Optimization of cultivation conditions for biosurfactant production by *Bacillus mojavensis* P1709, *Pseudomonas putida* PP021, and *Pseudomonas fluorescens* PCS-20 strains

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Abstract. Biosurfactants, being a promising group of compounds for agriculture, oil production and environmental protection, need further study. Including ways of their production by cultivation of microorganisms obtained from various sources. In this study, the effect of nutrient medium composition on the production of biosurfactants by *Bacillus mojavensis* P1709, *Pseudomonas putida* PP021 and *Pseudomonas fluorescens* PCS-20 strains was investigated. The emulsification index (E24) and surface tension (ST) of culture supernatants were determined to evaluate the surfactant properties of the biosurfactants. BH medium with glucose was found to be optimal for *B. mojavensis* strain P1709, providing an E24 of 61% and ST of 28.3 mN m^{-1}. For *P. putida* strain PP021 and *P. fluorescens* strain PCS-20, the best results were obtained on glycerol-nitrate medium with E24 85% and 80%, and ST 27.5 mN m^{-1} and 29.4 mN m^{-1}, respectively. Medium with hexane as the sole carbon source showed lower efficiency. Further cultivation of the selected strains on optimal media for 72 hours revealed that minimum ST values were reached by 36 hours for *B. mojavensis* P1709 (23.96 mN m^{-1}) and *P. fluorescens* PCS-20 (24.4 mN m-1), and by 72 hours (27.5 mN m^{-1}) for *P. putida* PP021. Despite reaching the plateau of cell growth, the decrease in ST continued, which may be due to changes in biosurfactant composition.

1 Introduction

Biosurfactants are secondary metabolites of microorganisms, which are surface-active compounds. Chemical structure - the presence of a hydrophilic “head” and a hydrophobic “tail” allows them to acquire amphiphilic properties. This allows them to reside at the interface between polar and non-polar phases, such as air-water or oil-water interfaces [1], and also form micellar structures. Science and industry are interested in their ability to change surface and interfacial tension, emulsify, disperse and solubilize various compounds - they are used as a promising tool for remediation of disturbed soils, increasing oil recovery, plant protection, drug delivery and many other challenges [2].

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Biosurfactants are secondary metabolites of various groups of microorganisms [3] and can be glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids and neutral lipids [4]. Representatives of the genera *Pseudomonas, Bacillus, Rhodococcus and Candida* are primarily used for the production of biosurfactants. These microorganisms are widely studied and used in various biotechnological applications due to their ability to produce powerful surfactants, but there is a wide variety of strains and sources of their production, and the habitat and substrate affect the characteristics of the resulting product - the biosurfactant [5].

Optimizing culture conditions is critical to maximizing the production of these valuable surfactants. Biosurfactants are primarily involved in increasing the solubility and availability of various water-immiscible substrates (vegetable and animal oils, hydrocarbons), which allows these substrates to be used for the production of biosurfactants [6].

The most important parameters of the culture process for biosurfactant production are the type of substrates, the amount and ratio of carbon and nitrogen in the medium, and the presence of various metal cations in the medium [7]. Studies show that carbon availability, nitrogen limiting conditions, and a C:N ratio of 20 are favorable for biosurfactant production [8]. Raza et al. (2016) noted that biosurfactant production is favored by low magnesium concentration and higher iron concentration [9].

Assessing the potential for biosurfactant production under different nutritional conditions will provide valuable information on the factors that control the biosynthesis of these surfactant compounds, which will ultimately facilitate the development of efficient and cost-effective bioprocesses for biosurfactant production. Based on our previous results, the present study aimed to evaluate the effects of three different culture media on biosurfactant production by selected strains of *B. mojavensis* P1709, *P. putida* PP021 and *P. fluorescens* PCS-20. In the course of this work, a) a medium for culturing the strain was selected and b) the duration of culturing the strain on the selected medium was selected.

2 Materials and methods

2.1 Strain preservation and inoculum preparation

The biosurfactant-producing strains used in this study, *Bacillus mojavensis* P1709, *Pseudomonas putida* PP021 and *Pseudomonas fluorescens* PCS-20, were previously isolated from the rhizosphere of *Lactuca sativa* and oil-contaminated soil samples through a comprehensive screening process. The most promising isolates were selected based on their ability to significantly reduce surface tension and form stable emulsions. After initial screening, the selected strains were preserved in a laboratory culture collection at -80°C in an appropriate nutrient medium for long-term storage.

For the present study, the preserved cultures were revitalized by streaking on cups of nutrient-rich agar such as Luria-Bertani (LB) with incubation at 28°C for 24 hours. Individual colonies were then used to inoculate the respective liquid media to prepare pre-cultures. Precultures were incubated at 28°C with agitation (150 rpm) for 16 hours until visually apparent turbidity. The pre-cultures served as inoculum for subsequent experiments on selection of media and culturing time for biosurfactant production by the selected strains.

2.2 Culturing media

Three different media were evaluated for their ability to support biosurfactant production by the selected strains. BH medium with glucose: Bushnell-Haas (BH) medium supplemented with 2% (wt./vol.) glucose as a carbon source. Glycerol-Nitrate Medium: a mineral-salt medium containing 2% (wt./volume) glycerol as a carbon source and 0.1% (wt./volume) sodium nitrate as a nitrogen source. Hexane-containing medium: mineral salt medium with 1% (by volume) hexane as the sole carbon source. Media were prepared according to standard protocols and sterilized by autoclaving at 121°C for 15 minutes. The pH of the medium was adjusted to 7.0±0.2 before sterilization.

2.3 Cultivation conditions

Pre-cultures of *B. mojavensis* P1709, *P. putida* PP021 and *P. fluorescens* PCS-20 were used to inoculate the respective culture media until the same optical density values were obtained. The inoculated media were incubated at 28°C under stirring (150 rpm) for 24 hours. Samples were collected at regular intervals (every 8 hours) for determination of optical density and biosurfactant production.

2.4 Preparation of Cell-free Culture Supernatants

After a 24-hour culture period, the microbial cultures were centrifuged at 3,000 g for 40 minutes at room temperature to separate the cells from the culture medium. The cell-free supernatants obtained were used to evaluate the biosurfactant production potential of the isolated strains under different culture conditions by analytical methods.

2.5 Analytical methods

Optical Density Measurement: the growth of microbial cultures was monitored by measuring optical density at a wavelength of 600 nm (OD600) using a spectrophotometer. Surface tension measurement: the surface tension of cell-free culture supernatants was measured using a tensiometer Kruss K-20 (KRUSS, Göppingen, Germany) by a Du Noüy ring method. The decrease in surface tension compared to unseeded medium was used as an indicator of biosurfactant production. Emulsification Index (E24): the cell-free culture supernatant was mixed with an equal volume of oil from the Middle Volga field and the mixture was stirred on shaking for 2 minutes. The height of the emulsified layer was measured after 24 hours and expressed as a percentage of the total height of the mixture.

Based on the results of these analyses, the most promising cultivation conditions for each strain were selected for further optimization and scale-up of biosurfactant production. In addition, for the selected optimal culturing conditions, changes in optical density and biosurfactant production (E24 and surface tension) were monitored over an extended culturing period of 72 hours, with samples collected every 8 hours.

3 Results and discussions

3.1 Selection of nutrient media

The production of biosurfactants by *B. mojavensis* P1709, *P. putida* PP021 and *P. fluorescens* PCS-20 strains was assessed by culturing them on three different media: BH medium with glucose, glycerol-nitrate medium, and medium with hexane for 24 hours at 28

°C (Table 1). We were guided by the emulsification index (E24) and surface tension (ST) values of the cell-free culture supernatants when selecting the culture media. As shown in Table 1, medium containing glucose as a carbon source was the most effective *for B. mojavensis* P1709, with an E24 of 61% and a surface tension (ST) of 28.3 mN m^{-1}. For *P. putida* PP021 and *P. fluorescens* PCS-20, the medium with glycerol and nitrate was better, providing E24 values of 85% and 80% and ST values of 27.5 mN m^{-1} and 29.4 mN m^{-1}, respectively (Table 1).

Table 1. Characteristics of cell-free supernatants obtained when strains were cultured on different media for 24 hours.

Carbon Source	Indicator	*B. mojavensis* P1709	*P. putida* PP021	*P. fluorescens* PCS-20
Glucose	E24, %	61±5	81±5	75±5
	ST, mN m^{-1}	28.3±0.22	28.8±0.18	31.5±0.21
Glycerol	E24, %	55±5	85±5	80±5
	ST, mN m^{-1}	29.3±0.54	27.5±0.43	29.4±0.34
Hexane	E24, %	30±2	66±5	54±5
	ST, mN m^{-1}	45.6±0.37	52.4±0.59	43.3±0.78

In contrast, the medium containing hexane as the sole carbon source did not perform better, with E24 values ranging from 30 to 66% and surface tension ranging from 43.3 to 52.4 mN m^{-1} for the three strains. Based on these results, the medium with hexane was excluded from further experiments, and BH medium with glucose and glycerol-nitrate medium were selected for further culturing and optimization studies. In general, these media are suitable for the production of a wide variety of different types of surfactants [10].

3.2 Evaluation of surface-active properties of culture supernatants

Surface tension measurements of culture supernatants revealed significant differences between strains during the initial stages of cultivation.

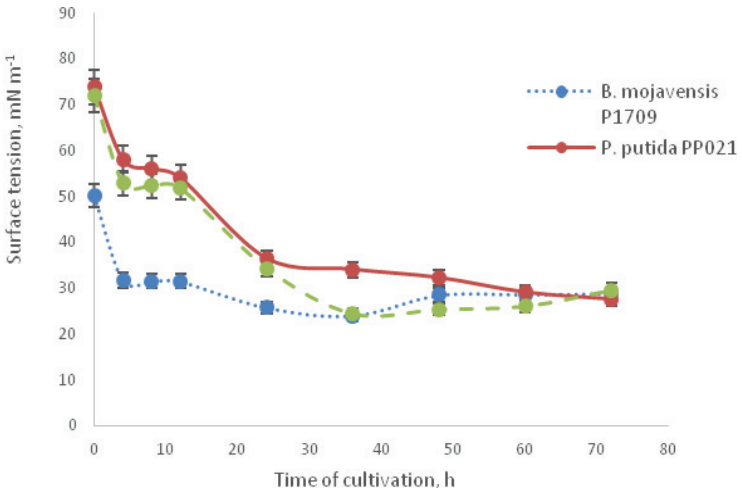

Fig. 1. Surface tension of the culture medium of *B. mojavensis* P1709, *P. putida* PP021, *P. fluorescens* PCS-20 strains.

As shown in Figure 1, supernatant of *P. fluorescens* PCS-20 had an initial surface tension of 72 mN m^{-1}, while that of *P. putida* PP021 was 73.8 mN m^{-1}. In contrast, *B. mojavensis* P1709 had a lower initial surface tension of 50.1 mN m^{-1}. These differences may be due to the use of different media for culturing the strains (BH medium with glucose and glycerol-nitrate medium).

A gradual decrease in surface tension was observed during the cultivation period. Already at the 4th hour of incubation the values decreased to 58.6 mN m^{-1} for *P. fluorescens* PCS-20 and 54.9 mN m^{-1} for *B. mojavensis* P1709. The minimum surface tension values were reached after 36 hours of cultivation, 24.4 mN m^{-1} for *P. fluorescens* PCS-20 and 23.96 mN m^{-1} for *B. mojavensis* P1709. For *P. putida* PP021, the minimum value of 27.5 mN m^{-1} was observed after 72 hours.

The obtained results are in agreement with the data given in the scientific literature for other effective biosurfactant-producing strains of the genera *Pseudomonas* and *Bacillus* [11–14]. This indicates a high potential of the studied strains in the production of biosurfactants that can be used for enhanced oil recovery.

3.3 Relationship between bacterial growth and biosurfactant production

At the next stage, work was carried out to select the optimal duration of culturing the strains until the highest biosurfactants yield was achieved. From the point of view of economic feasibility, the strains should be cultivated only as long as their biosurfactant activity continues to increase. The optical density (reflecting the number of cells) may continue to grow or already be at a plateau. Figure 2 shows the growth curves of the isolates, assessed dynamically.

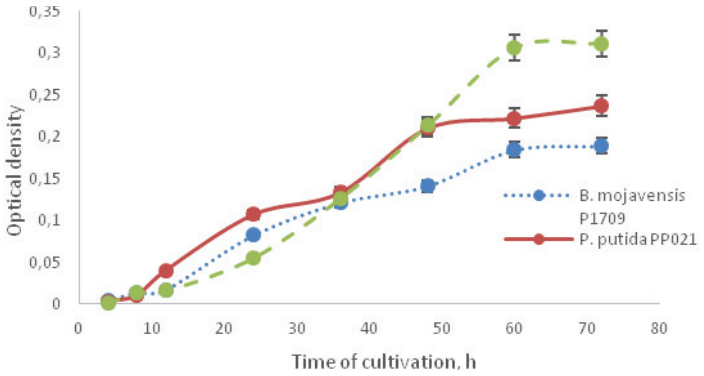

Fig. 2. Optical density of the culture medium of *B. mojavensis* P1709, *P. putida* PP021, *P. fluorescens* PCS-20 strains.

As shown in Figure 2, the optical density of the cultures reached a plateau at 48-60 hours of cultivation. Despite this, the surface tension of the supernatants continued to decrease. This phenomenon may be attributed to several factors, including the cessation of biosurfactant production by bacteria upon reaching a specific cell density, alterations in the ratio of various types of biosurfactants with distinct surface-active properties during cultivation, and the potential saturation of surfactant molecules at the interface [15].

Although no direct correlation was observed between bacterial growth and biosurfactant activity, the relative change in surface tension per unit cell density or per unit culturing time is an important economic characteristic for commercial biosurfactant production. Based on the results of this work, the culturing procedure of the selected strains can be optimized to 24-36 hours.

The results obtained indicate a high potential of the strains under study as biosurfactants producers, promising for application in oil production, wastewater treatment, plant protection in agricultural practice. Further research will be aimed at isolation, purification and identification of biosurfactants produced by the selected strains, studying their physicochemical and biological properties, as well as evaluating the efficiency of their application in various industries.

4 Conclusion

As a result of this study, BH medium with glucose was found to be optimal for *B. mojavensis* strain P1709, providing an emulsification index (E24) of 61% and a surface tension (ST) of 28.3 mN m^{-1}. For *P. putida* PP021 and *P. fluorescens* PCS-20 strains, the best results were obtained on glycerol-nitrate medium with E24 85% and 80% and ST 27.5 mN m^{-1} and 29.4 mN m^{-1}, respectively. Medium with hexane as the sole carbon source showed lower efficiency and was excluded from further experiments.

Further cultivation of the selected strains on optimal media for 72 hours with sampling every 8 hours allowed to establish that the minimum values of surface tension were reached by 36 hours for *B. mojavensis* P1709 (23.96 mN m^{-1}) and *P. fluorescens* PCS-20 (24.4 mN m^{-1}), and for *P. putida* PP021 - by 72 hours (27.5 mN m^{-1}). Despite reaching a plateau of cell growth by 48-60 hours, the decrease in surface tension continued, which may be due to a change in the ratio of different types of biosurfactants or the achievement of saturation of surface-active molecules at the interface.

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