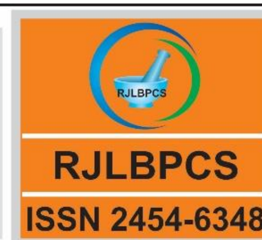

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STATISTICAL OPTIMIZATION OF FUNGAL LACCASE PRODUCED BY SOLID STATE FERMENTATION

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ABSTRACT: Effective utilization of agro based industrial waste for production of industrially important enzymes and value added products are promising renewable alternatives to reduce environmental issues. The interactive effect of different physical parameters including temperature, inoculum size, incubation time, moisture and pH on fungal laccase enzyme were investigated. Central composite design was implied to optimize the specific correlation between different physical parameters and the response i.e laccase enzyme. The increased laccase enzyme yield (281.62 U/mL) was obtained at pH 5 after 144 h of incubation at optimum temperature 30°C when properly moistened substrate (50%) of banana stalk was inoculated with 4mL of fungal culture. The satisfactory representation of quadratic model was authenticated by close values of regression coefficient R and modified R^2. The precision and reliability of present work was confirmed by 0.67 % i.e low value of coefficient of variation (CV). The present study enunciates the ability of new strain to induce laccase enzyme by solid state fermentation of lignocellulosic substrate and its statistical optimization.

KEYWORDS: Lignocellulose; Response surface Methodology (RSM); Central Composite Design (CCD); Laccase enzyme Optimization; Regression Coefficient.

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1.INTRODUCTION

Enzymes are highly multifaceted and stout biological catalytic stimulants having multiple emulous applications in industrial outgrowth in comparison to chemical catalytic agents. Production of most useful products is usually achieved by the utilization and commercialization of different enzyme catalyzed reactions. Currently enzymes with greater stability, catalytic efficiency and specificity are being produced by adoption of different techniques including screening from natural derivatives by exploiting their biological potential, random mutations and immobilization [1, 2]. Formation of different enzymes encompasses the selection of particular enzyme with best suitable strain having greater potential to produce extracellular enzymes. Moreover, enhanced enzyme yield can be obtained by improvement of particular strain by genetic engineering, optimized intensification of culture medium conditions and recovery process and synthesis of labile enzymatic product [3]. Different agricultural wastes are highly preferred to induce different enzyme by application of various microorganisms because of their renewable nature and economical value. The recalcitrant polymers of lignocellulosic waste material can be demineralized and depolymerized by the use of basidiomycetous WRF having spectacular degradation strategies depending on lignocellulosic material chemical composition and its morphology. Basidiomycetous fungi possess activating species different enzymes including oxidases and dehydrogenases and lesser molecular mass metabolites which act concomitantly for effective removal of complex lignin polymer from the ligocellulosic waste by giving special approach to carbohydrate contents of plant [4]. Comparison of substrate is most important parameter to determine the specific enzyme pattern of WRF. Ligninolytic and cellulolytic enzymes play an important role in breakdown of lignin waste material by holding on to the plant species and its nature [5, 6, 7, 8, 9]. Lignocellulosic biomass requires strong complex of enzyme having multiple characteristics to convert it into useful products by use of white rot fungi [10]. The application of white rot fungi for the synthesis of enzymes by biotechnological method has become a popular field of attention because of their capability to speed up the reactions in very short time. *Pleurotus* is a genus of gilled mushrooms originates from basidiomycetous ancestors including different species of edible mushrooms like *Pleurotus ostreatus* also known as oyster mushrooms because of their ability to form oyster like fruiting body [11, 12, 13]. *Pleurotus sapidus* also known as lung or phoenix oyster mushroom is quite similar to *Pleurotus ostreatus* regarding different properties with an exception of pale colour cap short in size than *Pleurotus ostreatus* having unique taste and preferably grows in warm weather [14]. Ligninolytic enzymes are of great biotechnological interest because of their enormous applications in textile and pharmaceutical and environmental aspect. Laccase is a multicopper blue oxidase enzyme (MCO) belongs to oxidoreductase family possess ability to catalyzes the oxidation of different compounds including phenolic and aromatic amines. It accelerates the conversion of phenoxyl radicals to ketone in phenolic structures [15, 16]. Laccases are glycoproteins in nature with a similar structure to

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ascorbate oxidase possessing three cupredoxin domains with molecular mass of 60 to 80 KDa having isolelectric point pI 3-6 [17]. Activity of enzymes is greatly influenced by many factors like pH, aeration, temperature, different nutritional factors like carbon and nitrogen sources, fungal strain and moisture level [12]. Efficient working of fungi can only be obtained by supervised control of different factors in order to get optimum conditions. RSM is advance set of mathematical and statistical technique that is applied to study the interactive effect of different factors at different levels with development of proper model to analyze different problems [18, 19, 20]. The objective of this study was to find out the ability of *Pleurotus sapidus* WC 529 to degrade lignocellulosic biomass and its utilization as efficient carbon source by inducing laccase enzyme through an exotic approach of (RSM) Response surface methodology and to investigate the effect of different physical parameters including temperature, inoculum size, incubation time, moisture and pH and their interactive effect on potentially induced laccase enzyme under central composite design using banana stalk.

2. MATERIALS AND METHODS

Biomass accumulation and Preparation

Six different lignocellulosic substrates including sugarcane baggase, wheat straw, rice straw, banana stalk, corn stover and corn cobs were obtained from Industrial biotechnology laboratory and CPC Rafhan Mill Faisalabad. All these substrates were washed, dried in oven at 70^0C followed by sun drying and then ground in an electric grinder to powder form of 40mm mesh size. These were stored in air tight plastic jars to keep them moisture free.

Fungal Strain and Inoculum Up-growth

Pure culture of *Pleurotus sapidus* WC 529 was obtained from Mushroom Laboratory, Department of Plant Pathology, University of Agriculture Faisalabad, Pakistan. Homogeneous spore suspension of *Pleurotus sapidus* WC 529 was prepared by growing the fungus in inoculum medium for 5-8 days. The inoculum medium was Kirk [21] supplemented with 1% (w/v) glucose. The medium pH was adjusted to 4.5 and it was sterilized at 121^0C temperature in laboratory scale autoclave (Sanyo, Japan) for 15 minutes. A loopful of *P. sapidus* WC 529 spores was then transferred to moderate cool sterilized inoculum medium under sterile conditions in laminar hood (Dalton, Japan) and the inoculated flask was incubated at 31^0C for 49 days in shaker (Sanyo- Gallemkemp, UK) with continuous shaking to obtain the spore concentration of 1×10^6 to 1×10^8 spores/mL [22].

SSF protocol for enzyme production and extraction

Experiments were conducted in triplicate using 250 mL Erlenmeyer flasks in a temperature controlled incubator (EYLA SLI-600ND, Japan). Lignocellulosic substrates were subjected to solid state fermentation. The flasks contained 5g of respective substrates were moistened with 10mL of the basal medium without glucose. Each flask was sterilized at 121^0C in an autoclave for 15 minutes and inoculated with 5mL of freshly prepared inoculum medium. The inoculated flasks were kept at

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30^0C for 1-10 days in temperature controlled incubator to determine the suitable substrate with high enzymatic activity. After the end of stipulated fermentation time period, the experimental and control flasks were harvested by adding 100mL of distilled water and kept in shaker for 30minutes. The filtrates were then centrifuged at 10,000 rpm for 10minutes at room temperature. The supernatants were collected carefully and stored in sterilized glass bottles for enzyme activity determinations.

Laccase enzyme assay

Enzyme activity of supernatant collected at the end of screening experiments and optimization steps was determined using a spectrophotometer (T 60, PG, Instruments, UK). Laccase enzyme activity was determined by method of Wolfenden and Wilson [23]. The oxidation of 2, 2 azinobis 3- ethyl-benzthiazoline 6 sulphonate (ABTS) was followed at 420 nm in a reaction mixture having 1mL of 1mM ABTS in 1mL of 50mM sodium malonate buffer (pH 4.5) and 100µL of enzyme extract. The enzyme activity was expressed as U/mL.

Process optimization using RSM under central composite design

Central composite design was used to appraise the effect of different parameters on laccase enzyme production by *Pleurotus sapidus* WC 529. The full experimental plan under investigation comprises the high and low levels of different independent variable including temperature (20-40°C), inoculum size (2-6mL), Incubation time (48-240 h), moisture (40-60%) and pH (2-6) respectively. The data obtained from RSM on response that is laccase was subjected to analysis of variance. A quadratic polynomial equation for estimating the response function i.e optimum laccase enzyme activity (U/mL) was expressed using second order polynomial according to equation 1 [24]

$$Y(x) = b_\circ + \sum_{i=1}^{k} bixi + \sum_{i=2}^{k}\sum_{i=1}^{j-1} bijxixj + \sum_{i=1}^{k} bijX2 + e$$

Where Y is predicted response i.e laccase enzyme yield, b exhibits regression coefficients. While i and j are linear and quadratic coefficients and k is the number of factors optimized during experiment and e is the random error.

3. RESULTS AND DISCUSSION

Six different lignocellulosic substrates banana stalk, sugarcane baggase, corn cobs, rice straw, corn stover and wheat straw were estimated to induce laccase enzyme by using a novel strain of *Pleurotus sapidus* WC 529 for ten days. The triplicate flasks were harvested after every 48 hours and maximum laccase enzyme activity 250.94 U/ mL noted on banana stalk after 8th days of incubation (Table 1). The enzyme activity is in agreement with earlier reports emphasizing that different *Pleurotus* species have potential to induce considerable amount of laccase enzyme with very minute amount of other lignin degrading enzymes [25, 26]. Among different white rot fungi *Pleurotus flabellatus djamor*, *Phanerochaete chrysosporium* and *P. eryngii* have been reported for most desirable quantities of laccase using different lignocellulosic biomass. The comprehensive quantity of competent enzymes is entirely dependent on nature of lignocellulosic substrate and potent fungal strain selection having ability to yield maximum enzyme under different environmental conditions. Moreover, selection of

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suitable lignocellulosic substrate is essential step for cost effective microbial enzyme production [27].

Table 1. Screening experiment for excerption of best laccase enzyme producer substrate by *Pleurotus sapidus* WC 529

Substrates	**Enzyme activities U/mL Incubation Days**				
	2	**4**	**6**	**8**	**10**
Corn Cobs	186.30±10.90	165.10±10.14	160.92±7.30	147.10±4.12	132.10±9.30
Corn Stover	100.25±9.15	183.45±9.30	173.11±8.18	175.25±4.30	138.26±8.13
Sugarcane Baggase	110.15±5.36	172.25±7.09	172.40±9.15	163.15±5.60	155.05±5.50
Banana Stalk	167.10±10.20	200.15±3.33	228.25±13.01	250.94±7.14	195.10±6.17
Wheat Straw	168.10±3.40	193.10±4.15	179.25±7.10	199.10±8.10	213.25±7.30
Rice Straw	174.20±7.08	190.12±6.13	186.43±7.08	100.11±9.15	165.11±9.13

Process Optimization using RSM

Response surface methodology is time saving, cost effective technique as compared to laborious classical technique including optimization of one factor at a time while RSM application gives the accurate optimized results of all concerned parameters influencing the yield of enzymes in a candid manner and the use of this statistical method is still in progress for increasing the enzyme production by using different fungal strains [27]. In this study, Solid state fermentation process was further optimized by using best laccase enzyme producer substrate banana stalk. Different physical parameters like temperature, inoculum size, incubation time, moisture and pH were optimized under central composite design through response surface methodology. The increased laccase enzyme activity 281.62 U/ mL was obtained at temperature 30^{0}C, inoculum size 4 mL, incubation time 144 h, 50% moisture content and pH 5 after optimization under CCD through response surface methodology (Table 2).

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Table 2. Process optimization for augmented Laccase Enzyme Induction by *Pleurotus sapidus* WC 529 through response surface methodology under central composite design

Runs	Temperature (^{0}C)	Inoculum size (mL)	Incubation Time (h)	Moisture (%)	pH	Laccase (U/mL)
1	35	4	144	50	4	240.06
2	30	3	144	50	4	253.03
3	20	6	48	60	6	230.88
4	40	6	240	60	6	242.9
5	40	6	48	60	2	253.7
6	30	4	144	50	4	263.82
7	20	6	240	40	6	250.22
8	40	6	240	40	2	219.51
9	20	6	48	40	2	231.3
10	20	2	240	60	6	259.66
11	40	2	48	40	2	234.89
12	20	2	48	40	6	243.49
13	30	4	144	50	4	263.85
14	30	4	144	45	4	251.14
15	30	4	96	50	4	269.85
16	30	4	144	55	4	256.9
17	20	2	240	40	2	239.9
18	20	6	240	60	2	235.9
19	40	2	240	40	6	256.2
20	30	4	192	50	4	269.23
21	30	4	144	50	4	259.8
22	30	4	144	50	4	261.1
23	30	4	144	50	3	279.09
24	30	4	144	50	5	281.62
25	40	2	48	60	6	238.07
26	30	5	144	50	4	249.52
27	40	6	48	40	6	227.86
28	25	4	144	50	4	243.75
29	30	4	144	50	4	261.1
30	30	4	144	50	4	260.88
31	40	2	240	60	2	247.17
32	20	2	48	60	2	255.15

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Statistical Synergy effect study among different varying levels

The experimental data was checked through ANOVA and the results were subjected to fit second order polynomial equation. Significance of model is mostly determined by large F- value 119.62 while t and P value is used to determine the significance of each coefficient (Table 3). Larger the t value and lower the P value indicates the highly significant corresponding coefficients. Predictability of model is confirmed by non-significant lack of fit 0.5202 for laccase enzyme. Variation of observed response due to different experimental factors and their interaction is determined by coefficient of variation. The variability and precision of experiments is confirmed by short value of coefficient of variation 0.67%.

Table 3. Analysis of variance table for quadratic polynomial model for laccase production by *Pleurotus sapidus* WC 529

Source	Sum of Squares	Df	Mean Square	F- value	P Value Prob> F
Model	6667.32	20	333.37	119.62	<0.0001 Significant
Temperature	47.67	1	47.67	17.10	0.0017
Inoculum size	427.79	1	427.79	153.50	<0.0001
Incubation	77.72	1	77.72	27.89	0.0003
Time	240.09	1	240.09	86.15	<0.0001
Moisture	66.10	1	66.10	23.72	0.0005
pH	19.23	1	19.23	6.90	0.0235
AB	11.56	1	11.56	4.15	0.0665
AC	44.56	1	44.56	15.99	0.0021
AD	9.36	1	9.36	3.36	0.0940
AE	44.02	1	44.02	15.80	0.0022
BC	4.97	1	4.97	1.78	0.2086
BD	4.91	1	4.91	1.76	0.2115
BE	26.16	1	26.16	9.39	0.108
CD	640.60	1	640.60	229.86	<0.0001
CE	329.24	1	329.24	118.14	<0.0001
DE	852.77	1	852.77	305.99	<0.0001
A2	207.16	1	207.16	74.33	<0.0001
B2	209.51	1	209.51	75.18	<0.0001
C2	101.10	1	101.10	36.28	<0.0001
D2	996.49	1	996.49	357.56	<0.0001
E2	30.66	11	2.79		
Residual	16.56	6	2.76	0.98	

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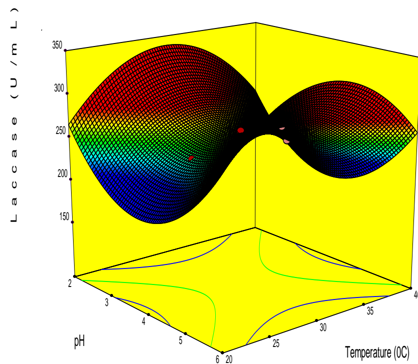
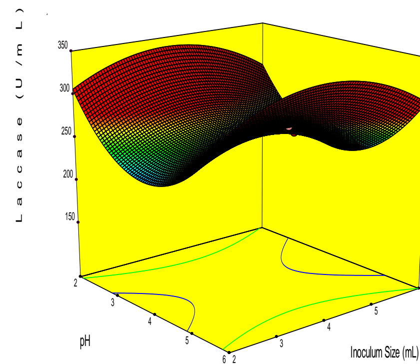
Lack of Fit	14.10	5	2.82		0.5202 not significant
Pure Error	6697.97	31			
Cor Total					

Compatibility of model with predicted response is determined by standard deviation value. Current results are in agreement with the previous study of Yasmeen [26]. The response surface plots for laccase enzyme production by *Pleurotus sapidus* are depicted in Fig 1 indicated that pH 5 has superlative effect on laccase enzyme and initial increase in pH showed tremendous effect on laccase enzyme yield but supplemented increase in pH resulted in lessened enzyme activity. The ability of white rot fungus to adapt with respect to different ecological conditions makes it magnanimous to tolerate different ranges of pH [24, 26, 28].



(A)



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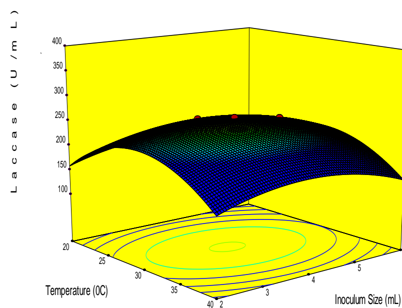

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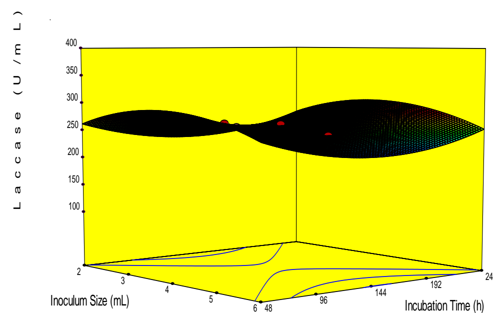

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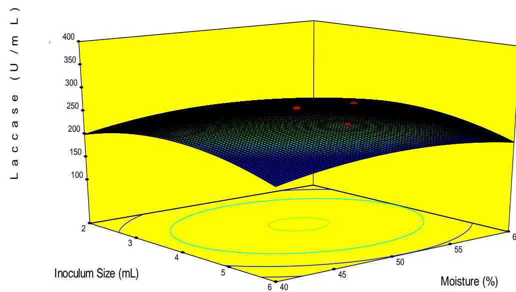

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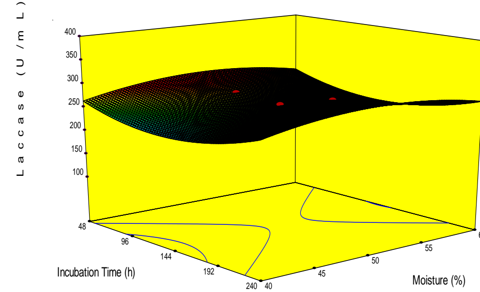

(F)

Fig 1. 3D Response Surface Plots (Created by Using Design of experts DOE. 9 showing the interactive effects (A) pH v/s Temperature (B) pH v/s Inoculum size (C)Temperature v/s Inoculum size (D) Inoculum size v/s Incubation time (E) Inoculum size v/s Moisture (F)Incubation Time v/s Moisture

High pH influences the enzyme activity by altering the conformational entity of enzyme [20, 24, 26]. The pH ranges 3.0- 5.0 have tremendous effect on augmented enzyme yield obtained by *Trametes versicolor* through (RSM) response surface methodology while optimized pH for laccase enzyme stimulation comes in the range of 4.5-6.0 [25, 29]. Appropriate growth of microorganisms and their metabolic activities are tremendously affected by most crucial factors of temperature and pH [26,30]. Enzyme activity can also be influenced by temperature and it is an important factor to be considered for best enzyme production. White rot fungi have tremendous ability to produce laccase enzyme under optimized conditions while suboptimal temperatures in the range of 15- 35^{0}C are sufficient for augmented laccase enzyme formation [26]. The results of present work are in close relation with the findings of Yasmeen [26] affirming that the best optimum temperature range for laccase enzyme production lies in the range of 25^{0}C to 37^{0}C. Inoculum size plays important role in the growth of microorganism under still culture conditions indicating that moderate level of inoculum has significant effect on growth of microorganism. Importance of exact inoculum size cannot be ignored. Low inoculum size and large amount of inoculum size is not sufficient to fulfill the nutrient requirements of microorganism as it results in lowered metabolic activity of microorganisms resulting in limited enzyme production due to depletion of nutrients creating condition of competitive inhibition [12,24, 31]. Increased laccase enzyme production was observed with moderate moisture level of 50%. Moisture content also plays an important role for the growth of microorganisms. Adequate moisture level is best for substrate because excess of moisture content and low level of moisture affect the enzyme yield. Microbial growth is greatly affected by low inoculum size resulting in inhibition of growth of microorganism due to limited contact of white rot fungus to crucial nutrients [32]. In Case of high moisture content substrate porosity is deeply affected as it ceases the oxygen transport resulting in lessened enzyme yield [26, 32]. Biosynthesis

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of fungus is also dependent on proper incubation time period. Microrganisms takes proper time for their growth to induce enzyme because of their primary metabolism and lengthy lag phase. Our results are in line with Aslam et al. [24] found maximum activity of laccase enzyme after five days of incubation under still culture condition induced by *Pleurotus nebrodensis*. The time taken by microorganism in order to produce laccase enzyme is due to long lag phase and primary metabolism [33]. Our current results suggest that optimization of different factors through RSM under central composite design has significant impact on laccase enzyme activity by considering many different factors at once and it also predicts that enhanced enzyme yield obtained followed by the interaction between different factors.

4. CONCLUSION

This study concluded that *Pleurotus sapidus* WC 529 produced laccase enzyme when grown on banana stalk under still culture conditions using novel approach of response surface methodology proved best for optimized production of laccase enzyme under central composite design. Most tremendous operating conditions and optimized levels for accelerated laccase enzyme yield were obtained by unique statistical optimization.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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