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Application of Experimental Designs for Optimization the Production of *Alcaligenes Faecalis* Nyso Laccase

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A sequential optimization strategy based on statistical experimental designs was implemented in order to enhance laccase production by a local isolate *Alcaligenes faecalis* NYSO in a submerged culture. To screen the parameters significantly influencing the laccase productivity, a 2-level Plackett-Burman design was applied. Among the studied variables, the pH, yeast extract, $(NH_4)_2SO_4$, glucose, and $CuSO_4.5H_2O$ were selected based on their high positive significant effect on laccase productivity. In order to find out the combination among the most significant variables that brings maximum yield, Response Surface Methodology was applied, where a 3-level Box-Behnken design was utilized to create a polynomial quadratic model correlating the relationship between the five variables and the laccase productivity. The optimal combination of the major medium constituents for laccase production was evaluated using the JMP program, was as follows: yeast extract, 0.896%; $(NH_4)_2SO_4$, 0.035%; $CuSO_4.5H_2O$, 0.0075%; $FeSO_4.7H_2O$, 0.000133%; glucose, 0.0943%, pH 10.6 and 30 ^oC for 24 hrs. The predicted optimum laccase activity was 791U ml^{-1} min^{-1}, which was 700 times the activity with basal medium. In addition, the further optimization for both pH, $CuSO_4.5H_2O$ concentration lead the yield to be 2435 U ml^{-1}min at pH 11.0, 200 mg $CuSO_4.5H_2O$ which achieved after 18 hrs incubation time.

Keywords: Laccase, Fractional Factorial Design, Response Surface Methodology, *Alcaligenes faecalis* NYSO

Introduction

The need to the maintenance of ecosystem and environment is essential concern nowadays and problems associated with environmental pollution demonstrate the necessity to improve remediation processes for detoxification of a wide variety of xenobiotic compounds which continuously discharged into the environment. Green chemistry technology is an environmental friendly novel trend; it was evaluated to overcome the obstacles associated with the traditional methods and improve the products quality[1]. Laccases (p-benzenediol: oxygen oxidoreductase, 1.10.3.2) are blue multicupper oxidases had a unique structure, it is a dimeric or tetrameric glycoprotein, which usually contains four cupper atoms per monomer distributed in three redox sites. The exploitation of laccase in biotechnological processes requires the production of high amounts of the enzyme at low cost and hence the current research focused and oriented towards the identification and optimization of such an efficient production system[2]. An isolation and identification of environmental friendly bacteria for lignin bioremediation become an essential because all the previous researchers concentrated on using fungal treatments. Bacterial laccases have great potential as biocatalysts due to their intrinsic properties of high thermal and alkaline pH stability than fungi[2]. The major obstacle to bacterial laccase commercial application is the lack of sufficient enzyme stocks. In view of the importance of laccases, the main objective of this work directed to design a new strategy for production of bacterial laccase.

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Material and Methods

Sample collection and isolate sources

Sludge, slime, black liquor and wastewater samples used in this study were collected aseptically from different discharged sites of paper and pulp industries and tanning & leather factories, Alexandria, Egypt. Stone and soil samples were collected from Muzdalifah and Mina, Saudi Arabia, the temperature of samples was 48 °C at the time of collection. The

samples were transported to the laboratory, stored in a cold room then analyzed.

Enrichment and isolation of laccase producing bacteria

For isolation of neutrophils, alkaliphiles bacterial producing laccase, the bacterial isolates were enriched using buffered liquid mineral medium Brunner (MMB) provided with 5.0 mM guaiacol[3] at pH 7.0/or 9.8 and incubated overnight at 55 °C/30 °C in a shaker incubator (200 rpm). To isolate pure cultures, the diluted enriched cultivated products were spreading onto MMB/guaiacol plates and incubated at 30 °C and/or 50 °C for 24 hrs.

Qualitative screening for laccase producing bacteria

Qualitative estimation was carried out by using a buffered or non-buffered diluted LB agar plates supplemented with 5.0 mM guaiacol. The appearance of reddish brown colonies after incubation overnight at 30 ^{o}C or 50 ^{o}C indicated a positive laccase production.

Quantitative screening

Sample preparation and fractionation

The cell pellets were collected after growing in MMB/guaiacol broth by centrifugation at 6000 rpm for 30 min followed by washing with phosphate buffer (0.1 M; pH 8.0) then sonicated. The cell lysate was used for quantitative determination of laccase activity[4] and protein concentration[5]. The cell-free supernatant was used for determination of the glucose concentration using glucose oxidation kit (Diamond Diagnostics, Egypt).

Laccase activity assay

Laccase activity has been estimated calorimetrically using (ABTS) as a substrate with an extinction coefficient (ε) 436=29,300 $M^{-1}cm^{-1}$ at 436 nm[4]. The reaction mixture (1.5 ml) contained appropriately diluted enzyme extract, 0.1 M McIlvaine buffer pH 4.0 supplemented with 10 mM $CuSO_4.5H_2O$, and 0.35 ml 20 mM of ABTS reagent, the reaction mixture was running in parallel with the control aliquot and incubated at 55 ^{o}C for 10 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidization 1.0 mmol of ABTS per minute under above standard assay conditions; the activities were expressed in U ml^{-1} min^{-1}.

Amplification of the 16S rDNA gene, sequencing, and similarity

Among the cultivated isolates, only 31 isolates appeared high frequency of laccase activity were chosen for further molecular identification. The genomic DNA extracted from the selected isolates followed by an amplification of the 16S rRNA gene via the polymerase chain reaction technique, using universal primers. Automated DNA sequencing of the purified PCR-fragments was performed. Subsequently, the sequence has been deposited in the GenBank to get an accession number.

Biochemical and morphological characterization of the selected isolate

The most potent laccase producing strain was subjected to further identification such as morphological observation by scanning electron microscopy (JSM 5300 JOEL, USA), and physio-biochemical characterization using a commercial kit (Macrobact GNB 24E kit) Oxoid. In addition, qualitative screening for some enzymes (cellulase, xylanase, pectinase, protease, amylase and lipase/esterase) production was carried out by plate assay method using 0.2% of the corresponding substrate.

Statistical optimization for laccase production

Plackett- Burman design

Plackett-Burman Design (PBD) was used to find factors that influence laccase production significantly[6]. Table-2, illustrates the matrix design of variables under investigation as well as levels of each variable with the corresponding response in terms of laccase yield. The result of the design was subject to multiple regression analysis using the JMP program for the data analysis. From the statistical analysis, the main effect chart was used to elucidate the significance of variables dependent on their nature; positive or negative effects on the production process as described by Amara[7]. The significance of variables was determined by calculating the *p*-value through standard regression analysis. Plackett-Burman factorial design based on the first-order polynomial model:

$$Y = \beta_o + \sum \beta_i X_i$$

Where Y is the response (laccase activity U ml^{-1} min^{-1}), β_o is the model intercepts, β_i is the linear coefficient, and X_i is the level of the independent variable. A verification experiment was performed through which the predicted optimum levels of the independent variables were examined and compared to the basal condition setting and the average of enzyme production was calculated

Response surface methodology (Box-Behnken Design)

The most significant variables were selected for further determination of their optimal level with

respect to enzyme activity (U $ml^{-1}min^{-1}$) as a response via Response surface methodology (RSM) design. Table 4 depicts the matrix design and used levels of the coded variables together with the relative response. Modeling and analysis were carried out using the statistical software design JMP program. Moreover, three-dimensional plots were constructed for visual observation of the trend of maximum response and the interactive effects of the significant variables on the response by STATISTICA 5.0 software[8]. The correlation between the five parameters and the response (laccase activity) was described by the following predictive quadratic polynomial equation:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+\beta_{12}(X_1X_2)+\beta_{13}(X_1X_3)+\beta_{14}(X_1X_4)+\beta_{15}(X_1X_5)+\beta_{23}(X_2X_3)+\beta_{24}(X_2X_4)+\beta_{25}(X_2X_5)+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{55}X_5^2$$

Where Y is the predicted response (laccase activity U ml^{-1} min^{-1}); β_0 is the model intercept; X_1, X_2, X_3, X_4, and X_5, are the independent variables, β_1, β_2, β_3, β_4, and β_5 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24} and β_{25} are cross product coefficients; and β_{11}, β_{22}, β_{33}, β_{44} and β_{55} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by a coefficient of determination, R^2. The mathematical model generated during RSM implementation was validated further experimentally.

Laccase production at different pH values and cupper sulfate concentrations

In case of pH, the optimized medium was prepared and adjusted to different values 9.6, 10, 10.6, 11, 11.6, 12 and 12.6 either initially (0.2M NaOH) or by glycine-NaOH buffer (0.1 M), whereas, different concentrations of cupper sulfate were tested (75, 100, 150, 200 and 250 mg) under optimum pH. The inoculated flasks were incubated under shaking condition (200 rpm) at 30 ^oC for 20 hrs. Aliquots (5.0 ml) were withdrawn for bacterial growth and laccase activity determinations.

Growth pattern in final formulated optimized medium

Bacterial growth, laccase production, glucose consumption and concentration of total soluble protein are monitored in the final optimized medium under optimized cultivation conditions.

Results and Discussion

Isolation, screening, and identification of the most potent isolate

It was found through the primary screening program for laccase production from enrichment cultivated isolates that 80 and 22 isolates out of 189 bacterial isolates exhibited laccase activity feature under alkaliphilic condition (pH 9.8) at 30 ^oC and 55 ^oC, respectively; whilst, 27 and 60 isolates had a preference to grow on media under neutral condition (pH 7.0) at 30 ^oC and 55 ^oC, respectively. Thirty-three bacterial isolates, which show could be used as a promising model for the study, were picked out for the secondary stage of screening and molecular identification. The phylogenetic relationship between these isolates was illustrated in Figure 1a according to partial sequences of their 16srRNA with their accession numbers. Subsequently, the biotype NYSO was selected as an experimental model for further studies, where it proved to be a true alkaliphile, mesophile, in addition to being the most potent laccase producer. The recorded results of molecular identification revealed that the selected biotype NYSO was more closely related to *Alcaligenes faecalis* strain KZJ01, with an identity 99%, so it could be identified as *Alcaligenes faecalis* NYSO (KP859538). Moreover, the physiological characteristic of the selected isolate NYSO was shown in Table 1 with reference to Bergey's Manual of Systematic Bacteriology which emphasize the molecular genetics identity. The morphological characteristics showed that the biotype NYSO has a short rod shape, non spore forming gram-negative and the cell size ranging from 0.7 μm in width and 2.2 μm in length (Figure 1b). Furthermore, the *Alcaligenes faecalis* NYSO (KP859538) had the ability to produce some commercially important enzymes other than laccase such as amylase, pectinase, xylanase, and cellulase. Despite that, it failed to utilize skim milk and tributyrin; this confirms the absence of protease and lipase enzymes from the enzymatic machinery of the experimental strain.

Statistical optimization of laccase production by *Alcaligenes faecalis* NYSO

In the first approach, a Plackett- Burman design was applied. The data in Table 2 indicate that there was a wide variation in laccase activity during the 24 runs ranging from 0 to 454 U $ml^{-1}min^{-1}$. The main effect of the examined variables was calculated and summarized graphically in Figure 2a. Regression coefficient analysis was shown in Table 3. The polynomial model which describes the correlation between the twenty factors and the laccase activity could be presented as follows:

$$Y_{activity} = 2.4184016 + 0.9980264\ X_1 - 0.16787\ X_2 - 0.266863\ X_3 + 0.5742785\ X_4 - 0.567084\ X_5 - 0.625158$$

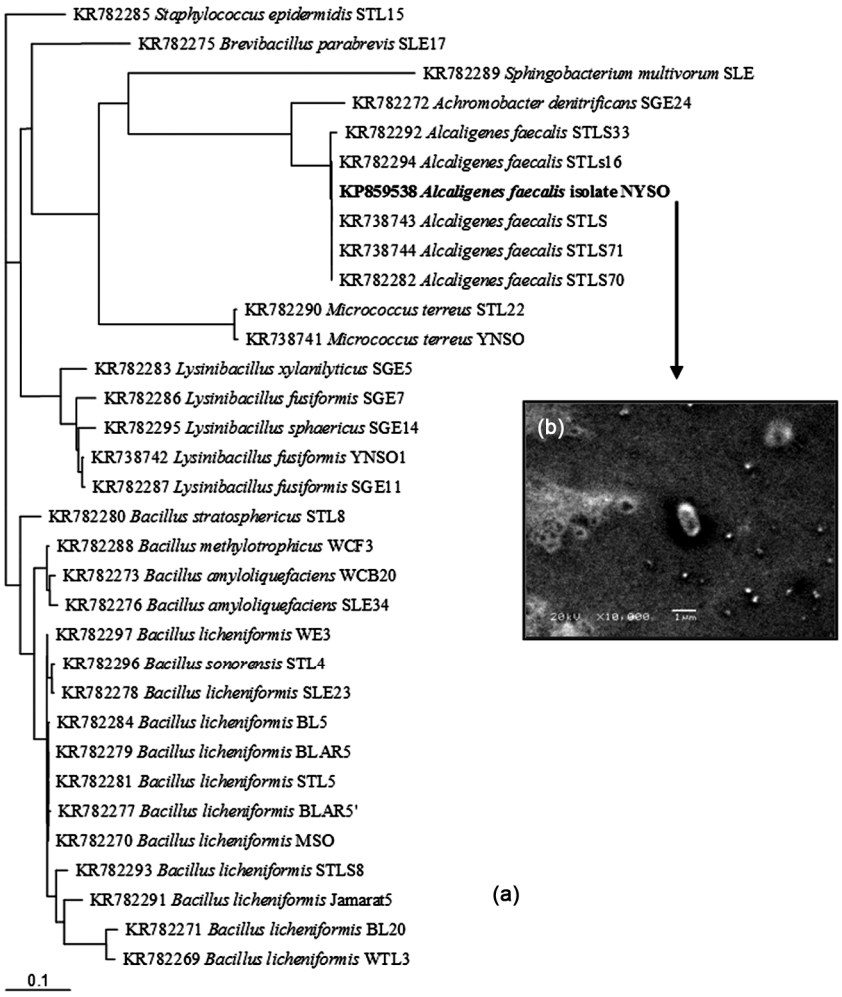

Fig. 1— a); Phylogenetic tree of the selected identified bacterial isolates involved in this study, b);The morphological characteristics of the selected isolate NYSO,

Table 1 — The biochemical characterisitics of *Alcaligenes faecalis* NYSO (KP859538)

S.N.	Test	Results	S.N.	Test	Results
1	Oxidase	+ve	15	TDA	- ve
2	Motility	+ ve	16	Gelatin	- ve
3	Nitrate	+ ve	17	Malonate	+ ve
4	Lysine	-ve	18	Inositol	- ve
5	Ornithine	- ve	19	Sorbitol	- ve
6	H_2S	+ ve	20	Rhamnose	- ve
7	Glucose	+ ve	21	Sucrose	- ve
8	Mannitol	- ve	22	Lactose	- ve
9	Xylose	- ve	23	Arabinose	- ve
10	ONPG	- ve	24	Adonitol	- ve
11	Indole	- ve	25	Raffinose	- ve
12	Urease	+ ve	26	Salicin	- ve
13	V-P	+ ve	27	Arginine	- ve
14	Citrate	+ ve	28	Catalase	+ ve

*ONPG=Hydrolysis of o-nitrophenyl-ß-d-galactopyranoside (ONPG) by action of ß- galactosidase
VP test = Voges-Proskauer test.

Table 2 — Randomized Plackett-Burman experimental design for evaluating factors influencing laccase production by *Alcaligenes faecalis* NYSO (KP859538).

Trials	Variables*																				Activity (Uml^{-1} min^{-1})
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17	X18	X19	X20	
1	1		-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	126.2799
2	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	0
3	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	0
4	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	0
5	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	0
6	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	148.1229
7	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	257.3379
8	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	12.28669
9	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	0
10	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	307.1672
11	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	0
12	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	0
13	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	68.25939
14	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	191.1263
15	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	163.1399
16	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	0
17	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	6.825939
18	1	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	454.6075
19	-1	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	78.49829
20	-1	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	144.0273
21	1	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	278.4983
22	-1	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	0
23	-1	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	263.4812
24	1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	23.89078

Variables*: X1-20 (tested variable); (-1) low level and (1) high level in g/l or values; X1 (yeast extract at levels 1-5); X2 (peptone at levels 1-5); X3 (casein hydrolysate at levels 1-5); X4 (glucose at levels 0.1-0.5; X5 (glutamic acid at levels 1-5); X6 (Na_2HPO4 at levels 1-5); X7 (KH2PO4 at levels 1-5); X8 (MgSO4.7H2O at levels 0.1-0.5); X9 [$(NH4)_2SO4$ at levels 0.1-0.5]; X10 (urea at levels 0.1-0.5); X11 (boric acid at levels 0-0.001); X12 (CuSO4.5H2O at levels 0-0.05); X13 (CaCL2.2H2O at levels 0.0-0.025); X14 (EDTA at levels 0-0.003); X15 (FeSO4.7H2O at levels 0-0.001); X16 (ZnSO4.7H2O at levels 0.0-0.001); X17 (MnCl.4H2O at levels 0.0-0.001); X18 (CoCl2.6H2O at levels 0,0-0.001); X19 (pH at values 8.6-10.6) and X20 (temp.. at values 30-37◦C)

X_6- 0.072866 X_7+ 0.1447472 X_8+0.4301934 X_9+ 0.0190741 X_{10}+ 0.0436668 X_{11}+ 2.4497618 X_{12}- 0.103631 X_{13}+ 0.0622652 X_{14}+ 0.5028155 X_{15}- 0.194691 X_{16}- 0.030219 X_{17}-0.528469 X_{18}+ 0.3297789 X_{19}- 0.622432 X_{20}.

From the statistical analysis, the greatest attention was paid to select six variables: $CuSO_4.5H_2O$, yeast extract, glucose, $FeSO_4.7H_2O$, $(NH_4)_2SO_4$ and pH, which had a significant effect on the laccase production and, they have confidence level > 90%. The analysis of variance using an ANOVA test was generated which gives p= 0.0056. This indicates that there is a statistically significant relationship between the variables at 99.6 % confidence level. The R- squared statistic indicates that the model is fitted explains 99.6% of the variability in laccase activity. A lot of investigators exploited the statistical experimental design for optimization of laccase production[9-10]. The present results are consistent with other studies that reported the cupper sulphate as laccase inducer, glucose and yeast extract revealed the most significant variables effect on laccase productivity among other variables[11-12]. According to Plackett-Burman and verification test results, a medium of the following composition (g%): Yeast extract, 0.5%; $CuSO_4.5H_2O$, 0.005%; glucose, 0.05%; $(NH_4)_2SO_4$, 0.05%; $FeSO_4.7H_2O$, 0.0001% was used as a basal medium for further optimization. The maximum enzyme activity (664.8 U $ml^{-1}min^{-1}$) was obtained after 24 hrs under shaking 200 rpm, at 30°C and pH, 10.6. These results presented about 600 fold increase in the enzyme activity compared to initial medium. In order to approach the optimum response region for laccase production in term of activity (U ml^{-1} min^{-1}), the significant independent variables (yeast extract, $(NH_4)_2SO_4$, $CuSO_4.5H_2O$,

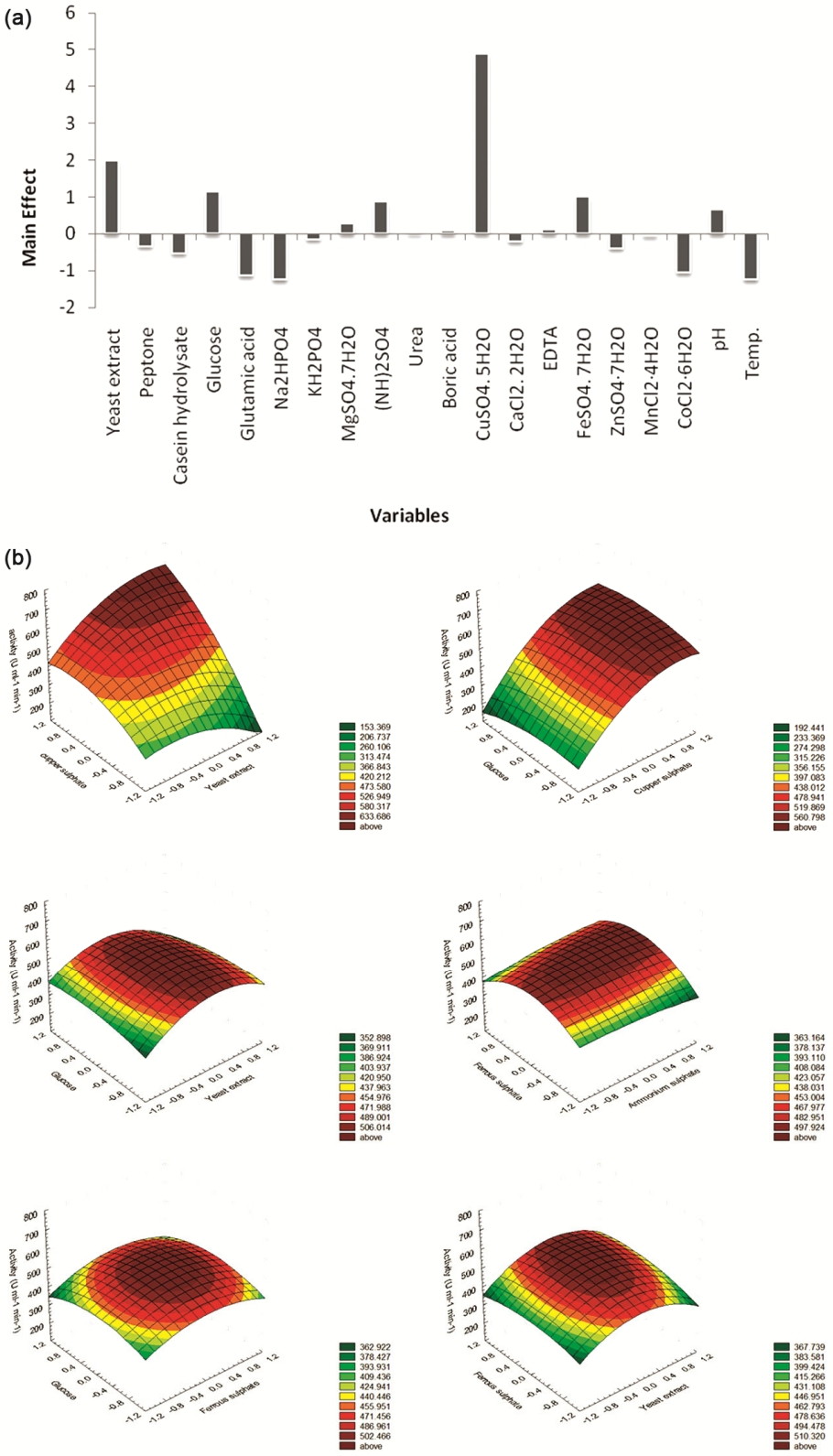

Fig. 2 — a): Column chart shown the main effect of culture variables according to the results of Plackett- Burman design, B): Three dimensional response surface representing laccase activity yield (U ml^{-1} min^{-1}) from *Alcaligenes faecalis* NYSO (KP859538) as affected by culture conditions.

Table 3 — Statistical analysis of Plackett- Burman design showing coefficient values, t- and p-values for each variable affecting on laccase production.

Variables	Coefficient	Main effect	Std Error	t-Stat	P-value	Confidence level (%)
Intercept	2.4184016		0.102887	23.51	0.0002	99.98
X1	0.9980264	1.996053	0.123927	8.05	0.004	99.6
X2	-0.16787	-0.33574	0.127564	-1.32	0.2797	72.1
X3	-0.266863	-0.53373	0.131922	-2.02	0.1363	86.4
X4	0.5742785	1.148557	0.137755	4.17	0.0251	97.49
X5	-0.567084	-1.13417	0.137501	-4.12	0.0258	97.42
X6	-0.625158	-1.25032	0.130137	-4.8	0.0172	98.28
X7	-0.072866	-0.14573	0.125673	-0.58	0.6027	39.73
X8	0.1447472	0.289494	0.123215	1.17	0.3249	67.51
X9	0.4301934	0.860387	0.11925	3.61	0.0366	96.34
X10	0.0190741	0.038148	0.120691	0.16	0.8845	11.55
X11	0.0436668	0.087334	0.120691	0.36	0.7415	25.85
X12	2.4497618	4.899524	0.11925	20.54	0.0003	99.97
X13	-0.103631	-0.20726	0.123215	-0.84	0.4621	53.79
X14	0.0622652	0.12453	0.125673	0.5	0.6543	34.57
X15	0.5028155	1.005631	0.130137	3.86	0.0307	96.93
X16	-0.194691	-0.38938	0.137501	-1.42	0.2518	74.82
X17	-0.030219	-0.06044	0.137755	-0.22	0.8404	15.96
X18	-0.528469	-1.05694	0.131922	-4.01	0.0279	97.3
X19	0.3297789	0.659558	0.127564	2.59	0.0814	91.9
X20	-0.622432	-1.24486	0.123927	-5.02	0.0152	98.5

$FeSO_4.7H_2O$, and glucose) were further explored through Response surface methodology (RSM) design. The main results of this study are presented in Figure 2b, which represents the expected laccase response and the correlation between variables in three-dimensional plots. It was observed that the highest levels of laccase activity were attained by increasing the concentration of $CuSO_4.5H_2O$, yeast extract, and glucose. The highest interaction was found between yeast extract/cupper sulphate> cupper/glucose> yeast extract/glucose at high levels. On the other hand, the lowest levels of ammonium sulfate /ferrous sulfate increase the titer of laccase activity (Figure 2b). For predicting the optimal point within experimental constraints, a second order polynomial function was fitted to the experimental results of laccase activity:

$$Y=525.70+15.54X_1+4.85X_2+154.49X_3+15.28X_4-4.626X_5-22.82X_1X_2+78.79X_1X_3+13.09X_1X_4-16.16X_1X_5-10.36X_2X_3+18.13\ X_2X_4-10.79\ X_2X_5+10.70\ X_3X_4+21.37\ X_3X_5+5.50\ X_4X_5-67.21\ X_1^2+44.72\ X_2^2-60.39\ X_3^2-17.73\ X_4^2-9.19\ X_5^2.$$

Where, X_1, X_2, X_3, X_4, and X_5, are yeast extract, $(NH_4)_2SO_4$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, and glucose. The value of the determination coefficient R^2 =0.9107 for laccase activity, being a measure of the fit of the model, indicates that about 8.92% of the total variations created by variables are not explaining laccase activity. The analysis of variance using an ANOVA test for Box- Behnken experiment was generated and gives p=0.0235, this is concluded that there is a statistically significant relationship between the studied variables at 95% confidence level (p=0.05) (Table 5). The optimal levels of the five studied variables found to be: yeast extract, 8.96 g/l; $(NH_4)_2SO_4$, 0.35 g/l; $CuSO_4.5H_2O$, 0.075 g/l; $FeSO_4.7H_2O$, 0.00133 g/l and glucose, 0.943 g/l with prediction calculated laccase activity equal to 734.21U $ml^{-1}min^{-1}$. In order to determine the accuracy of the quadratic polynomial, a verification experiment was carried out under predicted optimal conditions monitoring growth and enzyme activity. The bench scale experiment shows that the experimental laccase activity was 791.8088737 U $ml^{-1}min^{-1}$. The model accuracy was calculated as 107.845%. The exploitation of RSM for optimization of laccase production was carried by many investigators[13-14]. The highest concentrations of glucose and yeast extract were contributed to maximizing the laccase production in the present study as well as the result obtained by Zhang 2012[15] and Ronak Chhaya *et al.,*

Table 4 — Matrix designed for *Alcaligenes faecalis* NYSO (KP859538) Box-Behnken factorial experimental design.

Trials	Studied Variables *					Laccase ($Uml^{-1}min^{-1}$)
	Y.E.	$(NH4)_2$ SO4	CuSO4	$FeSO_4$	D-Glucose	
	X1	X2	X3	X4	X5	
1	-1	1	-1	1	-1	387
2	0	-1	0	0	0	572
3	1	1	-1	-1	-1	209
4	-1	1	1	1	1	562
5	1	0	0	0	0	515
6	0	0	0	0	0	707
7	0	0	-1	0	0	271
8	-1	1	-1	-1	1	354
9	-1	-1	-1	-1	-1	322
10	-1	-1	1	-1	1	518
11	1	-1	1	1	1	724
12	0	0	0	0	-1	582
13	-1	-1	1	1	-1	392
14	1	1	-1	1	1	183
15	1	-1	-1	-1	1	179
16	1	-1	-1	1	-1	213
17	0	0	0	1	0	551
18	0	0	0	0	1	429
19	0	0	0	-1	0	443
20	-1	0	0	0	0	380
21	-1	-1	-1	1	1	279
22	1	1	1	-1	1	570
23	1	1	1	1	-1	677
24	0	0	0	0	0	502
25	0	1	0	0	0	548
26	-1	1	1	-1	-1	447
27	0	0	1	0	0	638
28	1	-1	1	-1	-1	651
29	0	0	0	0	0	450

Variables*: X1-5 (studied variables); (-1) low, (0) middle and (1) high levels in g/l ; X1 (yeast extract at levels 3.5,6.5, 9.5); X2 [$(NH4)_2SO4$ at levels 0.35,0.65,0.95]; X3 (CuSO4.5H2O at levels 0.025,0.05,0.075); X4 (FeSO4.7H2O at levels 0.0005, 0.001.0.0015) and X5 (D-Glucose at levels 0.35,0.65,0.95)

2013[6] but contrary to the study exploited by Pratheebaa *et al.,* 2013[13]

Effect of pH values and cupper sulphate concentrations on laccase production

The effect of pH on the enzyme production and the growth of *Alcaligenes faecalis* NYSO were studied. The results elucidated that the bacterial growth was promoted when buffered optimized media used and maximum production was obtained (911.2 Uml^{-1} min^{-1}) at pH 11.0, but 55% of enzyme yield was lost at pH 12. Contrary to this study, other investigators reported that the optimum pH was either 7.0 or 5.0 for laccase production[16-17-18]. It was noticed that the concentration 200 mg of cupper sulfate was found as the optimum concentration based on the induction of laccase productivity to about 2435 U $ml^{-1}min^{-1}$. Further increment in $CuSO_4.5H_2O$ concentration (up to 250 mg) resulted in a decline in laccase production (data not shown). Hence, cupper sulphate might ubiquitously be employed as an efficient inducing metal for enhancing bacterial laccase production[11].

Monitoring of growth and laccase production by *Alcaligenes faecalis* NYSO

The growth of *Alcaligenes faecalis* NYSO was monitored in the fully optimized buffered medium of the following components: yeast extract, 0.896%; $(NH_4)_2SO_4$, 0.035%; $CuSO_4.5H_2O$, 0.02%; $FeSO_4.7H_2O$, 0.000133%; glucose, 0.0943%, pH 11 and cultivation temperature 30 ^oC along 30 hrs. Figure (3) shows the laccase production was directly proportional to the bacterial growth. The glucose consumption was climbed up (85.5 mg/dL) after one hour of incubation, then the utilization of glucose by bacterial cell was diminished gradually throughout the time to reach 57 mg/dL after 5 hrs, it was maintained constant with some fluctuation until the end of the incubation period. The maximum bacterial biomass was reached after 18 hrs accompanied by increasing in laccase titer and protein content to reach their maximum level (642.3U $ml^{-1}min^{-1}$and 4.5 mg ml^{-1}; respectively) after 18 hrs of incubation as well as the observation withdrawn by obtaining results of other investigators[18]. Unlike, the reported results of the present study, 48 hrs or more required for maximization of laccase enzyme yield and bacterial biomass by other investigators[16-19].

Table 5 — Statistical analysis of Box-Behnken design showing coefficient values, t- and p- values for each variable on laccase activity.

Term	Estimate	Std Error	t Ratio	Prob>|t|	Confidence level (%)
Intercept	525.70893	29.37568	17.9	<.0001	
X1&RS	15.547971	21.05132	0.74	0.4813	51.9
X2&RS	4.8540008	21.05132	0.23	0.8234	17.7
X3&RS	154.49374	21.05132	7.34	<.0001	99.99
X4&RS	15.282518	21.05132	0.73	0.4886	51.2
X5&RS	-4.626469	21.05132	-0.22	0.8316	16.9
X1*X2	-22.82423	22.3283	-1.02	0.3366	66.4
X1*X3	78.796928	22.3283	3.53	0.0077	99.3
X2*X3	-10.36689	22.3283	-0.46	0.6548	34.6
X1*X4	13.09727	22.3283	0.59	0.5737	42.7
X2*X4	18.131399	22.3283	0.81	0.4403	56
X3*X4	10.708191	22.3283	0.48	0.6444	35.6
X1*X5	-16.16894	22.3283	-0.72	0.4896	51.1
X2*X5	-10.79352	22.3283	-0.48	0.6418	35.9
X3*X5	21.37372	22.3283	0.96	0.3665	63.4
X4*X5	5.503413	22.3283	0.25	0.8115	18.9
X1*X1	-67.21839	57.02434	-1.18	0.2724	72.8
X2*X2	44.727007	57.02434	0.78	0.4554	54.5
X3*X3	-60.39245	57.02434	-1.06	0.3205	68
X4*X4	-17.73033	57.02434	-0.31	0.7638	23.7
X5*X5	-9.197907	57.02434	-0.16	0.8759	12.5

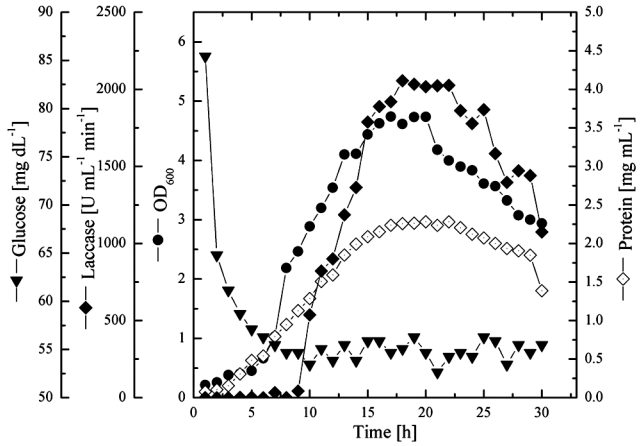

Fig. 3 — Monitoring of *Alcaligenes faecalis* NYSO (KP859538) growth and its laccase activity in optimized medium

Conclusion

The current study investigated the factors affecting the laccase production by a local isolate *Alcaligenes faecalis* NYSO. Twenty variables were screened through statistical experimental design (PBD) to select the most significant variables affected on laccase production. It was found that cupper sulfate, yeast extract, and glucose were the most significant factors affecting positively on enzyme production. Subsequently, further optimization for laccase production using response surface methodology (RSM) was applied and succeeded to increase the yield 700-fold compared to the initial basal medium. Additionally, further optimization was carried out for the inducing factor (cupper sulfate concentration), and pH values individually, it was found that 200 mg and pH 11.0 are the optimal ones; respectively. By monitoring the growth of the experimental bacterium and laccase production under final cultural optimized conditions found that the dependency of enzyme production on bacterial growth, where, the biomass reached a maximum after 18 hrs of incubation accompanied by increasing in laccase titer and protein content.

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