ISOLATION AND SCREENING OF ENDOPHYTIC FUNGUS FROM COCOA POD HUSK AS A SOURCE OF POLYPHENOL OXIDASE PRODUCER

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Abstract. Polyphenol oxidases are found in almost all fungal strains and they are considered to be excellent sources for industrial polyphenol oxidase production. Endophytic fungus in cocoa pod husk may be producing polyphenol oxidase with high bioactivity because cocoa pod husk contained polyphenol as inducer for fungus to produce this enzyme. The aim of this study is to get strain endophytic fungus from pod husk of Theobroma cacao fruit as source of polyphenol oxidase producer. Isolation was done by using Potato Dextrose Agar supplemented with 50 mg/l chloramphenicol to suppress bacterial growth, incubating during 5-14 days at room temperature. Screening fungus producing polyphenol oxidase was done by plate test method using tannic acid 0.5 % and gallic acid 0.1 %. Out of 4 culture tested, one culture was found to be polyphenol oxidase-positive and identified as genus of Trichothecium. The enzyme activity was 80 U/ml.

Keywords: Cocoa pod husk, endophytic fungus, polyphenol oxidase

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INTRODUCTION

Endophytic fungi are the fungi which spend the whole or part of their lifecycle colonizing inter- and/or intracellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Mín et al., 2010). Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture, and industry (Strobel and Daisy, 2003). Recent investigations have been intensified by the potentialities of endophytic fungal strains in production of bioactive metabolites like enzymes, i.e: Xylanase (Suto et al, 2002), Glucoamylase (Meliaiwati et al, 2006), Asparaginase (Theantana et al, 2007).

Cocoa (Theobroma cacao L.), familia Sterculaceae, are rich in polyphenol compounds (Othman et al, 2007), polyphenol oxidase (Lee, 1991), and endophytic fungi (Rubini et al, 2005). Cocoa podhusks are a waste product of the cocoa industry, and present a serious disposal problem. Cocoa podhusk also contained polyphenol as a mixture of condensed or polymerized flavonoids (Figuera, 1993; Sartini, 2007). Endophytic fungus in cocoa podhusk may be producing polyphenol oxidase with high bioactivity because cocoa podhusk contained polyphenol may be as inducer for fungus to produce this enzyme.

Polyphenol oxidase enzymes have significant applications in many areas such as food, medicine, and industry. In medicine, polyphenol oxidases are used for prevention of bacterial adhesion, treatment of Parkinson’s disease and control of melanin synthesis. In food processes, polyphenol oxidases have been mainly used for enhancement of the flavor in tea, coffee and cocoa (Astarci, 2003) depend on kind of polyphenol oxidase. Polyphenol oxidases consist of Catecholase (EC 1.10.3.2), laccase (EC 1.10.3.1), cresolase (EC 1.14.18.1 in plants) and tyrosinase (EC 1.14.18.1 in animals).

Discovery of novel polyphenol oxidase with different substrate specificities and improved stabilities is important for industrial applications. Microbes that produce polyphenol oxidase have been screened for either on solid media containing coloured indicator compounds that enable the visual detection of polyphenol oxidase production or with liquid cultivations monitored with enzyme activity measurements. The traditional screening reagents tannic and gallic acid can be use to detect polyphenol oxidase production, the positive of tannic and gallic acids reaction is a dark-brown coloured zone (Kiiskinen, 2004).

Thus in this study, we focus on the isolation of endophytic fungi from cocoa pod husk and screening them as source of polyphenol oxidase producer.

MATERIAL AND METHODS

Material. Potato Dextrose Agar (Oxoid), Gallic acid (Sigma), Tannic acid (Merck), Chloramphenicol, NaOCl 5.3% (Bayclin®), Ethanol 70%, Fruit of Theobroma cacao L., local variety from Soppeng Regency, South Celebes.

Isolation of endophytic fungi. Isolation of endophytic fungi was done by modification of Radu methods (2003). The mature fruit were thoroughly washed in running tap water. Pod husk were carefully cut 2 cm x 4 cm, after which they were surface sterilized by submerging them in 70% ethanol for 3 minutes, 5.3% sodium hypochlorite solution for 5 minutes, and 70% ethanol for 0.5 minutes, and washed again with sterilized water. After drying, the pod husk was cut 1 cm x 2 cm and placed on potato dextrose agar (PDA) supplemented with 50 mg/l chloramphenicol to suppress bacterial growth. All the plates were incubated at room temperature (28 °C) and periodically checked for purity.
Primary screening polyphenol oxidase-producing endophyte (modified Kiiskinen, 2003).

The pure endophytic fungus place onto PDA and incubation during 3 days at room temperature, after that steril tannic acid 0.5% and Gallic acid 0.1% were added onto the fungal colony and incubation 1-2 days in order to detect fungi that produced polyphenol oxidase.

Cultivation in liquid medium (modified Astarci, 2003). Fungal strains showing positive reactions in the plate-test screening were cultivated in PDA for 3 days at 28°C. Then, cultivated in 50 ml erlenmayer flasks containing 10 ml of preculture medium containing 0.4% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄ and 1.5% glucose at 50°C, 155 rpm in a shaker incubator. These pre cultures (10 ml) were used to inoculate 100 ml enzyme production media (1% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄ and 1% glucose, 0.034%, gallic acid, 0.0025% CuSO₄.5H₂O at 50°C, 155 rpm in 250 ml flasks. Incubations were performed in an orbital shaker at 155 rpm for 4-5 days, ambient temperature.

Polyphenol oxidase assay. Polyphenol oxidase activity was measured by continuous spectrophotometric rate determination (sigma Aldrich, 1994). One unit is equal to ΔA₂₆₅ nm of 0.001 per minute at pH 6.5 at 25°C in a 3 ml reaction mix containing catechol and L-ascorbic acid.

Calculation:

\[ \text{Unit/ml enzyme} = \frac{\Delta A_{265} \text{ nm Test} - \Delta A_{265} \text{ nm Blank}}{0.001 \times 0.1 \times \text{df}} \]

0.001 = The change in A₂₆₅nm per unit of polyphenol oxidase in a 3.00 ml reaction mixture at pH 6.5 at 25°C.

df = dilution factor

0.1 = volume (in milliliters) of enzyme used

RESULTS

1. Isolation and Identification of Endophytic Fungi

From 3 times isolating endophytic fungi from cocoa pod husk, we found five strain, i.e.: FE-KBK 1, FE-KBK 2, FE-KBK 3, FE-KBK 4, FE-KBK 5 (Figure 1).

2. Primary screening polyphenol oxidase-producing endophyte.

In order to find polyphenol oxidase producing endophytic fungi, a simple screening method was followed using Potato Dextrose Agar media containing indicator compound, Tannic acid 0.5% and Gallic acid 0.1%. From five isolates, FE-KBK 1 showed polyphenol oxidase positive because after Tannic acid 0.5% and Gallic acid 0.1% were added onto the fungal colony and incubation 1-2 days brown zone around colony was appeared (Figure 2).

3. Production of polyphenol oxidase in liquid media

In this study, the fungus was grown in 250 mL Erlenmayer flasks containing 100 ml production medium (1% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄, Gallic acid 0.034%, 0.0025% CuSO₄) at 28°C, 155 rpm for 5 days. Gallic acid and CuSO₄ were used as inducer for producing extracellular polyphenol oxidase. After three days incubation fermented media showed brown and 4th days incubation the media colour was dark brown, that it showed that in fermented media contained extracellular polyphenol oxidase. The result of average enzyme activity was 80 U/ml.

DISCUSSION

Isolating endophytic fungi from cocoa pod husk gave five strain fungi, four strain were identified by macroscopic and microscopic, recognized as genus of Trichotheccium (FE-KBK 1), Paecilomyces (FE-KBK 2), FE-KBK 3 (not yet
identified), FE-KBK 4 (Geomyces), FE-KBK 5 (Helicosporium). Endophytic fungi were isolated from healthy pods of cacao (Theobroma cacao) trees in natural forest ecosystems and agroecosystems in Latin America and West Africa, they found Pleosporales sp, Xylariaceae sp, Pycnoporus sp, Phlebioid sp (Crozier et al. 2006).

FE-KBK 3 was not identified by macroscopic and microscopic because it was lack of sporulation on PDA medium. Crozier (2006) also found that 65% endophytic fungi isolated could not be identified on the basis of traditional taxonomic techniques. Other endophyte assemblage studies carried out have revealed unidentifiable fungi, as a result of lack of sporulation on artificial culture media (Promputtha et al., 2005; Wang et al., 2005)

In order to find polyphenol oxidase producing fungi from the endophytes isolates, a simple screening method was followed using PDA media containing tannic acid and gallic acid. Of five isolates, FE-KBK 1 was polyphenol oxidase positive, because the brown colour appeared around the isolate. The oxidation of phenolic compound (tannic acid or gallic acid by polyphenol oxidase leading to production quinone compounds. These quinones self-polymerize or react with other substance to form high molecular weight black/brown pigment, melanins (Marusek et al., 2006).

Fungal polyphenol oxidase, such as Laccases, are extracellular enzymes secreted into the medium by filamentous fungi. They are generally produced during the secondary metabolism of different fungi. Several factors including type of cultivation (submerged or solid state), carbon limitation, nitrogen source, and concentration of microelements can influence their production. Low concentration of copper was also shown to exhibit inducible effect on their activity.

For production of polyphenol oxidase, all of the parameters such as temperature, pH, medium composition must be optimized. Also production of the enzyme is greatly influenced with the addition of some inducers. In this research we used composition production media, i.e.: 1 %Yeast extract, 1 % glucose, 0.1 % KH2PO4, 0.05 % MgSO4.7H2O, 0.0025 % CuSO4, Gallic acid 0.034 % as inducer at 28°C, 155 rpm for 5 days. The enzyme activity from crude enzyme (supernatant of liquid fermentation) was obtained 80 U/ml.

It showed that endophytic fungus isolated from cocoa podhusk, FE-KBK1, could produce polyphenol oxidase.

**FUTURE WORK**

Possible future studies include optimization of fermentation conditions to increase PPO production and purification of the enzyme.

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Figure 2. Oxidative polymerization of tannic acid (A) and gallic acid (B) to form brown zones in a round of fungal colony in Potato Dextrose Agar plate.