Isolation and Screening of *Trichosporon Asahii* for Biosurfactant Production and Optimization

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ABSTRACT

Biosurfactant are biomolecules compounds produced by microorganisms that have desirable characteristics such as being easily degradable and low toxicity over chemical surfactants. Despite their desired properties, their use industrially is limited due to production cost. In this study, optimization of biosurfactant production by *Trichosporon asahii* isolated from some mechanic workshops was carried out. Enrichment technique was used for the isolation of the organism while response surface methodology was used to optimize the production parameters to ensure cost-effective production. Four independent variables were optimized: carbon source (Rice bran), Nitrogen source (Cow dung), pH and inoculum size with the emulsification index as the response. Three biochemical test such as phenol-sulphuric, phosphate and biuret tests were used to characterize the produced biosurfactant. The result indicated that *Trichosporon asahii* isolated from oil contaminated soil can produce biosurfactant with emulsification index of 52.2% under optimized cultural conditions of 13.6 g/L of rice bran, 10.25 g/L of cow dung, 7 ml inoculum size and pH of 8.84 in 250 ml conical flask. Lowest surface tension reduction activity of 34.29 ± 0.10 and critical micelle concentration was attained at 6.4 mg/ml. The biochemical characterization showed that the produced biosurfactant was glycophospholipid. The regression coefficient (R^2) value of 56.5% implied good fitness of the model design used in the experiment. The study shows that the use of agro industrial waste and response surface methodology is efficient for the production and optimization of biosurfactant.

Keywords: Biosurfactant, *Trichosporon Asahii*, Optimization of Biosurfactant, Glycophospholipid Biosurfactant.

INTRODUCTION

Biosurfactant are a type of surface-active biological compounds that are produced by microorganisms, including bacteria, fungi, and yeast [1]. These molecules have regions that can either attract or repel water, making them effective in reducing surface tension and enhancing the solubility and emulsification of substances that do not dissolve in water. Biosurfactant are produced either extracellularly or as a part of the cell membranes of microorganisms [2].

Bacteria, particularly *Pseudomonas sp*. and *Bacillus sp.,* are the largest producers of biosurfactant, followed by fungi such as *Ascomycetes sp*. and *Basidiomycetes* [3]. However, yeast has gained greater visibility in biosurfactant production over bacteria and fungi due to its non-toxicity and non-pathogenicity characteristics [4]. Yeast can produce biosurfactant using oleaginous substrates like agricultural residues such as glycerol, oil, corn steeping liquor, etc. This capability makes the industrial use of biosurfactant

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derived from yeast viable and helps to cut down production costs [5].

Trichosporon asahii is a type of yeast that is commonly found in the environment, including soil, water, and air. *Trichosporon asahii* is known to thrive in various soil environments, indicating its resilience to various pH levels, temperature fluctuations, and nutrient availability. *T. asahii* can utilize oily substrates present in soil such as hydrocarbons and organic matter as a source for biosurfactant production.

Biosurfactant have several industrial applications, including their use in household cleaning products, personal hygiene products, and as antimicrobials, antitumor, and anti-inflammatory agents due to their bioactivity characteristics [6]. Biosurfactant can also improve soil quality by removing heavy metals. However, the high cost of production limits the large-scale production and use of biosurfactant. Therefore, it is crucial to develop an efficient and cost-effective bioprocess to improve the yield of biosurfactant from microorganisms.

To make the production of biosurfactant economically feasible, the cost of the fermentation media needs to be lowered as it currently constitutes approximately 50% of the final cost of the product [7]. Hence, incorporation of agro industrial waste product into the media has been proposed as a suitable substrate to enhance the viability of the large scale biosurfactant production and increase the competitiveness of these natural products [8].

While research has been carried out on the isolation and application of biosurfactant to oil and heavy metal recovery and remediation, not much has been done on isolation and optimization of biosurfactant yeast strain on waste engine oil. The present study aims to isolate and optimize biosurfactant production by using response surface methodology (RSM) with agro-industrial waste as the carbon source.

MATERIALS AND METHODS

Collection of Samples

The waste engine oil-polluted soil samples were collected from two different mechanic workshops in Onitsha. Anambra state. The rice bran was collected at Naira rice processing mill at Nise, Awka south local government area (LGA), while the slaughter-house waste (feces) sample was collected from Akwata slaughter in Nnobi, Idemili South LGA, Anambra state. The samples were filter sterilized and placed in different sterile polythene bags. The samples were transported to the department of Applied Microbiology and Brewing laboratory where isolation and preliminary identification was done. Other analysis was done at the Perfect Glory Research laboratory, Nnewi, Anambra state. All the culture media, reagents, chemicals and equipment used in the study were obtained from these laboratories and were of highest analytical grade.

Isolation and Identification of biosurfactant producing Yeast

For the isolation of the biosurfactant producing yeast, enrichment technique as described by [9] was employed. A 10 g of the mixed soil samples was suspended in 100 mL of mineral salt broth (K_2HPO_4 0.5g, Na_2So_4 2.0g, NH_4Cl 1.0g, $CaCl_2$ 0.15, $MgSo_4$ 0.02g) supplemented with one mL of crude oil and incubated at 30 °C for 5 days in a multipurpose shaker at 70 rpm. A ten-fold serial dilution was carried out. For the dilution, 5 test tubes were set up and 1 mL of the suspended sample was transferred into 9 mL of sterile distilled water in the first tube. A 1 mL of the mixture was taken from 10^{-1} dilution and was emptied into second tube. The same process was repeated for the remaining test tubes. A 0.1 mL of 10^{-5} dilution factors was inoculated onto Yeast-Malt agar plates in triplicate by spread plate method, and the plates incubated at 30 °C for 24 hours with 0.1w/v of tetracycline to eliminate bacteria growth. Ten colonies of *Trichosporon sp*. were isolated and identified based on the colony morphology on Potato Dextrose Agar, microscopic observation of yeast cells and biochemical characteristics such as Gram stain, Phenol-sulphuric

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

acid test, biuret test, phosphate test, Cetyl-trimethyl-ammonium-bromide (CTAB) and methylene blue test. The pure cultures were stored on potato dextrose agar slants at 4˚C.

Amplification of 16S rDNA region of selected yeast species by PCR Reaction

Genomic DNA was extracted from the samples using the Quick-DNATM Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented below. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up KitTM, Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. DNASTAR was used to analyse the. ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

ITS Primers Sequences

Name of Primer	Target	Sequence (5’ to 3’)
ITS-1	ITS rDNA sequence	TCCGTAGGTGAACCTGCGG
ITS-4	ITS rDNA sequence	TCCTCCGCTTATTGATATGC

Screening of biosurfactant producing strain

A 5 mL each of the ten 24-hour growth of the isolates were inoculated into each flask containing 100 mL of the modified mineral salt broth containing 1 mL of waste engine oil. The broths were then incubated at 30 °C for 4 days. At the end of the incubation period, the media were centrifuged at 4,000 rpm for 20 min for the removal of cells. The cell free supernatants were tested for the activity of biosurfactant using oil displacement test, emulsification assay, and surface tension screening methods. The isolates with high activity were considered effective biosurfactant producers [10].

Optimization of Fermentation Conditions for Biosurfactant Production using Response Surface Method.

The method described by [11] was used for optimization of fermentation conditions. In order to find the optimal conditions for biosurfactant synthesis, central composite design was used to optimize critical media component. A 2^4 full factorial central composite design (CCD) for four independent variables, each at five levels with eight-star points and one replicates at the center points were employed to fit a second-order polynomial model. The independent variables evaluated were carbon source (rice-bran) concentration (0-20g) designated as A, nitrogen source (slaughter waste) concentration (0 -40g) designated as B, pH (3 –15) designated as C and inoculum size (2-10 %) designated as D. Design Expert software version 13.0.5.0 was used to design the experiment. A total of 25 experiments were carried out while the emulsification index was the experimental response.

Biosurfactant production

The optimized conditions of 13.597 g of rice-bran, 10.245 g of cow dung, with pH of 8.842 as suggested by the software was poured in a 250 mL flask containing 100 mL of modified mineral salt brothand1 mL of crude oil and sterilized at 121 ℃ and 15 psi for 15 min. The sterilized medium was then inoculated with 7.0 % of the 24 hours old test culture and incubated at 30°C and 150 rpm in a multipurpose shaker for 4 days. After incubation, the fermentation broth sample was centrifuged at 4,000 rpm for 20 min and the emulsification activity was determined as described by [12].

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

Critical Micelle Concentration (CMC) Determination

The CMC was determined using different concentrations of the crude biosurfactant (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 16, 22 and 30 mg/ mL) and the surface tension of each concentration was determined using a glass capillary rise method at a temperature of 28 ± 2 °C after which the CMC of the biosurfactant was then determined. The CMC was estimated from the plot of surface tension against biosurfactant concentration [12] .

Statistical Analysis

All the experimental data obtained were expressed in mean ± standard deviation and presented in Tables and Figures using One-way analysis of variance (ANOVA). Values of $P < 0.05$ was considered statistically significant at 95% confidence intervals using GraphPad Prism version 8.0.2.

RESULT

Isolation and Screening of Biosurfactant Producing Yeast from oil contaminated Soil

Ten biosurfactant producers were isolated from oil contaminated soil labeled isolate 1-10. Among the isolates, isolate 4 showed overall best activity for the two test conducted with oil dispersive activity of 13.50 ± 0.10 and lowest surface reduction activity of 82.95 ± 0.10. It also showed the second best emulsification activity of 40.00 ± 0.10. Isolates 6 and 2 showed similar emulsification activity with isolate 4, however, they have low oil displacement activity and very poor surface tension reduction measurement. For surface tension capability, isolate 4 had the best result while isolate 10 had the least. For Emulsification assay, isolate 6 had the best activity while isolate 9 showed the least oil displacement activity as shown in table 4. Only the isolate 4 that yielded the best results was morphologically and molecularly identified as *Trichosporon asahii*. The screening result of the isolated yeast strains are shown in Table 1.

Table 1: Screening Result of the Biosurfactant Producing Yeast Strain

Strain code	Emulsification Assay (%)	Oil Displacement (cm)	Surface tension (mN/m)
Isolate 1	35.55 ± 1.00	11.85 ± 1.00	94.59 ± 0.15
Isolate 2	40.00 ± 1.04	4.30 ± 0.02	101.70 ± 0.20
Isolate 3	37.50 ± 1.00	11.71 ± 0.10	92.99 ± 0.00
Isolate 4	40.00 ± 0.10	13.50 ± 0.10	82.95 ± 0.10
Isolate 5	32.50 ± 0.10	1.02 ± 0.01	88.63 ± 0.20
Isolate 6	41.25 ± 0.01	2.54 ± 0.01	97.35 ± 0.00
Isolate 7	26.25 ± 1.00	0.50 ± 1.00	88.63 ± 0.20
Isolate 8	29.00 ± 1.10	1.13 ± 1.35	94.59 ± 1.50
Isolate 9	28.75 ± 0.00	8.04 ± 0.02	92.99 ± 0.02
Isolate 10	32.50 ± 1.00	1.23 ± 1.00	108.55 ± 1.50

Morphological, Biochemical and Molecular Characteristics of biosurfactant producing Yeast

The organism showed complex colonial features similar to yeast-like organisms. From the result of the gram

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

stain, the organism was observed to appear purple which is a characteristic of gram positive organisms with other observed features under the microscope as shown in Table 2. The result of the molecular description of the most potent biosurfactant producing yeast strain *Trichosporon asahii* is shown in Table 3. Figure 1 shows the plate for the Gel electrophoregram of the amplified ITS target gene of *Trichosporon asahii*. The isolate showed 99.82 % homology with other closely related strains of *Trichosporon asahii* available in Genbank database using the Accession no. of BLAST hit MG241533.1

Table 2: Morphological and Biochemical Characteristics of *Trichosporon sp.*

Parameters	**Observation**
General characteristics	
PDA	Circular, powdery, suedelike and irregular folds
Colour	Creamy
Pigmentation	–
Gram staining	Gram positive with hyphae
Lateral conidia	–
Arthroconidia	Barrel shaped
Appressoria	–
Biochemeical Characteristics	
Phenol sulphate test	+
Biuret	–
Phosphate test	+
Cetyl-trimethyl-ammonium-bromide	+

'+' indicates a positive result and '-' indicates a negative result; PDA: Potato Dextrose Agar.

Table 3: Molecular Description of the Most Potent Biosurfactant Producing Yeast Strain *Trichosporon asahii*

Sample ID Organism	**Chioma new *Trichosporon asahii***
Sequence length (bp)	543
% identity	99.82 %
Accession no of BLAST hit	MG241533.1
E-value	0.0
Alignment score	≥200
Highest query coverage (%)	100 %

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

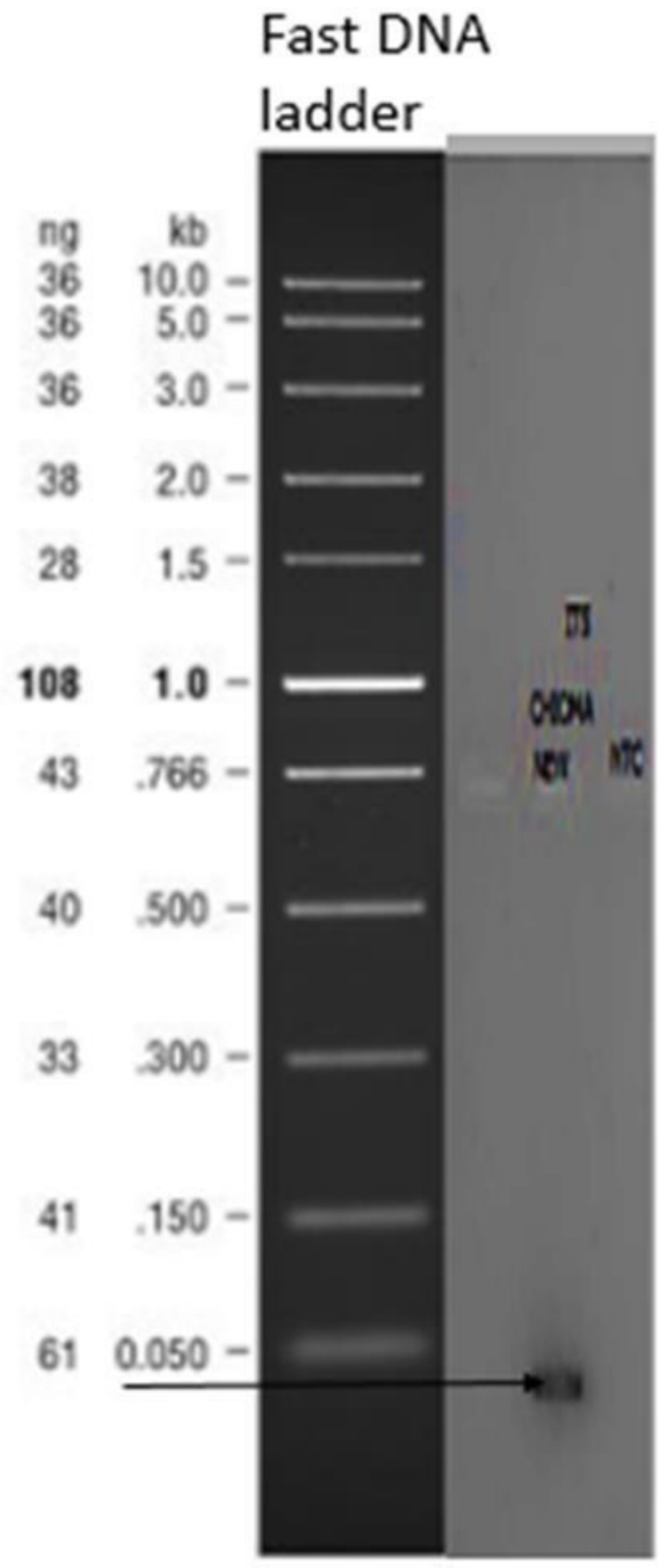

Figure 1: Gel electrophoregram of the amplified ITS target gene of *Trichosporon asahii*

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

Optimization of Biosurfactant Production by Trichosporon asahii

Multiple regression analysis using response surface methodology was done to fit the regression model to the experimental data and investigate the effect of the four variables selected. The central composite experimental design runs for optimization of biosurfactant production by *Trichosporon asahii* and actual responses of the dependent variable obtained are shown in Table 4. A total of 25 experiments were performed and emulsification index after each run were recorded as the response. The highest response was obtained to be 51.5 % for standard run 16, while the lowest was 35 % for standard run 4. In the predicted values, run 6 showed the highest emulsification response of 51.21 % while run 4 showed the least value of 35.36 %. Most of the runs showed differences in the experimental values when compared with the predicted values. However standard run 15, 3 and 4 for predicted and observed values showed similarities in their responses (Table 5). The three dimensional surface of the independent factors against emulsification index response are shown in Figure 2 while Figure 3 shows the distribution of predicted vs actual values of the regression model of emulsification index response. The regression model, which is a quadratic polynomial equation, gave the empirical interaction between the test variables and the response obtained as

Model Equation: Y1 (Emulsification index) = 50.1732 -1.18854A – 0.365604B – 0.205208C + 0.812813D – 0.009025AB + 0.146583AC + 0.0399375AD + 0.0204583BC + 0.0347187BD – 0.209271CD.

Where, Y1 is emulsification index, A is the concentration of rice bran, B is the concentration of cow dung, C is the pH and D is inoculum size.

Table 4: Coded Factors for the Central Composite Design (ccd) Using Response Surface Methodology

Optimum condition	**Coded level**	**Actual level**
Carbon source	0.72	13.60
Nitrogen source	– 0.95	10.25
Ph	– 0.053	8.84
Inoculum size	0.25	7.00
Response	Predictive value	Experimental value
Emulsification index	50.20	52.20

Table 5: Optimum Condition, Experimental and Predicted Value of Response at Optimized Conditions

Std	**Run**	**Space Type**	**Carbon Source (g)**	**Nitrogen Source (g)**	**pH**	**Inoculum Size (mL)**	**Emulsification assay Actual value (%)**	**Emulsification assay Predicted value %**
23	1	Axial	10	20	9	2	42.5	44.15
5	2	Factorial	5	10	12	4	50	46.61
4	3	Factorial	15	30	6	4	49.8	49.36
21	4	Axial	10	20	3	8	45.0	41.85
1	5	Factorial	5	10	6	4	42.5	44.73
16	6	Factorial	15	30	12	10	56.5	55.03
2	7	Factorial	15	10	6	2	40.01	41.53
17	8	Axial	0	20	9	8	40.10	42.44
14	9	Factorial	15	10	12	8	45.0	46.19

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

19	10	Axial	10	0	9	6	48.72	45.61
7	11	Factorial	5	30	12	2	47.5	44.69
20	12	Axial	10	40	9	6	40.0	43.07
24	13	Axial	10	20	9	8	42.5	44.52
6	14	Factorial	15	10	12	2	50	51.21
11	15	Factorial	10	30	6	10	52.2	50.24
22	16	Axial	10	20	15	6	42.9	46.82
18	17	Axial	20	20	9	6	50.0	46.24
8	18	Factorial	15	30	12	2	50.0	48.49
3	19	Factorial	5	30	6	2	41.01	40.35
9	20	Factorial	5	10	6	10	42.5	45.56
15	21	Factorial	5	30	12	8	41.02	41.03
10	22	Factorial	15	10	6	8	45.01	45.56
13	23	Factorial	5	10	12	8	41.5	40.40
12	24	Factorial	15	30	6	4	45.	44.94
25	25	Center	10	201	9	6	47.5	44.34

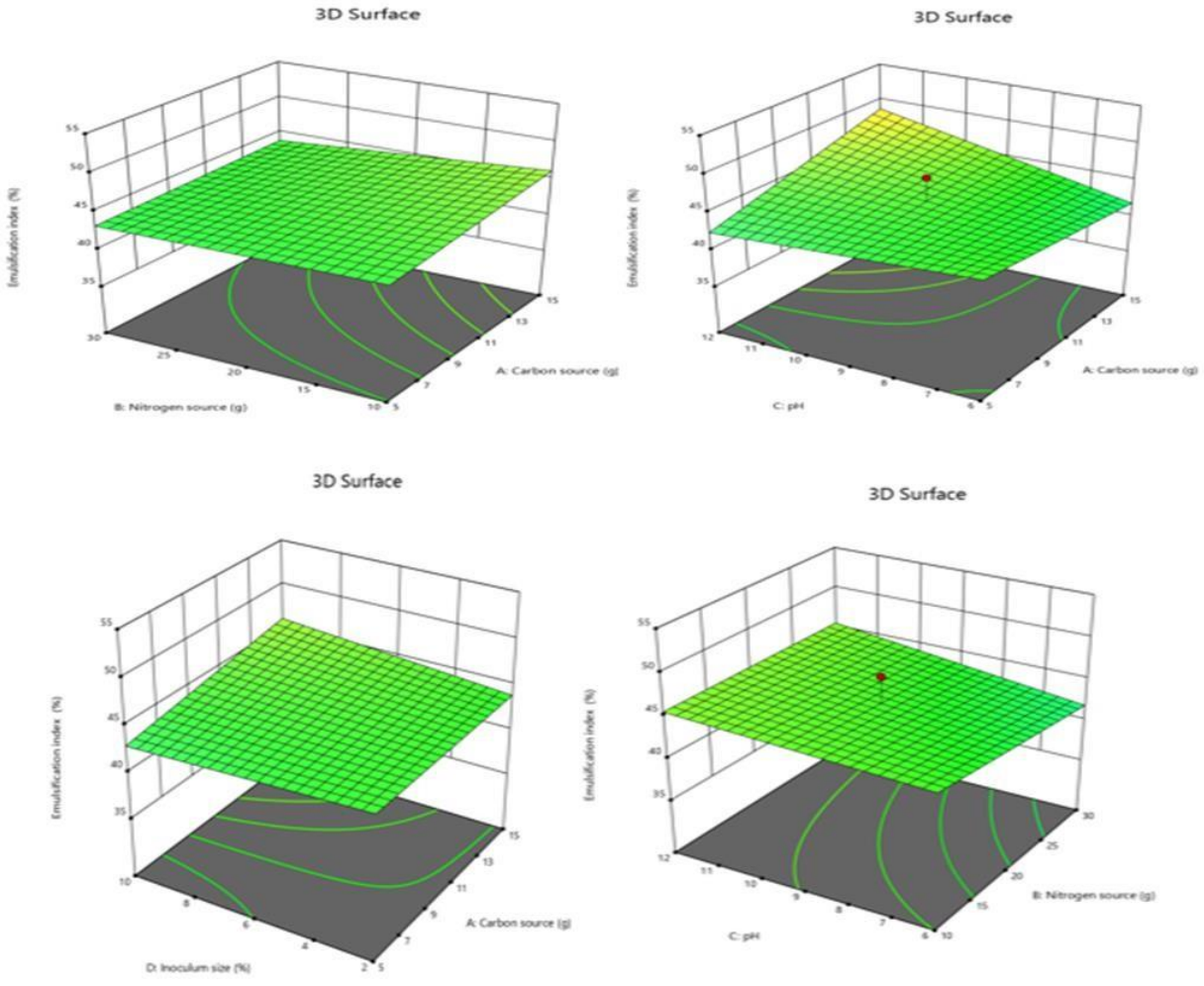

Figure 2: Graph plots showing the three-dimensional surface of the independent factors against emulsification index response

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

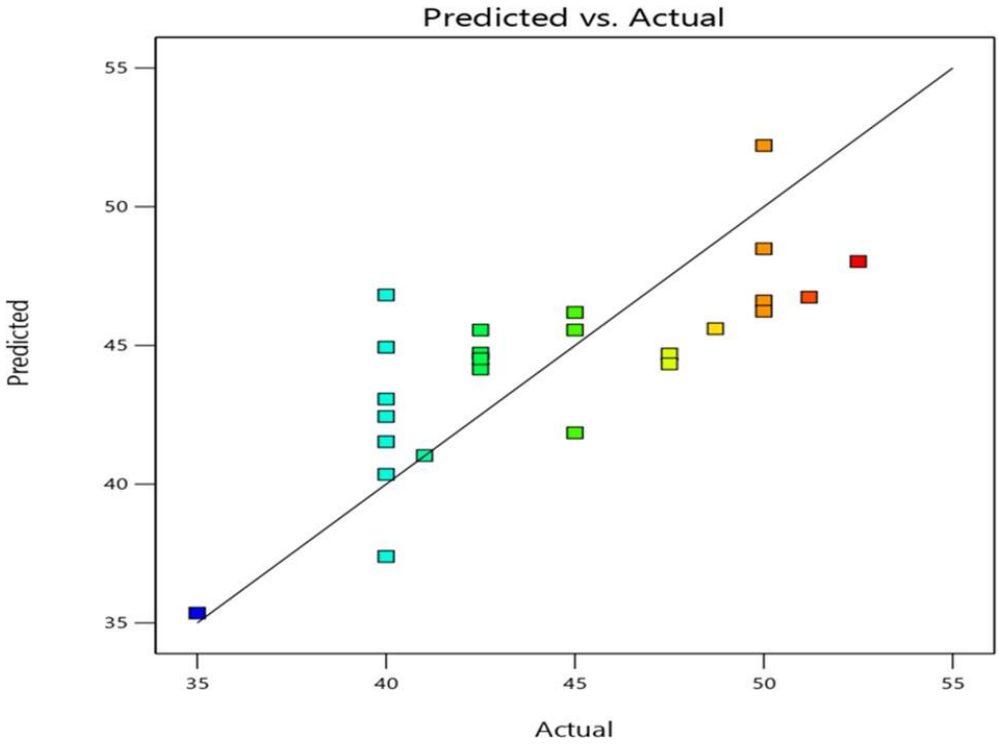

Figure 3: Distribution of predicted vs actual values of the regression model of emulsification index response

Critical Micelle Concentration (CMC) Determination

The result of the critical micelles concentration (CMC) of the produced biosurfactant is shown in Figure 4. From the figure, the 6.4 mg/mL concentration was found to be the CMC of the produced biosurfactant which decreases surface tension from 72.8 to 34.29 ± 0.01 mN/m. This value was the same when the concentration was further increased except for the concentration of 16.0 mg/mL that decreased the surface tension to 35.28 ± 0.15 mN/m.

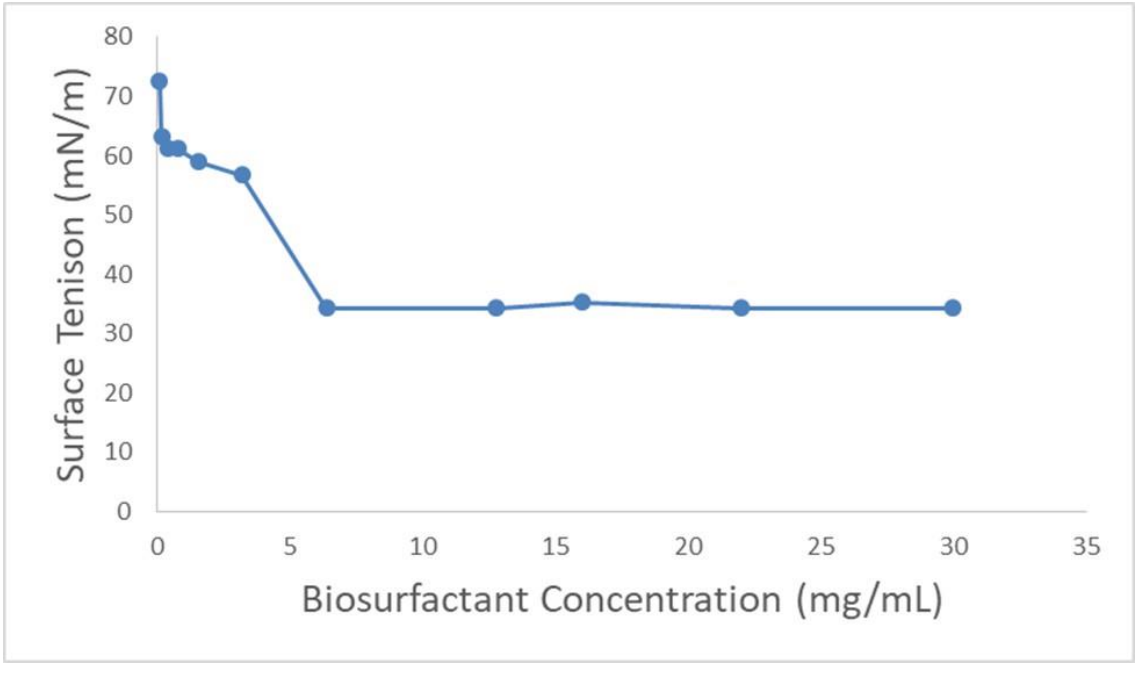

Figure 4: Critical micelles concentration of the biosurfactant produced by *Trichosporon asahii*

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

DISCUSSION

The isolation of *Trichosporon asahii* as a biosurfactant producer from the oil contaminated soil suggests that soil is a potential source of biosurfactant producers. This is in agreement with the study conducted by [13] that reported isolation of *Trichosporon asahii* from petroleum-contaminated soil in India. Studies by [14] also showed that *Trichosporon sp.* are widely distributed in the environment such as soil and occupy narrow ecologic niches.

The screening for biosurfactant production showed that the ten isolates can efficiently produce biosurfactant under submerged culture conditions. The result of the most potential biosurfactant producer as determined by emulsification assay (40.00 ± 0.10 %), oil displacement activity (13.50 ± 0.10 cm) and lowest surface tension (82.95 ± 0.01 mN/m) where similar with result of [13]. However, the oil displacement result and surface tension test obtained from this study differs from the study done by [14] that obtained 117 ± 1.5 cm and 30 ± 0.6 (mN/m) respectively. The dispersive, emulsification and lower surface tension properties observed with spent engine oil in this study, suggest the possible application of this biosurfactant in enhanced oil recovery and environmental biotechnology.

The results of the biochemical profile show that the produced biosurfactant yeast was positive to phenol and phosphate tests but negative to biuret. The results confirm that *Trichosporon asahii* is a producer of glycolipid and phospholipid. The findings disagreed with the work by [13] who reported that *Trichosporon asahii* produced sophorolipid biosurfactant. This also partially agreed to the study conducted by [15] using *Trichosporon asahii* in which the produced biosurfactant was positive to phenol-sulfuric acid test only but negative to biuret and phosphate suggesting that *Trichosporon asahii* is a producer of glycolipid. The molecular identification showed 99.82 % homology with other strains of *Trichosporon asahii.* Many studies have isolated different yeast genera as biosurfactant producers like *Rhodotorula*, *Saccharomyces*, [16-7], *Wickerhamomyces* [4] and *Trichosporon* [13].

To make the production of biosurfactant economically feasible, production must be optimized [18]. Four independent variables (carbon source, Nitrogen source, pH and Inoculum size) critical for the production process were optimized for biosurfactant production by *Trichosporon asahii* in the present study. Multiple regression analysis using response surface methodology was carried out to fit the regression model to the experimental data and investigate the effect of the four selected variables. Biosurfactant yield was based on the emulsification index of the supernatant with a hydrophobic substrate (crude oil). The accuracy of the models' prediction as shown by the graph of the plot of predicted and actual values indicated that the model prediction is near accurate at point 50.20, 52.20 for the predicted and actual value respectively. This is because points that fall on the diagonal line indicate that the models' predictions are accurate for those values. Analysis of variance (ANOVA) revealed that the model and linear values are statistically insignificant (p-value & gt; 0.05) implying they have no effect in the production process. The effect of the interactions on biosurfactant production showed that only concentration of the interaction of rice bran and pH, pH and inoculum size had a significant effect on the biosurfactant production (p-value & gt; 0.05), indicating that the levels of the interaction variables tested are at optimum and ideal conditions for growth and production of biosurfactant. Additionally, the non-significance of the lack of fit test indicated the adequacy of the model for optimum biosurfactant production by the isolate. The fit of the model was determined by the regression coefficient value, which was 56.50 %. This is indicative of considerable adjustment of the regression model to the experimental data, and that the model could explain 56.50 % variability between the predicted and experimental data and other factors could be responsible for the yield of biosurfactant. The optimum condition prediction obtained by the regression model were coded as 0.72, -0.95, -0.053, 0.25 for the concentration of rice bran, concentration of cow dung, pH and inoculum size in 250 mL conical flask respectively, which presents actual values as 13.60 g/L of rice bran, 10.25 g/L of cow dung, pH of 8.84 and 7.0 inoculum size. These results showed that the model is well fitted for optimum biosurfactant yield by *Trichosporon asahii.*

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue IV April 2024

The Critical micelle concentration (CMC) is an important physiochemical parameter used to evaluate biosurfactant activity, which indicates the minimum concentration of biosurfactant necessary to achieve the lowest stable surface tension. Efficient surfactants have low CMC values, i.e., less surfactant is required to decrease surface tension [19]. The Critical micelle concentration (CMC) in this study was obtained at 6.4 mg/mL concentration as represented in figure 4 and the result was the same when the concentration of the biosurfactant was increased above the CMC. This concentration reduced the surface tension from 72.48 to 34.29 mN/m. The low CMC value of the biosurfactant observed in this study could be due to its excellent formation and aggregation ability [20]. This result differs from the work of [13] who obtained a lower surface tension reduction of 30 ± 0.6 (mN/m) and another study by [20] that obtained a critical micelle concentration (CMC) of 2.2 mg/mL for *Trichosporon montevideense.*.

CONCLUSION

This study has shown that the oil contaminated soil collected from mechanic workshop in Onitsha, Anambra state is a reservoir of *Trichosporon asahii* an excellent producer of biosurfactant. The biosurfactant produced by *Trichosporon asahii* strain was found to be glycophospholipid producer based on the confirmation from biochemical test. The results of the optimization process open new future prospects as it can be useful in enhancing the large production of biosurfactant agents, making them attractive options for application at industrial levels.

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COMPETING INTEREST

The authors declare that they have no competing interest.

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