Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 137 (2019) 18-30 EISSN 2392-2192

Optimization of Biosurfactant production by a novel Rhizobacterial *Pseudomonas* species

E. Effiong*, O. K. Agwa and **G. O. Abu**

Department of Microbiology, Faculty of Science, University of Port Harcourt, Rivers State, Nigeria

*E-mail address: Eno.effiong2019@outlook.com

ABSTRACT

Optimization of biosurfactant has improved the value-chain, process development and cost of production associated with downstream synthesis. This study was designed to determine the optimal conditions for production of biosurfactant using hydrolyzed agroresidues under controlled conditions. Rhizobacterial isolate was obtained from *Paspalum* sp. growing on an aged crude oil impacted soil in Bodo, Rivers State, Nigeria. The bacterial isolates were identified using 16S rRNA molecular approach on a set of universal primers. One-Variable at a Time approach was applied for verification of pH, Carbon and nitrate sources respectively. Stat-ease Design-Expert version 12.0 was employed in the optimization of the variables while the operational conditions were fitted into a 20-run design matrix using α- level 2.0. Molecular identification confirmed the bacterial isolae to be *Pseudomonas* sp. with Accession number MH40927 with a gene molecular weight of 6.0kbp. Response for biomass, biosurfactant and Critical Miscelle Concentration (CMC) was observed to fit into a 2nd order Quadratic functions at $p<0.05$ with optimal conditions were pH = 7.0, Corn chaff = 2.0 g/L and Urea = 1.0 g/L. Biosurfactant = $+51.98 + 6.79A + 4.30B-5.45C + 0.2975AB + 0.4975AC + 1.63BC-6.46A^2 - 3.51B^2 - 6.94C^2$. This study further identified a directly proportional relationship between biosurfactant production and operational variables which represents a cheaper and feasible production roadmap for biosynthesis.

***Keywords*:** Optimization, Biosurfactant, Rhizobacteria, OVAT, Variables, Quadratic functions, *Pseudomonas, Gammaproteobacteria, Proteobacteria*

(Received 03 October 2019; Accepted 21 October 2019; Date of Publication 22 October 2019)

1. INTRODUCTION

Biosurfactants are surface-active compounds with the potential of reducing surficial and interfacial tensions (1). Their activity is due to the presence of the organic and inorganic moieties which act at interfaces (2). These compounds could assume a variety of shapes due to the presence of organic and inorganic compounds (3). The growing attention in the synthesis of these materials has increased in recent time because of the cost and sustainability of the conventional and synthetic surfactants which have impacted the microbiota (4). Furthermore, market feasibility and its vast applicability suggest a futuristic knowledge-economy with a drive for greener, cost-effective and sustainable bioremediation cocktails to replace the commercially available cleaning products. The Classification has been based on organism-type, molecular weight, chemical components, morphology and activity (5). Proteobacteria and Firmicutes floral diversity have been reported to synthesize these compounds (7;6) but elucidation of the structural components and extraction yields based on the niche and habitat have been under-reported by some peer review (9).

Gammaproteobacteria has a prominent taxonomic rank within the phylum *Proteobacteria*. They are Gram-negative, facultative anaerobes and are able to ferment simple sugars. They are richer in genera (~250) than all bacterial phyla (10). Some members of the phyla include *Escherichia*, *Yersinia*, *Vibrio*, *Klebsiella* and *Pseudomonas* (11). Members of the class *Gammaproteobacteria* are one of the ubiquitous groups of metabolically versatile, eukaryotic associated bacteria with effects on both health and survival of living things. Some members of these group have been described to possess chemotrophic mode of nutrition while others have been described to possess autotrophic and chemoorganotrophic mode of nutrition. They exist mainly as commensal, others may act as opportunistic pathogens in a wide array of host including plants and animals (12). One of the notable members of this phyla is *Pseudomonas* sp. a Gram-negative and non-spore forming rod occurring singly.

'Rhizosphere' is a word coined by Lorentz Hiltner implying the region around the root of a plant (13). It's a narrow region around the root of plant, controlling both physicochemical and biochemical conditions. They serve as anchorage systems, play conductive functions in nesting and acts as a protective regions for organisms (14). This parlance is used to refer to organisms that existed, tolerated exudates from plants and played key role as either rhizospheric organisms or rhizobacteria. This microbial flora is capable of resisting threats, and initiate an interaction between the chemical compounds secreted by the plant is referred to as exudate. They are competent in colonizing the rhizosphere, because they are secretions synthesized from plants which contain a wide array of organic substances and acts as either attractant or repellent. Other rhizodeposits are mucilages, border cells affect the microbial quality indices of plants and plays a significant role in the plant either as an attractant or repellant to both macro and microfauna (14,15).

Optimization of bioprocess could be achieved by either empirical or statistical approaches. The conventional one factor at a time approach is often characterized to be messy, cumbersome, and more likely to errors. These techniques have remained useful in several sectors. Response surface methodology (RSM) is a multivariate statistical tool used in the experimental modelling and predicting operational variables. It is most recognized for identifying the effect of a single variable and the effect it might have on the bioprocess. This tool has been employed in the area of media design and process validation, and have been credited to have better efficacy and time consumption than conventional approaches (17).

It reduces experimental challenges, improves the quality of statistical interpretation and variations in variables. As well as assists in the calculation of the variable response (18). Several software has been designed to carry out these levels of experimentation, some of which are the JMP, SAS, Minitab and Design expert. Box and Behnken design are a widely appraised approach and some peer-review have referred to it as the best for bioprocess optimization. The process is categorized into three major stages, Design of the experiments from statistically designed experiments, estimation of statistical coefficients and Mathematical Model Prediction (19). Some advances have been put into the design to improve the robustness of the data processing and output features.

2. MATERIALS AND METHODS

2. 1. Identification of plants obtained from polluted sites

Plant obtained from the study location were transported aseptically, in sample bags to the Department of Plant Science and Biotechnology, University of Port Harcourt. Rivers State for identification. The plants were later sent to the environmental laboratory of the department of microbiology for isolation of the microorganisms.

2. 2. Collection and hydrolysis of substrates

Cornchaff was obtained from Choba Market Rivers State, Nigeria, they were dried and hydrolysed using alkaline hydrolysis using 1.0 NaOH solution. The reaction was exposed to steaming using an autoclave at 121 ºC at 15 p.s.i. The hydrolysed material was washed, re-dried and distributed based on the weights used for the optimization of the biosurfactant production.

2. 3. Enrichment of soil samples

The soil samples were enriched in Bushnell Haas Media (BHM) by measuring 3.2 g of the salt was dissolved in one (1) Litre of distilled water, pH of the media was adjusted using 1.0 M HCl to pH 7.2. Ninety-eight mililiters of BHM was dispensed in a 250 ml conical flask to create room for adequate headspace, 1 % Bonny Light crude oil was introduced into the media and sterilization was performed at 121 ºC for 15 minutes and 15 psi. Upon cooling, the sterile set up was agitated with the aid of an orbital shaker incubator (Stuart, Germany S150) the samples were shaken at 170 r.p.m at 37 ºC (20).

2. 4. Molecular characterization of bacterial isolates.

Bacterial DNA extraction was done by liquid Nitrogen and crushing method using DNA miniprep extraction kit supplied by Inqaba South Africa. Five millilitres of an overnight broth culture of the bacterial isolate on nutrient agar was dislodged and crushed in liquid nitrogen, spun extracted at 14000 rpm for 3 min. The cells were re-suspended in 500 µL of normal saline and heated at 95 ºC for 20 min.

The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20 ºC for other downstream reactions. Four hundred (400) µL of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) µL of bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 µL, 800 µL was then transferred to a Zymo-Spin II Column in a collection tube and centrifuged at 10,000

x*g* for 1 minute, the flow-through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spuned. Two hundred (200) µL of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spuned at 10,000 x*g* for 1 minute followed by the addition of 500 µL of bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute. The Zymo-spin IIC column was transferred to a clean 1.5 µL centrifuge tube, 100 µL of DNA elution buffer was added to the column matrix and centrifuged at 10,000 x*g* for 30 seconds to elute the DNA. The ultrapure DNA was then stored at -20 ºC for other downstream reaction.

2. 5. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer

2. 6. 16S rRNA Amplification

The 16S rRNA region of the rRNA genes of the isolates was amplified using the 27F and 1492R primers (16SF: GTGCCAGCAGCCGCGCTAA and 16SR: AGACCCGGGAACGTA TTCAC) on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as a template.

The PCR conditions were as follows: Initial denaturation, 95 ºC for 5 minutes; denaturation, 95 ºC for 30 seconds annealing, 52 ºC for 30 seconds; extension, 72 ºC for 30 seconds for 35 cycles and final extension, 72 ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator. Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa.

2. 7. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

2. 8. Biosurfactant Production Studies

The isolates were grown on nutrient agar for 18h at 37 ºC. The bacterial isolates were dislodged using sterile distilled water. The bacterial culture broth was diluted and plated using spread-plate technique. The microbial titre was determine and 1.0 ml of the bacterial broth was seeded into the mineral salt media Cornchaff 3%, KH_2PO_4 4.03 mg/L, $MgSO_4$ 0.4 mg/L, NaCl 1.0 g/l, $CaCl_2 \cdot 2H_2O$ 0.1 g/l, Urea, 4.46 g/l, 0.1ml trace metal solution containing $MnSO_4.H_2O$ 1.5 g/L, $FeSO_4 \cdot 7H_2O$ 0.5 g/L, $CuSO_4 \cdot 2H_2O$ 0.1 g/l, $NaMoO \cdot 2H_2O$ 1.5 g/l, and H_3BO_3 0.3g/l as defined by Peekate and Abu (24). The inoculated culture media was incubated under 37 ºC in an orbital shaker at 120 r.p.m. The set up was monitored for 7 days.

2. 9. Optimization of growth conditions for High throughput bacterial strains.

Higher biosurfactant yield, the pH, Cornchaff, and Urea was considered as independent variables while the bioremediation indices would be applied as dependent variable. The experiment will be performed by Response Surface Methodology (RSM) using Box and Behnken Design (Emeko *et al*., 2015) to determine the optimum levels of the significant variables and the effects of their mutual interactions on oxalate production. Each independent variable was studied at three different levels (low, medium and high, coded as –1, 0 and +1, respectively) with the centre point of the design replicated three times for the estimation of error. Design Expert 10.0 software (Stat-Ease Inc. Minneapolis, USA) was used for experimental design and data analysis. From the experimental data according to this design, a second-order polynomial regression model equation will be derived to define the response in terms of the independent variables.

$$Y = \beta_2 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1 A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

where Y: Predicted response (Biomass, Biosurfactant and CMC)
A: pH, B: Cornchaff; C: Urea: β_0: Intercept, β_1, β_2, β_3 and β_4 are the linear coefficients, β_{11}, β_{22}, β_{33} and β_{44} are the squared coefficients, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction coefficients; A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD are the interactions between the variables as significant terms.

3. RESULTS AND DISCUSSION

3. 1. Molecular Identification of Rhizobacterial *Pseudomonas* sp.

The result of the gel electrophoresis in Figure 1.0 describes the genomic rRNA products from thermocyler of the PCR with a set of forward and reverse primers. The gene molecular weight was deduced from the ladder to be 6.0kbp from a 1.0kbp. The gel plates suggest a quality yield and amplification of the gene interest. The annotation and initials were drawn from the sample code and the source of the organism. Table 1.0 provides the details of the bioinformatics and putative identity of the isolates. The accession numbers are signature to identify the isolates have been deposited in the GenBank.

The phylogenetic construct in Figure 1.0 shows the evolutionary relatedness of the isolates to one already existing on the GenBank. The result of the molecular identification suggests the presence of novel *Pseudomonas* sp. from the rhizosphere of plants obtained from pre-exposed and aged soil. This study further agrees with the result reported by Astuti *et al*. (2019) whose study screened, characterized and produced biosurfactant by *Pseudoxanthomonas* sp.G3. In their study, the bacterial isolate was obtained from heavily polluted soil. They were able to screen over thirty-two (32) bacterial isolates. This findings from the current study, also agree Das, Yang, & Ma (22) that conducted an analysis of biosurfactants produced by *Pseudomonas* strain obtained from crude oil. In their study, bacteria were identified as *Pseudomonas* sp. *P. aeruginosa* LCD12 and *P. aeruginosa* D2 respectively by16S rRNA gene sequence analysis. Furthermore, Shahwar, Sheikh, & Jamil (23) isolated and characterized biosurfactant producing bacteria from produced water. The worked on three produced water contaminated soil from three different sites.

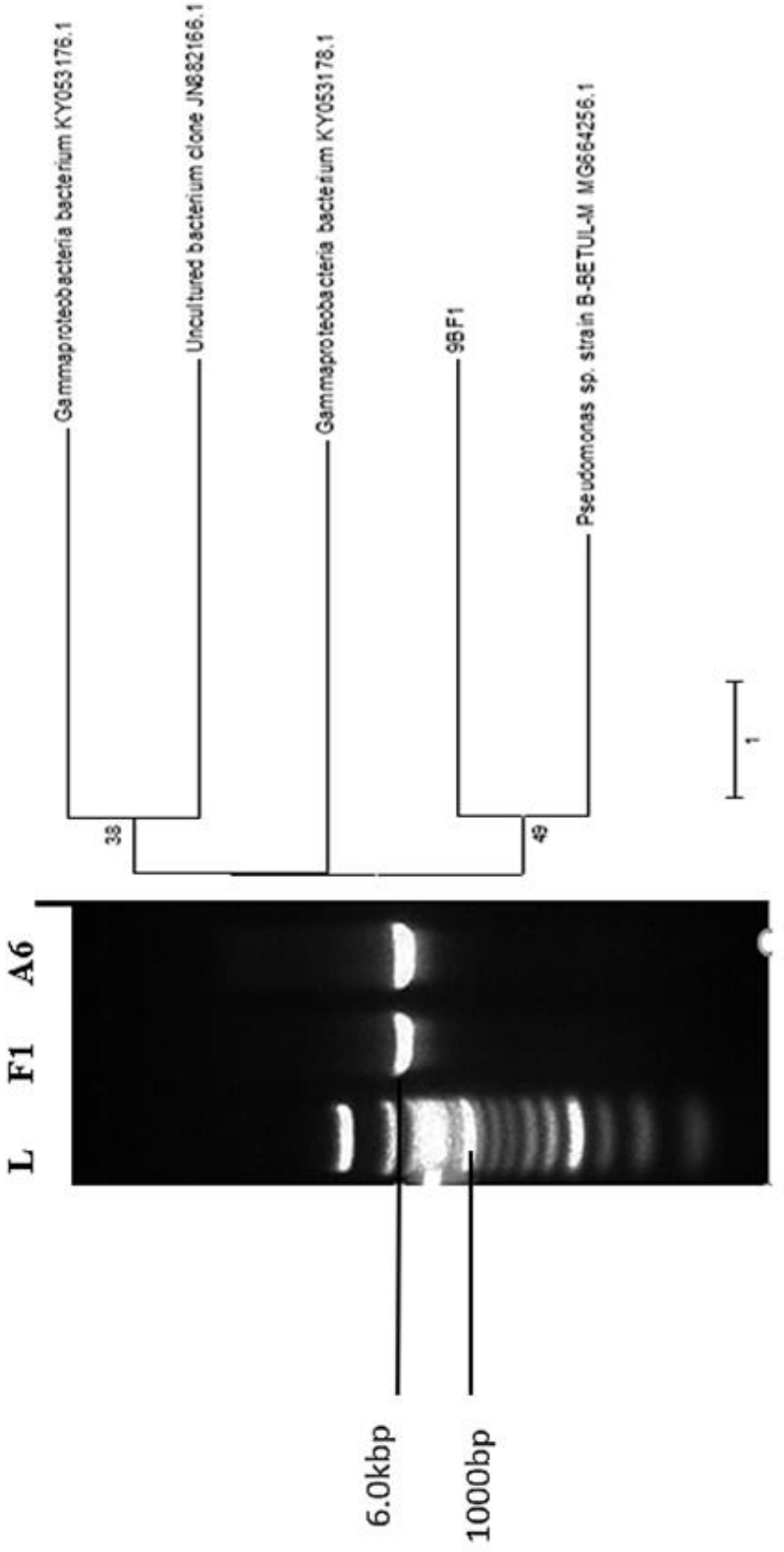

Figure 1. Gel electrophoresis of bacterial isolates Figure 2.0: Phylogenetic Construct for bacterial isolate

Over forty-seven hydrocarbon utilizing bacterial isolates. They employed a 16S approach in the identification. This study corroborates the predominance of *Pseudomonas* spp. in soil. This could further reduce or promote the process of eco-recovery. The report deferred from the report of (24) in whose report obtained *Pseudomonas fluorescens* from brackish water at Eagle Island, Port Harcourt. *Pseudomonas* spp. have by this study revealed their presence in most environments known to man.

Table 1. Bioinformatics and Identification profile of the isolates with the maximal production capacity.

Isolate code	BLASTn Id	Percentage similarity	Accession number
F1	*Pseudomonas* sp *BETUL-M*	**99%**	MH740927

3. 2. Modelling of biosurfactant production by *Pseudomonas* sp.

The Model F-value of 6.33 implies the model is significant. There is only a 0.40% chance that an F-value this large could occur due to noise as displayed in Figure 2.0. P-values less than 0.0500 indicate model terms are significant. In this case, A, AB, A^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Model F-value of 3.55 implies the model is significant. There is only a 3.07% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, C, A^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. A negative Predicted R^2 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher-order model may also predict better.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 5.562 indicates an adequate signal. This model can be used to navigate the design space. The Model F-value of 3.34 implies the model is significant. There is only a 3.71% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 1.16 implies the Lack of Fit is not significant relative to the pure error. There is a 43.73% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good is considered model to fit.

The report of (24) suggested that C:N ratio investigated ranged from 20–60, C:P ranged from 10–16, and pH ranged from 5.5 – 8.5. An Optimized combination of C:N = 20, C:P = 16, and pH = 5.5 achieved using the Response Surface Methodology resulted in maximum biosurfactant production as indicated by the low surface tension value. However, Rashedi, Jamshidi, Mazaheri, and Bonakdarpour (25) produced biosurfactant by *Pseudomonas* sp. They reported that paraffinic oil encouraged the production of rhamnolipids. They observed 150 mg/l of rhamnolipid and a 28% drop in the surface tension, but that glycerol offered better yield of

rhamnolipid. C/N ratio was best at 60g/l. This finding corroborates the report of this study, which fitted into a quadratic function and suggest that pH 7, Cornchaff 4 g/l and Urea 2 g/l as presented in Figure 2.

Table 2. Design matrix and responses for biomass, biosurfactant and CMC.

	Factors			Response 1.0: Biomass (Log_{10} Cfu/ml)		Response2: Biosurfactant mg/l		Response 3: CMC (g/l)	
Run	A: pH	B: Cornchaff (g/l)	C: Urea (g/l)	Actual	Predicted	Actual	Predicted	Actual	Predicted
1.0	7.0	4.0	3.7	7.1	6.61	27.6	23.20	0.31	0.3280
2.0	7.0	4.0	2.0	8.2	8.23	51.6	51.98	0.89	0.7964
3.0	5.5	2.0	3.0	4.3	5.04	12.5	16.70	0.11	0.1159
4.0	7.0	4.0	2.0	8.2	8.23	51.6	51.98	0.53	0.7964
5.0	8.5	2.0	1.0	6.3	6.84	40.4	43.84	0.63	0.6792
6.0	9.5	4.0	2.0	7.2	6.90	48.6	45.14	0.57	0.5365
7.0	7.0	4.0	0.3	7.4	6.86	50.4	41.52	0.80	0.6147
8.0	7.0	4.0	2.0	8.2	8.23	51.6	51.98	0.66	0.7964
9.0	5.5	6.0	1.0	7.0	7.55	36.7	36.60	0.41	0.4595
10	5.5	6.0	3.0	6.0	6.20	22.0	27.97	0.35	0.4191
11	5.5	2.0	1.0	5.3	5.74	17.8	31.84	0.21	0.4063
12	8.5	2.0	3.0	7.7	7.89	21.2	30.69	0.31	0.3787
13	4.5	4.0	2.0	6.1	5.31	32.1	22.29	0.43	0.2963
14	7.0	4.0	2.0	8.2	8.23	51.6	51.98	0.89	0.7964
15	7.0	4.0	2.0	8.2	8.23	51.6	51.98	0.89	0.7964
16	7.0	7.4	2.0	7.9	7.64	48.6	49.27	0.52	0.4861
17	8.5	6.0	1.0	6.6	6.59	44.6	49.78	0.37	0.4824
18	7.0	4.0	2.0	8.2	8.23	51.6	51.98	0.89	0.7964
19	7.0	0.6	2.0	7.7	6.88	48.8	34.80	0.53	0.3967
20	8.5	6.0	3.0	6.7	6.99	47.8	43.14	0.51	0.4319

Model for CMC formation

$CMC = +0.7964 + 0.0714A + 0.026B - 0.0852C - 0.0625AB - 0.0025AC + 0.0625BC - 0.1344A^2 - 0.1255B^2 - 0.1149C^2$

$Biosurfactant = +51.98 + 6.79A + 4.30B - 5.45C + 0.2975AB + 0.4975AC + 1.63BC - 6.46A^2 - 3.51B^2 - 6.94C^2$

$Biomass = +8.23 + 0.4735A + 0.2260B - 0.0748C - 0.5125AB + 0.4375AC - 0.1625BC - 0.7520A^2 - 0.3419B^2 - 0.5293C^2$

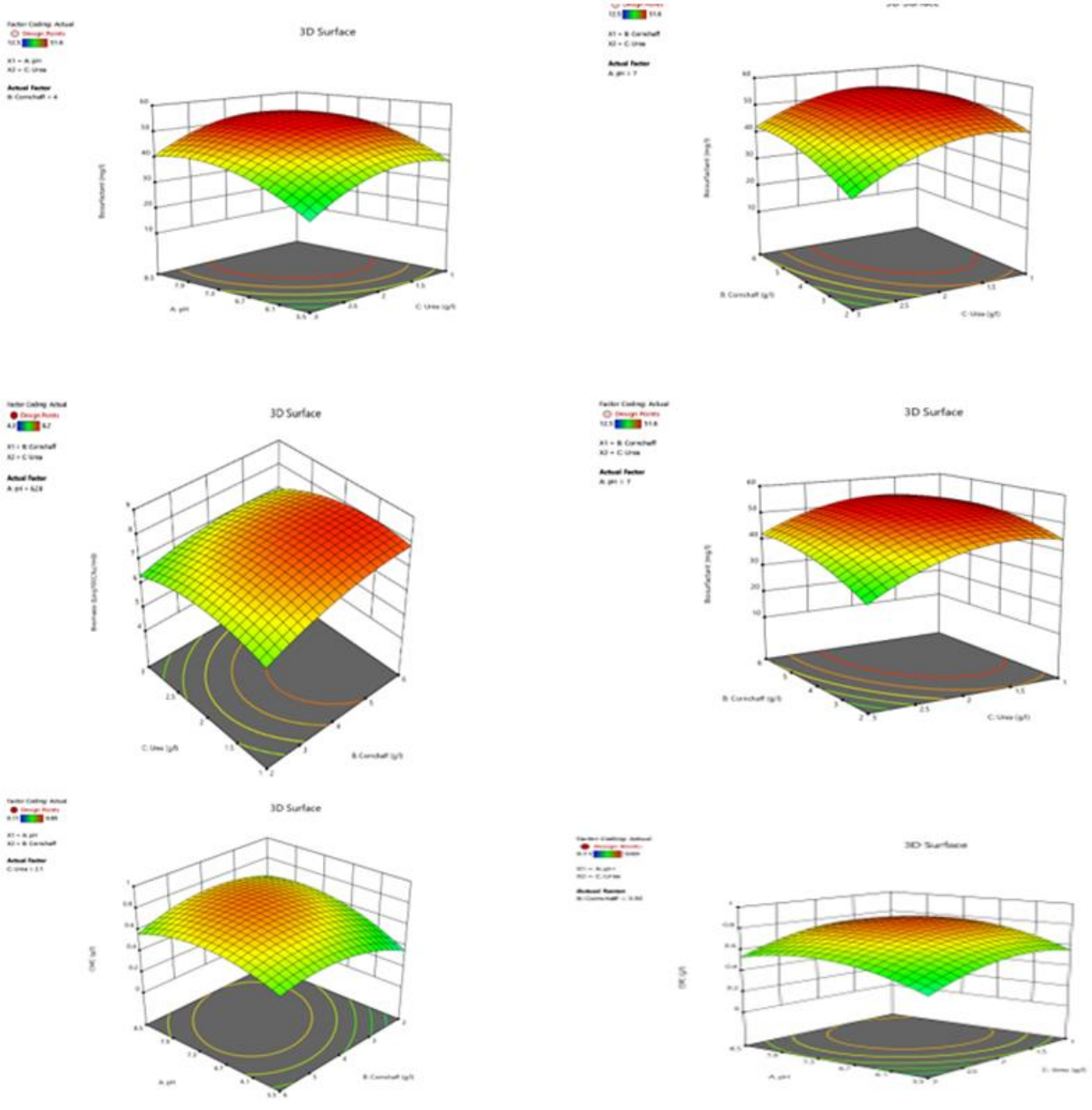

Figure 2. 3D-plots for Biosurfactant, biomass and CMC production at constant Urea/Corn chaff concentration of 2.0 g/l and varied corn chaff 4.0 g/l and pH = 7.0

Optimization allows for determination of operational variables in the selection stages of production. Cultivation of the feedstock is strongly dependent. The benefits of multivariate analysis hinge on lesser time consumption and the robustness of the data processing. According to Zhang & Dequan (31), there are no universal values for upstream cultivation and processing. These optimization studies have an unusual impact on the selection of process condition. From this study, Prehydrolyzed corn chaff was employed as a carbon source which was observed to have 4.0g/l as its optimal concentration Prob>F = 0.03 which is an ideal condition. This supports the report Abalos, Maximo, Manresa, & Bastida (27) on the basis of parameter, yield and the process conditions for biosurfactant production. Abbassi et al. (28) reported a 13.0 g/L biosurfactant, yield although lower than the yield obtained from our current study, About 30 g/L was obtained during the OVAT studies and 51g/L at pH7.0 and urea concentration of 2.0 g/l. at p< 0.04 which was an ideal model for industrial-scale production. Furthermore, Box and Behnken approach have been identified as a robust statistical analysis method, although Zhang and Dequan (31) support the multi-variate response analysis of several factors associated with the bioprocess and not primarily the product being assayed.

The result of the study agreed with other reports on the desirability of the model using quadratic functions and p<0.05. However, fit analysis suggests there might be low- noise ratio associated with collection and analysis of data. Furthermore, Abbasi et al. (28) reported an optimal condition at pH8.0, 28 ºC and 8% inoculum size using soya bean oil as carbon source, sodium nitrate as nitrate source and yeast extract at 9.3 and 3.92 g/L respective yielded a 12.6 g/l this further agrees to the factor that multivariate studies had a significant effect at an F-stat value of 26.7226 and prob>F < 0.0001 on the one-variable at a time yield as they reported a 1.46 times yield. In addition. Our findings strongly agree with the conditions reported by Deepika, Kalam, Ramu Sridhar, Podile, & Bramhachari (29) in whose report isolated *P. aeruginosa* from Mangrove swamp. Their study applied Plackett-Burman design approach using a simple polynomial function arrive at pH7.8 was and 9.17 g/L, with a production yield of 5.9 g/l, CMC of 0.1 g/L. Their also agrees with the report with the report of Kumar, Janardhan, Radha, Viswanath, & Narasimha (30) which reported the potency of glycerol and groundnut husk residues. The Box and Behnken have been appraised to be the simplest and the best approach to bioprocess optimization. From the study, the model of the optimization was observed to imply that there was a strong correlation between the process parameters were in tandem with the production and quality of the biosurfactant produced at the p-value < 0.05.

This study further buttresses the potency and viability of the waste-to-wealth technology as a measure of revenue generation as documented from the use of corn chaff and urea as source of biotechnological feedstock

4. CONCLUSIONS

The novel *Pseudomonas* sp. obtained from the study had a record biosurfactant production activity and could be harnessed in environmental application as an oil field chemical. The mathematical model suggests that the agro-residues could serve as an inexpensive substrate in bioprocess development. Operational conditions for cultivation and synthesis of biosurfactant is feasible and economic to handle in a controlled bioreactor system. The pH = 7.0, C = 4 g/l and N = 2 g/l. The Biomass yield and biosurfactant production suggest that the process of synthesis is growth-associated and regulated.

Recommendations

Agro waste residues have to be explored and analysed for compositional elements after hydrolysis. Synthetic industries must be encouraged to explore the locally available feedstock. The material safety data sheet for novel biomolecules could be advocated for especially for storage of eco-friendly and problem-oriented solutions. Further research could be done in the aspect of field-scale optimizations and productions. Reactor based optimization and recovery studies could be explored.

References

[1] Dhail S, Jasuja ND. Isolation of biosurfactant-producing marine bacteria. *African Journal of Environmental Science and Technology* 2012; 6(6): 263-6.

[2] Freitas BG, Brito JGM, Brasileiro PPF, Rufino RD, Luna JM, Santos VA, et al. Formulation of a commercial biosurfactant for application as a dispersant of petroleum and by-products spilled in oceans. *Front Microbiol.* 2016; 7(OCT): 1-9.

[3] Patowary K, Patowary R, Kalita MC, Deka S. Characterization of biosurfactant produced during degradation of hydrocarbons using crude oil as sole source of carbon. *Front Microbiol.* 2017; 8(FEB): 1–14.

[4] Abdel-Mawgoud AM, Lépine F, Déziel E. Rhamnolipids: diversity of structures, microbial origins and roles. *Applied Microbiology and Biotechnology* 2010 May 1; 86(5): 1323-36.

[5] Liang X, Guo C, Liao C, Liu S, Wick LY, Peng D, et al. Drivers and applications of integrated clean-up technologies for surfactant-enhanced remediation of environments contaminated with polycyclic aromatic hydrocarbons (PAHs). *Environ Pollut* 2017; 225: 129-140. http://dx.doi.org/10.1016/j.envpol.2017.03.045

[6] Santhini K, Parthasarathi R. Isolation and Screening of Biosurfactant Producing Microorganisms from Hydrocarbon Contaminated Soils from Automobile Workshop. 2014; 5(2): 158–67.

[7] Muthukamalam S, Sivagangavathi S, Dhrishya D, Sudha Rani S. Characterization of dioxygenases and biosurfactants produced by crude oil degrading soil bacteria. *Brazilian J Microbiol* 2017; 48(4): 637–47. http://dx.doi.org/10.1016/j.bjm.2017.02.007

[8] Rikalovic MG, Gojgić Cvijović G, Vrvic MM, Karadzic I. Production and characterization of rhamnolipids from Pseudomonas aeruginosa san-ai. *Journal of the Serbian Chemical Society* 2012; 77(1): 27-42.

[9] De-la-Pena C, Loyola-Vargas VM. Biotic Interactions in the Rhizosphere: A Diverse Cooperative Enterprise for Plant Productivity. P*lant Physiol.* 2014; 166(2): 701–19.

[10] Williams KP, Gillespie JJ, Sobral BWS, Nordberg EK, Snyder EE, Shallom JM, et al. Phylogeny of gammaproteobacteria. *J Bacteriol.* 2010; 192(9): 2305-14.

[11] Almagro G, Viale AM, Montero M, Rahimpour M, Muñoz FJ, Baroja-Fernández E, et al. Comparative genomic and phylogenetic analyses of gammaproteobacterial glg genes traced the origin of the Escherichia coli glycogen glgBXCAP operon to the last

common ancestor of the sister orders Enterobacteriales and Pasteurellales. *PLoS One* 2015; 10(1): 1–30.

[12] Keller-Costa T, Jousset A, van Overbeek L, van Elsas JD, Costa R. The freshwater sponge Ephydatia fluviatilis harbours diverse Pseudomonas species (Gammaproteobacteria, Pseudomonadales) with broad-spectrum antimicrobial activity. *PloS One* 2014 Feb 12; 9(2): e88429.

[13] Huang YH, Huang XJ, Chen XH, Cai QY, Chen S, Mo CH, et al. Biodegradation of di-butyl phthalate (DBP) by a novel endophytic bacterium Bacillus subtilis and its bioaugmentation for removing DBP from vegetation slurry. *J Environ Manage* 2018; 224: 1-9.

[14] Santoyo G, Moreno-hagelsieb G, Orozco-mosqueda C, Glick BR. Plant growth-promoting bacterial endophytes. *Microbiol Res* 2016; 183: 92–9. http://dx.doi.org/10.1016/j.micres.2015.11.008

[15] Ahemad M. Mechanisms and applications of plant growth promoting rhizobacteria : Current perspective. *J King Saud Univ – Sci* 2014; 26(1): 1–20. http://dx.doi.org/10.1016/j.jksus.2013.05.001

[16] S. Daliry, A. Hallajisani, J. Mohammadi Roshandeh, H. Nouri, A. Golzary. Investigation of optimal condition for Chlorella vulgarismicroalgae growth. *Global J. Environ. Sci. Manage.* 3(2): 217-230, 2017. DOI: 10.22034/gjesm.2017.03.02.010

[17] Majid M, Rasoul S, Raziyeh Z, Allal H, Philippe T, Frank D, et al. Optimization of biomass production of Acetobacter pasteurianus KU710511 as a potential starter for fruit vinegar production. *African J Biotechnol.* 2016; 15(27): 1429–41.

[18] Pankaj VP, Awasthi M. Optimization of Growth Condition for Chlorella Vulgaris Using Response Surface Methodology (Rsm). *International Journal of Engineering Science & Advanced Technology* 2014; 4(5): 492–500.

[19] Karanam SK, Babu IS, Rao GH. Process optimization for citric acid production from raw glycerol using response surface methodology. *Indian J Biotechnol.* 2008; 7(4): 496–501.

[20] Ekwuabu CB, Chikere CB, Akaranta O. Effect of Different Nutrient Amendments on Eco-Restoration of a Crude Oil Polluted Soil. InSPE African Health, Safety, Security, Environment, and Social Responsibility Conference and Exhibition 2016 Oct 4. Society of Petroleum Engineers.

[21] Indriani D, Isty A, Purwasena A, Eka R, Maghfirotul P, Yuichi A. Screening and characterization of biosurfactant produced by Pseudoxanthomonas sp . G3 and its applicability for enhanced oil recovery. *J Pet Explor Prod Technol* 2019; 9(3): 2279–89. http://dx.doi.org/10.1007/s13202-019-0619-8

[22] Das P, Yang XP, Ma LZ. Analysis of biosurfactants from industrially viable Pseudomonas strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity. *Frontiers in Microbiology* 2014 Dec 22; 5: 696.

[23] Shahwar D-E-, Sheikh RA, Jamil N. Isolation and Characterization of Biosurfactant Producing Bacteria Isolated from Produced Water. *Punjab Univ J Zool.* 2019; 34(1): 35–40.

[24] Peekate PL, Abu GO. Optimizing C: N ratio, C: P ratio, and pH for biosurfactant production by Pseudomonas fluorescens. *J. Adv. Microbiol.* 2017; 7(2): 1-4.

[25] Rashedi,H., and E. Jamshidi. Isolation and production of biosurfactant from Pseudomonas aeruginosa isolated from Iranian southern wells oil. *Int J Environ Sci Tech.* 2005; 2(2): 121–7.

[26] Xiangsheng Z, Lu D. Response surface analyses of rhamnolipid production by Pseudomonas aeruginosa strain with two response values. *African J Microbiol Res.* 2013; 7(22): 2757–63.

[27] Abalos A, Maximo F, Manresa MA, Bastida J. Utilization of response surface methodology to optimize the culture media for the production of rhamnolipids by Pseudomonas aeruginosa AT10. *J Chem Technol Biotechnol.* 2002; 77(7): 777–84.

[28] Abbasi H, Sharafi H, Alidost L, Bodagh A, Zahiri HS, Noghabi KA. Response surface optimization of biosurfactant produced by pseudomonas aeruginosa ma01 isolated from spoiled apples. *Prep Biochem Biotechnol.* 2013; 43(4): 398–414.

[29] Deepika K V., Kalam S, Ramu Sridhar P, Podile AR, Bramhachari P V. (2016) Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium Pseudomonas aeruginosa KVD-HR42 using response surface methodology. *Biocatal Agric Biotechnol.* 5: 38–47. http://dx.doi.org/10.1016/j.bcab.2015.11.006

[30] Kumar AP, Janardhan A, Radha S, Viswanath B, Narasimha G. Statistical approach to optimize production of biosurfactant by Pseudomonas aeruginosa 2297. *3 Biotech.* 2015; 5(1): 71–9. doi: 10.1007/s13205-014-0203-3

[31] Zhang X, Dequan L. Response surface analyses of rhamnolipid production by Pseudomonas aeruginosa strain with two response values. *African Journal of Microbiology Research* 2013 May 28; 7(22): 2757-63.