Optimization of extracellular peroxidsae production from *Coprinus* sp.

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Abstract: In this article, we describe the selection of medium components like pH, temperature, carbon sources and nitrogen sources for the optimal production of extracellular peroxidase by *Coprinus* sp. (AKL 02) with a series of experiments which is important for growth and enzyme production. The high quality enzyme elicited by the fungus has greater potential for industrial application especially waste water treatment.
Keywords: Extracellular peroxidase, *Coprinus* sp., fungi, enzyme.

Introduction

Peroxidase is an enzyme, which may contain heme that catalyze the transfer of oxygen from the hydrogen peroxidase to a suitable substrate and thus brings about oxidation of the substrate. The enzyme occurs in plants, animals and microorganisms. Its specificity, biological functions vary with sources of the enzyme.

The enzyme is an acidic protein (pH 3.5) and consists of a single polypeptide chain having the molecular weight of 41,600daltons. The enzyme contains one protohemin per molecule and exhibits the characteristic adsorption, circular dichroism, and magnetic circular dichroism spectra of a heme protein (Morita *et al*., 1988). Peroxidase has attracted industrial attention because of its usefulness as a catalyst in clinical examination, biosensor, environmental aspects and other applications.

In 1986, Yamada *et al.* discovered a novel peroxidase which is produced extracellular by a novel hyphomycete. Later, a similar peroxidase was also found in the culture filtrate of an ink cap Basidiomycetes *Coprinus cinereus* (Morita *et al*., 1988). During the past 12 years, unique applications of these peroxidase have been developed. Currently these fungi are known as new practical sources of peroxidase for industrial purposes in addition to horseradish roots.

The *Coprinus* peroxidase forms two characteristic intermediate compounds I and II, the rate constants for hydrogen peroxide and guaiacol had similar values to those for higher plant peroxidases (Morita *et al*., 1988). Extracellular peroxidase is the most important components of the extracellular lignin degrading system which is responsible for the initial attack of lignin by a non specific oxidation mechanism (Kirk & Farrell, 1987).

The newest varieties of class II fungal peroxidases with noligninolytic activities were discovered in the mid-1980s.These include ARP from an imperfect fungus *Arthromyces ramosus* isolated from soil and CIP from an inky cap basidiomycete *Coprinus cinereus* (Shinmen *et al*., 1986; Morita *et al*., 1988). There has been another variety of fungal peroxidase called CMP obtained from *C. macrorhizus*. However, this fungus is recognized as the same species as *C. cinereus* (Orton & Watling, 1979), and it was suggested that these two *Coprinus* peroxidases were identical.

Our recent fungal survey demonstrated peroxidase production by other *coprinus* species, such as *C. lagopus* UAMH 7499, *C. echinosporus* NBRC 30630, and several unidentified species investigated by others (Ikehata & Buchanan 2002; Ikehata *et al*., 2004). Among fungi tested in the present survey *Coprinus* species was found to be promising candidate for large scale peroxidase production. We are particularly interested in the peroxidase produced by *Coprinus* species (AKL 02), because this enzyme is apparently more stable at higher temperature than the enzyme produced by other fungal strains, although the enzyme productivity by the former fungi was lower. High thermal stability (up to 60- 70˚C) of an enzyme is generally preferable and especially beneficial for its application to industrial waste water treatment, because the temperature of industrial waste water tends to be above ambient, which would accelerate enzyme inactivation. In this article, we describe the selection of medium components like pH, temperature, carbon sources and nitrogen sources for the optimal production of extracellular peroxidase by *Coprinus* sp (AKL 02) with a series of growth experiments which is very important for growth and enzyme production (Ikehata *et al*., 2004; Hao *et al*., 2007).

Materials and methods

Isolation and screening for peroxidase enzyme

Fruiting body of *Coprinus* sp. (AKL 02) collected from banana plant at Keeriparai, Tamil nadu, India, was cut in 7mm mycelial disc and placed on a glucose malt extract salt agar medium. Plate was incubated at 28°C for 3 days and thereafter, 3ml of 1.7mM and 2.5 mM of ABTS and hydrogen peroxide respectively were overlapped on the plate and were kept in dark at 25°C for 5 minutes. Appearance of clear bluish green zone

around the fungus gave an indication of peroxidase production by the fungus.

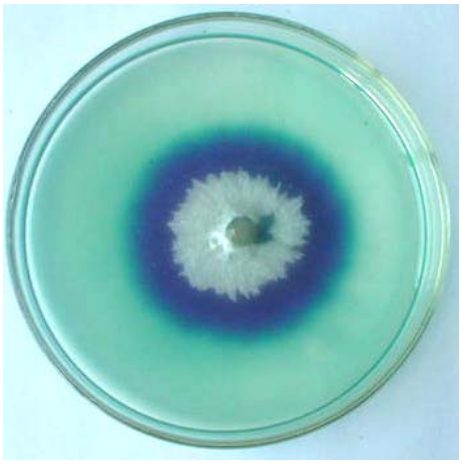
Enzyme production

Production of peroxidase by *Coprinus* sp. was carried out in a four different type of media viz. PDB, Malt extract broth, Cepeck Dox broth and finally with glucose malt extract salt medium containing the following: glucose 2% (w/v); malt extract 2% (w/v); $NaNO_3$ 0.2% (w/v); KH_2PO_4 0.2% (w/v); KCl 0.2% (w/v); $MgSO_4.7H_2O$ 0.1% (w/v); $FeSO_4.7H_2O$ 0.002% (w/v). The pH was adjusted to 6.5 and was sterilized by autoclaving at 121°C for 15 min. The medium (50ml in 250ml Erlenmeyer flasks) was inoculated with two mycelial discs (7mm) and maintained at 28°C in a static condition. Fermented broth was filtered on filter paper at 4°C at every 48hrs interval until the enzyme activity was declined and the supernatant was recovered and used as enzyme source. Further experiments were carried out in a suitable medium out of the above mentioned.

Effect of culture conditions on enzyme production

An attempt was also made to ascertain the optimum culture conditions such as pH, temperature, incubation period and carbon and nitrogen source requirements for their maximum enzyme activities. The peroxidase production of the selected isolate was recorded.

Fig.1. Peroxidase production the test fungus: plate assay (ABTS used as substrate)



Effect of pH: To determine the optimum pH of the growth medium for maximum enzyme production, the fungus was grown in medium with different pH (such as 4.0, 4.5, 5.0, up to 9.0) and the peroxidase activities were recorded.

Temperature: To determine the optimum temperature for enzyme production, the culture medium was incubated at 25, 28, 32 and 37°C temperature at an optimum pH. The effect of temperature on peroxidase production was recorded.

Carbon and nitrogen sources: The optimum carbon and nitrogen sources were studied at different concentrations (0.1, 0.5, 1.0, 1.5… 3.5% w/v) for the enzyme secretion. The culture medium was supplemented by replacing glucose with any other carbon source such as glycerol, D(+) galactose, lactose, sucrose, maltose, starch, mannitol, L (+) arabinose, fructose, inositol and CMC. Different nitrogen sources including NH_4NO_3, peptone, yeast extract, beef extract, soybean meal, $(NH_4)_2SO4$, $NH_4H_2PO_4$, NH_4Cl, $NaNO_3$, were also employed.

Analytical method

Extracellular peroxidase activity of cell free filtrate was assayed spectrophotometrically. The increase in the absorbance was measured at 414nm using 1.7mM ABTS (2,2 azino bis 3 ethyl benzo thiazolin 6 sulphonic acid), 2.5 mM hydrogen peroxide & 100mM of citrate phosphate buffer (these are final assay concentration for 1ml of reaction mixture). To 0.1ml of culture filtrate, 0.9ml of 1.7mM ABTS and 25µl of 100mM hydrogen peroxide was added and the OD was read at 414 nm for one minute. 0.1ml of distilled water, 0.9ml of 1.7mM ABTS and 25µl of 100mM hydrogen peroxide were kept as blank. Heat denatured enzyme served as control. One unit of peroxidase was defined as the change in absorbance of 1.0/ml/min at 414 nm.

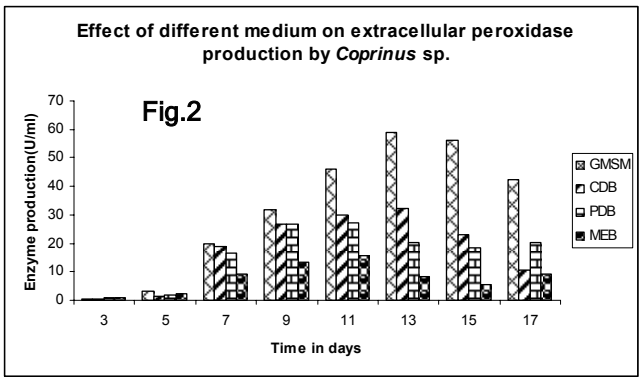
Results and discussion

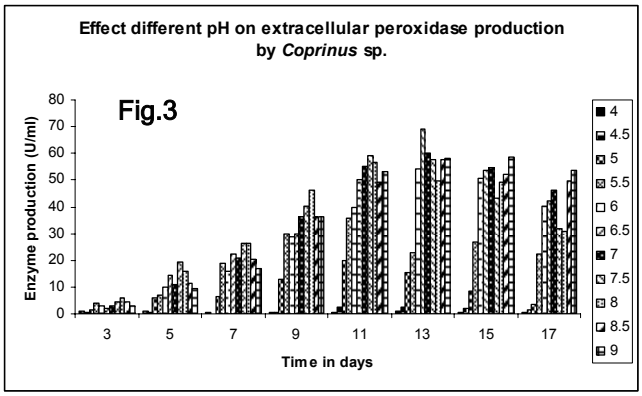
Microorganism

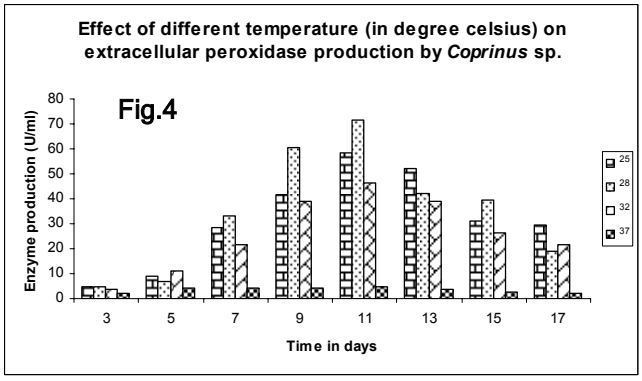
Five fungal isolates apparently with clear bluish green zone gave an indication of peroxidase producing organisms and thus selected from the preliminary screening for further studies. Among them, the fungal Isolate AKL 02, a potent peroxidase producer in liquid medium, was finally selected as test fungus for peroxidase production (Fig. 1). According to Pegler (1983), all morphological characters indicated that the test fungus strain AKL 02 was *Coprinus* sp.

Effect of different type of medium on peroxidase production by Coprinus sp.

Coprinus sp. showed maximum enzyme production in between 11-13 days of incubation period in GMSM (Glucose Malt extract Salt Medium) (59 U/ml) than other three type of medium (Fig. 2). Best enzyme production in GMSM can be attributed to the fact that it provides the complete pool of amino acids required for enzyme synthesis (Arora & Gill 2000; Fahraeus, 1952). Moreover, malt extract is rich in the aromatic amino acids tryptophan and tyrosine. Tryptophan is a precursor for the synthesis of a large number of N-substituted aromatic secondary metabolites of fungi (Turner & Aldridge 1983), many of which are substrates for MnP production (Urzua *et al.,* 1995). These may







then act as inducers for MnP in a way similar to veratryl alcohol and guaiacol, which are substrates as well as inducers for ligninase and laccase this observation gains further support from earlier studies where the addition of tryptophan to cultures of some white rot fungi increases the production of lignin peroxidase (Collins *et al*., 1997). In contrast to ligninase, which in general is best produced under nitrogen starvation conditions (Arora & Gill 2001); better production of MnP in nitrogen-rich media was reported in peptone and albumin media for *Bjerkandera* spp. strain BOS55, and in soybean medium for *Coprinus friesii* (Heintzkill *et al*., 1998; Mester *et al*., 1996).

Medium pH and temperature

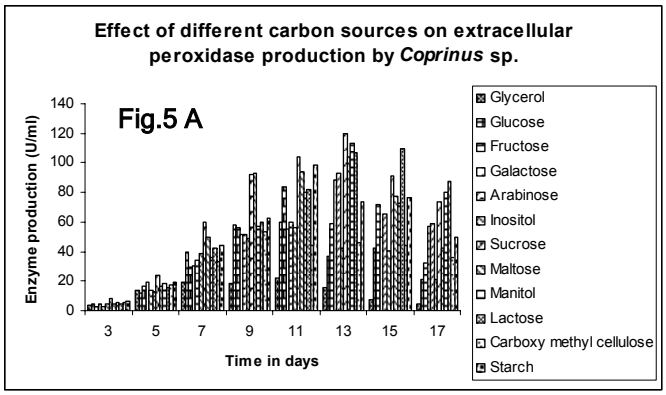
To ascertain the optimum incubation pH and temperature for maximum production of peroxidase, the selected isolate was incubated at different pH such as 4.0, 4.5, 5.0 up to 9.0 and at a temperature of 25, 28, 32 and 37°C. These two experiments were conducted separately.

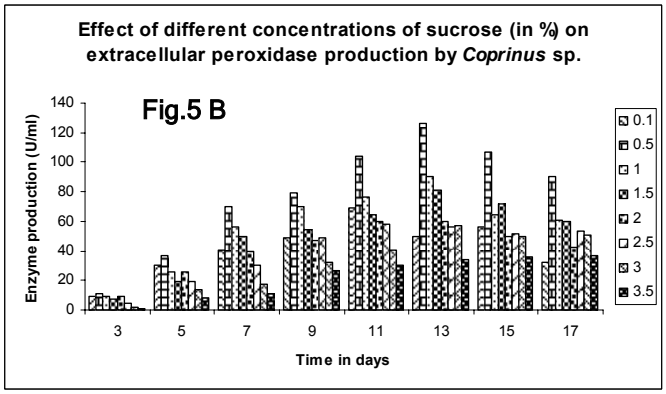
Optimum enzyme production *i.e*., 69.12 and 60 U/ml was noticed at pH 6.5 and 7.0 respectively and alkaline pH moderately supported the peroxidase production; acidic pH did not favpour for high enzyme production (Fig. 3). Similarly, Sakurai *et al.* (2002) also reported that the maximum enzyme production in *C. cinereus* was at neutral pH (7.0). Some basidiomycetes also secrete laccase and MnP in the medium at pH 6.5 (Songulashvili *et al*., 2007; Elisashvili *et al*., 2006). On the other hand, there are reports on acidic pH supporting the MnP production (Sugiura *et al.,* 2003; Baborova *et al.,* 2006; Rogalski *et al.,* 2006; Tsukihara *et al.,* 2006).

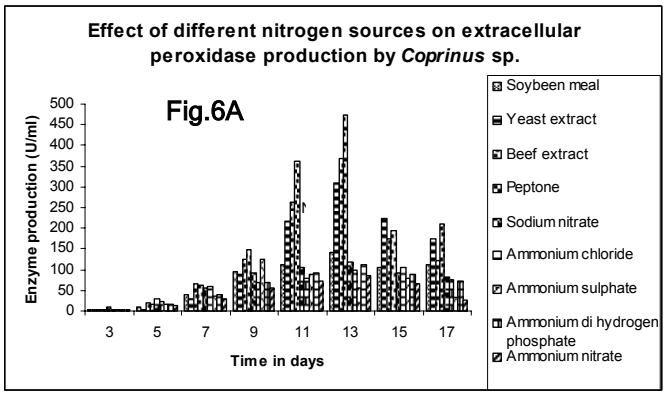
The *Coprinus* sp. showed maximum enzyme production at 28°C on 11th day (71.4U/ml) compared to that of other incubation temperature ranges (Fig. 4) which is in consensus with earlier findings i.e., the maximum enzyme yield ranges between 25-30°C (Ikehata & Buchanan 2002; Sakurai *et al.,* 2002; Ikehata *et al*., 2004; Vincentim & Ferraz, 2007).

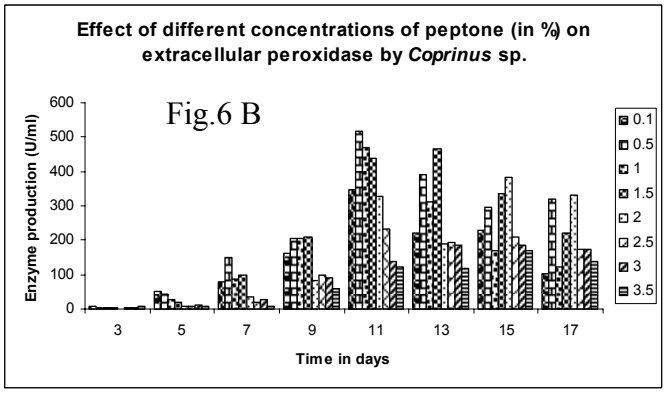
Carbon and nitrogen

The peroxidase production was found to vary with the different carbon sources. The maximum enzyme production (120U/ml) was recorded on 13th day of incubation with sucrose at the concentration of 0.5% in the medium. Moderate to good levels of enzyme activities were obtained with mannitol, lactose, maltose and starch (Fig. 5). On the other hand, various mono saccharide carbon sources and caboxyl methyl cellulose did not favour for peroxidase production. Glucose supported only 10-14 U/ml enzyme (by *Coprinus* sp UAMH10067) and 20-28 U/ml (by *Coprinus cinnereus* UAMH 4103). Similar trend was also noted by other workers (Ikehata & Buchanan 2002; Ikehata *et al.,* 2004). On the other hand, Tuncer *et al*., 1999 reported the enhancement of peroxidase production *in T.fusca* by xylan (5.6 U/ml).









Among the various organic and inorganic nitrogen sources, the maximum enzyme activity (473 U/ml) was obtained when peptone was used in the medium at the concentration of 0.5%. A moderate to good levels of enzyme activities were obtained when beef extract and yeast extract were used as nitrogen source (Fig. 6). When various inorganic nitrogen sources were tested, the peroxidase production was found repressed. Similar results were obtained by Stajic *et al*. (2006) with *P. eryngii* 616, *P. ostreatus* 493 and *P. ostreatus* 494 and also by Ikehata *et al*. (2004) with the *Coprinus* sp. On the other hand, Stajic *et al*. (2006) reported the enhancement of peroxidase production in *P. pulmonarious* by inorganic nitrogen sources like KNO_3 and $NH_4H_2PO_4$.

Conclusion

It is understood from the present study that optimum pH, temperature, carbon and nitrogen sources are the limiting factors for the maximum peroxidase production. The enzyme production rate can be increased, up to 700%, by optimization. Thus the enzyme elicitation by *Coprinus* sp (AKL 02) recorded an 8 fold (516 U/ml) increase over the previous report (Ikehata *et al.,* 2004; *Coprinus* sp. UAMH 10067 produced 68 U/ml).

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