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ORIGINAL PAPER

Utilization of rice straw for laccase production by *Streptomyces psammoticus* in solid-state fermentation

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Abstract Laccase production from a novel actinobacterial strain, *Streptomyces psammoticus*, MTCC 7334 was optimized in solid-state fermentation. The process parameters were initially optimized by the conventional "one factor at a time" approach, and the optimal levels of the factors that had considerable influence on enzyme production were identified by response surface methodology. Rice straw was identified as a suitable substrate for laccase production (17.3 U/g), followed by coffee pulp (15.8 U/g). Other optimized conditions were particle size, 500–1,000 μm (21.2 U/g); initial moisture content, 65% (26.8 U/g); pH of moistening solution, 8.0 (26.9 U/g); incubation temperature, 32°C (27.6 U/g) and inoculum size, 1.5×10^7 CFU (33.8 U/g). Yeast extract served as the best nitrogen source (34.8 U/g). No enhancement in enzyme yield was observed with carbon supplementation. The level of yeast extract, inoculum size and copper sulphate were optimized statistically. Statistical optimization performed using a central composite design resulted in threefold increase in laccase activity (55.4 U/g) as compared to the unoptimized medium (17.3 U/g). The upgrading of fermented rice straw for fodder enhancement is also discussed briefly.

Keywords Laccases · Rice straw · Solid-state fermentation · *Streptomyces psammoticus* · Central composite design

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Introduction

Laccases (benzenediol: oxygen oxidoreductases [EC 1.10.3.2]) are among the important enzymes that have attracted tremendous attention in recent years due to their important applications in varying industries. They are copper-containing enzymes which catalyse the oxidation of a great variety of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor, which is reduced to water [27]. The broad substrate specificity of laccases makes them useful for several biotechnological purposes, such as bioblEaching [1], dye degradation [22], and the removal of phenolics, xenobiotics and other aromatic compounds [6, 7]. Laccase activity has been demonstrated in several higher plants [14], non-filamentous bacteria [15], different fungal strains belonging to various classes [34] and also among different species of *Streptomyces* such as *S. cyaneus*, *S. coelicolor* and *S. lavendulae* [2, 13, 33]. Laccase production by microorganisms is usually associated with their lignin degrading ability, although laccases may play a role in some other functions such as sporulation, pigment production and fruiting body formation [17, 34].

The application of laccases in biotechnological processes requires the production of high amounts of enzyme at low cost and hence the current focus of laccase research is oriented towards the search for efficient production systems. Production of laccases from microbes has been carried out using submerged as well as solid-state fermentation (SSF) technologies. SSF offers many advantages over submerged fermentation, which include higher product titers, lower wastewater output, reduced energy requirements, simpler fermentation media, etc. [25]. Moreover, this technique offers the possibility of using by-products and wastes from food and agricultural industries

as the raw material for enzyme production, making the process much more efficient from both economical and environmental standpoints. SSF is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water [26]. Most of the work on ligninolytic enzyme production in SSF deals with fungi and large numbers of agro-industrial wastes have been used for ligninolytic enzyme production by different organisms [29]. The present work was directed at the utilization of rice straw for the production of laccases from a novel strain of *Streptomyces psammoticus* in SSF, thereby upgrading rice straw for use as a better fodder. The optimization of different cultural and nutritional parameters was attempted by the conventional "one factor at a time" approach, and the levels of the critical parameters were further optimized by response surface methodology. Optimization of media components for laccase production by statistical methods has been reported for many fungal strains [35, 36]. However similar studies with actinomycetes have not been reported; to the best of our knowledge this is the first report on the subject.

Materials and methods

Microorganism and inoculum preparation

Streptomyces psammoticus MTCC 7334 used for the present study is an aerobic filamentous bacterium isolated from a mangrove swamp [20]. The culture was grown and maintained on starch casein agar slants and subcultured regularly. One-week old fully grown slants were used for inoculum preparation. The culture was aseptically transferred to the inoculum medium with the following composition (g/l): glucose, 5.0; yeast extract, 3.0; $(NH_4)_2SO_4$, 0.1; Mg SO_4, 0.1; $CaCO_3$, 0.02 and 10 ml of a trace elements solution that contained 0.1% ferrous sulphate, 0.09% zinc sulphate and 0.02% manganese sulphate. The culture was allowed to grow in the above medium for 48 h and used as the inoculum.

Substrate preparation for SSF

Five grams of substrate were added to a 250 ml Erlenmeyer flask and was moistened with a salt solution containing (g/l): yeast extract, 1.0; $(NH_4)_2SO_4$, 0.2; Mg SO_4, 0.2; $CaCO_3$, 0.04; Cu SO_4, 0.002. Three ml of the moistening solution was added to the substrate and the initial moisture level in the substrate was adjusted to 50% by adding an adequate quantity of distilled water. After sterilization by autoclaving at 121°C for 45 min, the medium was cooled to room temperature and inoculated with 1×10^7 CFU of inoculum and incubated at 30°C for 96 h.

Optimization of fermentation process under SSF

Different agro industrial residues were screened to identify the suitable substrate for laccase production in solid-state fermentation. The substrates used were wheat bran, rice bran, rice straw, coffee pulp, coconut coir pith and sugarcane bagasse. The process parameters were also varied to optimize the laccase yield which included particle size of the substrate (300–>2000 μ), initial moisture content (54–75%), initial pH of the moistening solution (5–10), incubation temperature (25–40°C) and inoculum size (7.5×10^6–1.75×10^7 CFU). Supplementation with various organic nitrogen sources (beef extract, yeast extract, peptone, tryptone and corn steep solid at a concentration of 0.1% w/v), inorganic nitrogen sources (ammonium sulphate, ammonium chloride, diammonium hydrogen phosphate, potassium nitrate and sodium nitrate in such a way that the medium contained 0.004% Nitrogen) and additional carbon sources (glucose, galactose, sucrose, starch and xylan at 1% w/v) were also carried out. All the experiments were carried out in triplicates and standard deviation for all values was <±5%.

Scanning electron microscopy (SEM) and delignifying activity

Small quantity of rice straw fermented for 48 h with *S. psammoticus* was air dried. Very thin straw particles from the fermented matter were selected for observation under scanning electron microscopy (SEM). Unfermented rice straw was also observed under SEM and treated as the control. The remaining fermented matter as well as the unfermented rice straw was sterilized and re-inoculated with *Trichoderma reesei* and cellulase production was monitored. The total activity of cellulase was measured by the standard filter paper assay method [14].

Experimental design and statistical analysis

Effects of yeast extract, inoculum size and copper sulphate ($CuSO_4$) on laccase production were investigated using central composite design (CCD) in solid-state fermentation. The levels of yeast extract and inoculum level were determined based on the experience from single parameter optimization. The impact of $CuSO_4$, the widely used laccase inducer was not studied in the one factor approach and hence the levels were set randomly. CCDs are response surface designs that can fit a full quadratic model. The effect of each variable on enzyme production was studied at five different levels viz; $-\alpha$, -1, 0, $+1$, $+\alpha$. A set of 20 experiments was performed, in triplicates. All variables were taken at a central coded value considered as zero. The data obtained from RSM on laccase production were

subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second-order polynomial Eq. 1 that represents the behaviour of the system:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC, \quad (1)$$

where Y response variable, β_0 intercept, β_1, β_2, β_3 linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$ squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ interaction coefficients, and A, B, C, A^2, B^2, C^2, AB, AC, BC level of independent variables. Analysis of data and generation of response surface graphs were done using the statistical software Design Expert (Version 6.0.6, Stat-Ease, Minneapolis, MN, USA).

Enzyme extraction

The fermented material was extracted with distilled water to get a final extraction volume 100 ml. The contents were mixed thoroughly by keeping the flasks on a rotary shaker at 200 rpm for 1 h. The mixture was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was collected and used for enzyme assay.

Laccase assay

Laccase activity was measured by monitoring the oxidation of 500 μM ABTS (2, 2′-azino-bis-[3-ethyl benzothiazoline-6-sulphonic acid]) (Sigma, St. Louis, MO, USA) buffered with 0.2 M sodium phosphate buffer (pH 7.5) at 420 nm for 1 min [4]. The reaction mixture (3 ml) contained 1 ml of culture filtrate. One unit of enzyme activity was defined as 1 μM of ABTS oxidized per minute. To calculate enzyme activity an absorption coefficient of 3.6×10^4 M^{-1} cm^{-1} was used.

Biomass estimation

Biomass estimation was carried out by determining the *N*-acetyl glucosamine released by the acid hydrolysis of chitin present in the cell wall [30]. It was expressed as mg of glucosamine/g dry fermented matter.

Results and discussion

Solid-state fermentation was carried out for the production of laccases from *S. psammoticus* and the approach of single-parameter optimization was employed to optimize various cultural and nutritional parameters to enhance laccase production and the optimal level of some of the crucial factors were identified using response surface methodology.

Screening of substrates

Selection of an appropriate substrate is a key factor in SSF which determines the success of the process. It has been a practice to use lignocellulosic agroindustrial residues for the production of ligninolytic enzymes such as laccases. In the present study various agroindustrial residues that contain lignin in different proportions had been used for laccase production in SSF. Among the substrates screened rice straw was found to be the most suitable substrate for laccase biosynthesis followed by coffee pulp (Fig. 1). The use of wheat straw for the production of laccases has been reported widely from fungal as well as actinomycetes strains [2, 29]. However the utilization of rice straw, which contains 17% lignin [29], still remains to be a less exploited substrate for ligninolytic enzyme production. The filamentous nature of *S. psammoticus* was an added advantage which facilitated the penetration of straw particles and utilization of the nutrients; a characteristic usually appreciated in solid-state culture conditions. The present study has proved the utility of rice straw which is an inexpensive and easily available raw material as a suitable substrate for laccase production in SSF.

Delignification of rice straw

The use of rice straw for laccase production by *S. psammoticus* resulted in distortion of the cell wall layers. Figure 2b shows the various structural changes that occurred in rice straw during fermentation with *S. psammoticus*. The channelling and peeling appearance observed on the fermented straw (Fig. 2b) as compared to the unfermented straw (Fig. 2a) was a strong evidence of delignification in

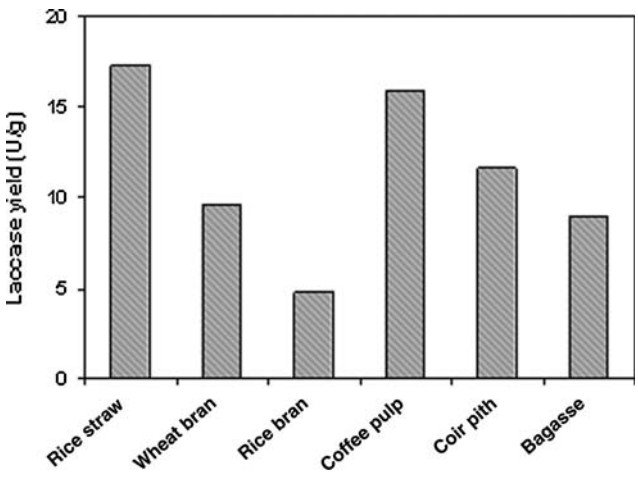

Fig. 1 Screening of agro industrial wastes for laccase production in solid-state fermentation. Fermentation conditions include moisture content 50%, particle size 300–500 μm and incubation temperature 30°C

the fibre. Delignifying ability of the culture was determined in an indirect way by assaying the amount of cellulase produced by *T. reesei* using the fermented and non-fermented rice straw. The experiment was designed based on the well-known fact that the accessibility to the cellulosic components will be more in delignified fibres [19]. In the present study it was observed that the amount of cellulase produced by *T. reesei* was higher with rice straw which was already subjected to fermentation with *S. psammoticus* (data not shown). Thus it could be concluded that substantial delignification had occurred on the rice straw particle upon fermentation with *S. psammoticus* which facilitated the availability of underlying cellulosic components to be acted upon by the cellulases of *T. reesei.* The above results were confirmed by the SEM photographs of the fermented and non-fermented rice straw (Fig. 2a, b).

Lignin present in the lignocellulosic materials not only acts as a barrier to the hydrolytic enzymes like cellulases and hemicellulases in attacking their respective substrates but also reduces the digestibility of the fibre by ruminants. Delignified straw is generally considered as a high-quality fodder with increased nutritive value and digestibility [3]. Upgradation of rice straw for ruminant feed has been studied by several workers [11] and hence the present work holds better prospects that the rice straw can very well be utilized for laccase production and simultaneously it can be upgraded for fodder production with enhanced digestibility and nutritive qualities.

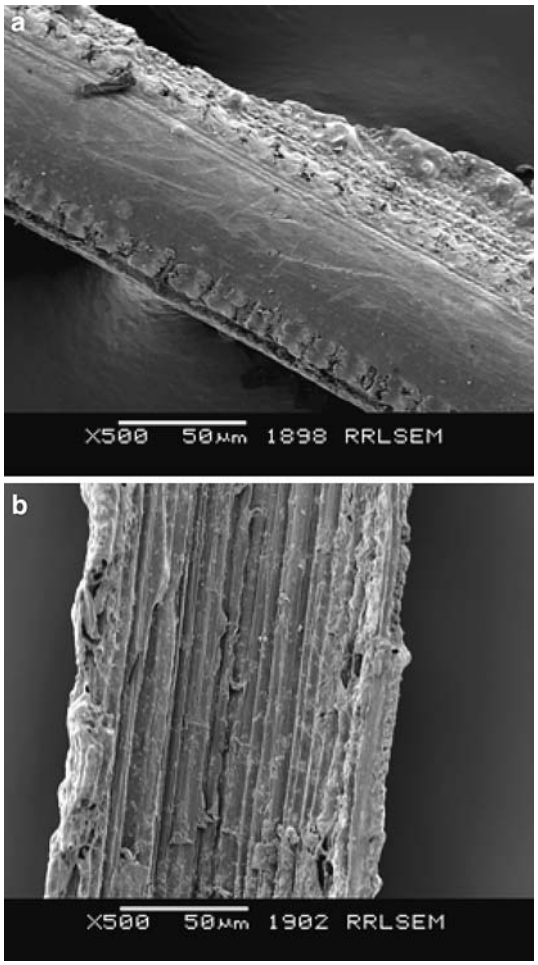

Fig. 2 Scanning electron micrographs of rice straw (**a**) surface characteristics of unfermented rice straw-control (**b**) appearance of rice straw after 48 h of fermentation with *S. psammoticus* under solid-state culture

Effect of particle size and initial moisture content

The adherence and penetration of microorganisms as well as enzyme action on the substrate clearly depend upon the physical properties of the substrate such as the accessible area, surface area, porosity, particle size, etc., of which particle size plays a major role because all the other physical properties of the substrate depends on it. In the present study particle size in the range of 500–1,000 µm was found to be optimum for laccase production (Fig. 3). The enzyme yield was low in the case of substrates with lower and higher particle size which was in congruence with the general concept that lower particle size results in substrate agglomeration, enhanced channelling problems and decreased heat transfer while larger particles reduce the production due to limited surface area for microbial attack [26]. When unsieved (mixed) substrate which contained different particle size was used, the enzyme production was better than that was obtained with lower and higher particle size substrates. This was probably due to the reason that the above-mentioned problems were comparatively less experienced with the mixed particle size.

Moisture is another key parameter to control the growth of microorganism and metabolite production in SSF.

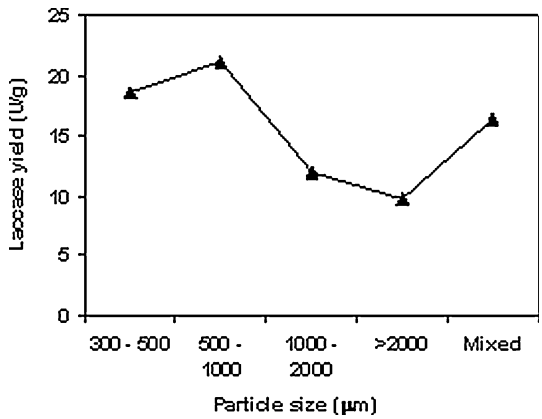

Fig. 3 Effect of particle size (*filled triangle*) on laccase production by *S. psammoticus.* The moisture content and incubation temperature for the experiment were 50% and 30°C, respectively. Inoculum size was 1×10^7 CFU

Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer while decrease in initial moisture level results in reduced solubility and low availability of nutrients to the culture. An initial moisture content of 65% was the optimum for laccase production by *S. psammoticus* (Fig. 4). Rice straw is comparatively a dry substrate and hence a low initial moisture level was observed to be inadequate for moistening the substrate evenly. However increasing the initial moisture content above the optimum also resulted in decreased enzyme yield due to the reduction in interparticle space and decreased porosity.

Effect of pH, incubation temperature and inoculum size

Optimum pH for maximal laccase production during SSF was observed at pH 8 (26.9 U/g). It has been already established that the laccase production by this organism in submerged fermentation occurred at pH 7.5 [20] and the current results confirmed that the enzyme production was favoured by neutral to alkaline pH range where as the acidic pH decreased the enzyme yield considerably (Fig. 5). Temperature is of much significance in the SSF systems because during fermentation there is a general increase in the temperature of the fermenting mass due to respiration [24]. Even though the impact of temperature is more prominent in the scale up processes it remains an inevitable factor in all fermentation systems due to its impact on microbial growth and metabolite production. Results of the present study (Fig. 6) suggested that an incubation temperature of 32°C (27.6 U/g) was the optimum for laccase production and considerable activity was observed also at 30°C (26.8 U/g). The effect of temperature on the growth of *S. psammoticus* as well as laccase production by this organism has been studied extensively

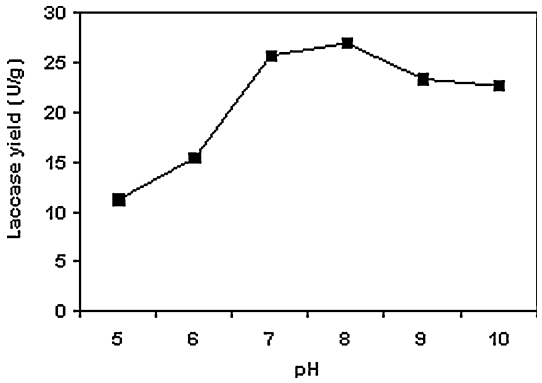

Fig. 5 Influence of initial pH (*filled square*) of moistening solution on laccase production under SSF. Culture conditions were particle size 500–1,000 μm, initial moisture content, 65%, incubation temperature 30°C and inoculum level 1×10^7 CFU

in our earlier work [21] and it can be concluded that temperature exerts a similar effect on growth and enzyme production irrespective of the mode of fermentation.

The optimization of inoculum size revealed that 1.5×10^7 CFU yielded maximum (33.4 U/g) laccase production (Fig. 7). The enzyme yield was reduced at lower and higher inoculum levels. A very low inoculum size was found to be inadequate for enzyme production while the inoculum level above optimum reduced the yield probably due to the competition for nutrients.

Effect of nitrogen and carbon supplements on laccase production

Nature and type of carbon and nitrogen sources are among the most important factors for any fermentation process [24]. There exist controversial reports on nitrogen requirement by ligninolytic organisms. The ligninolytic enzyme production (lignin peroxidase and manganese peroxidase) by the best-studied fungi *Phanerochaete chrysosporium*

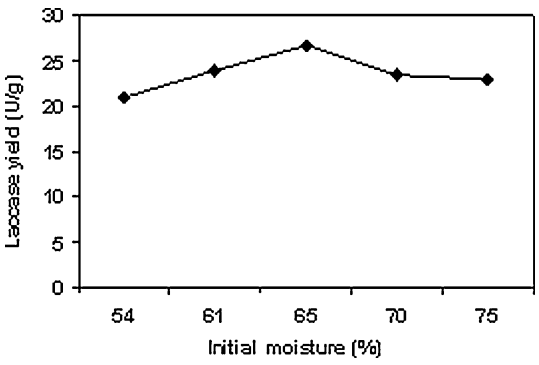

Fig. 4 Effect of initial moisture content (*filled diamond*) on laccase production by *S. psammoticus*. Particle size of the substrate was 500–1,000 μm, incubation temperature was 30°C and inoculum size was 1×10^7 CFU

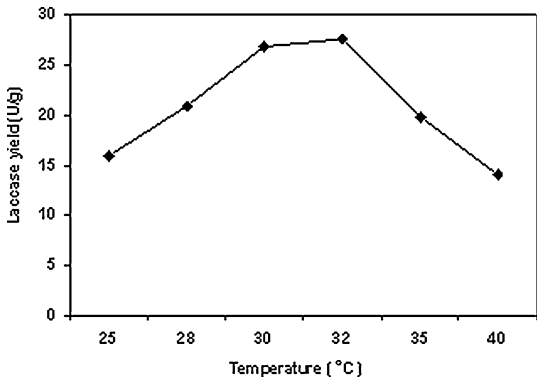

Fig. 6 Effect of incubation temperature (*filled diamond*) on laccase production by *S. psammoticus*. Culture conditions were particle size 500–1,000 μm, initial moisture content, 65%, pH of moistening solution 8.0 and inoculum size 1×10^7 CFU

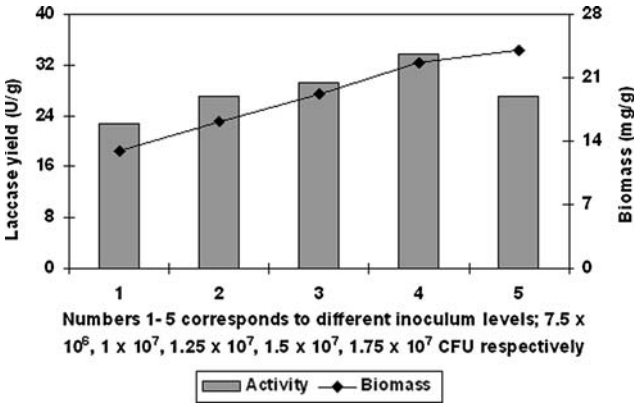

Fig. 7 Effect of inoculum size on laccase (*filled bar*) and biomass (*filled diamond*) production by *S. psammoticus* under SSF. Fermentation conditions include particle size 500–1,000 μm, initial moisture content-65%, pH of moistening solution 8.0 and incubation temperature 32°C

was found to be limited by nitrogen sources [5] where as the same organism has been reported to yield laccase in nitrogen containing media [31]. The differential effect of the source of nitrogen on laccase production has also been well established. Elisashvili et al. [8] have shown that medium with $(NH_4)_2SO_4$ has given highest levels of laccase activity in *Cerrena unicolor* while Kaal et al. [12] have established that laccase production in *Lentinus edodes* and *Pleurotus ostreatus* was enhanced by the organic nitrogen source, peptone. In the present study the replacement of yeast extract with other organic and inorganic nitrogen sources failed to elicit laccase production. This confirmed the suitability of yeast extract as the nitrogen source (34.8 U/g) for laccase production by *S. psammmoticus* and similar result has been reported from white rot fungal strain [28]. The influence of yeast extract on laccase production by the same organism has already been established in SmF [21]. The results (Table 1) also confirmed that the organic sources are better source of nitrogen than inorganic sources for laccase production by this strain.

The effect of different carbon sources on laccase production has been established in the case of fungal strains [32]. In the present study supplementation of the basal media with different carbon sources failed to exert any positive effect on laccase yield. However among the carbon sources tried glucose was comparatively a better source which yielded 32.1 U/g (Table 1). This was probably due to the reason that glucose is a readily utilizable substrate which would promote the biomass production. It has already been demonstrated that substrates which are efficiently and rapidly utilized by the organism results in high levels of laccase activity [10]. The carbon supplements used in the study might be repressing the genes that are involved in the metabolism of alternative carbon sources and that might be the reason for low laccase yield in the presence of these supplements.

Time course of laccase production by *S. psammoticus*

The time course of laccase production indicated that the maximum enzyme yield was achieved at 48 h of incubation. The enzyme production was observed to be in linear relation with the biomass production as it is presented in Fig. 8. The production of enzyme at an early incubation time and the linear relation of enzyme production with biomass were in compliance with the earlier report that ligninolytic enzyme production by actinomycetes is strictly a growth associated primary metabolic activity where as the fungi produce ligninolytic enzymes as a secondary metabolite [16, 18].

Statistical optimization

The single parameter optimization indicated that inoculum size had a profound effect on laccase production by this strain and hence it was selected as one of the critical factor for statistical optimization. The replacement of yeast extract with other nitrogen sources reduced the enzyme yield considerably which necessitated the need for finding out the exact level of yeast extract required for enzyme production. Copper sulphate ($CuSO_4$) is one of the widely reported inducer of laccases in many fungi [9, 23] and hence it was considered logical to have a better idea on the role of $CuSO_4$

Table 1 Effect of different organic and inorganic nitrogen sources and additional carbon supplements on laccase production by *S. psammoticus* in solid-state fermentation

Organic nitrogen supplements (0.1% w/v)	Peptone	Tryptone	Yeast extract	Beef extract	Corn steep solid
	26.9	25.8	**34.8**[a]	27.1	23.0
Inorganic nitrogen supplements (0.004% N)	$(NH_4)_2SO_4$	NH_4Cl	$NaNO_3$	KNO_3	$(NH_4)_2HPO_4$
	22.5	18.7	28.2	23.5	16.9
Carbon supplements (1% w/v)	Glucose	Galactose	Sucrose	Starch	Xylan
	32.1	27.0	17.5	19.4	26.5

[a] The medium that contained yeast extract as the nitrogen source was treated as the control for the above experiments. The control medium contained no carbon supplements

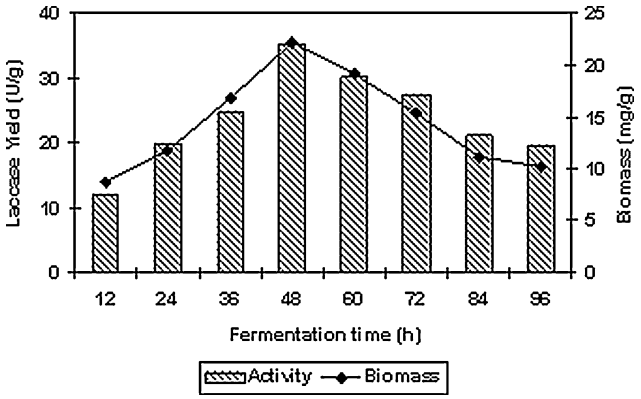

Fig. 8 Time course of laccase (*stripped bar*) and biomass (*filled diamond*) production by *S. psammoticus* under solid-state fermentation

on laccase production by this strain. Based on the above observations the three parameters viz; yeast extract concentration, inoculum level and $CuSO_4$ concentration were selected for statistical optimization to identify their optimal levels.

The minimum and maximum ranges of variables used, and the full experimental plan with respect to their values in actual and coded form is listed in Table 2. The results of response surface experiments (CCD) performed for optimizing the levels of yeast extract, inoculum size and copper sulphate are also presented in Table 2 in terms of enzyme yield. The ANOVA for the selected quadratic model showed that the model was significant with a Model $F = 11.63$ and $P > F$-value of 0.0003 (Table 3). The model terms A^2, B^2, AB, AC and BC exhibited confidence level above 95% ($P > F < 0.05$). This indicated that the squared effects of yeast extract concentration, inoculum level and the interaction effects of yeast extract and inoculum level, yeast extract and copper sulphate concentration, inoculum level and copper sulphate concentration were significant model terms. The coefficient of determination (R^2) was calculated as 0.9128 for laccase production, indicating that the statistical model can explain 91.28% of variability in the response. For a good statistical model R^2-value should be close to 1.0 where a value >0.75 indicates the aptness of the model. The model recorded an adequate precision of 10.137 which indicated an adequate signal to navigate the design space. The "Lack of Fit *F*-value" of 2.65 implied that the "Lack of Fit" was not significant and hence the model was fit.

Table 2 Experimental plan for central composite design (CCD) performed with *S. psammoticus* under solid-state fermentation for the selected parameters and the actual and predicted responses in terms of laccase yield

Run	A		B		C		Response	
	Yeast extract (%)		Inoculum size (ml)		$CuSO_4$ (mM)		Laccase yield (U/g)	
	Actual	Coded	Actual	Coded	Actual	Coded	Actual[a]	Predicted
1	0.20	−1	2.0	−1	2.0	−1	47.0	47.75
2	0.60	+α	3.5	0	3.0	0	31.3	27.16
3	0.35	0	3.5	0	3.0	0	43.0	38.15
4	0.35	0	3.5	0	4.68	+α	35.0	38.15
5	0.50	+1	5.0	+1	4.0	+1	47.2	45.26
6	0.35	0	0.98	−α	3.0	0	16.1	17.37
7	0.35	0	3.5	0	3.0	0	40.0	38.15
8	0.35	0	3.5	0	3.0	0	35.0	39.65
9	0.20	−1	5.0	+1	4.0	+1	16.1	14.00
10	0.20	−1	2.0	−1	4.0	+1	28.0	21.52
11	0.50	+1	2.0	−1	2.0	−1	24.2	25.11
12	0.35	0	3.5	0	1.32	−α	52.1	49.12
13	0.35	0	6.02	+α	3.0	0	15.0	15.41
14	0.50	+1	2.0	−1	4.0	+1	16.2	17.93
15	0.35	0	3.5	0	3.0	0	40.0	38.15
16	0.10	−α	3.5	0	3.0	0	14.1	19.91
17	0.35	0	3.5	0	3.0	0	33.1	38.15
18	0.50	+1	5.0	+1	2.0	−1	25.0	30.30
19	0.20	−1	5.0	+1	2.0	−1	21.0	18.09
20	0.35	0	3.5	0	3.0	0	38.1	38.15

[a] Mean of triplicate values

Table 3 Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of squares	*df*	Mean square	*F*-value	*P* > *F*
Model	2,512.11	9	279.12	11.63	0.0003
Yeast extract × Yeast extract (A^2)	384.74	1	384.74	16.03	0.0025
Inoculum size × Inoculum size (B^2)	853.29	1	853.29	35.55	0.0001
Yeast extract × Inoculum size (*AB*)	607.26	1	607.26	25.30	0.0005
Yeast extract × $CuSO_4$ (*AC*)	181.45	1	181.45	7.56	0.0205
Inoculum size × $CuSO_4$ (*BC*)	245.31	1	245.31	10.22	0.0095
Residual	240.05	10	24.01		
Corrected total	174.27	5			

The interaction between yeast extract and inoculum size indicated that at the higher inoculum level, the enzyme yield increased with the increase in yeast extract concentration while at lower inoculum levels there was a drop in enzyme yield with increase in yeast extract concentration (Fig. 9). Higher inoculum level might have resulted in rapid depletion of nutrients and hence higher levels of yeast extract were required for maintaining the biomass and enzyme production in such a situation. However when the inoculum level was low, the increase in yeast extract concentration beyond a particular concentration exerted a negative influence on laccase production.

It was obvious from the 3D surface curves (Fig. 10) that the laccase production increased with increase in yeast extract concentration and decrease in $CuSO_4$ concentration. At higher level of yeast extract (0.5%) the effect of different levels of $CuSO_4$ concentration on enzyme production was little while at lower levels of yeast extract concentration decreasing the level of $CuSO_4$ resulted in enhanced enzyme yield. Maximum enzyme yield was obtained when the yeast extract concentration was around 0.3% and $CuSO_4$ concentration at 2 mM. The interaction between inoculum size and $CuSO_4$ concentration (Fig. 11) exhibited almost a similar pattern as the one between yeast extract and $CuSO_4$ concentration. When the copper sulphate concentration was higher the increase in inoculum size resulted in increased enzyme production. At lower level of $CuSO_4$ (2 mM) increasing the inoculum size beyond a limit reduced the enzyme production and the maximum laccase production was observed when inoculum level was around 3.0 ml and the $CuSO_4$ concentration at 2 mM. The interactions of $CuSO_4$ with yeast extract and inoculum size indicated that maintaining a lower level of $CuSO_4$ was favourable for laccase production and this was probably due to the fact that higher levels of copper sulphate are toxic to most of the microorganisms.

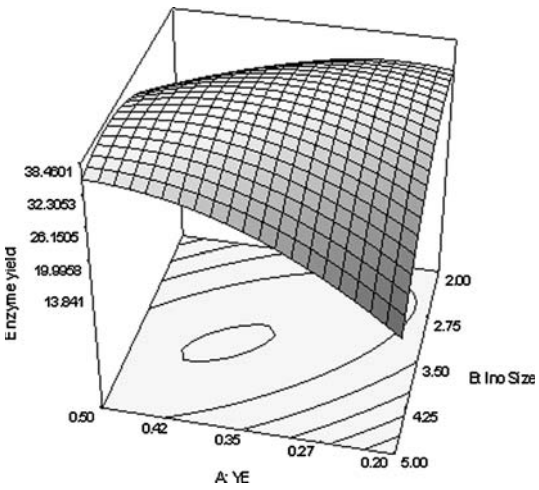

Fig. 9 Three-dimensional response surface plot for the interaction between yeast extract and inoculum size on laccase production by *S. psammoticus* under conditions optimized by RSM

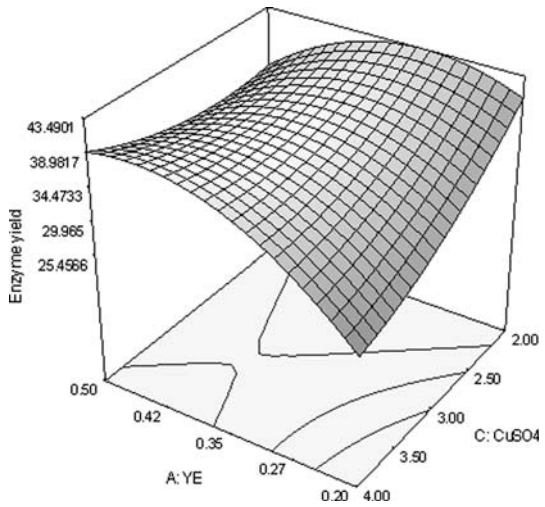

Fig. 10 *Response surface graph* showing the interaction between yeast extract and copper sulphate concentration on laccase production by *S. psammoticus* under conditions optimized by RSM

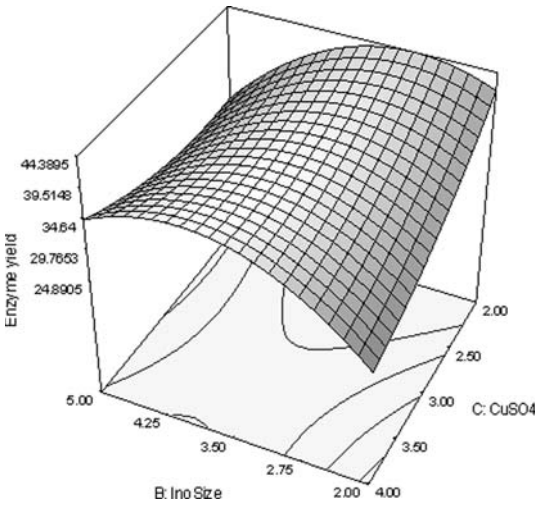

Fig. 11 *Response surface graph* showing the interaction between inoculum size and copper sulphate concentration on laccase production by *S. psammoticus* under conditions optimized by RSM

Validation of the model

Model validation was performed with three different solutions suggested by the software (Table 4). The results showed good agreement between the predicted and experimental values which indicated that the model had been validated successfully.

Conclusion

Solid-state fermentation is generally regarded as more suitable for the fungal system. The production of 55 U/g of laccase from *Streptomyces* species using rice straw as the substrate is a promising result and it suggests that solid-state culture is also functional with actinomycetes. The filamentous nature of these organisms might be favouring their growth on solid substrates. Optimization of the fermentation process by conventional procedures resulted in twofold increase in laccase production. The enzyme yield was enhanced further by response surface methodology and the model validation performed with RSM suggested that the model was valid with good reproducibility of the results. On the whole, threefold increase in laccase yield was attained after statistical optimization as compared to the unoptimized medium. The use of statistical approach for laccase production in submerged fermentation by the same strain has been studied earlier that resulted in a threefold increase in enzyme production (15.2 U/ml) in SmF [21]. Thus the present investigation has confirmed the aptness of using statistical methods for enhancing laccase production by this strain and it has also successfully evaluated the utility of rice straw, which is an inexpensive and easily available agro industrial waste for laccase production under solid-state fermentation.

Table 4 Validation of the RSM model with actual and predicted responses in terms of laccase yield

Yeast extract (g/l)	Inoculum size (ml)	$CuSO_4$ (mM)	Laccase activity (U/g) Predicted	Laccase yield (U/g) Actual[a]
0.20	2.06	2.00	47.7611	55.4
0.20	2.10	2.00	47.7424	54.5
0.50	4.83	4.00	45.3604	49.7

[a] Mean of triplicate values

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References

1. Arias ME, Arenas M, Rodriguez J, Soliveri J, Ball AS, Hernandez M (2003) Kraft pulp biobleaching and mediated oxidation of a non-phenolic substrate by laccase from *Streptomyces cyaneus* CECT3335. Appl Environ Microbiol 69:1953–1958
2. Berrocal MM, Rodriguez J, Ball AS, Perez-Leblic MI, Arias ME (1997) Solubilization and mineralization of [^{14}C] lignocellulose from wheat straw by *Streptomyces cyaneus* CECT 3335 during growth in solid state fermentation. Appl Microbiol Biotechnol 48:379–384
3. Bisaria R, Madan M, Vasudevan P (1997) Utilisation of agro-residues as animal feed through bioconversion. Bioresour Technol 59:5–8
4. Bourbonnais R, Paice MG (1990) Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of ABTS. Appl Microbiol Biotechnol 36:823–827
5. Buswell JA (1992) Fungal degradation of lignin. In: Arora DK, Rai B, Mukerji KG, Kundsen G (eds). Handbook of applied mycology, vol 1: soil and plants. Marcel Dekker, New York, pp 425–480
6. Crecchio C, Ruggiero P, Pizzigallo MDR (1995) Polyphenoloxidases immobilized in organic gels: properties and applications in the detoxification of aromatic compounds. Biotechnol Bioeng 48:585–591
7. Duran N, Esposito E (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl Catalysis B: Environ 28:83–99
8. Elisashvili V, Parlar H, Kachlishvili E, Chichua D, Bakradze M, Kokhreidze N (2001) Ligninolytic activity of Basidiomycetes grown under submerged and solid-state fermentation on plant raw material (sawdust of grapevine cuttings). Adv Food Sci 23:117–123
9. Galhaup C, Wagnera H, Hinterstoisser B, Haltrich D (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme Microb Technol 30:529–536
10. Galhaup C, Haltrich D (2001) Enhanced formation of laccase activity by the white rot fungus *Trametes pubenscens* in the presence of copper. Appl Microbiol Biotechnol 56:225–232
11. Huttermann A, Hamza AS, Chet I, Majcherczyk A, Fouad T, Badr A, Cohen R, Persky L, Hadar Y (2000) Recycling of

agricultural wastes by white-rot fungi for the production of fodder for ruminants. Agro-Food Ind Hi-Tech 11:29–32
12. Kaal EEJ, Field JA, Joyce TW (1995) Increasing ligninolytic enzyme activities in several white-rot basidiomycetes by nitrogen sufficient media. Bioresour Technol 53:133–139
13. Machczynski MC, Vijgenboom E, Samyn B, Canters GW (2004) Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. Protein Sci 13:2388–2397
14. Mandels M, Andreotti R, Roche C (1976) Measurement of saccharifying cellulase. Biotechnol Bioeng Symp 6:21–33
15. Martins LO, Soares CM, Pereira MM, Teixeira M, Costa T, Jones GH, Henriques AO (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J Biol Chem 277:18849–18859
16. Mayer AM (1987) Polyphenol oxidases in plants-recent progress. Phytochemistry 26:11–20
17. Mayer AM, Staples RR (2002) Laccases: new functions for an old enzyme. Phytochemistry 60:551–565
18. McCarthy AJ (1987) Lignocellulose—degrading actinomycetes. FEMS Microbiol Rev 46:145–163
19. McMillan JD (1994) Pretreatment of lignocellulosic biomass. In: Himmel ME, Baker JO, Overend RP (eds) Enzymatic conversion of biomass for fuels production. American Chemical Society, Washington, DC, pp 292–324
20. Niladevi KN, Prema P (2005) Mangrove actinomycetes as the source of ligninolytic enzymes. Actinomycetologica 19:40–47
21. Niladevi KN, Sukumaran RK, Jacob N, Anisha GS, Prema P (2007) Optimization of laccase production from a novel strain—*Streptomyces psammoticus* using response surface methodology. Microbiol Res. doi:10.1016/j.micres.2006.10.006
22. Nyanhongo GS, Gomesa J, Gubitzc GM, Zvauyab R, Readd J, Steinera W (2002) Decolorization of textile dyes by laccases froma newly isolatedstrain of *Trametes modesta*. Water Res 36:1449–1456
23. Palmieri G, Giardiana P, Bianco C, Fontanella B, Sannia G (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66:920–924
24. Pandey A, Radhakrishnan S (1992) Packed bed column bioreactor for production of enzymes. Enzyme Microb Technol 14:486–488
25. Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P (2001) Solid-state fermentation in biotechnology. Asiatech Publishers, New Delhi, p 221
26. Pandey A, Socool CR, Mitichell D (2000) New developments in solid-state fermentation. I. Bioprocess and products. Process Biochem 35:1153–1169
27. Piontek K, Antorini M, Choinowski T (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-A resolution containing a full complement of coppers. J Biol Chem 277:37663–37669
28. Revankar MS, Lele SS (2006) Enhanced production of laccase using a new isolate of white rot fungus *WR-1*. Process Biochem 41:581–588
29. Rodriguez Couto S, Sanroman MA (2005) Application of solid-state fermentation to ligninolytic enzyme production. Biochem Eng J 22:211–219
30. Sakurai Y, Lee TH, Shiota H (1977) On the convenient method of glucosamine estimation in koji. Agric Biol Chem 41:619–624
31. Srinivasan C, D'souza TM, Boominathan K, Reddy CA (1995) Demonstration of Laccase in the white rot basidiomycete *Phanerochaete chrysosporium* BKM-F1767. Appl Environ Microbiol 61:4274–4277
32. Stajic M, Persky L, Friesem D, Hadar Y, Wasser SP, Nevo E, Vukojevic J (2006) Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species. Enzyme Microb Technol 38:65–73
33. Suzuki T, Endo K, Ito M, Tsujibo H, Miyamoto K, Inamori YA (2003) Thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence and expression. Biosci Biotechnol Biochem 67:2167–2175
34. Thurston CF (1994) The structure and function of fungal laccases. Microbiology 40:19–26
35. Trupkin S, Levin L, Forchiassin F, Viale A (2003) Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolourization using response surface methodology. J Ind Microbiol Biotechnol 30:682–690
36. Vasconcelos AFD, Barbosa AM, Dekker RFH, Scarminio IS, Rezende MI (2000) Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response surface method. Process Biochem 35:1131–1138