Scientia Iranica, Vol. 14, No. 4, pp 373–378

SCIENTIA IRANICA

Research Note

Profiles of Phenolics and Antioxidant Activity of Pistachio Hulls During Solid-State Fermentation by *Phanerochaete chrysosporium*- Involvement of Lignin Peroxidase and Manganese Peroxidase

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The aim of the present work was to examine the changeable relationship between antioxidant activity and the phenolic content of dried pistachio hulls during Solid-State Fermentation (SSF) by *Phanerochaete chrysosporium*. Recently, in the literature, mobilization of phenolic antioxidants from soybean powders by *Lentinus edodes* has been reported [1]. *P. chrysosporium*, a well-studied white-rot fungus, was capable of producing lignin peroxidase (LiP) and manganese peroxidase (MnP) during the fermentation of pistachio hulls. Phenolic content, in the aqueous extract of the SSF of pistachio hulls, increased during the first 4 days of fermentation by 11% and decreased during the next 2 days. An increasing trend was again seen, which became evident during days 8 to 16 and which reached the highest level of 63 mg caffeic acid equivalent (CAE)/g of dried pistachio hulls. The lowest amount of phenolics was 49.28 mg of CAE/g dried pistachio hulls. The antioxidant activity of the water extract tested in terms of 2,2 diphenyl-1-picrylhydrazl (DPPH) assay. Antioxidant activity fluctuated similar to the phenolics content during the SSF period. The lowest antioxidant activity, in terms of the scavenging of DPPH, was 77%, while the highest level measured was 88%. The culture did not show any LiP and MnP activity during the first 2 days of the SSF. However, activity increased during the next 2 days and, for MnP, activity reached its highest level by day 14 (56.9 U/l), while LiP activity, which was 4.4 U/l on day 6 of fermentation, reached its highest (60.72 U/l). This work includes important points regarding the potential use of pistachio hulls as agricultural waste and as an inexpensive source of production of fungal peroxidases, while the existence of the changeable relationship between phenolics and antioxidant activity may favor the use of fermented pistachio hulls as possible functional food.

INTRODUCTION

Complex combinations of oxidation-reduction reactions occur in each living system, all of which have determining roles in drawing the whole picture of physiological processes, while any unpredicted change in the redox equilibrium position ultimately leads to cell abnormality. Molecular oxygen and its radical derivatives (superoxide anion, singlet state oxygen and hydroxyl radicals), along with peroxides and transition metals, have degenerative effects and most of them are constantly being produced in aerobic cells, due to an accident of chemistry and for specific metabolic functions [2]. Synthetic antioxidants, as food additives, are used at legal limits and are effective in preventing food from deteriorating [3]. The mechanisms of the formation of free radicals and other toxic substances in foods have been the subject of much research, while many works are undertaken to identify antioxidants of a natural origin [4,5].

Pistachio is one of the principal agriculture plants in Iran (the area under pistachio cultivation being about 430,000 acres) and Iran is also a major pistachio exporter worldwide ($> 130,000$ tons for year 2002) [6]. The antioxidant activity of the phenolics present in pistachio hulls has been reported [7]. Results obtained

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through the works of McCue and Shetty showed the phenolic antioxidant mobilization from the soybean by *Lentinus edodes* during solid state fermentation [8] and they found that the phenolic mobilization was linked to the activity of lignin degrading enzymes [9]. Lignin peroxidase (LiP) and manganese dependent peroxidase (MnP) are two major fungal peroxidases and are often produced simultaneously by *Phanerochaete chrysosporium*, which is the best characterized white-rot fungus [10-13]. The fungal peroxidases-catalyzed reactions are probably the most thoroughly studied enzymatic processes for waste treatment and, despite the great potential for its/their usage, a number of issues remain to be addressed before the enzymatic processes can be implemented on an industrial scale. Among these issues are the identification of low-cost sources for the production of enzymes in large quantities [14-17].

Pistachio hulls, as a good source of phenolics, having antioxidant activity, are traditionally used to make a kind of homemade jam in the pistachio growing areas of Iran. A more valuable method for the utilization of this agricultural waste is to use it as an inexpensive fermentation substrate for the fungal production of LiP and MnP enzymes. Indeed, the current disposal of much agricultural waste presents considerable economic and environmental problems. The objective of the present study is to investigate the bioconversion of the phenolics of pistachio hulls by *P. chrysosporium* in the Solid State Fermentation system (SSF). The antioxidant activities of pistachio hulls were determined in relation to changes in phenolic content, while the activities of LiP and MnP enzymes were also measured.

MATERIALS AND METHODS

Microorganism, Media and Cultivation

P. chrysosporium was purchased from Deutshe Sammlung Von Mikroorganism Zelkulturn (DSMZ) (Braunschweig, Germany) and was maintained on potato dextrose agar (PDA) slants at 4°C. Sub-cultures were routinely made every 2 months. One loop of the fungus growth on the PDA slant was transferred to a PDA plate, followed by incubation at 37°C for 5 days. The medium, which was covered with mycelium, was then cut into small squares (1-1.5 cm^2) and was transferred aseptically to a 250 ml, Erlenmeyer flask containing 100 ml of potato dextrose broth. The culture was incubated in the shaker at 60 rpm for 7 days. The mycelial mat produced was blended using a Waring blender. The homogenized mat of mycelium was inoculated into the flasks containing the dried pistachio hulls and the flasks were then incubated at 30°C for 20 days.

Fresh pistachio hulls were obtained from a wholesale market (Damghan city, south-east Tehran) and were dried by spreading them on a tray for 3 days under sunlight. The dried pistachio hulls were stored in a refrigerator until use (all experiments were carried out in one month). The dried hulls were blended in a laboratory blender and, for the SSF studies, 5 g of pistachio hull powder was transferred into a 500 ml Erlenmeyer flask. An appropriate amount of water was added to the flask content ($\sim$ 12 ml distilled-H_2O to provide a suitable moisture level at approximately 70% v/w) and the flasks were autoclaved at 121°C for 15 min. Then, the homogenized mycelial mat, prepared as above, was inoculated into each flask (20% v/w, about 2 ml of the homogenized mycelial mat for the flask content) and the flasks were then incubated at 30°C for 20 days. Preliminary tests showed that, for initiation of fungal growth, the addition of a small amount of soya powder was necessary, therefore, 0.5 g of soya powder were added to the content of each flask as a supplementary nutrient (soya used in this experiment was purchased from a local market).

Preparation of the Aqueous Extract from the Fermented Pistachio Hulls by the Fungus

100 ml of distilled-H_2O was added to the contents of the flask (500 ml) containing pistachio hulls fermented by *P. chrysosporium*. After mixing the contents in a Waring blender ($\sim$ 1 min), the mixture was centrifuged at 10000 g at 4°C for 20 min. The obtained supernatant ($\sim$70 ml) was then vacuum-filtered through a Whatman no. 1 filter paper.

Determination of Total Phenolics in the Fermented Pistachio Hulls (Aqueous Extract)

The total phenolic content of the fermented pistachio hulls extract was estimated by a photometric method, based on procedures described in [1,18]. Briefly, a 1 ml aliquot of the supernatant obtained from the aqueous extract, as above, was diluted (1:50). To this mixture, 0.5 ml of the Folin-Ciocalteu phenol reagent was added and, after 3 min, 1 ml of saturated sodium carbonate solution ($\sim$ 30%) was also added. The contents were mixed and left to stand at room temperature for 1 hr. Absorbance measurements were recorded at 725 nm, using a spectrophotometer and Caffeic acid was used for preparation of the standard curve (μg/ml).

Determination of Antioxidant Activity

The antioxidant activity of the aqueous extract obtained from the fermented pistachio hulls by the fungus was determined by a colorimetric assay, based on procedures given elsewhere [9]. In this method, the capacity of the prepared aqueous extracts to scavenge the stable

free radical 2,2-diphenyl-1-picrylhydrazl (DPPH) is monitored spectrophotometrically at 517 nm. Briefly, 0.8 ml of an ethanolic solution of DPPH (1 mM DPPH solution in 95% ethanol) was mixed with 0.2 ml of the sample solution and the mixture was left at room temperature for 30 min. The sample was then centrifuged at 16000 g for 5 min at room temperature and absorbance measurements were recorded at 517 nm. The antioxidant activity was obtained in terms of the radical scavenging activity and calculated as a percentage of DPPH discolorization, using the following equation:

$$(1 \quad A_E/A_D) \times 100,$$

where A_E is the absorbance of the sample solution (i.e., when the solution contains the extract) and A_D is the absorbance of the DPPH solution (0.8 ml) with 0.2 ml ethanol solution (95%) added.

Enzyme Assays

The manganese peroxidase activity was measured, according to the method described by Gandolfi-Boer et al. [19]. As pointed out elsewhere, the complex formation of Mn (III) ions with malonate can be monitored spectorophotometrically at 270 nm, while the pH of the reaction mixture is set at 4.5 (extinction coefficient = 11590 M ^{1}cm ^{1}) [20]. One unit (U) is the amount of enzyme that catalyzes the formation of Mn (III) -malonate complex (μmole/min). Lignin peroxidase activity was determined, according to the procedure described by Tien and Kirk [21]. LiP catalyzes oxidation of veratryl alcohol to veratraldehyde and H_2O_2 is used as the co-substrate (pH of the reaction mixture was 3. The fermentation process was carried out in three experiments and the results are the mean of the 3 samples taken separately from each of the three different flasks. Statistical tests were performed on the data obtained (a one-way analysis of variance, the criterion of significance being at a 0.05 level), when it was appropriate.

RESULTS

Fate of Phenolic Contents and Antioxidant Activity of Pistachio Hulls During SSF

The phenolic content in the aqueous extract of the SSF of pistachio hulls by *P. chrysosporium* increased during the first 4 days of fermentation by 11% (Figure 1). Total soluble phenolic content decreased by 5.1% after the next 48 hrs of fungus growth and changed from 56.8 mg CAE/g dried pistachio hulls to 54.02 mg CAE/g dried sample. In continuation of the fermentation, the phenolic content reached its highest level at 63 mg CAE/g dried sample, after 16 days of fermentation

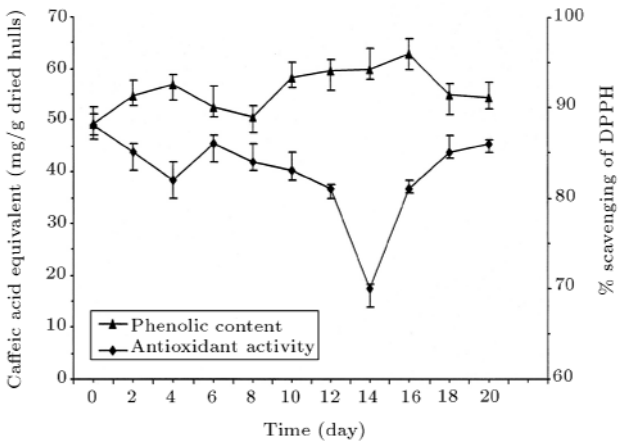

Figure 1. Time course of total phenolics content and antioxidant activity in the water extracts of the dried pistachio hulls bioprocessed by *P. chrysosporium* in the solid-state fermentation system. Each value in the plot is the mean of three experiments. CAE: Caffeic Acid Equivalent.

(Figure 1). In the course of the SSF process, the degree of association between different individual phenolics changed (statistical tests confirmed the significance of these differences).

Antioxidant activity in pistachio hull extract, measured in terms of DPPH scavenging power, fluctuated, similar to phenolic content, during the SSF period. At the beginning of the fermentation, up to 4 days, the DPPH scavenging activity decreased by 9%, from 88 to 80%. After 6 days of incubation, antioxidant activity rose by 7%. Thereafter, a decreasing trend in the change of the DPPH scavenging power began and continued slowly up to the 14th day of fermentation. The trend then increased, while activity reached its highest level of 86%, which was, more or less, similar to that percentage detected at the beginning of the fermentation process. In the literature, the particle size of the substrate has a definite role in SSF processes [22]. In the present study, when the particle size of the dried samples of pistachio hulls changed from 10 mm to 0.85 mm, the phenolic content of the extract increased to 49.01 mg CAE/g of dried sample. The data were obtained from the 8th day of fermentation (Figure 2). Error bars in the figure show that the phenolic content of pistachio hulls was significantly different, on the basis of the size of the particles. The distribution of moisture through the matrix of the substrate, during the SSF process, was probably not uniform (i.e., the uniformity of moisture distribution is less in the larger particles than that of the smaller sized particles of the substrate). The phenolic content decreased by 11% when the particle size of the dried pistachio hulls decreased almost 10 times, to 0.088 mm. The effect of the particle size of the substrate in the present study was not apparently

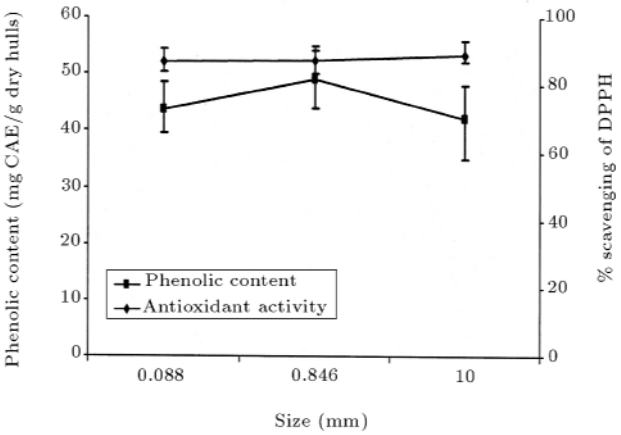

Figure 2. Effect of the particle size of the dried pistachio hulls on phenolics content and antioxidant activity in SSF system by *P. chrysosporium*. Each value in the plot is the mean of three experiments. CAE: Caffeic Acid Equivalent.

considerable on the DPPH scavenging activity (Figure 2).

Production of Lignin Degrading Enzymes (LiP and MnP) During SSF of Pistachio Hulls by *P. chrysosporium*

P. chrysosporium is a well-studied white-rot fungus growing on woody tissue and ligninolytic peroxidases are responsible for the strong degradative ability of the fungus [11]. Based on experimental findings, McCue et al. [8] hypothesized that the lignin decomposition ability of *L. edodes* may be involved in phenolic mobilization from defatted soybean powders during the SSF process. In the present work, therefore, the effect of *P. chrysosporium* bioconversion of the dried pistachio hulls on LiP and MnP activity was studied. Results of LiP and MnP activity, along with phenolic content, during the 20 days of the SSF, are given in Figures 3 and 4. In the first 2 days of the process, no activity was detected for either one of the two enzymes, while activity increased for both enzymes in the next 2 days. MnP activity, after 4 days of culture processing, was 16.7 U/l and, for the next 2 days, decreased by nearly 80%. Phenolic content, after 6 days of culture incubation, was at its lowest level and about 54 mg CAE/g dried pistachio hulls. The increasing trend of MnP activity, during the next 8 days of incubation (from day 6 to day 14), became evident and the activity reached its highest level, i.e., 56.9 U/l (Figure 3). Activity declined from day 16 and reached about 5.17 U/l after 20 days of the SSF process. This level of activity, again, corresponded to the phenolics at a low concentration.

As seen in Figure 4, LiP activity increased and reached 43.01 U/l after 4 days of culture processing. The declined levels of LiP activity in the next 10 days

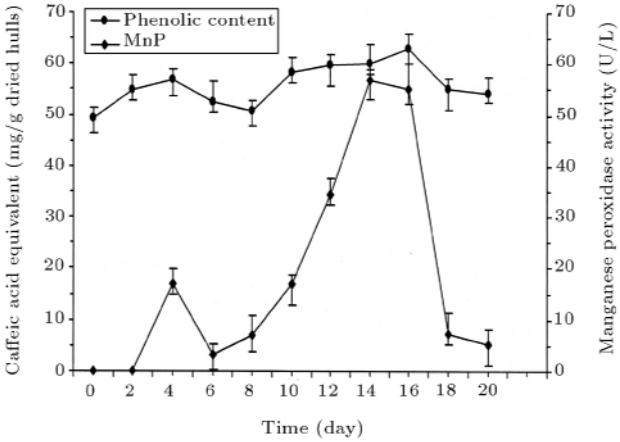

Figure 3. Manganese peroxidase activity in the SSF system of the dried pistachio hulls by *P. chlysosporium*. Total phenolics content is also presented. Each value in the plot is the mean of three experiments. CAE: Caffeic Acid Equivalent.

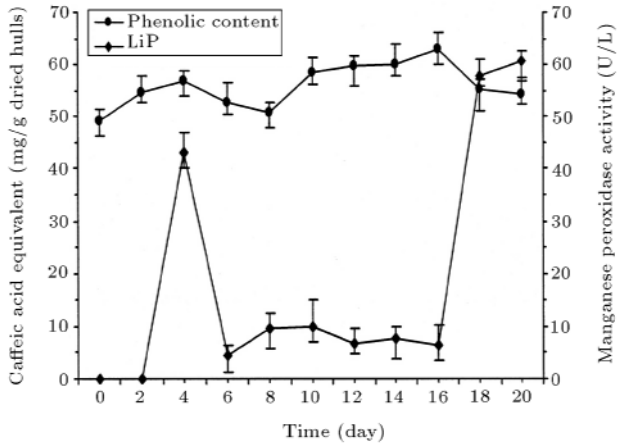

Figure 4. Lignin peroxidase activity in the SSF system of the dried pistachio hulls by *P. chrysosporium*. Total phenolics content is also presented. Each value in the plot is the mean of three experiments. CAE: Caffeic Acid Equivalent.

(day 6 to day 16) remained in a more or less steady position and a low level of activity was detected (6.42 U/l). Thereafter, LiP activity increased and reached 60.72 U/l at day 20 of the culture processing (Figure 3).

DISCUSSION

The isolation of LiP from ligninolytic cultures of the white-rot fungus, *P. chrysosporium*, in 1983, was a breakthrough in defining lignin biodegradation and made it possible to study this natural process at a molecular level [23,24]. LiP catalyzes the oxidation of nonphenolic lignin and lignin models and the presence of MnP in ligninolytic cultures of *P. chrysosporium* and other white-rot fungi have been shown to enhance lignin biodegradation [12]. MnP catalyzes the oxida-

tion of Mn(II) to Mn(III) and the latter species, in the presence of a suitable organic chelating agent, forms a complex capable of oxidizing phenolic lignin substructures [25].

Phenolic and nonphenolic compounds are both present in plant materials and, therefore, in agricultural waste such as pistachio hulls, and each has a role in expressing the antioxidant activities of the plant source. The results of the present study, regarding the fluctuations of pistachio hull phenolics during the SSF period, were in agreement with those reported by McCue and Shetty [9], who used *Lentinus edodes* for bioconversion of soybean phenolics. The increasing trend of phenolics in the present study was between days 8 to 16, while the highest level of MnP was at day 14 of the fermentation. The antioxidant activity was at its lowest level at day 14 of the SSF. The oxidative action of MnP corresponded to its depolymerizing effect on the polymeric phenolics matrix, with a release of phenolics in the free form. The result of these events is the declined antioxidant activity. While the polymerizing action of MnP on phenolics is also, possible and the formation of a new polymeric matrix may have a role in recovering antioxidant activity (day 16 to 20). From the pattern of the changes of the phenolics and antioxidant activity in the present study, the polymerizing action for LiP was less important and not detected. It may be possible for LiP to catalyze oxidation of the nonphenolic compounds of the pistachio hulls and, in this way, the enzyme plays a role in the observed decreasing pattern of antioxidant activity. The fluctuation pattern of the fungal peroxidases is a reflection of the changeable relationship present for profiles of phenolics and the antioxidants activity of pistachio hulls in the SSF system.

CONCLUSION

This research work was carried out to study the relationship between phenolic content and antioxidant activity of pistachio hulls bioprocessed by *P. chrysosporium* under SSF conditions. The presence of the changeable relationship was interesting and found to be under the influence of fungal LiP and MnP enzymes. Regarding lignin biodegradation, fungal peroxidase(s) have shown to induce polymerization/depolymerization [24]. In the present work, these enzyme activities were discussed in terms of the changeable relationship between phenolics and antioxidant activity. The importance of pistachio hulls as an inexpensive substrate for MnP production is mentioned. MnP and LiP are popular enzymes for use in the bio-treatment of industrial waste, especially those wastes containing refractory compounds not degraded by typical biological treatment. The use of bioprocessed pistachio hulls, a rich source of phenolics, having antioxidant activity, could be a novel approach for considering agricultural waste as a functional food.

REFERENCES

1. McCue, P. and Shetty, K. "A role for amylase and peroxidase-linked polymerization in phenolic antioxidant mobilization for health", *Process Biochemistry*, **39**, pp 1785-1791 (2004).
2. Prior, R. and Cao, G. "Analysis of botanical and dietary supplements for antioxidant capacity: A review", *JAOAC International*, **83**(4), pp 950-956 (2000).
3. Asmarai, A., Addis, P., Epley, R. and Krick, T. "Wild rice hull antioxidants", *J. Agric. Food Chem.*, **44**, pp 126-130 (1996).
4. Amarowicz, R., Pegg, R., Rahimi-Moghadam, P., Barl, B. and Weil, J. "Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies", *Food Chemistry*, **84**, pp 551-562 (2004).
5. Kuda, T., Tsunekawa, M., Hishi, T. and Araki, Y. "Antioxidant properties of dried Kayamo-nori, a brown alga *Scytosiphon lomentaria* (*Scytosiphonales phaeophyceae*)", *Food Chemistry*, **89**, pp 617-622 (2005).
6. "Anonymous", *Public Broadcasting System* (news from the National Radio-Tehran) (Oct. 31, 2005).
7. Vahabzadeh, F., Mehrnaz, M. and Mofarrah, E. "Antioxidant activity of pistachio hulls", *JAOCS*, **81**(6), pp 621-622 (2004).
8. McCue, P., Horii, A. and Shetty, K. "Mobilization of phenolic antioxidants from defatted soybean powders by *Lentinus edodes* during solid-state bioprocessing is associated with enhanced production of laccase", *Innovative Food Sci, and Emerging Technologies*, **5**, pp 385-392 (2004).
9. McCue, P. and Shetty, K. "A model for the involvement of lignin degradation enzymes in phenolic antioxidant mobilization from whole soybean during solid-state bioprocessing by Lentinus edodes", *Process Biochemistry*, **40**, pp 1143-1150 (2005).
10. Keyser, P., Kirk, K. and Zeikus, J. "Ligninolytic enzyme system of *Phanerochaete chrysosporium*: Synthesized in the absence of lignin in response to nitrogen starvation", *J. Bacteriol.*, **135**, pp 790-797 (1978).
11. Jansheker, H. and Fiechter, A. "Lignin: Biosynthesis, application and biodegradation", in *Advance in Biochemical Engineering*, **27**, Fiechter, A., Ed., Springer Verlag (ISBN: 0-387-12182-x) (1983).
12. Platt, M., Hadar, Y. and Chet, I. "The decolorization of the polymeric dye poly- blue (polyvinalamine sulfonate- anthroquinone) by lignin degrading fungi", *Appl. Microbiol. Biotechnol.*, **21**, pp 394-396 (1985).
13. Kirk, K. and Farrell, R. "Enzymatic combustion: The microbial degradation of lignin", *Ann. Rev. Microbiol.*, **41**, pp 465-505 (1987).

14. Aust, S. "Degradation of environmental pollutants by *Phanerochaete chrysosporium*", *Microbial Ecology*, **20**, pp 197-209 (1990).

15. Barr, D. and Aust, S. "Mechanisms white rot fungi use to degrade pollutans", *Environ. Sci. Technol.*, **28**(2), pp 78A-87A (1994).

16. Lacina, C., Germanian, G. and Apiros, A.N. "Utilization of fungi for bio-treatment of raw wastewaters", *African Journal of Biotechnology*, **21**(12), pp 620-30 (2003).

17. Ikehata, K., Buchanan, J. and Smith, D. "Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment", *J. Environ. Eng. Sci.*, **3**, pp 1-19 (2004).

18. Gutfinger, T. "Polyphenols in olive oils", *JAOCS*, **58**(11), pp 966-968 (1981).

19. Gandolfi-Boer, C., Obici, L., Souza, C. and Perelta, R. "Decolorization of synthetic dyes by solid state cultures of *Lentinula (Lentinus) edodes* producing manganase peroxidase as the main ligninolytic enzyme", *Biores. Technol.*, **94**, pp 107-112 (2004).

20. Wariish, H., Valli, K. and Gold, M. "Manganese (II) oxidation by manganese peroxides from the basidiomycete *Phanerochaete chrysosporium* kinetic mechanism and role of chelators", *J. Biol. Chem.*, **267**, pp 23688-23695 (1992).

21. Tien, M. and Kirk, K. "Lignine peroxidase of *Phanerochaete chrysosporium*", *Methods in Enzymology*, **161**, pp 238-240 (1988).

22. Roukas, T. "Citric acid production from carob pod by solid state fermentation", *Enzyme and Microbial Technol*, **24**, pp 54-59 (1999).

23. Glenn, J. and Gold, M. "Decolorization of several polymeric dyes by the lignin degrading basidiomycete *Phanerochaete chrysosporium*", *Appl. Environ. Microbiol.*, **45**, pp 1741-1747 (1983).

24. Schoemaker, H. and Leisola, M. "Degradation of lignin by *Phanerochaete chrysosporium*", *J. Biotechnol*, **13**, pp 101-109 (1990).

25. Perez, J. and Jeffries, T. "Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*", *Appl. Environ. Microbiol.*, **58**, pp 2402-2409 (1992).