Characterization of biosurfactants produced by the *Bacillus amyloliquefaciens* BKMA B-12464

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Abstract. Biosurfactants are amphiphilic substances with a wide range of uses. Biosurfactants are being researched in various areas of biotechnology. They are especially widely used in the development of new environmentally friendly methods for increasing oil production, as well as in agriculture as an effective biocontrol agent. However, the possibility of finding strains producing biosurfactants and the efficiency of their production is a separate task for research. The aim of this work was to evaluate the dynamics of biosurfactant production by the *Bacillus amyloliquefaciens* VKMA B-12464 strain, as well as to evaluate its chemical nature. During the research, the resulting biosurfactant was characterized by TLC and IR spectroscopy. It has been established that the biosurfactant produced by *Bacillus amyloliquefaciens* belongs to lipopeptides, and its highest yield is observed on the 7th day of cultivation.

1 Introduction

Biosurfactants are a group of surfactants produced by microorganisms in the course of their life activity [1]. Biological surfactants have properties similar to chemical ones - surface activity and emulsifying properties. In addition, they have such advantages as low critical micelle concentration (CMC), biodegradability, salt resistance and resistance to changes in the acidity of the environment. This makes them an attractive option for use in a variety of industries, including oil production [2]. Biosurfactants can affect crude oil in oil reservoirs by reducing oil-water interfacial tension, altering wettability, and emulsifying the crude oil [3].

Ecological safety and low toxicity to the environment and humans, as well as fungicidal properties, ensure their extensive research as plant protection products [4]. Biosurfactants can reduce plant disease by affecting the plasma membrane of phytopathogenic fungi, as well as increasing plant immunity to phytopathogens.

Researchers have found biosurfactant-producing microorganisms in aquatic environments, oil-contaminated soils, crude oil, and many rhizosphere and plant-associated microorganisms are biosurfactant producers. Biosurfactants are mainly produced by aerophilic bacteria (*Pseudomonas, Bacillus* and *Acinetobacter*), fungi (*Aspergillus* and

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Fusarium) and yeasts (*Candida* and *Pseudozyma*) in an aquatic environment. Raw materials such as hydrocarbons, carbohydrates, fats and oils are used as a carbon source.

Biosurfactants include a wide variety of amphiphiles, depending on the functional groups present [5]. According to the chemical composition, biosurfactants belong to glycolipids (rhamnolipids, sophorolipids, trehalolipids, mannosylerythritol lipids), lipopeptides (surfactin, lichenisin, iturin, fengycin, serravettin), fatty acids/phospholipids/neutral lipids (phosphatidylethanolamine, spicusporic acid), polymeric biosurfactants (emulsan, alasan, biodispersan, liposan) and solid biosurfactants (vesicles) [6].

Biosurfactants are classified using chromatographic and spectroscopic research methods. Studies show that the hydrophobic part is long chain fatty acids, while the hydrophilic part can be alcohol, amino acid, carbohydrates, phosphates, carboxylic acids, or cyclic peptides. Depending on their structure, biosurfactants can exhibit different activities. For example, low molecular weight biosurfactants effectively reduce surface and interfacial tension, while higher molecular weight biosurfactants can form a stable emulsion and have high emulsifying activity [7].

The aim of this study was to obtain and characterize biosurfactants produced by *Bacillus amyloliquefaciens* VKMA B-12464. Since *Bacillus amyloliquefaciens* was previously known as an effective biocontrol agent, the mechanism of suppression of pathogenic fungi mechanisms of suppression are being actively studied. Thus, this strain was tested for the possibility of producing biosurfactants.

2 Materials and methods

2.1 Biosurfactant Production

For increased biosurfactant production, strain *Bacillus amyloliquefaciens* BKMA B-12464 (provided by the ORGANIC PARK LLC, The Bionovatic) was cultivated in glycerol nitrate medium at 35 °C and 180 rpm for 6 days. The concentration of crude glycerol in the medium was 40 g L^{-1}. The medium except crude glycerol contained (g L^{-1}): $NaNO_3$ (4.0), $K_2HPO4{\cdot}3H_2O$ (4.0), KH_2PO_4 (3.0), $MgSO_4{\cdot}7H_2O$ (0.5), KCl (0.5), NaCl (0.5), $CaCl_2{\cdot}2H_2O$ (0.2). Then, the mixture of crude biosurfactants produced by the strain was extracted using acid precipitation and purified as described below. As a result, the acid precipitated fraction was obtained and used for further analyses.

2.2 Emulsification Test

Three millilitres of biosurfactant containing culture supernatant and 3mL of respective oils were added in test tubes followed by rapid vigorous vortexing for 2min. By adopting the formula given below the E24% was calculated.

E24% = (Height of the emulsified layer/total height of liquid column) × 100.

2.3 Extraction and purification of biosurfactant

For the extraction of biosurfactant, cell-free supernatant was obtained through centrifugation of culture broth for 20 min at 10,000 rpm at 4°C which served as the source of crude biosurfactant. To amend the pH at 2, 6N HCl was added to the clear supernatant. The supernatant was acidified to pH 2 using 6N HCl, then stored at 4°C overnight and centrifuged at 10,000 rpm and 4°C for 20 min. The precipitate was purified by dissolving

in a $CHCl_3$:CH_3OH (2:1, v/v) mixture followed by rotary evaporating under vacuum. The crude biosurfactant was quantified gravimetrically.

2.4 Chemical Characterization of the biosurfactant

For the extraction of biosurfactant, cell-free supernatant was obtained through centrifugation of culture broth for 20 min at 10,000 rpm at 4°C which served as the source of crude biosurfactant. To amend the pH at 2, 6N HCl was added to the clear supernatant. The supernatant was acidified to pH 2 using 6N HCl, then stored at 4°C overnight and centrifuged at 10,000 rpm and 4 °C for 20 min.

The precipitate was purified by dissolving in a CHCl3:CH3OH (2:1, v/v) mixture followed by rotary evaporating under vacuum. The crude biosurfactant was quantified gravimetrically. To determine the type and structure of biosurfactant, FT-IR was performed using a Lumos I instrument (Bruker, Billerica, MA, USA). The spectra were collected from wavenumbers 600 to 4000 (cm^{-1}).

3 Results and Discussions

The *Bacillus amyloliquefaciens* BKMA B-12464 strain was cultivated for 7 days, with sampling on days 3 (sample I), 5 (II) and 7 (III) days. It was found that the highest yield on the 7th day of cultivation, as well as the indicator of emulsifying activity (65%).

Table 1. Dynamics of the mass of biosurfactants and the E24 index.

Time of cultivation, day	Mass, mg l^{-1}	E24, %
3	11.1	31
5	7.1	33
7	34.0	65

Thus, this strain is cultivated for 7 days to achieve a high concentration of biosurfactants. The isolated biosurfactant was further analysed for its functional groups using FT-IR spectrum (Figure 1).

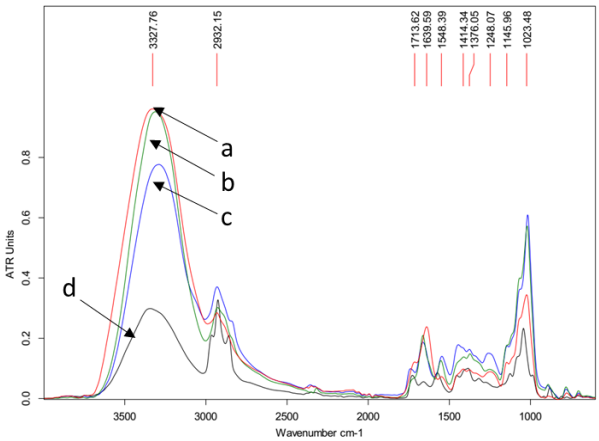

Fig. 1. FT-IR spectrum of the isolated surfactant from *Bacillus amyloliquefaciens* BKMA B-12464 (a – 1st day of fermentation, b – 2nd day of fermentation, c – 3rd day of fermentation, d – standard sample of surfactin).

FT-IR (Fourier Transform Infrared) spectroscopy of lipopeptide samples was performed using attenuated total reflection (ATR) with 4 cm^{-1} resolution on Bruker Lumos, then ATR the spectra were expressed in reciprocal centimetres (cm^{-1}). Spectral data were obtained in the range of 600-4000 cm^{-1}. The cumulative results were the mean of 256 scans. The collected data were used to form a conclusion about the chemical nature of the bonds and functional groups of microbial metabolites. In parallel, the FT-IR spectrum of a standard lipopeptide biosurfactant, surfactin, obtained from the microorganism *Bacillus subtilis* (Sigma, USA) was also obtained. The spectra of samples obtained on different days from our microorganisms were compared to confirm that the *Bacillus amylofasciens* product belongs to lipopeptides.

Infrared spectroscopy revealed the presence of lipopeptides compared to standard surfactin (Sigma, USA), and the chemical nature of surfactin molecules obtained from the FT-IR spectrum. The broad signal at 3327 cm^{-1} denotes both a hydroxyl group and compounds containing an amino group, which may refer to the cyclic hydrophilic peptide fragment of the lipopeptide. Other absorption peaks observed at 2932, 2851, 1713 cm^{-1} indicated stretching vibrations due to long alkyl chains, amino, peptide group of the compounds, respectively (Figure 1). The spectra of our compounds isolated on different days differ mainly in the relative intensity of the peaks in the region of absorption of hydroxyl and amine fragments, as well as the peptide fragment, which may indicate the heterogeneous nature of the formation of lipopeptide molecules at different stages of fermentation. Probably, the concentration of the product reached its maximum value on the 5th day, and on the 7th day it somewhat decreased, which is reflected in the reduced intensity of the characteristic absorption bands.

It is important to note that the intense absorption band at 3327 cm^{-1} in all samples, which is characteristic of NH bond stretching, indicated the presence of peptides. The CO-N bond stretching mode is indicated by a bar at 1639 cm^{-1} and the NH bond strain mode in combination with C-N stretching is indicated by a bar near 1548 cm^{-1}. The band at 1248 cm-1 for all samples was due to the adsorption of lactone carbonyl group that are characteristic of lipopeptide samples in biotechnologically obtained products [8]. These data are consistent with similar studies on the preparation of lipopeptides on various substrates and demonstrate the combination of aliphatics and carbonyl groups [9]. Spectra of obtained products are similar to lipopeptides that were extracted from *Bacillus amyloliquefaciens* SR1 using precipitation technique [10].

The chemical nature of the samples obtained at different stages of fermentation was further characterized by thin layer chromatography (Figure 2). This analytical method is standard for characterizing the composition and functionality of newly obtained products from various strains and for testing new conditions for growing microorganisms [11]. The resulting lipopeptides were applied to a silicon dioxide plate and elution was carried out in a solvent system that allows the most efficient separation of the components of the biotechnological production of biosurfactants (chloroform:methanol:water (13:3:0.4, v/v/v). Thus, separation of the compounds that make up the extracted product was observed and the presence of both hydrophobic fragments - lipid tails stained with iodine vapor, and hydrophilic groups - peptide heads stained with ninhydrin solution was demonstrated. In addition, the presence of carbohydrate fragments was revealed, probably getting into the extract from the nutrient medium and stained with a solution of para-anisaldehyde, which is a reagent. staining nucleophilic compounds. Such an observation allows both to confirm that the product belongs to lipopeptides and to draw attention to the need to improve extraction procedures for each individual target product, adjusted for the conditions of obtaining (composition of the medium and cultivation conditions).

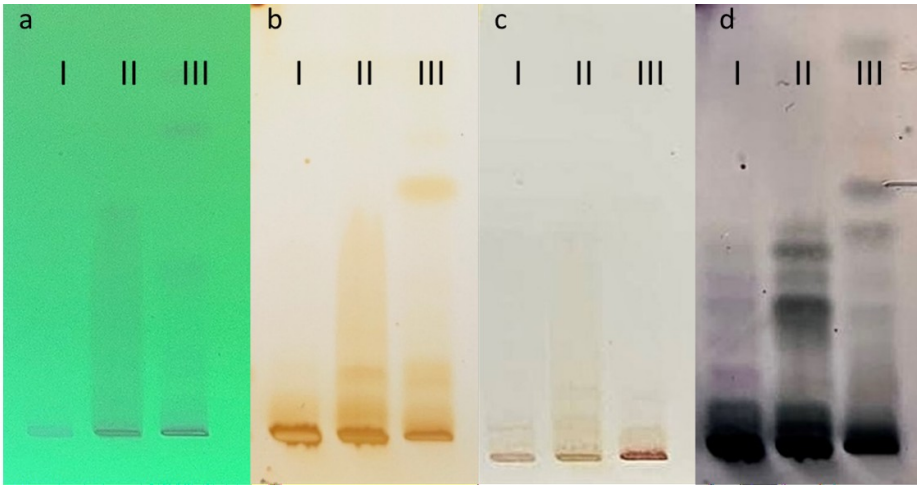

Fig. 2. TLC analysis of the biosurfactants produced by the *Bacillus amyloliquefaciens* BKMA B-12464 (I, II, III samples). Spots revealed: by UV light (a); after iodine vapor staining (b); after ninhydrin staining (c), after anisaldehyde staining (d).

The figure shows the multicomponent nature of the products, which is consistent with the literature - products in bacillus under different conditions are complex in composition and can include lipopeptides of various molecular weights and head-to-tail ratios [12]. The presence of individual spots for the product of this type of microorganisms is typical even for purified products, which was shown in a number of studies [13-14].

4 Conclusion

In the course of the study, the cultivation of the *Bacillus amyloliquefaciens* BKMA B-12464 strain was carried out and the dynamics of the increase in the mass of the biosurfactant was assessed on days 3, 5 and 7. It was found that the maximum yield of the biosufactant was observed on the 7th day, which is consistent with the data on the emulsification activity of E24. According to the analysis of IR and TLC, it was found that the isolated biosurfactant belongs to the class of lipopeptides. The obtained biosurfactant and the scheme of its cultivation will be used in further studies.

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