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ARTICLE

Statistical optimization of laccase production by *Aspergillus flavus* PUF5 through submerged fermentation using agro-waste as cheap substrate

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ABSTRACT The plan of this work was to valorize agro-wastes following statistical approach to the production of laccase enzyme by *Aspergillus flavus* PUF5 through submerged fermentation process (SmF). The process parameters influencing the enzyme production and initially identified using "one variable at a time (OVAT)" design. Among the variables screened, fermentation temperature, pH of the medium, concentration of yeast extract, and NaCl were found most considerable when waste ribbed gourd peel was used as substrate. The most favorable levels of these significant parameters were determined employing the central composite design (CCD). The goodness of fit of the model was checked by the determination coefficient (R^2). The contour plots revealed that the optimal values of the process conditions lie within the range; temperature: 25 °C, pH: 4, yeast extract concentration: 0.3% and NaCl: 0.07%. By using the optimal fermentation medium, the improved laccase production was found to be 15.96 U/ml; this was about 4.6-fold higher than the unoptimized media. **Acta Biol Szeged 61(1):25-33 (2017)**

KEY WORDS

agro-waste
laccase
response surface methodology
ribbed gourd
valorization

Introduction

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) are multicopper polyphenol oxidases, which mediate the oxidation of a wide range of phenolic compounds and aromatic amines with the concomitant four-electron reduction of O_2 to H_2O. The laccases are widely distributed in plants, fungi, bacteria and insects (Qiu et al. 2014). Among all these sources, laccases from fungi are of special interest, because of their aptitude to degrade lignocellulosic biomass by elaborating extracellular laccase enzyme (Ding et al. 2014). Laccase plays a major role in lignin degradation and their industrial and food application are increasing day by day (Osma et al. 2007). Laccases have broad substrate specificity, which makes them excellent candidates for biotechnological applications and provide green route in various biochemical processing such as oxidation of a wide variety of substrates like phenols, diphenols, polyphenols, substituted phenols, diamines, aromatic amines, and various non-phenolic compounds, delignification in paper pulping and pulp bleaching, degradation of textile dyes, bioremediation of herbicides, pesticides and xenobiotics generated by industrial processes, development of biosensors and biofuel cells, conversion of lignocellulosic for biofuel production and organic synthesis, juice and wine clarification (Sweeney and Xu 2012; Woolridge et al. 2014). From the industrial aspects, higher product yield and lower production cost are preferable during optimization of fermentation processes. The traditional optimization studies varying one parameter, while keeping the others at constant level (OVAT process), is simple, but it often fails to seek the optimum region, because the combinational effects of factors are not considered. In order to overcome this problem, optimization studies are done using response surface methodology (RSM). It is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factors (Roriz et al. 2009), which extracts the maximal information with the minimal number of runs. This technique has been widely used to determine the effects of several variables and to optimize different biotechnological processes such as optimization of media, process conditions, catalyzed reaction conditions, oxidation, production of different enzymes like amylases, lipases, proteases, cellulases, chitinases etc., (Beg et al. 2003; Das et al. 2013). Considering the importance of laccase enzyme, this study was carried out to optimize its production using a potent fungal strain *A. flavus* PUF 5 by the traditional OVAT process followed by statistical optimization of the fermentation process by response surface methodology based on central composite design (CCD) utilizing agricultural wastes as substrate.

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Materials and Methods

Microorganism and inoculum preparation

A potent laccase producing fungal strain *Aspergillus flavus* PUF5 prescreened from the soil was used in this study. This strain was identified by cultural, microscopic and molecular characterization (data not shown). The strain was grown on potato dextrose agar slants at 30 °C for 5 days (until good sporulation occurred) and stored at 4 °C until use. Inoculum was prepared by adding 5 ml of sterile distilled water to a fully sporulated culture slant. The spores were dislodged by gentle pipetting under aseptic conditions. The concentration of the prepared spore suspension was adjusted to about 5×10^9 spores/ml.

Optimization of submerged fermentation for laccase production by OVAT process

All the fermentation experiments were carried out in 250 ml Erlenmeyer flasks. Laccase production by *A. flavus* PUF5 was first optimized by 'one variable at a time' (OVAT) approach to find out the most important factors affecting the fermentation process. The mold was grown in Olga medium (Desai et al. 2011) with various agro-wastes (2%, w/v) to find the most suitable carbon source for laccase production. The effects of fermentation time and temperature on laccase production were investigated by incubating the flasks for up to 9 days at varying temperatures (25-40 °C); whereas the effect of pH was assessed by adjusting the pH (2-7) of the medium (before autoclaving) using 1 M HCl or NaOH. Screening of supplementary carbon source (1% w/v) and its concentration (0.2-1.4%) was studied by addition of different commercial carbohydrates in the culture flasks, while the effect of additional nitrogen sources was assessed by supplementation the fermentation media with different commercial nitrogen sources (0.5% w/v). The effect of metal salts on laccase production was studied using different metal salts like $MgSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, NaCl, etc., with a final concentration of 0.05% in the fermentation medium.

Fermentation kinetics

The specific rate of enzyme production (q_p) was determined as the maximum enzyme activity/g of fungal biomass/h. The growth of fungus was determined on the basis of dry biomass (mg/ml), which was also used to calculate the value of specific growth rate as

$$\mu\ (h^{-1}) = \ln (m_t / m_o)/T$$

Where, m_t is fungal biomass at a given time T (h), m_o is the baseline spore biomass at the start of the fermentation (Sterner and Elser 2002).

Statistical experimental design and data analysis

Four major factors that significantly affected the laccase production through OVAT process were further optimized using the response surface methodology (RSM). The central composite design (CCD) of response surface method was used to obtain data that fits a full second order polynomial model. The CCD with three factors and five levels including six replicates at the center point was used to fit the response surface. The proportion of variance explained by the polynomial models obtained was given by multiple coefficient of determination, R^2. The fitted polynomial equation was expressed as three-dimensional response surface plots to find the concentration of each factor for maximum laccase production. These diagram shows relationship between the responses and the experimental levels of each factor used in the design. To optimize level of each factor for maximum response 'Numerical optimization' process was employed. The combination of different optimized parameters, which gave maximum laccase yield, was tested experimentally to validate the model.

Statistical analysis was performed using the statistical software Design-Expert Version 6.0.10, Stat-Ease, Minneapolis, USA.

Assay of enzyme and fungal biomass

Laccase activity was measured by the method adopted by Desai et al. (2011). The laccase activity in U/ml is calculated using the guaiacol at 450 nm. The specific laccase activity was determined as U/mg of protein, where amount of soluble protein was determined by Lowry method (Lowry et al. 1951). Fungal biomass estimation was carried out by determining the amount of N-acetyl glucosamine released by the acid hydrolysis of the chitin, present in the cell wall of the fungi, following the process of Ramachandran et al. (2005).

Results and Discussion

Screening of different agro-wastes for maximum laccase production

Biotechnological applications require large amounts of low-cost enzymes. Therefore, one of the appropriate approaches for this purpose is to utilize the potential lignocellulosic waste residues, which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of laccase enzymes. In the current study, different lignocellulosic wastes (2%) (like wheat bran, rice bran, orange peel, tea waste, potato peel, ribbed

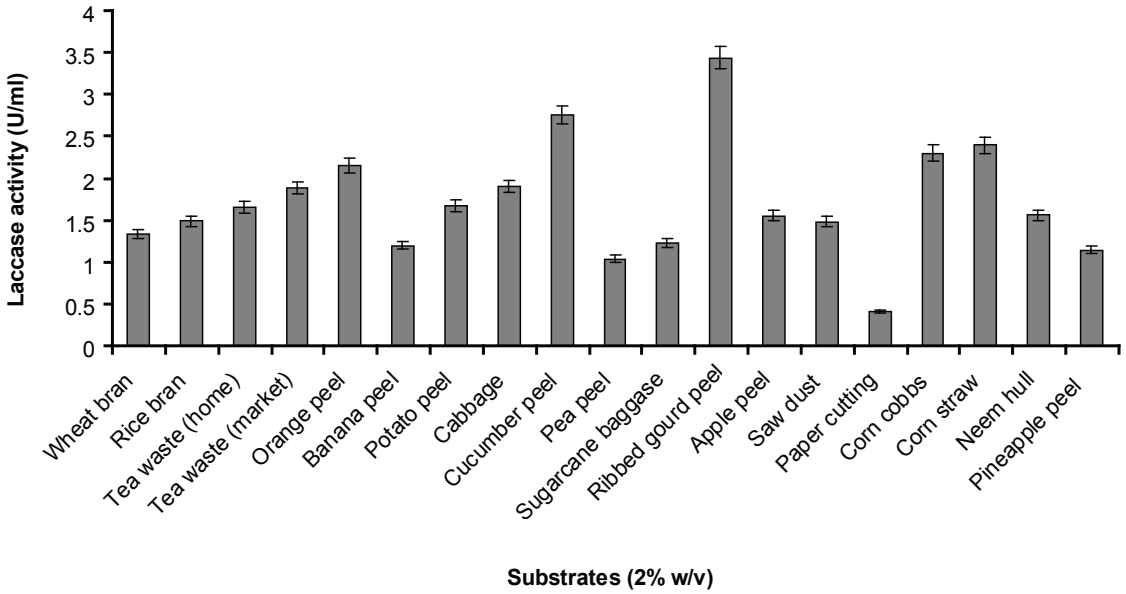

Figure 1. Effect of different lignocellulosic wastes/residues on production of laccase by *A. flavus* PUF5 through submerged fermentation.

gourd peel, sawdust, corn straw, corn cobbs, etc) were used instead of glucose in the media for laccase production. The pH of media was adjusted at 5. Results (Fig. 1) showed that the maximum laccase activity of 3.44 U/ml was recorded with ribbed gourd peel followed by cucumber peel (2.76 U/ml) and corn straw (2.40 U/ml). In recent times, agricultural waste products like chestnut shell were often used for laccase production (Dong et al. 2014). Thus, the value added to agronomic waste residue will not only reduce the disposal problem, but the chances of the environmental pollution will also be reduced.

Time course for laccase production

The growth kinetics and production of laccase from ribbed gourd peel in shake flask batch culture was examined by growing the fungi in Olga medium as described in Materials and Methods. The flasks were incubated for 9 days and laccase activity was measured at 24 h intervals. The results (Fig. 2) suggested that under normal condition laccase secretion started on the 3rd day of incubation and the maximum activity (5.74 U/ml) was achieved on the 7th day suggesting its constitutive production, which was also reported by Scheel et al. (2000). Besides laccase production, fungal biomass was also gradually increased and reached maximum (9.2 mg/ml) with specific growth rate (μ) of 26.91/h on the 7th day of fermentation, suggesting that laccase production was growth associated. It was found that the amount of soluble protein (138.3 μg/ml, after 7 days of fermentation) was also increased in accordance with the fungal growth and laccase production. The result revealed that the laccase production was growth associated, and the enzyme secretion was dependent on the fungal biomass and the specific growth rate of *A. flavus* PUF5.

Earlier reports on *Trametes hirusta* indicated maximum laccase enzyme activity (7.614 U/ml) after an incubation period of 20 days using wheat bran (Bakkiyaraj et al. 2013). In another study, *Ganoderma lucidum* was reported to obtain maximum laccase activity of 2.7 U/ml after 14th days of incubation with wheat bran (Songulashvili et al. 2007).

Optimization of fermentation temperature and initial medium pH

Among the different fermentation parameters for production of enzymes, incubation temperature and the pH of substrate play a vital role in the metabolic activity of microbial cell. During optimization of culture condition for laccase from *A. flavus* PUF5, the maximum laccase activity was detected at a temperature of 30 °C (Fig. 3A). Further increase in temperature resulted decrease in laccase activity, which is incongruence with a report on *Agaricus* sp. where the maximum laccase is produced at 30 °C (Yang et al. 2011). In general, fermentation temperature between 25 °C and 30 °C is optimal for fungal laccase production (Kunamneni et al. 2007). It was found that at elevated temperatures the activity of ligninolytic enzymes was diminished (Viswanath et al. 2014).

The pH of the medium is another vital parameter affecting the production of enzymes as well as growth of microorganisms. The effect of initial culture pH on growth

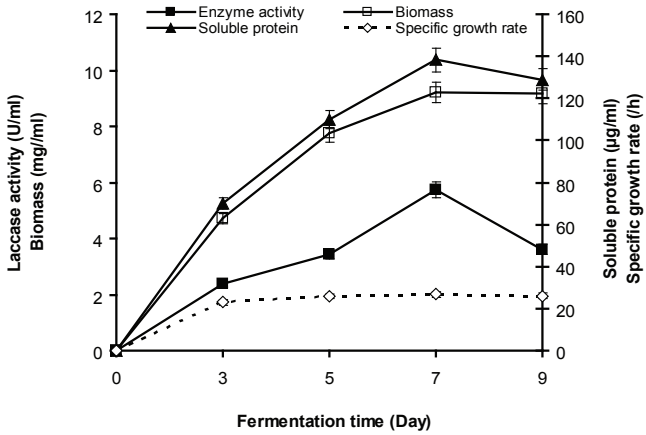

Figure 2. Optimization of fermentation time for maximum laccase production by *A. flavus* PUF5 through submerged fermentation.

of *A. flavus* PUF 5 and laccase production are summarized in Figure 3B. The results clearly indicated that maximum laccase production (5.93 U/ml) took place at pH 5 and its formation occurred over a broad range of pH values. With the increase in fermentation pH, both the fungal biomass and the amount soluble protein were also gradually decreased. Being proteins, enzymes contain different ionizable groups and their structures and functions are highly affected by the pH of the culture medium. Thus, development of an optimal pH control strategy is helpful in obtaining higher enzyme production by efficient fungal strains. It has been reported that at medium pH of 5, excess laccase production was observed in *A. bisporus* (Yang et al. 2011). Fungal laccases are reported to be generally active at low pH values (pH 3-5) (More et al. 2011). This observation is also in accordance with the optimum pH of 5 for laccase production by *T. versicolor* (Minussi et al. 2007).

Effect of substrate concentration on laccase production

Substrate concentration is another important parameter for suitable microbial growth and production of primary metabolites like enzymes. For optimization of substrate concentration, varied amount of ribbed gourd peel was subjected to fermentation and the result indicated that 4% substrate concentration was optimum for maximum laccase production (5.81 U/ml). The amount of fungal biomass and soluble protein also gradually increased (Fig. 4).

Effect of supplementary carbon and nitrogen sources on laccase production

Comparison of fermentation kinetics related to enzyme production and growth of the organism were studied in presence of different types of carbohydrate like glucose, sucrose, maltose, carboxymethyl cellulose (CMC), and soluble starch (1.0%, w/v) in the culture media in attempt to study the effect of supplementary carbon source, as well as inducer for biosynthesis of laccase enzymes by *A. flavus* PUF 5. Table 1 shows that easily utilizable carbohydrate like glucose supported highest specific fungal growth (µ: 29.70) and produced maximum soluble proteins (239 µg/ml), but the specific rate of enzyme production (q_p) was significantly reduced. Laccase production was maximum (9.21 U/ml) in presence of soluble starch (1%) followed by carboxymethyl cellulose. During further optimization of starch concentration, it was found that laccase production was gradually increased with the increase of starch concentration up to a certain level (1%) above, which enzyme production was markedly decreased (Fig. 5A). This can be attributed to the high viscosity of culture broth, which can interfere with the O_2 transfer rate leading to the limitation of dissolved oxygen required for the growth of

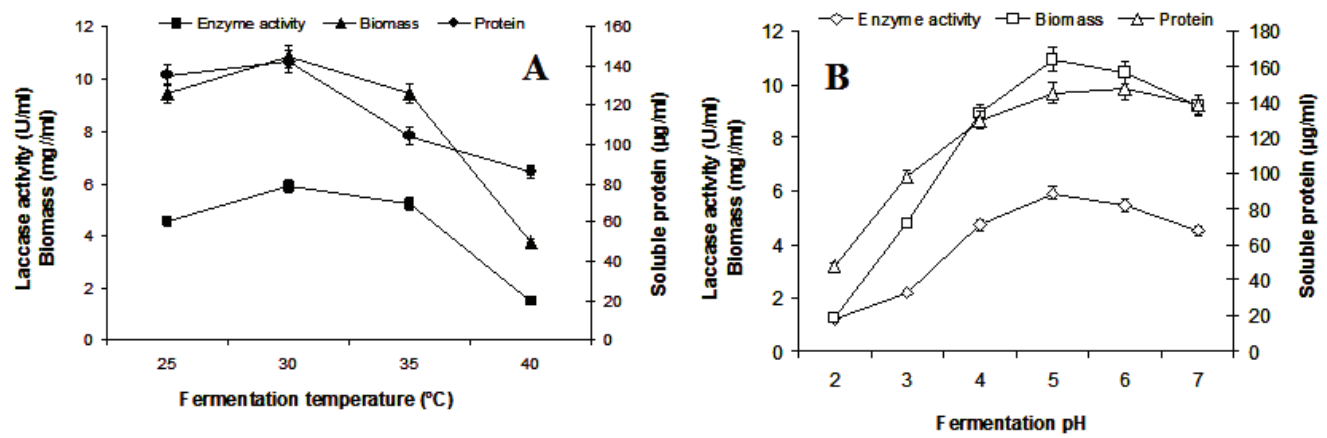

Figure 3. Effect of fermentation temperature (A) and pH (B) on laccase production by *A. flavus* PUF5 through submerged fermentation.

organism (Das et al. 2013).

Nitrogen is involved in amino acid synthesis, which makes up proteins and other value added substances. In the present study, different inorganic and organic nitrogen sources (0.5%, w/v) *viz.* urea, sodium nitrate, ammonium sulfate, yeast extract, peptone etc., was supplemented in broth and it was found that laccase production was greatly varied with the type of nitrogen source used. Among the tested nitrogen sources after 7 days of fermentation maximum enzyme production (9.77 U/ml) was achieved with yeast extract as compared to control (Fig. 5B), which may be due to higher growth of the organism (Anwer et al. 2012). Generally, a high carbon to nitrogen ratio is required for laccase production. Vahidi et al. (2004) reported that when yeast extract was used as nitrogen source it increased laccase production. Casein also has been reported to significantly increase laccase activity (7.08 U/ml) after 13 days of fermentation by *Pleurotus sajor-caju* PS-2001 (Bettin et al. 2009). *Pleurotus ostreatus* fungi showed the highest laccase activity with ammonium chloride (Stajic et al. 2006).

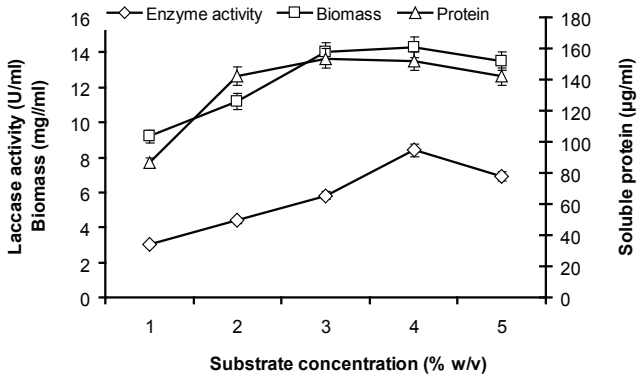

Figure 4. Optimization of substrate concentration for maximum laccase production by *A. flavus* PUF5 through submerged fermentation.

Effect of metal salt on laccase production

In this study, different metal salts like $MgSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, NaCl, etc., with a final concentration of 0.05% were added in the fermentation medium. Among them, NaCl supported the maximum laccase production followed by $CuSO_4$ and $MgSO_4$ (Fig. 6). The phenomenon of increased laccase production with addition of different metal ions has already been established in different reports. Laccase production by *Ganoderma applanatum* and *Peniophora* sp. was stimulated 49.2-fold and 19.7-fold, respectively, by adding copper (Fonseca et al. 2010). The stimulating effect of copper was attributed to the regulation of laccase gene transcription (Collins and Dobson 1997). Potassium at 1.0 mM also yielded the highest production of laccase in *Schizophyllum commune* (Irshad and Asgher 2011).

Optimization of laccase production through response surface methodology

From OVAT process, four most influencing parameters like fermentation temperature (ºC), fermentation pH, concentration of yeast extract, and concentration of NaCl were chosen and subjected to further optimization through response sur-

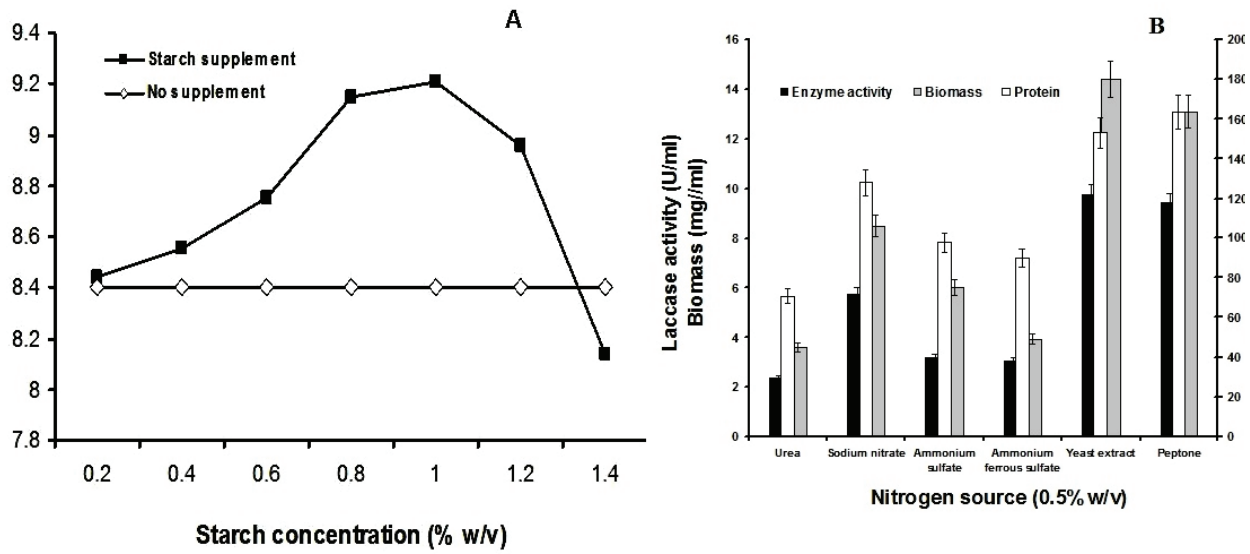

Figure 5. Optimization of supplementary starch (A) and nitrogen (B) sources for maximum laccase production by *A. flavus* PUF5 through submerged fermentation.

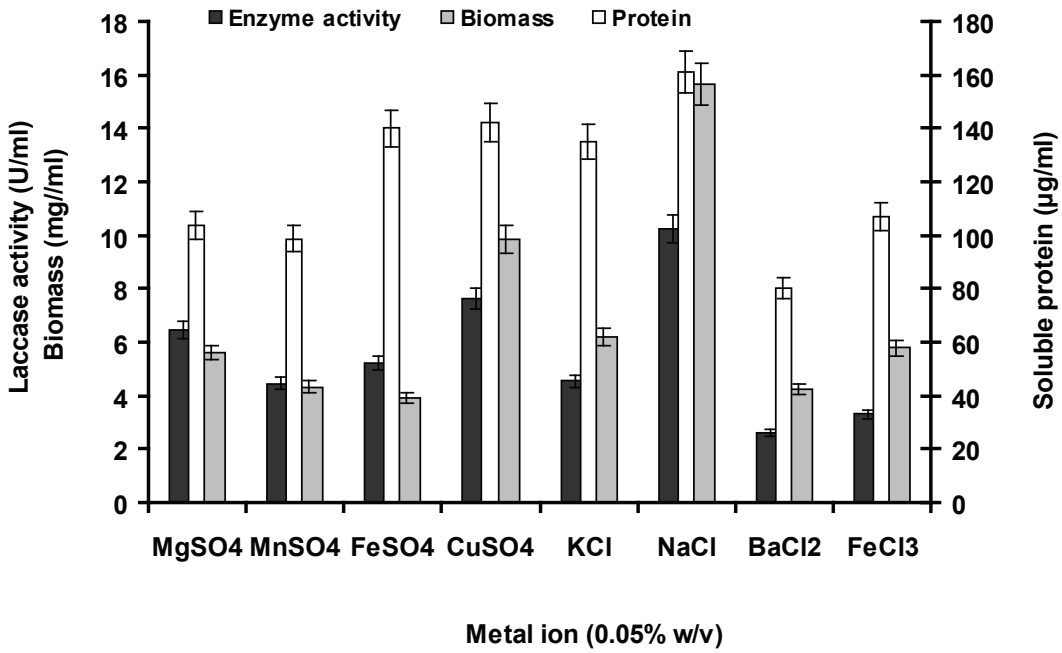

Figure 6. Optimization metal salts for maximum laccase production by *A. flavus* PUF5 through submerged fermentation.

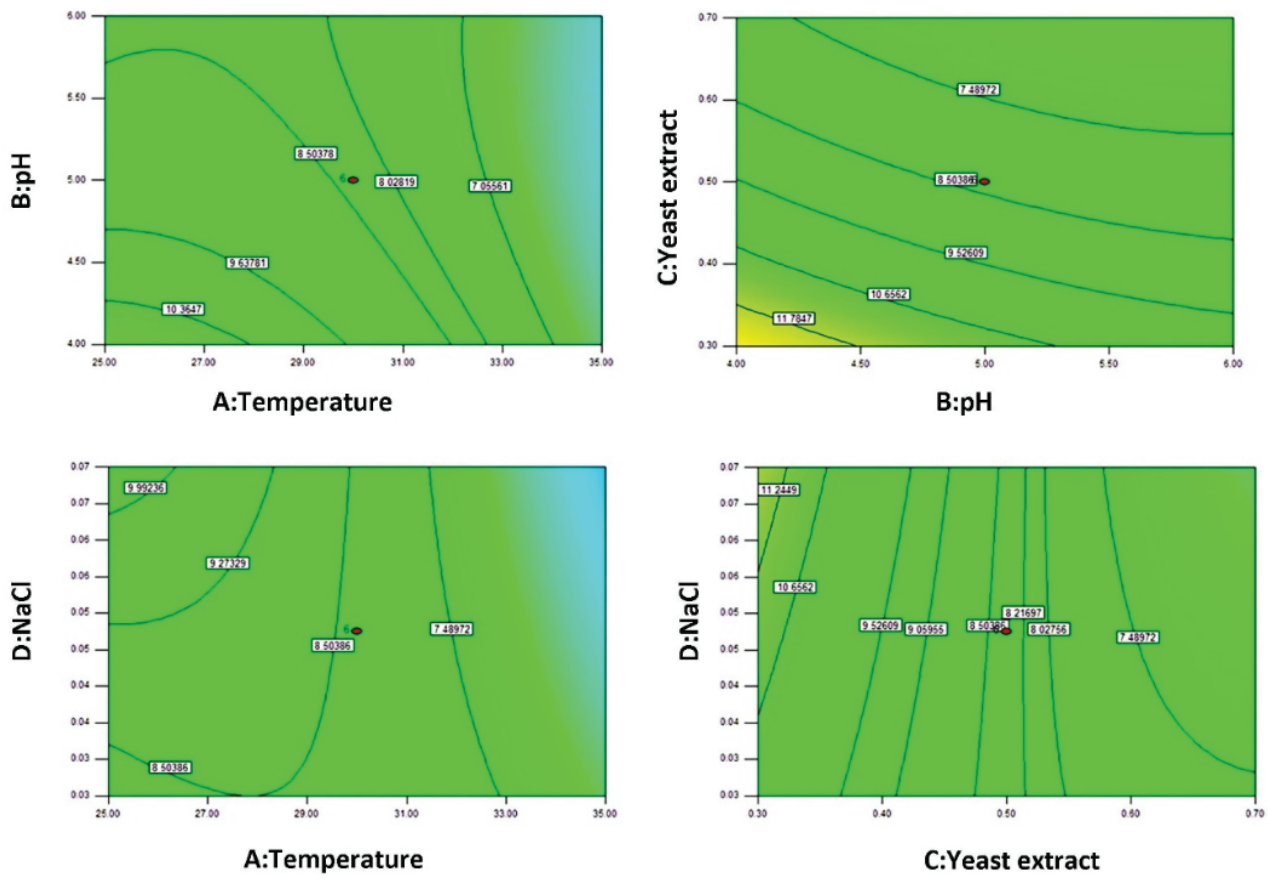

Figure 7. Contour plots showing the effects of different factors on laccase production.

Table 1. Effect of different carbon sources on laccase production by *A. flavus* PUF5.

Carbon source (1%)	Laccase activity (U/ml)	Protein (µg/ml)	Biomass (mg/ml)	Specific growth rate (µ)	Specific rate of enzyme production (q_p)
glucose	5.16	239	14.7	29.70	0.06
fructose	2.2	170	10.59	27.75	0.036
sucrose	3.6	131	7.32	25.55	0.086
maltose	2.27	86	5.41	23.75	0.076
carboxymethyl cellulose	8.47	133.8	12.8	28.88	0.111
soluble starch	9.21	142.7	13.6	29.24	0.113

Table 2. Central composite experiments design matrix with experimental and predicted values for laccase production from *A. flavus* PUF5.

Run	Selected factor (level with coded value)					
	Temperature (°C)	pH	Yeast extract (%)	NaCl (%)	Observed response	Predicted response
1	35 (+1)	4 (-1)	0.70 (+1)	0.03 (-1)	5.8	6.14
2	25 (-1)	6 (+1)	0.30 (-1)	0.07 (+1)	12.4	12.34
3	35 (+1)	4 (-1)	0.30 (-1)	0.03 (-1)	9.1	9.11
4	25 (-1)	6 (+1)	0.70 (+1)	0.03 (-1)	6.9	6.88
5	35 (+1)	6 (+1)	0.70 (+1)	0.03 (-1)	6.6	6.76
6	30 (0)	5 (0)	0.50 (0)	0.09 (+2)	8.9	8.52
7	35 (+1)	6 (+1)	0.30 (-1)	0.03 (-1)	7.7	7.82
8	20 (-2)	5 (0)	0.50 (0)	0.05 (0)	7.6	8.08
9	35 (+1)	4 (-1)	0.70 (+1)	0.07 (+1)	2.9	3.44
10	30 (0)	5 (0)	0.50 (0)	0.05 (0)	8.5	8.35
11	25 (-1)	4 (-1)	0.70 (+1)	0.07 (+1)	9.2	9.36
12	30 (0)	5 (0)	0.50 (0)	0.05 (0)	8.1	8.35
13	35 (+1)	6 (+1)	0.70 (+1)	0.07 (+1)	6.3	6.68
14	30 (0)	3 (-2)	0.50 (0)	0.05 (0)	12	11.55
15	30 (0)	5 (0)	0.10 (-2)	0.05 (0)	15	14.93
16	30 (0)	5 (0)	0.50 (0)	0.05 (0)	8.7	8.35
17	40 (+2)	5 (0)	0.50 (0)	0.05 (0)	1.3	0.55
18	30 (0)	5 (0)	0.50 (0)	0.05 (0)	8.2	8.35
19	30 (0)	7 (+2)	0.50 (0)	0.05 (0)	8	8.18
20	30 (0)	5 (0)	0.50 (0)	0.01 (-2)	8.2	8.32
21	25 (-1)	6 (+1)	0.30 (-1)	0.03 (-1)	10	9.44
22	25 (-1)	6 (+1)	0.70 (+1)	0.07 (+1)	7.3	7.27
23	25 (-1)	4 (-1)	0.30 (-1)	0.07 (+1)	16.5	16.32
24	25 (-1)	4 (-1)	0.70 (+1)	0.03 (-1)	8.4	8.01
25	35 (+1)	6 (+1)	0.70 (+1)	0.07 (+1)	3.2	3.11
26	30 (0)	5 (0)	0.50 (0)	0.05 (0)	8.6	8.35
27	30 (0)	5 (0)	0.90 (+2)	0.05 (0)	7.1	6.90
28	35 (+1)	4 (-1)	0.30 (-1)	0.07 (+1)	8.6	8.91
29	30 (0)	5 (0)	0.50 (0)	0.05 (0)	8.1	8.35
30	25 (-1)	4 (-1)	0.30 (-1)	0.03 (-1)	12.1	12.48

face methodology. Experiments were performed, according to the CCD experimental design given in Table 2 in order to search for the optimum combination of process parameters for the maximum laccase production. The experimental data showed a good fit with the second order polynomial equations, which were statistically acceptable at $P<0.05$ level. ANOVA for laccase production indicated 'F-value' of 100.19, which implied that the model was appropriate. Model terms having 'Prob>F' values less than 0.05 are considered to be significant. In this case, fermentation temperature, pH and concentration of yeast extract were significant model terms. A similar result was observed by Sun et al. (2013), where fermentation temperature and pH were found to be significant factors for laccase production by *Coriolus hirsutus*. Youshuang et al. (2011) also found yeast extract to be a significant nitrogen source for laccase production by *Trametes versicolor* sdu-4 during optimization process through CCD under submerged fermentation. The goodness of fit of the model was

Table 3. Analysis of variance (ANOVA) for laccase production in second-order polynomial model.

Source	Sum of squares	DF	F-value	Prob>F
Model	281.79	14	100.19	<0.0001
Temperature	85.13	1	423.75	<0.0001
pH	17.00	1	84.63	<0.0001
Yeast extract	96.80	1	481.87	<0.0001
NaCl	0.060	1	0.30	0.5828
AB	3.06	1	15.24	0.0014
AC	2.25	1	11.20	0.0044
AD	16.40	1	81.65	<0.0001
BC	3.61	1	17.97	0.0007
BD	0.90	1	4.49	0.0511
CD	6.25	1	31.11	<0.0001
A^2	27.89	1	138.82	<0.0001
B^2	3.94	1	19.63	0.0005
C^2	11.29	1	56.22	<0.0001
D^2	7.619E-003	1	0.038	0.8482
Residual	3.01	15		
Cor Total	284.80	29		

R^2 - 0.9894, Adj R^2 - 0.9795, Pred R^2 - 0.9454. Adequate precision - 49.744

checked by the determination coefficient (R^2). According to Table 3, the R^2 value of 0.9894 was in good agreement with the adjusted R^2 value of 0.9795. The coefficient value of variation (CV) was also low as 5.35 indicate that the deviations between experimental and predicted values are low. The 'adequate precision' value of 49.744 indicated an adequate signal and suggested that the model can be used to navigate the design space (Table 1). The contour plots were plotted to study the interaction among various physicochemical factors used and to find out the optimum concentration of each factor for maximum laccase production from *A. flavus* PUF5. The contour plots for laccase production are shown in Figure 7. It shows that increase in fermentation temperature and pH of the medium, leads to decrease in laccase production, whether increased NaCl concentration supported laccase yield. Result also indicated that a lower concentration of yeast extract supported maximum laccase production. The studies of the contour plot also reveal the best optimal values of the process conditions lies within the range; temperature: 25 °C, pH: 4, yeast extract concentration: 0.3% and NaCl: 0.07%. Under these conditions, confirmation experiments were conducted in three replicates. The observed mean laccase activity was 15.96 U/ml. This value was largely consistent with the predicted value of 16.325 U/ml.

Conclusion

The results of this work disclosed the potential of *Aspergillus flavus* PUF5 to produce laccases in a liquid medium when waste ribbed gourd peel was used as substrate. Among the different additional sources of nitrogen tested, yeast extract was the most appropriate for laccase production, especially at a concentration of 0.3% (w/v) in the culture medium, under the conditions of the present assays. The addition of NaCl 0.07% (w/v) to the medium increased not only the laccase production, but also soluble protein and fungal biomass in the medium. In addition, it was observed that soluble starch, a relatively cheap carbohydrate, also induced laccase production, particularly when added to the medium at a concentration of 1% (w/v). The most significant parameters and their favorable levels were further optimized employing the central composite design and the results revealed that the optimal values of the process conditions lie within the range; temperature: 25 °C, pH: 4, yeast extract concentration: 0.3% and NaCl: 0.07%. The improved laccase production was found to be 15.96 U/ml, which was about 4.6-fold higher than the unoptimized media. The results show a good alternate of valorization of agro-waste in to value added product and preventing environmental pollution.

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