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# Improvement of Biosurfactant Production by Microbial Strains Through Supplementation of Hydrophobic Substrates

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Biosurfactants are amphiphilic tensioactive natural products that are capable of lowering the surface and interfacial tensions of the growth medium. Efficient biosurfactants are characterized by their ability to enhance the aqueous solubility of hydrophobic compounds and to emulsify hydrocarbons in aqueous medium. Improvement in the fermentation technology, strain selection and use of cheaper and renewable substrates have a vital role in enhancing the production processes of biosurfactant industries. However, large scale production of biosurfactants has not reached a satisfactory economical level due to their low yields. Several studies have reported significant effect of carbon sources on the productivity of biosurfactants by different strains. In the current study medium composition optimization approach was investigated for optimal biosurfactant production using a combination of hydrophobic and hydrophilic carbon sources by *Bacillus subtillis* CN2 strain, previously isolated from hydrocarbon contaminated soil. The study demonstrated that both quantity and type of carbon sources prompted a significant difference in the amount and activity of the biosurfactant produced. The hydrophobic carbon sources were found to be superior to hydrophilic ones in promoting biosurfactants production and surface activity superiority. The strain produced 10-fold more biosurfactant when growing on oil than when grown on glycerol and significantly higher surface activity as determined from the emulsification index. In addition to using the hydrophobic substrate sunflower oil as a sole substrate, addition of sunflower oil (5%, wt/v) in to the growth medium after depletion of hydrophilic substrate (glycerol) stimulated the production of biosurfactant by more than 200%. Both the type and concentration of the carbon source were shown to be essential determinants of biosurfactant yield and physicochemical properties. The result of our study showed that the presence of optimal hydrophobic substrates in the growth medium triggered release of more biosurfactant through their inductive effect, which shows a promising potential of the approach for large scale viable biosurfactant production.

## 1. Introduction

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between two immiscible liquids such as oil/water or air/water interfaces, causing reduction of surface and interfacial tension of liquids, and the ability to form micellar systems or microemulsions between two immiscible phases and conferring them excellent detergency, emulsifying, foaming, and dispersing properties (Santos *et al.,* 2016). Biosurfactants are surface-active biomolecules produced by microbes (bacteria, fungi, and yeast), currently gaining considerable attention owing to their high biodegradability, low toxicity, higher foaming; specific activity at extreme temperatures, pH, and salinity, wide range of industrial applications, structural diversity and ability to be synthesized from renewable feed-stocks (Ribeiro *et al.,* 2019; Singh *et al.,* 2019). The global biosurfactants market was estimated at USD 4.20 Billion in 2017 and is projected to reach USD 5.52 Billion by 2022, at a compound annual growth rate of 5.6% during the forecast period. The increasing demand for green solutions primarily in the personal care, and home care industries is expected to drive the growth of the global biosurfactants market (https://www.marketsandmarkets.com/Market-Reports/biosurfactant-market-163644922.html). However, biosurfactants are not yet widely exploited in industry, the application of biosurfactants depends on whether they can be produced economically at large-scale. Presently,

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biosurfactants are not competitive with chemical surfactants from an economic point of view, since expensive substrates are required for their production (Silva *et al.,* 2014; Bezza and Chirwa, 2017). The major factor restraining the growth of the global biosurfactants market is the high production cost as compared to conventional chemical surfactants and bio-based surfactants. The high raw material cost, lower productivity, and cost associated with the product purification steps are some of the factors responsible for the high overall manufacturing cost of biosurfactants (Najmi *et al.,* 2018). The type, quantity, and quality of biosurfactants are mostly influenced by the nature of the carbon substrate, and the concentration of nitrogen, phosphorous, magnesium, iron, and manganese ions in the medium and culture conditions, which include pH, temperature, agitation, dilution rate, etc. (Shekhar *et al.,* 2015).

The cost of biosurfactant production can be reduced by strain improvement, optimizing medium composition by statistical methods or by using alternative low-cost substrates. The choice of cheap raw materials is important to the overall economy of the process as they account for 50% of the final production cost and also reduce the expenses with waste treatment (dos Santos *et al.,* 2010). Optimizing factors that affect growth in biosurfactant producing organisms with potential for commercial exploitation is of paramount importance (Abouseoud *et al.,* 2008). One of the most accepted methodologies used for optimizing medium composition is response surface methodology (RSM), which is widely used for optimization of the process parameters. This statistical technique has been successfully used to optimize medium composition for the synthesis of metabolites and biodegradation processes (Moshtagh *et al.,* 2018). Nitrogen is reasonably considered to be a nutrient source for the production of biosurfactant and the effect of total mass of carbon sources to mass of nitrogen, C/N, ratio is one of the most important parameters in biological systems and has been extensively studied in many fermentation processes (Heryani and Putra, 2017). Improved biosurfactant synthesis have been reported by some microorganisms when growing on water-immiscible substrates such as n-alkanes and olive oil through inductive effect (Viramontes-Ramos *et al.,* 2010). In the current study optimization of quantities of water miscible and water immiscible carbon sources and C/N ratio on optimal biosurfactant production by *B.subtilis* CN2 strain, an efficient biosurfactant producer previously isolated in our lab (Bezza and Chirwa, 2015), was examined using response surface methodology and effect of type of hydrocarbon on the quantity and surface activity of the biosurfactant was examined.

## 2. Materials and Methods

### 2.1 Biosurfactant Production

The biosurfactant production was carried out in 500-mL Erlenmeyer flasks containing 100 mL of a mineral medium consisting of 0. The mineral salt medium (MSM) was composed of (g/L): $(NH_4)_2SO_4$, 6; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.4; $Na_2HPO_4 \cdot 2H_2O$, 7.59; $KH_2PO_4$, 4.43; and 2 mL/L of trace element solution. The trace element solution consisted of (g/L): EDTA (disodium salt), 20.1; $FeCl_3 \cdot 6H_2O$, 16; $CoCl_2 \cdot 6H_2O$, 0.18; $ZnSO_4 \cdot 7H_2O$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.16 and $MnSO_4 \cdot H_2O$, 0.10. (Bezza and Chirwa, 2015), using an increasing concentration of sunflower oil as hydrophobic (3- 9 %, wt/v) and glycerol (2- 8 %, wt/v) as a hydrophilic carbon sources the carbon source and $NH_4NO_3$ addition was adjusted to carbon sources accordingly to an increasing ratio of C/N (3 to 20). The initial pH of the medium was adjusted to 7.0 using 1 M NaOH. Inoculation volumes corresponding to about 0.1 g/L of exponential-phase cells were used and the flasks were incubated at 250 rpm and 37 °C for 5 days. In all experiments, the biosurfactant production was indirectly evaluated through the surface activity determination through emulsification index ($E_{24}$) evaluation of the cell-free supernatant (collected after centrifugation of culture at 12,000 rpm, 10min) with *n*-octane.

### 2.2 Experimental design

The three-level, three-factorial Box-Behnken experimental design with categoric factor of 0 was employed to study the combined effect of supplementation of various amounts of hydrophobic and hydrophilic carbon sources in percentage by weight and C/N ratio on the biosurfactant surface activity using evaluation of emulsification index of cell free supernatant (response). The design was composed of three levels (low, medium and high, being coded as −1, 0 and +1) and a total of 17 runs were carried out in duplicate to optimize the level of chosen variables. For the purpose of statistical computations, the three independent variables were denoted as $X_1$, $X_2$, and $X_3$, respectively (Table 1). According to the preliminary experiments, the range and levels used in the experiments are selected and corresponding experiments were performed (Table 2). For each experiment, the response (activity of biosurfactant) was evaluated in duplicates, and the average was used to calculate the coefficients. For reverse surface methodology (RSM), the most commonly used second-order polynomial equation developed to fit the experimental data and determine the relevant model terms can be written as (Eqn. 1): where Y is the estimated response, $\beta_0, \beta_i, \beta_{ii}\ and\ \beta_{ij}$ regression coefficients for the intercept, linearity, square, and interaction, respectively, $X_i$, $X_j$ (i=1–3, j=1–3 and i ≠ j). The results

were analysed by applying the coefficient of determination ($R^2$), response plots and analysis of variance (*ANOVA*).

$$Y = \beta_0 + \sum_{n=1}^{3} X_i\beta_i + \sum_{i=1}^{3} \beta_{ii}X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3} \beta_{ij}\ X_iX_j \quad \text{...(Eqn. 1)}$$

*Table 1 List of independent factors and corresponding levels*

| Variables | Real values of coded levels | | |
|---|---|---|---|
| | -1 | 0 | +1 |
| C/N Ratio, X1, (wt/wt) | 4 | 12 | 20 |
| Hydrophobic Carbon, X2, (%, w/v) | 3 | 6 | 9 |
| Hydrophilic Carbon, X3, (%, w/v) | 2 | 5 | 8 |

*Table 2 Box–Behnken design matrix and corresponding experimental responses*

| | Factor 1 | Factor 2 | Factor 3 | Response 1 |
|---|---|---|---|---|
| Runs | X1:C/N | X2: Hydrphobic | X3: Hydrophilic | Emulsification Index |
| | | (%, w/v) | (%, w/v) | ($E_{24}$, %) |
| 1 | 4 | 3 | 5 | 34 |
| 2 | 12 | 6 | 5 | 77 |
| 3 | 12 | 9 | 8 | 89 |
| 4 | 20 | 6 | 8 | 88 |
| 5 | 12 | 6 | 5 | 84 |
| 6 | 12 | 3 | 2 | 66 |
| 7 | 20 | 9 | 5 | 94 |
| 8 | 12 | 6 | 5 | 88 |
| 9 | 12 | 6 | 5 | 90 |
| 10 | 12 | 6 | 5 | 87 |
| 11 | 12 | 9 | 2 | 96 |
| 12 | 4 | 6 | 8 | 33 |
| 13 | 12 | 3 | 8 | 67 |
| 14 | 4 | 9 | 5 | 44 |
| 15 | 20 | 3 | 5 | 77 |
| 16 | 4 | 6 | 2 | 33 |
| 17 | 20 | 6 | 2 | 97 |

### 2.3 Emulsifying activity determination

Emulsifying activity was determined by the addition of 2 ml of *n*-octane to the same volume of cell-free culture supernatants or biosurfactant solutions in glass test tubes. The tubes were mixed with a vortex at high speed for 2 min and kept at 37°C for 24 h. The stability of the emulsion was determined after 24 h, and the emulsification index ($E_{24}$) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). In order to study the ability of the biosurfactant to form stable emulsions with different hydrophobic substrates, *n*-octane was replaced in the emulsification assays by the solvents: hexadecane, ethyl acetate, dichloromethane and n-hexane. All emulsification assays were done in duplicates.

## 3 Results and Discussion

### 3.1 Statistical analysis

In the current study, the combined effects of C/N ratio, amount of hydrophobic and hydrophilic carbon sources on optimal biosurfactant production was investigated. The coefficients of the full regression model equation

and their statistical significance were determined and evaluated using Design-Expert 11.0.2.0 software from State-Ease Inc. The final model after excluding insignificant terms in relations of actual value for biosurfactant activity (Y), is presented in Eqs. (2). Analysis of variance (ANOVA) of this response demonstrated that the model is highly significant ($p < 0.001$) and F-value of 34,56 as displayed in Table 3.

*Table 3: Analysis of variance (ANOVA) for RSM quadratic model and respective model terms of the emulsification index surface activity*

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 0,0009 | 9 | 0,0001 | 35,34 | < 0.0001 | significant |
| X1-C/N | 0,0006 | 1 | 0,0006 | 216,30 | < 0.0001 | |
| X2-Hydrophobic | 0,0000 | 1 | 0,0000 | 14,00 | 0,0072 | |
| X3-Hydrophilic | 2,976E-08 | 1 | 2,976E-08 | 0,0109 | 0,9196 | |
| X1X2 | 4,700E-06 | 1 | 4,700E-06 | 1,73 | 0,2301 | |
| X1X3 | 2,767E-09 | 1 | 2,767E-09 | 0,0010 | 0,9754 | |
| X2X3 | 2,732E-07 | 1 | 2,732E-07 | 0,1004 | 0,7605 | |
| X1² | 0,0002 | 1 | 0,0002 | 81,55 | < 0.0001 | |
| X2² | 3,356E-08 | 1 | 3,356E-08 | 0,0123 | 0,9147 | |
| X3² | 6,574E-06 | 1 | 6,574E-06 | 2,42 | 0,1640 | |
| **Residual** | 0,0000 | 7 | 2,720E-06 | | | |
| Lack of Fit | 0,0000 | 3 | 5,622E-06 | 10,34 | 0,0235 | significant |
| Pure Error | 2,175E-06 | 4 | 5,436E-07 | | | |
| **Cor Total** | 0,0009 | 16 | | | | |

$$Y = 0.1084 - 0.031X1 - 0.009X2 + 0.0261X1^2 \quad \text{(Eqn. 2)}$$

Meanwhile, X1, X2, $X1^2$ are discovered as significant model terms for efficient biosurfactant production. C/N ratio (X1) and quadratic term of C/N ratio ($X1^2$) have the greatest effect on biosurfactant production with an F-value of 216,33 and 81,55 followed by, the hydrophobic (sunflower oil) carbon source (X2) with an F-value 14 respectively. The C/N ratio and hydrophobic (sunflower oil) carbon source presented a positive effect on biosurfactant activity and quantity. Therefore, the surface activity and performance of the biosurfactant was found to increase with increasing C/N ratio and hydrophobic substrate (oil) carbon source. The 3D response surface plot of Figure 1a shows the effect of C/N ratio and hydrophobic substrate concentration on the yield and activity of biosurfactants produced by *B. subtilis* CN2*.* It can be observed from Figure 1 that increase in C/N ratio from 4 to 16 increases biosurfactant activity from 40 to 98. The maximum surface activity of the biosurfactant was observed at C/N ratio ranging from 14 to 16 and hydrophobic substrate concentration of ~9% (w/v). The results demonstrate that higher dosages of hydrophobic substrate sources and C/N ratio would induce optimal productivity of biosurfactants.

The hydrophobic carbon sources were found to be superior to hydrophilic ones in promoting biosurfactant production and getting enhanced surface activity of the biosurfactant synthesized. The strain produced 10-fold more biosurfactant when growing on oil than when grown on glycerol and significantly higher surface activity as determined from the emulsification index. In addition to using the liquid hydrophobic substrate, sunflower oil, addition of sunflower oil (5%, wt/v) in to the growth medium after depletion of hydrophilic substrate (glycerol) stimulated the production of biosurfactant by more than 200%. Similar observation of synthesis of biosurfactant of better surface activity after addition of water immiscible substrate have previously reported by (Prieto *et al.,* 2008; Gudiña *et al.,* 2015). The biosurfactant grown on sunflower oil only formed stable emulsions with arrange oh hydrocarbons and hydrophobic solvents (n-hexadecane, dichloromethane and ethyl acetate), demonstrating its ability to stabilize emulsions with different hydrocarbons and potential application in a variety of biotechnological applications. On the other hand, the biosurfactant produced using hydrophilic substrate (glycerol) showed very low surface activity and poor emulsion. As depicted in Figure 2, emulsification index of the biosurfactant produced by *B. subtilis* CN2 on n-hexadecane demonstrated an

increasing surface activity with increasing percentage of hydrophobic substrate with respect to the total carbon source. Similar observations have previously been reported by Gudiña et al., (2015).

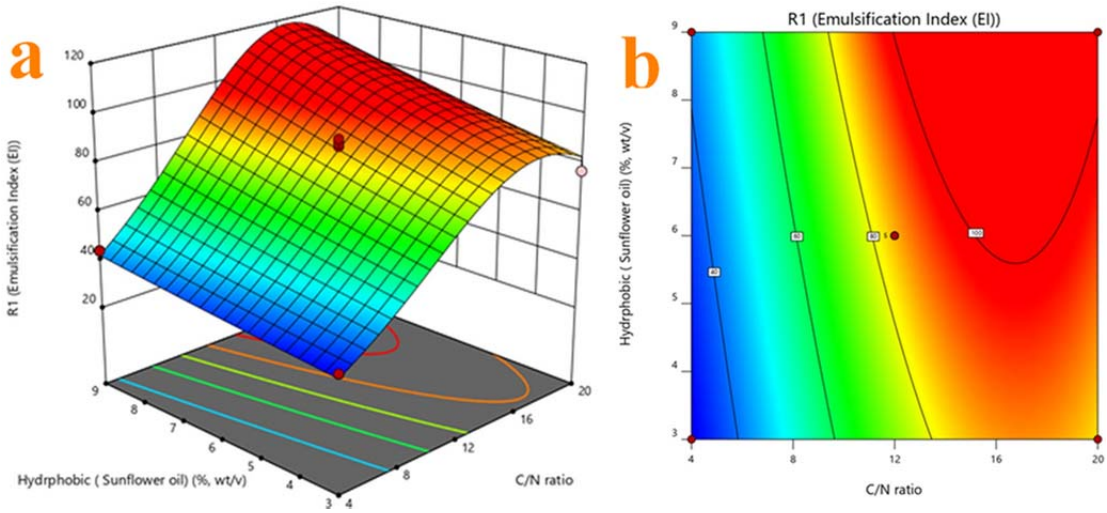


*Figure 1. 3-D Response surface plot (a) and counter plot (b) showing the interactive effect of C/N ratio and hydrophobic (sunflower oil) substrate on biosurfactant surface activity, emulsification index ($E_{24}$), while keeping the hydrophilic concentration at 5%, w/v.*

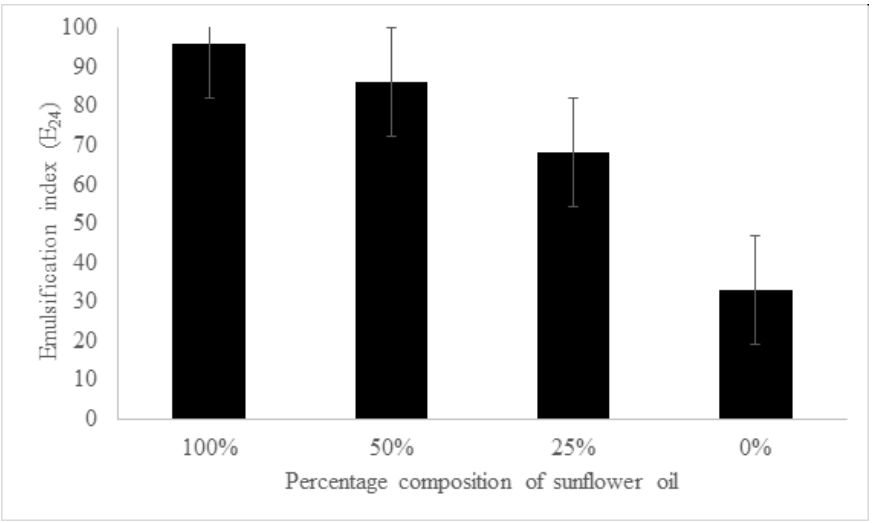


*Figure 2. Emulsifying indices of the biosurfactant produced by B. subtilis CN2 with n-hexadecane grown on 100%, 50%, 25%, 0% hydrophobic (sunflower oil) carbon with respect to the total carbon sources*

## 4. Conclusions

The current study conducted to optimize of quantities of hydrophobic and hydrophilic carbon sources and C/N ratio for optimal biosurfactant production by the *B.subtilis* CN2 strain using response surface methodology and study the effect of type of hydrocarbon on the quantity and surface activity of the biosurfactant. The model identified that within the studied range of experiments, the C/N ratio and hydrophobic substrate amount in the growth medium showed a significant effect on the activity and quality of the biosurfactant synthesized. While the effect of hydrophilic substrates was insignificant on the surface activity and quality of biosurfactant synthesized. That is, with increment of both hydrophobic substrate and C/N ratio, the biosurfactant surface activity increases. It was observed that increase in C/N ratio from 4 to 16 increased biosurfactant activity of the biosurfactant from 40 to 98%. The maximum surface activity of the biosurfactant was observed at C/N ratio

ranging from 14 to 16 and hydrophobic substrate concentration of ~9% (w/v). The results demonstrate that higher dosages of hydrophobic substrate sources and C/N ratio would induce optimal productivity of biosurfactants.

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