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RESEARCH ARTICLE

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Optimization of Growth Medium and Fermentation Conditions for the Production of Laccase3 from *Cryphonectria parasitica* Using Recombinant *Saccharomyces cerevisiae*

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ABSTRACT

Statistical experimental methods were used to optimize the medium for mass production of a novel laccase3 (Lac3) by recombinant *Saccharomyces cerevisiae* TYEGLAC3-1. The basic medium was composed of glucose, casamino acids, yeast nitrogen base without amino acids (YNB w/o AA), tryptophan, and adenine. A one-factor-at-a-time approach followed by the fractional factorial design identified galactose, glutamic acid, and ammonium sulfate, as significant carbon, nitrogen, and mineral sources, respectively. The steepest ascent method and response surface methodology (RSM) determined that the optimal medium was (g/L): galactose, 19.16; glutamic acid, 5.0; and YNB w/o AA, 10.46. In this medium, the Lac3 activity (277.04 mU/mL) was 13.5 times higher than that of the basic medium (20.50 mU/mL). The effect of temperature, pH, agitation (rpm), and aeration (vvm) was further examined in a batch fermenter. The best Lac3 activity was 1176.04 mU/mL at 25 °C, pH 3.5, 100 rpm, and 1 vvm in batch culture.

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1. Introduction

Laccases (bezenediol: oxygen oxidoreductase; EC 1.10.3.2) are multi-copper-binding phenoloxidases that are found in plants as well as many insects and a variety of fungi [1–3]. Laccases are particularly widespread in ligninolytic basidiomycetes, and more than 125 different basidiomyceteous laccase genes have been described [4]. Laccases, using oxygen as the final electron acceptor, catalyze the reduction of oxygen to water accompanied by the oxidation of a number of aromatic substances such as diphenols, methoxy-substituted monophenols, and aromatic amines [5]. Many laccases contain a total of four copper ions and are characterized by the presence of one type-1, one type-2, and two type-3 copper ions. A single electron is removed from the substrate by the type-1 copper ion, which is subsequently transferred to the type-2/type-3 copper site, where molecular oxygen is reduced to water [6]. Because laccases can oxidize a broad range of polyphenolic substrates without the use of costly cofactors such as NADH or NADPH, industrial applications for laccases include delignification [7], the purification of colored waste water [8], textile dye decoloration [9], beverage and food treatment [10], the sulfurization and solubilization of coal for use in enzyme-based biosensors [11], the bioremediation of toxic environmental pollutants [12], and the pretreatment of lignocellulosic bioenergy resources [13,14]. In addition, recent studies have shown that laccase substrate specificity can be further broadened in the presence of redox mediators [15]. Therefore, given the versatility and broad-spectrum substrate specificity, laccases could become among the most important biocatalysts in biotechnology [3].

During the past several decades, numerous laccases have been isolated and characterized, mainly from fungal species and a few types of bacteria [16–18]. Among the many different laccase types, fungal laccases seem to have more favorable properties for commercial applications than other plant and bacterial enzymes due to the high reduction potential of their type-1 copper [19]. However, most fungal laccases have not been industrially useful due to low production levels. Thus, the heterologous expression of fungal laccases is inevitable for mass production and application. Laccases are

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notoriously difficult to produce in nonfungal expression systems [20]. Thus, several fungi, including *Saccharomyces cerevisiae* [20–22], *Trichoderma reeesei* [23], *Aspergillus oryzae* [24], *Pichia pastoris* [25], *Kluyveromyces lactis* [21], *Aspergillus sojae* [26], and *Aspergillus niger* [27] have been used for the heterologous expression of laccase in the past.

Recently, we characterized a new laccase, laccase3 (Lac3), from the chestnut blight fungus *Cryphonectria parasitica,* which is induced by the presence of tannic acid [28] and were able to express Lac3 using *S. cerevisiae* as a heterologous host [29]. Given that this enzyme is specific to tannic acid, Lac3 represents a new laccase type isolated from a necrotic but not wood-decaying fungus. Therefore, the heterologous production of a functional Lac3 was worthwhile to better characterize the enzyme and pursue its potential industrial applications. However, heterologous production of Lac3 using *S. cerevisiae* resulted in harmful effects on the growth of *S. cerevisiae* [29].

In this study, the optimization of heterologous production of Lac3 using recombinant *S. cerevisiae* was performed.

2. Materials and methods

2.1. Chemicals and enzymes

Unless specified otherwise, all chemicals, media, and enzymes used in this study were purchased from Sigma Chemical Co. (St. Louis, MO), Difco Laboratories (Detroit, MI), or Boehringer Mannheim (Mannheim, Germany), respectively.

2.2. Strains and culture conditions

Plasmids were maintained and propagated in *Escherichia coli* HB101 or DH5α as described in Green and Sambrook [30]. The recombinant *S. cerevisiae* TYEGLAC3-1 strain transformed with an episomal vector for the expression of cDNA encoding Lac3 from *C. parasitica* was obtained from our previous study [29], and was used for the heterologous production of Lac3 [28].

S. cerevisiae TYEGLAC3-1 was maintained in uracil-deficient (ura^-) selective medium (yeast nitrogen base without amino acids (YNB w/o AA), 6.7 g/L; adenine and tryptophan, 0.03 g/L; casamino acids, 5 g/L; dextrose, 20 g/L; and agar, 20 g/L). A primary inoculum was prepared from 5.0 mL ura^- selective medium cultured for 48 h, then a 50 mL conical tube containing 5 mL ura^- selective broth was inoculated with 10% (v/v) primary inoculum and cultured for 16 h. Expression cultures were carried out with 10% (v/v) seed culture in a 250-mL Erlenmeyer flask containing 50 mL ura^- selective broth and grown at 30 °C with continuous agitation (200 rpm) for 48 h, after which culture supernatants were obtained by centrifugation at 3,000×*g* and were assayed for laccase activity as described previously [31]. Dry cell weight (DCW) was determined according to a standard curve obtained by the comparison of optical density at 660 nm and the measured dried cell weight (DCW) after dried at 105 °C until no change in the weight was detected. Residual carbohydrate such as galactose was measured as previously described [32].

2.3. Medium optimization using the one-factor-at-a-time method

2.3.1. Selection of carbon, nitrogen, and other salts (sulfate and phosphate) sources

The basal medium before optimization consisted of ura^- selective broth. For carbon source screening, dextrose was replaced with the same concentration of fructose, galactose, lactose, maltose, and sucrose while keeping other components in the medium constant. For nitrogen source screening, casamino acids were replaced with the same concentration of L-glutamic acid, L-aspartic acid, 3,5-dinitro salicylic acid, 2-hydroxy-3,5-dinitro-benzoic acid, and nitric acid, while keeping other components in the medium constant. The best carbon and nitrogen sources were determined according to Lac3 production measured by laccase activity in the culture filtrate. Other salts such as sulfate and phosphate sources were compared with 0.5% ammonium sulfate contained in the YNB w/o AA medium. For sulfate and phosphate screening, ammonium sulfate was replaced by ferric sulfate, iron sulfate, potassium sulfate, sodium sulfate, potassium phosphate dibasic, ammonium phosphate monobasic, sodium phosphate monobasic, di-sodium hydrogenphosphate-12-H_2O, or magnesium phosphate while keeping other components in the medium constant except for the replacement of YNB w/o AA with YNB w/o AA and ammonium sulfate. The best salt sources were determined according to Lac3 production and cell growth.

2.4. Statistical optimization of the medium

2.4.1. Screening of significant variables using the Plackett–Burman design

The Plackett–Burman design was used to choose the important factors that affect Lac3 production. The five variables in ura^- selective broth (YNB w/o AA, adenine, tryptophan, glutamic acid, and galactose) were studied at two levels to screen the most significant parameters affecting Lac3 production. The low

level (−1) and high level (+1) for each factor are listed in Table 1. The statistical software Design Expert pro (Stat-Ease Inc. Minneapolis, MN) was used for experimental design and to determine the significance of each variable *via* *t*-test.

2.4.2. Fractional factorial design

Based on the results from the different carbon, nitrogen, and salt sources screens through the one-factor-at-a-time classical method and different components by the Plackett–Burman design, galactose, glutamic acid, and YNB w/o AA were found to be the best sources for Lac3 production, respectively. Galactose as the carbon source, glutamic acid as the nitrogen source, and YNB w/o AA were taken as independent variables for response surface methodology (RSM) optimization. Each variable was studied at three levels (+1, 0, −1): for galactose: 24, 20, and 16 g/L; glutamic acid: 6, 5, and 4 g/L; and YNB w/o AA: 8.04, 6.70, and 5.36 g/L.

2.4.3. Method of steepest ascent and central composite design (CCD)

Using the method of steepest ascent, the optimal region was determined when laccase activity exhibited a turning point in the response. The difference and design are summarized in Table 2. The central composite design (CCD) under the RSM was employed to illustrate the nature of the response surface. This approach identified the key factors themselves or their interactions by acquiring the response required to fit the polynomial. In this study, CCD contained an imbedded full or fractional factorial matrix with center points and "star points" around the center point that allow estimation of the curvature [33]. One unit was designated to the distance from the center of the design space to a factional point, while α was designated to the distance from the center of the design space to a star point. If the factorial was a full factorial, then $\alpha = [2^k]^{1/4}$. The star points represent new extreme values for each factor in this design. The total number of experimental combinations is 2^k+2k+n_0, where k is the number of independent variables and n_0 is the number of repetitions of the experiments at the center point.

For statistical calculations the independent variables were coded as follows:

$$x_i = \frac{(X_i - X_o)}{\delta X_i} \quad (1)$$

where X_i is the experimental value of variable; Xo is the midpoint of X_i, δX_i is the step change in X_i, and x_i is the coded value for X_i, $i = 1 - k$.

Data from the CCD were analyzed by multiple regressions through the least-squares method to fit the following equation:

$$Y = \beta_o + \sum_{I=1}^{K} \beta_i x_i + \sum_i^{\kappa} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response, β_0 the intercept term, β_i the linear coefficient, β_{ii} the squared coefficient, and β_{ij} the interaction coefficient. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2.

In CCD, galactose, glutamic acid, and YNB w/o AA were the three independent variables as the axial points with five replicated central points. The experimental design and the corresponding results are summarized in Tables 3 and 4. After having obtained the response of 17 experiments listed in

Table 1. The Plackett–Burman design for five variables.

	Variables						
Trial	A	B	C	D	E	Lac3 (mU/mL)	DCW[a] (g/L)
M1	+	−	+	−	−	159.17	6.06
M2	+	+	−	+	−	121.13	6.11
M3	−	+	+	−	+	160.95	5.87
M4	+	−	+	+	−	158.75	6.36
M5	+	+	−	+	+	120.30	5.92
M6	+	+	+	−	+	163.91	6.89
M7	−	+	+	+	−	160.89	6.09
M8	−	−	+	+	+	153.58	5.89
M9	−	−	−	+	+	110.98	5.18
M10	+	−	−	−	+	112.95	5.64
M11	−	+	−	−	−	120.84	4.98
M12	−	−	−	−	−	111.09	5.04

A: galactose (+: 24 g/L, −: 16 g/L); B: glutamic acid (+: 6 g/L, −: 4 g/L); C: yeast nitrogen base w/o amino acids (+: 8.04 g/L, −: 5.36 g/L); D: tryptophan(+: 0.045 g/L, −: 0.015 g/L); E: adenine(+: 0.045 g/L, −: 0.015 g/L).
[a]DCW indicates dry cell weight.

Table 2. Experimental design for the optimization of Lac3 production medium through the steepest ascent method (SAM).

	Galactose	Glutamic acid	Yeast nitrogen base w/o amino acids
Center point	20	5	6.7
Origin step	4	1	1.34
Main effect	−16.81	0.17	67.2
Coefficient	−8.405	0.085	33.6
r value		4.335	
Slope value	−1.052	0.011	4.206
New step	−4.208	0.011	5.636

Table 3. Run table of the experimental design for the optimization of Lac3 production medium through the steepest ascent method (SAM).

No.	Galactose (g/L)	Glutamic acid (g/L)	Yeast nitrogen base w/o amino acids (g/L)
Center point	20	5	6.7
1	19.160	5.002	7.826
2	18.320	5.004	8.952
3	17.480	5.006	10.078
4	16.640	5.008	11.204
5	15.800	5.010	12.330

Table 4. Concentration of the respective medium components at each level (central composite design for the response surface method).

		Level (g/L)				
Xn	Independent variables	$-\alpha$	−1	0	+1	$+\alpha$
X_1	Galactose	12.72	15.33	19.16	22.99	25.61
X_2	Glutamic acid	3.32	4.00	5.00	6.00	6.68
X_3	Yeast nitrogen base w/o amino acids	5.196	6.26	7.83	9.40	10.46

α: 1.682.

Table 5, the statistical software Design Expert pro was used to determine an empirical relationship between Lac3 production and the three dependent variables. Each experimental design was carried out in duplicate.

2.5. Optimization of fermentation conditions using optimized medium and a 2.5-L fermenter

Optimization of batch fermentation was carried out using a 2.5-L fermenter (Kobio Tech Co. Daejeon, Korea) containing 1.5 L optimized medium inoculated with 10% (v/v) seed culture. Temperature, pH, airflow, and agitation were varied one at a time. Temperature was varied from 25 to 40 °C at 5 °C intervals. The pH was changed from 3 to 5 at 0.5 intervals. Two different airflows of 1 vvm (volume of air/volume of fluid/min) and 2 vvm were compared. Three different agitation speeds at 100, 200, and 300 rpm were compared for optimal Lac3 production.

2.6. Laccase assay

The laccase activity in the culture filtrate was determined by spectrophotometric assay using 10 mM 2,6-dimethoxyphenol (DMOP) as substrate in sodium tartrate buffer at pH 3.4 [34]. A laccase unit was defined as a 1.0/min A_{468} increase at 25 °C as described previously [34].

3. Results and discussion

3.1. Selection of carbon, nitrogen, and mineral sources

The effects of different carbon sources on Lac3 production by recombinant *S. cerevisiae* (TYEGLAC3-1) cells were examined by one-factor-at-a-time analysis. Among the six different carbon sources, galactose was the best carbon source with the laccase activity at 39.30 mU/mL, which was an approximate two-fold increase from that of the basal ura$^-$ selective medium using glucose (data not shown). In addition, there was no significant difference in DCW between glucose and galactose. The effects of different nitrogen sources on Lac3 production were also examined. Among the six different nitrogen sources, glutamic acid resulted in the highest laccase activity at 186.78 mU/mL when galactose was employed as the carbon source (data not shown). Compared to Lac3 activity in ura$^-$ selective medium at 20.50 mU/mL, a greater than nine-fold increase in Lac3 activity was achieved using galactose and glutamic acid as the carbon and nitrogen sources, respectively. Interestingly, aspartic acid resulted in Lac3 activity at 175.79 mU/mL; however, the DCW of the corresponding culture was significantly reduced to 4.77 g/L, which was 75% to 80% of the growth in ura$^-$ selective medium and glutamic acid media, respectively. These results clearly indicate differences in nutrient composition affect the growth of cells and subsequent Lac3 production.

To determine the appropriate sulfur and phosphate sources, 0.5% ammonium sulfate in YNB w/o AA was replaced with the same concentration of ferric sulfate, iron sulfate, potassium sulfate, sodium sulfate, potassium phosphate dibasic, ammonium phosphate monobasic, sodium phosphate monobasic, di-sodium hydrogenphosphate-12-H_2O, magnesium phosphate with galactose and glutamic acid as the carbon and nitrogen sources, respectively. Among the salts tested, all showed Lac3 activity at concentrations less than 40.00 mU/mL and no significant differences were observed. However, when the 0.5% ammonium sulfate as YNB w/o AA was employed, a Lac3 activity at 185.32 mU/mL was obtained, which is similar to that of medium containing galactose, glutamic acid, and YNB w/o AA.

Thus, the galactose, glutamic acid, and YNB w/o AA medium, was chosen as the sources of carbon, nitrogen, and minerals, respectively, for further optimization.

3.2. Screening of significant variables by the Plackett–Burman design

The Plackett–Burman design can be used to screen the most important factors from a set of process variables; as such, it is a useful tool to distinguish variables whose effects can be ignored for further optimization. Five variables were screened by the Plackett–Burman design and 12 experiments were conducted as outlined in Table 1. The greatest amount of Lac3 production was 163.91 mU/mL observed in M6 medium. High levels of Lac3 activity were observed in M1, M3, M4, M6, M7, and M8 media containing high concentrations of YNB w/o AA. The variables indicating statistically significant effects on Lac3 activity were selected based on the mean square values. As shown in Table 6, the YNB w/o AA, glutamic acid, and galactose with mean square values of 5631.51, 143.59, and 26.60,

Table 5. Experimental matrix for the central composite design experiment and determined Lac3 production and dry cell weight in TYEGLAC3-1 cells.

	Culture condition					
Exp No.	Galactose	Glutamic acid	Yeast nitrogen base w/o amino acids	Predicted Lac3 (mU/mL)	Experimental Lac3 (mU/mL)	DCW (g/L)
1	−1	−1	−1	121.56	130.43	5.68
2	+1	−1	−1	116.82	129.52	5.10
3	−1	+1	−1	142.80	156.52	5.18
4	+1	+1	−1	143.87	154.22	5.23
5	−1	−1	+1	200.74	197.11	5.76
6	+1	−1	+1	192.52	185.55	5.93
7	−1	+1	+1	204.11	198.15	5.73
8	+1	+1	+1	201.89	199.95	6.06
9	−α	0	0	172.85	168.51	5.39
10	+α	0	0	166.87	161.79	6.13
11	0	−α	0	121.49	117.99	6.33
12	0	+α	0	147.07	140.43	6.41
13	0	0	−α	147.82	123.81	5.83
14	0	0	+α	262.87	277.04	6.64
15	0	0	0	239.54	238.93	5.77
16	0	0	0	239.54	246.39	5.79
17	0	0	0	239.54	234.95	5.72

Each variable of culture conditions was studied at five levels (+α, +1, 0, −1, and −α): for galactose: 25.61, 22.99, 19.16, 15.33, and 12.72 g/L; glutamic acid: 6.68, 6, 5, 4, and 3.32 g/L; and YNB w/o AA: 10.46, 9.40, 7.83, 6.26, and 5.196 g/L.

Table 6. Analysis of Lac3 shown in Table 1.

	A	B	C	D	E
∑(H)	836.20	848.02	957.25	825.64	822.67
∑(L)	818.34	806.51	697.29	828.90	831.87
Difference	17.87	41.51	259.96	−3.27	−9.20
Effect	2.98	6.92	43.33	−0.54	−1.53
Mean square	26.60	143.59	5631.51	0.89	7.06

H and L represent data using high and low levels, respectively.

Table 7. Experimental matrix for the three-factor fractional factorial design (FFD) and Lac3 production in TYEGLAC3-1 cells.

	A	B	C	Lac3 (mU/mL)	DCW[a] (g/L)
1	−	−	−	133.76	0.71
2	+	−	−	99.25	0.72
3	−	+	−	101.71	0.7
4	+	+	−	160.13	0.77
5	−	−	+	243.29	0.83
6	+	−	+	152.61	0.98
7	−	+	+	184.10	0.87
8	+	+	+	183.63	0.89
9	0	0	0	193.45	0.82
10	0	0	0	198.83	0.82

A: galactose (+: 24 g/L, 0: 20 g/L, and −: 16 g/L); B: glutamic acid (+: 6 g/L, 0: 5 g/L, −: 4 g/L); C: yeast nitrogen base w/o amino acids (+: 8.04 g/L, 0: 6.7 g/L, −: 5.36 g/L).
[a]DCW indicates dry cell weight.

respectively, were found to be three most influencing factors for the production of Lac3 and were chosen for further optimization.

3.3. Determination of variable effects using fractional factorial design

To combine the different states of the three factors (galactose, glutamic acid, and YNB w/o AA), a fractional factorial approach was applied through 10 experimental runs. The corresponding fractional factorial experimental design matrix for the production of Lac3 and cell growth as determined by DCW is shown in Table 7 and was further analyzed using the statistical software Design Expert pro. The following second-order polynomial equation was found to represent Lac3 production adequately (A, B, and C represent galactose, glutamic acid, and YNB w/o AA, respectively), where Y (mU/mL)$=157.31-8.41\times A+0.083\times B+33.60\times C+22.89\times A\times B-14.38\times A\times C-7.13\times B\times C3$.

According to the sign of the main effect to improve Lac3 production, it was indicated that an increase in both glutamic acid and YNB w/o AA concentration had a positive effect on Lac3 production, while an increase in galactose had a negative effect (Table 2). In addition, according to the sign of the interaction between the variables, the galactose-glutamic acid interaction had a positive effect of 22.89 whereas the galactose-YNB w/o AA interaction and the glutamic acid-YNB w/o AA interaction exhibited negative effects. These results were in good agreement with the data from screening of the nitrogen source while galactose was employed as the carbon source and demonstrated a remarkable increase from the comparison (39.30–186.78 mU/mL).

3.4. Determination of the central point levels by steepest ascent experiments

The RSM regression equation can reflect the approximate real situation only in the neighborhood regions close to the highest point of the response surface. Therefore, the real levels of the central point (the coded 0 level for the three significant variables) should be determined. As can be seen from Table 2, the initial concentrations of glutamic acid and YNB w/o AA had a significant positive effect and should be increased to approach the highest Lac3 production levels. However, the galactose should be

decreased for further optimization. According to the effect direction of the three factors, the steepest ascent method was designed using the central points of the fractional factorial design as the starting point of SAM. Based on the determined *r*-value, glutamic acid and YNB w/o AA increased to 0.002 and 1.126, respectively, from the starting point (Table 3). Galactose decreased (0.840) from the starting point. The highest amount of Lac3 activity at 229.88 mU/mL was obtained at step 2 and consisted of 18.320, 5.004, and 8.952 g/L of galactose, glutamic acid, and YNB w/o AA, respectively, suggesting that this point was near the region of maximum Lac3 production, and was, therefore, chosen for further analysis.

3.5. Effects of galactose, glutamic acid, and yeast nitrogen base w/o amino acids on Lac3 production

CCD is a very useful tool to determine the optimal level of medium components and their interactions. The levels of the three variables for the CCD experiment were selected according to the results of the SAM path. The concentrations of the major nutrients tested are presented in Table 4 and the CCD design matrix is presented in Table 5. Using Design Expert pro software, a second-order polynomial model for Lac3 production was obtained from a regression analysis of the CCD results (A, B, and C represent galactose, glutamic acid, and YNB w/o AA, respectively), where Y (Lac3 production)$= 239.54 - 1.78 \times A + 7.61 \times B + 34.25 \times C + 1.49 \times A \times B - 0.82 \times A \times C - 4.42 \times B \times C$.

The model adequacy was verified by the F test and the determination coefficient R^2. The high *F*-value (17.36) and a very low probability ($p > F = .0005$) indicated that the present model was in good prediction of the experimental results. Therefore, the obtained mathematical model was adequate. In addition, the goodness of fit of the model was expressed by the coefficient of determination, R^2 (0.9571), indicating that 95.71% of the variability in the response could be explained by the model and only about 4.29% of the total variation was not explained by the model.

The effects of galactose, glutamic acid, and YNB w/o AA concentrations on Lac3 production were further analyzed by RSM. The three-dimensional response surfaces for the optimization of medium components were plotted as presented in Figure 1 illustrates the effects of galactose, glutamic acid, and YNB w/o AA on Lac3 production. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant. From the 3 D response surface plots and the 2 D corresponding contour plots (Figure 1), the optimal values of the independent variables and the corresponding response could be predicted, and the interaction between each independent variable pair could be understood. An elliptical contour indicated that there was a perfect interaction between the independent variables, including glutamic acid and YNB w/o AA, as well as galactose and YNB w/o AA. Figure 1 shows a nearly circular contour, indicating little interaction between galactose and glutamic acid. The maximum predicted value was identified by the surface confined in the smallest ellipse in the contour diagram and it was determined to be 19.16, 5.0, and 10.46 g/L of galactose, glutamic acid, and YNB w/o AA, respectively (Table 5). To verify the RSM results, laccase activity was examined based on the CCD design matrix in Table 5. Experimental laccase activity was highest in accordance with the optimal value derived from RSM in respect of Lac3 production rate. A total of 277.04 mU/mL of Lac3 activity was produced in the optimal medium of 19.16, 5.0, and 10.46 g/L of galactose, glutamic acid, and YNB w/o AA, respectively (Table 5).

3.6. Optimization of fermenting parameters based on the optimal fermentation medium

In order to optimize the fermenting conditions using the optimal fermentation medium, temperature was varied from 25 to 40 °C while other parameters such as agitation, aeration, and pH were fixed at 200 rpm, 2 vvm, and pH 5.0, respectively. The response indicated that Lac3 production decreased as the temperature increased from 25 °C. At 25 °C, 388.19 mU/mL of Lac3 was produced after 21 h fermentation. The optimum temperature for the Lac3 production differed from those of *Trametes sp*., *F. fraxinea*, and *Lentinula edodes* fungal species. In addition, Lac3 production was different from that of yeast growth at 30 °C, but was coincident with the optimum growth temperature of *C. parasitica* where the Lac3 gene originated, indicating that the optimum temperature reflected both growth conditions and Lac3 enzymatic activity.

The optimum pH was examined by varying the pH from 3 to 5 under 200 rpm, 2 vvm, and 25 °C conditions. Maximum Lac3 production was achieved at pH 3.5, which was within the optimal pH range for purified laccase activity. The maximum yield of Lac3 activity was 588.02 mU/mL under 200 rpm, 2 vvm, and pH 3.5 at 25 °C conditions.

A pair-wise comparison of aeration between 1 and 2 vvm was conducted under conditions of 200 rpm and pH 3.5 at 25 °C. Compared to 588.03 mU/mL at 2 vvm, Lac3 production increased

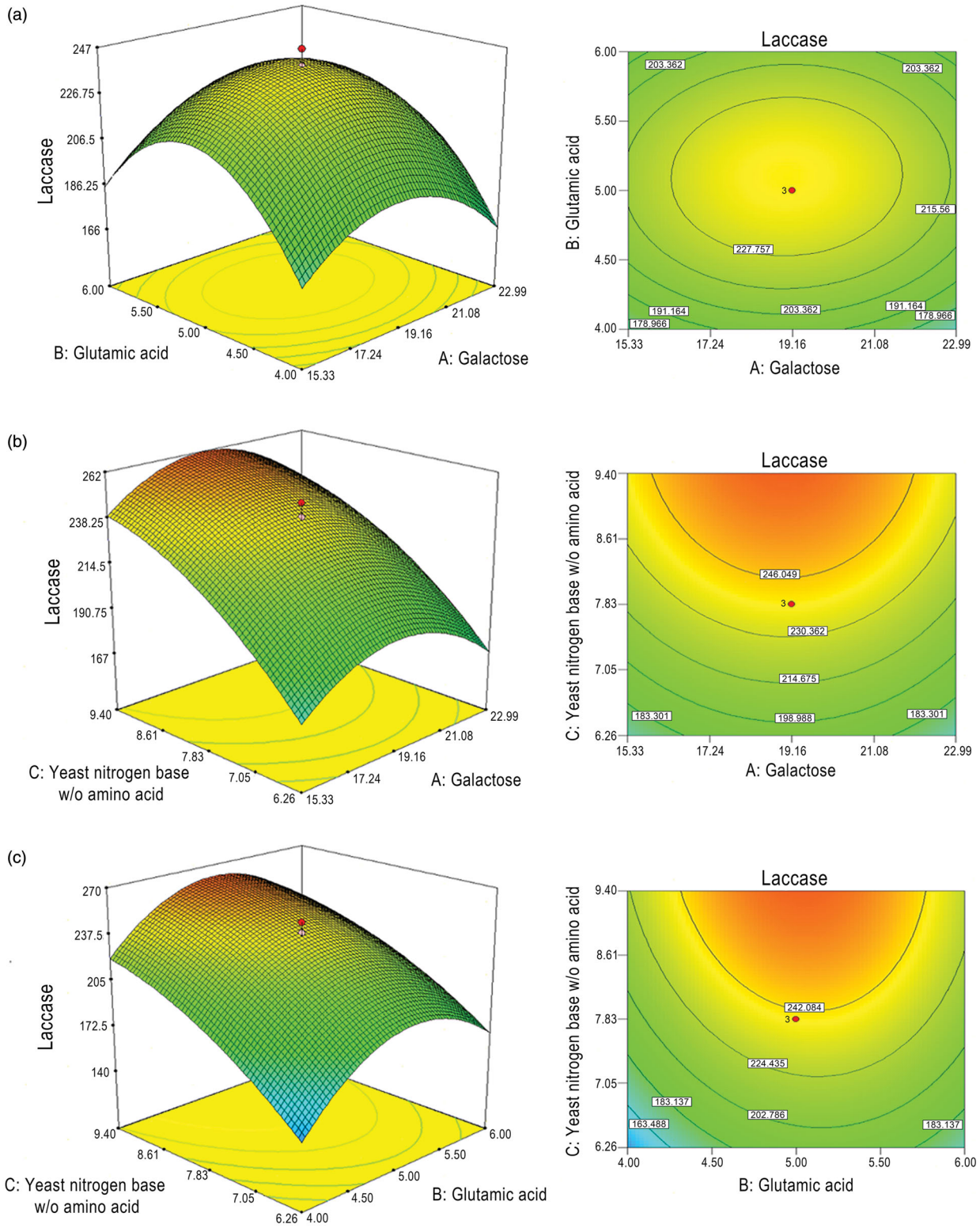

Figure 1. Three-dimensional mesh plot and 2 D contour plot of Lac3 production. (a) The effect of galactose (A) and glutamic acid (B) on Lac3 production with other components set at center level; (b) The effect of galactose (A) and YNB w/o AA (C) on Lac3 production with other components set at center level; (c) The effect of glutamic acid (B) and YNB w/o AA (C) on Lac3 production with other components set at center level.

to 1003 mU/mL at 1 vvm, which was a 1.7-fold increase by decreasing the vvm.

Three different agitation speeds, 100, 200, and 300 rpm were examined under the conditions of 1 vvm and pH 3.5 at 25 °C for increased Lac3 production. The corresponding Lac3 activities were 1157.04, 1003.88, and 960.94 mU/mL for 100, 200, and 300 rpm, respectively.

The optimum culture conditions of temperature, agitation, aeration, and pH were determined to be 25 °C, 100 rpm, 1 vvm, and pH 3.5, respectively, for the production of Lac3. The maximum Lac3 activity

was 1157.04 mU/mL at the optimal conditions using optimal fermentation medium. The cell mass increased up to 54 h after inoculation at which point 2.99 g/L (36% residual amount) galactose still remained.

In conclusion, the optimum fermentation medium components were determined for maximum Lac3 production using statistical analyses. The validated Lac3 production was 277.04 mU/mL in the optimal medium of 19.16, 5.0, and 10.46 g/L galactose, glutamic acid, and YNB w/o AA, respectively. Optimizing the culture conditions such as temperature, pH, aeration, and agitation further increased the maximum yield of Lac3 production. Under optimized culture conditions of 25 °C, 100 rpm, 1 vvm, and pH 3.5, the maximum Lac3 production was at 1157.04 mU/mL. This represents an approximately 56-fold increase over production using conventional media and culture conditions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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