Prickly Palm Cactus Husk as a Raw Material for Production of Ligninolytic Enzymes by *Aspergillus niger*

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Abstract Prickly palm cactus husk was used as a solid-state fermentation support-substrate for production of the ligninolytic enzymes laccase, peroxide manganese, and lignin peroxidase by *Aspergillus niger*. Effects of water activity, temperature, and fermentation time on enzymatic production were evaluated using a central composite rotatable design. Response surface methodology revealed that maximum enzyme production was achieved at 73.38 h of fermentation, a water activity of 0.87 A_w , at 28.74°C for laccase, at 65.33 h, 0.89 A_w , and 28.96°C for lignin peroxidase, and at 70.44 h, 0.91 A_w , and 28.84°C for manganese peroxidase. Optimized enzyme production was 9,023.67 UI/L for laccase, 2,234.75 UI/L for lignin peroxidase, and 8,534.81 UI/L for manganese peroxidase. Thermostability and pH stability were observed for all enzymes. Enzymatic deactivation kinetic experiments indicated that enzymes remained active after freezing of crude extracts.

Keywords: fermentation, biotransformation, statistical, semi-arid, optimisation

Introduction

The cactus pear has been grown with relative success in semi-arid northeastern Brazilian since the early 20th century, and in arid and semi-arid regions of the United States, Mexico, and South Africa (1). The prickly pear fodder is well adapted to conditions of semi-arid Brazil, enduring long periods of drought due to physiological properties characterized by an efficient photosynthetic process. Its has been known that (2,3) the cactus pear in semi-arid Brazil is used in diverse livestock production systems due to a high level of total digestive nutrients, including carbohydrates, ash, vitamins, and iron. Lignin degradation is of economic importance for making available substances of interest to industry, livestock production, and agriculture. Lignin degradation can be understood as a process involving the ligninolytic enzymes laccase (Lac), peroxide manganese (MnP), and lignin peroxidase (LiP) that all catalyze glycosidic linkages of the polymer, thereby causing collapse (4). It has been known that (5,6) the prickly pear cactus can be an important low cost source for production of ligninolytic enzymes and to amplify applications.

Enzyme production is a field of biotechnology for which in the last decades there has been an increasing trend towards use of solid-state fermentation (SSF) techniques for enzyme production (7–9).

Statistical approaches have been used for establishment of conditions for high yield enzyme production. Response surface methodology (RSM) is advantageous over conventional methods available due to fewer experiment numbers, suitability for multiple factor experiments, and searching for common relationships between factors to identify the most suitable production conditions for a bioprocess response. RSM has been extensively used to optimize processes, such as production of enzymes (10–11).

The objective of this work was to investigate use of the prickly pear cactus (*Nopale cochenillifera*) as the main raw material for ligninolytic enzyme production with solid-state fermentation using *Aspergillus niger*. A composite central rotatable design (CCRD) and response surface methodology (RSM) were used to determine optimum production conditions for ligninolytic enzymes, which can contribute to transformation of a low commercial value biomass into a high aggregate value product.

Materials and Methods

Materials and microorganism Potato dextrose agar medium (PDA) was purchased from Himedia (Mumbai, India). Magnesium sulfate, Tween 80, ethanol, sodium lactate, and sodium hydroxide were acquired from Vetec (Duque de Caxias, Brazil). Syringaldazine (4-hidroxi-3,5-azino-metoxibenzaldide), veratryl alcohol, phenol red, albumin, and hydrogen peroxide (H_2O_2) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Sodium citrate buffer, citrate phosphate buffer, and succinate buffer were acquired from Synth (Diadema, Brazil). The fungus *Aspergillus niger* was provided by the Laboratory for Agro-industrial Residue Use (ATCC 1004) State University of Southwest Bahia at the Itapetinga campus, Bahia, Brazil.

This study did not involve any experimentation with human or animal subjects performed by any of the authors.

Solid state fermentation Prickly pear cactus husk samples were collected at the Agrostology field of the State University of Southwest Bahia at the Itapetinga campus, Bahia, Brazil, in April of 2013. After cutting, in the form of horizontal strips, husks were dried in a drying oven (SL 102; Solab, Piracicaba, Brazil) at 70°C for 24 h, ground in a Willey mill (ACB; Labor, São Paulo, Brazil) to obtain a 2 mm size powder, and sterilized in an autoclave (CS30; Prismatec, Itu, Brazil). *Aspergillus niger* was inoculated into a potato dextrose agar medium (PDA) and incubated for 7 days at 35°C in a temperature control incubator (SL 222; Solab) then spores were collected using a 0.01% Tween 80 solution. Spores in suspension were counted using a Neubauer chamber and a binocular microscope (L1000; BIOVAL, São Paulo, Brazil) (12).

Fermentation was conducted in Erlenmeyer flasks containing 10 g of sterilized powder containing dry forage cactus pear. Suspended spores were added to the substrate at the standart concentration of 10^8 spores per g of dry substrate. Sterile water was added to the dry substrate to water activity values of 0.786, 0.865, and 0.903 A_w (Aqualab PRE CAP; AquaLab, São José dos Campus, Brazil). Cultivation was performed at 25, 30, and 35°C for 24, 72, and 120 h following the experimental design. Experiments were performed in a temperature control incubator (TE-317; Tecnal, Piracicaba, Brazil) with forced air ventilation. After the fermentative process, 10 mL of a 50 mM (pH 4.8) sodium citrate buffer solution was added for each 1 g of substrate. The enzyme extract resulting from the fermentation was collected and centrifuged at 100×g for 10 min at 4°C (CT-6000R; Cientec, Piracicaba, Brazil).

Experimental design A 2^{3-1} fractional factorial design (13) with 18 runs, including 4 repetitions for the central point was implemented in order to evaluate the influence of fermentation temperature, water content, and fermentation time on production of Lac, LiP, and MnP (Table 1).

Determination of the laccase enzymatic activity (Lac) The activity

Table 1. Design approach of RSM

Run	Coded values			Real values		
	X1 (h) ¹	X2 (a_w) ¹	X3 (°C) ¹	X1 (h)	X2 (a_w)	X3 (°C)
1	-1	-1	-1	24	0.786	25
2	-1	-1	+1	24	0.786	35
3	+1	-1	-1	120	0.786	25
4	+1	-1	+1	120	0.786	35
5	-1	+1	-1	24	0.903	25
6	-1	+1	+1	24	0.903	35
7	+1	+1	-1	120	0.903	25
8	+1	+1	+1	120	0.903	35
9	0	-1	0	72	0.786	30
10	0	+1	0	72	0.903	30
11	-1	0	0	24	0.865	30
12	+1	0	0	120	0.865	30
13	0	0	-1	72	0.865	25
14	0	0	+1	72	0.865	35
15	0	0	0	72	0.865	30
16	0	0	0	72	0.865	30
17	0	0	0	72	0.865	30
18	0	0	0	72	0.865	30

¹Variable X1 corresponds to fermentation time, X2 to water activity, and X3 to fermentation temperature.

of laccase was determined using 0.1 mL of an ethanol solution of 1 mM syringaldazine. The oxidation reaction was conducted in an ethanol solution containing 0.3 mL of a 50 mM citrate-phosphate buffer (pH 5.0), and 0.6 mL of an enzymatic extract. The reaction was quantified after 10 min based on measurement of the absorbance at 525 nm carried out using a spectrophotometer (SF200DM-UV; Vis. Bel Photonics, Osasco, Brazil). The enzymatic activity was calculated based on the molar absorptivity of the oxidized substrate (14). Analyses were performed in triplicate and the enzymatic activity results were expressed as average values.

Determination of the lignin peroxidase enzymatic activity (LiP) LiP was evaluated by UV spectrometry. The reactive mixture contained 1.8 mL of enzyme extract, 0.5 mL of 50 mM citrate-phosphate buffer (pH 4.5), and 0.1 mL of 2 mM veratryl alcohol. The initial time of reaction (time=0) was the moment at which 0.1 mL of H_2O_2 was added. Absorbance measurements using a spectrophotometer (SF200DM-UV; Vis. Bel Photonics) were performed at time=0 and after 10 min of reaction. Aliquots of 1 mL were taken from each tube containing a boiled sample, whereas unboiled samples were subjected to measurement of the absorbance at 310 nm carried out using a spectrophotometer (SF200DM-UV; Vis. Bel Photonics). The color that develops in the presence of LiP was compared with a blank assay where inactivated culture supernatants were used to replace activated culture supernatants. Mixtures with LiP show a change in the blueness from a greenish blue to purple blue. The speed of colour change has a direct connection with the LiP activity (15). Analyses were performed in triplicate and enzymatic activity results were expressed as average values.

Determination of the manganese peroxidase enzymatic activity (MnP)

The manganese peroxide enzymatic activity was determined based on oxidation phenol red at 30°C. Reaction mixtures contained 1.5 mL of 25 mM sodium lactate, 0.5 mL of 1 mM MnSO₄, 0.5 mL of 1% albumin, 0.1 mg of phenol red, and 0.5 mL of the enzymatic extract filtrate in 20 mM sodium succinate buffer (pH 4.5). The reaction was started by the addition of H₂O₂ to final concentration of 0.1 mM after 10 min of reaction, 1 mL of the mixture was removed and 30 µL of a 6.5 mM sodium hydroxide solution was added. Reaction kinetics were evaluated based on measurement of the absorbance at 610 nm carried out using a spectrophotometer (SF200DM-UV; Vis. Bel Photonics). The activity of MnP was calculated based on molar absorptivity of oxidized phenol red (16). Analyses were performed in triplicate and enzymatic activity results were expressed as average values.

Determination of thermostability and pH stability Thermal stability was determined based on measurement of enzymatic activity after incubation (TE-0541/1; Tecnal) of enzymes at different temperatures, as follows: 20, 40, 60, 80, and 100°C. Aliquots were removed after 10, 30, 60, 120, and 150 min and immediately packed in ice for enzymatic determination as described above. Results were expressed as residual activity (%) -the enzymatic activity that remained after the incubation period (13).

The pH stability was determined based on measurement of enzymatic activity after incubation at 25°C of the enzyme solution in a sodium citrate buffer solution at pH 3, 4, 5, 6, 7, and 8. Aliquots were removed after 10, 30, 60, 120, and 150 min of reaction and stored in freezer prior to analysis. Results were expressed as residual activity (%). The enzymatic activity of each enzyme was evaluated in triplicate (13).

Kinetics of enzymatic deactivation Aliquots of enzymatic extracts stored at 4 and -25°C were removed at time intervals of 24, 72, 96, 120, and 144 h for determination of enzymatic activity. The enzymatic activity of each enzyme was evaluated in triplicate (13).

Statistical analysis Analysis of variance (ANOVA), regression, and plotting of response surfaces were performed to obtain optimum conditions for enzymatic activity. Experimental results were subjected to ANOVA and effects were considered significant at $p < 0.01$. Experimental coefficients were adjusted using a second order polynomial model (Eq. 1) and regression coefficients were obtained using multiple linear regression:

$$EA = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k \quad (1)$$

where β_0 , β_i , β_{ii} , β_{ij} , and β_{ijk} represent the overall constant process effect, the linear and quadratic effects of X_i , and the interaction effects between $X_i X_j$, and $X_i X_j X_k$ on enzymatic activity, respectively. ANOVA for models was performed and model significance was examined using Fisher's F -test. The best conditions for enzymatic

activity were determined for experimental conditions based on RSM. The model parameters were tested using t test with $p < 0.1$.

All statistical analysis was conducted using SAS software version 9.0 (SAS Institute Inc., Cary, NC, USA). To validate the adjusted model, optimized values of the independent variables X_1 (fermentation time), X_2 (water activity), and X_3 (fermentation temperature) were used in the same initial experimental procedure in order to verify the predictive power based on comparison of predicted and experimental results (17).

Results and Discussion

Response surface method Based on statistical analysis, Lac, LiP, and MnP activity data fit significantly ($p < 0.01$) to second-order models. Lack of fit was not significant ($p > 0.01$). The second-order models (models containing all 2 parameter interactions) were not accepted by the mentioned tests, which were improved by eliminating the model term until the established conditions were fulfilled (model parameter significant at $p < 0.1$ by t -test). All non-significant factors at 10% significance level were then grouped into the error term and a new reduced model was obtained for the response variables through a regression. Reduced models obtained can be described by Eq. (2-4) using coded values:

$$AC1 = -37537 + 299.706X_1 + 14232X_2 + 2160.687X_3 - 0.8434X_1^2 - 199.971X_2^2 - 39.611X_3^2 \quad (2)$$

$$AC2 = -77988 + 478.7626X_1 + 5548.862X_3 - 3.3395X_1^2 - 95.7501X_3^2 \quad (3)$$

$$AC3 = -43897 + 225.249X_1 + 2957.28701X_3 - 1.523X_1^2 - 50.074X_3^2 \quad (4)$$

where AC1, AC2, and AC3 are Lac, LiP, and MnP activities, respectively. X_1 , X_2 , and X_3 correspond to fermentation time, water activity, and fermentation temperature, respectively.

The outcome of an ANOVA can be visualized using a Pareto Chart (Fig. 1). The absolute value of the magnitude of the standardized estimated effect (the estimated effect divided by the standard error) of each factor was plotted in decreasing order and compared with the minimum magnitude of a statistically significant factor ($p = 0.10$), represented by the vertical dashed line. Besides, from the Pareto Chart it can be verified that fermentation time, and water activity, and temperature contributed most for production of MnP ($p = 0.1$). LiP production was influenced by time and temperature ($p = 0.1$). Lac production was influenced only the fermentation time significantly ($p = 0.1$).

High moisture content results in decreased substrate porosity, which prevents oxygen penetration and low moisture content, can lead to poor nutrient accessibility, resulting in slow microbial growth (18). Although, the water activity of a medium has been considered to be a fundamental parameter for mass transfer of water and solutes across microbial cell membranes, the effect of water activity on ligninolytic enzyme production was less than the effects of temperature and fermentation time (Fig. 1).

Fig. 1. A Pareto chart showing significance of time of fermentation, temperature of fermentation, and water activity for production of Lac, LiP, and MnP. T is time, TP is temperature, and A_w is water activity.

The importance of fermentation time is related to the disponibility of nutrients, and other culture conditions, for microbial growth and production of enzymes. Production of enzymes is slow in the beginning, and then accelerates until a maximum value is achieved and, from there, the concentration of generated products reduces the activity. The response surfaces for each model here presented (Eq. 2-4) were combinations of the effects of fermentation time and water activity on enzyme activity (Fig. 2).

The optimal activity point for Lac production was 73.38 h of fermentation with a water activity of 0.87 A_w and a temperature of 28.74°C. For LiP, 65.33 h with a water activity of 0.89 A_w and a temperature of 28.96°C were optimum. The MnP optimum activity was at 70.44 h with a water activity of 0.91 A_w and a temperature of 28.84°C. Optimized production of Lac was 9,023.67 UI/L, LiP was 2,234.75 UI/L, and MnP was 8,534.81 UI/L. The increase of Lac production was of 120%, whereas production values of LiP and MnP were of 300 and 400%, respectively, for the lowest production level of each enzyme.

Other types of agro-industrial residues and microorganisms have also been studied for ligninolytic enzyme production (19), emphasizing a trend toward reduction of production cost.

A low production level of ligninolytic enzymes was related to fermentation using potato peelings and *Pleurotus ostreatus* HK-35 with reported values of 6,708.3 and 2,503.6 U/L for Lac and MnP, respectively (20). In another study in which fermentation was conducted with *Pleurotus eryngii* using grape waste, the highest Lac activity was 2,247.62±75 U/L, whereas for MnP the value was 198.44±65 U/L (21). Thus, use of prickly palm cactus husks inoculated with *Aspergillus niger* was more efficient for production of the ligninolytic enzymes Lac, LiP, and MnP under conditions used in this study. Among the agroindustrial wastes evaluated for fermentation with *Phanerochaete chrysosporium*, brewery wastes and pomace have served as sources for production of MnP, LiP, and Lac (8,19).

Fig. 2. Response surface plots for Lac (A), LiP (B) and MnP (C) enzymes as a function of fermentation time and water activity.

Use of solid state fermentation with a ligninocellulosic biomass and inducers has also contributed to an increase in ligninolytic enzyme production in order to reduce cost (22,23). However, use of inducers may not increase enzymatic production, emphasizing the importance of optimizing the fermentation process and the ability of microorganisms to produce enzymes (24).

The bioreactor design for application of solid state fermentation is other important approach for maximizing ligninolytic enzyme production to provide an environment for microorganism growth and activity, considering aeration, humidity, agitation, and temperature (25). In addition, particle size, porosity, and chemical composition

Fig. 3. Thermostability of Lac (A), LiP (B) and MnP (C) enzymes.

may be considered for selection of a support substrate (25-27).

Biochemical characterization of ligninolytic enzymes Enzymatic characterization is important in order to determine characteristics, such as temperature and pH stability, which are the most relevant parameters for industrial application, and to maintain the efficiency of enzymatic activity during catalysis.

Based on thermal stability, ligninolytic enzymes retained approximately 90% of activity after incubation at 60°C (Fig. 3A-3C). The residual activity was higher than 70% for Lac and MnP, whereas for LiP residual activity was approximately 55% with incubated at 80°C for 150 min. That is a technically important characteristic because the majority of industrial processes require temperatures of approximately 50°C, denoting a favorable application of these enzymes for industrial applications. Although incubation at 100°C caused a pronounced

Fig. 4. pH stability of Lac (A), LiP (B) and MnP (C) enzymes.

activity reduction for all enzymes, the residual activity was higher than 40% after 150 min of incubation (Fig. 3A-3C).

The thermal stability of Lac from *Aspergillus niger* described in this study was higher than reported in other studies. The maximal thermal stability of Lac from *Ganoderma lucidum* was at 30°C and decreased abruptly above 60°C (22). Even less stability was observed for Lac from *Paraconiothyrium variable* with a 50% reduction in activity above 50°C after 1 h (24). Contrary to a report that, in general, typical fungal laccases exhibit a half-life of less than 10 min at 80°C (28), the laccase studied herein maintained more than 70% of activity above 150 min at 80°C.

The thermostability of MnP produced from different microorganisms has been shown to be lower than reported herein. Manganese

Fig. 5. Inactivation kinetics of Lac, LiP and MnP enzymes based on storage at 4°C (A) and at -25°C (B).

peroxidase from *Phanerochaete chrysosporium* was inactivated totally at 65°C (29), whereas the enzyme from *Trichophyton rubrum* LSK-27 was even more thermosensible, losing 80% of activity with incubation at 60°C for 10 min with complete inactivation at 70°C after 5 min (30).

The Lac enzyme (Fig. 4A) was more stable in a pH range between 5 and 7, whereas LiP (Fig. 4B) and MnP (Fig. 4C) were more stable at pH 4 to 6. These enzymes were more stable at pH 5 without loss of activity.

The best relative activities were obtained at pH 5 which, in principle, was close to the isoelectric point of the enzymes between pH 4.5 and 5.5 (13).

Results of pH stability reported herein were based on values reported previously (22,29). *Aspergillus terreus* LD-1 produced MnP that was stable in a pH range from 11 to 12.5. The enzyme was inactive at pH 7 or lower (31). Enzymes tested remained active over a wide pH range, which may have been related to the raw state of the enzymatic extracts tested, or to fermentation properties in solid state with fibrous fungi, which have been reported as most efficient for production of enzymes stable against temperature and pH changes

(Fig. 4).

The pH value affects the ionization state of amino acid residues in the enzyme active site, promoting changes in the tridimensional structure of the active site that may interfere with enzymatic activity. This makes enzymes most active only in a range of pH values (32). An enzymatic deactivation kinetics study (Fig. 5A and 5B) was performed for evaluation of storage time and temperature of enzymatic extracts. Storage under freezing at -25°C completely preserved the enzymatic activity, whereas storage at 4°C caused a significant loss of activity.

Higher stability at storage under 4°C was observed for MnP from *Phanerochaete chrysosporium* without loss of activity for 1 week (29).

Freezing affects protein original structures based on interference with the water activity and provoking polypeptide chains to adopt a new structure. After freezing, enzymes may acquire a new conformation that can affect enzymatic activity. The freezing process interrupts chemical reactions and biological activities.

Use of prickly pear as a substrate and the fungus *Aspergillus niger* was satisfactory for production of the ligninolytic enzymes Lac, LiP, and MnP, resulting in increases of 120, 300, 400%, respectively, under optimized conditions. Prickly pear as a cheap organic substrate that is common in northeast Brazil is a potential source for production of enzymes of industrial interest. Future research should evaluate the prickly pear as a cheap substrate for production of other enzymes.

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Disclosure The authors declare no conflict of interest.

References

1. Nascimento CES, Rodal MJN, Cavalcanti AC. Phytosociology of the remaining xerophytic woodland associated to an environmental gradient at the banks of the São Francisco river - Petrolina, Pernambuco, Brazil. *Braz. J. Bot.* 26: 271-287 (2003)
2. Do Santos, TC, Diniz, GA, De Brito, AR, Pires, AJV, Franco, M. Effect of solid state fermentation on nutritional content and evaluation of degradability in cactus pear. *Rev. Caatinga* 28: 248-254 (2015)
3. Vasconcelos AGV, Lira MA, Cavalcanti VAL, Santos MVF, Câmara T, Willandino L. Micropropagation of prickly-pear cv. Miúda (*Nopalea cochenillifera* - Salm Dyck). *Rev. Bras. Cienc. Avic.* 2: 28-31 (2007)
4. Mane JD, Modi S, Nagawade S, Phadnis SP, Bhandari VM. Treatment of spentwash using chemically modified bagasse and colour removal studies. *Bioresour. Technol.* 97: 1752-1755 (2006)
5. Ahmad Z, Butt MS, Rias M. Partial purification and characterization of xylanase produced from *aspegillus niger* using wheat bran. *Pak. J. Agr. Sci.* 50: 433-437 (2013)
6. Park C, Kawaguchi T, Sumitani J, Takada G, Izumori K, Arai M. Cloning and sequencing of an exoglucanase gene from *Streptomyces* sp. M23, and its expression in *Streptomyces lividans* TK-24. *J. Biosci. Bioeng.* 99: 434-436 (2005)
7. Fleuri LF, Novelli PK, Delgado CHO. Biochemical characterisation and

- application of lipases produced by *Aspergillus* sp. on solid-state fermentation using three substrates. *Int. J. Food Sci. Tech.* 49: 2585-2591 (2015)
8. Gassara F, Ajila CM, Brar SK, Tyagi RD, Verma M, Valero J. Influence of aeration and agitation modes on solid-state fermentation of apple pomace waste by *Phanerochaete chrysosporium* to produce ligninolytic enzymes and co-extract polyphenols. *Int. J. Food Sci. Tech.* 48: 2119-2126 (2013)
 9. Tao NG, Shi WQ, Liu YJ, Huang SR. Production of feed enzymes from citrus processing waste by solid-state fermentation with *Eupenicillium javanicum*. *Int. J. Food Sci. Tech.* 46: 1073-1079 (2011)
 10. Hasan-Beikdashti M, Forootanfar H, Safiarian MS. Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*. *J. Taiwan Inst. Chem. E.* 43: 670-677 (2012)
 11. Liu J, Zhang Y. Optimisation of lipase production by a mutant of *Candida antarctica* DSM-3855 using response surface methodology. *Int. J. Food Sci. Tech.* 46: 695-701 (2011)
 12. Santos TC, Gomes DPP, Bonomo RCF, Franco M. Optimisation of solid state fermentation of potato peel for the production of cellulolytic enzymes. *Food Chem.* 133: 1299-1304 (2012)
 13. Santos TC, Filho GA, Oliveira AC, Rocha TJO, Machado FPP, Bonomo RCF, Mota KIA, Franco M. Application of Response Surface Methodology for Producing Cellulolytic Enzymes by Solid-state Fermentation from the Purple Mombin (*Spondias purpurea* L.) Residue. *Food Sci Biotechnol.* 22: 1-7 (2013)
 14. Szklarz G, Antibus RK, Sinsabaugh RL, Linkins AE. Production of phenoloxidases and peroxidases by wood-rotting fungi. *Mycologia* 81: 234-240 (1989)
 15. Archibald FS. A new assay for lignin-type peroxidases employing the dye azure. *Appl. Environ. Microb.* 58: 3110-3116 (1992)
 16. Vares T, Kalsi M, Hatakka A. Lignin peroxidases, manganese peroxidases, and other ligninolytic enzymes produced by *Phlebia radiata* during solid-state fermentation of wheat straw. *Appl. Environ. Microb.* 615: 3515-3520 (1995)
 17. Granato D, Ribeiro JCB, Castro IA, Masson ML. Sensory evaluation and physicochemical optimization of soy-based desserts using response surface methodology. *Food Chem.* 121: 899-906 (2010)
 18. Pandey A. Solid-state fermentation. *Biochem. Eng. J.* 13: 81-84 (2003)
 19. Gassara F, Brara SK, Tyagia RD, Verma M, Surampalli RY. Screening of agro-industrial wastes to produce ligninolytic enzymes by *Phanerochaete chrysosporium*. *Biochem. Eng. J.* 49: 388-394 (2010)
 20. Ozcirak S, Ürek RO. Utilization of potato peelings for the production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. *New Biotechnol.* 29: 107-108 (2012)
 21. Akpinar M, Ürek RO. Production of ligninolytic enzymes by *Pleurotus eryngii* through solid-state fermentation using grape waste. *Prep. Biochem. Biotech.* 42: 582-597 (2012)
 22. Manavalan T, Manavalan A, Thangavelu KP, Heese K. Characterization of optimized production, purification and application of laccase from *Ganoderma lucidum*. *Biochem Eng. J.* 70: 106-114 (2013)
 23. Osma JF, Toca-Herrera JL, Rodriguez-Couto S. Cost analysis in laccase production. *J. Environ. Manage.* 92: 2907-2912 (2011)
 24. Forootanfar H, Faramarzi MA, Shahverdi AR, Yazdi MT. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile*. *Bioresource Technol.* 102: 1808-1814 (2011)
 25. Couto SR, Sanromán MA. Application of solid-state fermentation to ligninolytic enzyme production. *Biochem. Eng. J.* 22: 211-219 (2005)
 26. Amin F, Bhatti HN, Bhatti IA, Asgher M. Utilization of wheat bran for enhanced production of exo-polygalacturonase by *Penicillium notatum* using response surface methodology. *Pak. J. Agr. Sci.* 50: 469-477 (2013)
 27. Santos TC, Cavalcanti IS, Bonomo RCF, Santana NB, Franco M. Optimization of productions of cellulolytic enzymes by *Aspergillus niger* using residue of mango a substrate. *Ciência Rural* 41: 2210-2216 (2011)
 28. Sadhasivam S, Savitha S, Swaminathan K, Lin FH. Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. *Process Biochem.* 43: 736-742 (2008)
 29. Ürek RO, Pazarlioglu NK. Purification and partial characterization of manganese peroxidase from immobilized *Phanerochaete chrysosporiu*. *Process Biochem.* 39: 2061-2068 (2004)
 30. Bermek R, Yazici H, Öztürk H. Purification and characterization of manganese peroxidase from wood-degrading fungus *Trichophyton rubrum* LSK-27. *Enzyme Microb. Tech.* 35: 87-92 (2004)
 31. Kanayama N, Suzuki T, Kawa K. Purification and characterization of an alkaline manganese peroxidase from *Aspergillus terreus* LD-1. *J. Biosci. Bioeng.* 93: 405-410 (2002)
 32. Shuler ML, Kargi F. *Bioprocess Engineering: Basic Concepts*. 2nd ed. Prentice Hall, Upper Saddle River, NJ, USA. p. 479 (2001)