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Solid State Production of Manganese Peroxidases Using Arecanut Husk as Substrate

Akhila Rajan[1], Jayalakshmi Gopinatha Kurup[2] and Tholath Emilia Abraham[3*]
[1]Department of Biotechnology; Kerala University; Karaiavattom; Trivandrum 695581 - India [2,3]Chemical Science Division; National Institute of Interdisciplinary Science and Technology; CSIR, Trivandrum 695 019 - India

ABSTRACT

*The lignocellulosic biomass from arecanut husk (*Areca catechu Linnaeus*) was evaluated as a new substrate for cultivation of* Phanerochaete chrysosporium *and* Phanerochaete *sp for solid state fermentation of manganese peroxidase (MnP). Arecanut had a moisture content of 79.84 % for ripe nut husk whereas green nut husk had 68.39 % moisture and a pH of 5.0, 3.0 and 7.0 for raw, ripe and dry husk. Reducing sugar content was 14.31, 19.21 and 1.77(mg/g of husk) for raw, ripe and dry nut husk, respectively. Non reducing sugar was 1.04(mg/g of husk) for raw and 0.68 (mg/g of husk) for dry husk. Solid state fermentation carried out at different pH showed optimum enzyme production at pH 6.0 (52.60 IU/g) for* P.chrysosporium *and pH 5.0 (44.08 IU/g) for* Phanerochaete *sp. Optimum temperature was 30 ± 2° C for both the organisms. Lower concentration of $MnSO_4$ (0.1 mM $MnSO_4$) induced maximum enzyme production in* P.chrysosporium *whereas* Phanerochaete *sp. required 1 mM $MnSO_4$ for induction. Absence of carbon and nitrogen stimulated enzyme production in* P.chrysosporium *while* Phanerochaete sp. *needed nitrogen. Enzyme was partially purified by ammonium sulphate precipitation followed by ion exchange chromatography.*

Key words: Arecanut husk, Solid state fermentation, Manganese peroxidase, *Phanerocheate chrysosporium*, *Phanerocheate sp.*

INTRODUCTION

In recent years, there has been an increasing trend towards the utilization of organic wastes such as residues from the agricultural, forestry and alimentary industries as raw materials to produce value-added products by solid-state fermentation (Kalogeris et al., 2003). The use of such wastes besides providing alternative substrates helps to solve environmental problems, which are otherwise caused by their disposal. Solid state fermentation is generally defined as the growth of microorganisms on moist solid substrates in the absence or near absence of free water, employing either a natural support or an inert support as a solid material (Pandey et al., 2000). Though historically established centuries ago, this technique of fermentation could gain a fresh attention from researchers since the past one decade, mainly because of a number of major advantages which SSF offers over liquid (submerged) fermentation (SmF), particularly in the areas of solid waste management, biomass energy conservation and its application to produce secondary metabolites. Advantages of SSF over submerged fermentation are high product titers, low expenditures incurred, reduced energy requirement due to lack of medium agitation and of rigorous control of fermentation parameters, low cost incurred in downstream processing due to

* Author for correspondence:emiliatea@yahoo.com

highly concentrated leachates produced (Losane et al.,1994). The selection of an adequate support for performing solid-state cultivation is essential, since the success of the process depends on it. The use of inexpensive substrates like agro-industrial wastes in SSF leads to lower production costs (Pandey et al., 1994).

SSF has been used in the production of food, feed, enzymes, organic acids and antibiotics, degradation of toxic wastes, production of mushrooms, and liquid or gaseous fuel from starchy and cellulosic substrates (Balasubramanya et al., 1994). The application of the ligninolytic enzymes to several industrial bioprocesses like biobleaching and depolarization of industrial effluents on a large scale requires the utilization of an efficient production system. SSF processes have been shown to be particularly suitable for the production of enzymes by filamentous fungi (Pandey et al., 1999a; Moo-Young et al., 1983) since they reproduce the natural living conditions of such fungi (Pandey et al., 1999b). Only a small number of microorganisms, particularly white rot fungi secrete extracellular lignin enzymes (Martin et al., 1999) with powerful capacity to degrade lignin. These materials are typically starch- or (ligno-) cellulose-based agricultural products or agro-industrial sources such as grains and grain byproducts (Pandey et al., 1992). Furthermore, most of these materials contain lignin or/and cellulose and hemicellulose, which act as inducers of the ligninolytic activity. Thus, for example, the production of lignin peroxidases was favoured by the utilization of organic wastes rich in lignin (Rodr´ıguez Couto et al., 2003). Moreover, most of them are rich in sugars, which make the whole process much more economical. Wheat bran has been widely employed as a substrate in the production of ligninolytic enzymes by SSF technique (Barbosa et al., 1996; Kalogeris et al., 2003; Malt'seva et al., 1989; Hofrichter et al., 1999).

Palm trees are monocots grown along the coastal areas and yields commercially important products such as nuts, fiber and oil. Arecanut, also known as betelnut is the kernel obtained from the fruit of arecanut (*Areca catechu* L.). The arecanut husk is about 15-30% of the weight of the raw nut. The husk fiber is composed of cellulose with varying proportions of hemicellulose (35–64.8%), lignin (13.0–26.0%), pectin and protopectin (Ramachandra et al., 2004). The average filament length of areca husk fiber is 4 cm and is too short compared to other biofibers. The fibers are biosoftened for the production of furnishing fabrics and textiles by blending with cotton, viscose and polyester (Rajan et al., 2005). It is also used in making thick boards, fluffy cushions and non-woven fabrics (Ghosh et al., 1975). Fresh and ripe areca nut husk is yellow to orange in colour having fibrous sweet pulp containing around 18 g of reducing sugars per kilogram that can be used by the micro organisms. The present paper describes the results of the production of manganese peroxidase (MnP) from *Phanerocheate* species by SSF technique employing arecanut husk as a support–substrate.

MATERIALS AND METHODS

Raw material

Substrate

Areca nuts of various maturities belonging to the variety 'Mangala' developed by Central Plantation Crops Research Institute (CPCRI) which gives golden-orange colored nuts were procured from a farm in Trivandrum, Kerala, India. The nuts were dehusked manually and dried. Average length of arecanut husk fibers obtained was 5.5 cm. Dried arecanut husk was used as the solid substrate for manganese peroxidase production.

Microorganisms used for solid state fermentation

Phanerochaete chrysosporium (MTCC 787, Chandigarh, India) and Phanerochaete sp. was used for the fermentation.

Growth Media for Enzyme Production

Vogel's Mineral Medium consisting of Vogel's salt and Trace elements (Glenn and Gold, 1983). Vogel's salt 4ml (50x), trace elements 2ml (100x) and 4g sucrose dissolved in 180ml of distilled water and made upto 200ml.

Estimation of moisture content

About 2 g of husk fiber was dried at 110± 2^{o}C for 5 h in a hot air oven, cooled for 1 h in a desiccator and weighed. From the difference in weight of the husk, the percentage of moisture was calculated.

Estimation of pH of the arecanut husk fiber extract

About 4 g of husk fiber was macerated with 100 ml distilled water using a mortar and pestle, filtered through crude filter paper, centrifuged the

filtrate at 6000 rpm for 10 min and the pH of the supernatant was checked.

Estimation of reducing sugar content

Three hundred microlitres of the above supernatent was taken, diluted to 1 ml with distilled water, added 2 ml DNS reagent, mixed well and incubated for 5 min in a boiling water bath, cooled and the volume was made up to 10 ml using distilled water. Standard glucose solution (0.5 mg/ml) at different concentrations (50-500 mg) was also treated in the same way and a standard graph of concentration versus OD at 540 nm was plotted in a spectrophotometer (Shimadzu UV2100, Japan). The concentration of reducing sugar of the test sample was calculated from the standard graph.

Estimation of non-reducing sugar content

Two milliliters of the above husk extract supernatant was diluted to 8 ml with distilled water and hydrolyzed using 100 ml of 2N HCl, for 25 min in a boiling water bath, cooled, neutralized using 2N NaOH and the reducing sugar content was estimated by DNS method at 540 nm in a spectrophotometer. The concentration of non-reducing sugar is obtained by multiplying the concentration of reducing sugar with 0.9 (for anhydrous glucose unit)

MnP Assay

MnP activity was monitored with Phenol red as substrate at 30±2°C (Glenn and Gold, 1983).The reaction mixture contained 25 mM lactate, 0.1mM $MnSO_4$, 1 mg BSA, 1 mg phenol red and 0.2 ml culture filtrate in 20 mM sodium acetate buffer (pH 4.5) in a total volume of 2 ml. The reaction was started with the addition of 0.1 M H_2O_2 and stopped after one minute with 100 µl of 10 % NaOH. The absorption at 610 nm was measured against a blank without any manganese in the reaction mixture. The molar extinction coefficient of the oxidized phenol red is 22 mM^{-1} cm^{-1}. One IU is the amount of enzyme needed to form 1 micromol of oxidized phenol red/ml/min under the assay conditions.

Estimation of protein

The protein concentrations of the enzyme samples were measured using Lowry's method (1951) with Folin-Phenol reagent and bovine serum albumin as standard.

Solid-state Fermentation

Arecanut husk was used as the matrix for solid-state fermentation. A preweighed quantity of the substrate supplemented with 60% moisture and 5 ml inoculum of *Phanerochaete chrysosporium* and Phanerochaete sp. were kept at 30±2°C for 15 days. The enzyme was extracted by adding 50 ml distilled water and shaking at 200 rpm for 1 h. The extract was filtered through filter paper and the filtrate was centrifuged at 6000 rpm for 10 min. The supernatant was used for MnP assay. The activity is expressed in micromole/min/g of arecanut husk.

Optimization of pH and temperature for SSF

Solid-state fermentation was carried out at varying pH of 5.0, 6.0, 7.0 (Phosphate buffer 0.1 M) along with a control (pH 6.5). The temperature was maintained at 20°C, 30° C and 40° C.

Optimization of manganese concentration for SSF

To enhance the production of MnP, SSF was done by supplementing manganese as $MnSO_4$ at concentrations of 1.0, 0.5 and 0.1 mM and a control without manganese.

Purification of MnP

The enzyme produced by *P. chrysosporium* and *Phanerochaete sp* was purified by ammonium sulphate precipitation, followed by ion exchange chromatography using DEAE sephadex column. All the purification steps were carried out at 4°C. Enzyme grade ammonium sulphate (20-90%) was added gradually to precipitate the desired protein. The pellet obtained by centrifugation was dissolved in tris buffer (50 mM, pH 8.0) and dialysed for 16 h against the same buffer and lyophilized.

An aliquot of the enzyme in tris buffer containing 0.01 M NaCl was used for ion exchange chromatography. The enzyme was tested for binding with DEAE sephadex and CM cellulose in tris buffer buffer (50 mM, pH 8.0 containing 0.01 M NaCl) for 4 h and found that the enzyme was bound to DEAE sephadex. Hence DEAE sephadex chromatographic separation was chosen. The glass column (L/D ratio 2:1) contained 10 g of DEAE sephadex pre-swollen in the tris buffer. The proteins were eluted with stepwise addition of 90 ml each of 0.1,0.5 and 1.0 M NaCl in tris buffer (50 mM, pH 8.0) and 3 ml fractions were collected. The fractions containing maximum MnP activity was taken, dialysed and lyophilized.

Statistical Analysis
The experimental results are means ±SD of three experiments. The results were processed using Microsoft excel 2003 and the data was subjected to one way analysis of variance. (ANOVA) and the significance of differences between the sample means were calculated by Duncan's version 7.5.1, SPSS Inc., Chicago,IL. P values 0.05 were regarded as significant and P values 0.01 as very significant.

RESULTS AND DISCUSSION

Maximum enzyme production was obtained on 13th day of incubation for both the organisms, and the activity of enzyme produced by *P. chrysosporium* was 42.08 IU/g of husk and that produced by *Phanerochaete* sp. had an activity of 47.20 IU/g. The organism took more time to get acclimatized to the stress condition mainly due to the deficiency of moisture. However, this stress condition along with the deficiency of sufficient nutrients was found to enhance the enzyme production.

Physicochemical properties of arecanut husk
The moisture content of sun dried, ripe and raw arecanut husk was 8.05, 79.84 and 68.39% respectively (Table.1). The pH of the extract was found to be acidic, i.e. 3.0 for ripe nut (orange), 5.5 for raw nut (green) husk and 7.0 for dry husk. The reducing sugar content per gram of husk was 1.77 mg for dry husk, 19.21 mg for ripe nut husk and 14.31 mg for raw nut husk. The non-reducing sugar content per gram of the husk was 0.68 mg for dry nut, 0.98 mg for ripe nut and 1.04 mg for raw nut husk.

Table 1 - Properties of arecanut husk.

Properties	Dry	Ripe	Raw
Length (cm)	5.5±0.5	5.8±0.5	5.9±0.5
Moisture content (%)	8.05±0.1	79.84±0.1	68.39±0.1
pH of extract	7.0±0.1	3.0±0.1	5.0±0.1
Reducing sugar content (mg/g of fiber)	1.77±0.1	19.21±0.1	14.31±0.1
Non-reducing sugar content (mg/g of fiber)	0.68±0.01	0.98±0.01	1.04±0.01

As the fruit ripens the moisture content increases and is subsequently lost on drying. The reducing sugar content also increases on ripening but the non-reducing sugar content decreases due to hydrolysis to simple sugars (Kresic, 2004).

Optimization of pH for SSF
Maximum enzyme activity of 52.60 IU/g of husk was observed at a pH of 6.0 for *P. chrysosporium.* For *Phanerochaete sp.* the maximum activity of 44.08 IU/g of husk was found at pH 5.0 (Fig.1).

Optimization of temperature for SSF
Maximum activity of enzyme was observed at 30^{o}±2°C for both the organisms. MnP activity of 41.04 IU/g of husk was observed for *P. chrysosporium* and for *Phanerochaete sp*, it was 46.16 IU/g of husk (Fig.2).

Optimization of manganese concentration for SSF
A maximum MnP activity of 54.03 IU/g of husk was observed for *P.chrysosporium* at manganese concentration of 0.1 mM, whereas in the case of *Phanerochaete* sp. the maximum activity of 38.40 IU/ml was observed at a higher manganese concentration of 0.1 mM. Manganese acts as an inducer for the production of MnP which is also involved in the catalytic activity of the enzyme (Fig.3).

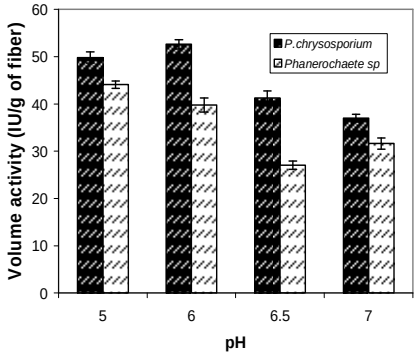

Figure 1 - Optimization of pH for SSF.

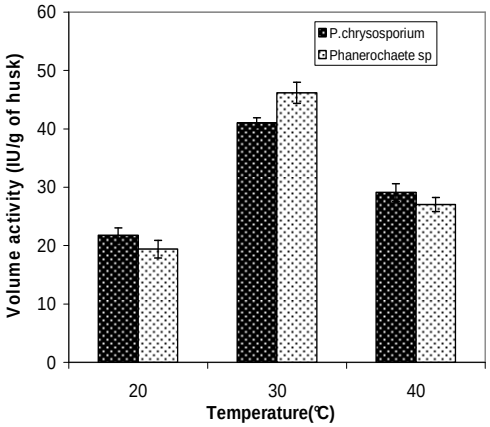

Figure 2 - Optimization of temperature for SSF.

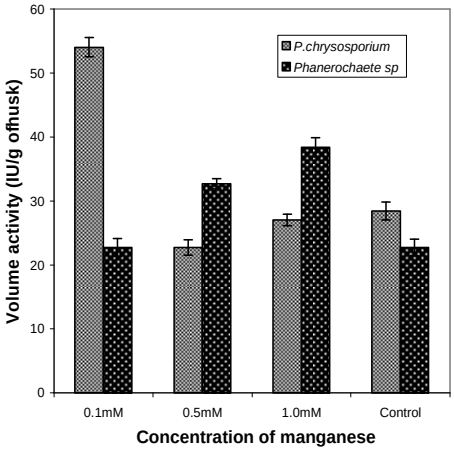

Figure 3 - Optimization of manganese concentration for SSF.

Partial Purification of enzyme

The enzyme produced by *P.chrysosporium* precipitated at 65% $(NH_4)_2SO_4$ saturation and that produced by *Phanerochaete* sp. precipitated at saturation of 60%. This showed that the *P.chrysosporium* MnP was of comparatively low molecular weight than that of *Phanerochaete* sp. enzyme. During ion exchange chromatography, with the DEAE sephadex column, the enzyme produced by *P. chrysosporium* eluted out with tris buffer (pH 8.0) containing 0.1 M NaCl, (Fig.4) which showed that this enzyme was less anionic than the enzyme from *Phanerocheate sp*. since it could only be eluted with tris buffer (pH 8.0) containing 1.0 M NaCl (Fig.5) as it was highly anionic and strongly bound to DEAE sephadex at the pH of 8.0. For the enzyme produced by *P.chrysosporium*, the purification was 2.68 fold and the yield was 5.56%. For enzyme produced by *Phanerocheate sp*, the purification was 1.77 fold and the yield was 13.03% (Table.2).

SSF on lignocellulosic materials such as arecanut husk composed of cellulose with varying proportions of hemicellulose (35–64.8%) and lignin (13.0–26.0%) simulates the natural habitats of *Phanerochaete* sp, a white-rot fungus where the production of lignin-modifying oxidoreductases such as manganese peroxidase is environment-induced.

The moisture content of ripe and raw arecanut husk was 79.84 and 68.39% respectively and pH in the acidic range of 3-5.0, with 19 mg reducing sugar and 1mg non reducing sugar per gram which is ideal for direct use in SSF. Sun dried husk has only a moisture content of 8.05 % and has a neutral pH and low levels of sugars and hence fresh husk has to be used. The MnP enzyme that is produced (41- 46.16 U/g of husk) by the *Phanerochaete* is cationic, was concentrated by ammonium sulphate fractional precipitation and purified by DEAE sephadex column. The MnP when concentrated by counter current extraction in large scale can be utilized for various purposes like delignification of paper pulp, bleaching of dyes, treatment of wastewater and in the manufacture of biosensors. This method facilitates the production of other peroxidase enzymes in a cost effective way and also an effective method of waste utilization of arecanut processing industry.

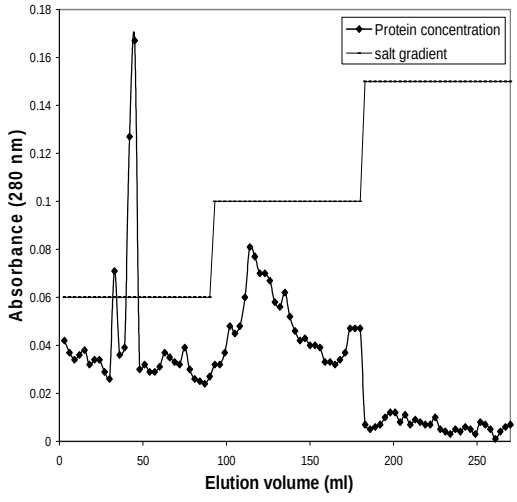

Figure 4 - Purification of MnP from *P.chrysosporium* by ion exchange chromatography.

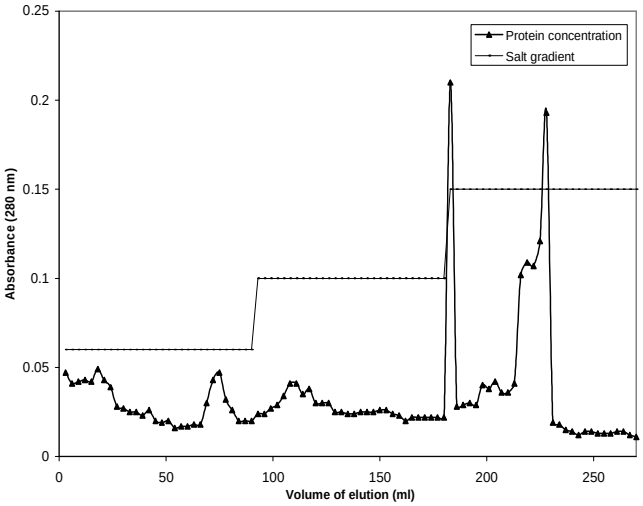

Figure 5 - Purification of MnP from *Phanerochaete sp* by ion exchange chromatography.

Table 2 – Purification of MnP

Steps	Total volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Fold purification	Yield (%)
Phanerochaete chrysosporium						
Crude	78	6431.99	68.25	94.24	-	100%
After IEC	6	358.26	4.25	252.9	2.68	5.56%
Phanerocheate sp.						
Crude	74	5891.88	63.51	92.68	-	100%
After IEC	15	767.7	14.04	164.04	1.77	13.03%

CONCLUSIONS

SSF on lignocellulosic materials such as arecanut husk, composed of cellulose with varying proportions of hemicellulose and lignin, simulates and induces *Phanerochaete, a* white-rot fungus for the production of lignin-modifying oxidoreductases such as manganese peroxidase. Fresh ripened arecanut husk has to be used in SSF systems, which has the right moisture content, pH and requires minimal nutrient addition. This waste raw material is very promising for the production of value-added products such as enzymes and biopharmaceuticals.

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