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OPTIMIZATION OF *BACILLUS LICHENIFORMIS* DSM13 FOR BIOSURFACTANT PRODUCTION USING RESPONSE SURFACE METHODOLOGY

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Biosurfactants are surface-active compounds that can reduce surface tension in both aqueous solutions and hydrocarbon mixtures, which in recent times have become more valuable due to their lower toxicity and are generally referred to as green or organic surfactants. Such products are much better than chemical surfactants in terms of their enhanced biodegradation rates and the bioavailability of organic contaminants. Fungi, yeast and bacteria are mainly capable of producing microbial biosurfactants. Bacteria, especially Bacillus, are one of the most frequently applied and studied biosurfactant producers. This study investigated the kinetics of cell growth, the production of biosurfactants as well as the effect of and interactions between the (A) pH within the range of 4.1 to 9.8, (B) glucose concentration between 3.0 and 36.9 g/l, (C) surface tension and (D) emulsification index to maximize biosurfactant production. The analysis was carried out using a central composite design (CCD) model with four factors and five levels. The optimized medium (pH=8 and glucose concentration = 38 g/l) decreased the surface tension to 60 mN/m and increased the product yield up to 2.7 g/l.

Keywords: cell growth, effect of pH, glucose concentration

1. Introduction

Surfactants are surface-active compounds that reduce the surface tension between two liquids. Surfactant molecules are comprised of hydrophobic and hydrophilic, that is, water-hating and water-loving components, respectively. They are also regarded as detergents because of their wetting potential and emulsifying as well as foaming agents. Biosurfactants are also surfactants but from a microbial source. They have proven to be more sustainable due to their ability to leave less of or even no chemical residues after their use. Biosurfactants have been commonly used in industries such as the petroleum, cosmetics, antimicrobial, pharmaceutical and bioremediation industries [1]-[4].

Biosurfactants, also known as biological surfactants, are structurally varied molecules with high surface and emulsifying activities [5]-[6]. Glycolipids, lipopeptides and polymeric biosurfactants are the three major groups of biosurfactants. Biosurfactants have many advantages over conventional surfactants, including their ease of renewability, large-scale production, economic viability, cheaper substrates, more significant degree of foaming, good selectivity, turbidity, good biocompatibility, effectiveness at high temperatures or pH levels, chemical diversity and environmentally-friendliness [7]-[8]. Biodegradation is one of the most effective methods for combating environmental degradation mainly as a result of petroleum hydrocarbons, which pose a significant danger to ecosystems. This includes bacteria that use toxic materials as carbon sources, resulting in the breakdown of polluted components into low-molecular-weight or less harmful chemicals with no adverse effects [9].

Biosurfactants can decrease the surface tension of water to between 35 and 27 mN/m, which has been recorded by biosurfactant-producing bacteria [10]-[11], as well as increase the emulsifying activity from 20 to 30% for different hydrocarbon compounds during experiments on the emulsification index. A CCD was used to create appropriate testing levels for our response surface methodology (RSM). This analysis will create relevant parameters by making observations or taking measurements to determine the best combination of media that produces the desired response as well as characterizing the reaction so the conventional medium optimization strategy of modifying one independent variable while keeping the rest constant can be applied [12].

The main aim of our research is to produce biosurfactants to study their antifungal effects on crops in agriculture. Therefore, the first step is to produce biosurfactants efficiently. For this purpose, media optimization is commenced by using statistical optimization for cell growth in the fermentation broth to

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increase the biosurfactant yield, i.e. decrease the surface tension and increase the emulsification index of the biosurfactant produced by *Bacillus licheniformis* DSM13.

2. Materials and Methods

2.1. Microorganism and cultivation of strain

The *Bacillus licheniformis* DSM13 strain was purchased from Hungary's National Collection of Agricultural and Industrial Microorganisms. The biosurfactant fermentations were conducted in 500 ml shake flasks (Erlenmeyer flasks with cotton plugs covered with aluminum foil). The inoculum was incubated for two days at 150 rpm and 37°C in a rotary shaker (New Brunswick Excella E24) by applying an inoculation ratio of 10 %. During the biosurfactant fermentation, the starting total volume, including the inoculum, was 150 ml, with an aeration greater than the working volume.

For the biosurfactant fermentation, a minimal medium was used; 1 liter of minimal media (pH=6) contained 1.0 g NH_4NO_3, 34.0 g glucose (Hungrana Kft., Szabadegyháza, Hungary), 6.0 g KH_2PO_4, 2.7 g Na_2HPO_4, 0.1 g $MgSO_4*7H_2O$, $1.2x10^{-3}$ g $CaCl_2$, $1.65x10^{-3}$ g $FeSO_4*7H_2O$, $1.5x10^{-3}$ g $MnSO_4*4H_2O$ and $2.2x10^{-3}$ g Na-EDTA (Reanal Laborvegyszer Kft., Hungary) Joshi et al. [13]-[14].

2.2. Unoptimized (reference) fermentation

Scale-up tests were conducted in a 1 l benchtop fermenter Biostat Q bioreactor (B Braun Co) filled up to 700 ml. During batch fermentation, the temperature was maintained at 37°C, the agitation rate at 300 rpm and the aeration rate at 0.5 VVM without pH control. A collection vessel was added to the exhaust air of the fermenter to collect the foam produced during the fermentation.

2.3. Statistical analysis of CCD (RSM) for optimization

Using a CCD for two variables, the power of the response surface approach to maximize biosurfactant production by *Bacillus licheniformis* was investigated in this study, which has served as the foundation for the simulated experimental plan and subsequent analysis. The randomized empirical findings were statistically analyzed using the statistical program TIBCO Statistica (version 13 for Windows) to detect the significant differences between the independent variables, namely (A) glucose concentration between 10 and 36 g/l and (B) pH between 4 and 9, to achieve maximum biosurfactant production by *B. licheniformis* DSM13 (*Table 1*).

Table 1. CCD runs showing factors and their levels

Standard run	2**(2) central composite, nc=4 ns=4 n0=2 Runs=10	
	Glucose [g/l]	pH
3	34.00	5.00
8	20.00	9.83
1	10.00	5.00
6	36.97	7.00
7	20.00	4.17
2	10.00	9.00
5	3.03	7.00
4	34.00	9.00
10 (C)	20.00	7.00
9 (C)	20.00	7.00

2.4. Biomass analysis

A CamSpec M501 spectrophotometer was used to determine the biomass content via optical density measurements at a wavelength of 600 nm (OD_{600}).

2.5. Surface tension analysis

Surface tension is the force per unit length measured in Millinewtons per meter (mN/m). The surface tension was measured using a stalagmometer (Wilmad-LabGlass LG-5050-100) according to the method of Czinkóczky et al. [11].

2.6. pH analysis

Initially, the pH of the media was set by adjusting it to several pH values ranging from 4 to 9 with 5M HCl or 6M NaOH. The pH was measured by a METTLER TOLEDO FiveEasy™ pH meter.

2.7. Emulsification index (E24)

The emulsification index measurement applied was established by Plaza et al. [15]. The emulsification index was determined from the supernatant of the fermented broth at intervals corresponding to the sampling frequency during the fermentation. In a test tube, 2 ml of crude oil and 2 ml of cell-free media (supernatant) were introduced and homogenized for 2 minutes by vortexing at 4000 rpm. The emulsifying activity was once more determined after 24 hours as follows: the relative height of the two liquid layers was documented by photos and the pixel size divided to obtain the values of the emulsification index [16]. E24 (%) is defined as [(total height of the emulsified layer) / (total height of the liquid layer) x 100] after 24h of vortexing.

2.8. Isolation and purification of the biosurfactant

Acid precipitation was used to isolate the biosurfactants. The bacterial cells were first removed by centrifugation and the remaining supernatant containing the biosurfactant was acidified with 2M HCl solution until the pH reduced to 2. The mixture was then incubated at 4 °C for 24 hours. The precipitate was collected by centrifugation at 10,000 rpm and 4°C for 30 minutes. The residue was then resolved in distilled water and the pH reset to neutral before being freeze-dried by a Martin Christ Alpha 1-4 LSCbasic lyophilizer [13].

3. Results

3.1. Unoptimized (reference) fermentation

An unoptimized fermentation according to Section 2.2. was carried out and the parameters that were measured during the process are shown in *Fig.1*. The highest reduction in the surface tension (ST) was observed 4 - 30 hours after the fermentation commenced. ST ranged from 75.5 to 68.3 mN/m. After 8 hours, the foam began to overflow and was collected in a vessel for further processing. The collected foam had a ST of 60 mN/m, indicating that the biosurfactants produced are mainly collected in the foam phase.

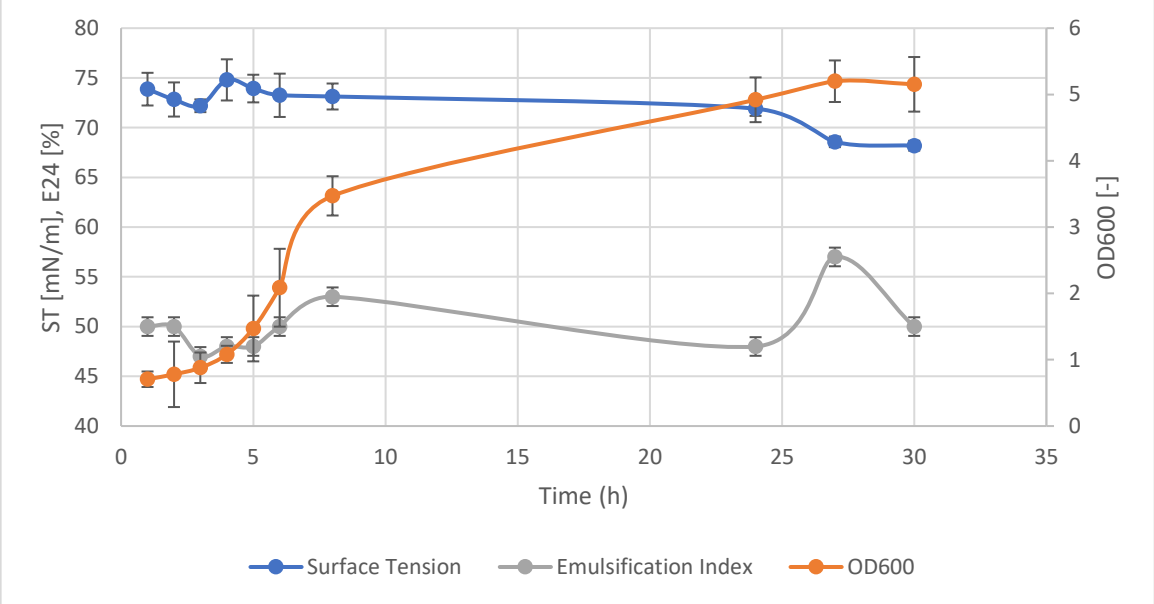

Figure 1. Growth, surface activity and emulsification index (E24) of *Bacillus licheniformis* in a 1 l fermenter during growth on a mineral salt medium

3.2. RSM

The effects of the glucose concentration and pH is shown in *Fig.2*. The highest biomass growth (OD) was recorded at a glucose concentration of 40 g/l and a rather higher pH value of approximately 9 resulting in an optical density of 4.5.

The mixed effect of the pH and glucose concentration regarding the surface tension is shown in *Fig.3*. The minimum surface tension was achieved while fermenting at pH 7.

According to *Fig.4*, the smallest product amount was achieved when the pH was extremely high at 11 and the glucose concentration was the lowest, while at a higher glucose concentration, the highest product yield was still achieved. This indicates that the gradient of the surface is minimal and very high at low and high glucose concentrations, respectively; moreover, depends on the pH. Therefore, the pH was the most important factor leading to an increase in the amount of biosurfactant produced.

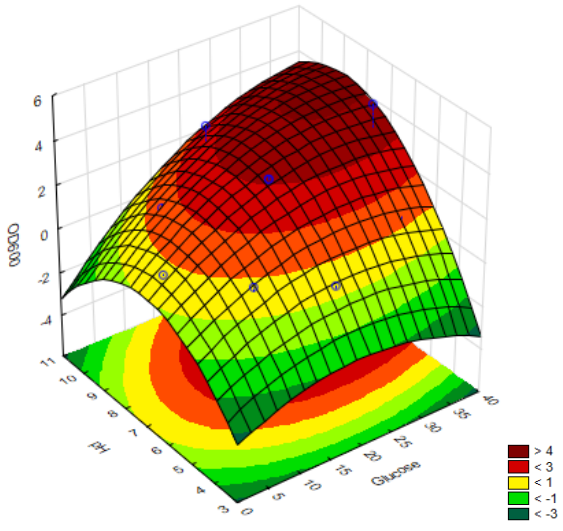

Figure 2. 3D RSM plot of the interactive effects of glucose concentration (g/l) and pH on biomass production

The model predicted the following ideal conditions to maximize the biosurfactant productivity using *Bacillus licheniformis* DSM13: pH=8 and glucose concentration=40g/l. The anticipated optimum point was confirmed experimentally, moreover, the observed and isolated product was 2.7 g/l. These findings show a strong connection between the predicted and actual experimental values, moreover, this model accurately represents biosurfactant production in the presence of *Bacillus licheniformis* DSM13. Since the highest product yield (i.e. lowest surface tension, *Fig.3*) was observed at different conditions, it is recommended to examine product purity in the future as the presence of contaminants can depend on the pH because of pH-dependent precipitation during product isolation.

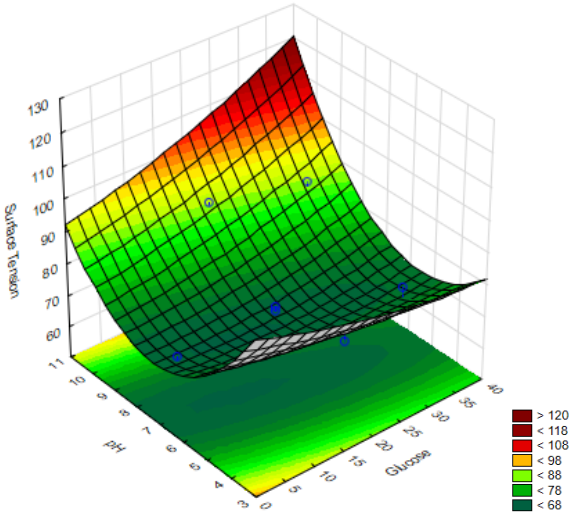

Figure 3. 3D RSM plot of the interactive effects of the glucose concentration (g/l) and pH on the surface tension (mN/m)

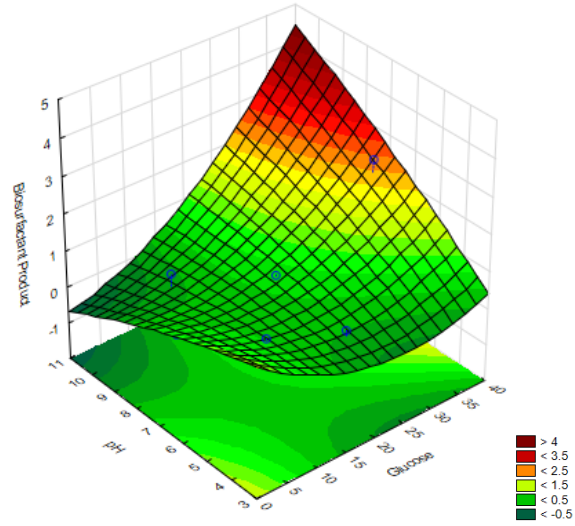

Figure 4. 3D RSM plot of the collaborative effects of the glucose concentration (g/l) and pH on the biosurfactant product

4. Conclusions

Biosurfactants are emerging as suitable alternatives to their predominant, less sustainable, petroleum-derived counterparts. In this study, the CCD, in conjunction with the response surface approach, is used to predict the optimization of *Bacillus licheniformis* DSM13 effectively. An increase in the glucose concentration yielded a high biosurfactant concentration while also increasing the fermentation time. At a lower pH level, the strain achieved a low biomass yield and lower biosurfactant yield but no change in the surface tension was observed. It was observed that the best operating conditions for biosurfactant production with *Bacillus licheniformis* DSM13 was a higher pH (pH=8) and a glucose concentration of 38 g/l. Future process validation to optimize biosurfactant manufacturing techniques is advised as follow-up research.

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