International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

Modelling of Biosurfactant Production by *Pseudomonas Aeruginosa* Using Red Cashew (Anacardium Accidentale) Pomace as Substrate; A Response Surface Methodology Approach

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***Abstract*—** *Biosurfactants are amphiphilic compounds produced by microorganisms as secondary metabolite. Its unique properties such as low toxicity, higher biodegradability, mild process conditions, higher foaming capacity, temperature, pH and salinity stability and synthesis under user-friendly conditions makes them possible to replace synthetic surfactants which are mainly used in food, cosmetics and pharmaceutical industries and in environmental applications. In this study, biosurfactant production by P. aeruginosa was investigated as well as the influence of fermentation factors (stay time, pH and salinity), the result showed that increased in pH to alkaline medium favoured the biosurfactant yield, as the stay time increased to 4days the yield increased, the optimum salinity was found to be 1.5w/v. Optimization of the process was carried out using response surface methodology adopting Box-behnkien design. The selected factors were stay time, pH and salinity and the optimum time for biosurfactant production is 6 days, optimum pH of 8 and a salinity level of 1.0% (w/v), also a second order polynomial model was generated which was found significant across all model criteria*.

***Keywords*—** *Biosurfactant, response surface methodology, model, pseudomonas aeruginosa, pomace, optimization, Red Cashew*.

I. INTRODUCTION

Biosurfactants are naturally surface-active compounds derived from microorganisms (Anandaraj and Thivakaran, 2010). They are amphiphilic compounds produced mostly on microbial cell surfaces or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface and interfacial tensions between two immiscible fluids like oil and water (Anyanwu *et al*., 2011). Considerable attention has been given in the past to the production of surface-active molecules of biological origin because of their potential utilization in food processing, pharmacology, cosmetic, biomedical and petroleum industries (Emine and Aysun, 2009). The upsurge on replacement of synthetic surfactant with their biological counterparts (Biosurfactants) is due to the latter's better characteristics such as low toxicity, higher biodegradability and mild process conditions, higher foaming capacity, temperature, pH and salinity stability and synthesis under user-friendly conditions (Parveen *et al.*, 2011; Chandran and Das, 2010). On the other hand, different microorganisms are known to synthesize different types of biosurfactants when grown on several carbon sources, therefore the type, quality and quantity of biosurfactant produced are also influenced by the nature of the carbon substrate and the culture conditions such as pH, temperature, agitation and dilution rate in continuous culture (Lakshmipathy *et al.*, 2010). In this study, biosurfactant production by *P. aeruginosa* was optimized based on number of fermentation days, pH and salinity conditions. *Pseudomonas* species has been identified to degrade hydrocarbons and produce biosurfactants predominately glycolipids (Beal and Betts, 2000). *P. aeruginosa*-derived biosurfactant production is applicable to many purposes, including for the microbe-enhanced oil recovery (MEOR) and bioremediation.

II. MATERIALS AND METHODS

2.1. Equipment and Materials

The cashew used were bought from Ofagbe main market, washed and taken to the laboratory for pomace preparation.

2.2 Preparation of Basal Mineral Medium (B.M.M) and Culture Media

The basal mineral medium was prepared as described by Atlas (2010). The trace element solution was prepared first by adding components (0.232g H_3BO_3, 0.174g $ZnSO_4.7H_2O$, 0.116g $FeSO_4(NH_4)_2SO_4.6H_2O$, 0.096g $CoSO_4.7H_2O$, 0.022g $(NH_4)_6Mo_7O_2.4H_2O$, 8.0mg $CuSO_4.5H_2O$, 8.0mg $MnSO_4.4H_2O$) to distilled water and bringing its volume to 1.0L. The solution was then mixed thoroughly. The basal mineral medium (B.M.M) was prepared by adding components (12.5g K_2HPO_4, 3.8g $KHPO_4$, 1.0g $(NH_4)_2SO_4$, 0.1g $MgSO_4.7H_2O$ plus 5.0mL of the trace elements solution) to distilled water and bringing the volume to 900.0mL mark. The solution was mixed thoroughly; it was then autoclaved at 121^{o}C, 15psi for 15min and cooled to 45°C. Subsequently, the carbon source was prepared by adding 14g of the red cashew pomace to distilled water, bringing its volume to the 100.0mL mark and mixing thoroughly. 100ml of the carbon source (red cashew pomace) was added to 900ml the sterilized basal mineral medium and it was shaken, 200mL were distributed into ten different conical flasks. 1mL of seed culture containing *Pseudomonas aeruginosa* was inoculated in each flask.

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ISSN (Online): 2455-9024

2.3 Extraction of Biosurfactants

The culture broth was centrifuged at 4000rpm for 15min to remove the cells as well as debris and the supernant was used for the extraction. The supernant was then precipitated by acidification with hydrochloric acid to pH 2.0. Chloroform: methanol (2:1) was added. This mixture was shaken and left overnight for evaporation. White coloured sediment was obtained as a result i.e. the crude biosurfactant. This was dried and weighed (Anandaraj and Thivakarn, 2010).

2.4. Stay Time Dependence on Biosurfactant Production

The effect of the stay time (inoculum time) on biosurfactant production was investigated at intervals of (2-10) days. 200mL of the culture broth was measured into 5 distinct flasks followed by the addition of 1mL of Pseudomonas aeruginosa seed culture at pH 7 and salinity of 2%, these flasks were labelled with respective inoculum time of 2, 4, 6, 8 and 10 days and allowed to ferment for the various days, at the end of each stay time, the flasks were taken for biosurfactant extraction and quantification.

2.5. Effect of pH in Biosurfactant Production:

1mL of seed culture containing *Pseudomonas aeruginosa* was added to 200mL of culture broth and the pH was adjusted to 2, 4, 6, 8, 10 and 10 in respective flasks so as to determine the impact on the microbial activity in respect of biosurfactant production. The media was then allowed to stay for 4 days as *optimum* time of biosurfactant production at salinity of 2%. At the end of the 4 days each flask was subjected to biosurfactant extraction and quantification.

2.6. Effect of Salinity on Biosurfactant Production.

Effect of salinity on biosurfactant production was done by using 0.05M NaCl to vary the salinity concentration to distinct (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4) % (w/v) at pH 8. The culture was then allowed to stay for 4 days before biosurfactant extraction and quantification.

III. Result and Discussion

3.1. Effect of Stay Time Biosurfactant Production.

The impact of stay time on biosurfactant production was evaluated so as to determine how long the *P. aeruginosa* can survive in the culture broth in respect of biosurfactant weight (BSW) produced, also to know the optimum time for maximum BSW. The broth fermentation was done in 2 days intervals at the range of (2-19) days. The result for stay time effect is presented in fig. 1.

From the plot, it is evident that the as the number of stay time increased from 0 to 4 days, the quantity of biosurfactant produced also increased from 0 to 0.81g, this could be link to the fact that as the days prolong, the *P. aeruginosa* have more time to feed on the substrate, grow and excrete the biosurfactant but after 4 days the *P. aeruginosa* activities began to decline which could be as a result of their death. Consequently, 4 days was taken as the optimum stay time.

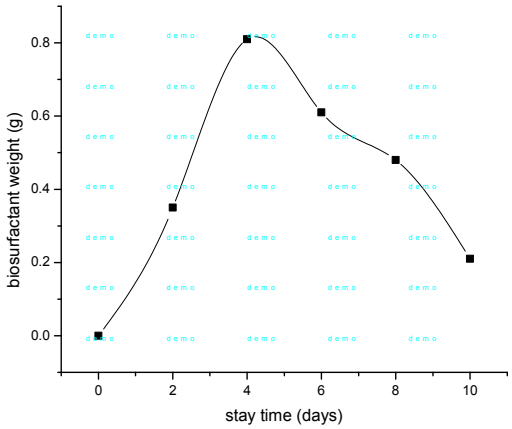

Fig. 1. Stay time effect on biosurfactant.

3.2. Effect of pH on Biosurfactant Production:

The result for the influence of pH on biosurfactant weight produced is shown in fig. 2, the BSW increased gradually as the pH moved 6, but a sharp increase was noticed in BSW at about pH 7.5-8.5 before a fall in BSW started, by implication, pH of narrow alkaline range (7.5-8.5) is suitable for synthesis of secondary metabolite in *P. aeruginosa*. Generally, the intracellular pH of most microorganisms are maintained near neutrality regardless of the pH in outside medium (Riebeling et al, 1975), The maximum biosurfactant production was observed at pH 8.0(optimum pH) as shown in fig. 2.

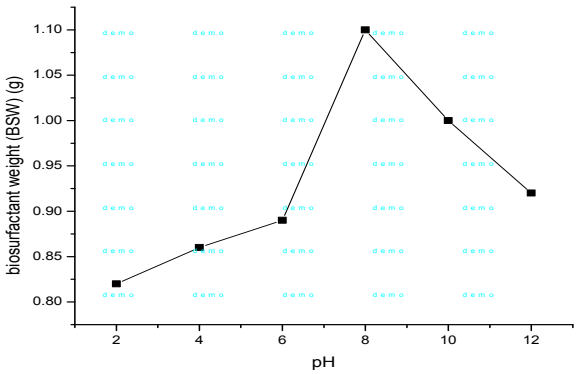

Fig. 2.

3.3. Effect of Salinity on Biosurfactant Production:

The concentration of salt in certain media can influence the production of biosurfactant from microorganisms. In ths study, different concentration of 0.05M NaCl was added to the media and the result is presented in Fig. 3, it was observed that optimum production of biosurfactant was at 1.5% (w/v), this means higher salinity inhibit the microoganisms activity as well as their growth hence decline in biosurfactant weight. Similar view had been reported by (Gomathy and Senthil kumar, 2013).

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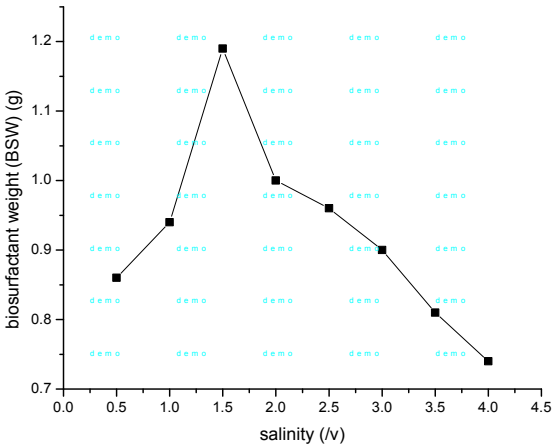

Fig. 3. Salinity effect plot on biosurfactant production.

IV. Response Surface Analysis

The experimental design of Box-Behnken (BBD) was used in this work as shown in table

The bio-surfactant production variables investigated are; inoculum time (days), pH and medium salinity (%w/v), this gave a total of 15 experimental runs as generated by Minitab statistical software V16. The uncoded values for these variable taken from the optimal points in the single factors investigation. The surfaces were varied under three equidistance level (-1, 0, 1) in respect of biosurfactant weight (Y).

4.0. Data Analysis.

Data from the experimental matrix were subjected to analysis of variance to test the level of significance of the model and the influence magnitude of the surface on the response, also regression analysis and residual plots were made to get the model terms coefficients and model verification using Minitab software version 16.

TABLE I. Experimental surfaces coded and uncoded levels.

Surface variables	Designations	levels		
		-1	0	1
Stay Time(days)	X_1	2	4	6
pH	X_2	8	10	12
salinity (%w/v)	X_3	1.0	1.5	2.0
% Dye removed	Y			

4.1. Model Determination

The 15 matrix runs using uncoded values along with corresponding response (Y, biosurfactant weight) were shown in table V. This result was used to generate a regression quadratic polynomial that described the fermentation process. The model takes the form of

$$Y_{surfactant\ weight} = \beta_0 + \sum_{i=1}^{n}\beta_i X_i + \sum_{i=1}^{n}\beta_{ii} X_{ii} + \sum_{i<j}^{n}\beta_{ij} X_i X_j \qquad (1)$$

Where β_0 is the model constant, β_i = linear coefficient, β_{ii} = squared coefficient, β_{ij} = interaction coefficient, respectively (Park et al., 2014, Abalos et al., 2002).

TABLE II. Runs matrix for uncoded factor with response values.

Std Order	Run Order	PtType	Blocks	Stay Time (Days)	pH	Salinity (%w/v)	BSW (g)
14	1	0	1	4	8	1.5	0.87
3	2	2	1	2	10	1.5	0.62
2	3	2	1	6	6	1.5	0.83
6	4	2	1	6	8	1.0	1.19
9	5	2	1	4	6	1.0	0.74
4	6	2	1	6	10	1.5	1.00
10	7	2	1	4	10	1.0	0.80
12	8	2	1	4	10	2.0	0.65
15	9	0	1	4	8	1.5	0.89
5	10	2	1	2	8	1.0	1.10
1	11	2	1	2	6	1.5	0.64
11	12	2	1	4	6	2.0	0.57
7	13	2	1	2	8	2.0	0.75
8	14	2	1	6	8	2.0	1.13
13	15	0	1	4	8	1.5	0.88

From table II data, the optimum biosurfactant weight (BSW) is 1.94g yielded by stay time of 6 days, pH of 8 and salinity of 1.0% as marked yellow. This result was tested by regression analysis and analysis of variance (ANOVA) and the outcome are presented in table III and IV respectively.

TABLE III.Estimated regression coefficients for BSW (g) using coded values.

Term	Coef	SE Coef	T	P
Constant	0.880000	0.013601	64.699	0.000
STAY TIME (DAYS)	0.135000	0.008329	16.208	0.000
pH	0.036250	0.008329	4.352	0.007
SALINITY (%w/v)	-0.096250	0.008329	-11.556	0.000
STAY TIME (DAYS)*STAY TIME (DAYS)	0.127500	0.012260	10.400	0.000
pH*pH	-0.235000	0.012260	-19.168	0.000
SALINITY (%w/v)*SALINITY (%w/v	0.045000	0.012260	3.670	0.014
STAY TIME (DAYS)*pH	0.047500	0.011779	4.033	0.010
STAY TIME (DAYS)*SALINITY (%w/v)	0.062500	0.011779	5.306	0.003
pH*SALINITY (%w/v)	0.005000	0.011779	0.424	0.689
R-Sq = 99.50% R-Sq(pred) = 92.44% R-Sq(adj) = 98.59%				

All the fermentation term for biosurfactant synthesis were found significant except the pH*salinity interaction effect as indicated in table III, this was buttressed in ANOVA table IV of significance. The coefficient of determination was found to R^2 = 99.50, this shows that the model for the biosurfactant production process was adequate in describing it. From the predicted R2 = 92.44, the model has a good predicting power since the difference between R^2, R^2 pred and R^2 adj is less than 20% (Park et al., 2014, Zhang and Dequan, 2013).

Response surface model

Using the terms coded values of coefficients in table III, the quadratic term generated by the Minitab software is as followed.

$$Y = 0.88 + 0.135A + 0.0363B - 0.0963C + 0.128A^2$$
$$-0.235B^2 + 0.045C^2 + 0.0475AB + 0.0625AC + 0.005BC$$

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Where A = stay time, B = pH, C = medium salinity.

$$Y_{BSW} = -1.2763 - 0.3763 STAY\,TIME + 0.903 pH - 1.023 SALINITY$$
$$+0.0319(STAY\,TIME)^2 - 0.0588(pH)^2 + 0.180(SALINITY)^2$$
$$+0.0119 STAY\,TIME * pH + 0.063 STAY\,TIME * SALINITY + 0.005 pH * SALINITY$$

At R^2 = 99.50

TABLE IV. Analysis of variance for BSW (g).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	0.548158	0.548158	0.060906	109.74	0.000
Linear	3	0.230425	0.230425	0.076808	138.39	0.000
STAY TIME (DAYS)	1	0.145800	0.145800	0.145800	262.70	0.000
Ph	1	0.010512	0.010512	0.010512	18.94	0.007
SALINITY (%w/v)	1	0.074112	0.074113	0.074113	133.54	0.000
Square	3	0.292983	0.292983	0.097661	175.97	0.000
STAY TIME (DAYS)*STAY TIME (DAYS)	1	0.074298	0.060023	0.060023	108.15	0.000
pH*pH	1	0.211209	0.203908	0.203908	367.40	0.000
SALINITY (%w/v)*SALINITY (%w/v)	1	0.007477	0.007477	0.007477	13.47	0.014
Interaction	3	0.024750	0.024750	0.008250	14.86	0.003
STAY TIME (DAYS)*pH	1	0.009025	0.009025	0.009025	16.26	0.010
STAY TIME (DAYS)*SALINITY (%w/v)	1	0.015625	0.015625	0.015625	28.15	0.006
pH*SALINITY (%w/v)	1	0.000100	0.000100	0.000100	0.18	0.689
Residual Error	5	0.002775	0.002775	0.000555		
Lack-of-Fit	3	0.002575	0.002575	0.000858	8.58	o.106
Pure Error	2	0.000200	0.000200	0.000100		
Total	14	0.550933				

Table IV showed that the regression model was significant with p = 0.000, also the linear, square, and interaction terms were all significant at p = (0.000, 0.000 and 0.003) respectively. There was also an indication of insignificance in lack of fit, which show that about 10.6% of the result were due to noise, and the model can fit in similar data that are not in the experiment (Abalos et al., 2002).

Fig. 4 showed the residual plot using four in one plot, this was to determine the normality of the model with the experimental data, from the plots residual were linearly fitted, the fitted point were evenly distributed and the observed order were random.

4.2 Verification Analysis.

The optimum points from table I (6, 8 and 1.0) for stay time, pH and salinity respectively were used for a new run and the result presented in table V, the BSW gotten was 1.20g, which is a close call to that 1.19g in table III, similar observation had been made by Zhang and Dequan, (2013).

TABLE V. Model verification test result.

Stay time (days)	Ph	Salinity %w/v)	BSW (g)
6	8	1.0	1.20

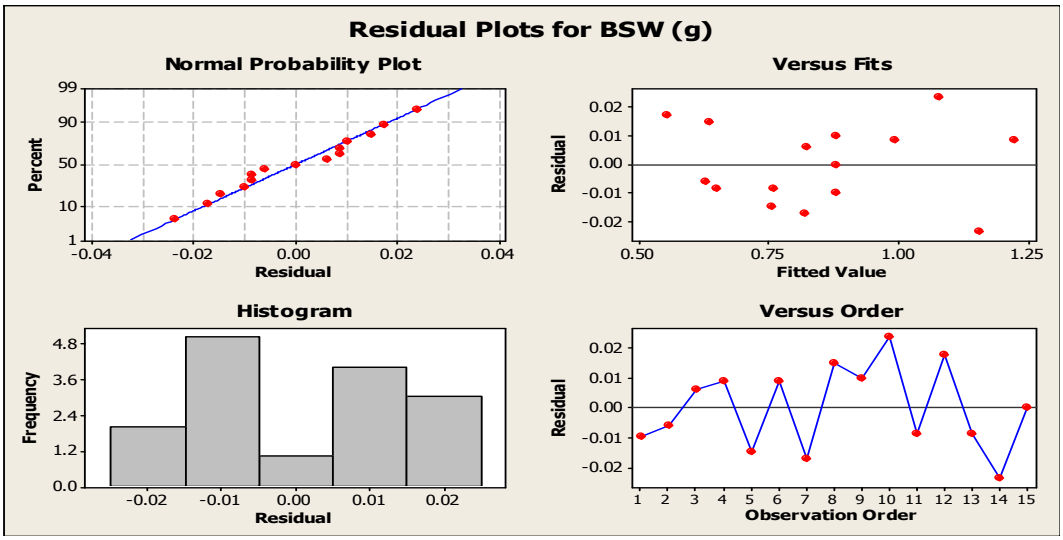

Fig. 4. Residual plot for biosurfactant production using red cashew.

4.3. Interactions Effect Plots

3-D surface and contour plots were made so as to visualize the interaction effects of the fermentation process as shown in Fig. 5a-b, 6a-b and fig. 7a-b respectively.

The interaction effect from fig. 5a was synergetic to biosurfactant production, as stay time increased toward the 6 days with increase in salinity from 1.0, amount of biosurfactant yielded also increased until a decline was noticed after about 1.1.45% of salinity. This interaction effect is high as shown in the closed contour line in Fig. 6a. this effect showed that the longer the pomace stays in the higher salinity the lower the biosurfactant produced, consequently, higher salinity inhibit the microbes activities (Khalifeh et al., 2013). Similarly, in Fig. 6a and 6b, increased in pH with mild increased in salinity favoured BSW yield.

That means at little increase in salinity the microbes activities are enhanced with more alkaline pH.

This was proven by the interaction effect of pH and stay time, the longer the days the pomace stayed in the higher pH the more favourable it is to biosurfactant yield as in Fig.7a &b.

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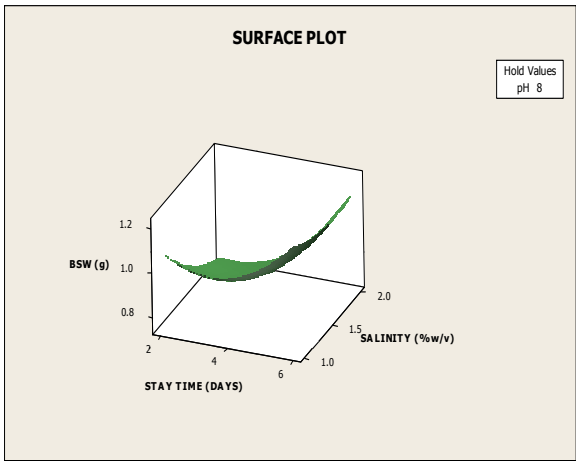
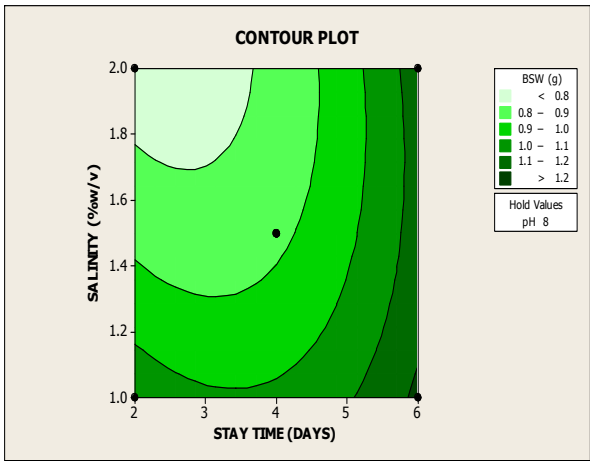
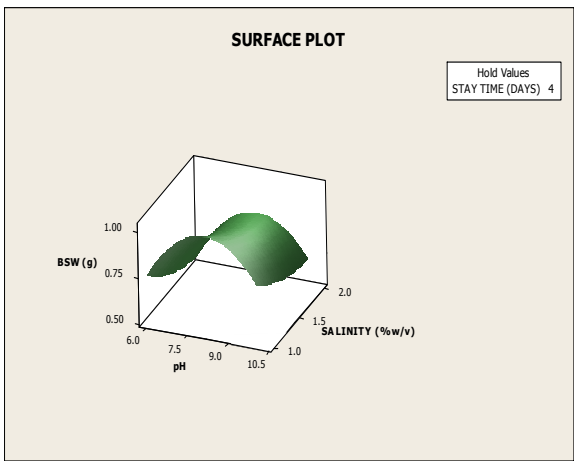


5a

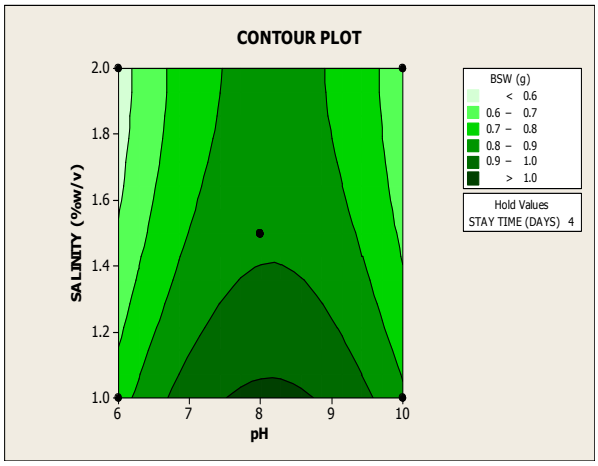


5b

Fig. 5a&b. stay time and salinity Vs BSW plot.

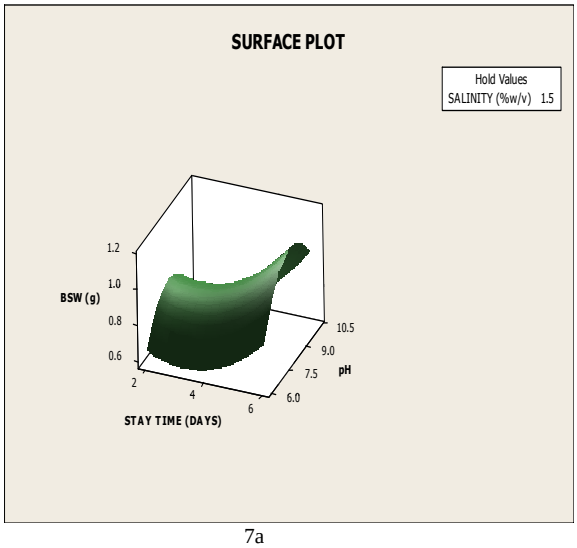


6a

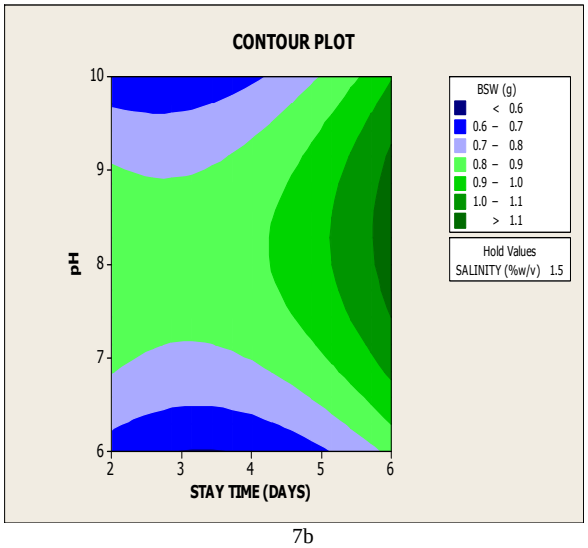


6b

Fig. 6. pH and salinity effect plot.



7a



7b

Fig. 7. Stay time and pH Vs BSW plot.

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4.4. Optimization of Fermentation Process

The minitab v16 response optimizer was employed in this stage using the optimum parameters in table as starting point adopting the target of 5.0g with minimum of 0.7g.

The predicted response are; BSW (g) = 1.22784 at desirability of 0.122754
Hence the global solution for optimized parameters are;
Stay time (days) = 6, pH = 8.3434, salinity (%w/v) = 1.0 as shown in fig. 8.

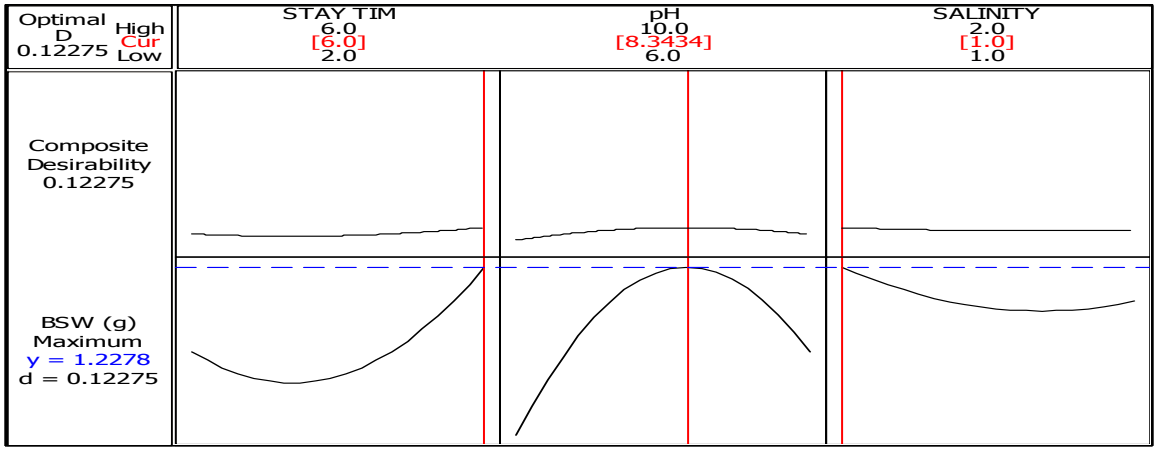

Fig. 8. Optimization plot.

4.5. Validation Test.

The predicted values were used to run the fermentation process again in replicate and the result shown a good correlations as in table VI.

TABLE VI. Optimized values verification result.

Stay time (days)	pH	Salinity (%w/v)	Y1	Y2	avgY (g)
6	8.3	1.0	1.21	1.22	1.215

The value of avgY (BSW) is very near the predicted resulted, this shows that *Pseudomonas aeruginosa* activities is most favoured in salinity of 1.0%, alkaline pH ≥ 8.3 and time of 6 days.

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