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Extracellular Laccase Production by Co-culture between *Galactomyces reesii* IFO 10823 and Filamentous Fungal Strains Isolated from Fungus Comb Using Natural Inducer

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***Abstract*—**Extracellular laccases are copper-containing microbial enzymes with many industrial biotechnological applications. This study evaluated the ability of nutrients in coconut coir to enhance the yield of extracellular laccase of *Galactomyces reesii* IFO 10823 and develop a co-culture between this yeast and other filamentous fungi isolated from the fungus comb of *Macrotermes* sp. The co-culture between *G. reesii* IFO 10823 and *M. indicus* FJ-M-5 (G3) gave the highest activity at 580.20 U/mL. When grown in fermentation media prepared from coconut coir and distilled water at 70% of initial moisture without supplement addition, G3 produced extracellular laccase of 113.99 U/mL.

***Keywords*—**Extracellular laccase, production, yeast, natural inducer.

I. Introduction

TERMITE-fungal mutualistic relationships are widespread and include commensalism, which often involves the production of selective hydrolytic enzymes by the fungi to support the digestive systems of the termites. Termites and fungi share a long history of association in natural habitats where they exist in similar environmental conditions [9].

Termite-associated fungi are mainly ligninolytic enzyme producing microorganisms in the tropical rain forest regions including Thailand [26]. The success of the termites is undoubtedly attributed to their engagement in a mutualistic symbiosis with *Termitomyces* fungi which aid in the decomposition of plant biomass [12], [14]. The fungus comb is housed on a special substrate in the termite's nest, which is maintained by the host through the continuous supplementation of digested plant biomass that has passed through the digestive tract along with asexual fungus spores [15], [13], [25].

Ligninolytic enzymes such as extracellular laccase (Lac) and manganese peroxidase (MnP) are well known members of the oxidoreductase group. They play a significant role in biochemical oxygen demand (BOD) removal and color reduction in wastewater treatment including paper mill effluent and palm oil mill effluent [5]-[7], [21]. Moreover, lignin degrading enzymes are also applied on microbial fuel cell (MFC) cathodes to enhance electricity generation [23].

Laccase (EC 1.10.3.2) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic center, usually called multicopper oxidases. Laccases can directly catalyze phenolic and aromatic compounds like lignin in the biomass. Laccases have been successfully applied to the detergent industry, textile industry and also as microbial fuel cells [18].

Modern microbial laccase producing processes use chemical inducers such as copper sulfate ($CuSO_4$) for enhancing the enzyme yield [17]; however, high levels of copper sulfate can be corrosive to the skin and eyes. Copper sulfate solution is also a fungicide used to control bacterial and fungal diseases of fruit, vegetables, nuts and field crops [22]. Previous studies indicated that some plant material can be used as a natural inducer for laccase production in cultural media without the need for chemical addition [4].

Coconut palm (*Cocos nucifera* L.) is an important economic plant in Southern Thailand. Thailand is the world's sixth largest producer of coconuts with a coconut palm plantation area of around 206,000 hectares. Thailand produces coconut oil, coconut milk and activated carbon of around 1,960, 179,297, and 8,829 million tons per year, respectively Biomass waste as coconut coir results from the processing units of the coconut industry at 37,704 million tons per year [3].

Fig. 1 Coconut coir from coconut palm orchard in Thailand

The genera *Macrotermes*, *Odontotermes* and *Microtermes* consist of subterranean termites that are widespread in

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Thailand. These termite groups degrade plant material using the enzymatic system of their fungal symbiosis [26]. This study examined the extracellular laccase production by co-culture between the ligninolytic yeast *Galactomyces reesii* IFO 10823 [11] and filamentous fungal strains *Aspergillus* sp. PSFNRH, *Penicillium citrinum* MF410, *Mucor indicus* FJ-M-5 and *M. amphibiorum* RSC1 isolated from fungus comb in the genus *Macrotermes*, using coconut coir as a natural inducer.

II. Experimental Details

A. Substrate Preparation

Coconut coir (Fig. 1) was collected from coconut palm orchards in Phatthalung Province, Southern Thailand. The substrate was sterilized at 121°C for 15 mins and dried at 80°C in an oven for moisture removal until constant weight.

B. Microorganism and Culture Condition

G. reesii IFO 10823, *Aspergillus* sp. PSFNRH, *P. citrinum* MF410, *M. indicus* FJ-M-5, and *M. amphibiorum* RSC1 isolated from the fungus comb (Fig. 2) of *Macrotermes* sp. were obtained from the laboratory at Thaksin University, Phatthalung Campus, Thailand.

Cultures were inoculated in malt extract broth (malt extract 20 g/L, sucrose 20 g/L, peptone 6 g/L) supplemented with 25 µg/mL of chloramphenicol to avoid bacterial contamination. They were grown under aerobic conditions at room temperature (25±1°C) with shaking at 150 rpm for 7 days. The cultures were then stored at 4°C pending further study.

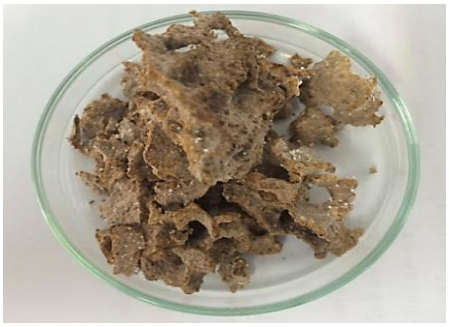

Fig. 2 Fungus comb of *Macrotermes* sp.

C. Consortia Preparation

The consortia were prepared in malt extract broth by co-culture between *G. reesii* and another fungal strain isolated from the fungus comb. The compositions of the consortia are shown in Table I.

D. Extracellular Laccase Production

The consortia and pure yeast culture were inoculated in coconut coir medium (dried coconut 10 g/L, malt extract 1 g/L, sucrose 1 g/L and peptone 0.3 g/L). They were grown under aerobic conditions at room temperature (25±1°C) with shaking at 150 rpm for 7 days.

TABLE I
THE COMPOSITION OF CONSORTIA

Consortia code	Composition
G1	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH
G2	*G. reesii* IFO 10823 *P. citrinum* MF410
G3	*G. reesii* IFO 10823 *M. indicus* FJ-M-5
G4	*G. reesii* IFO 10823 *M. amphibiorum* RSC1
G5	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH *P. citrinum* MF410
G6	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH *M. indicus* FJ-M-5
G7	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH *M. amphibiorum* RSC1
G8	*G. reesii* IFO 10823 *P. citrinum* MF410 *M. indicus* FJ-M-5
G9	*G. reesii* IFO 10823 *P. citrinum* MF410 *M. amphibiorum* RSC1
G10	*G. reesii* IFO 10823 *M. indicus* FJ-M-5 *M. amphibiorum* RSC1
G11	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH *P. citrinum* MF410 *M. indicus* FJ-M-5
G12	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH *P. citrinum* MF410 *M. amphibiorum* RSC1
G13	*G. reesii* IFO 10823 *P. citrinum* MF410 *M. indicus* FJ-M-5 *M. amphibiorum* RSC1
G14	*G. reesii* IFO 10823 *Aspergillus* sp. PSFNRH *P. citrinum* MF410 *M. indicus* FJ-M-5 *M. amphibiorum* RSC1

E. Enzyme Assay

For crude enzyme preparation, all culture media were passed through filter paper to remove the large pieces of biomass. The filtered culture media were then centrifuged at 9,000 rpm for 10 mins. The supernatants were collected in 1.5 mL sterile microcentrifuge tubes and stored at 4±1 °C until required for use.

Extracellular laccase (EC 1.10.3.2) activity was determined at 30±1 °C using 1 mM ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M sodium acetate buffer (pH 3.6). The absorbance increase of the assay mixture was monitored at 436 nm (ε = 29,300 $M^{-1}cm^{-1}$) using a spectrophotometer (Shimadzu UV-3600, Japan).

The enzyme activity was defined as the amount of enzyme needed to produce 1 µmol of product per min.

F. Fermentation

Fermentation was performed in 250 mL Erlenmeyer flasks with 6 mL of distilled water added to 10 g of dried coconut coir. The highest enzyme producing fungus was added to the media as 1 mL of consortia (with the initial moisture content

about 70%) and incubated at room temperature (25±1 °C) in static condition for 7 days. The culture was then measured for laccase activity by ABTS assay.

III. Results and Discussion

A. Extracellular Laccase Activity of Yeast Strain

Coconut coir is the fibrous material that separates from the mesocarp of the coconut fruit. Analysis of the chemical composition of coconut coir indicated high lignin content at 42.10%, cellulose 32.69% and hemicellulose 22.56% [20].

Previous studies found that a pure culture of *G. reesii* IFO 10823 produced extracellular laccase [11]. However, this constitutive enzyme production was possibly affected by the action of lignin and other nutrients in coconut coir.

G. reesii IFO 10823 showed a significant enzyme increase to 180.75 U/mL, some 2.13-fold higher than our previous findings. Thus, coconut coir is a suitable natural inducer for laccase production by *G. reesii* IFO 10823.

B. Enzyme Production by Co-Culture

Results indicated that the G3 co-culture between *G. reesii* IFO 10823 and *M. indicus* FJ-M-5 was the most suitable for enzyme production using coconut coir as the substrate and natural inducer. G3 showed the highest laccase activity at 580 U/mL (Table II and Fig. 3), which was 3.21-fold higher than the pure culture. Moreover, G3 also gave higher enzyme activity than white rot fungi (Table III).

TABLE II
The Extracellular Laccase Activity Of Fungal Groups

Consortia code	Extracellular laccase activity (U/mL)
G1	335.15
G2	246.19
G3	580.20
G4	184.76
G5	206.83
G6	354.95
G7	346.79
G8	483.73
G9	190.44
G10	266.44
G11	378.91
G12	324.91
G13	442.78
G14	173.83

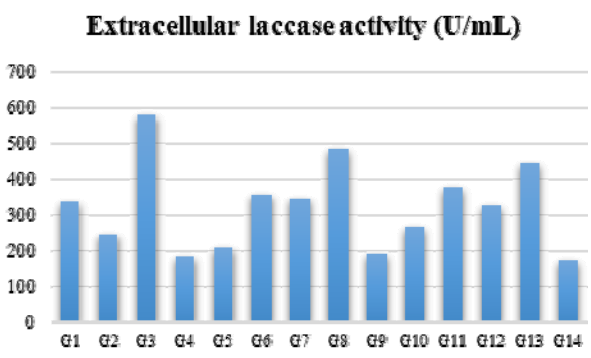

Fig. 3 Comparison of extracellular laccase yield of co-culture

TABLE III
The Laccase Activity of White Rot Fungi

Fungi	Laccase (U/mL)	Reference
Ganoderma applanatum strain F *Peniophora* sp. BAFC 633 *Pycnoporus sanguineus* BAFC 2126 *Coriolus versicolor*	1.87 4.14 3.14 0.68	Fonseca et al. [17]
Funalia trogii	29.23	Birhanli et al. [8]
Xylaria sp.	20.56	Castano et al. [10]

B. Solid State Fermentation

The ability of microbial enzymes to attack plant cell walls has been widely exploited in biotechnological applications. Filamentous fungi are involved in the efficient decomposition of plant biomass and degrade complex plant polysaccharide structures as the carbon source by producing hydrolytic enzymes. Owing to the development of industrial fermentation process, the production of microbial enzymes is important proportion of the biotechnological industrial total yields [24]. Among the enzyme producing processes, solid state fermentation (SSF) is the most favored because of several advantages. The SSF process may be defined as the fermentation process for cultivation of microorganisms on a non-soluble substrate, which is used as a source of nutrients as well as a physical support under controlled conditions without a free-flowing aqueous phase [2].

Coconut coir was used as a novel solid support for microbial anchorage, giving higher levels of laccase production in solid state fermentation. Coconut coir was reported as a promising substrate for the production of cellulase by *Aspergillus niger* NCIM 1005 [19]. Coconut coir was also used to produce cellulose and β-glucosidase by a co-culture between *A. ellipticus* and *A. fumigatus* [1]. Coconut coir is enriched with carbon and nitrogen sources, and also contains high amounts of phosphorus (P), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^-), sulfate (SO_4^{2+}) and sodium (Na^+) [16] that can support fungal growth; therefore, the potential of *Galactomyces* strains was evaluated for the production of plant cell wall degrading enzymes using coconut coir as the growth medium. To our knowledge, this is the first report describing laccase preparation by co-culture between laccase producing yeast *G. reesii* IFO 10823 and another filamentous fungi under solid state fermentation of coconut coir without nutrient or chemical addition.

Various strategies have been applied to increase laccase production under solid state fermentation, including supplementing the solid substrate with nutritional components to act as enzyme inducers. Enhanced production of laccase was observed by *Ganoderma applanatum* strain F, *Peniophora* sp. BAFC 633, *Pycnoporus sanguineus* BAFC 2126, and *Coriolus versicolor* using a production medium mixed with fermentation media and copper sulfate [17]. The fermentation results presented in this paper indicate that the co-culture G3 can grow on coconut coir at 70% of initial moisture without any supplementation by other nutrients and produce extracellular laccase of 113.99 U/mL.

IV. Conclusion

Coconut coir was used for extracellular laccase production by *G. reesii* IFO 10823 to determine the optimal mixture of fungal types to enhance enzyme yield. Results regarding coconut coir usage in solid state fermentation without chemical addition indicate the possibility of low cost development on an environmentally friendly industrial scale.

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