Rice bran as an efficient substrate for laccase production from thermotolerant basidiomycete *Coriolus versicolor* strain RC3

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Suitable substrates for laccase production by the thermotolerant basidiomycete *Coriolus versicolor* strain RC3 were screened using solid and liquid media. In liquid basal medium, 1%(w/v) rice bran as a carbon source was found to be the most efficient substrate for laccase production compared to 1%(w/v) glucose, wheat bran and rice straw meal. After 15 days cultivation at 37°C in shake flask culture, the extracellular laccase activity was found to be 0.22U/ml with rice bran, while 0.09, 0.01 and 0.01U/ml were obtained from wheat bran, glucose and rice straw meal, respectively. The optimum concentration of rice bran was 1%(w/v). Comparison of laccase production on three different selected solid substrates including rubber wood meal (*Heavea* sp.), Hang nok yoong wood meal (*Delonix regia*) and rice bran, were carried out using 5g of solid substrate supplemented with 15ml of distilled water and cultivated at 37°C in the dark for 30 days. Laccase production from *C. versicolor* strain RC3 was 1.98, 0.06 and 0.07U/g substrate from rice bran, rubber wood meal and Hang nok yoong wood meal, respectively. The highest laccase productivity with rice bran in liquid medium was 22U/g substrate at 15 days cultivation. This was 11 times higher than the maximum activity obtained at 30 days on solid substrate cultivation.

Key words: enzyme production, solid state fermentation, *Coriolus versicolor*

Introduction

Laccase (E.C. 1.10.3.2, *p*-benzenedial: oxygen oxidoreductases) is an oxidoreductase able to catalyse the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water

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(Thurston, 1994). Laccases are found in plants, insects and bacteria, but the most important sources of these enzymes are basidiomycetes (Abdel-Raheem and Shearer, 2002; Risna and Suhirman, 2002; Urairuj *et al*., 2003). Fungal laccases are considered to play a role in lignin degradation and/or the removal of potentially toxic phenols arising during morphogenesis, sporulation, or phytopathogenesis and fungal virulence (Gianfreda *et al*., 1999). The role of laccases in lignin and phenolic compound degradation has been evaluated in a large number of biotechnological applications such as dye degradation (Wong and Yu, 1999) and bioremediation of some toxic chemical wastes (Glen and Gold, 1983; Swammy and Ramsay, 1999; Mayer and Staples, 2002). Laccases are also likely to be applied in the pulp and paper industries (Pratima, 1999), wastewater and soil treatments (Nelson and Elisa, 2000) and also biosensor developments (Nelson *et al*., 2002; Kulys and Vidziunaite, 2002). The potential use of laccases in biotechnology has stimulated the need to discover suitable enzymes in large quantities. Laccase production may be affected by fermentation factors such as, medium composition, pH, temperature and aeration. There have been reports describing increased production of extracellular laccases in many species of white rot fungi when grown on natural substrates, such as cotton stalk (Ardon *et al*., 1996), molasses waste water (Kahraman and Gurdal, 2002), wheat bran (Souza *et al*., 2002) and barley bran (Couto *et al*., 2002). Utilization of industrial and agricultural wastes for laccase production is an effective way to reduce production costs and also simultaneously utilise these substrates efficiently (Risna and Suhirman, 2002). This paper describes the selection of suitable substrate for laccase production by *Coriolus versicolor* strain RC3 in solid liquid media.

Materials and methods

Fungal strain

Coriolus versicolor strain RC3 is a thermotolerant white rot fungus isolated from Chiang Mai province, Thailand (Khanongnuch *et al*., 2004). It was cultivated at 37°C on potato dextrose agar (PDA) and stored at 4°C. The culture has been deposited at Laboratory of Applied Microbiology, Biology Department, Faculty of Science, Chiang Mai University, Thailand.

Laccase production on solid substrate

Mycelial plugs from 3-day-old cultures of *C. versicolor* RC3 on PDA were transferred to 250mL Erlenmeyer flasks containing 5g solid substrate and

15mL distilled water. The solid substrates were rubber wood meal (*Heavea* sp.), Hang nok yoong wood meal (*Delonix regia*) and rice bran. Cultures were incubated at 37°C in the dark for 36 days. Enzymes were extracted by adding 50mL of distilled water to the culture and mixing at 4°C for 1 hour. The mixtures were filtered through cotton cloth and the filtrates used as enzyme solutions.

Laccase production in liquid culture

Coriolus versicolor strain RC3 laccase production in liquid culture was investigated using rice bran, wheat bran, glucose and rice straw meal as the sole carbon sources. Composition of liquid medium consisted of 5g carbon source, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.2g NH_4NO_3, 0.1g yeast extract, 0.01g $CaCl_2$, 1mg $CuSO_4.5H_2O$, 1 mg $FeSO_4.7H_2O$ and 1mg $MnSO_4$ per liter of water. Five mycelial plugs were inoculated into 250ml Erlenmeyer flasks containing 50mL of liquid medium with each carbon source and cultured at 37°C on a rotary shaker (150 rpm) for 15 days.

Enzyme assays

Laccase activity was determined by oxidation of 2,6-dimethoxyphenol (DMP) at room temperature. The reaction mixture contained 0.05mL of 4mM DMP, 0.5mL of 20mM acetate buffer pH 5.0, 0.35mL distilled water and 0.1mL enzyme solution. DMP oxidation was monitored by determination of an increasing in absorbance at 470nm (ε_{470}; 49.6 mM^{-1} cm-1). One unit of laccase activity was defined as 1µmole of DMP oxidized product formed per minute. Laccase production on solid substrate was expressed as unit per gram of substrate. Xylanase, β-mannanase and cellulase production were determined by analysis of reducing sugar released during hydrolysis of oat spelt xylan, locust bean gum and carboxymethylcellulose (CMC), respectively. The reducing sugar formed was determined by dinitrosalicylic method (Miller, 1959). One unit of enzyme activity was defined as amount of enzyme that released 1 µmole of reducing sugar per minute.

Results and discussion

Laccase production on solid media

The highest laccase activity after 36 days of cultivation was obtained on rice bran (1.98U/g substrate), those of rubber wood meal and Hang nok yoong

wood meal were 0.06 and 0.07U/g substrate, respectively. In addition to laccase production, *C. versicolor* strain RC3 also produced the high levels of xylanase, β-mannanase and cellulase, especially on rice bran. The enzyme production of β-mannanase, cellulase and xylanase were found up to 236.3, 167.0 and 173.4U/g substrate, respectively, after 7 days cultivation. It can be suggested that *C. versicolor* strain RC3 utilize xylan, cellulose and mannan in rice bran as carbon sources during the initial growth phase. Rice bran contains total carbohydrate 82%(w/w) approximately and the main composition (31%) was hemicellulose (Claye *et al*., 1996). The main component of rice bran hemicellulose was expected to be arabinoxylan as most sugars found are xylose and arabinose (Mod *et al*., 1978) and rice bran was also used as a sole carbon source for xylanase production by *Streptomycete actuosus* A-151 (Wang *et al*., 2003).

When the levels of those carbon sources decreases, laccase synthesis was induced by phenolic compounds containing in rice bran, leading to increasing of laccase production. This induction mechanism may help fungus to degrade lignin or aromatic compounds in rice bran to supply further nutrients especially carbon and nitrogen. The similar pattern in production of laccase and hemicellulytic enzyme was also found with several white- and brown rot fungi cultivated on *Eucalyptus grandis* wood chips (Machuca and Ferraz, 2001). *Trametes versicolor* produced the highest xylanase and cellulase from solid state culture at 15 days, while those of laccase and peroxidase were at 60 days (Machuca and Ferraz, 2001).

The fungal response in enzyme production support the previous work as the deprivation of nitrogen and carbon sources is considered as a major factor in triggering ligninolytic system of white rot fungi. (Leatham and Kirk, 1983; Mester *et al*., 1996). Laccases were found in the initial state of cultivation on rubber and Hang nok yoong wood meal, while other hemicellulolytic enzymes were also produced at high levels and were markedly lower than those on rice bran. The production of laccases in the presence of hemicellulolytic enzymes such as xylanase or cellulase may reflect the low level of available carbon sources such as mannan and xylan as described previously.

A comparison of laccase production on the three substrates is given in Fig. 2. Cultivation on rice bran clearly exhibited the highest laccase production and, if the time course of enzyme production in Fig. 1(A) is considered, it is evident that laccase activity still increased after 36 days cultivation and this have to be further studied.

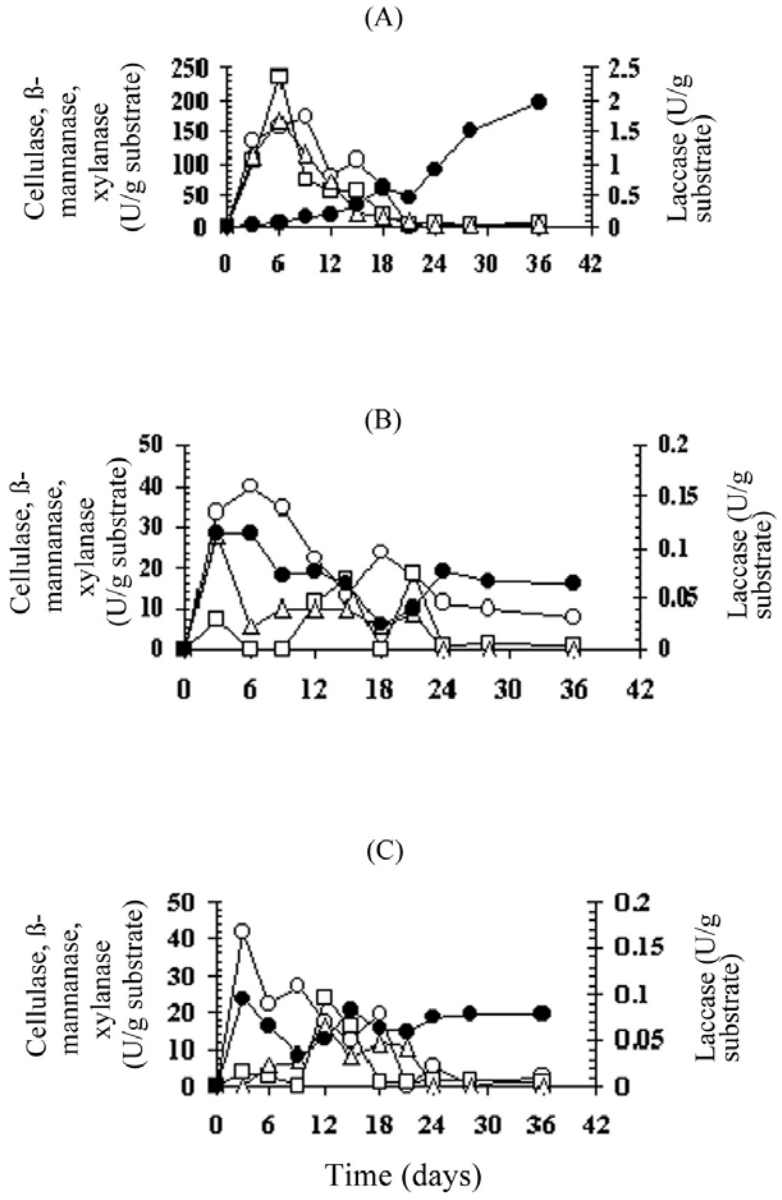

Fig. 1. Enzyme production on solid substrate cultivation of *C. versicolor* RC3 on rice bran (A), Hang nok yoong wood meal (B) and rubber wood meal (C): • = laccase, o = xylanase, □ = β-mannanase and Δ = cellulase.

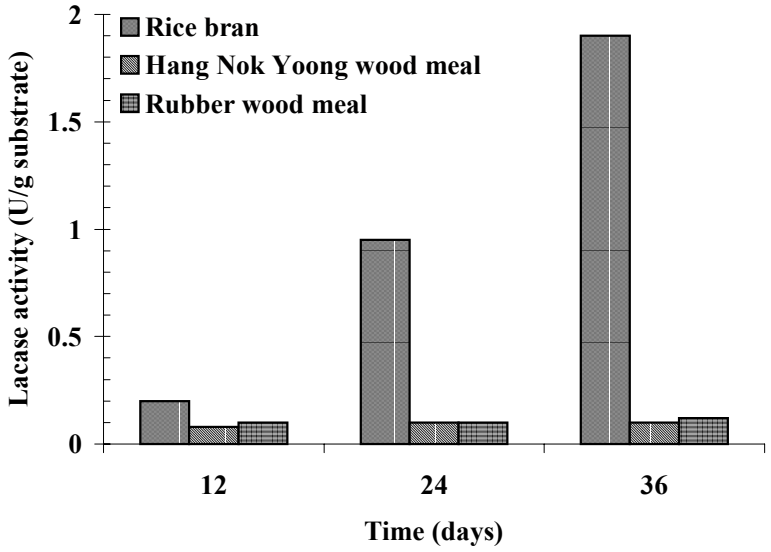

Fig. 2. Comparison of laccase production by *C. versicolor* RC3 on different solid substrates.

Laccase production in liquid media

Laccase activity obtained from rice bran liquid medium after 15 days of cultivation was 0.22U/mL, while activity levels obtained from wheat bran, glucose and rice straw meal liquid medium were 0.09, 0.01 and 0.01U/mL, respectively (Fig. 3). *Coriolus versicolor* strain RC3 produced laccase on rice bran liquid medium with levels 2.4 times higher than on wheat bran liquid medium and 22 times higher than on glucose and rice straw meal liquid medium. These results indicate that rice bran exhibits a higher inductive capability in liquid medium than other carbon sources as also occurred on the solid substrates. However, addition of rice straw in mineral salt broth (MSB) increased laccase production by white rot fungus *Daedalea flavida* MTCC145 from 0.06 U/mL to 9.04 U/mL (Arora and Gill, 2001).

Effects of rice bran concentration in liquid medium on laccase production is shown in Fig. 4. After 7 days, laccase production was 0.19U/mL from 2%(w/v) rice bran, while 0.08 and 0.17U/mL were obtained when using 0.5 and 1%(w/v) rice bran, respectively. Productivity of laccase using 2%(w/v) and 1%(w/v) rice bran was 9.5 and 17U/g rice bran, respectively. It was concluded that the optimum concentration of rice bran was 1%(w/v). Rice bran at 2%(w/v) might be caused the excess level of carbon and nitrogen, which

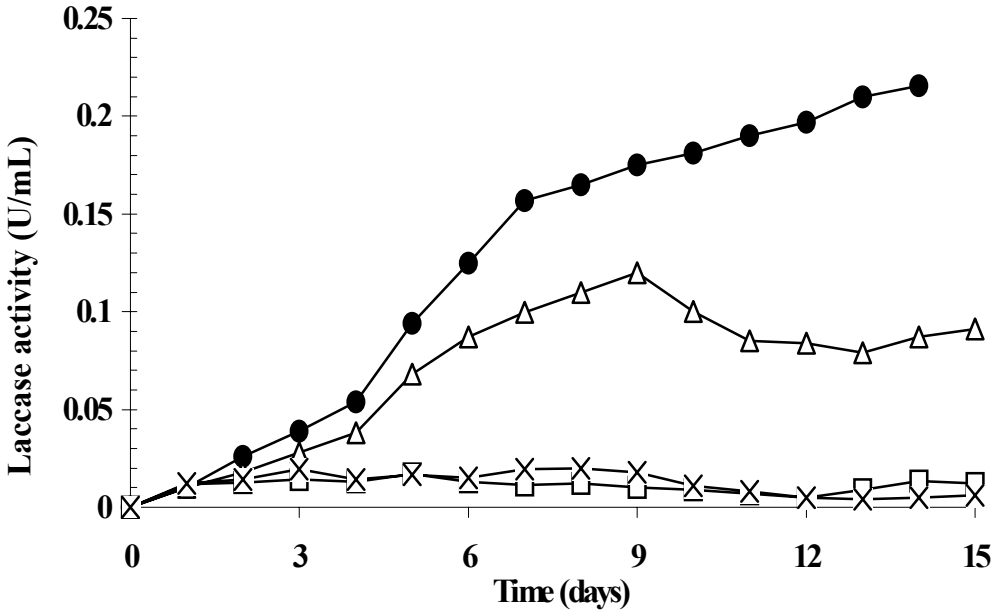

Fig. 3. Laccase production from *C. versicolor* RC3 by liquid cultivation in different carbon sources for 15 days: • = rice bran, □ = glucose, Δ = wheat bran and x = rice straw meal

directly affected on ligninolytic enzyme production (Leatham and Kirk, 1983; Mester *et al*., 1996).

Laccase production in both solid substrate and liquid medium, rice bran was higher than other substrates. The inductive capability of rice bran to laccase production may be related with its phenolic compounds such as ferulic acid, vanillic acid which were reported to be an inducer for laccase production by white rot fungi (Bollag and Leonowize, 1984; Munoz *et al*., 1997). Ferulic acid is found approximately 0.1%(w/v) and easily prepared in large quantity from rice bran (Taniguchi *et al*., 1999). Eight strains of white rot fungi *Pycnoporus cinnabarinus* produced laccase in higher level with the average value of 0.33-9.50U/mL in basal medium supplemented with 0.5mM ferulic acid compare with 0.06-4.03U/mL in control group (Herpoel *et al*., 2000).

There are many reports concerning using rice bran as substrate for the production of many biological compounds such as alpha amylase (Ikramul *et al*., 2003) and antibiotics (Yang, 1996). Many agricutural wastes are also investigated to use as substrates for laccase production by white rot fungi

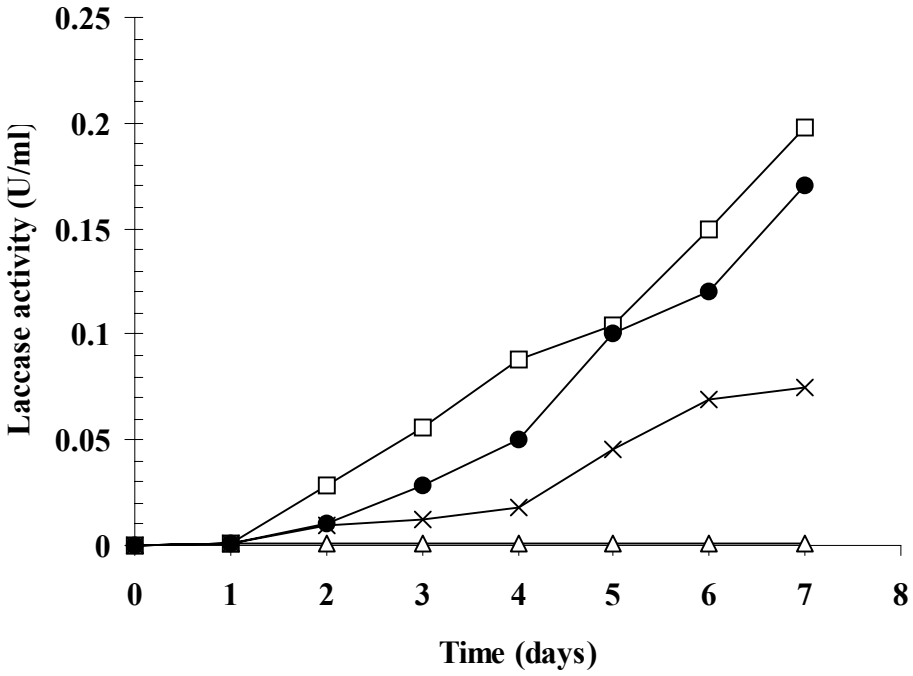

Fig. 4. Effect of different concentration of rice bran in liquid medium to laccase production: Δ = 0% (control), □ = 2% (w/v), • = 1%(w/v), x; 0.5%(w/v).

including grape seeds, grape stalks, barley bran (Lorenzo *et al*., 2002), cotton stalk, molasses waste water (Kahraman and Gurdal, 2002) and wheat bran (Souza *et al*., 2002). This work is a first report concerning rice bran utilizing for laccase production. However, laccase production in both solid and liquid medium cultivation did not reach the maximum level of laccase activity and the prolonged cultivation is needed to observe the production both on solid and liquid culture. Purification and characterization of laccase from *C. versicolor* strain RC3 are in progress.

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