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Optimization of process parameters using response surface methodology (RSM) for laccase enzyme production using *Aspergillus nidulans* in solid state fermentation utilizing agro-industrial waste

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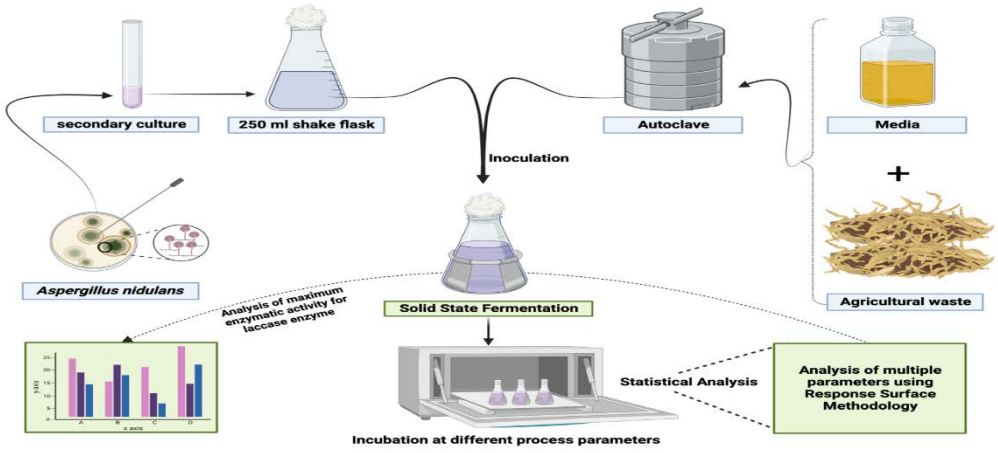
Article

ABSTRACT



The utilization of microbial laccase for the biological delignification of biomass is regarded as an environmentally sustainable procedure. Present study aimed to optimize the process parameters to achieve improved laccase production. Mustard oil cake was utilized as a solid substrate, and *Aspergillus nidulans* was employed as the fungal strain for the production of Laccase enzyme. To optimize the different physical and chemical factors for laccase production, specifically moisture content, incubation time, nutrient pH, incubation temperature, salt concentration, and additional carbon and nitrogen sources were considered. Additionally, response surface methodology was employed to optimize both the media and process parameters. The highest observed enzyme activity was 6.99 U/mL under optimum conditions. Experimental data showed that moisture content of 100% (w/v), incubation temperature of 28°C, pH range of 5-6, 6% $MgSO_4$ (w/v) and ammonium chloride when used as supplementary salts in the production medium resulted in the maximum production of laccase. The production medium was incubated for a duration of 96 hours once optimized. To maximize laccase production, this study optimized the different media components using conventional OFAT method and further optimized the critical parameters using the response surface methodology (RSM).

Keywords: Solid state fermentation, agriculture waste, laccase enzyme, lignocellulosic, response surface methodology

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INTRODUCTION

Laccases [benzenediol: oxygen oxidoreductases; EC 1.10.3.2] are multicopper oxidoreductase enzymes that have the potential for oxidizing a wide variety of structurally different substrates, including amines, phenols and their compounds.[1] It represents the major subclass of blue MCO viz. multicopper oxidases, which contains copper that is utilized for catalysis of an extensive variety

of aromatic and phenolic substrates just by using oxygen molecules as an electron acceptor along with the consequent conversion of oxygen molecules into water molecules. Because of its broad specificity towards the substrate, laccase enzyme is used widely in industrial areas like paper, textile, and biopharmaceuticals.[2] Also, this enzyme is applied in various environmental technologies and biotechnological approaches, including bio-bleaching, decolorization, and removal of xenobiotics leading to bioremediation, bio-pulping, bioleaching, transformation of antibiotics and steroids.[3] The major disadvantages of industrial laccase enzyme production have been noticed: its high production cost, low enzymatic activity, and low yield.[4] Therefore, production strategies are required by which the laccase enzyme formation cost reduces with an increment in its enzymatic activity using a cost-effective process. In recent times, the submerged fermentation process (SmF) is often used most commonly in making a variety of enzyme categories along with laccase enzymes.[5] In SmF-type fermentation technology, the uniform dispensation of nutrient sources occurs, resulting in the complete absorption of nutrients by microorganisms cultured in the liquid medium. Due to the complete contact of microorganisms and media, the consumption rate of nutrient media by microorganisms increases rapidly, making this process expensive.[6] In the past decade, Solid state fermentation (SSF) process has been utilized widely in the making and processing of many enzymes because of its simpler technique with various advantages like low energy consumption, low production rate, low pollution rate and high recovery rate of products.[7] In this fermentation technology, microorganisms are cultivated inside a fermentation tank with negligible unbound water content.[8] An insoluble material commonly recognized as a solid tray is generally incorporated in SSF that can act as physical material support and also simulates the natural environment for microorganisms to grow compared to SmF. Also, SSF provides the possibility of using waste materials and by-products from food industries and agricultural sectors like sugarcane bagasse, rice bran, apple pomace, crude olive pomace, brewer's spent grain, orange pomace, wheat bran, vine shoots mixture, olive oil, corn stalk, bagasse of sweet-sorghum, spent coffee waste, cassava peel, jatropha cake, banana peel and citrus peel as a feedstock in SSF that can be consumed by feeding different microbial strains for various enzyme production which makes it more economical and eco-friendly.[17] Researchers also suggested that lignolytic by-products has been considered as one of the major source of agricultural waste products, which includes 3 major polymeric compounds, i.e., lignin, hemicellulose and cellulose that can be depolymerized directly by using laccase enzyme as natural substrate.[9] A wide variety of lignocellulosic materials contain inductive substances like phenolic compounds and flavonoids that can be used directly in solid state fermentation to increase fungal laccase enzyme production.[10] This work aims to utilize inexpensive agro-industrial waste residues like wheat bran, mustard oil cake and rice bran as a feedstock in SSF to cultivate fungal strain *A. nidulans* in the production of laccase enzyme. In this experiment, the effect of various parameters like moisture content, pH range, carbon source, temperature range, salt concentration, and nitrogen source has been studied experimentally as well as with the help of statistical tools, i.e., response surface methodology (RSM) for laccase enzyme production using agro-industrial waste products.

MATERIALS AND METHODS

Microorganism

Aspergillus nidulans is a versatile fungal that can rapidly grow on all common mycological media such as potato dextrose agar (PDA), malt extract, etc. Laccases are one of the most important enzymes produced as a primary metabolite in fungi[11]. Therefore, this strain was chosen to experiment with the production of laccase enzyme by utilizing rice bran, wheat bran and mustard oil cake as a solid substrate in SSF. The culture of *A. nidulans* was cultivated over potato dextrose broth and revived after 2-3 weeks at regular intervals. Once the revived microbial strain for the experiment was inoculated in the petri plates containing PDA media, culture plates were covered with para-film and incubated at 28°C in Biochemical Oxygen Demand (BOD) incubator. On another hand, the culture was grown on Yeast-Peptone-Dextrose (YPD) media which contains chemical constituents like distilled water, peptone, dextrose and yeast. The culture was then incubated at 28°C in a BOD incubator for a duration of 5 days. A broth of *A. nidulans* has been prepared for inoculum development that was used in further experiments in flask-level solid state fermentation.[12]

Selection of agro-industrial waste residues

A wide range of agro-industrial waste residues were collected from various industrial areas. Using standard procedures, waste products were collected from the sites and have been screened to be used as a substrate for cultivating the fungal strain for laccase production.[13] Three agricultural waste residues, viz. wheat bran, mustard cake and rice bran, were selected for the experiment based on their availability and cost, which was further washed and dried, followed by grinding. After grinding, the substrates were autoclaved at 121°C with 15 psi pressure and then seeded with fungal strain. It has been noticed that optimal growth of fungal strain was established after 3 days of incubation which was further extracted and assayed. One agricultural waste, i.e., mustard oil cake, has been observed in maximum laccase production at flask level solid state fermenter.[14]

Inoculum preparation

Inoculum was prepared using analytical grade chemicals and then transferred 5 mL suspension culture into 250 mL conical flask containing 95 mL of sterile inoculum medium. The composition of the inoculum medium was (g/L): Glucose (20 g/L), NH_4NO_3 (3 g/L), $MgSO_4.7H_2O$ (0.5 g/L), KCl (0.5 g/L), K_2HPO_4 (1 g/L), $FeSO_4.7H_2O$ (0.01 g/L), with a pH of solution 6.0. The flasks were incubated on a rotary shaker at 200 rpm at 30°C for 48 h.

Enzyme assay

Guaiacol has been used for the laccase assay in which the reddish-brown colour developed due to oxidation of guaiacol by laccase enzyme is used to measure enzyme activity at 470 nm spectrum. In this experiment, the reaction mixture for enzyme assay was prepared using different chemical constituents, i.e., guaiacol (2 mM) – 1 mL, sodium acetate buffer (10 mM) – 3 mL, and enzyme source (fungal supernatant) – 1 mL. A blank containing 1 mL of distilled water instead of an enzyme was also prepared. The mixture was incubated at 30°C for 15 min, and the absorbance was recorded

at 470 nm using a UV spectrophotometer. In this experiment, enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min[15].

The effect of various process parameters in laccase production

The solid substrates viz. wheat bran (WB), rice bran (RB) and mustard oil cake (MOC) were procured from the local market and were used to observe as potential solid substrates for the production of laccase. Mustard oil cake was found to be an excellent solid substrate for laccase production using *A. nidulans* at initial experiments in shake flask SSF. Further, several bioprocess parameters such as moisture content, temperature, pH, salt concentration, carbon source and nitrogen source were assessed and optimized for maximum laccase yield, as discussed below. [16]

Experiments were performed to identify the best moisture content for laccase production. It was noticed that the growth of filamentous fungi requires moisture content for higher fungal cultivation (Tudor et al., 2012). In this experiment, a different range of moisture content was provided to the solid substrate in SSF to observe the effect of it on the growth of filamentous fungi. Different moisture content, i.e., 80%, 100%, 120% and 150% (w/v), were used to analyze the effect on fungal growth and laccase production. Also, temperature was considered one of the important parameters that can affect the laccase production in SSF. In this experiment, different temperature ranges, i.e., 25℃, 28℃, 30℃ and 32℃ had been considered and enzyme assay was observed at all different temperatures. Statistics of laccase enzyme formation with a discrete temperature range are represented in the result section. pH and salt concentration were also optimized to study its effect on laccase expression. The effect of additional nutrient sources, i.e., carbon and nitrogen, have been optimized for enhanced laccase production. Recently, researchers suggested that the presence of the carbon source acts as an essential energy source for the growth of the microbial strain, including bacteria, fungi and algae (Gabriel-Ajobiewe et al., 2021). In this experiment, filamentous fungi grew by providing extra energy through various C-sources, including dextrose, maltose, fructose, etc. Many researchers also found that nitrogen is considered a macronutrient that can be used by the cells to synthesize DNA content and plays a major role in amino acid synthesis (Gabriel-Ajobiewe et al., 2021). In this experiment, nitrogen source was provided in two forms, i.e., ammonium nitrate and urea, with varied concentrations to analyze maximum fungal growth. The result section represents an assay of laccase enzyme formation with various carbon and nitrogen sources.

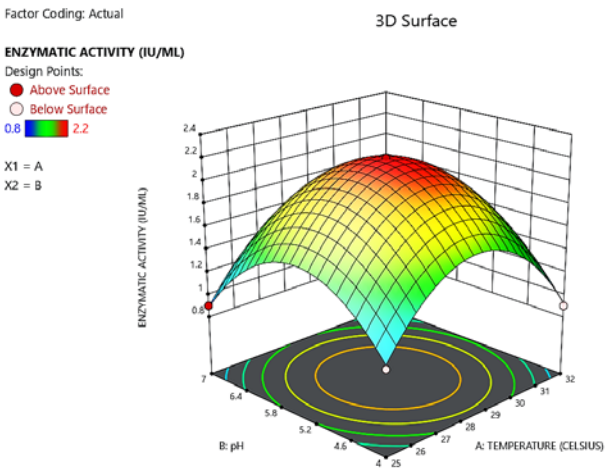
Statistical Analysis – Response Surface Methodology

Response surface methodology (RSM) is a set of experiments performed to fit the predictive model, including various continuous variables. The main purpose of using RSM includes determining variable settings for which the mean response is optimized. All the data incorporated in this study were statistically analyzed by response surface methodology to optimize three independent variables, i.e., temperature, moisture content and pH. The most optimum operating conditions were obtained from response surface methodology, and this outcome showed that optimum temperature (28°C), moisture content (100% v/v) and (pH 5) is a favourable condition for laccase production with observed laccase activity ~2.2 U/mL. Fig. 1, 2 and 3 shows 3D surface graphs and counter plots to analyze RSM for laccase production using *A. nidulans*.

The response was calculated by using the equations given below:

- **equation 1** = - 49.47517 + 3.11182 A + 2.74503 B + 0.004762 A*B - 0.055204 A^2 - 0.265000 B^2.
- **equation 2** = -10.47636 + 2.82491 B + 0.089476 C- 3.37804e-17 B*C - 0.055204 B^2- 0.265000 C^2.
- **equation 3** = -35.67619 + 2.50102 A + 0.029209 C - 1.73018e-16 A*C - 0.043878 A^2 - 0.000120 B^2

where temperature-A, pH-B, Moisture content-C.



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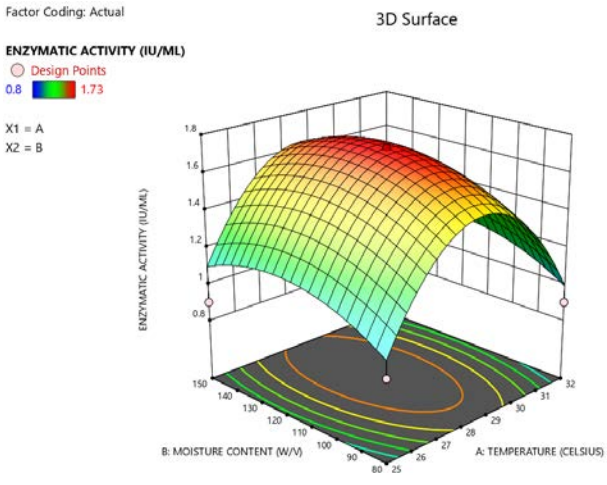

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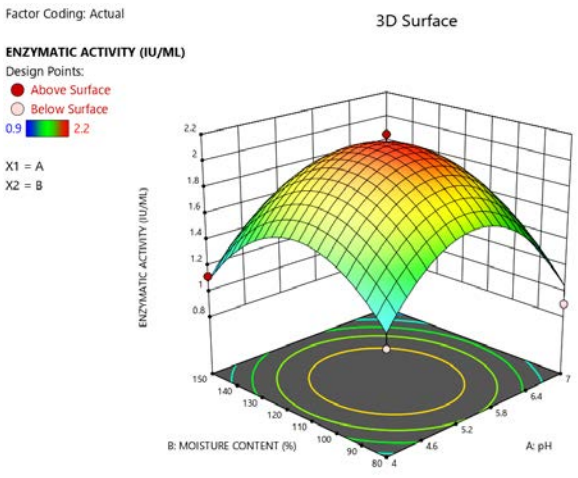
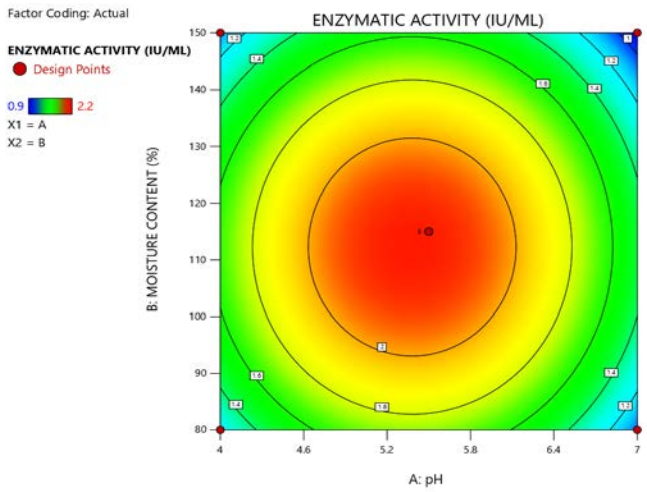


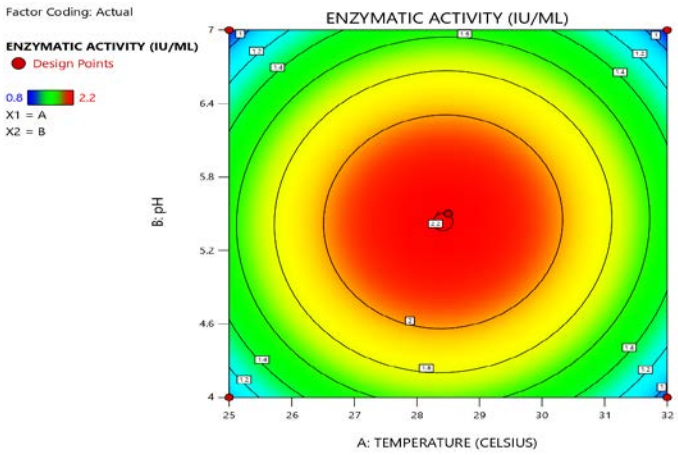
(c)

Figure 1. Response surface three-dimensional (3D) plots showing the interaction between a) pH versus temperature b) temperature versus moisture content c) moisture content versus pH



(c)

Figure 2. Surface and contour plots showing the combined effect of a) pH versus temperature b) temperature versus moisture content c) Moisture content versus pH for laccase production using *A. nidulans*



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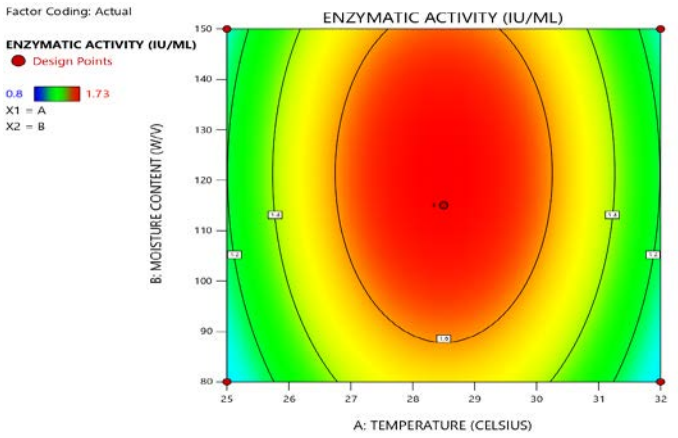

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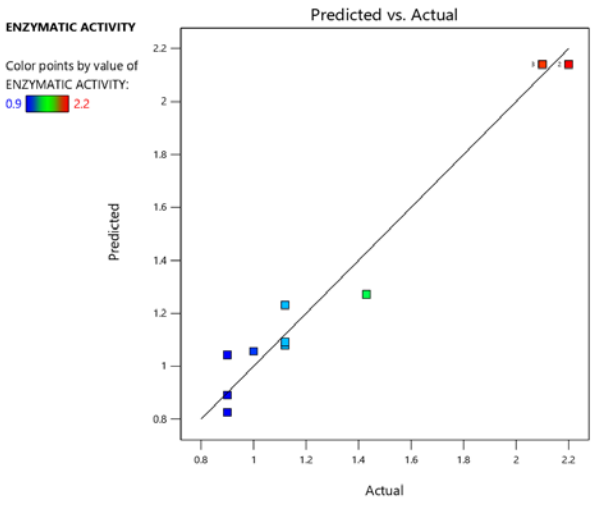

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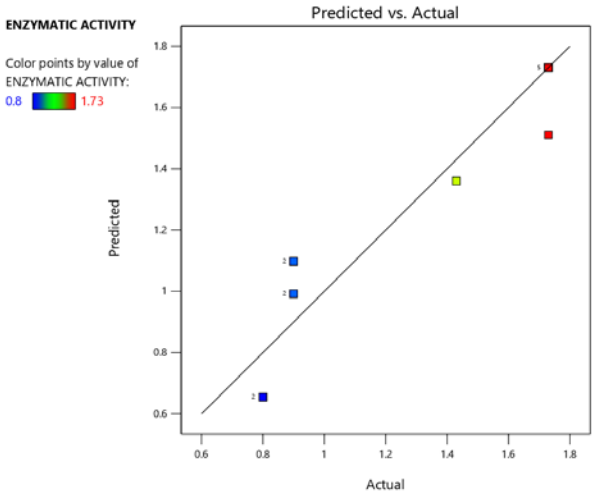

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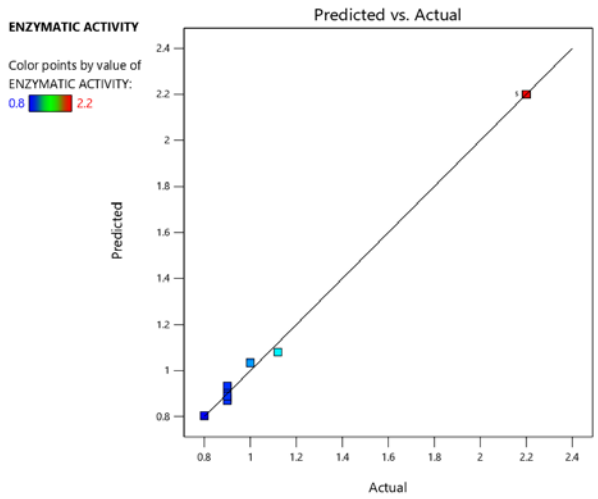

(c)

Figure 3. Predicted vs. actual value graphs showing the combined effect of a) pH versus temperature b) Temperature versus moisture content c) Moisture content versus pH for laccase production using *A. nidulans*

Table 1. Analysis of variance (ANOVA) for response surface model for the optimization of physical parameters (temperature, moisture content and pH) for laccase production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Optimization of pH and temperature						
Model	5.04	5	1.01	1208.71	< 0.0001	Significant
pH	0.0073	1	0.0073	8.74	0.0212	
Temperature	0.0211	1	0.0211	25.34	0.0015	
pH * temperature	0.0025	1	0.0025	3.00	0.1270	
pH ^{2}	3.18	1	3.18	3815.64	< 0.0001	
Temperature ^{2}	2.47	1	2.47	2966.27	< 0.0001	
Residual	0.0058	7	0.0008			
Cor Total	5.04	12				
Optimization of temperature and moisture content						
Model	2.07	5	0.4149	15.23	0.0012	Significant
Temperature	4.441E-16	1	4.441E-16	1.630E-14	1.0000	
Moisture content	0.0225	1	0.0225	0.8259	0.3937	
Temperature * moisture content	0.0000	1	0.0000	0.0000	1.0000	
Temperature ^{2}	2.01	1	2.01	73.77	< 0.0001	
moisture content ^{2}	0.1513	1	0.1513	5.56	0.0506	
Residual	0.1907	7	0.0272			
Cor Total	2.27	12				
Optimization of pH and moisture content						
Model	3.74	5	0.7479	64.38	< 0.0001	Significant
pH	0.0705	1	0.0705	6.07	0.0432	
Moisture content	0.0462	1	0.0462	3.98	0.0863	
moisture content * pH	4.441E-16	1	4.441E-16	3.823E-14	1.0000	
pH ^{2}	2.43	1	2.43	208.91	< 0.0001	
moisture content ^{2}	1.66	1	1.66	142.69	< 0.0001	
Residual	0.0813	7	0.0116			
Cor Total	3.82	12				

RESULT AND DISCUSSION

During this research work, it has been observed that different agro-industrial wastes may be used as a solid substrate for laccase production. Initial screening reveals that mustard oil cake has excellent potential for laccase enzyme production when used as a solid substrate in SSF. The result indicates that when used as a solid substrate, mustard cake produces the maximum absorbance at 540 nm wavelength with 100% initial moisture content. It has also been found that moisture content plays a crucial role in the growth of microorganisms and, subsequently, in the production of laccase enzyme.[18] Temperature is another physical parameter that also plays a significant role in the growth of fungal strain and product formation. In this work, it has been analyzed that the optimum temperature at which the growth of the fungus was maximum is about 28°C. Furthermore, according to the experimental outcomes, it has been observed that additional carbon and nitrogen sources support the growth of *A. nidulans* and result in the enhanced production of laccase. Dextrose was used as the carbon source, and urea as a nitrogen source, promoting fungus growth. Different physical and chemical parameters effects on producing laccase were also studied. While experimenting, the major constituents that had been analyzed in different comparable ranges, viz. temperature, moisture content, salt concentration, pH, carbon, and nitrogen source, have the potency to alter the growth of filamentous fungal strain and laccase enzyme. The end results of various physical and chemical parameters are given below.[19]

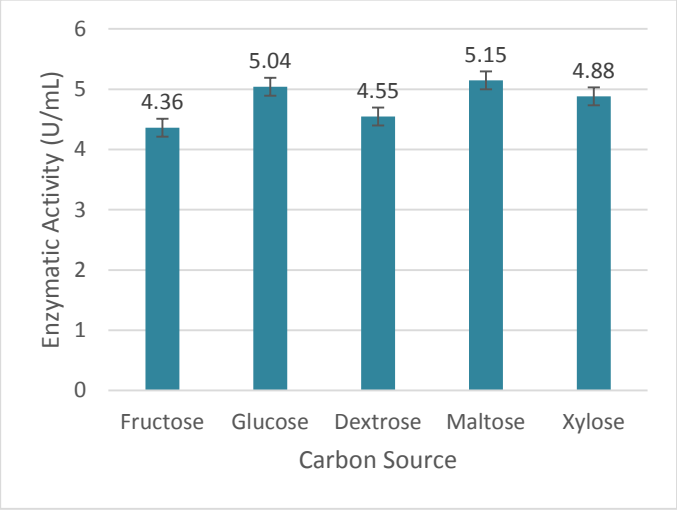

Figure 4. Effects of supplementary carbon source in the laccase production

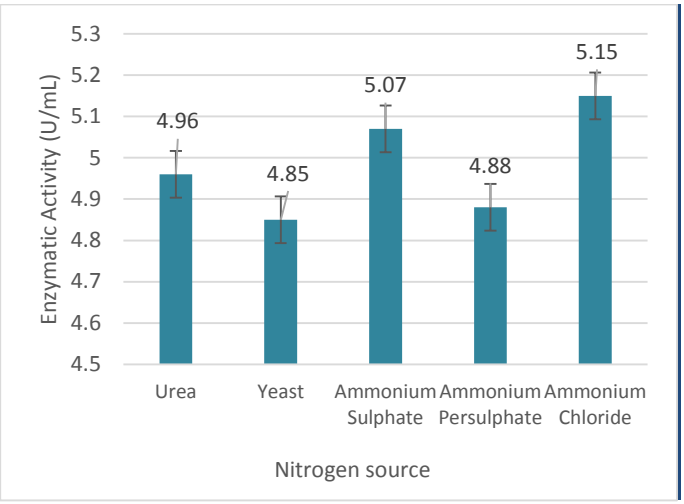

Figure 5. Effects of supplementary nitrogen source in the laccase production

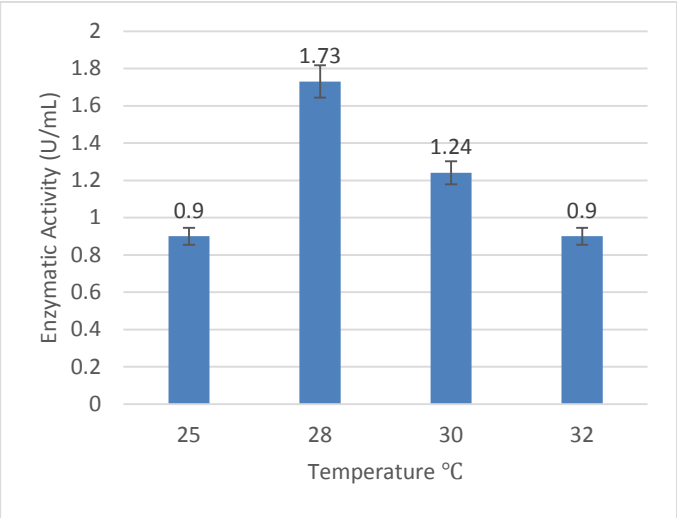

Figure 6. Effects of temperature on the growth of microorganisms and production of an enzyme

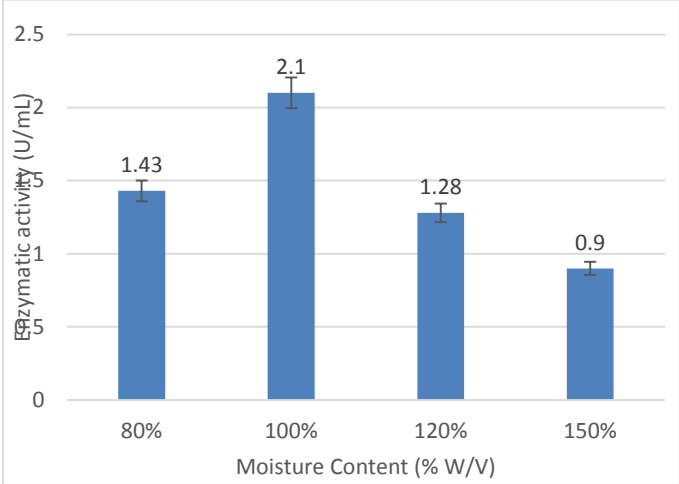

Figure 7. Effect of moisture content in solid state fermentation for laccase enzyme production using fungal strain *A. nidulans*

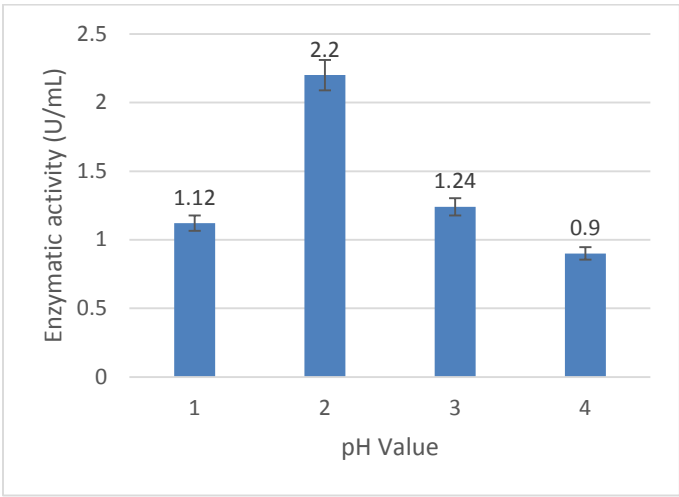

Figure 8. Effect of pH on the laccase enzyme production

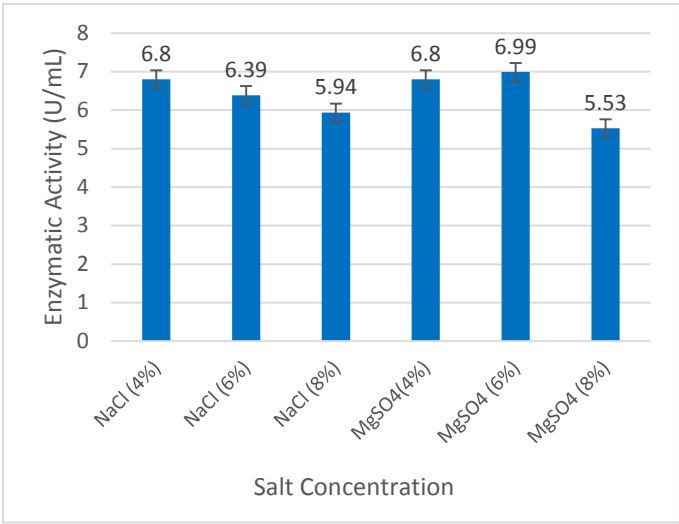

Figure 9. Effect of additional salt concentration in shake flask SSF for laccase enzyme production

Optimization of carbon source

Experimental data suggest that additional carbon source in the fermentative media promotes microorganism growth when used in appropriate concentration. Different carbon sources, viz. glucose, dextrose, fructose, xylose, and maltose, were provided as a media constituent in shake flask SSF (Fig. 4.). Experiments were performed in five flasks containing additional carbon sources along with substrates to study the effect of it on laccase production. When maltose was used as an additional carbon source, it was observed that maximum growth of fungus occurred, leading to maximum laccase enzyme production, i.e., 5.11 U/mL after being incubated for 96 hours.[20]

Optimization of nitrogen source

In the experiment, a Nitrogen source is to be provided to support microbial growth. The effect of nitrogen was studied in flask-level SSF in which different nitrogen sources 0.5% (w/v) concentration were added. All the flasks were incubated at 28℃ and analyzed for desired product formation (Figure 5.). The effect of various nitrogen sources, viz. yeast extract, ammonium persulphate, urea, ammonium sulfate and ammonium chloride, had been studied in shake flask SSF. The experiment concluded that the maximal enzymatic activity of about 5.15 U/mL was observed when incubated for 96 hours.[21]

Optimization of temperature

The experiment optimized the temperature to analyze the enzymatic activity at distinct ranges. Four different shake flasks with 100% moisture content were inoculated with *A. nidulans* and incubated at four different temperature ranges of about 25℃, 28℃, 30℃ and 32℃(Fig. 6.). The maximal enzymatic activity of about 1.73 U/mL has been obtained at 28℃ after 96 h of incubation time. The combined effect of temperature with pH and moisture content was also optimized using statistical tools, i.e., described in section 3 of the paper.[12]

Optimization of moisture content

Moisture content was optimized at different concentrations, and all other supplementary nutrients at 28℃ for 72 hours. 80% w/v, 100% w/v, 120% w/v and 150% w/v levels were used to perform the experiment. During these experiments, 100% w/v moisture content was observed to favor enhanced enzyme production. The combined effect of moisture content with pH and temperature was also optimized using RSM, described in section 3. This has also been reported that an increased amount of moisture content prevents the growth of filamentous fungi. However, lower moisture content, i.e., 80% (w/v), decreases water availability, resulting in less growth of fungi and decreased enzymatic activity. Moisture content, surface area, porosity and particle size also play a crucial role in filamentous fungus growth and enzyme production through SSF. The effects of moisture content on enzyme production are mentioned in Fig. 7[22].

Optimization of pH

pH optimization has been done to analyze the impact of different pH values on the growth of microorganisms and laccase enzyme production. To perform the experiment, four shake flasks with pH 4, 5, 6 and 7 were considered (Fig. 8.). Lactic acid and NaOH were used simultaneously to balance the pH in each flask. After balancing, all flasks were inoculated with *A. nidulans* and incubated at 30℃. The maximum laccase enzyme production was observed at pH 5 with enzymatic activity of about 2.2 U/mL. The combined effect of pH with other parameters was also optimized using the statistical tool RSM described in section 3.[23]

Optimization of salt concentration

Many researchers have found that the fungus growth largely depends upon the type of salt and its concentration in the media. The experiment, $MgSO_4$ and NaCl were used as additional salt at a varied concentration range of about 4%, 6% and 8% (Fig. 9). Finally, it was observed that the increasing concentration of salts greatly affects the enzymatic activity. The maximal enzymatic activity was found to be 6.99 U/mL when $MgSO_4$ was used at a concentration of about 6%.

CONCLUSION

In this study, different solid substrates were screened for laccase enzyme production. However, mustard oil cake showed better fungal growth and enhanced laccase enzyme production. Since this agro-waste is easily available and economically significant, that can be used as a major solid substrate for producing laccase by *A. nidulans* using solid-state fermentation. The effect of various physical & chemical parameters that affect the laccase enzyme production, i.e., moisture content, incubation time, pH of nutrient, incubation temperature, the concentration of salts, supplementary carbon & nitrogen source have also been optimized using solid state fermentation Statistical tool response surface methodology was also used to optimize different physical parameters which affect the laccase production using SSF. The study reveals that 100 % (w/v) of moisture content, 28°C incubation temperature, pH range of 5-6, and additional salt solution of 6% (w/v) $MgSO_4$ and ammonium chloride as an additional nitrogen content, respectively, showed maximum production after 96 hours of incubation period. During the present research work, adding additional carbon and nitrogen sources with salt concentration played a crucial role in the growth of fungi and laccase production in solid-state fermentation.

CONFLICT OF INTEREST STATEMENT

It is declared that there is no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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