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Research Article

STATISTICAL OPTIMIZATION OF LACCASE PRODUCTION BY *ENTEROBACTER CLOACAE* STRAIN T137 UTILIZING WHEAT BRAN AS SUBSTRATE IN SUBMERGED FERMENTATION (SmF) BY PLACKETT-BURMAN METHOD

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ABSTRACT

The work aimed to produce laccase enzyme by using wheat bran as substrate by a statistical approach by *Enterobacter cloacae* strain T137. The study was conducted through the submerged fermentation process (SmF) by using the regression model of Plackett-Burman experiments. The significant factors influencing the laccase production was analyzed by ANOVA and Pareto method. Among the variables screened, inoculum size, trace element solution, magnesium sulfate, pH of the medium, agitation rate and incubation time found was significant when the basal salt medium was supplemented with wheat bran as the substrate. The aptness of the model was determined by the coefficient (R^2) of the Plackett-Burman factorial model.

Keywords: Agro-wastes, *Enterobacter cloaeae* strain T137 laccase, Plakett-Burman design, ANOVA.

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are the most extensively studied group of enzymes in common with multicopper oxidases. They catalyze the one-electron reaction of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water [1]. Laccases are more commonly distributed in many prokaryotes. The first laccase was reported in the bacterium *Azospirillum lipoferum*. Thereafter the laccases were discovered in the number of bacteria such as *Bacillus subtilis, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Pseudomonas syringae, Pseudomonas aeruginosa,* etc. [2]. Laccases were known to catalyze the oxidation of various phenolic compounds and aromatic amines [3]. To date, laccases were best produced from white-rot fungi but there also few bacteria exist and produced laccases [4] and actinomycetes [5]. In recent years, laccases have attracted growing scientific interest due to the immense industrial applications [6]. Laccases were studied for the removal of several xenobiotics from the polluted water streams [7], oxidations of phenolic compound residues in wine manufacture [8], maintaining the stability of vegetable and fruit juices [9]. Laccases are used in denim washing [10] and textile dye decolourization [11]. The ever increasing demand for laccases requires the production process to be more economical. Therefore identifying cheap raw materials for enzyme production could be viewed as a solution to make the entire process cost effective [12]. From the commercial point of view, upper product yield and lesser production costs are desirable during the fermentation processes. With this view, the present study was aimed to screen different agro-wastes through single parameter optimization (OVAT method) followed by statistical optimization of the fermentation process using basal salt medium (BSM) supplemented with wheat bran as the substrate in submerged fermentation by Plakett-Burman method and also to work out the foremost important process parameters early within the enhancement of enzyme production [13, 14].

2. MATERIAL AND METHODS

2.1. Microorganism and its Maintenance

Enterobacter cloacae strain T137 used in the present study was a facultative aerobic Gram-negative bacterium. It was isolated from the Devarayanadurga forest soil sample [15]. The strain was screened for laccase activity and maintained in Luria Bertani copper broth (LB/Cu^{2+}) with 15% glycerol as a stock culture for further studies [16].

2.2. Medium and cultural conditions for submerged fermentation (SmF)

The Submerged fermentation was carried out in the basal salt medium and had the following composition (g/L): Yeast extract (YE) - 6.0; Ammonium sulfate ($(NH_4)_2SO_4$)-0.1; Magnesium sulfate ($MgSO_4$)-0.1; Calcium carbonate ($CaCO_3$)-0.02; Copper sulfate ($CuSO_4$)-0.001; and 1.0 ml of trace elements solution contained 0.1% Ferrous sulfate ($FeSO_4$); 0.09% Zinc sulfate ($ZnSO_4$) and 0.02% Manganous sulfate ($MnSO_4$); pH 7.5 [17]. Culture flasks were oxygenated by agitating at 150 rpm/min in an orbital shaker incubator at 30°C for 2 days. The bacteria grown for 48 h were used as the inoculum (10%) for laccase enzyme production.

2.3. Enzyme assay

The laccase activity in the crude enzyme extract from the bacterial culture broth was determined by using guaiacol as a substrate [18] in 30 mM Tris-HCl buffer (pH 8.0), and incubated at 37°C for 10 min. The development of reddish-brown color by the oxidation of guaiacol was used to measure enzyme activity at 470 nm in a UV-visible spectrophotometer. All experiments of laccase activity were performed in triplicates, with an average standard deviation of laccase activity less than 5%, and the data was analyzed by using Minitab 18.0 software. The laccase activity was calculated as per the following formula:

$$E.A = \frac{A \times V}{t \times e \times v}$$

Where;

E.A-Enzyme activity, A-Absorbance, V-Total mixture volume (3 ml), v-enzyme volume (0.5 ml), t-incubation time (10 min), e-extinction coefficient for guaiacol (6740 $M^{-1}cm^{-1}$)

2.4. Analytical methods

The culture supernatant obtained by centrifugation at 10,000 rpm for 20 min at 4°C was used for the following analyses. pH measurement of the culture filtrate was done by digital pH meter. Biomass was estimated by determining the dry weight of the bacteria after submerged fermentation in a microwave oven [19]. Total soluble protein was determined by Lowry's method using bovine serum albumin as the standard [20].

2.5. Cultural and nutritional parameters optimazation

To study the cultural and nutritional parameters influencing the production of laccase enzyme in submerged fermentation, initially, 10 g/L of different agro-industrial residues were screened such as rice bran, wheat bran, rice straw, sugarcane bagasse, coffee bean husk, and coir pith [17]. The effect of NaCl concentration ranged between 1 to 10% on laccase enzyme production by *Enterobacter cloacae* strain T137 was also determined in the medium. The medium without NaCl was used as a control for the experiments [21]. The effect of initial media pH ranged from 5.0 to 10.0 on laccase enzyme production in the production medium was optimized by using acid (1 M HCl) and alkali (1 M NaOH). Temperature influence on laccase enzyme production was optimized by incubating all the bacterial cultures at different temperatures ranging from 28°C to 45°C. The effect of agitation rate on laccase production was optimized at different rpm levels with an interval of 20 rpm and ranged between 100-200 rpm [17]. The influence of inoculum size varied in the range of 1 to 5%. The influence of organic nitrogen sources on laccase production was optimized by the addition of beef extract, peptone, yeast extract, and tryptone. The effect of inorganic nitrogen sources on laccase enzyme production was optimized by ammonium sulfate, sodium nitrate, potassium nitrate, and ammonium nitrate [17]. The organic and inorganic nitrogen sources (1%) were added to the production medium and the effect of the incubation period ranged 24 h to 144 h on laccase production was also optimized for the *Enterobacter cloacae* strain T137 was also determined in the medium [21].

2.6. Statistical optimization of laccase production by the Plackett-Burman method

Statistical design of experiments was used to optimize laccase production [22]. In the very beginning screening of variables was determined according to Paratoe's rule as the first step followed by optimization of important variables in the second step. The fractional factorial technique like Plackett-Burman design [23] an extensively used method for the screening of physicochemical parameters affecting the laccase production by *Enterobacter cloacae* strain T137 in submerged fermentation. Experiments to be performed in triplicates were chosen as per the design matrix. The design matrix was focused on the number of processes parameters to be examined. From the design matrix, the values of every row represented a test and every column represented a variable and their levels were varied [17]. The general number of experiments to be performed out

was n+1. Where the term "n" represents the total number of process variables included in the experiment. From the design matrix, the individual process variable was examined at two different levels like high (+) and low (-). The important variables were identified early within the process by the Pareto method and residual plot analysis of the Plackett-Burman experiments. The statistical software Minitab-18 was used for experimental design and data analysis.

3. RESULTS AND DISCUSSION

The various agro residues screened for laccase enzyme production by *Enterobacter cloacae* strain T137 are shown in the table (Table 1, Fig. 1a and Fig. 1b). It is obvious that culture medium supplemented with wheat bran resulted in higher laccase production (5.043 U/mL) compared to other agro residues. Further, a total of eleven parameters of cultural and Nutritional parameters were selected from single parameter optimization (OVAT) for laccase production from *Enterobacter cloacae* strain T137 by Placket-Burman design experiments and their levels are shown in the Table (Table 2).

Table 1: Screening of Agro-wastes for laccase enzyme production by *Enterobacter cloacae* strain T137

Agro-wastes	Laccase activity (U/ml)
Rice bran	4.272±0.56
Wheat bran	5.043±0.119
Rice straw	2.165±0.207
Bagasse	2.788±0.389
Coffee husk	3.975±0.106
Coir pith	3.679±0.514

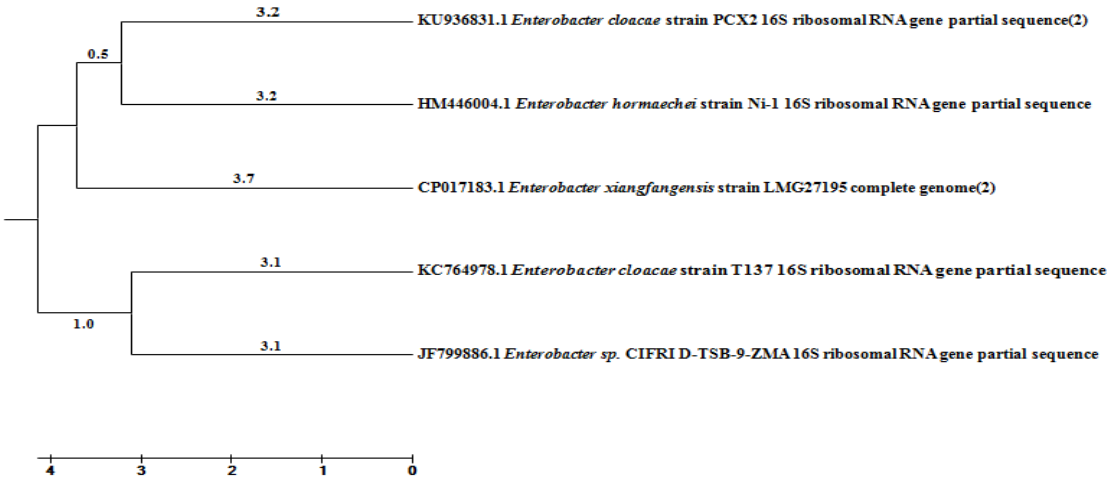


Fig. 1a: Phylogenetic relationship of *Enterobacter cloacae* strain T137 with other strains

Fig. 1b: Inoculation of *Enterobacter cloacae* strain T137 in production medium with different agro-residues for laccase enzyme production

Table 2: Cultural and Nutritional parameters for laccase production from *Enterobacter cloacae* strain T137 by Placket-Burman design experiment

Factors		Cultural and nutritional parameters†										
		X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
Coded levels	Low (-)	5.0	28°C	24 h	1%	100 rpm	2 g/L	2 g/L	0.5 mM	0.5 mM	0.5 mM	5mL/L
	High (+)	8.0	40°C	72 h	2%	140 rpm	10 g/L	10 g/L	1.0 mM	1.5 mM	2.0 mM	15mL/L

Cultural and nutritional parameters†; X_1 to X_{11} denotes: X_1-pH; X_2-Temperature (Temp); X_3-Incubation time (IT); X_4-Inoculum size (IS: 1%=1×10^6 CFU; 2%=3×10^8 CFU*); X_5-Agitation rate (AR); X_6-Carbon source (Carb); X_7-Nitrogen source (Nitro); X_8-Magnesium sulfate (MgS); X_9-Ammonium sulphate (AS); X_{10}-Copper sulphate (CS); X_{11}-Trace elements (TE); CFU*- Colony forming unit.*

Table 3: Plakett-Burman matrix (randomized) for 12 runs and 11 variables

Run	Blk	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	1	+	+	-	+	-	-	-	+	+	+	-
2	1	-	-	-	+	+	+	-	+	+	-	+
3	1	+	+	-	+	+	-	+	-	-	-	+
4	1	-	+	-	-	-	+	+	+	-	+	+
5	1	+	-	+	+	-	+	-	-	-	+	+
6	1	-	-	+	+	+	-	+	+	-	+	-
7	1	+	+	+	-	+	+	-	+	-	-	-
8	1	-	+	+	-	+	-	-	-	+	+	+
9	1	+	-	+	-	-	-	+	+	+	-	+
10	1	-	-	-	-	-	-	-	-	-	-	-
11	1	-	+	+	+	-	+	+	-	+	-	-
12	1	+	-	-	-	+	+	+	-	+	+	-

The design matrix selected for the screening of variables (X1-X11) for laccase production by statistical optimazation is shown in the table (Table 3). From the Plakett-Burman experiments, trial 8 showed the highest enzyme production, whereas trials 1 and 8 showed the lowest enzyme production when compared to other trials (Table 4). The data on enzyme production was subjected to multiple linear regression analyses using Minitab-18 to find out an estimate, t-value, and p-values are entered and shown (Table 5) and the first-order linear regression equation is derived (Eqn. 1).

Y-Laccase activity (U/mL) = 1.917 + 0.0 X4_1x107 + 2.633 X4_3x108 + 0.0 X11_5 + 2.400 X11_15+ 0.0 X8_0.5 – 1.900 X8_1.0 + 0.0 X1_5 – 1.500 X1_8 + 0.0 X5_100+ 1.467 X5_140 + 0.0 X3_24 + 1.267 X3_48…. (Eqn. 1)

The Pareto chart offers an appropriate way to observe the results obtained by the Plackett-Burman design and the order of significance of the variable affecting laccase production (Fig.2). It is also observed that the supplementation of nitrogen sources at a very high level reduced the synthesis of the laccase enzyme. This is in good agreement with the study of inducer tannic acid to red gram husk in enhancing the production of tannase enzyme by *Aspergillus foetidus* MTCC 3557 [14]. During the fermentation, the main effects of the parameters on laccase production were estimated by subtracting the mean responses of parameters at their lower levels from their corresponding higher levels and finally dividing by the total number of trials [24, 25]. Inoculum size, Trace elements, Magnesium sulfate, pH of the medium, Agitation rate, incubation time were found to have a p-value less than 0.05, which indicated that they significantly affect the laccase produc-tion (Table 5). The adequacy of the model was tested and the parameters with statistically significant effects were identified using Fisher's test for analysis of variance (ANOVA). The analysis of variance for the selected factorial model showed that the model was significant with a Model F-value (Table 5). Factors having a confidence level greater than 95 % ($P>F \leq 0.05$) were considered to have a significant effect on the response

and were selected for further studies. Inoculum size with Prob> F value of 0.001 was found to be the most influencing factor followed by trace elements concentration (0.002) and Magnesium sulfate (0.006) in the medium, pH of the production medium (0.015), agitation rate (0.016), and incubation time with a value of 0.028 (Table 5). The Pareto chart offers an appropriate way to observe the results obtained by the Plackett-Burman design and the order of significance of the variable affecting laccase production (Fig. 2). The factors such as incubation time, inoculum size, agitation rate, carbon source, and copper sulfate ($CuSO_4$) had a negative impact on laccase production. While the other factors such as pH, nitrogen source such as ammonium nitrate, concentrations of magnesium sulfate ($MgSO_4$), ammonium sulfate, and trace elements exerted a positive influence on enzyme production as indicated by observation order of residual plot analysis of Plakett-Burman experiments for laccase production (Fig. 3).

Table 4: Optimization of media components for laccase production from *Enterobacter cloacae* strain T137 by Plackett-Burman design matrix

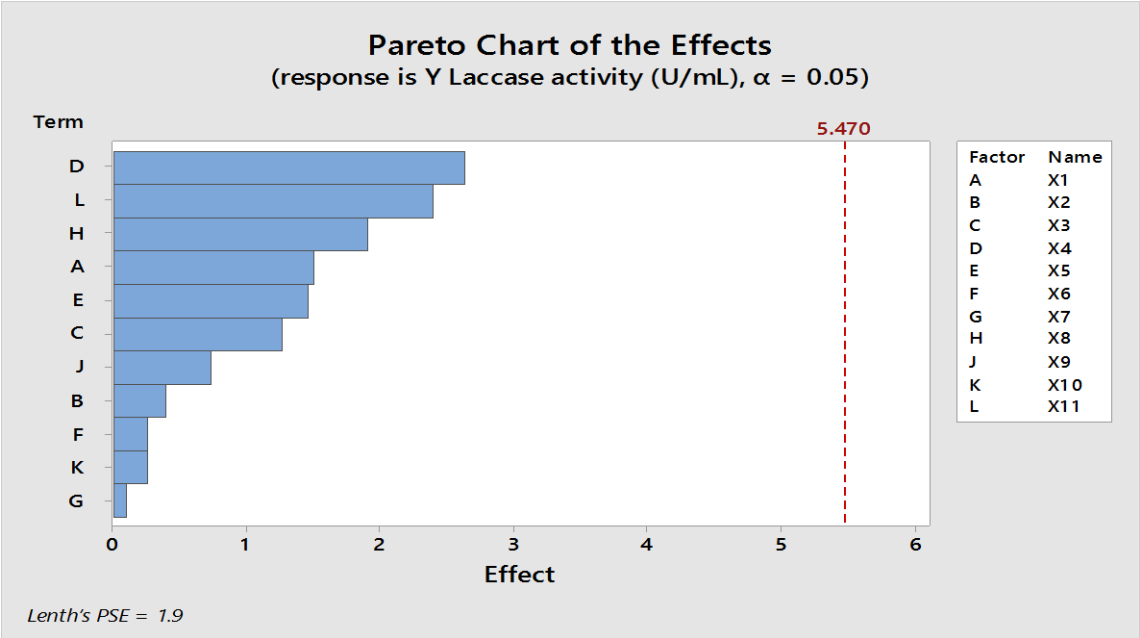
Run No.	Cultural and nutritional parameters†											Laccase activity (U/mL)
	X_1 (pH)	X_2 (Temp)	X_3 (IT)	X_4 (IS)	X_5 (AR)	X_6 (Carb)	X_7 (Nitro)	X_8 (MgS)	X_9 (AS)	X_{10} (CS)	X_{11} (TE)	
1	8.0	40	24	3%	100	2	2	1.0	1.5	2.0	5	1.5
2	5.0	28	24	3%	140	10	2	1.0	1.5	0.5	15	7.0
3	8.0	40	24	3%	140	2	5	0.5	0.5	0.5	15	6.7
4	5.0	40	24	1%	100	10	5	1.0	0.5	2.0	15	2.2
5	8.0	28	48	3%	100	10	2	0.5	0.5	2.0	15	6.2
6	5.0	28	48	3%	140	2	5	1.0	0.5	2.0	5	4.5
7	8.0	40	48	1%	140	10	2	1.0	0.5	0.5	5	1.4
8	5.0	40	48	1%	140	2	2	0.5	1.5	2.0	15	7.4
9	8.0	28	48	1%	100	2	5	1.0	1.5	0.5	15	2.3
10	5.0	28	24	1%	100	2	2	0.5	0.5	0.5	5	1.4
11	5.0	40	48	3%	100	10	5	0.5	1.5	0.5	5	6.6
12	8.0	28	24	1%	140	10	5	0.5	1.5	2.0	5	2.0

Cultural and nutritional parameters†; X_1 to X_{11} denotes: X_1-pH; X_2-Temperature (Temp); X_3-Incubation time (IT); X_4-Inoculum size (IS: 1%=1×10^6 CFU; 2%=3×10^8 CFU*) X_5-Agitation rate (AR); X_6-Carbon source (Carb); X_7-Nitrogen source (Nitro); X_8-Magnesium sulfate (MgS); X_9-Ammonium sulphate (AS); X_{10}-Copper sulphate (CS); X_{11}-Trace elements (TE); CFU*- Colony forming unit.*

Table 5: ANOVA for Plackett-Burman factorial model for laccase activity

Source	Degrees of freedom (DF)	Adjusted sums of squares (Adj SS)	Adjusted mean squares (Adj MS)	F-Value	P-Value
Inoculum size (X_4)	1	20.803	20.8033	40.79	0.001
Trace elements (X_{11})	1	17.280	17.2800	33.88	0.002
Magnesium sulphate (X_8)	1	10.830	10.8300	21.24	0.006
pH of the media (X_1)	1	6.750	6.7500	13.24	0.015
Agitation rate (X_5)	1	6.453	6.4533	12.65	0.016
Incubation time (X_3)	1	4.813	4.8133	9.44	0.028
Error	5	2.550	0.5100	---	---
Total	11	69.480	---	---	---

Keys: X_1-pH;X_3-Incubation time; X_4-Inoculum size; X_5-Agitation rate; X_8-Magnesium sulphate; X_{11}-Trace elements respectively Model summary: R-sqared (R^2) = 96.33%; R-sqared (R^2 pred) =78.86%; R- sqared (R^2 adj) = 91.93%.



Where all variables are arranged according to their significance and studied at low level (-) and high level (+). The variables X_1 to X_{11} denotes: X_1-pH; X_2-Temperature; X_3-Incubation time; X_4-Inoculum size; X_5-Agitation rate; X_6-Carbon source (wheat bran); X_7-Nitrogen source (Ammonium nitrate); X_8-Magnesium sulphate; X_9-Ammonium sulphate; X_{10}-Copper sulphate; X_{11}-Trace elements respectively

Fig.2: Pareto plot for Plakett-Burman Design of Experiments for Laccase production from *Enterobacter cloacae* strain T137

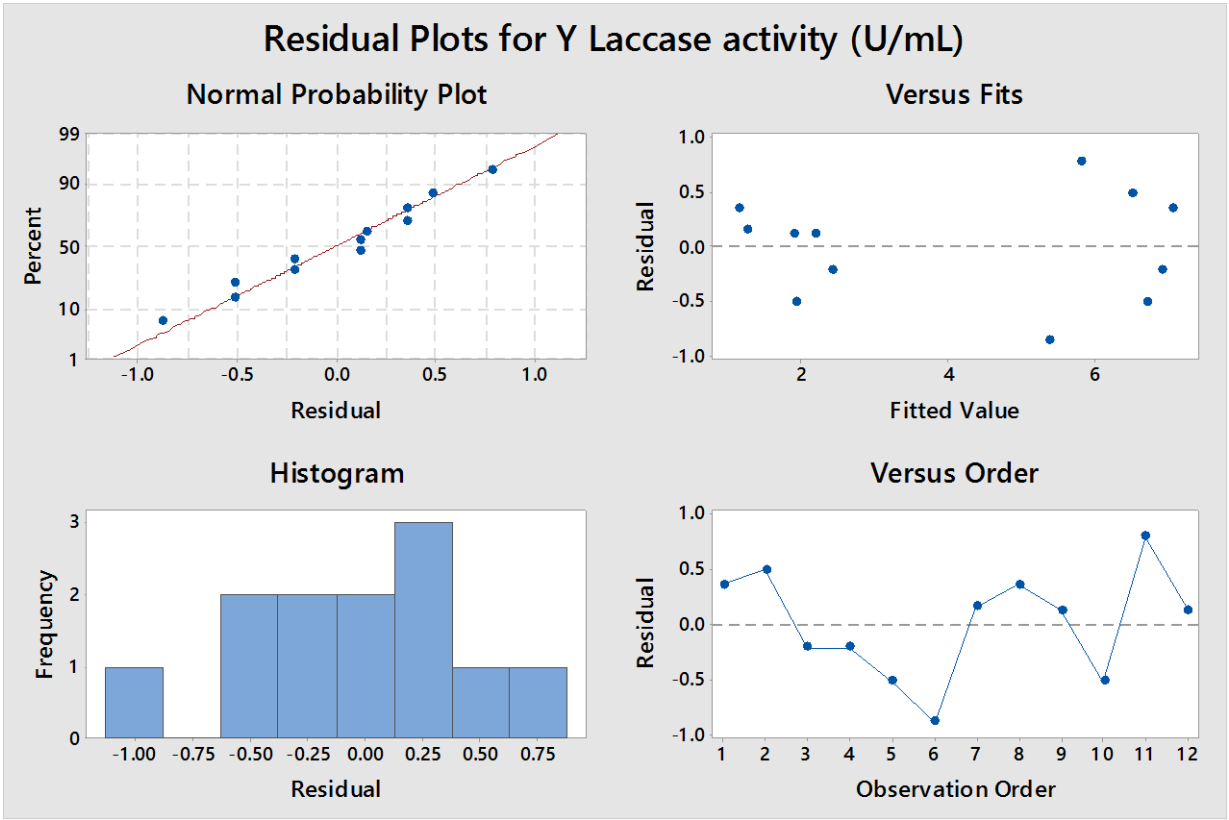


Fig. 3: Residual plot analysis of laccase production by *Enterobacter cloacae* strain T137 in submerged fermentation by Plakett-Burman factorial model

4. CONCLUSION

Optimization of process parameters for laccase production by *Enterobacter cloacae* strain T137 in submerged fermentation revealed that the laccase production by this isolate was influenced by the presence of a low percentage of sodium chloride (NaCl) and alkaline pH of 8.0 in the production medium. The present study had also focused on the prospective use of wheat bran as low-cost agro-residue for laccase production by *Enterobacter cloacae* strain T137. The application of statistical design for screening and optimization of process parameters allows quick identification of important factors early in the process. In the present study, Plakett-Burman experiments are useful in studying the physicochemical factors that supported the enhanced production of laccase by *Enterobacter cloacae* strain T137 under submerged fermentation. The study result showed that an improvement of laccase yield over the single parameter optimization by*Enterobacter cloacae* strain T137.

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Conflict of interest

The authors declare no conflict of interest.

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