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Research Article

BIOCONVERSION OF AGRICULTURAL BIOMASS FOR INDUSTRIAL ENZYME PRODUCTION AND POLLUTION CONTROL

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ABSTRACT

The areca nut husk constitutes about 60- 80% of the total weight and volume of the fresh fruit. It is a solid residue generated as waste and large quantity is disposed as such. The areca nut husk fibres are predominantly composed of cellulose and hemicelluloses (35-64.8%), lignin (13.0- 26.0%) and pectin. Manganese-dependent peroxidases are a family of extracellular glycosylated haem proteins, which are produced by most litter harbouring fungi. Manganese peroxidase (MnP) was discovered in 1985 by Michael H. Gold and Ronald Crawford in the fungus *Phanerochaetechrysosporium*. Manganese Peroxidase enzyme catalyse oxidation-reduction reactions and also acts as a chelating agent. The lignocelluloses biomass from areca nut husk (*Areca catechu Linnaeus*) was evaluated as a new substrate for cultivation of *Fusarium oxysporum* and *Fusarium verticillioides* for manganese peroxidase (MnP) production. Solid state fermentation carried out at different pH showed optimum enzyme production at pH 6.0 for *Fusarium oxysporum* (54.3±0.50IU/g) and pH 5.0 for *Fusarium verticillioides* (49.15±0.15 IU/g). Incubation temperature of 30^0C and shaking condition was ideal for both the organisms. The study showed that potential soil fungi can be cultured on cost effective substrates such as areca nut husk and can be effectively exploited for the production of Manganese Peroxidase enzyme in large scale.

INTRODUCTION

Manganese Peroxidase (MnP, EC 1.11.1.13) belongs to the family of oxidoreductases, acting on peroxide as acceptor (peroxidases). The systematic name of this enzyme class is Mn(II): hydrogen-peroxide oxidoreductase. Manganese peroxidase is a heme glycoprotein that catalyzes the oxidation of Mn2+ to Mn3+ in the presence of H_2O_2. Mn3+ is efficiently stabilized in aqueous solution by -hydroxy acids (Giardina *et al*., 2000). Potential applications for MnP include biomechanical pulping, pulp bleaching, dye decolorization, bioremediation and production of high-value chemicals from residual lignin from biorefineries and pulp and paper side-streams. This enzyme has been found useful in industrial applications such as remediation of azo-dyes, pesticides and other harmful chemicals. It can also be used to degrade the lignin in biomass so that the sugars can be converted into biofuels. Applications of MnP are limited due to slow growth and low productivity of native enzyme producers and lack of an efficient recombinant production process (Järvinen, 2012). The enzyme can mineralize a wide variety of recalcitrant xenobiotic compounds and organo pollutants having structural similarity with the lignin (Hatakka, 2001 and Hofrichter, 2002).

Utilization of organic wastes such as residues from the agricultural and forestry as raw materials to produce value-added products using microorganisms is the most upcoming trend in recent years (Kalogeris *et al*., 2003). Besides providing alternative substrates, such wastes help to solve environmental problems, which are otherwise caused by their disposal. Areca nut, also known as betel nut is the kernel obtained from the fruit of areca nut (*Areca catechu* L.). About 15-30% of the weight of the raw nut is the areca nut husk. The husk fibre is composed of cellulose with varying proportions of hemi cellulose (35-64.8%), lignin (13.0-26.0%), pectin and proto-pectin. The average filament length of areca husk fiber is 4 cm

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and is too short compared to other bio-fibres. The fibres are bio-softened for the production of furnishing fabrics and textiles by blending with cotton, viscose and polyester. It is also used in making thick boards, fluffy cushions and non-woven fabrics (Rajan *et al*., 2010). The present paper describes the results of the production of manganese peroxidase (MnP) from *Fusarium oxysporum* and *Fusarium verticillioides* using areca nut husk as a support substrate.

MATERIALS AND METHODOLOGY

Isolation and Identification of Fungi from Litter Samples

Litter samples were collected from some selected Areca nut plantation areas of Kodagu District, Karnataka State.Isolation was done using Potato Dextrose Agar (PDA) with 0.01% guaiacol and incubating at 28ºC. The guaiacol positive reaction will be indicated by the formation of a reddish-brown halo. The fungi were identified by observing under microscope and identified using standard manuals. The identification was further confirmed by Molecular Characterization. The isolated fungal strains will be maintained on 2% (w/v) Malt Extract Agar slants at 4ºC and the fungi will be activated at 26ºC (Erden *et al*., 2009).

Optimization of Growth Media for Manganese Peroxidase Enzyme production

The growth condition of fungi for the production of manganese peroxidase enzyme was optimized with respect to pH 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. The stationary and shaking type (150 rpm/minute) of incubation methods was selected for the culturing of fungi. To enhance the production of MnP, the substrate was supplemented with manganese as $MnSO_4$ at concentrations of 1.0, 0.5 and 0.1 mM (Rajan, A. *et al*., 2010).

Preparation of the Substrate

Areca nuts of different maturity were procured from a farm in Sunticoppa, Kodagu District. The nuts were de husked manually, dried and powdered. Moisture content (Osonol *et.al*. 2002) and pH (Zadrazil and Brunnert, 1982) was estimated. Twenty-five grams (25g) of substrate was weighed into each 250 ml conical flask and pH was adjusted with 75ml Phosphate buffer 0.1 M. The flasks were immediately covered with aluminium foil and sterilized in the autoclave at 121^0 C for 15 minutes.

The substrates were prepared in three replicates (Adenipekun and Fasidi, 2005).

Inoculation

The flasks were inoculated at the centre of the substrate with two agar plugs (7mm in diameter) of vigorously growing mycelia disc and covered immediately. They were kept for incubation at 20°C, 30° C and 40° C under stationary and rotary type of incubation methods for 20 days (Bhargava and Orskov, 1987).

Extraction and Estimation of Mn Peroxidase

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 20 min at 4^0C. The supernatant was used for MnP assay The MnP assay was performed on the basis of oxidation of phenol red. The reaction mixture containing 1 ml of sodium citrate buffer (50 mM) (pH 4.5), 1 ml of lactic acid (50 Mm) (pH 5), 0.4 ml manganese sulfate (0.1 mM), 0.7 ml phenol red (0.1mM),0.4 ml hydrogen peroxide(50 µM), 1 ml gelatin (1mg/ml) and 0.5 ml of crude enzyme was taken. The reaction was initiated by adding hydrogen peroxide and incubated at 30^0C.40µl of 5N sodium hydroxide will be added to 1 ml of reaction mixture. Absorbance will be measured at 610 nm against a blank without any manganese in the reaction mixture. After every min the same step will be separated with 1 ml of the reaction mixture up to 4 minute. The molar extinction coefficient of the oxidized phenol red is 22 mM^{-1} cm^{-1}. One unit the enzyme activity (IU) is the amount of enzyme needed to form 1 micromole of oxidized phenol red/ml/min under the assay conditions. The activity is expressed in micromole/min/g of areca nut husk (Modified method of Dev & Thankamani, 2012).

RESULTS AND DISCUSSION

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.

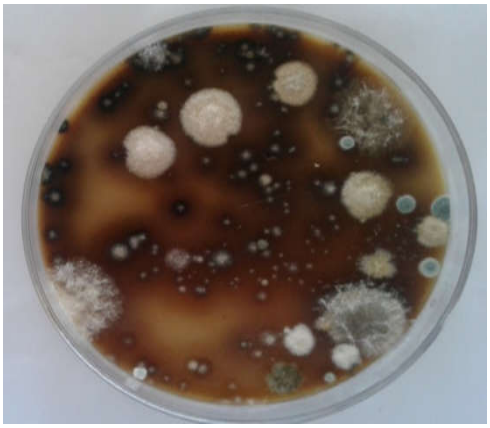

Fig 1 Mnp Producing Fungi on PDA +Guiacol Media

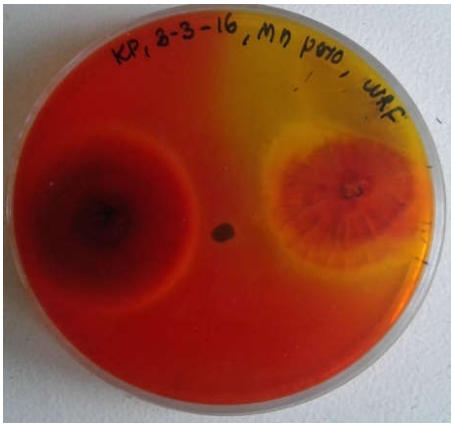

Fig 2 Confirmatary Test on PDA Supplemented with Phenol Red

Statistical Analysis

The experimental results analysed are means ±SD of three experiments. All the results were analysed using IBM SPSS Software version 20. Univariate ANOVA was performed with the Duncan's multiple range test with significant mean at P≤0.05.

The screening of fungi for the production of manganese peroxidase enzyme was done using Potato Dextrose Agar (PDA) with 0.01% guaiacol. The brown diffusion zone was considered to be the result of oxidation of the acids, and here after this reaction will be referred to as "oxidase" reaction. Confirmatory test for the manganese peroxidase enzyme was done on Potato Dextrose Agar with 0.02 % phenol red. Two strains showing large red hallow zone around the fungal mat because of oxidation of phenol red was considered as the positive result and taken for further study.

The isolated fungi were identified based on morphological characters. Microscopic observation and molecular characterization as *Fusarium oxysporum* and *Fusarium verticillioides* respectively

Manganese Peroxidaseproduction on areca nut husk under optimized Growth conditions

The moisture content of sun dried arecanut husk was 12.34% and the pH of the extract was found to be 6.8 for dry husk. As the fruit ripens the moisture content increases and is subsequently lost on drying. The pH of raw and ripened fruit is acidic and it turns out towards basic on drying (Kresic, 2004).

Optimization of pH

Maximum enzyme activity of 49.4±0.20IU/g of husk was observed at a pH of 6.0 for *Fusarium oxysporum*. For *Fusarium verticillioides* the maximum activity of 51.7±0.50 IU/g of husk was found at pH 5.0 on the 16th day under shaking method of incubation (Table 1). Rajan *et al*., (2010), carried out SSF by *P. chrysosporium* using areca husk and found out maximum enzyme activity of 52.60 IU/g at a pH of 6.0 and by *Phanerochaete sp*. the maximum activity of 44.08 IU/g of husk was found at pH 5.0

Table1 Effect of pH on Mn Peroxidase production

Isolate	pH	Method of incubation	Enzyme Activity in IU/gof husk
Fusarium oxysporum	5	Shaking	34.1 ± 0.10^{c}
		Stationary	30.9 ± 0.10^{a}
	6	Shaking	49.4 ± 0.20^{i}
		Stationary	32.7 ± 0.30^{b}
	7	Shaking	36.1 ± 0.10^{d}
		Stationary	32.65 ± 0.35^{b}
Fusarium verticillioides	5	Shaking	51.7 ± 0.50^{j}
		Stationary	42.4 ± 0.20^{f}
	6	Shaking	44.2 ± 0.20^{g}
		Stationary	41.1 ± 0.10^{e}
	7	Shaking	44.3 ± 0.30^{g}
		Stationary	45.5 ± 0.30^{h}

Note: Each value is a mean of 3 replicates. Means with different superscripts in each column are significantly different at P≤0.05 according to Duncan's multiple range test.

Optimization of temperature

Maximum activity of enzyme was observed at 30°C for both the organisms. MnP activity of 51.15±0.25 IU/g of husk at pH 6 was observed for *Fusarium oxysporum* and for *Fusarium verticillioides*, it was 45.15±0.15 IU/g of husk at pH 5 on the 16th day under shaking method of incubation (Table.2). Sparrat *et al*., (2000) observed that maximum MnP production at a temperature of 37°C by *P. chrysosporium* using olive and sugar beet wastes.

Table 2 Effect of Temperature on Mn Peroxidase production

Isolate	Incubation Temperature	Method of incubation	Enzyme Activity in IU/g of husk
Fusarium oxysporum	20°C	Shaking	32.5 ± 0.30^{c}
		Stationary	28.9 ± 0.10^{b}
	30°C	Shaking	51.15 ± 0.25^{h}
		Stationary	32.7 ± 0.30^{c}
	40°C	Shaking	36.1 ± 0.10^{d}
		Stationary	32.65 ± 0.35^{c}
Fusariumverticillioides	20°C	Shaking	28.7 ± 0.50^{b}
		Stationary	22.4 ± 0.20^{a}
	30°C	Shaking	45.15 ± 0.15^{g}
		Stationary	41.1 ± 0.10^{e}
	40°C	Shaking	42.3 ± 0.30^{f}
		Stationary	35.5 ± 0.30^{d}

Note: Each value is a mean of 3 replicates. Means with different superscripts in each column are significantly different at P≤0.05 according to Duncan's multiple range test.

Optimization of manganese concentration

A maximum MnP activity of 54.3±0.50 IU/g of husk was observed for *Fusarium oxysporum* at manganese concentration of 1 mM, at pH 6, whereas in the case of *Fusarium verticillioides,* the maximum activity of 49.15±0.15IU/g was observed at a lower manganese concentration of 0.5mMat pH 5 on the 16th day under shaking method of incubation at 30°C. Manganese acts as an inducer for the production of MnP which is also involved in the catalytic activity of the enzyme (Table 3). Li *et al*., (1995), observed that the expression of manganese-peroxidase is induced by the presence of manganese, hydrogen peroxide and lignin in white-rot fungi such as *Phanerochaetechrysosporium. P.chrysosporium* exhibited maximum MnP activity of 54.03 IU/g on areca husk 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.

Table 3 Effect of manganese on Mn Peroxidase production

Isolate	Manganese inducer	Method of incubation	Enzyme Activity in IU/g of husk
Fusarium oxysporum	1.0mM	Shaking	54.3 ± 0.50^{i}
		Stationary	33.9 ± 0.10^{b}
	0.5 mM	Shaking	45.65 ± 0.25^{g}
		Stationary	32.7 ± 0.30^{a}
	0.1 mM	Shaking	39.1 ± 0.10^{e}
		Stationary	32.65 ± 0.35^{a}
Fusarium verticillioides	1.0mM	Shaking	$34.7\pm0.50b^{c}$
		Stationary	32.4 ± 0.20^{a}
	0.5 mM	Shaking	49.15 ± 0.15^{h}
		Stationary	41.5 ± 0.30^{f}
	0.1 mM	Shaking	37.8 ± 0.80^{d}
		Stationary	35.55 ± 0.05^{c}

Note: Each value is a mean of 3 replicates. Means with different superscripts in each column are significantly different at P≤0.05 according to Duncan's multiple range test.

Sahni & Phutela (2013), pretreated the paddy straw with *Coriolus versicolor* MTCC 138 (standard) and *Fusarium* sp. isolated from compost and plant debris. On 20th day of incubation lignin loss observed was 27.1 and 17.5% in paddy straw pre-treated with *C. versicolor* MTCC 138 and *Fusarium* sp., respectively. Solid-state fermentation (SSF) was performed using soy and wheat bran to investigate the growth and production of lignocellulosic enzymes from *Fomes sclerodermeus,* Maximal manganese peroxidase (MnP) activity(14.5 U g ^{1}) was observed on 15 days after inoculation (Papinutti & Forchiassin, 2007).

CONCLUSION

Arecanut husk, a ligno-cellulosic material simulates and induces the growth of fungus which are involved in the production of lignin-modifying oxido-reductases such as manganese peroxidase. Areca nut husk, avery low cost substrate, having the right moisture content and pH is very promising for the production of value-added products such as enzymes and biopharmaceuticals using *Fusarium oxysporum* and *Fusarium verticillioides*. Fungal pre-treatment for ligno-cellulosic substrates when pre-treated with lignolytic fungi can be used for efficient degradation of lignocellulosic biomass. The degradation of ligno-cellulosic biomass is an alternative energy technology for the bioconversion of biomass to combat the greater demands of industrially important enzymes and control pollution.

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