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Extracellular laccase from *Pleurotus sajor-caju*: Fermentative conditions and influence of nitrogenous sources

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The present paper describes the production of extracellular laccase, a lignolytic enzyme, through fermentation of molasses salt medium (MSM) by *Pleurotus sajor-caju*, an edible mushroom, under stationary condition. MSM supported better growth of the fungus as well as laccase production compared to the other two media used, *viz*., whey and potato dextrose broth. Both laccase and edible biomass production attained the maximum value after 15 d of fermentation and depended on the fungal species, physico-chemical conditions of fermentation, and nature and composition of the fermentation media. Increase in laccase and biomass production to the extent of 9.1-48.0% and 4.2-76.4%, respectively was achieved by supplementing the medium with different organic and inorganic nitrogen sources. The positive effects of supplementation of MSM with nitrogen sources on enzyme production resulted from higher biomass yield. About 2.5-fold increase in laccase activity was obtained in comparison with the non-optimized medium. The results establish the importance of *P. sajor-caju* for the production of lignolytic extracellular enzyme, laccase.

Keywords: Fermentation kinetics, laccase, molasses salt medium, nitrogenous sources, *Pleurotus sajor-caju*, whey

Introduction

Laccase (benzenediol:oxygen oxidoreductases; EC 1.10.3.2.) belongs to the member of multinuclear copper-containing oxidases; it catalyses the monoelectronic oxidation of substrates at the expense of molecular oxygen. Lignocellulolytic enzyme, laccase depolymerizes lignin and ultimately converts it to CO_2 and H_2O[1]. It finds wide application in the textile to the pulp and paper industries, food processing and bioremediation[2,3]. Laccases are mostly extracellular enzymes produced by different groups of fungi, of which basidiomycetes fungi secrete substantial amount of the enzyme[4,5]. Several factors influence laccase production, such as, fermentation conditions, carbon and nitrogen sources of the growth media[6,7]. A number of strategies can be adopted along with media and process optimization to improve economics of fermentation.

In fermentation technology, improvement in the productivity of microbial metabolite is achieved by manipulating the physico-chemical parameters of the fermentation process, such as, medium composition, physical state of the medium, fermentation conditions and harvesting time[8-10]. One of the important approaches, therefore, is to search for the enzyme producers along with the selection of appropriate production medium and physiochemical environment for the growth of organism. Carbon and nitrogen sources of the production medium play significant roles in this respect by way of enhancing cell proliferation and metabolite biosynthesis[11]. Both nature as well as quantity of available nitrogen sources in the culture medium play important role in influencing the growth, biomass composition and/or extracellular ligninolytic enzyme production by wood-rotting basidiomycetes[12,13]. It has been reported that nitrogen source in the culture medium not only influences the amount and type of ligninolytic enzymes produced by several white-rot fungi, but also the proportion of laccase isoenzymes[14].

In recent years, much attention has been focused on the utilization of agro-wastes as cheap carbon source for the fermentation media to reduce the cost of production of the desired products. Different by-products of agro-industries that are produced in huge quantity worldwide have been successfully used as a carbon source for the production of different enzymes including lignocellulolytic enzyme[6,15]. The economics

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of fermentation depends largely on the cost of substrates of the production medium. Molasses and whey, by-products of sugar cane and dairy industries, respectively are considered as the two important cheap carbon sources of the fermentation medium. Molasses contains about 50% sucrose and the rest includes non-sugar organic matters, proteins, minerals, vitamins and other minor constituents, which are valuable sources as growth factors for the organism. On the other hand, whey, the largest by-product of the dairy industries, contains about 50% of the total solid of milk and leads to environmental pollution. Whey has been utilized as the main ingredient of the fermentation medium for the production of different useful substances[16,17].

The present paper describes the fermentative production of extracellular laccase by different fungi with special reference to *Pleurotus sajor-caju*, an edible mushroom, using some cheap medium constituents including molasses or whey as the main carbon source. The influence of both organic as well as inorganic nitrogenous sources on laccase production has also been included in the present study.

Materials and Methods

Materials

All the chemicals and biochemicals used in the study were purchased from E. Merck, Germany, and the ingredients of microbiological medium were procured from HiMedia, India. Molasses was purchased from local market and whey was procured from sweetmeat manufacturers.

Microorganisms

P. sajor-caju (MTCC 141), *Phanerochaete chrysosporium* (MTCC 787), *Rhizopus oryzae* (MTCC 262), *Aspergillus versicolor* (MTCC 280) and *Aspergillus niger* (MTCC 281) used in this study were obtained from the Institute of Microbial Technology, Chandigarh, India and maintained on potato dextrose agar slants (20% potato extract, 2% dextrose and 2% agar) and subcultured at regular intervals (30 d).

Fermentation Medium

The following three fermentation media were initially used to study the growth and production of laccase by the fungi.

Whey Medium (WM)

After adjusting to pH 4.6, fresh whey was deproteinized by heating at 95°C for 30 min. The precipitated proteins were removed by centrifugation at 4°C in a Sorvall RC-6 refrigerated centrifuge at 12000 rpm for 15 min. In the deproteinized whey, then lactose concentration was adjusted to 4.6% (w/v) and 0.02% yeast extract was added to it.

Potato Dextrose Broth (PDB)

Potato dextrose broth contained 20% potato extract and 2% dextrose.

Molasses Salt Medium (MSM)

The medium contained 0.1% K_2HPO_4, 0.001% $FeSO_4.7H_2O$, 0.001% $MgSO_4.7H_2O$, 0.02% yeast extract and molasses. The amount of molasses added to the medium so as to obtain 4% (w/v) sucrose concentration.

Nitrogen supplements in the medium were used at a concentration equivalent to nitrogen content of 0.8% diammonium hydrogen phosphate[8]. The nitrogen source was added to the medium after autoclaving (121°C for 15 min), separately. Urea was sterilized by filtration (millipore filter 0.22 μm).

Each medium (pH 5.6) of 50 mL was dispensed in different 250 mL Erlenmeyer flasks and sterilized by autoclaving at 121°C for 15 min.

Determination of Sugar

Concentrations of sucrose and lactose were determined by the phenol sulfuric acid method using sucrose and lactose, respectively, as standards[18].

Determination of Laccase Activity

Laccase activity of the mycelia grown on potato dextrose agar plates was initially noted according to Viswanath *et al*[19] with little modification by visualizing reddish brown zones on potato dextrose agar plates containing 0.02% guaiacol due to the oxidative polymerization of guaiacol. Quantitative assay of laccase activity in the cell free fermentated broth was done spectrophotometrically using guaiacol, a widely used substrate, following the procedures described by Das *et al*[20]. The reaction mixture (1 mL) contained 10 mM guaiacol, 100 mM sodium acetate buffer (pH 5.0), 10% (v/v) acetone and 50 μL appropriately diluted fermented broth. Oxidation of guaiacol was determined by the increase in absorbance at 470 nm (ϵ = 6740 M^{-1} cm^{-1}) at 30°C for 5 min of incubation. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol min^{-1} of substrate under assay conditions.

Inoculum and Fermentation

Inoculum was prepared by growing the organisms onto potato dextrose agar (PDA) plates at 25°C for

7 d. Mycelia-covered agar disks of 5 mm diameter[21] were cut with a sterile cork borer. Individual flask containing the medium was inoculated with one disk and incubated at 25°C under stationary condition for 15 d unless stated otherwise. At the end of the desired incubation period, mycelia were collected by filtration, washed thrice with double distilled water, dried by lyophilization and weight taken. Laccase activity of the fermented broth after separation of the mycelia was determined and kept at –20°C until further use.

Statistical Analysis

All results reported here are the means of five replicate experiments and the data were subjected to the statistical as well as the analysis of variance (ANOVA) using MATLAB 7.9.0 (R 2009b) and SPSS for windows version 20.0 (SPSS Inc. Chicago, IL, U.S.A.) statistical software.

Results and Discussion

Screening of Enzyme Producing Organisms and Fermentation Media

Initially, five different fungi, *viz*., *P. sajor-caju* (MTCC 141), *P. chrysosporium* (MTCC 787), *R. oryzae* (MTCC 262), *A. versicolor* (MTCC 280) and *A. niger* (MTCC 281), were tested for their ability to produce laccase in potato dextrose agar plates. Only *P. sajor-caju* and *P. chrysosporium* were found to produce laccase as indicated by the development of reddish brown zones around the growth of the organism on the agar plate containing guaiacol.

In order to study the influence of low cost media on laccase production, the organisms were grown in three different media, *viz*., PDB, WM and MSM, as described above. At the end of fermentation, dry wt of the biomass produced and the laccase activity in the cell free fermented broth were determined. Both biomass and laccase production are greatly influenced by the composition of the growth medium. Higher yield of biomass and laccase production was observed in case of *P. sajor-caju* in comparison to *P. chrysosporium*. And the maximum yield of biomass as well as laccase production by *P. sajor-caju* was obtained using MSM as fermentation medium, followed by PDB and WM (Table 1). Moreover, differences in laccase as well as biomass production in MSM by *P. sajor-caju* in comparison to *P. chrysosporium* were highly significant ($p < 0.001$). In earlier studies, white rot fungi, *P. chrysosporium* had failed to produce detectable levels of laccase under the culture conditions generally employed in the laboratories[22,23]. However, it is interesting to note that, in the present study, increase in laccase activity was noted with the increase in biomass production in all cases (Table 1). MSM as fermentation medium supported better growth of the fungus and enzyme production in comparison to other media studied. Sucrose present in molasses is initially hydrolyzed to glucose and fructose, and then metabolized by the organism to support the growth. This might be one of the reasons for obtaining higher production of laccase as Mansur *et al*[24] has reported earlier that the use of fructose instead of glucose in the fermentation medium resulted in a 100-fold increase in the specific laccase activity of basidiomycetes. On the other hand, extracellular laccase activity was not detected in the culture filtrate of *R. oryzae* (MTCC 262), *A. versicolor* (MTCC 280) and *A. niger* (MTCC 281) grown in MSM (data not shown). Considering both laccase production and economics of fermentation, *P. sajor-caju* and MSM were chosen as producer organism and fermentation medium, respectively for further studies.

In order to obtain better yield of a desired enzyme, it is essential to monitor the production of the enzyme along with the growth of the organism and corresponding changes in pH and sugar concentration

Table 1—Biomass as well as laccase production by *P. sajor-caju* (MTCC 141) and *P. chrysosporium* (MTCC 787) using three different media

Organisms	Biomass production (g L^{-1})			Laccase production (IU L^{-1})*		
	MSM	WM	PDB	MSM	WM	PDB
P. sajor-caju	5.64±0.22[a]	5.28±0.18	4.65±0.13	198±4.45[c](35.1)	177±3.80(33.5)	121±3.56(26.02)
P. chrysosporium	4.85±0.15[b]	4.13±0.10	4.72±0.14	101±3.02[d](22.47)	98±2.54(23.72)	105±3.04(21.3)

* Figures in the parenthesis indicate laccase production/g of biomass
Data represent an average of five independent experiments
Within the column, the different letter indicates significant differences at $p < 0.001$ levels
MSM = Molasses salt medium; WM = Whey medium; PDB = Potato dextrose broth

of the fermentation medium. The growth of *P. sajor-caju* was a slow process even under optimum conditions. The organism reached stationary phase after 15 d of incubation and the phase of rapid growth was extended between 6 and 12 d (Fig. 1). Interestingly, the extracellular laccase production increased with the time of fermentation and the maximum activity (198 IU L^{-1}) was noted at about 15^{th} d (Fig. 1). Thereafter, the laccase activity did not change significantly.

The pH of the fermentation medium greatly influences the microbial growth and product formation. Hence, the change in pH of MSM was followed during the entire course of fermentation of *P. sajor-caju*, but no remarkable change in pH was noted (data not shown). The change in sucrose concentration during the entire experimental period was marked by a steady fall from 40 to 17.58 g L^{-1}, which was obviously due to bioconversion of the sugar to biomass. The rate of sucrose utilization was rapid till the development of maximum mycelial growth (Fig. 2).

Effect of Nitrogen Source

The production of ligninolytic enzymes is greatly affected by the nature and amount of the nutrients, especially nitrogen (N) and microelements, in the growth medium[12]. However, there are conflicting reports and some fungi show better laccase activity in the nitrogen-rich media, while some exhibit higher production under nitrogen-limited conditions[25,26]. Therefore, it was decided to incorporate different nitrogen sources into MSM in order to enhance laccase production. Altogether nine different nitrogenous compounds were tested and these included inorganic as well as simple to complex organic compounds. Initially it was observed that the maximum biomass and laccase production by *P. sajor-caju* was obtained when MSM was supplemented with diammonium hydrogen phosphate at a concentration of 0.8% (data not shown). Hence, nitrogenous source was used at a concentration equivalent to 0.8% diammonium hydrogen phosphate. It had been observed that supplementation of MSM with nitrogenous compounds enhanced both laccase as well as biomass production in comparison to unsupplemented one (Table 2). Depending on the nitrogenous sources used, enhancement in laccase and biomass production varied from 9.1% (in case of ammonium sulfate) to 148% (in case of peptone) and 4.2% (in case of ammonium sulfate) to 76.4% (in case of peptone), respectively (Table 2). Further, laccase as

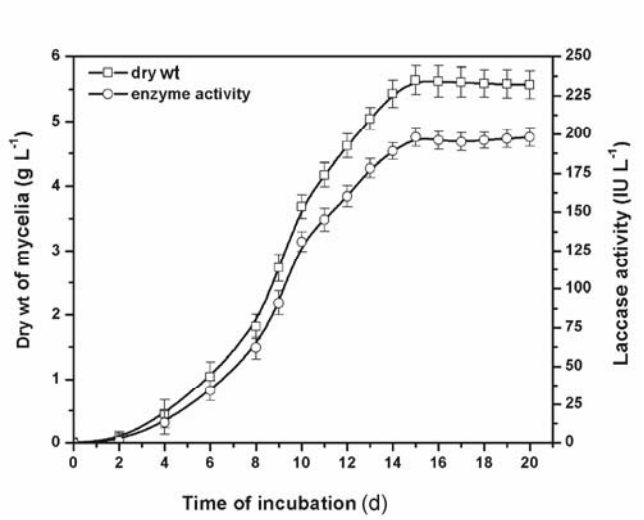

Fig. 1—Biomass as well as laccase production by *P. sajor-caju* in MSM. [Data represent an average of five independent experiments; ±SD shown by error bar.]

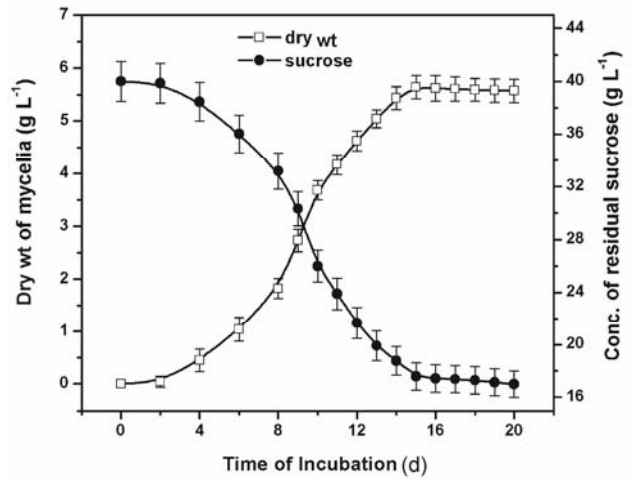

Fig. 2—Kinetics of *P. sajor-caju* in fermentation of MSM in relation to biomass as well as laccase production. [Data represent an average of five independent experiments; ±SD shown by error bar.]

well as biomass produced per liter of MSM supplemented with organic nitrogenous compounds were found to be higher compared to that of inorganic nitrogenous compounds. However, diammonium hydrogen phosphate is a good promoter of growth as well as enzyme production and was comparable to organic sources. Growth as well as extracellular laccase production in ammonium sulphate or ammonium chloride supplemented media was much lower compared to diammonium hydrogen phosphate. Probably phosphate had no role in this respect as addition of equivalent amount of phosphate did not

Table 2—Effect of supplementation on molasses salt medium (MSM) with different nitrogenous compounds on growth and laccase production by *P. sajor-caju*

Nitrogen source*	Dry wt of biomass produced (g L^{-1})	Biomass yield (g 100 g^{-1} sucrose utilized)	Final pH of broth	Final sugar conc. (g L^{-1})	Laccase production (IU L^{-1})	Increase in laccase production (%)	Laccase production (g $biomass^{-1}$)
Control	5.64±0.22[a]	25.15	5.5	17.58±1.07[c]	198±4.45[e]	-	35.1
Sodium nitrate	6.96±0.27	44.13	5.7	15.77±0.96	328±6.32	66	47.1
Ammonium sulfate	5.98±0.24	34.24	5.8	17.46±1.06	216±4.83	9.1	36.1
Ammonium chloride	5.88±0.23	33.07	5.8	17.78±1.09	218±4.84	10.10	37
Urea	6.21±0.25	38.26	6.2	16.23±0.99	295±5.60	49	47.5
Diammonium hydrogen phosphate	9.27±0.36[b]	88.36	5.6	10.49±0.64[d]	456±8.24[f]	130.3	49.1
Peptone	9.95±0.39	105.40	5.5	9.44±0.55	491±9.02	148	49.3
Glutamic acid	8.18±0.32	61.68	5.2	13.26±0.78	433±7.72	118.7	52.9
Glycine	7.82±0.29	55.42	5.4	14.11±0.83	422±7.45	113.1	53.9
Alanine	7.63±0.28	52.43	5.3	14.55±0.89	409±7.11	106.6	53.6

*MSM supplemented with nitrogenous sources
Control contains only MSM
Data represent an average of five independent experiments
Within the column, the different letter indicates significant differences at $p < 0.001$ levels

increase either of the parameters (data not shown). It is well known that the kind and quantity of the nitrogen source in the culture medium can influence the growth of microorganisms as well as fermentation products, and our results corroborate the earlier findings in this regard[12,14]. Among the organic nitrogen sources used in the present experiment, the maximum increase in growth as well as laccase production was noted with peptone, followed by glutamic acid, glycine and alanine. It was also reported earlier that organic nitrogen sources, ***e.g.***, peptone and glutamic acid strongly enhance the laccase production[7,12,14].

In addition, different nitrogen sources did not influence significantly the medium pH, which ranged between 5.2 and 6.2 during the growth of fungus. This shows the good buffering capacity of the media (Table 2). It is also interesting to note here that the extracellular laccase production increases with increase in biomass production (Table 2). More than 2.5-fold (130.3%) increase in laccase production could be achieved by supplementing molasses salt medium with 0.8% diammonium hydrogen phosphate, a cheap nitrogen source. Addition of nitrogenous compound to the MSM enhances biomass production and is reflected in higher sugar utilization (Table 2). The amount of biomass produced *vis-a-vis* sugar utilized can better be expressed by the term "Economic Coefficient" or "Biomass Yield", which is defined as the biomass produced per 100 g of sugar utilized, ***i.e.***, sucrose in the present case. The yield of biomass was found to be the highest with peptone supplemented MSM, followed by diammonium hydrogen phosphate and glutamic acid.

The present investigation establishes the production of industrially important lignolytic enzyme laccase as well as edible biomass by fermenting MSM with *P. sajor-caju*. The physico-chemical parameters that affected the fermentation process had also been optimized. Addition of organic and inorganic nitrogen sources to the inexpensive MSM increases laccase and biomass production to a great extent. Incorporation of diammonium hydrogen phosphate, a cheap nitrogen source, to the medium increased the production of laccase and biomass by 130.3% and 64.4%, respectively.

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