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Statistical Analysis of Experimental Variables for the Production of Lactic acid using Lactobacillus casei from Waste Potato Starch by Box-Behnken Design

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Abstract: Production of lactic acid is studied from potato waste using bacterial species *Lactobacillus casei*. α-amylase and glucoamylase are used with 1:1 ratio as enzyme mixture in this investigation for simultaneous saccharification and fermentation. Amount of potato waste, enzyme mixture concentration, yeast extract concentration, NH_4Cl and inoculum size are the experimental variables used in this investigation to study their effect on the production of lactic acid. All sets of experiments are carried out at 37°C. Their effect is analyzed statistically by using Box-Behnken Design. The linear, squared and interaction effect of these experimental variables on the production of lactic acid are explained. The linear effect of potato waste substrate, enzyme mixture, yeast extract and NH_4Cl are found to be highly significant on the production of lactic acid than the effect of inoculum size. Similarly the squared effect of potato waste substrate, enzyme mixture, yeast extract and NH_4Cl are found to be highly significant on the production of lactic acid than the effect of inoculum size. The interaction effect of yeast extract and NH_4Cl is alone found to be significant on the production of lactic acid based on the *P* value. The second order polynomial model developed by Box-Behnken Design is highly significant and adequate to represent the actual relationship between the response and the experimental variables. The regression coefficient of this model 0.9785 and the normal probability plot ensure the same. The experimental and predicted values of Lactic acid production is compared and found to concur with one another.
Keywords: Lactic acid, *Lactobacillus casei*, potato waste substrate, α-amylase and glucoamylase, Box-Behnken Design.

INTRODUCTION

Food and agro based industries produce large volumes of wastes. Consequently, disposal and pollution problems are increased. Also a great loss of valuable biomass and nutrients are resulted. Effective utilization, recycling and reprocessing of these waste can be beneficial uses rather than their discharge to the environment which might cause harmful environment effects[1].

In many cases, these food residues have a good potential for conversation into useful by-

product, or a raw material for other product. Organic acid is an example of such a valuable by-product, through the fermentation of high carbohydrate containing industrial substrates[2,3]. For instance, potato processing plant releases an appreciable amount of starch in wastewater streams. Also potatoes, which do not fit the standard quality criterion, are discarded directly. This could be utilized as a cheap substrate for microorganisms to produce high value of organic acid like lactic acid.

Lactic acid (2-hydroxypropionic acid, CH3CHOHCOOH) is the most widely utilized multifunctional organic acid, of which 85% is used in food and food related applications[4,5]. Lactic acid occurs naturally as two optical isomers, D-(−)-and L-(+)-lactic acids. Since elevated levels of the D-isomer are harmful to humans, L-(+)-lactic acid is the preferred isomer in the food and pharmaceutical industries[6, 7, 8]. One of its most promising applications is its use for the manufacture of biodegradable and biocompatible polylactate polymers, an environmentally friendly alternative to non-biodegradable plastics derived from petrochemicals[4,9,10,11]. Also lactic acid is used as an ingredient, intermediate and feedstock in food processing and beverages industries. Since it possesses excellent biomedical applications, it has increasing demand in Food, Pharmaceutical and Chemical Industries and for production of Poly lactic acid polymers. Due to the large potential demand for lactic acid, many large corporations have been involved in the production and process development for lactic acid.

Lactic acid is currently manufactured either through chemical or microbial route via fermentative mode. The chemical production of lactic acid always results in a mixture of the two isomers, while fermentation can yield either form alone, or a mixture of the two isomers in different proportions, depending on the microorganism, substrate and growth conditions used[6,12,13]. Another significant advantage over chemical synthesis is that biological production can use cheap raw materials, such as whey, molasses, starch waste, beet- and cane-sugar and other carbohydrate rich materials[12,14,15,16]. In commercial processes, sugars and starches have been widely used as substrates for the biological production of lactic acid. The global production of this organic acid is estimated to be 100 million pounds/year and is expected to grow by 8.6% annually[17]. In India, the annual production capacity of lactic acid is 6000 T and an estimated gap of 2300 T in supply by the year 2015 is predicted, if the present level of production is not increased. Wastes containing starch generated from food processing plants may be regarded as a viable option for meeting this growing demand for lactic acid, if appropriate biotechnological experiments are used. A sustainable approach for production of lactic acid would include utilization of starch containing agro wastes through a fermentative mode by Lactic acid bacteria. Lactic acid bacteria are among the best-studied microorganisms for human health beneficial effects and for fermentation. Progress has also been made in the construction of food grade genetically modified Lactic acid bacteria. The desirable characteristics of the microorganisms for industrial use are their ability to rapidly and completely ferment cheap raw materials, requiring minimal amount of nitrogenous substances.

In two stage hydrolysis and fermentation, the starch substrate is treated first with α-amylase for liquefaction and then it is treated again with glucoamylase for saccharification at elevated temperature for faster liberation of hexoses[18]. But the liberation of hexoses is slower in simultaneous saccharification and fermentation using the enzyme mixture of α-amylase and glucoamylase. Although it is slower, the conversion of the liberated hexoses to lactic acid is faster than the two step fermentation. Hence the negative influence of reducing sugar is nullified and the yield of lactic acid is increased.

Lactic acid production is carried out using fungal as well as bacterial cultures but simultaneous saccharification and fermentation by bacterial system for the production of Lactic acid is limited in literature[19]. The ability of Lactobacillus species to transform a wide range of carbohydrates to lactic acid is well known[20,21,22,23,24,25,26,27,28,29,30,31,32,33,34]. Minimal supplementation of inorganic nutrients to inexpensive substrates like whey, cellulose, cassava powder, rice straw and starchy would increase lactic acid production[11,35,36,37]. Potato waste is found to be the best solid substrate by simultaneous saccharification and fermentation for production of L (+) Lactic acid.

In this present investigation, an attempt is made to study the ability of lactic acid producing bacteria *Lactobacillus casei* by simultaneous saccharification and fermentation. It is a fastidious bacterium. Potato waste is used as the substrate. The enzymes α-amylase and glucoamylase are used in this investigation. The effect of amount potato waste substrate, concentration of enzyme mixture, yeast extract, NH_4Cl and inoculum size on the production of lactic acid is analyzed statistically. Box-Behnken Design (BBD) is employed for the statistical analysis.

MATERIALS AND METHODS

MICROORGANISM AND INOCULUM PREPARATION

Lactic acid producing culture *Lactobacillus casei* (MTCC 1423) National Collection of Industrial Microorganism, National Chemicals Laboratory, Pune, India is used in this present study. MRS agar medium is used to maintain the culture and subcultured in every two weeks. A temperature of 37°C is used for inoculum preparation[18]. Lactobacillus MRS Agar is recommended for cultivation of all Lactobacillus species. The composition is given below in Table 1.

Table 1: The composition of MRS Medium

Ingredients	gm/L
Protease peptone	10
Beef extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2
Agar	12
Final pH (at 25°C)	6.5±0.2

ENZYMES

Commercial amylases, α-amylase (2000 IU/mL) and glucoamylase (4000 IU/mL) (National Scientific Suppliers, India) are used for hydrolysis of potato starch.

FERMENTATION MEDIUM

The medium consisted of potato waste in distilled water enriched with yeast extract and $NH_4Cl.CaCO_3$ (60%, w/w of starch) is added for burring. The medium is autoclaved at 121°C for 15 min and the enzymes are added to the medium along with the inoculum (24-hr-old). The inoculated flaks are incubated at 37°C for 60 hr.

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION WITH BOX-BEHNKEN DESIGN (BBD)

Effect of potato waste concentration, enzyme mixture concentration, yeast extract concentration, NH_4Cl concentration and inoculum size are studied on the effect of lactic acid production. Box-Behnken Design[38] (BBD) is chosen to analyze statistically and to develop the second order polynomial model for the effects these five variables on the production of lactic acid since it requires reduced number of actual experiments without significant loss of information. Also, it is a good statistical tool in response surface methodology because it permits: (i) estimation of the parameters of the quadratic model, (ii) building of sequential design, (iii) determination of lack of fit of the model and (iv) use of blocks. This second order polynomial model demonstrates the rapport between potato waste, enzyme mixture, yeast extract, NH_4Cl and inoculum concentration on lactic acid production.

The number of experiments (n) required for the development of BBD is defined as $n = 2k (k - 1) + C_o$ where k is the number of experimental variables and C_o is the number of experiments repeated at the center point ($k = 5$; $C_o = 6$). As a result, a total of 46 set of experiments have to be performed. All other experimental conditions are kept constant during the experiments and the runs are randomized to exclude any bias.

Potato waste, enzyme mixture, yeast extract, NH_4Cl and inoculum concentration are the independent variables studied in the experimentation of production of lactic acid. These five variables are tested at different levels by associated plus signs (+1) with high levels, zero (0) indicating centre value and minus signs (−1) with low levels. Table 2 shows the levels and coded values of independent variables used in the experimental design for production of lactic acid.

For statistical calculation, the variables are coded according to the following Equation 1, where Z_j is the coded value of the independent variable, X_i is its real value, X_o is its real value at the center point and Δ_j is the step change in the variable X_i. Table 3 shows the experimental design and response value for the production of lactic acid.

$$Z_j = \frac{X_i - X_o}{\Delta_j} \quad i = 1, 2, 3, 4, 5 \text{ ----- (1)}$$

The second-order polynomial regression model is given as Equation 2 to express Y as a function of the independent variables as follows whereas β_o is a constant, while β_i, β_{ii} and β_{ij} are the linear, quadratic and interactive coefficients respectively. X_i and X_j are the levels of the independent variables.

$$Y = \beta_O + \sum_{i=1}^{5}\beta_i X_i + \sum_{i=1}^{5}\beta_{ii} X_i^2 + \sum\sum_{\substack{i=1 \\ i<j}}^{5}\beta_{ij} X_i X_j \quad \text{----- (2)}$$

Table 2: Parameter levels and coded values used in the experimental design for the production of Lactic acid

Factors	Symbol	Range and Level		
		-1	0	+1
Potato Waste (g/L)	PW	50	75	100
Enzyme Mixture (mL/L)	EM	5	10	15
Yeast Extract (g/L)	YE	4	8	12
Ammonium Chloride NH_4Cl (g/L)	AC	1	3	5
Inoculum Size (CFU×10^9/100mL)	IS	4	6	8

The accuracy and ability of the above polynomial model could be evaluated by the coefficient of determination R^2 and *F*-test. The significance of the regression coefficient is tested by Student's *t*-test. The 'MINITABTM' (version 15) software is used for regression analysis of experimental data and response.

ANALYSIS

Samples are withdrawn after 60 h of incubation period and treated with 1 M H_2SO_4 to release the lactic acid from medium as it is formed as calcium lactate with buffering agent, CaCO3. Lactic acid extracted out from medium and the extract is diluted to the required level with distilled water and the amount of total lactic acid is estimated according to the colorimetric method of Barker and Summerson[39] and is expressed as mg/mL of the fermentation medium. The amount of reducing sugar is determined by the 3, 5 dinitro salicylic acid method[40]. Starch is estimated by Nampoothiri et al.,[41] description using aqueous iodine solution as reagent. The color development is measured using UV spectrophotometer (Elico Limited) at 620 nm

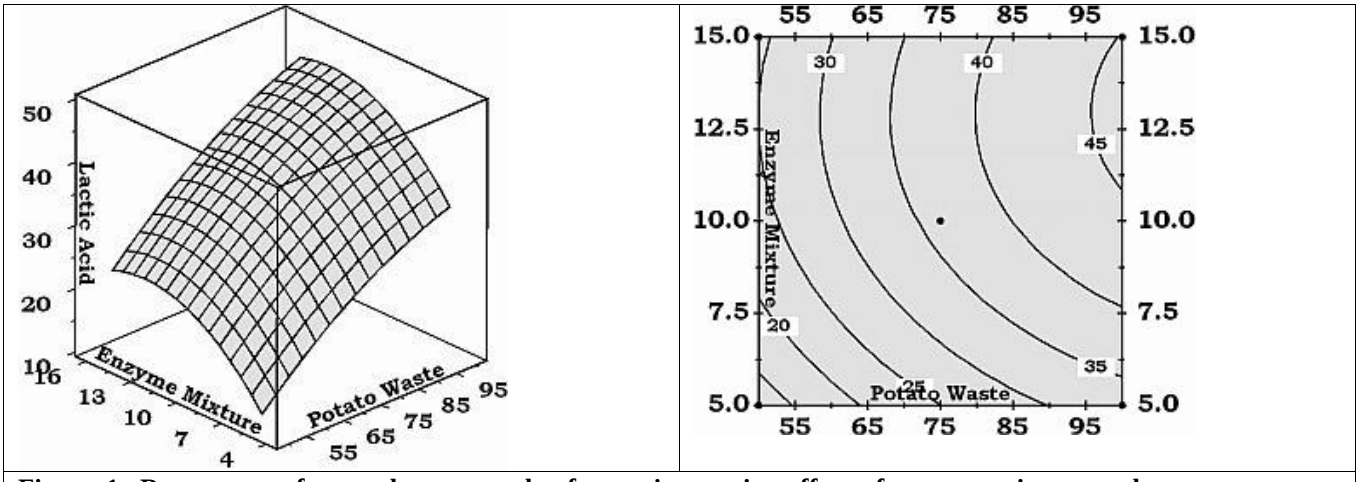

Figure 1: Response surface and contour plot for an interactive effect of enzyme mixture and potato waste on production of lactic acid

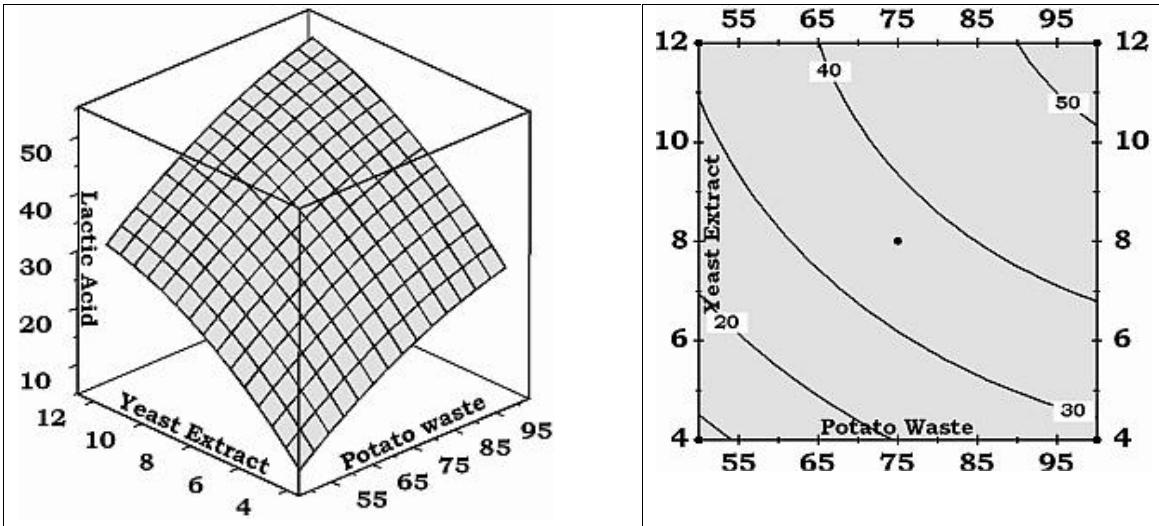

Figure 2: Response surface and contour plot for an interactive effect of yeast extract and potato waste on production of lactic acid

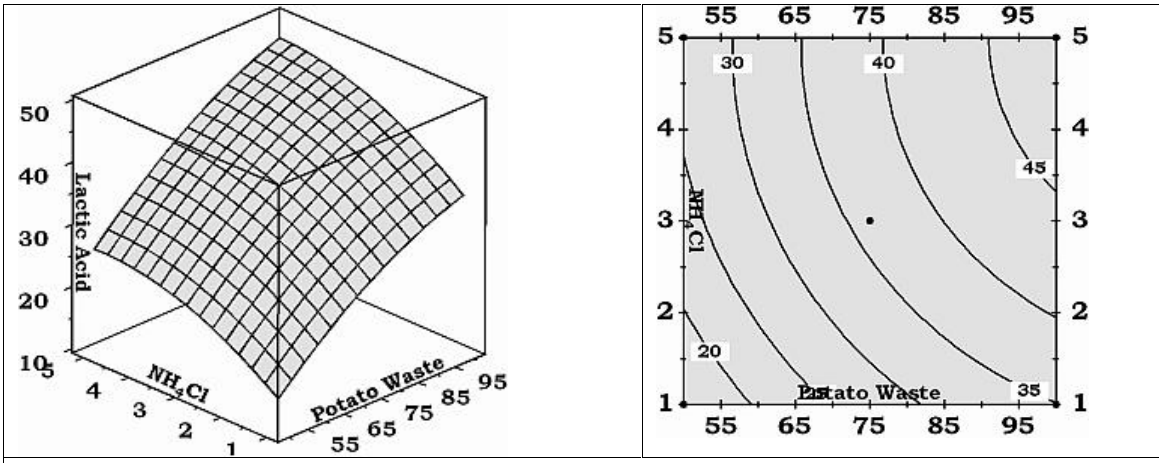

Figure 3: Response surface and contour plot for an interactive effect of NH_4Cl and potato waste on production of lactic acid

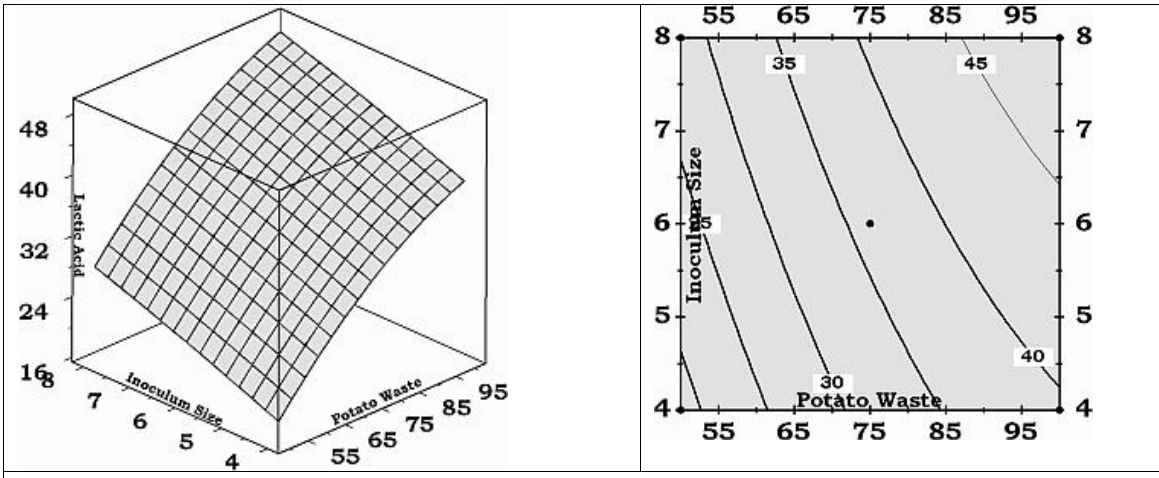

Figure 4: Response surface and contour plot for an interactive effect of inoculum size and potato waste on production of lactic acid

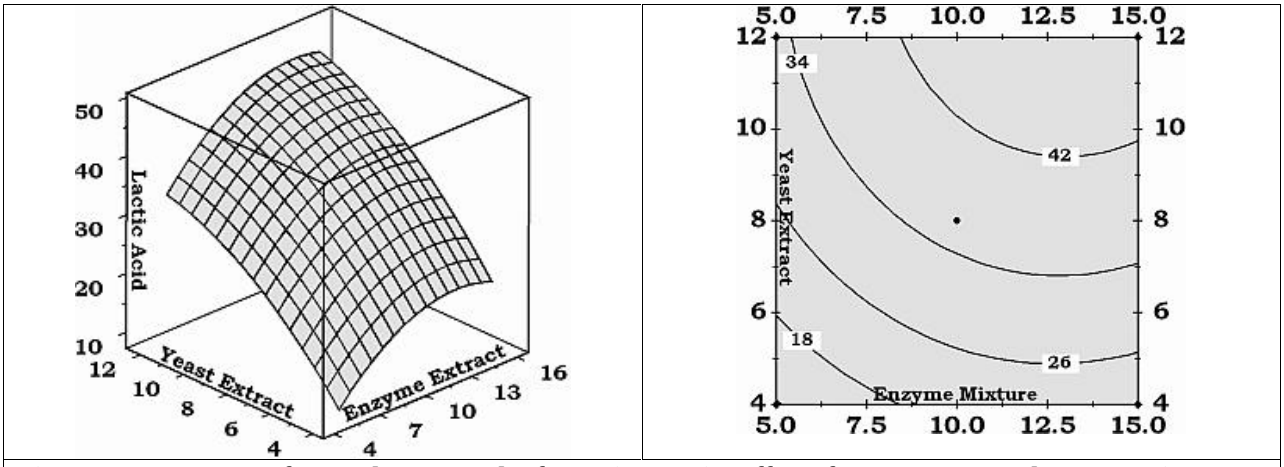

Figure 5: Response surface and contour plot for an interactive effect of yeast extract and enzyme mixture on production of lactic acid

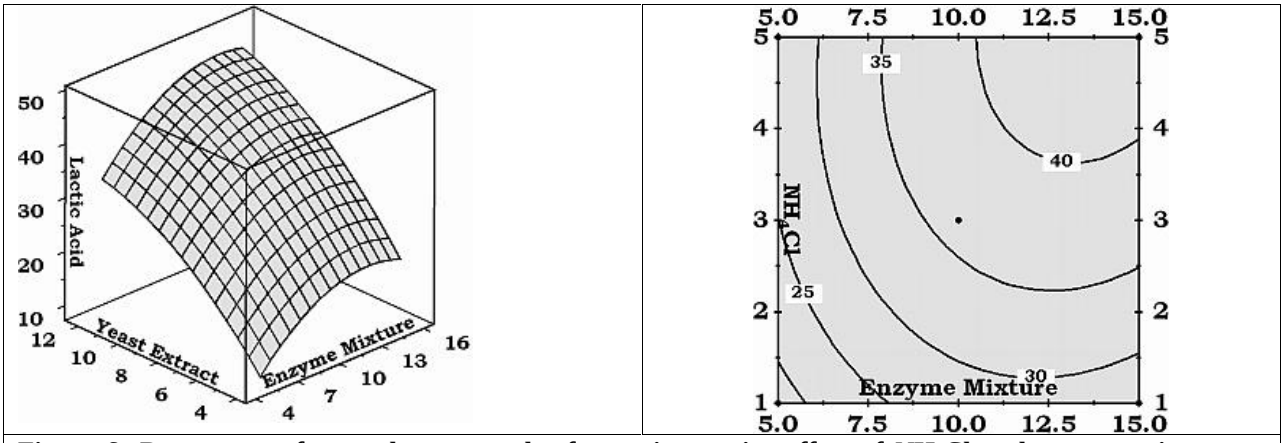

Figure 6: Response surface and contour plot for an interactive effect of NH_4Cl and enzyme mixture on production of lactic acid

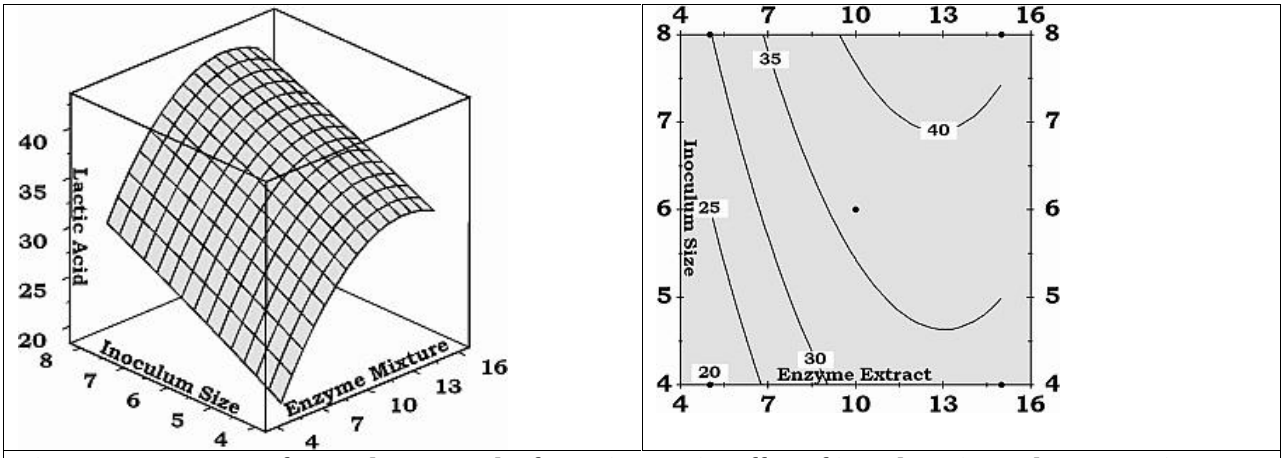

Figure 7: Response surface and contour plot for an interactive effect of inoculum size and enzyme mixture on production of lactic acid

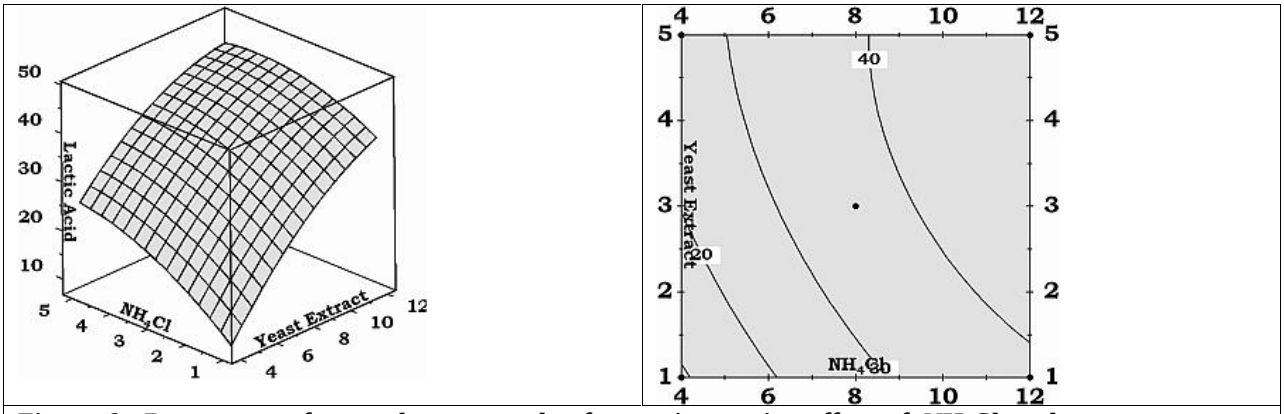

Figure 8: Response surface and contour plot for an interactive effect of NH_4Cl and yeast extract on production of lactic acid

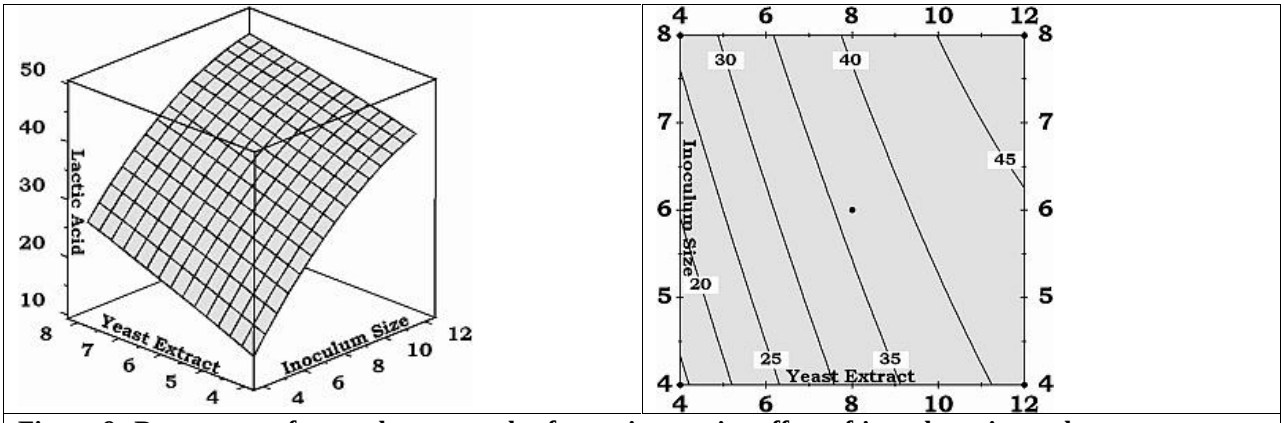

Figure 9: Response surface and contour plot for an interactive effect of inoculum size and yeast extract on production of lactic acid

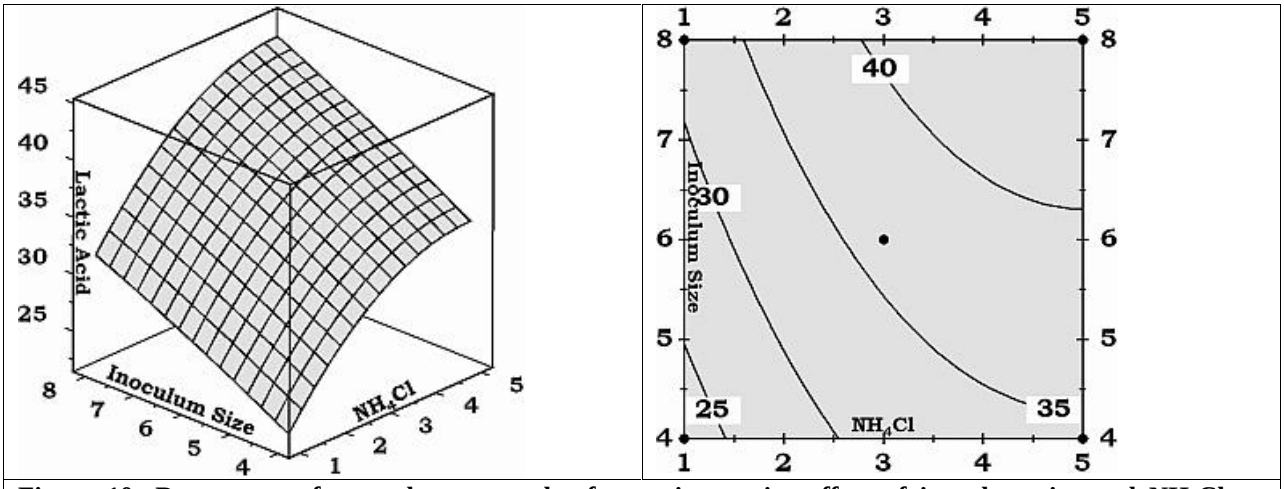

Figure 10: Response surface and contour plot for an interactive effect of inoculum size and NH_4Cl on production of lactic acid

Table 3: Experimental design and response value for the production of Lactic acid

Run	Blk	Coded values					Uncoded values					Lactic acid Production
		PW	EM	YE	AC	IS	PW	EM	YE	AC	IS	
01	1	+	0	0	+	0	100	10	8	5	6	46.23
02	1	0	+	-	0	0	75	15	4	3	6	23.00
03	1	+	+	0	0	0	100	15	8	3	6	44.82
04	1	0	0	0	0	0	75	10	8	3	6	38.13
05	1	0	0	+	-	0	75	10	12	1	6	40.00
06	1	0	0	+	+	0	75	10	12	5	6	46.00
07	1	0	0	0	+	-	75	10	8	5	4	35.87
08	1	0	0	0	0	0	75	10	8	3	6	35.33
09	1	-	+	0	0	0	50	15	8	3	6	24.24
10	1	0	0	0	0	0	75	10	8	3	6	36.32
11	1	0	+	0	0	-	75	15	8	3	4	33.85
12	1	+	0	-	0	0	100	10	4	3	6	28.00
13	1	0	0	0	+	+	75	10	8	5	8	44.93
14	1	+	-	0	0	0	100	5	8	3	6	31.23
15	1	-	0	0	0	-	50	10	8	3	4	18.47
16	1	0	-	-	0	0	75	5	4	3	6	10.00
17	1	-	0	0	+	0	50	10	8	5	6	24.34
18	1	-	0	0	-	0	50	10	8	1	6	17.98
19	1	0	+	0	+	0	75	15	8	5	6	40.21
20	1	0	0	+	0	-	75	10	12	3	4	43.00
21	1	+	0	+	0	0	100	10	12	3	6	52.00
22	1	0	-	0	+	0	75	5	8	5	6	28.64
23	1	0	0	0	0	0	75	10	8	3	6	34.52
24	1	+	0	0	-	0	100	10	8	1	6	38.64
25	1	0	-	0	0	+	75	5	8	3	8	29.73
26	1	0	0	0	0	0	75	10	8	3	6	36.41
27	1	0	-	0	-	0	75	5	8	1	6	20.19
28	1	0	+	0	0	+	75	15	8	3	8	42.24
29	1	0	0	-	-	0	75	10	4	1	6	08.00
30	1	-	0	-	0	0	50	10	4	3	6	07.00
31	1	0	0	-	+	0	75	10	4	5	6	24.00
32	1	0	+	+	0	0	75	15	12	3	6	45.00
33	1	-	-	0	0	0	50	5	8	3	6	11.52
34	1	+	0	0	0	-	100	10	8	3	4	38.78
35	1	0	0	0	-	+	75	10	8	1	8	27.35
36	1	-	0	0	0	+	50	10	8	3	8	28.63
37	1	0	0	-	0	+	75	10	4	3	8	26.00
38	1	0	+	0	-	0	75	15	8	1	6	24.58
39	1	0	0	0	-	-	75	10	8	1	4	18.75
40	1	+	0	0	0	+	100	10	8	3	8	48.21
41	1	0	-	+	0	0	75	5	12	3	6	29.52
42	1	0	0	+	0	+	75	10	12	3	8	49.00
43	1	0	0	-	0	-	75	10	4	3	4	14.00
44	1	0	0	0	0	0	75	10	8	3	6	37.22
45	1	0	-	0	0	-	75	5	8	3	4	19.47
46	1	-	0	+	0	0	50	10	12	3	6	30.15

Table 4: Estimated regression coefficients and corresponding statistical t- and P-values for the production of Lactic acid

Term	Coefficient	SE Coefficient	T	P
Constant	-131.451	24.464	-5.373	0.000
Potato Waste	1.005	0.275	3.648	0.001
Enzyme Mixture	4.789	1.295	3.699	0.001

Yeast extract	8.523	1.619	5.265	0.000
NH_4Cl	7.483	3.162	2.366	0.026
Inoculum Size	5.225	3.443	1.518	0.142
Potato Waste*Potato Waste	-0.004	0.001	-3.334	0.003
Enzyme Mixture*Enzyme Mixture	-0.210	0.031	-6.767	0.000
Yeast extract*Yeast extract	-0.243	0.049	-5.004	0.000
NH_4Cl*NH_4Cl	-0.742	0.194	-3.820	0.001
Inoculum Size*Inoculum Size	-0.063	0.194	-0.326	0.747
Potato Waste*Enzyme Mixture	0.002	0.009	0.190	0.851
Potato Waste*Yeast extract	0.002	0.012	0.185	0.855
Potato Waste*NH_4Cl	0.006	0.023	0.268	0.791
Potato Waste*Inoculum Size	-0.004	0.023	-0.159	0.875
Enzyme Mixture*Yeast extract	0.031	0.057	0.540	0.594
Enzyme Mixture*NH_4Cl	0.179	0.115	1.564	0.130
Enzyme Mixture*Inoculum Size	-0.047	0.115	-0.407	0.687
Yeast extract*NH_4Cl	-0.312	0.143	-2.179	0.039
Yeast extract*Inoculum Size	-0.187	0.143	-1.307	0.203
NH_4Cl*Inoculum Size	0.029	0.287	0.100	0.921

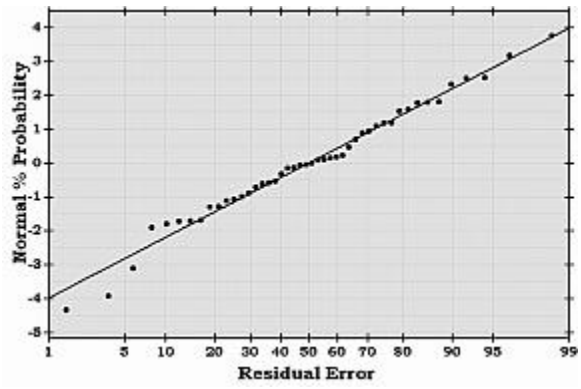

Figure 11: Normal % probability verses residual error

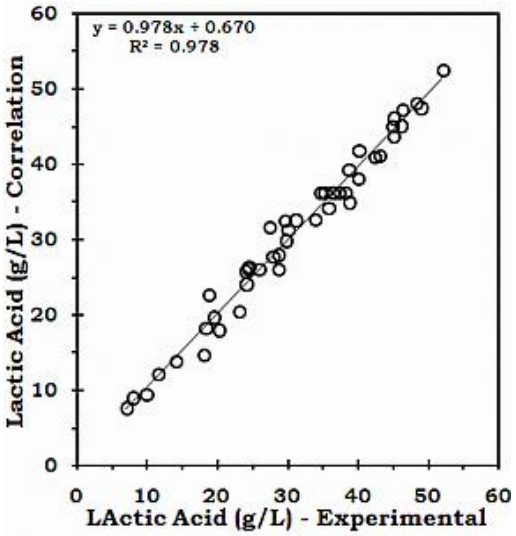

Figure 12: Comparison of experimental and correlation values of production of lactic acid

Table 5: Analysis of variance model regression for the production of Lactic acid

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	5980.18	5980.19	299.01	56.77	0.000
Linear	5	5580.11	215.51	43.10	8.18	0.000
Square	5	349.84	349.84	69.97	13.28	0.000
Interaction	10	50.23	50.23	5.02	0.95	0.505
Residual Error	25	131.67	131.68	5.27		
Lack-of-Fit	20	123.36	123.36	6.17	3.71	0.076
Pure Error	5	8.31	8.31	1.66		
Total	45	6111.86				

S = 2.29499 PRESS = 505.415

R^2 = 97.85%; R^2 Predicted = 91.73% and R^2 Adjusted = 96.12%

DF, degree of freedom; SS, sum of squares; MS, mean of squares; F, F-value; P, significance level of P-value (a significance level <0.05)

Table 6: Experimental and predicted values for the production of Lactic acid

Run	Coded values					Uncoded values					Lactic acid	
	PW	EM	YE	AC	IS	PW	EM	YE	AC	IS	Production	Correlation
01	+	0	0	+	0	100	10	8	5	6	46.23	47.34
02	0	+	-	0	0	75	15	4	3	6	23.00	20.49
03	+	+	0	0	0	100	15	8	3	6	44.82	45.14
04	0	0	0	0	0	75	10	8	3	6	38.13	36.32
05	0	0	+	-	0	75	10	12	1	6	40.00	38.21
06	0	0	+	+	0	75	10	12	5	6	46.00	45.05
07	0	0	0	+	-	75	10	8	5	4	35.87	34.29
08	0	0	0	0	0	75	10	8	3	6	35.33	36.32
09	-	+	0	0	0	50	15	8	3	6	24.24	24.01
10	0	0	0	0	0	75	10	8	3	6	36.32	36.32
11	0	+	0	0	-	75	15	8	3	4	33.85	32.76
12	+	0	-	0	0	100	10	4	3	6	28.00	27.81
13	0	0	0	+	+	75	10	8	5	8	44.93	43.75
14	+	-	0	0	0	100	5	8	3	6	31.23	32.50
15	-	0	0	0	-	50	10	8	3	4	18.47	18.33
16	0	-	-	0	0	75	5	4	3	6	10.00	09.53
17	-	0	0	+	0	50	10	8	5	6	24.34	26.03
18	-	0	0	-	0	50	10	8	1	6	17.98	14.80
19	0	+	0	+	0	75	15	8	5	6	40.21	41.92
20	0	0	+	0	-	75	10	12	3	4	43.00	41.23
21	+	0	+	0	0	100	10	12	3	6	52.00	52.57
22	0	-	0	+	0	75	5	8	5	6	28.64	26.12
23	0	0	0	0	0	75	10	8	3	6	34.52	36.32
24	+	0	0	-	0	100	10	8	1	6	38.64	34.88
25	0	-	0	0	+	75	5	8	3	8	29.73	29.80
26	0	0	0	0	0	75	10	8	3	6	36.41	36.32
27	0	-	0	-	0	75	5	8	1	6	20.19	17.87
28	0	+	0	0	+	75	15	8	3	8	42.24	41.07
29	0	0	-	-	0	75	10	4	1	6	08.00	08.88
30	-	0	-	0	0	50	10	4	3	6	07.00	07.54
31	0	0	-	+	0	75	10	4	5	6	24.00	25.72
32	0	+	+	0	0	75	15	12	3	6	45.00	46.07
33	-	-	0	0	0	50	5	8	3	6	11.52	12.24
34	+	0	0	0	-	100	10	8	3	4	38.78	39.39
35	0	0	0	-	+	75	10	8	1	8	27.35	31.68
36	-	0	0	0	+	50	10	8	3	8	28.63	27.93
37	0	0	-	0	+	75	10	4	3	8	26.00	26.13
38	0	+	0	-	0	75	15	8	1	6	24.58	26.48
39	0	0	0	-	-	75	10	8	1	4	18.75	22.68
40	+	0	0	0	+	100	10	8	3	8	48.21	48.26
41	0	-	+	0	0	75	5	12	3	6	29.52	32.62
42	0	0	+	0	+	75	10	12	3	8	49.00	47.47
43	0	0	-	0	-	75	10	4	3	4	14.00	13.89
44	0	0	0	0	0	75	10	8	3	6	37.22	36.32
45	0	-	0	0	-	75	5	8	3	4	19.47	19.62
46	-	0	+	0	0	50	10	12	3	6	30.15	31.45

RESULTS AND DISCUSSION

Three-dimensional response plots and their corresponding contour plots for the production of lactic acid are shown in Figures 1 – 10**.** The graphical representation provides a method to visualize the relation between the response and experimental variables at its each level. The linear, quadratic and interaction product terms in the second-order polynomial model are used to generate this three dimensional response surface graph and a two-dimensional contour plot. While the three-dimensional response surface graph can assist the researcher to determine the direction of an increase in desired response and the nature of the fitted surface as a maximum, minimum or saddle point, it is difficult to determine the levels of variables from such a graph. This can be more readily being achieved from a contour plot of the same variables. Table 3 gives the estimated regression coefficients and corresponding statistical t- and P-values for the production of lactic acid.

Figure 1 shows the response surface and contour plot for an interactive effect of enzyme mixture and potato waste on production of lactic acid while yeast extract, NH_4Cl and inoculum size are fixed at its middle level. The interaction relationship between the two chosen variables and the response variable could be easily understood by examining the contour plots. The P value (0.8510 > 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is insignificant on the production of lactic acid, even if the shape of the respective contour plot seems to be slight elliptical nature.

It could be seen from Figure 1, the production of lactic acid increases with an increase in amount of potato waste subtract since it is sole carbon source. The same trend is noticed even an increase in concentration of enzyme mixture. Similarly the lactic production is found to be increased with an increase in concentration of enzyme mixture and reaches a constant value. Regardless of amount of potato waste, the highest concentration of enzyme mixture shows the highest production. However at the lowest substrate loading, enzyme needed is comparatively lower. Rojan et al.,[18] reported the same as, higher enzyme concentration may required more substrate for hydrolyzes, as a result the amount of fermentable sugars in the medium is increased.

Also it is observed from Figure 1, the potato waste subtract has the positive influence on the production of lactic acid. It is due to the conversion of starch in the potato waste into hexoses in presence of amylase enzyme mixture. Rojan et al.,[18] suggested that in two stage hydrolysis and fermentation, the starch substrate is treated first with α-amylase for liquefaction and then it is treated again with glucoamylase for saccharification at elevated temperature for faster liberation of hexoses. But the liberation of hexoses is slower in simultaneous saccharification and fermentation using the enzyme mixture of α-amylase and glucoamylase. Although it is slower, the conversion of the liberated hexoses to lactic acid is faster than the two step fermentation. Hence the negative influence of reducing sugar is nullified. The increase of lactic acid production with an increase in enzyme mixture might be the reason of activity of enzyme on the production of lactic acid. After an increase, the production of lactic acid reaches an optimum level even the concentration of enzyme mixture is increased. This may due to the enzyme may not show their extreme activity for the production of lactic acid. But the available activity from enzyme mixture is expected to help the controlled release of sugars, which is very crucial for the fermentation. Even at high amount of starch concentration, the same is approached. If enzyme activity is rigorous, the feedback inhibition due to high reducing sugar level would expect. Rojan et al.,[18] reported strongly that the conversion percentage of starch to reducing sugar in simultaneous saccharification and fermentation is higher than the two stage hydrolysis and fermentation.

Figure 2 describes the response surface and contour plot for an interactive effect of yeast extract and potato waste on production of lactic acid while enzyme mixture, NH_4Cl and inoculum size are fixed at its middle level. The P value (0.8550 > 0.05) in Table 4 indicates that the interaction effect of yeast extract and potato waste is insignificant on the production of lactic acid. The circular nature of the contour plot ensures the same.

From Figure 2, it is observed that the lactic acid production is increased with an increase in yeast extract concentration. Even an increase in potato waste, the same increasing trend is observed.

The positive influence of yeast extract on the production of lactic acid is somewhat higher than the influence of enzyme concentration on lactic acid production. For growth and fermentation[18,19, 41] the lactic acid bacteria requires complex nutrient supplement. Nancib et al.,[42] reports that vitamin B is the main growth promoters in the yeast extract which helps to enhance the lactic acid production.

The response surface and contour plot for an interactive effect of NH_4Cl and potato waste on

production of lactic acid is presented in Figure 3 where the enzyme mixture, yeast extract and inoculum size are fixed at its middle level. As in the case of Figure 1 and Figure 2, the P value (0.7910 > 0.05) in Table 4 indicates that the interaction effect of yeast extract and potato waste is also insignificant on the production of lactic acid. The clear circular nature of the contour plot ensures the same.

NH_4Cl is the inorganic nitrogen source for the lactic acid producing bacteria's. It has the positive influence on the production of lactic acid irrespective of increase in the amount of potato waste. But the influence is not that much pronounced as in the case of effect of yeast extract on the lactic production.

Figure 4 depicts the response surface and contour plot for an interactive effect of inoculum concentration and potato waste on production of lactic acid while enzyme mixture, yeast extract and NH_4Cl are fixed at its middle level. The P value (0.8750 > 0.05) in Table 4 indicates that the interaction effect of inoculum size and potato waste is insignificant on the production of lactic acid. The circular nature of the contour plot ensures the same.

Figure 4 shows that the production of lactic acid increases with an increase in amount of potato waste subtract. The same trend is noticed even an increase in inoculum concentration. Similarly the lactic production is found to be increased with an increase in inoculum concentration. Irrespective of the amount of potato waste, inoculum concentration shows a constant increase in lactic acid production. At both the lower and higher levels of potato waste a higher concentration of inoculum resulted in better lactic acid production. It indicates its effect on promoting growth of organism which leads to increase an increase in lactic acid. Higher amount of potato waste substrate requires always the higher inoculum concentration. Also at higher substrate concentration, the inoculum had an additive effect with increase in cell number results increased lactic acid production.

Response surface and contour plot for an interactive effect of yeast extract and enzyme mixture on production of lactic acid is shown in Figure 5 in which amount of potato waste, NH_4Cl and inoculum concentration are fixed at its middle level. The P value (0.5940 > 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is insignificant on the production of lactic acid. The circular shape of the respective contour plot ensures the same. It could be seen from Figure 5, the production of lactic acid increases with an increase in amount of yeast extract. The same trend is noticed even an increase in concentration of enzyme mixture. Similarly the lactic production is found to be increased with an increase in concentration of enzyme mixture irrespective of amount of yeast extract.

Figure 6 shows the response surface and contour plot for an interactive effect of NH_4Cl and enzyme mixture on production of lactic acid while potato waste substrate, yeast extract and inoculum concentration are fixed at its middle level. The P value (0.130 > 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is insignificant on the production of lactic acid. The circular shape of the respective contour plot ensures the same.

Figure 7 depicts the response surface and contour plot for an interactive effect of inoculum size and enzyme mixture on production of lactic acid where potato waste substrate, yeast extract and NH_4Cl are fixed at its middle level. The P value (0.687 > 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is insignificant on the production of lactic acid. The circular shape of the respective contour plot ensures the same.

Response surface and contour plot for an interactive effect of NH_4Cl and yeast extract on production of lactic acid is given in Figure 8 where potato waste substrate, yeast extract and inoculum concentration are fixed at its middle level. The interaction between NH_4Cl and yeast extract in found to be significant. The necessity of the inorganic nitrogen source as nutrient is higher when a higher inoculum size is used since *Lactobacilli* are fastidious organisms requiring complex nutrients. With an increase in number of the viable cells, it is expected that there would be a corresponding increase in demand for nutrients, which could explain this observation. Though the P value (0.039 < 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is significant on the production of lactic acid, the near circular shape of the respective contour plot does not ensures the same.

Figure 9 depicts the response surface and contour plot for an interactive effect of inoculum size and yeast extract on production of lactic acid in which potato waste substrate, Enzyme mixture and NH_4Cl are fixed at its middle level. The P value (0.2030 < 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is insignificant on the production of lactic acid. The circular shape of the respective contour plot also ensures the same.

Figure 10 shows the response surface and contour plot for an interactive effect of inoculum size and NH_4Cl on production of lactic acid where the potato waste, enzyme mixture and yeast extract are fixed at its middle level. The P value (0.921 < 0.05) in Table 4 indicates that the interaction effect of enzyme mixture and potato waste is insignificant on the production of lactic acid. The circular shape of the respective contour plot also ensures the same.

The statistical significance of the ratio of mean square variation due to regression and mean square residual error is tested using ANOVA. It is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters of the model. According to ANOVA reported in Table 4, the *F* values for all regressions were higher. The large value of *F* indicates that most of the variation in the response can be explained by the regression equation. The associated *p* value is used to estimate whether *F* is large enough to indicate statistical significance. If $p > F$ value is lower than 0.05, then it indicates that the model is statistically significant. The *P* values in Table 4 for all the regressions were lower than zero. This means that at least one of the terms in the regression equation has a significant correlation with the response variable. The ANOVA Table 5 also shows a term for residual error, which measures the amount of variation in the response data left unexplained by the model. The analysis shows that the form of the model chosen to explain the relationship between the factors and the response is correct[43].

The ANOVA result for the production of lactic acid shows the *F* value to be 15.53, which implies that the terms in the model have a significant effect on the response.The model gives R^2 value of 97085 % and an adjusted R^2 valule of 96.12%. Therefore ,it can be assumed that the proposed model doesnot explain at least 2.15% of the experimental results. The probability p(~0.0000) is less than 0.05 .This indicates that the model terms are significant at 95% probability level. Any factor or interaction of factors with $p < 0.05$ is significant. The linear, square and interaction effects in the ANOVA Table 4 obtained from the response surface quadratic model indicates its significance. The value of the predicted R^2 is the measure of the variation in data explained by the model. The predicted R^2 = 0.9173 implies that the present model has a large block effect. *A* value of (predicted R^2 −adjusted R^2) > ±0.20, indicates a problem with either the data or the model (Montgomery, 2004). The final mathematical equation in terms of experimental variables (confidence level above 95%) as determined by Box-Behnken Design is given Equation 3.

A normal probability plot is shown in Figure 5. The data points on this plot lie reasonably close to a straight line, lending support to the conclusion that experimental variables have the significant effects and that the underlying assumptions of the analysis are satisfied. Figure 6 shows the relationship between the experimental and predicted values for the production of lactic acid. It is seen in Figure 6 that the developed models are adequate because the residuals for the prediction of each response are minimum, since the residuals tend to be close to the diagonal line. Table 6 represents the comparison of experimental and predicted values of Lactic acid production. The F_{test} value for the production of lactic acid is greater than tabulated $F_{0.05\ (45,\ 184)}$ - test (1.43) which ensures that the second order polynomial equation is highly significant and adequate to represent the actual relationship between the response and the variables.

Equation 3.

$$\text{Lactic Acid} = -131.45 + 1.005PW + 4.789EM + 8.523YE + 7.483AM + 5.225IS - 0.004PW^2 - 0.210EM^2 - 0.243YE^2 - 0.742AC^2 - 0.063IS^2 + 0.002(PW*EM) + 0.002(PW*YE) + 0.006(PW*AC) - 0.004(PE*IS) + 0.031(EM*YE) + 0.179(EM*AC) - 0.047(EM*IS) - 0.312(YE*AC) - 0.187(YE*IS) + 0.029(AC*IS) \quad (3)$$

CONCLUSION

The production of lactic acid is investigated from potato waste by Simultaneous saccharification and fermentation. *Lactobacillus casei* bacterial species is used in this investigation along with α-amylase and glucoamylase as enzyme mixture. Potato waste substrate, enzyme mixture, yeast extract, NH_4Cl and inoculum size are the experimental variables used in this investigation to study their effect on the production of lactic acid. Box-Behnken Design is used for statistical analysis. The linear effect of potato waste substrate, enzyme mixture, yeast extract and NH_4Cl are found to be highly significant on the production of lactic acid than the effect of inoculum size. Similarly the squared effect of potato waste substrate, enzyme mixture, yeast extract and NH_4Cl are found to be highly significant on the production of lactic acid than the effect of inoculum size. The interaction effect of yeast extract and NH_4Cl is alone found to be significant on the production of lactic acid based on the *P* value. The second order polynomial model developed by Box-Behnken Design is highly significant and adequate to represent the actual relationship between the response and the experimental variables. The regression coefficient of this model 0.9785 and the normal probability plot ensure the same. The experimental and predicted values of Lactic acid production is compared and found to concur with one another.

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