Appl Biochem Biotechnol (2011) 163:423–432
DOI 10.1007/s12010-010-9050-9

Induction and Purification by Three-Phase Partitioning of Aryl Alcohol Oxidase (AAO) from *Pleurotus ostreatus*

Vaidyanathan Vinoth Kumar · Vinohar Stephen Rapheal

Received: 22 April 2010 / Accepted: 19 July 2010 /
Published online: 22 September 2010

Abstract Aryl alcohol oxidase (AAO) is an extracellular flavoenzyme involved in lignin degradation by white rot fungi. Screening of lignolytic and AAO activity from twenty different fungal species were carried out. Among them, seven species showed lignolytic activity and three of them (*Pleurotus ostreatus, Pleurotus eous*, and *Pleurotus platypus*) were found to be AAO positive. Maximal AAO activity was observed in batch cultures of *P. ostreatus* and was found to be induced by aromatic amino acids and aryl alcohols up to a level of 289 U/l. Purification of AAO was carried out by three-phase partitioning (TPP). The 67 kDa enzyme was purified up to 10.19-fold by TPP with an overall recovery of 10.95%. Optimum pH and temperature for *P. ostreatus* AAO activity was found to be around 6 and 40 °C, respectively. From the LB plot, K_m value of AAO for oxidizing veratryl alcohol was determined to be 0.6 mM. Results of the study indicate that *P. ostreatus* is the best producers of AAO, and they could be employed as promising fungal species for biotechnological applications.

Keywords Aryl alcohol oxidase · Inducer · *Pleurotus ostreatus* · Three-phase partitioning

Introduction

The lignin degradation systems of fungi mainly comprise the triad of laccase, lignin peroxidase, and manganese peroxidase. Those enzyme systems exhibit differential characteristics depending on the species, strains, and culture conditions [1, 2]. It is considered that these lignin degrading enzymes do not function independently but mutually interact with each other as well as with other oxidases such as aryl alcohol oxidase (AAO) which belongs to the family oxido reductase, i.e., glucose methanol choline oxidases [3, 4]. The AAO (E.C. 1.1.3.7) activity was initially reported in *Polystictus versicolor* [5] and more over it was identified in several fungal species namely, *Pleurotus* species [4, 6–11], *Fusarium* species [12, 13], *Geotrichum candidum* [14], *Amauroderma boleticeum* [15],

V. V. Kumar · V. S. Rapheal (✉)
Department of Biotechnology, Kumaraguru College of Technology, Coimbatore 641006, India
e-mail: stevekct@gmail.com

Phanerochaete chrysosporium [16], and *Bjerkandera adusta* [17]. Recently, the AAO activity has also been reported in a bacterial species *Comamonas* [18]. The AAO genes from *Pleurotus eryngii* [19] and *Pleurotus pulmonarius* [10] have been cloned and sequenced.

On account of the industrial usage of AAO, methods for enhancing its production from basidiomycetes have been gaining importance. Two approaches have been followed for the production of high amount of AAO namely, overexpression for the production of AAO [20] and optimizing the culture conditions, especially by using inducers [4, 16, 21]. Inducers are usually natural substrates or substrate analogs for the enzyme. Inducers reported to be used for the AAO production are tyrosine [4], vannilyl and veratryl alcohol [16], kraft lignin [12], benzyl alcohol [12, 16], and phenyl alanine [21].

The problems with conventional purification methods includes small feed volumes at a time and high cost of up to 60–90% of the downstream processing [22]. three-phase partitioning (TPP) provides an alternative, efficient, economical, and scalable technique for purification of proteins and carbohydrate polymers [23, 24]. TPP has been used both for upstream and downstream protein purification processes or it can be used as a one-step purification protocol [24].

Reports of AAO being employed for the decolorization of textile dye [9, 18] and other industrial applications like bioethanol production, paper pulp manufacturing, source of renewable fuels, chemicals, and materials [25] emphasize the importance for developing high-yielding low-cost AAO from fungi. Owing to the importance of wide applications, the study was carried out to screen highly efficient AAO-producing fungi and to investigate the effect of different inducers on AAO production. To date, there is no literature report on the purification of lignolytic enzymes using TPP.

Materials and Methods

Chemicals and Fungi

Guaiacol, *p*-anisyl and veratryl alcohol were obtained from Himedia, Mumbai, India. Benzyl alcohol was obtained from SD Fine Chemicals, Mumbai, India. Reference proteins were procured from Sigma Chemical Co., St. Louis, USA. All other reagents and chemicals used were of analytical grade. The fungal species used for the study are listed in Table 1 and were maintained on potato dextrose agar (PDA) slants.

Lignolytic and AAO Screening Assay

Initially organisms were screened for lignolytic activity using guaiacol as indicator compound. Intense reddish brown color was produced in the medium around the fungal colonies and was taken as the positive reaction for the presence of lignolytic enzyme activity as previously reported [26]. Screening of lignolytic organisms was done on plates using PDA supplemented with 0.02% guaiacol. Twenty different fungal species were inoculated in sterile Petri plates containing the above media and were incubated at 30 °C for a period of 7 days. Cultures showing definite color changes were considered lignolytic.

AAO quantitative screening work was done on 250 ml Erlenmeyer flasks containing 80 ml of the culture media (pH 6.3) containing malt extract, 1%; yeast extract, 0.4%; glucose, 0.4%, and 5 mM L-tyrosine as previously reported [4]. The media was inoculated with 7-day-old sporulated culture suspension prepared from the cultures grown in

Table 1 Presence of lignolytic and AAO activity among the different fungal species

S. No.	Species	AAO	Lignolytic
1	*Agaricus bisporus*	−	+
2	*Aspergillus niger* MTCC 1344	−	−
3	*Aspergillus terrus* MTCC 2580	−	−
4	*Aspergillus flavus*	−	−
5	*Beauveria bassiana* MTCC 6100	−	−
6	*Beauveria feline* MTCC 6294	−	−
7	*Fusarium solani*	−	+
8	*Metarhizium anisopilae* MTCC 892	−	−
9	*Paecilomyces fumosoroseus* MTCC 6292	−	−
10	*Paecilomyces lilacinus* MTCC 1422	−	−
11	*Penicillium decumbens*	−	−
12	*Penicillium chrysogenum* NCIM 709	−	+
13	*Penicillium* sp.	−	−
14	*Pleurotus eous*	+	+
15	*Pleurotus floridanus*	−	+
16	*Pleurotus ostreatus*	+	+
17	*Pleurotus platypus*	+	+
18	*Trichoderma harzianum* MTCC 936	−	−
19	*Trichoderma viridie* MTCC 2535	−	−
20	*Verticillium lecani*	−	−

The fungal isolate were obtained and maintained as pure cultures from the following culture collection centers

PSGMC PSG Medical College (Coimbatore, India), *TNAU* Tamil Nadu Agriculture University (Mushroom Department, Coimbatore, India), *MTCC* Microbial Type Culture Collection (Punjab, India), *NCIM* National Collection of Industrial Microorganism (Pune, India)

petriplates. For growing basidiomycetes in suspension, inoculum was prepared by homogenizing the 6-day-old mycelium obtained from the Petri plates. The flasks were then incubated at 30 °C for 24 days. Culture filtrates was assayed for AAO activity.

AAO activity was assayed spectrophotometrically using veratryl alcohol as a substrate. The reaction mixture consisted of 1 mM veratryl alcohol, 50 mM potassium phosphate buffer (pH 6.0), and the suitable dilution of the enzyme extracts. Oxidation of the substrate at 30 °C was monitored based on the absorbance increase at 310 nm resulting from the formation of veratraldehyde (ε_{310}-9300/M.cm). One unit of the enzyme activity was defined as the amount of enzymes required to produce 1 μmol of veratraldehyde per minute under the assay conditions [4].

Induction Studies

Several natural and synthetic substrates have been reported to enhance AAO activity in basidiomycetes [4, 16]. Based on the previous reports the following inducers were used in the present study: phenyl alanine and tyrosine (5 mM), veratryl alcohol (7 mM), benzyl alcohol (10 mM), and anisyl alcohol (8 mM). The compounds tested as inducers were sterilized by filtration. The AAO-producing fungi were cultured for 24 days on previously

mentioned media in the presence of inducers. AAO activity was determined at 3-day intervals, by centrifuging the culture at 10,000×*g* for 30 min, and the supernatant was filtered through Whatman No. 1 filter paper and used for the assay.

Characterization Studies

The effect of pH on the activity of the AAO was determined by assaying the enzyme activity at different pH values ranging from 2.0 to 9.0 using 0.01 M of the following buffer systems: acetate (2.0, 3.0, 4.0, and 5.0), phosphate (6.0 and 7.0), and tris–HCl (8.0 and 9.0). The effect of temperature on activity of the AAO was determined by performing the standard assay procedure at different temperatures ranging from 25 to 60 °C. The kinetic constants of the enzyme (V_{max} and K_m) were determined by varying the concentrations of veratryl alcohol (0.3–2 mM).

Three-Phase Partitioning

TPP was carried out by the method of Sharma and Gupta [24]. The crude enzyme solutions was saturated with 30% (*w*/*v*) of ammonium sulfate and *t*-butanol was added in the ratio of 1:1 (*v*/*v*; ratio of crude extract to organic solvents) and the tubes were kept at 30 °C for 1 h for complete phase formation. The tubes were centrifuged at 2,000×*g* for 10 min and the three phases formed were collected separately. The enzyme is usually precipitated in the middle layer. To enrich the enzyme concentration, lower aqueous layer obtained in the first stage was again saturated with 30% salt (such that final concentration was 60% *w*/*v*) followed by addition of an equal volume of t-butanol and the tubes were centrifuged and interfacial precipitate was separated as described previously. Protein concentration was determined by the Lowry's method using bovine serum albumin (BSA) as the standard [27].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

For calculation of the protein molecular mass, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 10% gel, as described by Laemmli [28]. Reference proteins (rabbit muscle phosphorylase b, 97.4 kDa; BSA, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; and soybean trypsin inhibitor, 21 kDa) were used as molecular markers according to the instructions provided by the manufacturer (High Molecular Weight Markers Kit, Cat no. SDS-6H). After running the gel, the proteins were stained by Coomassie brilliant blue R-250.

Results and Discussion

Screening of AAO-Producing Fungi

Primary and secondary screening was carried out for the AAO-producing fungi. Out of 20 fungal species, intense reddish brown color zone was formed around the colonies of *Pleurotus ostreatus*, *Pleurotus florida, Pleurotus platypus, Pleurotus eous, Fusarium solani, Agaricus bisporus*, and *Penicillium chrysogenum* was found to be lignolytic (Table 1). These fungal cultures were further screened for AAO production, and only three species viz. *P. ostreatus, P. eous*, and *P. platypus* were found to produce extracellular AAO

(Table 1). AAO activities of the fungi grown on culture medium for 18 days growth period were found to be as follows: *P. ostreatus* (40 U/lit), *P. platypus* (32 U/lit), *P. eous* (18 U/l) (data not shown).

Color reactions due to guaiacol oxidation have been reported to be simple indicator method to screen for lignolytic activity [29]. Budallo et al. reported six lignolytic positive fungal species from different environmental samples obtained using guaiacol as indicator [26]. Extracellular AAO has been characterized from the culture liquid of *Amauroderma*, *Bjerkendra, Fusarium, Geotrichum*, and *Pleurotus* species [4–15, 17] and from the mycelial extracts of *Phanerochaete chrysosporium* [16]. In the earlier reports, southern blot method was applied for the screening of AAO gene in 30 basidiomycetes. However, only *Pleurotus floridanus, Pleurotus sajorcaju, P. eryngii, P. ostreatus*, and *P. pulmonarius* and *Bjerkendra adusta*, showed positive results [30]. They also mentioned the occurrence of AAO gene in *P. floridanus*, but we failed to detect AAO production in this fungus. This may be due to the fact that the composition of the culture medium is known to substantially affect the production of this enzyme. The time course of AAO activity showed a pattern similar to that described by Okamoto et al. [4] although the maximal activity levels were higher.

Induction of AAO Activity

The AAO-producing *P. ostreatus* was grown separately on culture medium containing one of the following inducers: veratryl alcohol, benzyl alcohol, anisyl alcohol, tyrosine and phenyl alanine. Compared with the control, inducers increased the AAO production in *P. ostreatus* in the following order: tyrosine>veratryl alcohol>benzyl alcohol>phenyl alanine >*p*-anisyl alcohol. The results concerning AAO production are shown in Fig. 1. Among the aromatic amino acids, tyrosine induces maximum AAO activity (220 U/l) on the 18th day. Among the aryl alcohols, veratryl alcohol enhanced the maximum AAO production and also shortened the time for AAO production. Combination of tyrosine and veratryl alcohol yielded maximum amount of AAO from *P. ostreatus* (289 U/lit). The findings emphasize the importance of tyrosine and veratryl alcohol are the promising inducers for AAO production and *P. ostreatus* consistently showed maximal AAO activity. In the biosynthetic pathway of *P. ostreatus*, tyrosine was metabolized into veratryl alcohol (shikimic acid

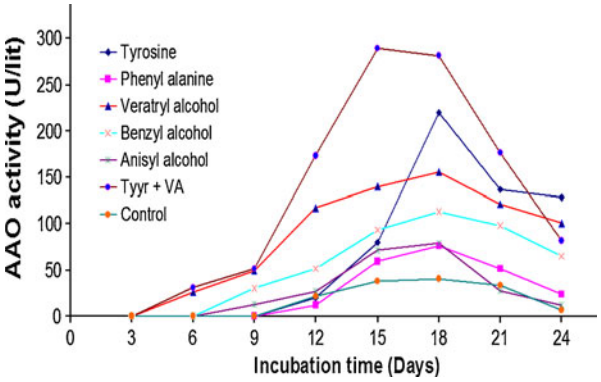

Fig. 1 Induction of *Pleurotus ostreatus.* AAO by different aromatic amino acids and aryl alcohols *P. ostreatus* was grown for 18 days in cultural medium at pH 6.3, and 30 °C in the presence of tyrosine and phenyl alanine (5 mM), vertaryl alcohol (7 mM), benzyl alcohol (10 mM), anisyl alcohol (9 mM) as inducers. Control cultures did not contain any added inducers. Results represent the average of three independent experiments

pathway), so more accumulation of veratryl alcohol in the fermentation medium results in high AAO production.

Studies have shown aromatic amino acids and aryl alcohols to be the most efficient elicitors of AAO are [4, 16, 21]. For example, AAO induction by tyrosine was reported in *P. ostreatus* [4], and by phenyl alanine in *B. adusta* [21]. Although 10 mM benzyl alcohol has been described as the best elicitor of *P. chryosporium* [16], in *Fusarium proliferatum* it was needed to decrease the concentration of this compound to 0.5 mM to observe a positive effect in AAO production. The AAO from *P. chryosporium* is to be inducible by aryl alcohols. About 4.0-, 2.6-, and 2.2-fold increases in the specific activity of intracellular AAO were observed in the media containing 0.1% vannilyl, veratryl, and benzyl alcohol respectively [16]. In *Pleurotus* species, AAO appears to be constitutive because it is produced in different growth phases and culture conditions [31]. In *Pleurotus pulmonaris*, AAO activity was occurred in the extracellular medium, attaining nearly 500 U/l after 9 days [11]. Based on reports in the literature, a double origin of AAO inducers can be postulated, firstly, from direct synthesis by the fungi (shikimic acid pathway) [32] and reduction of some of these aromatic aldehydes and acids to the corresponding alcohols has been reported in lignolytic fungi [5, 33, 34].

Three-Phase Partitioning

In the past, various methods were used to purify AAO by conventional or special chromatographic methods such as anion exchange column (DEAE-Toyopearl 650 M & Butyl-Toyopearl 650 M) and gel filtration (Sephacryl S-100). These separation techniques are time consuming, expensive and require pretreatment of samples [4]. Hence, the trend has been to develop protocols with limited number of steps for protein purification to make the process economical. TPP is a non-chromatographic separation technique, that has been successfully utilized for purification of biomolecules provided the phase constituents (solvents and salts) in the concentration used, must not be toxic or inhibitory to the biomolecules and the biomolecules must partition wholly or predominantly into one of the phases.

Efforts were also made to optimize the ammonium sulfate concentration, ratio of volume of *t*-butanol to fermented media and temperature for efficient yield and fold purification. The optimum condition for TPP was carried out at 30 °C and 30% ammonium sulfate along with 1:1 ratio of *t*-butanol was added to aqueous crude extract. The optimum concentration determined here is similar to the condition proposed as the best concentration to operate TPP for various systems reported [23, 24, 35]. The

Table 2 Overall purification of AAO by TPP

Steps	Total activity (U)	Protein concentration (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	8.4	54	0.155	1	100
TPP—1st precipitate*	0.22	1.74	0.126	0.8	2.61
TPP—2nd precipitate^{+}	0.92	0.58	1.58	10.19	10.95

*The crude extract was saturated with 30% (*w*/*v*) ammonium sulfate followed by addition of 1:1 *tert*-butanol

$^{+}$ First step aqueous phase was saturated with 30% (*w*/*v*) ammonium sulfate followed by addition of 1:1 *tert*-butanol

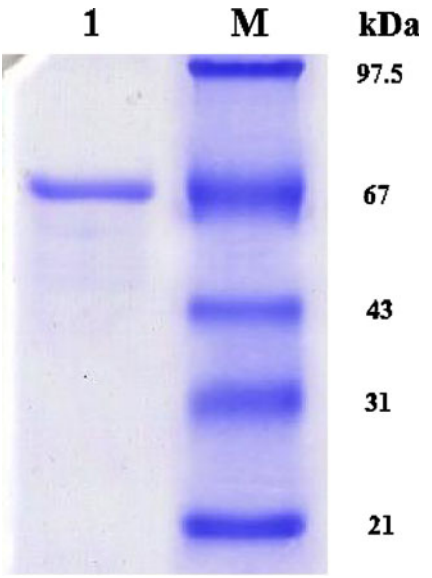

Fig. 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified aryl alcohol oxidase (AAO) TPP purified AAO was subjected to SDS-PAGE, followed by staining with Coomassie brilliant blue R-250. *Lane 1*, AAO; *M* molecular weight marker proteins

interfacial layer obtained after first TPP, contained high molecular weight contaminants, cell debris and precipitates 2.61% of AAO activity. Saxena et al. [36] reported that the aqueous phase containing most of the desired protein when subjected to a second cycle of TPP results in considerable purification of the target protein. The aqueous phase was subjected to second stage of TPP under same conditions leading to a recovery of over 10.95% AAO activity and 10.19-fold purification. Quite some successful applications of second TPP have been described. By using TPP technique several enzymes and proteins have been purified with different purification folds and yields [23, 24, 35]. The overall purification of AAO by TPP is given in Table 2. The specific activity of the purified AAO was 1.58 U/mg of protein and the molecular mass of AAO was estimated to be 67 kDa by SDS-PAGE (Fig. 2). The molecular mass of the enzyme reported here is similar to that of the AAO from *Geotrichum candidum* (65.3 kDa) [14]. Also values of 66 kDa have been described for AAO from *Comomonas* sp. [18].

Comparison of TPP with Other Conventional Purification Techniques

One of the critical factors in the industrial purification of enzymes using TPP is the selection of the appropriate system. An overall yield of 2.1% and a 35.1-fold purification

Table 3 Comparison of yield and fold purification in other literatures

Fungal species	Purification methodology	Yield (%)	Fold purification	Unit operations (Steps)	Reference
Pleurotus ostreatus K16-2	Chromatography	2.1	35.1	4	[4]
Pleurotus sajorcaju	Chromatography	8.3	36.4	5	[6]
Geotrichum candidum	Chromatography	16	20	4	[14]
P. ostreatus	TPP	10.96	10.19	1	Present study

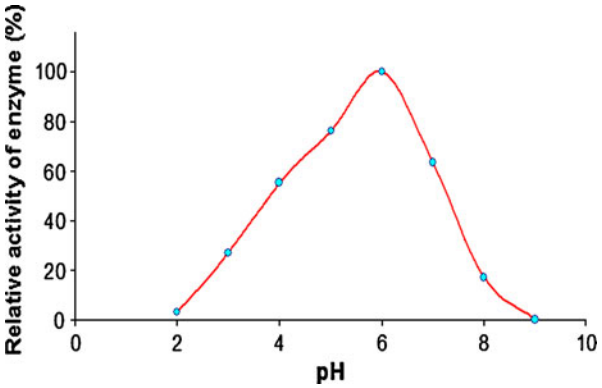

Fig. 3 Effect of pH on AAO activity *Pleurotus ostreatus* was grown for 18 days in standard conditions in culture medium at pH 6.3, and 30 °C. The enzyme extracts were prepared and incubated in buffers of varying pH and relative AAO activity were determined. Results represent the average of three independent experiments

were obtained when the AAO crude extract was applied to conventional purification of four-unit operations [4]. In contrast, the TPP process that includes addition of ammonium sulfate and *t*-butanol, one-step operation produces an overall AAO yield of 10.96% having a 10.19-fold purification with much lower salt concentrations. It is clear that a reduction of unit operations with a higher enzyme yield (i.e., 10.96%) is obtained from the TPP process compared with that from the multi-step chromatography process. TPP produce a less favorable increase in the purity of AAO (10.19) compared with that produced after the ion-exchange chromatography step (35.1), but this difference is small considering the increased yield and reduced cost of the TPP. Table 3 compares the enzyme activity yield and fold purification obtained in this study to those reported for AAO by different authors in the literature. Dhananjay and Mullimani have studied in detail the purification of α-galactosidase in TPP and made direct comparison with the chromatographic process, and they concluded that purification of α-galactosidase with TPP is cost effective (economically viable) with high enzyme yield in minimum operation unit steps [35].

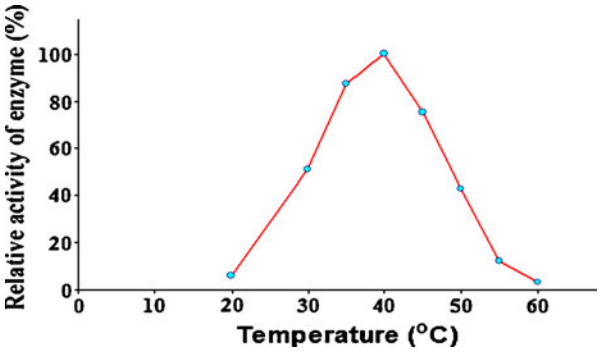

Fig. 4 Effect of temperature on AAO activity *Pleurotus species* were grown for 18 days in standard conditions in defined medium at pH 6.3, and 30 °C. The enzyme extracts were prepared and incubated in buffers (pH 6.0) of varying temperature and relative AAO activity were determined. Results represent the average of three independent experiments

Characterization Studies

The effect of pH on the AAO activity in *P. ostreatus* is presented in Fig. 3. Optimum pH was found to be around 5.5 to 6. As it can be observed, the AAO was active over a broad pH range, displaying over 63% of its activity in the pH range of 4.0–7.0. A further increased of these pH values decreased the relative activity down to 17%. This optimum pH was in agreement with the studies conducted by several authors using different strains [4, 8, 14, 37]. Therefore, AAO can be a potential candidate for different applications in the industries demanding acid pH. The *P. ostreatus* AAO was active over a broad temperature range of 30–60 °C with an optimum temperature of 40 °C (Fig. 4), and it completely losses its activity at 60 °C. The optimum temperature of AAO, as reported for other species was between 35 and 60 °C [4, 8, 14]. The kinetic parameters of AAO for veratryl alcohol were obtained by a double reciprocal Lineweaver Burk plot. The K_m and V_{max} values for *P. ostreatus* oxidizing veratryl alcohol is 0.6 mM and 2 U/mg, respectively. A K_m value of 1.4 was reported for *P. eryngii* [8], and it is evident that and *P. ostreatus* produces an AAO that has 2.5-fold higher affinity for veratryl alcohol.

Conclusion

In conclusion, our results showed that *P. ostreatus* is able to produce AAO efficiently, in the presence of the inducers tyrosine and veratryl alcohol. Among the inducers tested, we detected a quick induction of AAO activity by veratryl alcohol, while combination of tyrosine and veratryl alcohol shows resulted in maximal induction. This present work gave a study of the purification of AAO from fermented media of *P. ostreatus* using TPP. In addition the catalytic parameters of the enzyme were also investigated, to shed light on its suitability for industrial applications.

References

1. Rogalski, J., Lundell, T. K., Leonowicz, A., & Hatakka, A. I. (1991). *Phytochemistry, 30*, 2869–2872.
2. Heinzkill, M., & Messner, K. (1997). The ligninolytic system of fungi. In T. Anke (Ed.), *Fungal biotechnology* (pp. 213–227). Weinheim: Chapman & Hall.
3. Ander, P., & Marzullo, L. (1997). *Journal of Biotechnology, 53*, 115–131.
4. Okamoto, K., & Yanase, H. (2002). *Mycoscience, 43*, 391–395.
5. Farmer, V. C., Henderson, M. E. K., & Russell, J. D. (1960). *The Biochemical Journal, 74*, 257–262.
6. Bourbonnais, R., & Paice, M. G. (1988). *The Biochemical Journal, 255*, 445–450.
7. Sannia, G., Limongi, P., Cocca, E., Buonocore, F., Nitti, G., & Giardina, P. (1991). *Biochimica et Biophysica Acta, 1073*, 114–119.
8. Guillén, F., Martínez, A. T., & Martínez, M. J. (1990). *Applied Microbiology and Biotechnology, 32*, 465–469.
9. Eichlerova, I., Homolka, L., & Nerud, F. (2006). *Process Biochemistry, 41*, 941–946.
10. Varela, E., Bockle, B., Romero, A., Martinez, A. T., & Martinez, M. J. (2000). *BBA Protein Structure Molecular Enzymology, 1476*, 129–138.
11. Gutiérrez, A., Caramelo, L., Prieto, A., Martínez, M. J., & Martínez, A. T. (1994). *Applied and Environmental Microbiology, 60*, 1783–1788.
12. Regalado, F., Perestelo, A., Rodrõiguez, A., Carnicero, F. J., Sosa, G., Fuente, D. L., et al. (1999). *Applied Microbiology and Biotechnology, 51*, 388–390.
13. Saparrat, M. C. N., Martínez, M. J., Tournier, H. A., Cabello, M. N., & Arambarri, A. M. (2000). *World Journal of Microbiology & Biotechnology, 16*, 799–803.
14. Kim, S. J., Suzuki, N., Uematsu, Y., & Shoda, M. (2001). *Journal of Bioscience and Bioengineering, 91*, 166–172.

15. Saparrat, N. M. C., Martinez, M. J., Cabello, M. N., & Arambarri, A. M. (2002). *Revista Iberoamericana de Micología, 19*, 181–185.
16. Asada, Y., Watanabe, A., Ohtsu, Y., & Kuwahara, M. (1995). *Bioscience, Biotechnology, and Biochemistry, 59*, 1339–1341.
17. Muheim, A., Waldner, R., Leisola, M. S. A., & Fiechter, A. (1990). *Enzyme and Microbial Technology, 12*, 204–209.
18. Jadhav, U. U., Dawkar, V. V., Tamboli, D. P., & Govindwar, S. P. (2009). *Biotechnology and Bioprocess Engineering, 14*, 369–376.
19. Varela, E., Martínez, A. T., & Martínez, M. J. (1999). *The Biochemical Journal, 341*, 113–117.
20. Varela, E., Guillen, F., Martıınez, A. T., & Martinez, M. J. (2001). *Biochimica et Biophysica Acta, 1546*, 107–113.
21. Lapadatescu, C., & Bonnarme, P. (1999). *Biotechnology Letters, 21*, 763–769.
22. Kula, M. R., Kroner, K. L., & Hustedt, H. (1982). *Advances in Biochemical Engineering, 24*, 73–118.
23. Dennison, C., & Lovrein, R. (1997). *Protein Expression and Purification, 11*, 149–161.
24. Sharma, S., & Gupta, M. N. (2001). *Biotechnology Letters, 23*, 1625–1627.
25. Martinez, A. T., Speranza, M., Ruiz-Duenas, F. J., Ferreira, P., Camarero, S., Guillen, F., et al. (2005). *International Microbiology, 8*, 195–204.
26. Budallo, V., Chandra, M. S., Pallavi, H., & Reddy, B. R. (2008). *African Journal of Biotechnology, 7*(8), 1129–1133.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. L. (1951). *The Journal of Biological Chemistry, 193*, 265–275.
28. Laemmli, U. K. (1970). *Nature (London), 227*, 680.
29. Kiiskinen, L. L., Ratto, M., & Kruus, K. (2004). *Journal of Applied Microbiology, 97*, 640–646.
30. Varela, E., Martınez, A. T., & Martınez, M. J. (2000). *Journal of Biotechnology, 83*, 245–251.
31. Guillen, F., Martinez, A. T., & Martinez, M. J. (1992). *European Journal of Biochemistry, 209*, 603–611.
32. Turner, W. B., & Aldridge, D. C. (1983). *Metabolites from the shikimic acid pathway. In: Fungal metabolites II* (pp. 6–13). London: Academic Press Limited.
33. Guillen, F., Martinez, A. T., Martinez, M. J., & Evans, C. S. (1994). *Applied Microbiology and Biotechnology, 41*, 465–470.
34. Guillen, F., & Evans, C. S. (1994). *Applied and Environmental Microbiology, 60*(8), 2811–2817.
35. Dhananjay, S. K., & Mulimani, V. H. (2009). *Journal of Industrial Microbiology & Biotechnology, 36*, 123–128.
36. Saxena, L., Iyer, B. K., & Ananthanarayan, L. (2007). *Process Biochemistry, 42*, 491–495.
37. Ferreira, P., Medina, M., Guillen, F., Martinez, M. J., van Berkel, W. J. H., & Martinez, A. T. (2005). *The Biochemical Journal, 389*, 731–738.