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Research article

Structural identification of lipopeptide biosurfactants produced by *Bacillus subtilis* strains grown on the media obtained from renewable natural resources

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ABSTRACT

The aim of the study was to identify and characterize lipopeptide (LP) biosurfactants produced by two *Bacillus subtilis* strains (KP7 and I′-1a) grown on various media prepared from renewable natural resources: two different brewery wastewaters (BW#4 and BW#6), 2% beet molasses (M), apple peels extract (APE) supplemented with 0.25% of yeast extract (YE) or 0.25% peptone (P), and similarly supplemented carrot peels extract (CPE). In all used media both strains retained their individual LP production signature characterized by surfactin and iturin overproduction exhibited by KP7 and I′-1a strain, respectively. The production level and the structural diversity of synthesized LPs were dependent on the medium composition. In the CPE+YE medium it was higher than the yield obtained in Luria-Bertani (140.6 and 100.3 mg L^{-1}, respectively). Surfactins were produced by both strains as a mixture of four homologues (C13-C16) with the domination of variant C14. All other broths prepared from renewable resources strongly stimulated the iturin production by I′-1a strain with the exception of BW media. The highest iturin concentration (428.7 mg L^{-1}) obtained in the CPE+P culture of I′-1a strain was about seven-fold higher than in LB. In all cultures only iturin A was identified. Among four iturin homologues (C13-16) produced by I′-1a strain, the highest relative contents of C16 variant (70–80%) were calculated for samples obtained from APE+P and CPE+P media. The obtained data indicate that the waste composition has an influence on both the types and amounts of biosurfactants produced by studied *B. subtilis* strains.

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1. Introduction

Now, several industries have been increasingly directed towards biotechnology and circular bioeconomy (Communication EU, 13.2.2012 COM, 2012). Worldwide interest in biosurfactants(considered as “green technology” products)has significantly grown in recent years due to their more environmentally friendly properties compared to the chemical surfactants (Paraszkiewicz, 2016). The worldwide biosurfactant production is expected to reach 476,512.2 tons by 2018 with the global surfactant market worth more than US $41 billion (Kapadia and Yagnik, 2013; Dhanarajan and Sen, 2015). Commercial biosurfactant production is discussed frequently in the literature, debating the large difference in the monetary inputs versus actual financial gain. The development of cheaper processes of biosurfactant production and the use of low-cost raw material are important factors that account for 10–30% of the overall cost (Kosaric and Vardar-Sukan, 2015). Agro-industrial wastes are considered as a promising substrate for microbial synthesis of biosurfactants and can solve various industrial waste management problems (Makkar et al., 2011). Consequently, research has focused on the usage of several effective renewable natural resources to overcome the financial hurdles in the biosurfactant production industry. Currently, substrates such as wheat straw, rice straw, cassava, cassava flour, sugarcane molasses, bagasse of sugarcane, beet molasses, bran, and corn are being tested for biosurfactant production at the commercial level (Banat

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et al., 2014; Mnif et al., 2012). Satpute et al. (2017) discuss a novel and efficient way of using agro-industrial, dairy and food processing waste for biosurfactant production. It is very important to recycle and reuse various renewable substrates as ingredients to recover industrial-scale products (Kosaric and Vardar-Sukan, 2015). At the same time, it is very important to choose top-quality substrates in terms of nutritional value that can allow the growth of desired microorganisms along with plentiful production of microbial surface active agents.

Bacillus subtilis is a well-studied producer of a variety of cyclic LP biosurfactants from which the best characterized are compounds from surfactin, iturin and fengycin families. Bacilli LPs exhibit interesting physiological, biocidal, physicochemical, and surface-active properties (Raaijmakers et al., 2010; Wang et al., 2015; Diaz De Rienzo et al., 2016). Surfactins are mostly used as emulsifying and foaming agents in remediation technologies and iturin derivatives display a strong *in vitro* and *in vivo* antimicrobial activity against fungi belonging to various families. Due to their natural diversity, LPs are synthesized by particular strains as mixtures of isoforms and homologues differing in the amino acid composition of the peptide sequence and in the length of the fatty acid chain, respectively (Pecci et al., 2010). Studying the relationships between yields and molecular structures of synthesized LPs and the kind of wastes/by-products used as growth media components is considered to be important for the development of cost effective technologies enabling the production of novel biochemicals with different properties and their potential use in industrial applications.

In this context, the above paper addresses the potential of various agro-industrial wastes for surfactin and iturin production. Moreover, structural diversity of the LP biosurfactants produced by two studied *B. subtilis* strains has been determined.

2. Materials

2.1. Culturing of Bacillus strains on different media

The research aim, objectives, and methods are briefly described in Table 1. In detail, two strains of *Bacillus subtilis* named KP7 and I′-1a were used in this study. KP7 strain was obtained from the collection of the Department of Industrial Microbiology and Biotechnology, University of Łódź, and I′-1astrain was supplied by the Institute for Ecology of Industrial Areas in Katowice, Poland. Taxonomic identification and preliminary characterization of strains KP7 and I′-1a have been described previously by Płaza et al. (2015). The strains were preserved at −70 °C in Luria–Bertani (LB) medium supplemented with 20% (v/v) glycerol. Bacterial 24-h-old cultures obtained from LB medium and adjusted to OD_{600nm} 0.9; (approximately 10^7–10^8 CFU mL) were introduced as a 2% inoculum to 300 mL Erlenmeyer flasks containing 150 mL of the selected medium. The cultures were grown aerobically for 96 h with constant shaking (120 rpm) at 28 °C.The following media were used to evaluate LPs production: Luria-Bertani (LB) broth; Cooper's broth (Al-Ajlani et al., 2007), two different brewery wastewaters (BW) obtained from beer production based on barley malt and wheat malt (signed BW#4 and BW#6, respectively), 2% beet molasses (M), apple peels extract (APE) supplemented with 0.25% of yeast extract (YE) or peptone (P) and the carrot peels extract (CPE) with the same supplements. Supernatants obtained by centrifugation (10.000×*g*, 20 min) of 96 h cultures were used for LPs isolation by the method based on the QuEChERS technique (Siewiera et al., 2015; Paraszkiewicz et al., 2017). To obtain the vegetable extracts, proper quantities of carrot and apple peels were boiled for 30 min. After centrifugation, the remaining solid particles were removed by filtration. Distilled water was added to the extracts to a final volume of 1000 ml. All media (with pH adjusted to 7) were sterilized before being used.

2.2. Lipopeptides isolation and mass spectrometry analysis

Supernatants obtained by centrifugation (10.000×*g*, 20 min) of 96 h cultures were used for LPs isolation according to the method described by Płaza et al. (2015). LP concentrations in culture supernatants as well as their structures were determined by liquid chromatography – mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/TOF) techniques (Bernat et al., 2016). Surfactin and iturin standards were obtained from Sigma-Aldrich (Germany) and all other chemicals were purchased from Avantor Performance Materials Poland S.A., Sigma-Aldrich (Germany), Serva (Germany) or Fluka (Germany).

2.3. Statistical analysis

All the experiments were performed in triplicates and analysed individually. An average standard deviation was calculated.

3. Results and discussion

Data presented in Table 2 reveal that in all growth media used in the study *B. subtilis* strains were able to synthesise both surfactin and iturin. Moreover, regardless of the used growth medium, KP7 strain produced more surfactin than iturin, while I′-1a strain overproduced iturin with only traces of surfactin. The same, in all

Table 1
Research aim, objectives and methods of the study.

Research principle		Description
Research aim		Determination of the potential of various agro-industrial wastes for surfactin and iturin production. Determination of the structural diversity of the biosurfactants produced by two *B. subtilis* strains cultured in standard and low-cost media
Research objectives	*Bacillus subtilis* strains	KP7 - surfactin overproducer, producing in LB medium surfactin and iturin in the ratio 17.6: 1; I′-1a – iturin overproducer, producing in LB medium surfactin and iturin in the ratio 1: 19.8
	Cost-effective culture media containing agro-industrial wastes	Two different brewery wastewaters (BW) obtained from beer production based on barley malt and wheat malt (signed BW#4 and BW#6, respectively); 2% beet molasses (signed M); the apple peels extract (APE) and the carrot peels extract (CPE) supplemented with 0.25% of yeast extract (YE) or peptone (P)
	Control (standard) growth media	Luria–Bertani (LB) medium and Cooper's medium
Methods	Biosurfactant isolation	Modified QuEChERS technique
	Biosurfactant analysis	LC-MS/MS[a] MALDI-TOF/TOF[b]

[a] Liquid chromatography – mass spectrometry.
[b] Matrix-assisted laser desorption time-of-flight mass spectrometry.

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