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Production of cellulase and laccase enzymes by *Oudemansiella radicata* using agro wastes under solid-state and submerged conditions

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***Oudemansiella radicata* (Relhan ex Fr.) is one of the medicinally important edible mushrooms belonging to Tricholomataceae. In the present study we reported the efficient production of cellulase and laccase enzymes by *O. radicata* under submerged (SMF) and solid-state (SSF) fermentations. *O. radicata* had grown well both in submerged and solid-state conditions. Under submerged condition the maximum cellulase activity (490 units / ml / min) was observed. In the case of SSF the maximum cellulase activity was observed as 400 units / ml / min in wheat bran, followed by rice bran (367 units / ml / min). In the case of laccase production, the maximum activity was observed as 1.476 units / ml / min on 14th day at pH 7 under SMF. In solid substrate fermentation the maximum activity was noticed as 25.784 units / ml / min in rice bran. The next higher activity was 11.473 units / ml / min in wheat bran, and the least activity was recorded with saw dust. Higher levels of laccase and cellulase activity were seen in solid-state fermentation than in submerged fermentation. Hence the present results clearly explain that *O. radicata* is a potential candidate for the production of industrially important enzymes using agro-wastes.**

Key words: Cellulase, Laccase, *Oudemansiella radicata* , Solid-state, Submerged

The enzyme production is a growing field of biotechnology. Annual world sales figures are close to billion dollars (Layman *et al.,* 1990) with increasing number of patents and research articles related to this field. Since the biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of ligninolytic enzymes (Elisashvili *et al.,* 2001, Reddy *et al.,* 2003, Moldes *et al.,* 2004).

Cellulose is the most abundant renewable biopolymer on the earth. It is a dominant material from agriculture wastes generated from both natural and man-made activities (Bhat, 1997, Reeta Rani Singhania, 2006). However, for many

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large-scale bioconversion processes, the costs of enzymes are still a major factor influencing the economics, and to reduce production costs and enhance the formation of cellulase and hemicellulase, which are both essential for the utilization of the carbohydrate components of lignocellulosics, different strategies can be applied (Biely, 1985). Laccase is a polyphenol oxidase that can use oxygen to oxidise different types of aromatic molecules and to form lignin type of aromatic polymers from phenolic compounds. Laccases are produced by white-rot fungi, which use them to degrade lignin, the aromatic polymer found in all plant materials (Garcia *et al.*, 2000). Laccase is the most widely distributed of the large blue copper-containing oxidases, being found in many fungi, higher plants and insects (Gianfreda *et al.*, 1999).

SSF is traditionally defined as those processes in which microbial growth and products formation occur on the surfaces of solid substrates in the near absence of free water. Due to this low amount of water available in solid-state bioprocessing, the class of micro-organisms that are most commonly used is fungi (Pandey *et al.*, 2000). Submerged fermentation (SmF) is used for industrial production of cellulase (Maryam Latifian *et al.*, 2007). Production of cellulase in SSF using various substrates, microorganisms and nutrient solutions has been reported (Yang *et al.*, 2004; Awafo *et al.*, 2000).

Oudemansiella radicata (Relhan ex Fr.) is one of edible and medicinally important mushrooms belonging to Tricholomataceae, Agaricales has been known to be inhabited on the soil surface or rotted woods of the broad-leaved trees from summer to autumn (Lee, 1988). The fruiting body of *O. radicata* has been reported to possess oudenone which is one of medicinally important chemical compounds (Umezawa *et al.*, 1973; Tsantrizos *et al.*, 1999). It has been reported that 10,000 – 20,000 kg of the dried body *O. radicata* is produced every year in China, and there is a great need to supply the market with high-quality *O. radicata* products due to its medicinal value (Tsantrizos & Zhou, 1995). The present study deals with the cultivation of edible fungus *O. radicata* and its potential to produce cellulase and laccase under submerged and SSF. In solid state fermentation studies, the effect of different substrates such as wheat bran, rice bran, paddy straw and saw dust on the production of cellulase and laccase has been studied. The conditions required for optimal production have also been studied.

Materials and methods

Microorganism

Oudemansiella radicata used in the study was obtained from Tamil Nadu Agriculture University, Coimbatore, India. They were maintained on potato-dextrose agar (Hi-Media, Bombay) and were subcultured every fortnight and stored at 25°C until further use.

Raw materials

Agro-waste materials such as rice bran saw dust, paddy straw and wheat bran were obtained from processing unit and Marketing Corporation, Chennai, India. They were further mechanically dehydrated until the moisture content of dried materials reached 4±1%, which generally took 3 – 4 hours. Dried

materials were packed in polythene pouches for further studies (Vinod Kumar Joshi, 2006).

Submerged fermentation

Liquid culture was grown in Erlenmeyer flasks (100 ml) containing 30 ml of the medium (Potato dextrose broth or Nitrogen limiting medium). Mycelium was cultured in Nitrogen limiting medium: Glucose, 15 g; Malt extract, 0.4 g; $MnCl_2$, 0.3 g; $FeSO_4.7H_2O$, 0.004 g; $MgSO_4.7H_2O$, 0.04 g; 3'3 dimethyl butyric acid, 20 mM; Distilled water, 1000 ml.

Solid-state fermentation

The collected agro-waste materials were used as substrates for solid-state fermentation. The substrates were separately transferred into Erlenmeyer flasks and distilled water was added and soaked over night. The next day, water was drained off, and care was taken to retain sufficient moisture. The flasks were then sterilized in an autoclave. After cooling, all the flasks were inoculated with the fungal isolates under aseptic condition, and then incubated for the complete growth of mycelium. After 7, 14 days of incubation the flasks were taken and phosphate buffer (pH 7) was added under aseptic condition to the bottles containing different solid substrates. It was mixed well with glass rod and again buffer solution was added to give a final volume of 50 mL. The flasks were kept on the shaker for 30 minutes at 120 rpm, and then the flasks were kept in cold room overnight. Later, the contents were filtered with a muslin cloth, and centrifuged for 20 minutes at 4000 rpm. The clear supernatant (crude enzyme) was collected in separate containers and it was estimated for cellulase and laccase.

Estimation of extracellular cellulase

The culture was centrifuged at 3000 rpm for 20 minutes and the supernatant was used as the enzyme source. Two ml of the enzyme, 4 ml of the substrate [100 ml of citrate buffer (Na_2HPO_4, 2.658 g; Citric acid, 0.1334 g; Distilled water, 100 ml; pH, 7.6;) and 0.5 mg carboxy methyl cellulase (CMC)] were mixed. The tubes were then incubated in water bath at 40 - 50°C for 1 hour. After incubation, 1 ml of the aliquot was taken and 1 ml of the alkaline copper reagent was added. The tubes were again incubated for 20 minutes in a boiling water bath. Tubes were cooled and 1 ml of the arsenomolybdate reagent (Conc. sulphuric acid, 21 ml; Distilled water, 450 ml; Sodium arsenate, 5 g in 25 ml of water) was added to the reaction mixture and the colour development was read at 590 nm. The amount of reducing sugars was estimated and the result was expressed as the amount of reducing sugar released per mg of dry weight.

Estimation of extracellular laccase

Basal Medium (KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.35 g; $MnSO_4.4H_2O$, 0.3 g; $ZnSO_4.7H_2O$, 0.3 g; $FeSO_4.7H_2O$, 0.3 g; MgMoO4, 0.3 g; Ammonium Citrate, 0.2 g; Thiamine, 0.2 g; Niacin, 0.2 g; Riboflavin, 100 µg; Pantothenic acid, 100 µg; P-amino benzoic acid, 100 µg; Cyanocobalamine, 100 µg; Biotin, 50 µg; Pyridoxine, 50 µg; Folic acid, 50 µg; Glucose, 20 g; Distilled water, 100 ml; pH, 6.0) was prepared and 20 ml of medium was distributed in 100 ml conical flasks. After sterilization these flasks were inoculated with two mycelial discs (7 mm) and kept for incubation for 14 days. Extra cellular laccase activity of

culture filtrate was assayed spectrophotometrically on 7th and 14th day. The increase in absorbance was measured at 530 nm using 1 mM 2, 2-azinobis-3-ethylbenz thiozoline-6-sulfonic acid (ABTS) as substrate. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 mM of substrate/minute. To 0.1 ml of culture filtrate, 1 ml of substrate ABTS was added and the optical density (O.D) was read at 530 nm at 30 seconds intervals upto 3 minutes, 0.1 ml of distilled water and 1 ml of substrate ABTS acts as blank.

Calculations

$$\text{Units / ml enzyme} = \frac{(\text{rA530 nm / min Test} - \text{rA530 nm / min Blank})\ (\text{df})}{(0.001)\ (0.5)}$$

rA = Relative absorbance, df = Dilution factor,
0.001 = The change in A530nm/minute per unit of Laccase at pH 6.5 at 30°C in a 3 ml reaction mixture, 0.5 = Volume (in milliliter) of enzyme used

$$\text{Units / mg solid} = \frac{\text{Units / ml enzyme}}{\text{Solid / ml enzyme}}$$

Results and Discussion

In the present study, test fungus was grown in the broth medium and the dry weight of the mycelium was determined at seven days interval. Effect of different hydrogen ion concentration on the mycelial growth of *O. radicata* under submerged cultivation condition was presented in (Fig. 1). The results of the present study clearly indicated that maximum growth (520 mg / 20 ml) was observed on 7th day itself, whereas on 14th and 21st day there was drastic change in their growth under submerged conditions. Cellulase activity by *O. radicata* under submerged and solid-state condition was studied. Cellulase production by the test fungi on different days of incubation under submerged fermentation are presented in Fig. 2. Under submerged condition it showed maximum activity of cellulase (490 units /ml / min) on 7th day while on the 14th day it was only 402 units which were observed as nearly 20% reduction. Cellulase activity was observed maximum both in wheat bran and rice bran. Cellulase production is the most important step in the economical production of ethanol, single cell protein and other chemicals from renewable cellulosic materials (Liming Xia *et al.,* 1999). Similar observation was made with *Pycnoporus cinnabarinus,* a white rot fungus (Eggert *et al.,* 1996) and also demonstrated that higher production of laccase in *Phlebia radiata* which was supported by cereal based liquid medium.

In SSF *O. radicata* showed maximum cellulase activity (400 units / ml / min) was observed in wheat bran, followed by rice bran (367 units / ml) and it was in par with paddy straw (357 units / ml / min). Saw dust showed least activity of 207 units/ml/min (Fig. 3). Production

of cellulase in SSF using various substrates, microorganisms and nutrient solutions has been reported (Awafo *et al.,* 2000; Yang *et al.,* 2004). A few works have shown lignocellulosic wastes will stimulate enzyme production by basidiomycetes (Kapich *et al.,* 2004; Elisashvili *et al.,* 2006). It has been reported that SSF is an attractive process to produce cellulase economically due to its lower capital investment and lower operating expenses (Chahal *et al.,* 1991). Previously Liming Xia *et al.,* (1999) studied different dosages of wheat bran for cellulase production by solid state fermentation with 70% water. Moo-Young, (1992) reported the actions of cellulases and xylanases are synergetic over substrate, especially for microorganisms isolated from environments where wood and agro-residues are biodegraded.

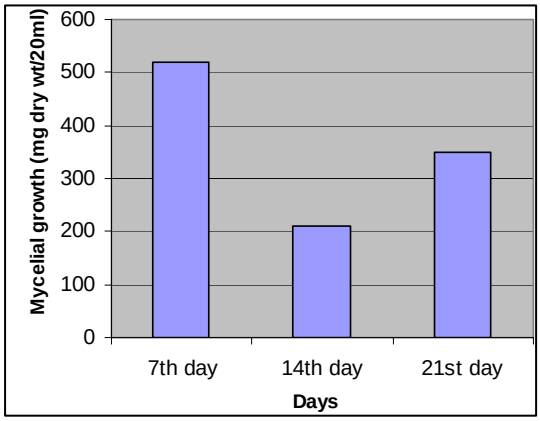

Fig. 1. Effect of incubation period on the mycelial growth of *Oudemansiella radicata* under submerged cultivation

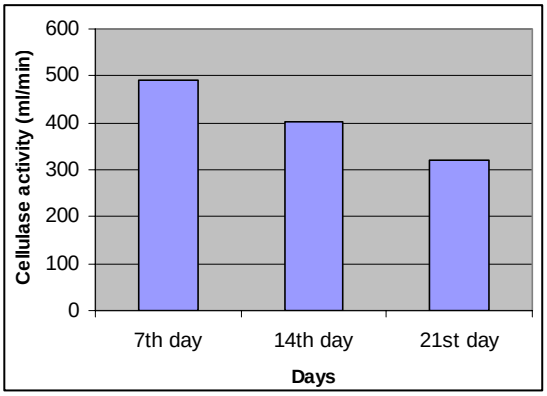

Fig. 2. Estimation of cellulase activity of *O. radicata* under submerged condition

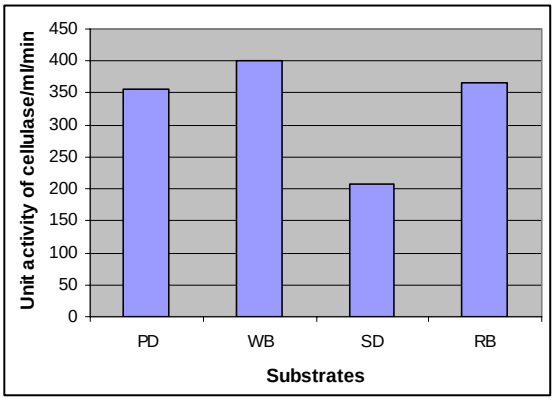

Fig. 3. Estimation of cellulase activity by *O. radicata* under various solid substrates after seven days of incubation. PD: Paddy straw, WB: Wheat bran, SD: Saw dust, RB: Rice bran.

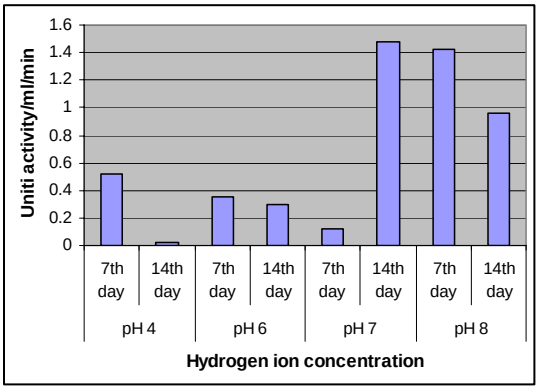

Fig. 4. Effect of hydrogen ion concentration on the laccase activity of *O. radicata* under submerged cultivation

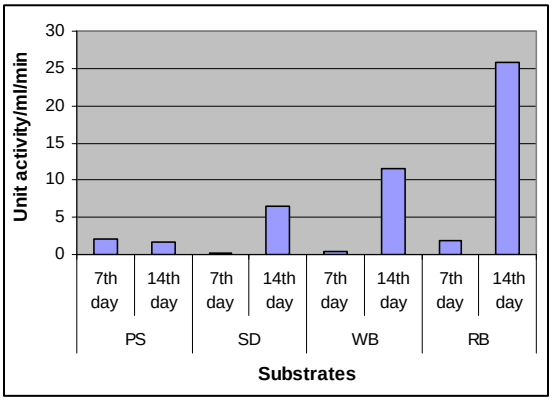

Fig. 5. Estimation of laccase activity of *O. radicata* under various solid substrates. PD: Paddy straw, WB: Wheat bran, SD: Saw dust, RB: Rice bran.

In the case of laccase activity using the same test fungus both under submerged and solid-state conditions, the maximum laccase production (1.476 units / ml / min) on 14th day at pH 7 under SMF. Similar results were also observed on 7th day at pH 8 (Fig. 4). The production of laccase with different substrates in solid state fermentation is shown in Table 5. Balaraju *et al.*, (2007a & b) studied the activity of laccase and cellulase from *P. ostreatus* and compared with that of other basidiomycete *Rigdoporus lignosus*, where it was shown that enzymes are highly destabilized by prolonged exposure to low pH or high temperature. In solid substrate fermentation using the present fungal organism, maximum activity (25.784 units /ml /min) was observed in rice bran on 14th day. The next higher activity (11.473 units /ml /min) was observed in wheat bran on 14th day, and the least activity was recorded with saw dust (Fig. 5). The present study clearly indicates that *O. radicata* can be used extensively in production of industrially important cellulase and laccase enzymes. And also it shows that solid-state fermentation is more effective and economical. However, further studies are essential for optimizing conditions for large scale production.

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