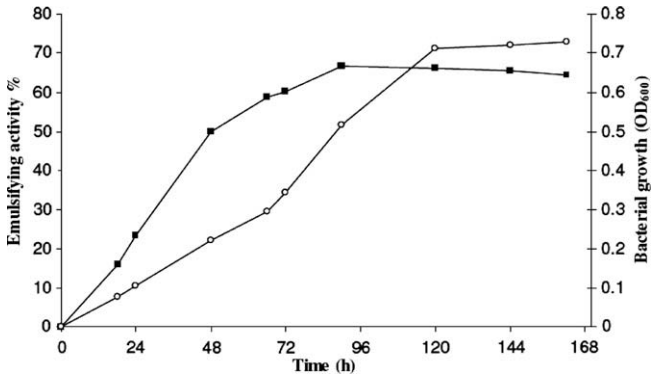

Fig. 1. Emulsification index (EI_{24}) (■) during growth (○) of *P. fluorescens* BD5 on mineral salt medium with 2% glucose as carbon source at 28 °C.

3.3. Fatty acid and amino acid analysis

Both pseudofactins were separately hydrolyzed and the hydrolyzate was analyzed by GC–MS for the content of FAMEs and by TLC for the content of amino acids. The lipophilic fraction of the hydrolysis products, after extraction to hexane, was analyzed and the chromatogram showed that the FAMEs from both lipopeptides contained only one FAME with a retention time of 19.76 min. The mass spectrum was characteristic for FAME with a saturated long chain, i.e. palmitic acid. In contrast to this finding, lipopeptides produced by species of *Bacillus*, such as surfactin and lichenysin A, usually have 3-OH–C_{14} and 3-OH–C_{15} FA (Youssef et al., 2005) while the cyclic lipopeptide produced by *Pseudomonas* has also 3-OH–FA but from C_{10} to C_{12} (Laycock et al., 1991; Raaijmakers et al., 2006; Sinnaeve et al., 2009).

TLC analysis of the hydrolysis products identified five spots from pseudofactin I and four spots from pseudofactin II. Based on the comparison of R_f of the amino acid standards and amino acids from lipopeptides it is clear that pseudofactin I contains L-Gly, L-Thr, L-Leu, L-Ser and L-Val, whereas pseudofactin II has L-Gly, L-Thr, L-Leu, and L-Ser.

3.4. Structural characterization of the purified lipopeptide

The molecular mass of the purified active compounds, i.e. fractions 3 and 4 was measured using MALDI TOF mass spectrometry.

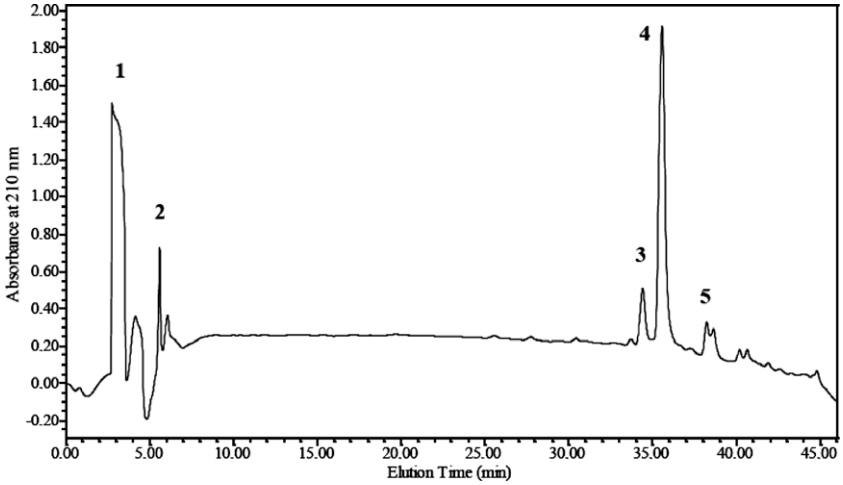

Fig. 2. RP-HPLC chromatogram of compounds with retention times 2.87, 5.78, 34.38, 35.57 and 38.29 min obtained from *P. fluorescens* by using linear gradient of acetonitrile (0.1% TFA) and water. Absorbance was measured at 210 nm.

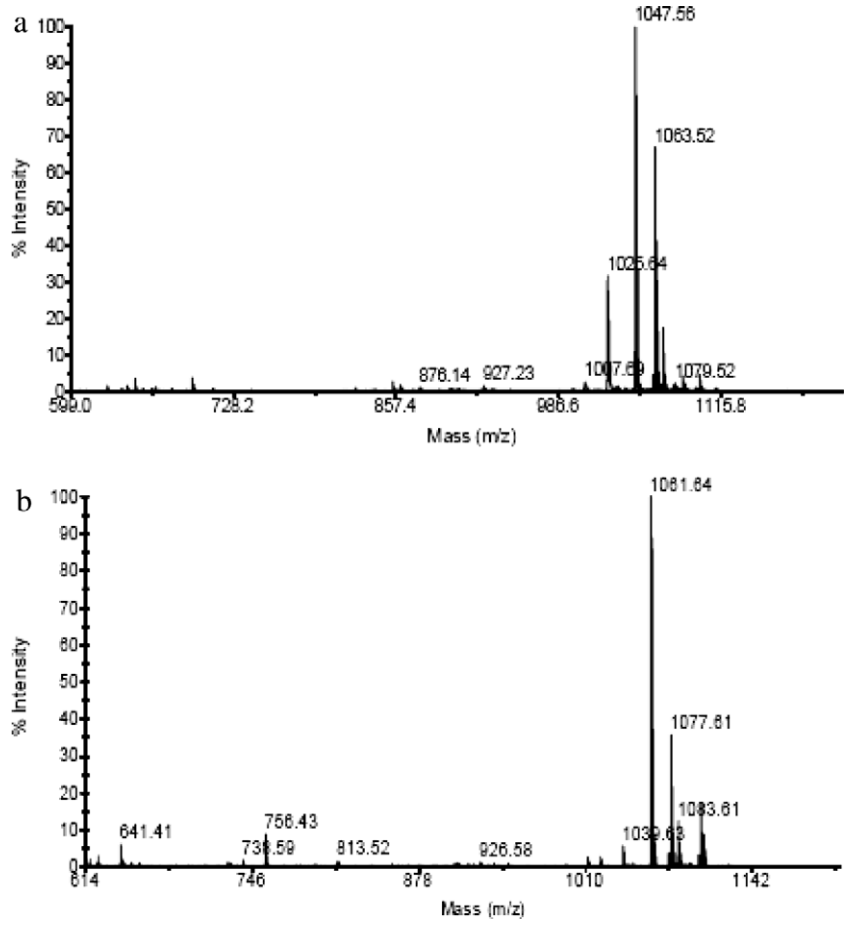

Fig. 3. MALDI-TOF/TOF mass spectrum of the purified biosurfactant from *P. fluorescens* BD5. MALDI-TOF/TOF mass spectrum of peak 34.38 min (a) and 35.57 min (b), respectively.

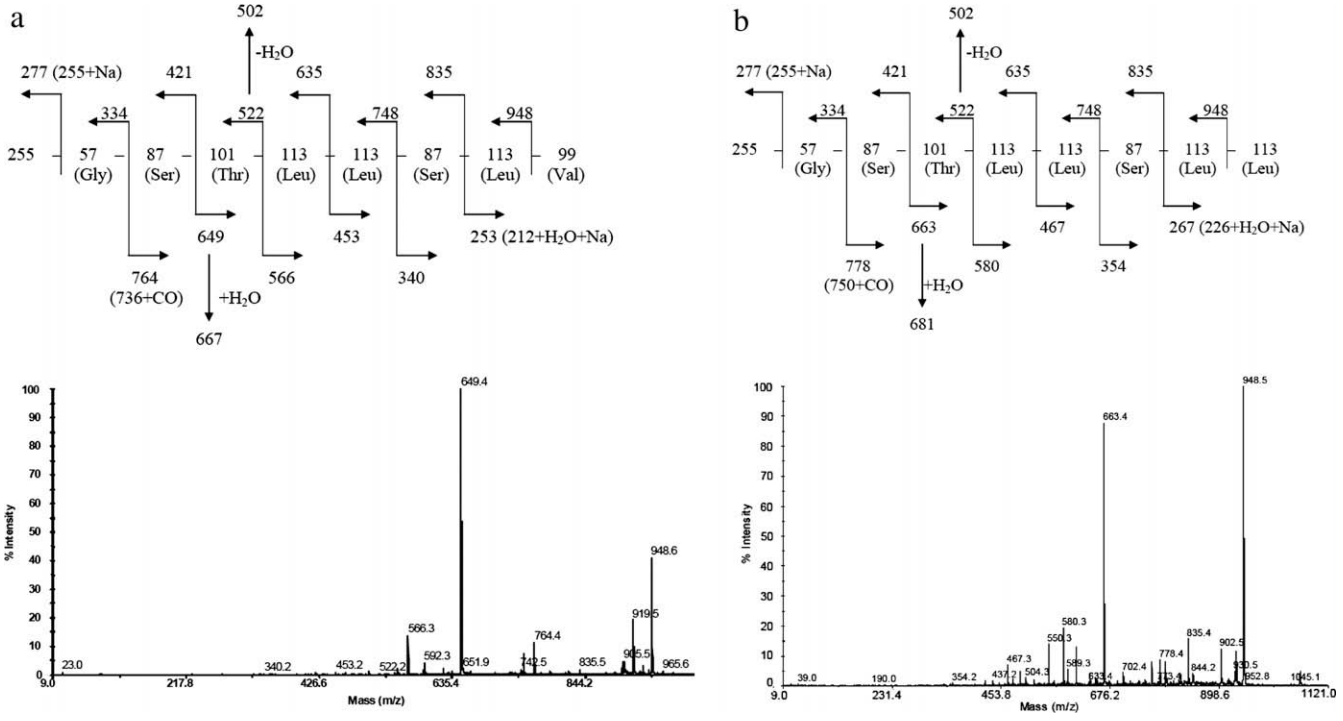

Fig. 4. Tandem MS spectra of the precursor ion at *m/z* 1047.6 (a) and *m/z* 1061.6 (b) from MALDI-TOF/TOF.

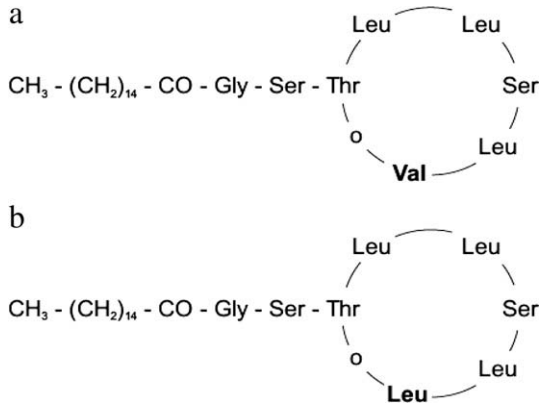

Fig. 5. The structures of pseudofactin I (a) and pseudofactin II (b) based on mass spectrometric and amino acid analyses.

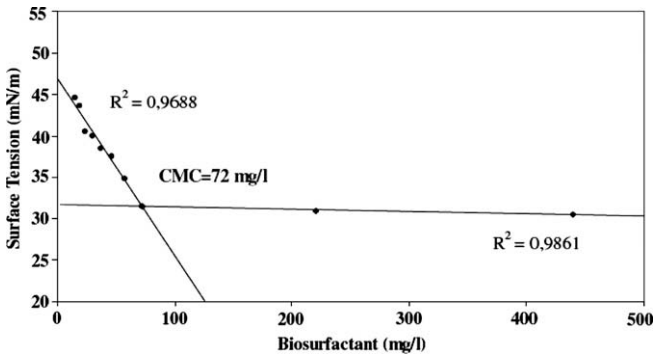

Fig. 6. Effect of pseudofactin II concentration on surface tension. The CMC was determined from the intersection of regression lines that describe two parts of the curve, below and above CMC.

Pseudomolecular ions were observed at *m/z* 1025.6, 1047.6 and 1063.6 (Fig. 3a), and at *m/z* 1039.6, 1061.6 and 1077.6 (Fig. 3b), which were assigned to the protonated molecular ion and to the adducts of sodium and potassium ions, respectively. Two peaks, at *m/z* 1047.6 and 1061.6 revealed differences of 14 Da, i.e. difference by one methylene group. The difference between the two structures is located in the last amino acid from the *C*-terminal, which is valine for pseudofactin I and leucine for pseudofactin II. The *m/z* 1047.6 and 1061.6 ions were used as precursor ions for further TOF–MS/MS analysis (Fig. 4a and b). The C-terminal carboxylic group of the last amino acid (Val or Leu) forms a macrolactone with the hydroxyl of Thr3. The ring formation was deduced from reduced mass ions at *m/z* 1047 and 1061 Da, which are 18 Da lower than in the case of the linear chain. The sequence of amino acids in the peptide moiety was established from these spectra (Fig. 5a and b).

Based on the isolation procedure, the high surface activity we suggested the presence of compounds related to viscosin (Laycock et al., 1991) and its derivatives massetolides (De Souza et al., 2003) or pseudophomins (Pedras et al., 2003), which are cyclic acylated nonapeptides from *Pseudomonas viscosa* (Raaijmakers et al., 2006). This hypothesis was confirmed by the MS^2 analysis. A set of daughter ions was observed in the MS/MS spectrum of the parent ion at *m/z* 1047, i.e. $[M + H]^+$ ion from the latter larger peak from RP-HPLC. The fragmentation pattern led us to deduce the partial sequence palmitoyl-Gly–Ser–Thr–Leu–Leu–Ser–Leu–Leu (Fig. 5b). The same procedure was used with the minor peak in which the AA sequence was very similar except for the 14 Da mentioned above, which was due to the replacement of Val by Leu. This fact was also confirmed by AA analysis.

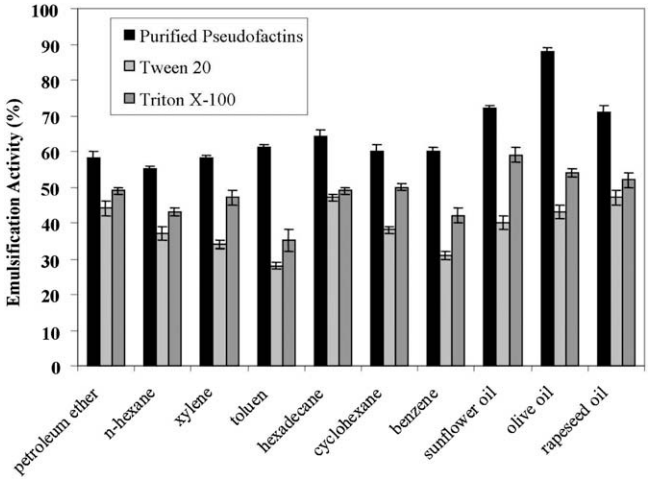

Fig. 7. Comparison of emulsifying activity of purified pseudofactin II and synthetic surfactants Tween 20 and Triton X-100.

Structural analyses of the two biosurfactants of *P. fluorescens* BD5 revealed that both are novel cyclic lipopeptides with a palmitic acid connected to the *N*-terminal group of 8-amino acid peptide moiety. The mass spectrometric results led to the structures of pseudofactin I and II shown in Fig. 5a and b.

3.5. Surface tension, CMC and emulsification index of the biosurfactants

Average yield of purified pseudofactin II was about 10 mg from one liter of culture. As pseudofactin I was 12-fold less abundant (Fig. 2), only pseudofactin II was used for CMC and emulsification experiments. Surface tension of purified pseudofactin II was measured by a du Nouy'a ring tensiometer. It was found to reduce the surface tension of the water from 72 to 31.5 mN/m. Critical micelle concentration (CMC) determined with the aid of a series of concentrations was around 72 mg/l (Fig. 6). Generally, the surface tension of purified biosurfactants has been reported to range from 72 to about 27–35 mN/m (Suk et al., 1999; Kim et al., 2000; Lee et al., 2007). A previously described biosurfactant with a palmitic acid tail isolated from *Rhodococcus* sp. (Peng et al., 2008) showed similar reduction of the surface tension of water to 30.7 mN/m but lower CMC value of 23.7 mg/l. The various isoforms of the longest-known biosurfactant surfactin produced by different strains of *Bacillus subtilis* have been reported to lower the surface tension to 25–40 mN/m (Peypoux et al., 1994; Abdel-Mawgoud et al., 2008).

The emulsifying properties of the pseudofactin II from *P. fluorescens* BD5 were studied with several hydrophobic substrates and compared with two commercial surfactants Tween 20 and Triton X-100 (Fig. 7). The results indicated that the emulsification activity of pseudofactin II was better than that of the two chemical surfactants since it more effectively emulsified aromatic and aliphatic hydrocarbons and several plant oils, and was in most cases similar to the range of 59–66% exhibited by *B. subtilis* surfactin against kerosene, diesel and motor oil, as reported by Abdel-Mawgoud et al. (2008).

4. Conclusion

In this paper, the biosurfactants produced by *P. fluorescens* BD5 strain were isolated by RP-HPLC and chemically characterized as two cyclic lipopeptides, pseudofactin I and II. When compared to synthetic surfactants, e.g. Tween 20 and Triton X-100, pseudofactin II showed comparable physico-chemical properties in terms of the

surface activities and emulsifying abilities. These new biosurfactants from *P. fluorescens* BD5 may be potentially useful in a wide variety of biotechnological and medicine applications. Further studies of biological activity of pseudofactins are needed to determine their applicability as antibacterial, antifungal, antiviral or anticancer agents.

Acknowledgements

We are grateful to Dr. Karel Sigler for critical reading and valuable discussions of the manuscript. This work was supported by grant KB/48/13639/IT1-B/U/08 from the Polish National Centre for Research and Development, EU POIG.01.01.02-00-016/2008 and by the Institutional Research Concept AV0Z50200510.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.02.109.

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Supplementary materiál

Fig. S1. Thin-layer chromatography analysis of lipopeptide produced by BD5. Developed with chloroform/methanol/water (65:25:4 vol/vol/vol); detected with iodine (plate a), ninhydrin (plate b) and bromothymol blue (plate c).

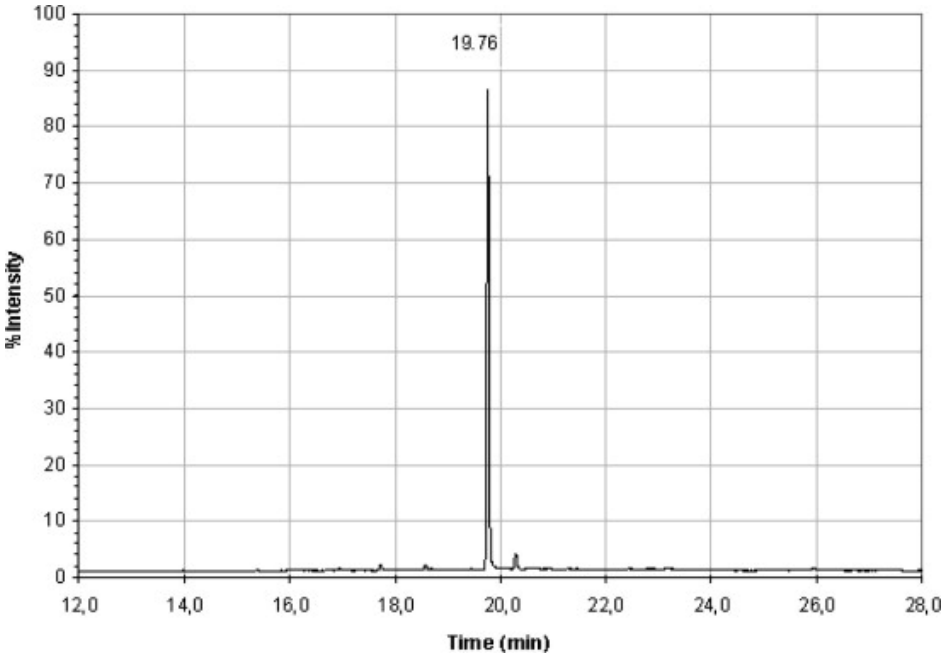

Fig. S2. The GC–MS of FAMEs from pseudofactin II.