IJSETT International Journal "International Journal for Science and Emerging Technologies with Latest Trends" 11(1): 10-21 (2013)
ISSN No. (Online):2250-3641
ISSN No. (Print): 2277-8136

Optimization of Bioremediation of Cheese Whey with the Activity of Klebsiella Pneumonia Using Response Surface Methodology

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(Received 23 August 2013 Accepted 8 September 2013)

Abstract -Response surface methodology was used to study the optimization of bioconversion of cheese whey to 2,3-Butanediol using Klebsiella pneumonia at room temperature. 3-Level factorial design was employed to correlate the bioremediation parameters with the biomass of K. pneumonia and concentration of 2,3-BD as response. Quadratic polynomial equation was developed to achieve optimal performance of the process. The result showed that the optimum condition for the bioremediation process in unaerated and airlifted batch reactors for concentration of cheese was 39.98g/L and 39.94g/L, and fermentation time was 95.5hours which resulted in biomass of k. pneumonia 1.4633mg/L and 3.6580mg/L, and 2,3-BD production of 10.696g/L and 17.997g/L respectively. 2,3-BD production in unaerated and airlifted batch reactors amounted to 36.88% and 62.06% respectively. The study has shown that k. pneumonia not only utilized cheese whey as a source of food and energy but optimally better for remediation of cheese whey in airlift batch reactors.
Keywords-bioremediation, cheese whey, optimization, response surface methodology, 2,3-butanediol

1. INTRODUCTION

The environmental sustainability of the human society largely depends on the management of the natural environment. Contaminated soil and water from industrial or agricultural activities posed a potential health threat to animals and humans with detrimental effect on economic systems by making land and water unsuitable for agriculture and other economic purposes.

Cheese whey, the greenish translucent liquid is a milk serum of dairy industry and obtained by application of acid, heat and rennet after milk coagulation and removal of the curd which contains large percentage of lactose as well as moieties of protein, fat and mineral salts [4, 5]. It has been reported that whey is one of the major source of biochemical oxygen demand (BOD) that indicates high pollution potential [2, 4, 5, 25]. Whey disposal is a costly and time consuming process to manage and its direct disposal into sewage water causes inherent danger to water pollution (Ambreen et al, 2012). Being a cheaper organic carbon and nitrogen source, its utilization for production of valued added products is a great management and better for economic of the world, especially the developing countries [13].

In Iran, about 1.8 million tons of whey produced as the by-product of cheese producing factories every

year and converted into ethanol due to its low price compared to the price of other raw materials which makes whey utilization the considerable attention in the world [13].

Bioremediation, the utilization of microorganisms and plants with the ability to degrade or immobilize toxic compounds has been proposed as a cheaper alternative to conventional methods such as Physical and chemical remediation techniques [6, 7, 15]. The biodegradation-enhancing effect of whey was primarily attributed to an increased microbial biomass stimulated by the readily available carbon source which depends on the nature of the products [23]. Cheese whey has been used as a cheap source of food and energy for the production of valuable products by microbes [9, 10, 14, 19, 24, 27].

Many researchers have work on cheese whey for the production of citric acid by *Aspergillus niger* in submerged fermentation, ethanol by *Kluyveromyces marxianus and Kluyveromyces fragilis,* Xanthan gum *by Xanthomonas campestris,* lactic acid by *E- coli, Lactobacillus casei,* 2,3-butanediol by *K. pneumonia*, single cell protein by *Trichosporon sp* as a source of carbon for the making valuable products and growth of these microbes [1, 3, 5, 11, 13, 14, 17, 18, 19, 20, 24]. The degradation studies also showed a more complex dependence of carbon sources and growth factors such as B-vitamins, on the degradation of an aromatic compound (phenanthrene) compared to an aliphatic compound (hexadecane) (Jonsson & Östberg, 2011).

Botheju and Bakke [8] and Ghaly et al [12] reported that aeration and addition of nutrients significantly increased the population of the microorganisms, enhanced substrate consumption, prevent pH drops, reduced accumulation of intermediate products, enhanced methane yield, and minimized sulfides content thereby minimizing toxic effect of sulfide. Researchers have also shown that aeration increased the rate of product formation and biomass concentration [5, 13, 21]. Azeez [5] investigated biodegradation kinetics parameters in the production of 2, 3 – butanediol from cheese whey without study the optimal conditions for the process.

The initiation of this research work is to optimize the bioconversion of cheese whey to 2, 3-butanediol using response surface methodology which will give more insight on bioremediation and management of cheese whey as a waste water so as to liberate the environment from the danger of BOD that might be posted on its disposal.

2.0 MATERIALS AND METHOD

2.1. Cheese Whey Used

The cheese whey used for this study was obtained from a local cheese making industry and quantified by Azeez [5] with the proximate composition of Protein (12.90 %), Fat and Oil (2.10 %), Ash (7.95 %), Water (4.54 %) and Lactose (72.50 %).

2.2. Isolation of Biomass Concentration of Klebsiella pneumonia

The method of Azeez [5] was employed to determine the biomass concentration of *Klebsiella pneumonia (K. pneumonia)* and concentration of 2, 3-butanediol (2, 3-BD) produced. The culture of *K. pneumonia* was obtained from Medical Laboratory, Bowen University of Teaching Hospital, Ogbomoso tagged in a slide. Cheese whey broth samples contains 20g/L, 30g/L and 40g/L were prepared, boiled, filtered and oil content was removed and discarded. 150ml of each of the whey broth were autoclaved at 121^0C and 15psia for 15 minutes. The pH of the whey broth was standardized at 6.40 after sterilization. *K. pneumonia* was inoculated into the nutrient agar plates for production of the bacterium enmasse for 24hours and 1ml amounted to 0.05mg/L of *K. pneumonia* inoculated into 20g/L, 30g/L, and 40g/L of the cheese whey at 28^0C for 96 hours in non-aerated batch reactors and air-stirred batch reactors. Samples were taken to determine biomass concentration of *K. pneumonia*. The dry weight analysis method was used to determine biomass concentration of *K. pneumonia* in which 10ml of each of samples of were taken at variable time and centrifuged at a 2500rpm for every 20minutes. The supernatant was decanted into a glass container and *K. pneumonia* cells that settled down at the bottom of the centrifuge tube were scooped and dried in an oven at a temperature of 60^0C for 8hours to a constant weight and recorded. The weight obtained was taken as the dry weight of *K. pneumonia* in the sample analyzed.

2.3. Extraction of 2, 3-Butanediol from spent medium

The method of Azeez [5] was employed to isolate 2,3-BD and *K. pneumonia* cells from spent medium by centrifugation as earlier described. The supernatant were separated from the cells and 5ml of each of supernatant of sample was saturated with sodium chloride salt after chemical assay of 2, 3-BD in fermentation broths using spectrophotometer. 2,3-BD was extracted from saturated supernatant with ethyl acetate of equal volume. The ethyl acetate layer was separated from aqueous layer by separating funnel, then dehydrated with anhydrous Glauber's salt and 2,3-BD with colourless, transparent, sharp taste and syrup in nature was removed under reduced pressure.

2.4. Experimental design

A 3 – level factorial design (3-LFD) with response surface methodology of Design – Expert software version 6.0.8 (2002 East Hennepin ave., Suite 480 Minneapolis, MN 55413, stat Ease, Inc.) was used. The present experimental study consisted of two steps:

(1) The 3-level factorial design (3-LFD) aimed at determining the effects of 2 factors on the bioremediation of cheese whey.

(2) Determining the effect of aeration rate on the biodegradation process of cheese whey spent medium and fermentation time in both unaerated and airlifted batch reactors.

Two factors were considered to perform response surface methodology with 3-LFD: initial concentration of cheese whey (C), and fermentation time (t), with three different levels for each of the factors. The values of the chosen factors were 20 g/L, 30 g/L and 40 g/L for initial Cheese whey concentration and 0, 48 and 96 hours for fermentation time as shown in the Table 1. The range of these values was considered since it characterized the optimum range for the *K. pneumonia* and the expected range in which the process could be operated.

Table 1: Factor Levels of the Independent Variables for Bioremediation

Factors	low Level (-1)	Medium (0)	High Level (+1)
Concentration of Cheese Whey (g/L)	20	30	40
Fermentation time (hr)	0	48	96

All the experiments were done in triplicates and the average of biomass concentration of K. pneumonia (Y_1) and concentration of 2,3-Butanediol (Y_2) production obtained were taken as the response function (Y) of the factors. The Second degree polynomials equation (1) which contains factors with interaction terms were used to calculate the predicted response:

$$Y_i = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y_i is the predicted response of biomass concentration of *K. pneumonia* and concentration of 2, 3-Butanediol as dependent variables; n is the number of independent variables (factors), x_i and x_j are the concentration of cheese whey and fermentation time respectively; ε is the random error; β_0 the constant coefficient, and β_i, β_{ij} and β_{ii} the coefficients of linear, interaction and quadratic term, respectively. Numerical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quadratic models were represented as contour plots (3D) and response surface curves were generated for variables.

3.0 RESULTS AND DISCUSSION

3.1. Model Fitting

In this study, the experimental design consisted of 13 runs and the independent variables were studied at three different levels of the experimental design with the results of 3-LFD of response surface for the experimental and predicted values as shown in the Table 2.

The two responses (Biomass concentration of *K. pneumonia* and Concentration of 2,3-BD production) were correlated with the two factors (Fermentation time and concentration of cheese whey) using the second-order polynomial, as represented by Eq. (1).

Table 2: 3-LFD Experimental Value for Optimization of Two factors (Each on 3 Level) for the Biomass of 2 K. pneumonia and 2,3-BD Production

			Airlifted batch reactors				Unaerated batch reactors			
			Biomass of *K. pneumonia*		2,3-BD		Biomass of *K. pneumonia*		2,3-BD	
Run	t (hr)	C (g/L)	Actual Value	Predicted Value	Actual Value	Predicted Value	Actual Value	Predicted Value	Actual Value	Predicted Value
1	48	30	1.85	1.85034483	7.65	7.6506897	0.05	0.0370977	0	-0.145402
2	48	30	1.85	1.85034483	7.65	7.6506897	0.7	0.7003448	4.62	4.5903448
3	0	30	2.68	2.67747126	13.31	13.308276	1.13	1.1274713	7	7.2174713
4	96	40	1.2	1.32413793	4.71	5.1782759	0.8	0.7887644	4.23	3.9412644
5	48	40	1.75	1.68876437	8.83	8.5958621	0.05	0.0508046	4.62	4.5903448
6	48	30	2.5	2.37413793	10.6	10.128276	0.7	0.7003448	4.62	4.5903448
7	96	30	0.05	-0.0129023	0	-0.234138	0.7	0.7003448	4.62	4.5903448
8	96	20	1.85	1.85034483	7.65	7.6506897	0.05	0.0620977	0	0.2145977
9	48	30	1.85	1.85034483	7.65	7.6506897	0.7	0.7003448	4.62	4.5903448
10	0	20	0.05	0.05080460	0	-0.001724	0.7	0.7003448	4.62	4.5903448
11	48	30	0.05	0.11209770	0	0.2358621	0.9	0.8741379	6.76	6.4741379
12	48	20	1.85	1.85034483	7.65	7.6506897	0.5	0.5241379	2.48	2.9141379
13	0	40	3.6	3.66376436	17.79	18.025862	1.45	1.4637644	10.63	10.701264

From the experimental data, quadratic regression models were obtained as shown in Equations (2), (3), (4) and (5) for production of biomass of *K. pneumonia* and 2,3-BD in unaerated and airlifted batch processes:

Biomass concentration (mg/L) in unaerated batch process:

$$Y_1 = 2.4425x10^{-3} + 1.9741x10^{-3}C + 5.6926x10^{-3}t - 1.2069x10^{-5}C^2 - 4.8267x10^{-5}t^2 + 3.3854x10^{-4}Ct \quad (2)$$

Concentration of 2,3-BD in unaerated batch process:

$$Y_2 = 0.3249 + 0.044276C + 0.018245t - 1.0379x10^{-3}C^2 - 4.4106x10^{-4}t^2 + 3.333x10^{-3}Ct \quad (3)$$

Biomass concentration in airlifted batch process:

$$Y_1 = -0.14756 + 6.9741x10^{-4}C + 0.018713t - 1.2069x10^{-5}C^2 - 2.1103x10^{-4}t^2 + 9.6354x10^{-4}Ct \quad (4)$$

Concentration of 2,3-BD in airlifted batch process:

$$Y_2 = -0.68345 + 0.021948C + 0.040205t + 2.5862x10^{-5}C^2 - 4.3291x10^{-4}t^2 + 4.6667x10^{-3}Ct \quad (5)$$

The p-value was used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables [22, 26]. P-value less than 0.05 indicated that significant model terms are function of the response surface (Y).

The significant model terms are C, t, C^2, t^2 and Ct for biomass production in the processes due to $p < 0.05$ as presented in Table 3. In the two processes, C^2 is the only insignificant model term for 2, 3 – butanediol production while other model terms are significant. The mathematical model reduces to (6), (7), (8) and (9) by truncation of insignificant terms with $p > 0.05$:

Biomass concentration (mg/L) in unaerated batch process:

$$Y_1 = 2.4425x10^{-3} + 1.9741x10^{-3}C + 5.6926x10^{-3}t - 1.2069x10^{-5}C^2 - 4.8267x10^{-5}t^2 + 3.3854x10^{-4}Ct \quad (6)$$

Concentration of 2,3-BD in unaerated batch process:

$$Y_2 = 0.3249 + 0.044276C + 0.018245t - 4.4106x10^{-4}t^2 + 3.333x10^{-3}Ct \quad (7)$$

Biomass concentration in airlifted batch process:

$$Y_1 = -0.14756 + 6.9741x10^{-4}C + 0.018713t - 1.2069x10^{-5}C^2 - 2.1103x10^{-4}t^2 + 9.6354x10^{-4}Ct \quad (8)$$

Concentration of 2, 3-BD in airlifted batch process:

$$Y_2 = -0.68345 + 0.021948C + 0.040205t - 4.3291x10^{-4}t^2 + 4.6667x10^{-3}Ct \quad (9)$$

Models (6), (7), (8) and (9) can be used to navigate design process for respective cases due to "Adeq precision" which measures the signal to the noise ratio as the magnitude greater than 4 in all cases presented in the Table 3.

Table 3: Model Coefficient Estimated by Linear Regression

	Biomass Concentration			2,3-BD Concentration		
Model Term	Coefficient	F - Value	P > F	Coefficient	F - Value	P > F
Unaerated batch reactors						
constant	0.00244253	1534.12122	< 0.0001	0.32494253	325.1154	< 0.0001
C	0.00197414	681.405084	< 0.0001	-0.04427586	275.9057	< 0.0001
t	0.00569265	6448.10078	< 0.0001	0.01824473	1155.895	< 0.0001
C^2	-1.207E-05	22.745	0.0020377	0.0010379	3.7642	0.09351
t^2	-4.827E-05	126.663115	< 0.0001	-0.00044106	41.39447	0.00036
Ct	0.00033854	391.692038	< 0.0001	0.00333333	148.6173	< 0.0001
Airlifted batch reactors						
constant	-0.1475575	406.782856	< 0.0001	-0.68344828	688.1856	< 0.0001
C	0.00697414	246.893405	< 0.0001	0.02194828	388.2142	< 0.0001
t	0.01871348	1545.04387	< 0.0001	0.04020474	2806.837	< 0.0001
C^2	-1.207E-05	16.7640	0.00461	2.5862E-05	4.8600	0.063305
t^2	-0.000211	97.4743977	< 0.0001	-0.00043291	29.02213	0.00102
Ct	0.00096354	127.738878	< 0.0001	0.00466667	211.9951	< 0.0001

The ANOVA of the quadratic regression model presented in Table 4 shows that the model was highly significant in both unaerated and airlifted batch processes for biomass of *K. pneumonia* production and production of 2, 3-BD with the F-value for the model was 1534.12 and 325.12, and 406.78 and 688.19 respectively. The probability of all the cases of response is less than (< 0.0001) which indicated that the models are significant ($p < 0.05$) as seen in the Table 4.

Fitness of the model was determined by the regression coefficient (R^2) which was 0.9999 for biomass production and 0.9957 for 2, 3-BD production in an unaerated batch reactors, and 0.9966 for biomass production and 0.9980 for 2,3-BD production in airlifted batch reactors as illustrated in the Figure 1a, b, 2a and b.

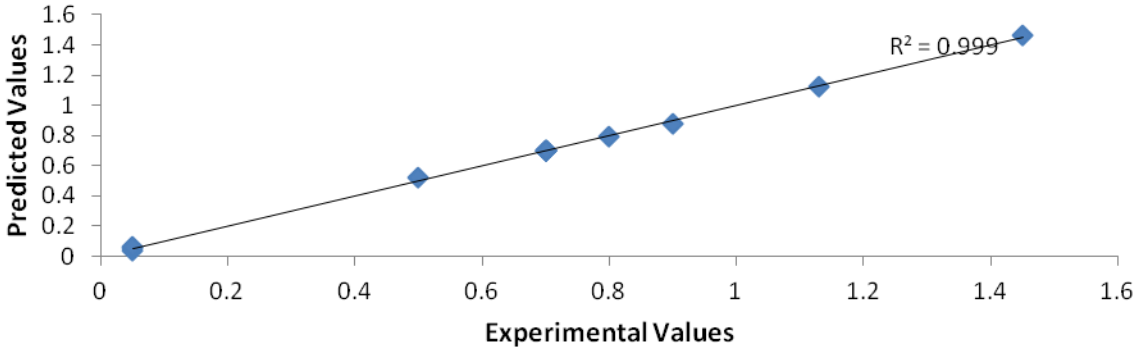

Figure 1a: Biomass Response surface Plot of Predicted against Experimental Values for unaerated Batch Process

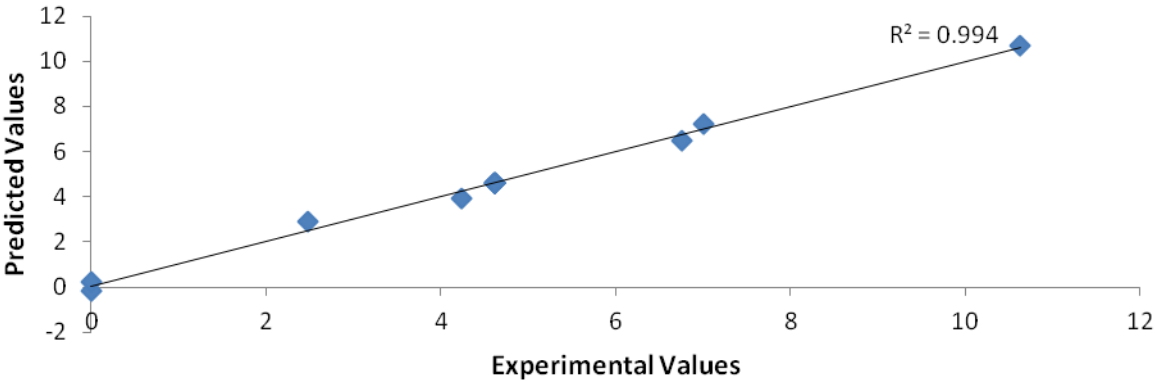

Figure 1b: 2, 3 - Butanediol Response surface Plot of Predicted against Experimental Values for unaerated Batch Process

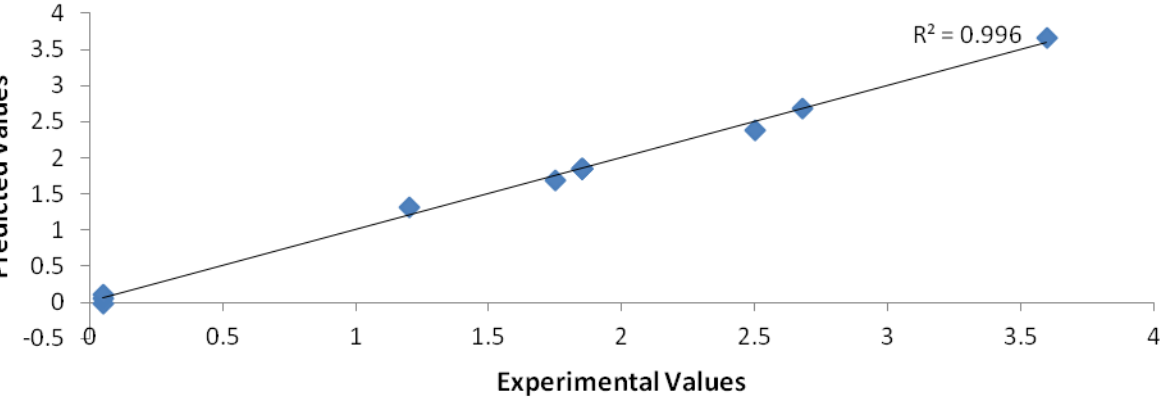

Figure 2a: Biomass Response surface Plot of Predicted against Experimental Values for airlifted Batch Process

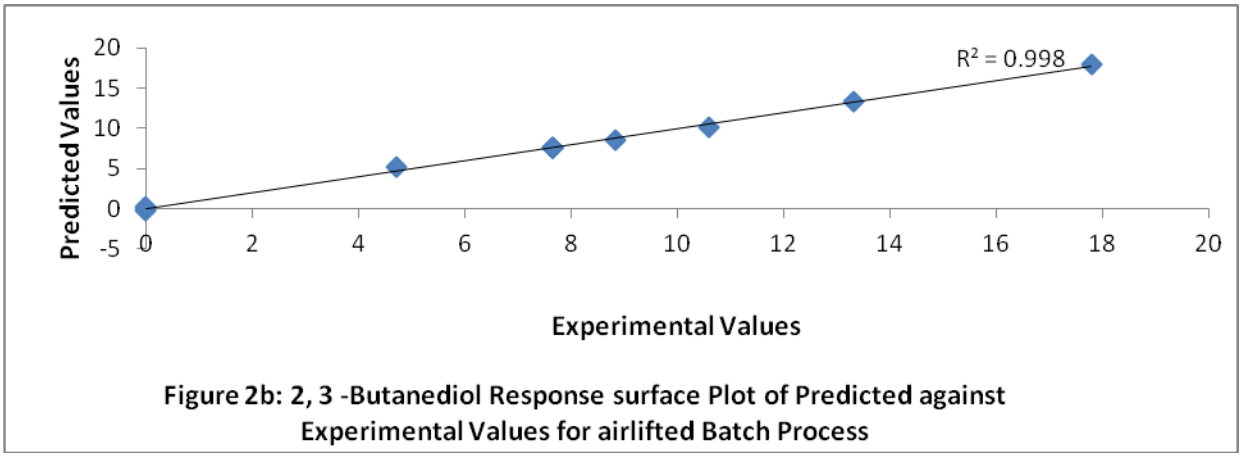

Figure 2b: 2, 3 -Butanediol Response surface Plot of Predicted against Experimental Values for airlifted Batch Process

More so, the model in each processes are in good agreement with the predicted model due to closed magnitude of the "pred R^{2}" and "Adj R^{2}" as shown in the Table 4.

Table 4: Analysis of Variance (ANOVA) for quadratic Model

Source	sum of square	Degree of freedom	Mean Square	F-value	P>F	R^2	Adj R^2	Pred R^2	Adeq Precision
Biomass production of *K. pneumonia* in unaerated batch reactors									
Model	2.068	5	0.4137	1534.12	< 0.0001	0.999	0.9984	0.9907	127.881
Residual	0.002	7	0.00027						
Lack of Fit	0.002	3	0.00063						
Pure error	0	4	0						
Total	2.07	12							
Biomass production of *K. pneumonia* in airlifted batch reactors									
Model	13.62	5	2.7247	406.78	< 0.0001	0.996	0.9941	0.9651	66.126
Residual	0.047	7	0.0067						
Lack of Fit	0.047	3	0.01563						
Pure error	0	4	0						
Total	13.67	12							
2,3-Butanediol production in unaerated batch reactors									
Model	112	5	22.401	325.12	< 0.0001	0.994	0.9927	0.9573	60.824
Residual	0.482	7	0.0689						
Lack of Fit	0.482	3	0.16077						
Pure error	0	4	0						
Total	112.5	12							
2,3-Butanediol production in airlifted batch reactors									
Model	325.8	5	65.1532	688.19	< 0.0001	0.998	0.9965	0.9793	87.354
Residual	0.663	7	0.09467						
Lack of Fit	0.663	3	0.22091						
Pure error	0	4	0						
Total	326.4	12							

Figure 3a, b, 4a and b shows the 3-D response surface plots for unaerated and airlifted batch processes as representation of the regression equation used to visualize the relationship between the response and experimental levels of each factor. As shown in these plots, increased biomass of *K. pneumonia* production was observed with increased utilization of cheese whey as a substrate and increased bioremediation time for both processes.

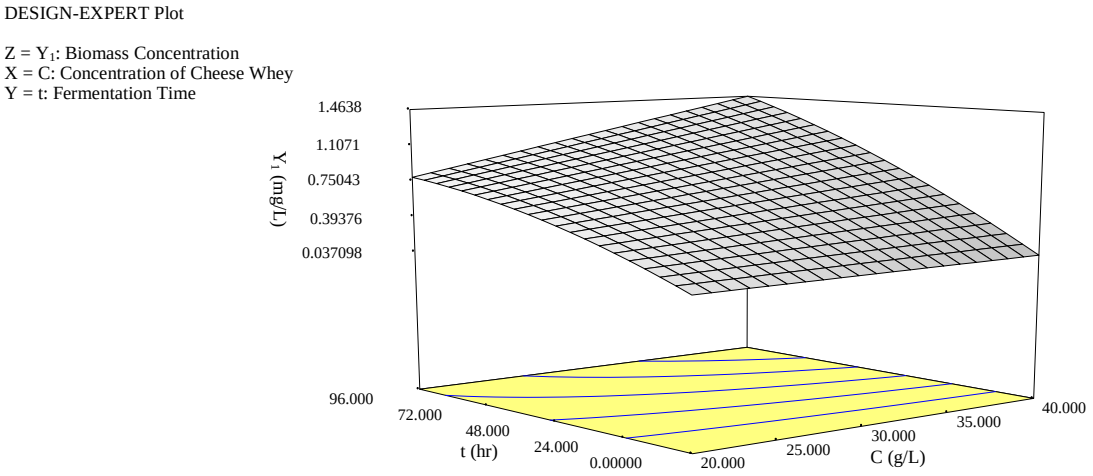

Figure 3a: Biomass response of cheese whey concentration and fermentation time in unaerated batch reactors

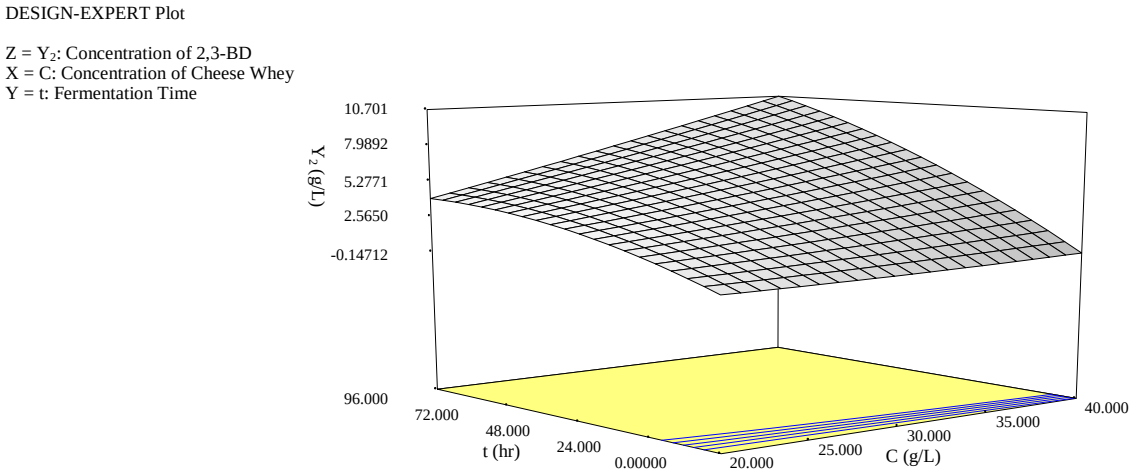

Figure 3b: 2, 3 - BD response of cheese whey concentration and fermentation time in unaerated batch reactors

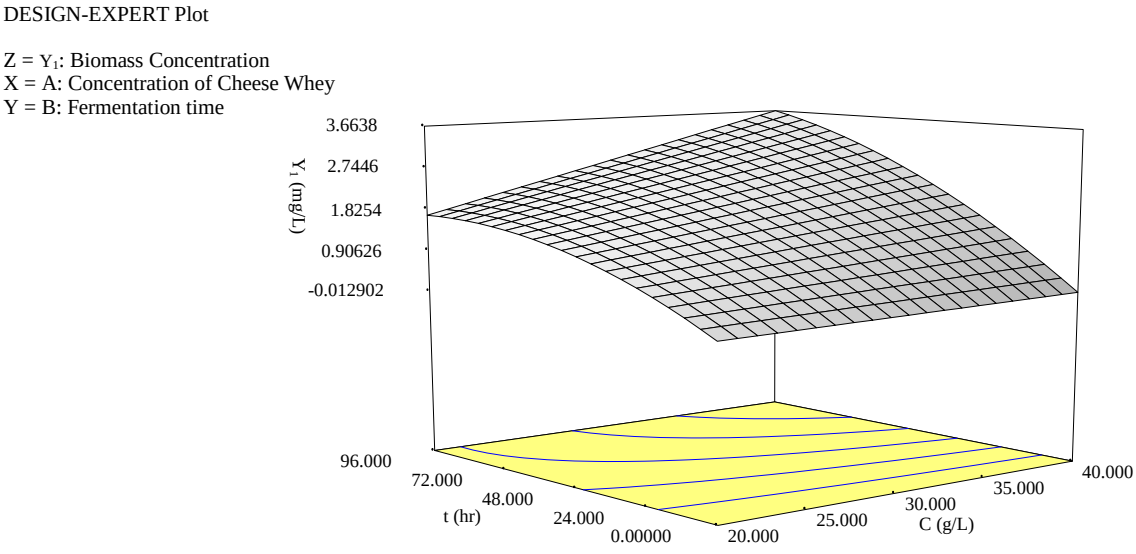

Figure 4a: Biomass response of cheese whey concentration and fermentation time in airlifted batch reactors

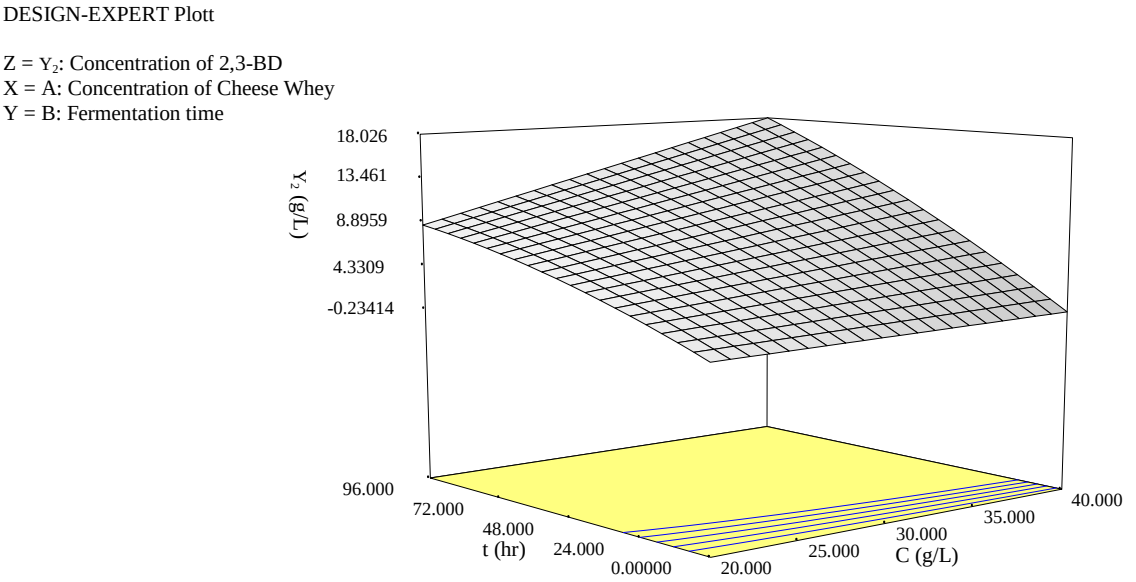

Figure 4b: 2, 3 - BD response of cheese whey concentration and fermentation time in airlifted batch reactors

The optimal conditions for production of biomass of *K. pneumonia* was 39.98 g/L of cheese whey utilized and 95.5 hours for unaerated batch process and 39.94 g/L of cheese whey utilized and 95.5 hours for airlifted batch process. The optimum yield of k. pneumonia was 1.4633mg/L and 3.6580mg/L for unaerated and airlifted batch process respectively. Figure 3b and 4b show the effect of the factors on the 2, 3-BD production which represents the response surface performance as a function of concentration of cheese whey and fermentation time. The optimal production of 2, 3 - BD was 10.696 and 17.997g/L which indicated 36.88 and 62.06 percent of lactose utilized in an unaerated and airlifted batch process as presented in the Table 5. The optimization result is closer to experimental result reported by Azeez [5] in which about 36.6 and 61.3 percent of lactose utilized in unaerated and aerated

condition respectively. With increased lactose level of 20 to 40 g/L, biomass yield and 2, 3 – BD production increases due to favourable conditions of the *K. pneumonia* and high concentration of cheese whey utilized and production of 2, 3 – BD in airlifted batch reactors was due to enhanced effect of aerated conditions of *K. pneumonia*.

Table 5: Optimal Solution for Bioconversion of Cheese whey to 2, 3-BD

Process	Fermentation time	Cheese whey Concentration	Biomass concentration of K. Pneumonia	concentration of 2,3-BD
Airlifted batch	95.5	39.94	3.6580	17.997
Unaerated batch	95.7	39.98	1.4633	10.696

The optimal region for factors of biomass *K. pneumonia* and 2, 3 – BD response using were shown in the overlay plot of Figure 5 and 6 for unaerated batch and airlifted batch reactors respectively.

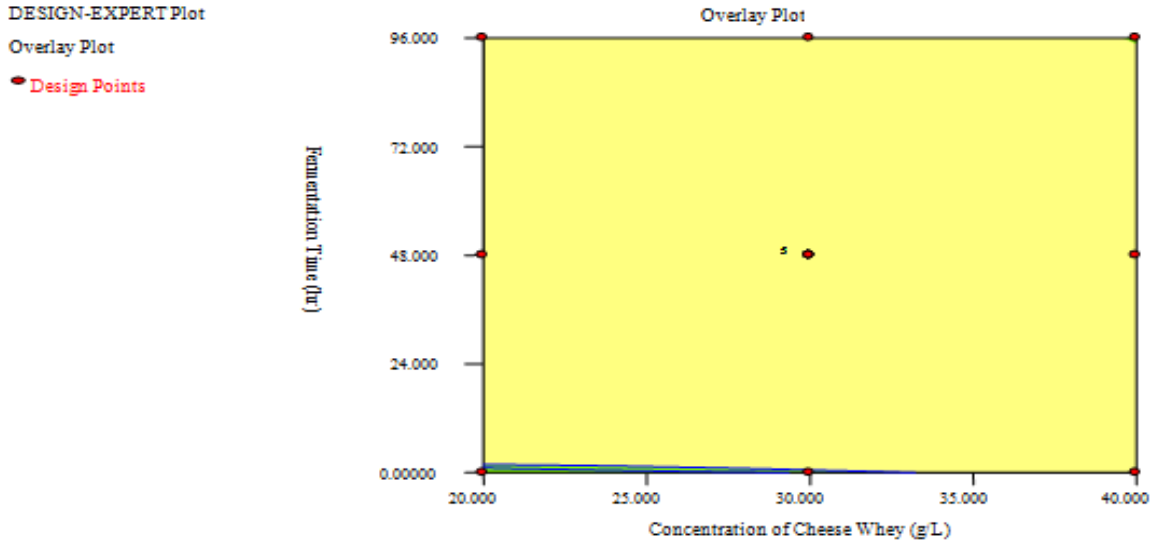

Figure 5: Overlay plot of factors of response in unaerated batch reactors

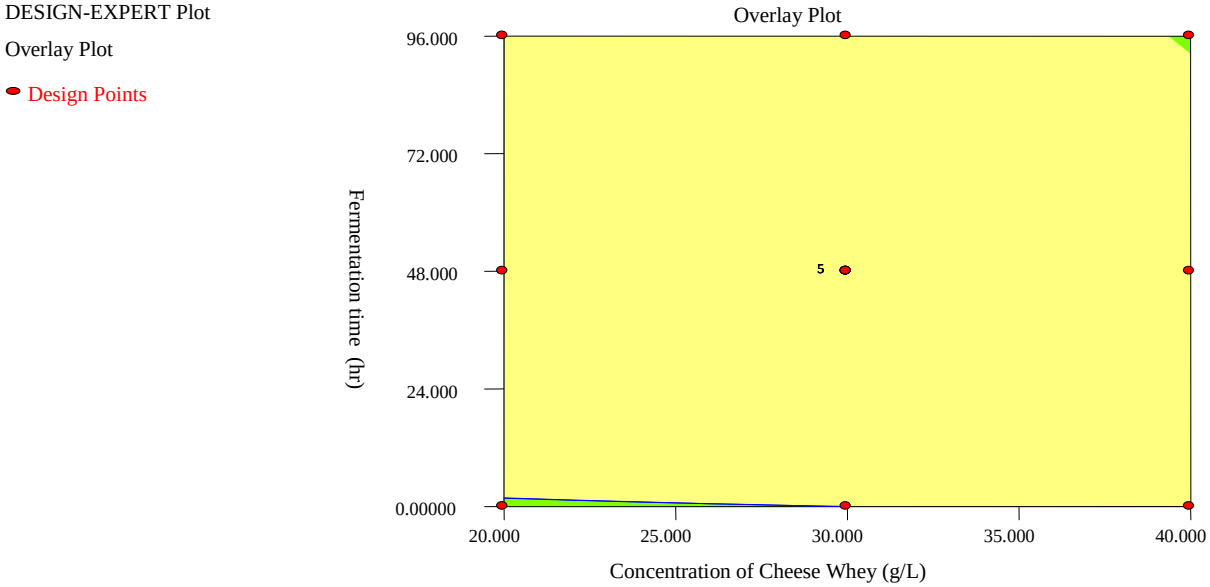

Figure 6: Overlay plot of factors of response in airlifted batch reactors

CONCLUSIONS

3-level factorial design and response surface methodology can be used for the purpose of finding the maximum production of 2, 3- butanediol by *K. pneumonia*. This design based on the analysis of 13 experiments, involving the anaerobic fermentation of lactose contained in cheese whey, was performed. The effects of two factors fermentation time and initial lactose concentration were estimated. After the ANOVA test on the complete quadratic model, all the negligible effects were removed in order to improve the model predictive performance. A response surface quadratic model was obtained as a function of the only significant effects of C, t, t^2 and Ct. High magnitudes of an adjusted R^2 value testified a good model correlation performance for both unaerated and aerated conditions. The optimization showed that the best set of operating parameters to operate the fermenter at room temperature of 28^0C for pH of 6.40 was 40 g/l for initial lactose concentration and 0.05mg/l for *K. pneumonia* concentration. The effect of aeration rate on the fermentation process based on the variation of 2, 3-butanediol, lactose and cell (biomass) concentration with time in the unaerated batch reactors and airlift bioreactor. The results show that the best condition was the aeration rate of butanediol production of 62.06 percent in concentrated cheese whey at the optimal conditions with 100 g/L lactose utilized. This study clearly shows that 3-LFD is undoubtedly a good technique for studying the effect of major process parameters on response factor by significantly reducing the number of experiments in the batch bioremediation process.

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