Research Article

OPTIMIZATION OF MANGANESE PEROXIDASE PRODUCTION IN SUBMERGED MEDIUM BY INDIGENOUS LITTER DECOMPOSING BASIDIOMYCETE *AGARICUS HETEROCYSTIS*

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ABSTRACT

Optimization of manganese peroxidase production by an indigenous litter decomposing basidiomycetes *Agaricus heterocystis* was studied under submerged fermentation. The physical parameters namely, pH, temperature, and the nutritional parameters like suitable carbon and nitrogen sources and aminoacids were studied for the higher enzyme production. Of the different temperature (20, 25, 30, 35 and 40°C) tested for the optimal MnP production, 30°C showed the maximum activity of 45.19 ± 1.08 U/ml on 17th day. The optimum pH for the MnP production was found to be pH 5.5. Among the different carbon sources tested fructose supported maximum (52.17 ± 0.97 U/ml) MnP production, where as peptone supported the maximum activity (53.86 ± 1.09 U/ml at day 19) among the different nitrogen sources tested. Of the different amino acids tested, tryptophan enhanced the maximum enzyme activity (54.39 ± 0.87 U/ml). The above result indicates that the *Agaricus heterocystis*can be used as a biotechnological tool.

Keywords: Manganese peroxidase, *Agaricus heterocystis*, Physical parameters, Nutritional parameters.

INTRODUCTION

Basidiomycetes are the principal organisms responsible for lignocellulose degradation.[1] Wood-rotting Basidiomycetous fungi are usually divided into white-rot, brown-rot and litter-decomposing fungi.[2–4] Basidiomycetous white-rot fungi and related litter-decomposing fungi are the only organisms capable of mineralizing lignin efficiently.[5] Basidiomyceteslitter-decomposing fungi occur ubiquitously in forests and grass lands, where they colonize the upper layer of soil and humus layers. They live on dead plant material such as leaves, needles, twinges and grass residues. To break the protecting lignin barrier in lignocellulose, they produce the similar spectrum of extracellular oxidoreductase namely manganese-dependent peroxidase (MnP) (EC 1.11.1.13) and laccase (EC 1.10.3.2) as wood decayingbasidiomycetes.[6–8]

Manganese peroxidases (MnP) are extracellular haeme containing glycoprotein produced only by ligninolytic (wood-rotting and litter-degrading) basidiomycetes, especially during the secondary metabolism.[9] They catalyse the H_2O_2-dependent oxidation of Mn2+ to a highly reactive Mn3+.[10] The complex is a highly reactive oxidant that can freely diffuse away from the enzyme's active centre because of its low molecular weight. Hence, it non-specifically oxidizes a variety of phenolic and non-phenolic substances, including lignin and toxic pollutants.[11] By removing lignin, fungi are able to access plant polysaccharides (hemicelluloses, cellulose), which serve as their primary source of carbon and energy. Hence, these ligninolytic enzymesare used in various biotechnological applications in pulp and paper, food, textile and dye industries, bioremediation, cosmetics, analytic biochemistry and many others.

The main issue in delaying their implementation at industrial scale is the low yield of ligninolytic enzymes in most fungi. The ligninolytic machinery in most basidiomycetes is highly regulated by many typical fermentation factors such as medium composition, nature of carbon source, pH of fermentation broth, fermentation temperature, amount and nature of nitrogen source and presence of inducers (Cu2+, Mn2+, etc.).[12–15] MnP production is generally optimal at high oxygen tension, but is repressed by agitation in submerged liquid culture.[16,17] It is evident that the potential applications of these enzymes in industrial and environmental technologies require huge amounts of these enzymes at low cost.

However, no studies on optimization of culture conditions for production of MnP by an indigenous edible litter-decomposing basidiomycetes *Agaricus heterocystis* has been reported to date. Hence, in the present attempt, the test fungus was used to study the optimization of nutritional and environmental factors for the higher MnP production.

MATERIALS AND METHODS

Organism and inoculum preparation

Fruiting body of the *Agaricus heterocystis* was isolated from south eastern part, IIT Madras, Chennai, India, and the culture was maintained onpotato dextrose agar medium(PDA) at room temperature. Inoculum of *A. heterocystis* was prepared from mycelia grown on the same medium incubated at room temperature for 4–6 days. From the plate, 7-mm diameter mycelial disc was used as the inocula.

Optimization of medium on MnP production

The modified Asther *et al.,*[18] media composed of glycerol (10 g l^{-1}), ammonium tartarate (1.84 g l^{-1}), sodium tartarate (2.3 g l^{-1}), KH_2PO_4 (2 g l^{-1}), $MgSO_4$ ($7H_2O$) (0.7 g l^{-1}), $CaCl_2$ ($2H_2O$)(0.14 g l^{-1}), $FeSO_4$ ($7H_2O$) (0.07 g l^{-1}), $ZnSO_4$ ($7H_2O$) (0.046 g l^{-1}), $MnSO_4$ ($7H_2O$) (0.035 g l^{-1}), $CuSO_4$ ($5H_2O$) (0.007 g l^{-1}), thiamine (0.0025 g l^{-1}), yeast extract (1 g l^{-1}), veratryl alcohol (0.067 g l^{-1}) and Tween 80 (0.5 g l^{-1}) were used throughout the optimization strategies for manganese peroxidase production. Incubation was carried out on static condition at 30 ± 1°C in 250 ml Erlenmeyer flask containing 30 ml of the medium inoculated with 7 mm agar plug from 6-day-old mycelia grown on malt-extract agar. Periodic harvesting of the mycelia was performed using the filter paper. An aliquot of supernatant was collected aseptically and culture filtrates were used as enzyme sources.

Optimization of nutritional parameters on MnP production

Optimization of MnP production by *A. heterocystis* was studied using different carbon sources such as fructose, lactose, sucrose, maltose, starch; various nitrogen sources such as ammonium nitrate, urea, beef extract, peptone, yeast extract; and various concentrations of aminoacids such as glycine, proline, alanine, tryptophan, methionine were used. Optimization of physiological parameters such as pH (4.0–8.0) and temperature (20–40°C) were carried out. All chemicals used in this research were of analytical grade and were used without further purification.

Enzyme activity assays

Manganese peroxidase activity was determined by monitoring the oxidation of guaiacol (2-methoxyphenol) as the substrate at 465 nm with extinction coefficient, ε465 = 12100 M^{-1} cm^{-1}.[19] The reaction mixture contained 0.5 M sodium succinate buffer (pH 4.5), 4 Mm guaiacol, 1 mM $MnSO_4$, 600 µl of mycelial culture filtrate and 1 mM H_2O_2.Reaction mixture without culture filtrate served as the blank.One unit enzyme activity was defined as the amount of enzyme that oxidifies 1 µM of substrate per minute at 25°C. The activities were expressed in U/ml. The data represented are means of three replicates (mean ± SD).

RESULTS AND DISCUSSION

Effect of physical parameters (temperature, pH) on MnP production

The optimum temperature for maximum MnP production by *A.heterocystis* was found to be 30°C on day 17 with an activity of 45.19 ± 1.08 U/ml (Fig. 1). Very little ligninolytic activities were observed at temperatures above 30°C probably due to the fact that increasing the temperature could have inhibited the fungal growth and hence, low/decreased enzyme activities. The same trend has also been demonstrated by Zadrazil *et al*.,[20] when *Pleurotus* species and *Dichomitus squalens* were cultivated at temperatures higher than 30°C. Similar results have been reported by Nakamura *et al.,*[21] whereby, maximum lignolytic activity from cultures of *B.adusta* were attained at 30°C; but above 37°C, there was no activity observed. Also, Iqbal *et al.,*[15] found substantial decrease in ligninolytic enzymes of *Trametes versicolor* IBL-04 when cultivated at temperatures higher than 30°C.

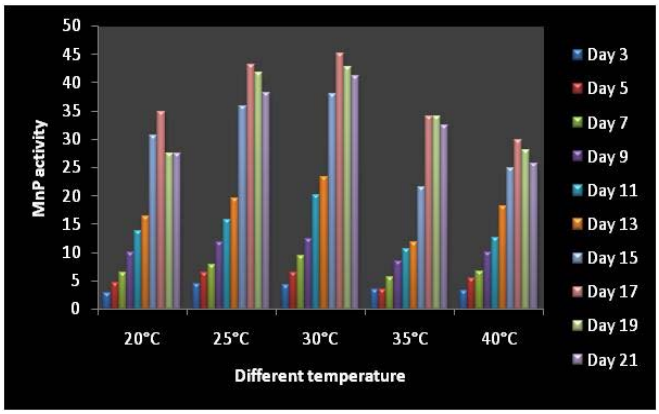

Figure 1: Effect of temperature on MnP production

Maximum MnP produced was 48.33 ± 0.87 U/ml at pH 5.5 on day 17 (Fig. 2). Activities in the most acidic medium (pH 3.5) were low compared to slightly acidic medium. These findings are in agreement with previous reports as most fungal enzymes have maximum activity when the initial pH of the nutrient medium ranges from 4 to 6.[22–24]

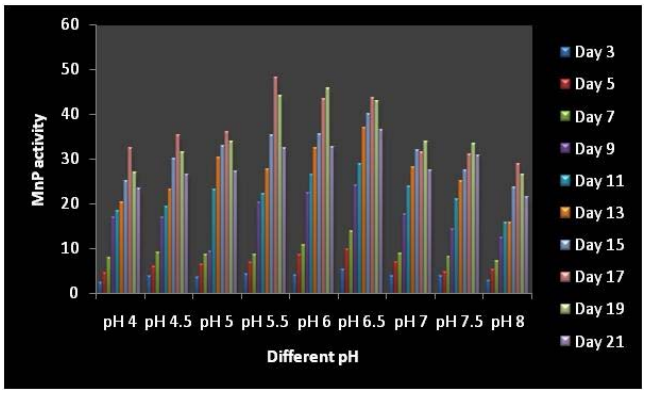

Figure 2: Effect of pH on MnP production

Effects of nitrogen on MnP production

Among the various organic and inorganic nitrogen sources, the highest MnP produced was 53.86 ± 1.09 U/ml at day 19 (Fig. 3) in the peptone containing culture medium. Nitrogen concentration in the submerged culture medium plays an important role in the production and activation of lignolytic enzymes. High nitrogen conditions have the effect of increasing fungal growth and biomass yield, thus increased enzyme production could have been a result of increased fungal biomass. The results obtained here are consistent with some previous findings, for example, supplementing organic nitrogen (peptone or casein) increase the MnP production in *Pleurotus ostreatus*.[25–27] Levin and Forchiassin[28] found high MnP production in the high nitrogen submerged culture of *Trametes trogii*. On the

other hand, Stajic *et al.,*[29] reported the enhancement of peroxidase production in *P.pulmonarious* by inorganic nitrogen sources like $KNO_3$and $NH_4H_2PO_4$.

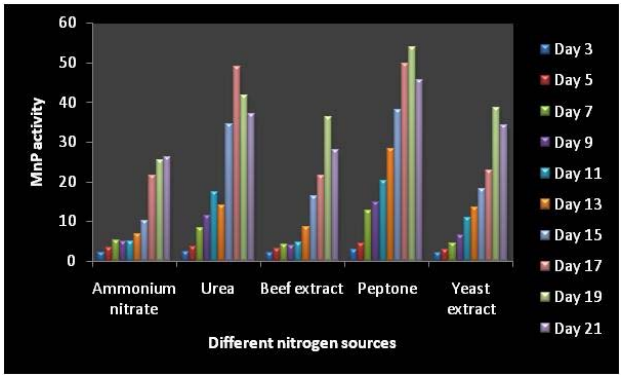

Figure 3: Effect of nitrogen on MnP production

Effect of carbon on MnP production

The MnP production was found to vary with the different carbon sources. The maximum enzyme production (52.17 ± 0.97 U/ml) was recorded on 19th day of incubation with fructose at the concentration of 0.1% in the medium. Moderate levels of enzyme activities were obtained with mannitol, lactose, sucrose and starch (Fig.4).Increased enzyme activity in media containing these simple sugars can be explained by the high production rate of secondary metabolites when their producing organisms grow in complex media,[30] whereas Mansure *et al.,*[31] showed that the use of fructose instead of glucose resulted in a 100-fold increase in the specific lignolytic activity of basidiomycetes. Fasidi[32] found that glucose and fructose stimulated mycelial biomass production in *Volvariella esculenta.* The lignolytic enzyme activity obtained in cultivation of *Pleurotus sajor-caju*in media containing 0.5 g/l fructose or glucose (37and 36 U/ml, respectively) was significantly higher than those obtained with lactose[33].

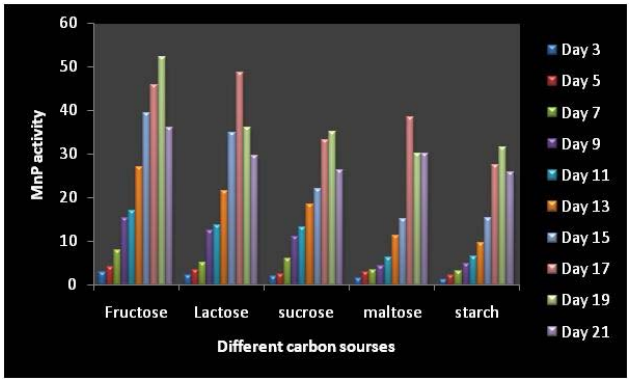

Figure 4: Effect of carbon on MnP production

Effect of amino acids on MnP production

The highest MnP production (54.39 ± 0.87 U/ml) was recorded on 17th day of incubation with tryptophan at the concentration of 0.01% in the medium (Fig.5). Lycine, alanine, methionine and proline (Fig. 5) showed moderate effect on MnP production. Levin and Forchiassin[28] reported that the addition of tryptophan increased enzyme production in the cultures of *T.trogii* BAFC 463. Dhawan and Kuhad[34] tested 23 amino acids and 6 vitamins for their effects on lignolytic enzyme production by *Cyathus bulleri* 195062 and showed the positive effects of methionine and tryptophan. Chandra *et al.,*[35] reported that asparagine and aspartic acid have been employed in increasing the mycelial growth and fruit body production in *Agaricus bisporus.*

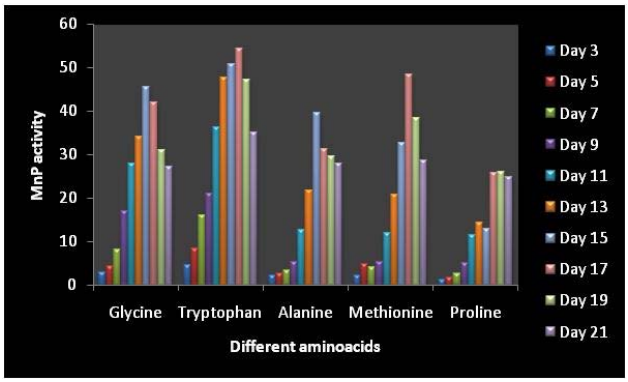

Figure 5: Effect of amino acids on MnP production

CONCLUSION

There has been growing interest in studying the ligninolytic enzymes from fungi with the expectation of finding more effective systems for their application in various biotechnological approaches. It can be concluded that one of the key factors to increase the yield of ligninolytic enzymes is the optimization of the production medium.This study attempted to optimize culturing conditions in order to improve MnP activities in submerged culture of *A.heterocystis*. Varying the physicochemical parameters such as incubation temperature and initial medium pH improved the amounts of enzymes produced. Furthermore, altering the media compositions including addition of carbon, nitrogen, aminoacids enhanced the enzyme yields.An overall two-fold increase in MnP production was attained as compared to the initial medium. The substrates and inducers are safe, cheap and could be suggested for prospective application for the higher production of enzyme. This work provides baseline information on growth parameters optimization for *A.heterocystis* under submerged culture conditions. These findings have implication in the culture condition choice and design for further investigation at large scale.

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