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Statistical Optimization of Medium Components by Response Surface Methodology for Enhanced Production of Lactic acid by *Lactobacillus Plantarum*

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ABSTRACT

Lactic acid production is carried out in submerged fermentation using agro waste as substrates by *lactobacillus plantarum*. Various agro wastes are selected and pretreated using acid and alkali. The results show that the alkali treated banana peel and acid treated press mud yields better results. Experiments were performed with the selected substrates. Initial screening of nutrient was performed using Plackett–Burman design and the variables with statistically significant effects on lactic acid were identified. Peptone, $MnSO_4$ and $CaCl_2$ were found to influence lactic acid production significantly for banana peel. Peptone, $MnSO_4$, $CaCl_2$, sodium acetate and $MnCl_2.7H_2O$ were found to influence lactic acid production significantly for pressmud. These variables were selected for further optimization studies using Box-Behnken design (BBD). At the optimized conditions, validation of model was carried out. At these conditions a maximum lactic acid production of 42 and 55 g/L for pressmud and banana peel respectively was achieved.

1. Introduction

Lactic acid is a versatile organic acid used as acidulant, flavor, pickling agent and preservative in food, pharmaceutical, leather and textile industries, for the production of base chemicals like acetaldehyde, acrylic acid, propionic acid, etc., and for polymerization to biodegradable polylactic acid (PLA). Lactic acid exists as two optical isomers, d-and l-lactic acid. Both isomeric forms of lactic acid can be polymerized and polymers with different properties can be produced depending on the composition. Of the 80,000 tons of lactic acid produced worldwide every year, about 90 % are made by fermentation employing lactic acid bacteria and the rest is produced synthetically by the hydrolysis of lactonitrile [1]. Fermentative production has the advantage that an optically pure product can be obtained by choosing a strain of lactic acid bacteria producing only one of the isomers, whereas synthetic production always results in a racemic mixture of lactic acid. It is also possible to use renewable resources as substrates, such as starch and cellulose in fermentative production [2,3]. Cellulose, hemicellulose and starch are the most abundant compounds in the world, and when hydrolyzed to fermentable sugars they can be utilized by a number of microorganisms. Generally, lactic acid is produced using refined sugars as carbon source. The main obstacle in the production of lactic acid in large scale is the cost of raw material. The use of starchy material in the place of refined sugars reduces the cost of production. Cassava ranks the fourth among the staple food crops in the world and is consumed by more than 800 million people [4]. The waste, cassava bagasse obtained from the cassava tubers in the starch industries can be used as the carbon source because of its high starch content (more than 50 %).

Fermentative lactic acid production from renewable resources comprises the following steps: pretreatment of substrate including hydrolysis to sugars, fermentation of sugars to lactic acid or simultaneous saccharification and fermentation, separation of bacteria and solid particles from the broth, and purification of lactic acid. The negative impact of large concentration of sugar can be avoided by simultaneous saccharification and fermentation where the sugar formed is utilized as soon as it is formed [5,6].

Cheap raw materials are necessary for the feasible economic production of lactic acid because polymer producers and other industry users usually require large quantities of lactic acid at relatively low cost. Raw materials for lactic acid production should not only be of low cost, but with low levels of contaminants and low toxic materials capable of being fermented with little or no pre-treatment resulting in rapid production rate and high yield with little or no by-product formation and be available throughout the year [7,8]. Lactic acid can be produced by several microorganisms classified into bacteria, fungi, yeast, cyanobacteria, and algae. Each biocatalyst has achieved one or more improvements over the others, such as a broader substrate range, improved yield and productivity, reduction of nutritional requirements, or improved optical purity of lactic acid. Most lactic acid bacteria, including *lactobacilli*, are considered to be safe for industrial lactic acid production because they have had a long history of industrial-scale production without adverse health effects on either consumers or production workers. Commercially important lactic acid bacteria strains, such as *lactobacillus* strains, have been particularly useful due to their high acid tolerance and their ability to be engineered for selective production of D –or L -lactic acid [9-11].

Statistical methods provide an alternative methodology to optimize a particular process by considering the mutual interactions among the variables and give an estimate of combined effect of these variables on final results. Response surface methodology (RSM) is one such technique based on the fundamental principles of statistics, such as randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. This is generally known as full factorial design [12]. Application of factorial designs and RSM is a common practice in biotechnology for the optimization of media components and culture conditions [13-15]. These optimization methods involve three major steps; performing statistically designed experiments, estimating coefficients in a mathematical model, predicting the response, and checking the adequacy of the model. In this study, nine different pretreated agro waste was used for lactic acid production by *lactobacillus plantarum*.

2. Experimental Methods

2.1. Microorganisms and Culture Conditions

Lactobacillus plantarum (MTCC 1407) was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained on Malt dextrose agar slants at 4 °C and the slants were sub-cultured every month.

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2.2. Media Components

Malt dextrose agar (MDA) and medium component were purchased from Hi-Media Limited, India. HPLC grade acetonitrile (ACN) and ethanol were purchased from Rankem, New Delhi, India. All the chemicals used were of analytical grade.

2.3. Inoculum Preparation

Actively growing slants were used to prepare the spore suspension of *lactobacillius plantarum* in sterile water. A spore suspension 106 spores mL^{-1} prepared from such slants was used to inoculated into conical flasks containing the seed medium: as given in Table 1 in 1000 mL of distilled water. The pH is adjusted to 6. These cultures were incubated at 30 °C for 48 h in a shaking incubator at 120 rpm. 5 percent of this pre-culture was used to inoculate into the production medium. Fermentation experiments were carried out at 30 °C for 4 days using *lactobacillius plantarum* in 250 mL Erlenmeyer flasks containing 100 mL of production media, as per the experimental design.

2.4 Raw Material Processing

Nine different agro wastes viz. pressmud, peels of banana, sugarcane bagasse, cassava peel, peel of sweet sorghum, rice bran, wheat bran, peel of potato and pineapple peel were screened for lactic acid production by *lactobacillius plantarum*.

2.5 Pretreatment of Agricultural Waste

Samples of each agricultural waste, shown in Table 2, were sun dried and then reduced to very small sized particles by grinding, using a serrated disk grinder. The particles were then sieved to obtain an average particle size of 300 μm for each sample. When lignocellulosic biomass feedstock is used, a pretreatment process is required prior to fermentation. Physical (grinding and irradiation) and chemical (acid and alkali), processes have been performed to reduce the particle size and crystalinity, to increase solubilization of hemicelluloses and lignin, and to enhance the accessibility of cellulose to the enzyme in the following enzymatic hydrolysis step.

2.5.1 Acid Pretreatment

Dilute acid pretreatment is performed by adding dilute acid (<4 % HCl) at elevated temperatures between 130 and 200 °C for 2–80 min [16-18]. During acid hydrolysis of lignocellulosic biomass, it is important to select proper pretreatment conditions to maximize the solubilization of hemicellulose and minimize the formation of inhibitors such as furfural and hydroxymethylfurfural (HMF).

2.5.2 Alkali Pretreatment

Alkaline pretreatment (NaOH or lime) can swell the pores of the cellulosic biomass at temperatures ranging from 25 °C to 85 °C. Alkali pretreatment can reduce the degree of polymerization and crystallinity, increase the surface area, and increase the solubilization of lignin and hemicelluloses [19-20]. The solid/liquid ratio of alkali treatment is about 10–20 %. The pretreatment time ranges from 1 to 30 h depending on the pretreatment temperature and alkali loading.

2.6 Selection of Agricultural Waste for Maximum Lactic Acid Production

Spore suspension was inoculated into 50 mL liquid medium in a 250 mL flask containing 5 % of pretreated (by acid and alkali) substrate as shown in Table 2. Cultivations were performed on a rotary shaker at the speed of 200 rpm, and at 30 °C for 4 days.

Table 1 Nutrient medium for the planckett burman technique for low and high level value

Sl.No	Chemical components	Low Level (g/L)	High level (g/L)
1	Peptone	2.5	10
2	Yeast extract	1.25	5
3	Beef extract	1.25	5
4	Sodium acetate	1.25	5
5	K_2HPO_4	0.5	2.0
6	$NaH_2PO_4.2\ H_2O$	0.5	2.0
7	Diammonium citrate	0.5	2.0
8	NH_4HCO_3	0.19	0.75
9	$(NH_4)_2HPO_4$	0.058	0.23
10	$(NH_4)_2SO_4$	0.05	0.2
11	Diamine citrate	0.05	0.2
12	$MnSO_4$	0.013	0.05
13	$MgSO_4$	0.03	0.1
14	$CaCl_2$	0.005	0.02
15	$FeSO_4.7H_2O$	0.005	0.02
16	$MnCl_2.7H_2O$	0.005	0.02

Table 2 Effect of pretreatment on the nine different agro waste for the production of Lactic acid

S.No	Substrate	Lactic acid (g/L)	
		NaOH	HCl
1	Banana peel	23.1	11
2	Cassava peel	9.2	10
3	Sweet sorhgam	6.7	10.7
4	Sugarcane baggase	7.77	10.45
5	Rice brain	9.1	10.89
6	Wheat brain	11.3	13.12
7	Press mud	17.34	24.12
8	Pineapple	10.9	11.8
9	Mango peel	12.1	13.1

Table 3 Plackett-Burman design for medium optimization, positive (+1) and negative (-1) levels of independent variables used in trials and measure response

Run Order	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	Lactic Acid Production (g/L)	
																	Banana Peel	Pressmud
1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	18	18
2	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	22	24
3	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	5	6
4	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	16	18
5	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	25	25
6	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	31	31
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	33	31
8	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	30	29
9	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	14	16
10	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	21	21
11	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	23	24
12	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	20	21
13	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	26	27
14	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	29	26
15	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	18	19
16	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	15	17
17	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	17	18
18	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	14	14
19	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	22	22
20	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	21	21

2.7 Experimental Design and Data Analysis Identification of Suitable Variables Using Plackett - Burman Design (PBD)

PBD is a powerful screening method to recognize the most important factors with few number of experiments. This design is valuable in the initial phase of experiments to identify the active factors when full philosophy about the process is missing. Initially, Plackett- Burman design was used to identify the critical factors of media having significant effect on the Lactic acid production. The medium components were given in Table 1. Twelve experiments were carried out with fifteen assigned variables. The factors were examined at two levels -1 for low level and +1 for high level. The name of variables, its code and actual value was demonstrated in Table 3. All the experiments were carried out in triplicate and its average were taken as response.

2.8 Response Surface Methodology

The levels of the significant parameters and the interaction effects between various variables that influence the lactic acid production were analyzed and optimized by Box – Behnken methodology. The experimental design used for the study was shown in Table 4 and 5 for banana peel and pressmud respectively. All the experiments were done in triplicate and the average of lactic acid production obtained was taken as the dependent variable or response (Y). The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software (Version 7.1.5, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial equation is

$$Y = \beta 0+ \Sigma \beta iXi + \Sigma \beta ii\ Xi2+ \Sigma\beta i\ jXi\ Xj \qquad (1)$$

Where Yi is the predicted response, xi, xj are input variables which influence the response variable Y; βo is the offset term; β is the i th linear coefficient; βii is the ith quadratic coefficient and βij is the ijth interaction coefficient.

Table 4 Experimental code and levels of factors in BBD for lactic acid production using banana peel

Medium	Levels, g/L		
	-1	0	1
Peptone (A)	1	6	11
$MnSO_4$ (B)	0.02	0.04	0.06
$CaCl_2$ (C)	0.004	0.012	0.02

2.9 Analytical Methods

The samples containing lactic acid, residual sucrose, glucose and fructose were diluted two fold with distilled water, and 1.5 mL was centrifuged at 4 °C at 12,000 rpm for 15 min and used for HPLC analysis (Agilent Technologies, 1200 series, Germany) equipped with DAD (210 nm) detector and Refractive Index Detector. The concentration of lactate and lactic acid was determined using RI and DAD (= 210 nm) detector respectively [9] .The analytical conditions were as follows: column Zorbax SB-Aq, 4.6 mm ID × 150 mm, 5 m (Agilent Technologies, Germany); mobile phase 1 % acetonitrile (Sigma–Aldrich) and 99 % 20 mM KH_2 PO_4 solution at pH 2.0 maintained with the help of ortho-phosphoric acid with a flow rate of 1 mL/min; Column temperature 35 °C.

Table 5 Experimental code and levels of factors in BBD for lactic acid production using pressmud

Medium	Levels, g/L		
	-1	0	1
Peptone	1	6	11
$MnSO_4$	0.01	0.03	0.05
$CaCl_2$	0.004	0.012	0.02
$MnCl_2.7H_20$	0.004	0.012	0.02
Sodium Acetate	1	3	5

3. Results and Discussion

3.1 Selection of Agricultural Waste for Maximum Lactic Acid Production

Effects of various kinds of pretreated agro wastes on lactic acid production using *lactobacillius plantarum* were studied. From Table 2, it is evident that the highest production of lactic acid was obtained using pressmud and banana peel. A maximum lactic acid production of 24.12 and 23.12 g/L was obtained for pressmud treated with acid and banana peel treated with alkali respectively. Hence these two substrate, with corresponding pretreatment technique, was selected for further studies.

3.2 Screening of Medium using Plackett - Burman Technique

PB design was adopted to optimize various medium components for the production of lactic acid fermentation by *lactobacillius plantarum*. Various media components were investigated for their effect in the process of lactic acid production. The medium components for the independent variables and their respective high and low concentrations used in PB optimization study with respect to lactic acid production. Fifteen nutrients were chosen for PB design with a 12 experimental runs PB design was used to study the effect among the fifteen constituents of the medium [6].

The two substrates yielding maximum production of lactic acid was selected for further studies. The results in Table 3 show wide variation of lactic acid yield in the twelve trials (5 to 33 g/L) for banana peel and pressmud. It reflects the importance of medium optimization to enhance the lactic acid yield. The medium components were screened and those with a p– value of < 0.1 using 90 % confident level were accepted as significant factors affecting the production of lactic acid. Based on pareto chart (Fig. 1 for banana peel and Fig. 2 for pressmud), the variables which were found to be dominant on the production of lactic acid in their order are: Peptone, $MnSO_4.7H_2O$ and $CaCl_2$ for banana peel and Peptone, $MnSO_4.7H_2O$, $MnCl_2.7H_2O$, Sodium acetate and $CaCl_2$ for pressmud. Hence these medium components were selected for further optimization using Box – Behnken design. These results indicated that the Plackett – Burman design is a powerful tool for identification of the variables that could significantly affect lactic acid production.

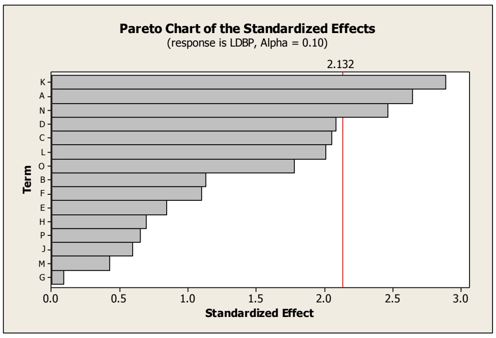

Fig. 1 pareto chart for the screening of lactic acid production by banana peel as substrate using lactobacillus delburickii

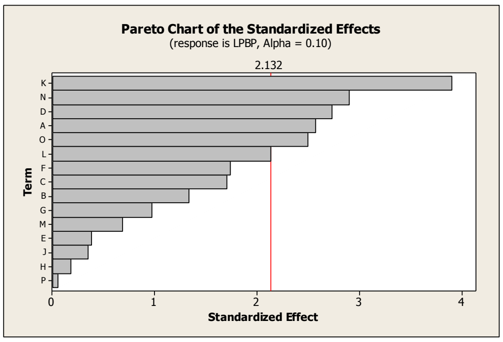

Fig. 2 Pareto chart for the screening of lactic acid production by pressmud as substrate using lactobacillus delburickii

3.3. Optimization of Process Parameters using BBD and RSM for Lactic Acid Production using Lactobacillus Plantarum

The effect of various medium constituents was studied using RSM. Optimization of medium constituents using *lactobacillius plantarum* was done keeping the other nutrients concentration as constant level for both the substrate. These medium constituents mostly influence the fungal growth and secondary metabolite production. RSM is a sequential procedure with an initial objective to lead the experimenter rapidly and efficiently along a path of improvement toward the general vicinity of the optimum. Response surface methodology (RSM) was used to optimize the fermentation medium for enhancing lactic acid production. The Box Behnken design and RSM were applied to determine the optimal for each significant variable. To identify the optimum levels for different medium constituents influencing lactic acid production, submerged fermentation was carried out in conical flasks containing optimized nutrients. The individual and interactive effects of these variables were studied by conducting the fermentation run at randomly selected and different levels of all five factors.

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Table 6 Box-Behnken design matrix for optimization of 3 nutrients for lactic acid production using banana peel

Exp. No.	A	B	C	Lactic acid (g/L)
1	-1	-1	0	30.53
2	-1	1	0	32.04
3	1	-1	0	31.30
4	1	1	0	37.34
5	0	-1	-1	29.13
6	0	-1	1	25.39
7	0	1	-1	31.06
8	0	1	1	31.89
9	-1	0	-1	27.78
10	1	0	-1	33.78
11	-1	0	1	27.68
12	1	0	1	31.42
13	0	0	0	36.83
14	0	0	0	37.02
15	0	0	0	36.93

Table 7 Box-Behnken design matrix for optimization of nutrients for lactic acid production using pressmud

S.No.	A	B	C	D	E	Actual Activity	Predicted Activity
1	-1	-1	0	0	0	16.12	18.01
2	+1	-1	0	0	0	28.11	25.04
3	-1	+1	0	0	0	17.83	19.73
4	+1	+1	0	0	0	32.13	29.06
5	0	0	-1	-1	0	18.20	14.53
6	0	0	+1	-1	0	21.94	26.83
7	0	0	-1	+1	0	23.30	21.15
8	0	0	+1	+1	0	24.65	24.58
9	0	-1	0	0	-1	19.59	21.48
10	0	-1	0	0	-1	24.92	21.48
11	0	-1	0	0	+1	25.60	25.38
12	0	+1	0	0	+1	26.77	28.10
13	-1	0	-1	0	0	14.77	18.31
14	+1	0	-1	0	0	6.64	20.75
15	-1	0	+1	0	0	23.24	20.43
16	+1	0	+1	0	0	33.08	34.36
17	0	0	0	-1	-1	24.82	27.53
18	0	0	0	+1	-1	27.71	28.78
19	0	0	0	-1	+1	28.13	30.35
20	0	0	0	+1	+1	32.88	33.47
21	0	-1	-1	0	0	13.94	11.46
22	0	+1	-1	0	0	17.05	13.88
23	0	-1	+1	0	0	17.67	18.87
24	0	+1	+1	0	0	21.68	22.20
25	-1	0	+1	-1	0	20.09	13.60
26	+1	0	0	-1	0	32.09	27.62
27	-1	0	0	+1	0	23.31	21.62
28	+1	0	0	+1	0	36.49	36.82
29	0	0	-1	0	-1	21.22	18.31
30	0	0	+1	0	-1	27.75	28.67
31	0	0	-1	0	+1	27.82	24.55
32	0	0	+1	0	+1	29.36	29.92
33	-1	0	0	0	-1	24.63	26.71
34	+1	0	0	0	-1	35.30	32.98
35	-1	0	0	0	+1	26.96	28.55
36	+1	0	0	0	+1	41.45	38.65
37	0	-1	0	-1	0	13.97	16.81
38	0	+1	0	-1	0	21.58	23.55
39	0	-1	0	+1	0	21.48	22.87
40	0	+1	0	+1	0	21.34	21.86
41	0	0	0	0	0	49.60	49.39
42	0	0	0	0	0	48.98	49.39
43	0	0	0	0	0	50.39	49.39
44	0	0	0	0	0	50.26	49.39
45	0	0	0	0	0	49.65	49.39
46	0	0	0	0	0	50.70	49.39

The response was measured in terms of lactic acid production. The total of 15 and 46 experiments was used to optimize the medium constituents of banana peel and pressmud respectively. These nutrients were tested at three coded levels namely -1, 0 and +1. The optimum levels of the selected variables were obtained by solving the regression equation using MATLAB software and by analyzing the response surface and contour plots. The experimental and the predicted values were presented along with the BBD experimental design. The results were presented in Table 6 and 7 for banana peel and pressmud respectively. The results demonstrated markedly varied results, ranging from 25.39 to 37.34 mg/L and 6.6 to 50.6 g/L in lactic acid production for banana peel and pressmud respectively. The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results were presented in Table 8 and 9 for banana peel and pressmud respectively. The model F value of 9.81 and 13.28 implies that the model was significant for banana peel and pressmud respectively. The R^2 value closer to 1 denoted better correlation between the observed and predicted response for both the substrate. The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (5.02 and 15.62) indicated that the experiments performed were highly reliable for banana peel and pressmud respectively. The p values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. The results obtained from the BBD were fitted to a second order polynomial equation to explain the dependence of lactic acid production on the medium components banana peel and pressmud.

Production of Lactic acid (Banana Peel) = $38.53 + 1.25A + 1.12B - 0.88C - 2.27A^2 - 4.02B^2 - 6.02C^2 - 0.25AB - 0.25AC + 1.5BC$

Production of Lactic acid (Pressmud) = 49.3892 +4.09235A + 1.43445B + 3.93252C + 1.09215D + 1.87593E - 9.78473A*A - 16.6436B*B - 16.1432C*C - 11.4735D*D - 7.88315E*E + 0.574997A*B + 2.86991A*C + 3.50872A*D + 0.957030 A*E + 0.225780B*C - 1.93672B*D -0.0753037B*E - 2.22072C*D - 1.24844 C*E + 0.466405D*E

Where A, B, C, D and E are the coded values of peptone, $MnSO_4$, $CaCl_2$, $MnCl_2.7H_20$ and Sodium acetate respectively.

The value of "R^2" for the production of lactic acid was 0.9646 and 0.914 for banana peel and pressmud respectively, which indicates a good agreement between experimental and predicted values. The corresponding analysis of variance (ANOVA) is presented in Table 8 and 9 for banana peel and pressmud respectively. The F- value is measure of variation of the data about the mean. Generally, the calculated F value should be several times greater than the tabulated value, if the model is a good prediction of their experimental results and the estimated factors effects are real. Also the high F-value and a very low probability (P>F = 0.0001) indicates that the present model is in a good prediction of the experimental results. Values of prob >F less than 0.0500 indicates model terms are significant.

In the present study, all terms are significant model terms. High F-values and non-significant lack of fit indicated that model was a good fit. The pred R-squared of 0.6499 and 6499 is in a reasonable agreement with the adj R- squared value of 0.9464 and 0.914 for banana peel and pressmud respectively.

Table 8 Analysis of variance for lactic acid production using banana peel by Box-Behnken Design matrix

Source	Coefficient Estimate	df	Standard Error	F Value	p-value Prob > F
Model	38.53	9	0.93	59.39	0.0002
A-Peptone	1.25	1	0.57	85.66	0.0002
B-$MnSO_4$	1.12	1	0.57	87.51	0.0002
C-CaCl2	-0.88	1	0.57	9.88	0.0256
AB	-0.25	1	0.8	14.06	0.0133
AC	-0.25	1	0.8	3.5	0.1203
BC	1.5	1	0.8	14.31	0.0128
A^2	-2.27	1	0.84	28.01	0.0032
B^2	-4.02	1	0.84	61.3	0.0005
C^2	-6.02	1	0.84	263.12	< 0.0001
Residual	1.82	5	0.36		
Lack of Fit	1.81	3	0.6	66.63	0.0148
Pure Error	0.018	2	0.009		
Cor Total	196.8	14			

Std. Dev.- 1.6 1 R-Squared - 0.9464, Adj R-Squared -0.85, Pred R-Squared -0.6499, C.V. % - 5.02 Mean – 31.97, PRESS – 197.46, Adeq Precision - 10.069

Table 9 Analysis of variance for lactic acid production using pressmud by Box-Behnken design matrix. Using banana peel

Source	Coefficient Estimate	df	Standard Error	F Value	p-value Prob > F
Model	49.39	20	1.74	13.28	< 0.0001
A-$MnSO_4$	4.09	1	1.09	14.07	0.0009
B-Sodium Acetate	1.43	1	1.11	1.68	0.2065
C-$CaCl_2$	3.93	1	1.06	13.74	0.001
D-Peptone	1.09	1	1.09	1	0.3265
E-$MnCl_2.7H_20$	1.88	1	1.13	2.77	0.1085
AB	0.57	1	2.15	0.072	0.7911
AC	2.87	1	2.04	1.97	0.1722
AD	3.51	1	2.28	2.36	0.1369
AE	0.96	1	2.15	0.2	0.6597
BC	0.23	1	2.15	0.011	0.9171
BD	-1.94	1	2.15	0.81	0.3758
BE	-0.075	1	2.4	0.0099	0.9752
CD	-2.22	1	2.04	1.18	0.2872
CE	-1.25	1	2.15	0.34	0.5662
DE	0.47	1	2.15	0.047	0.8298
A^2	-9.78	1	1.45	45.54	< 0.0001
B^2	-16.64	1	1.5	123.01	< 0.0001
C^2	-16.14	1	1.45	124.15	< 0.0001
D^2	-11.47	1	1.45	62.62	< 0.0001
E^2	-7.88	1	1.5	27.6	< 0.0001
Residual	461.21	25	18.45		
Lack of Fit	445.06	19	23.42	8.7	0.0066
Pure Error	16.16	6	2.69		
Cor Total	5362.16	45			

Std. Dev.- 4.3, R-Squared - 0.914, Adj R-Squared -0.8452, Pred R-Squared -0.6499, C.V. % - 15.62 Mean - 27.5, PRESS - 1877.23, Adeq Precision - 13.069

3.4 Interaction among the Nutrients

The 3D response surface over 2D contour plot is the graphical representation of the regression equation. The main goal of response surface is to efficiently hunt for the optimum value of the variables such that the response is maximized. Response surface curve were made for variation in the yields of lactic acid production as a function of concentration of two nutrients and other nutrient being at their constant levels. From the response surface plot, it is very easy and convenient to understand the interactions among the nutrients and also to locate their optimum concentration. It can be observed that each of three variables used in present study has its individual effect on lactic acid by *lactobacillius plantarum.*

Fig. 3 provide a visual interpretation of the interaction between two factors for pressmud. In the response surface plot, lactic acid is held at an intermediate level and levels of sodium acetate and $MnSO_4$ are varied from -1 to +1. It can be seen that the lactic acid yield increases with increase in concentration of sodium acetate and $MnSO_4$ up to midlevel, further increase the medium concentration the yield will be decreased. So the levels of peptone has to be varied between 2 to 8 g/L and yeast extract has to be varied from 0.03 to 0.04 to optimize lactic acid production.

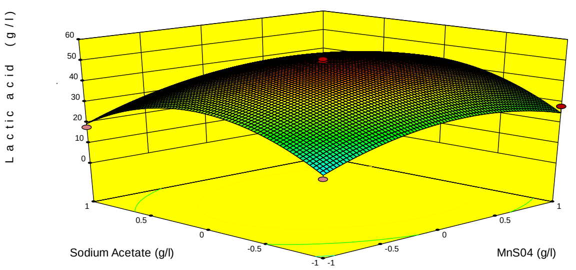

Fig. 3 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between sodiumacetate and $MnSO_4$

Figure 4 and 5, showed the effect of $CaCl_2$ and $MnSO_4$ on lactic acid production for banana peel and pressmud respectively. It was observed that gradual increase in the concentration of $CaCl_2$ at low level (0.02 g) to its higher level (0.06 g) and $MnSO_4$ at low level (0.02 g) to its higher level (0.06 g) increases the production of lactic acid. Further rise in concentration leads to significant decrease in lactic acid production. The maximum lactic acid production was found to be in the midlevel of nutrient concentration after increase of nutrient concentration causes negative effect on lactic acid production.

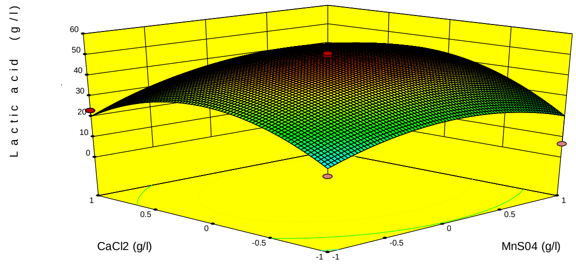

Fig. 4 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between $CaCl_2$ and $MnSO_4$

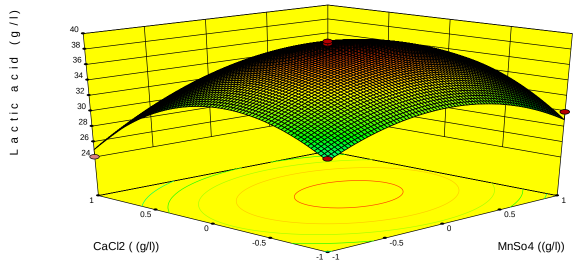

Fig. 5 Response surface plot over contour plot of lactic acid production by pressmud as substrate using lactobacillus planturean, showing interaction between $CaCl_2$ and $MnSO_4$

Fig. 6 and 7, represents the interactive effect of peptone and $MnSO_4$ on overall lactic acid production for banana peel and pressmud respectively. The increase in peptone concentration from 1 to 6 g/L, increases lactic acid production, after that there was decline in lactic acid yields with increase in peptone level.

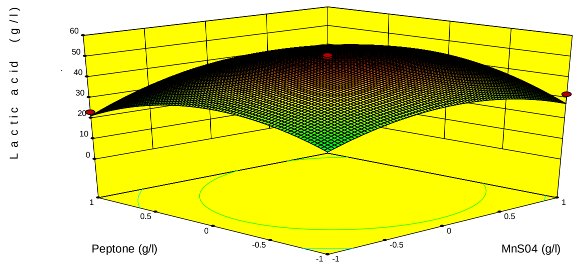

Fig. 6 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between peptone and $MnSO_4$

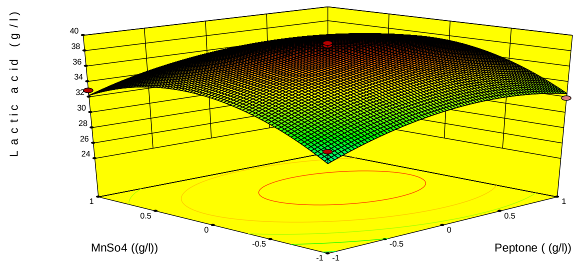

Fig. 7 Response surface plot over contour plot of lactic acid production by pressmud as substrate using lactobacillus planturean, showing interaction between peptone and $MnSO_4$

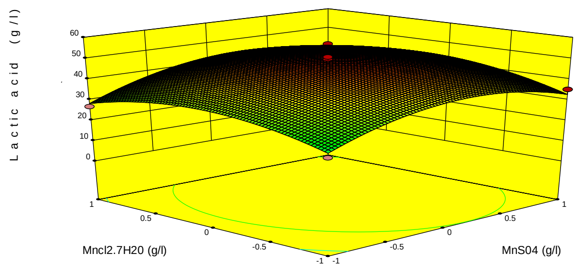

Fig. 8 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between $MnCl_2.7H_2O$ and $MnSO_4$

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Fig. 8 shows the interpretation of the interaction between $MnCl_2.7H_2O$ and $MnSO_4$ for pressmud. In the response surface plot the maximum lactic acid is held at a midlevel of the graph. It can be seen that the lactic acid yield increases with increase in concentration of $MnCl_2.7H_2O$ and $MnSO_4$ up to midlevel, further increase the medium concentration the yield will be decreased. So the levels of $MnCl_2.7H_2O$ has to be varied between 2 to 8 g/L and yeast extract has to be varied from 0.003 to 0.014 to optimize lactic acid production.

Fig. 9 represents with increase in concentration of peptone and with increase in concentration of sodium acetate, the yield of lactic acid increases considerably up to midlevel for pressmud as substrate. The lactic acid decrease further increase in concentration of medium. The surface plot indicates the levels of peptone and sodium acetate that have to be used to get optimum conditions as 3 to 8 g/L for peptone and 2 to 4 g/L for sodium acetate. Similar observation was seen Figs. 10-12 for in between $MnCl_2.7H_2O$ vs sodium acetate, $CaCl_2$ and peptone for pressmud as substrate for the production of lactic acid.

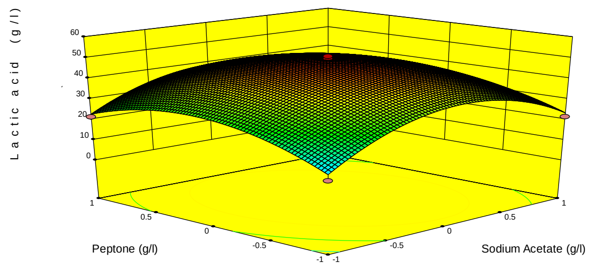

Fig. 9 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between sodiumacetate and peptone

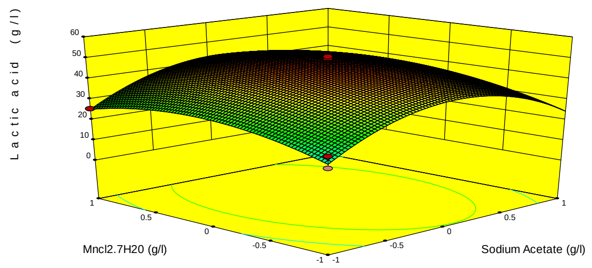

Fig. 10 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between sodiumacetate and $MnCl_2.7H_2O$

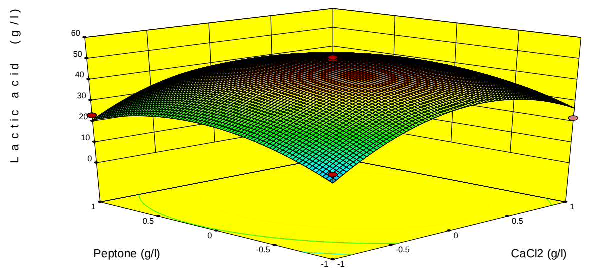

Fig. 11 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between peptone and $CaCl_2$

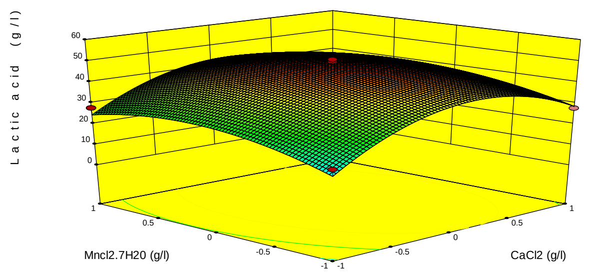

Fig. 12 Response surface plot over contour plot of lactic acid production by banana peel as substrate using lactobacillus planturean, showing interaction between $CaCl_2$ and $MnCl_2.7H_2O$

Fig. 9 and 13 shows the interaction of peptone and $CaCl_2$ for banana peel and pressmud respectively. From the figures it is evident that the higher lactic acid production was achieved at midlevel of nutrient concentration for both the substrate. Fig. 4, showed the effect of peptone and $MnSO_4$ on lactic acid production for pressmud. It was observed that gradual increase in the concentration of peptone at low level (1.00 g) to its higher level (11.0 g) and $MnSO_4$ at low level (0.02 g) to its higher level (0.06 g) increases the production of lactic acid. Further rise in concentration leads to significant decrease in lactic acid production. The maximum lactic acid production was found to be in the midlevel of nutrient concentration after increase of nutrient concentration causes negative effect on lactic acid production. The optimum values for both the substrate were given in Table 8.

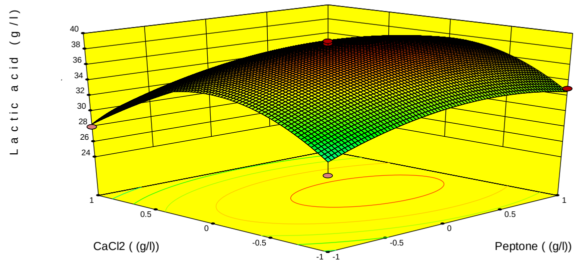

Fig. 13 Response surface plot over contour plot of lactic acid production by pressmud as substrate using lactobacillus planturean, showing interaction between peptone and $CaCl_2$

Table 10 Medium composition of two substrates at the optimized conditions

S.No	Medium	Substrate	
		Banana Peel	Pressmud
1	Peptone	6.89	6.5
2	$MnSO_4$	0.068	0.032
3	$CaCl_2$	0.076	0.014
4	$MnCl_2.7H_2O$	-	0.013
5	Sodium Acetate	-	3.2

4. Validation of the model

The validation experiment was carried out in 250 mL Erlenmeyer flask under the optimum combination of the medium components predicted by the polynomial model. The optimum values for banana peel and pressmud was given in Table 9. The model predicted a maximum response of 39 g/L and 52 g/L of lactic acid production for banana peel and pressmud respectively. At these optimized conditions, a maximum lactic acid production (experimental) of 42 and 55 g/L was obtained for banana peel and pressmud respectively, which is higher than the predicted lactic acid production, thereby validating the proposed model.

5. Conclusion

The production of lactic acid is investigated for nine different pretreated agro waste using *lactobacillius plantarum*. Among these pressmud and banana peel yields better production. The screening of medium was done using these two substrates by Planckett Burmen technigue. The screened medium was optimized using Box-Behnken design. The value of R^2 for the production of lactic acid was found to be 0.9646 and 0.914 for banana peel and pressmud respectively. It indicates a good agreement between experimental and predicted values. On experimental validation, a maximum lactic acid production of of 42 and 55 g/L was obtained for pressmud and banana peel respectively.

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