Journal of Bioscience and Bioengineering
VOL. 107 No. 2, 108 – 112, 2009

www.elsevier.com/locate/jbiosc

Production and characterization of alcohol oxidase from *Penicillium purpurescens* AIU 063

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Received 5 August 2008; accepted 21 October 2008

A filamentous fungus belonging to *Penicillium purpurescens* was isolated as a strain grown well in methanol medium. The strain produced an alcohol oxidase (AOD), which oxidizes short-chain primary alcohols and ethylene glycol, by incubation with a medium containing not only methanol, but also ethanol, ethylene glycol, glycerol or glucose. This AOD belonged to the same group as AOD from methylotrophic yeast, but some properties were different from AODs from methylotrophic yeast and other fungi. Thus, this enzyme consisted of four identical subunits of 66 kDa, while AODs from methylotrophic yeasts were of eight subunits. The enzyme activity was enhanced to two-fold by incubation at pH 6.0 and 40 °C for 60 min, whereas the K_m values for ethanol and ethylene glycol did not change.

[**Key words:** Alcohol oxidase; *Penicillium purpurescens*; Ethylene glycol; Glycolaldehyde; Primary alcohol; Methylotrophic yeast]

Alcohol oxidase (AOD) (EC1.1.3.13) is well known as the key enzyme of the methanol metabolism in methylotrophic yeasts such as *Candida* and *Pichia*. The characteristics of AODs and the regulation of the gene encoding AODs have been studied in detail in methylotrophic yeasts (1–9). However, the function and characteristics of AOD in fungi have not been widely studied. In the production of AODs by filamentous fungi, Ko et al. (10) have reported that *Thermoascus aurantiacus* NBRC 31963 inductively produced two AODs by incubating with the pectin medium. This strain could not grow in the methanol medium, but the metabolic products from pectin contributed to the AOD production. We also isolated *Aspergillus ochraceus* AIU 031 as a producer of AOD by incubating in a medium containing cholesterol as a sole carbon source (11). In this strain, metabolic products from cholesterol contributed to the AOD production, but the product amount of AOD was enhanced by incubating in the methanol medium. Recently, Kondo et al. (12) reported that *Paecilomyces variotii* IRI 017, isolated as a formaldehyde-resistant fungus, produced AOD, when it was incubated in a methanol medium. These studies indicate that some fungi can inductively produce AOD under the specific conditions of alcohol or alcohol-derivative production. Those investigations also revealed that intracellular AODs from fungal strains were constructed of 4 or 6 identical subunits, while AODs from methylotrophic yeasts were constructed by 8 identical subunits. Thus, the structure of fungal AODs is different from that of yeast AODs. To investigate in detail the function and characteristics of AOD in fungal strains, we newly isolated a filamentous fungus grown well in a medium containing methanol as a carbon source. The present paper describes the production of AOD by a newly isolated filamentous fungus belonging to *Penicillium purpurescens*. The remarkable properties of AOD produced by this strain are also reported using purified enzyme.

MATERIALS AND METHODS

Chemicals Methanol, ethylene glycol and glycolaldehyde were purchased from Wako Pure Chemical Industries (Osaka). Horseradish peroxidase (EC 1.11.1.7) was the gift of Amano Enzyme Inc. (Nagoya). All other chemicals used were of analytical grade and commercially available.

Isolation of fungi In the first step, enrichment culture was carried out three times using the cholesterol medium under the same condition as in our previous report (11), and fungal strains were isolated on agar plates of the cholesterol medium. Each isolated strain was then incubated at 30 °C for 3 d in a 500-ml flask containing 100 ml of a methanol medium consisting of 0.2% methanol, 0.1% NaH_2PO_4, 0.2% K_2HPO_4, 0.2% NH_4NO_3, 0.02% $MgSO_4{\cdot}7H_2O$ and 0.05% yeast extract, pH 6.5. The oxidase activities on methanol, ethanol and isopropanol were assayed using the cell-free extract, which was prepared by disrupting the cells below 5 °C for 6 min with a Multi-beads shocker (Yasui Kikai, Osaka). A strain exhibiting high oxidase activity on methanol and ethanol was selected.

Identification of isolated strain The isolated strain was grown on Czapek's agar with yeast extract (CYA), malt extract agar (MEA) and 25% glycerol agar (G25N). Preparation of these media is detailed by Pitt (13). Morphological and physiological observations were made on cultures that had been grown 7 d at 5, 25 or 37 °C in the dark (13).

Enzyme assay AOD activity was assayed by measuring the rate of hydrogen peroxide formation at 30 °C as follows. The standard reaction mixture contained 173 μmol of ethanol, 0.6 μmol of 4-aminoantipyrine, 1.94 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate, 6.7 units of peroxidase, 0.1 mmol of potassium phosphate, pH 7.0, and an appropriate amount of enzyme, in a final volume of 1.0 ml. The formation of hydrogen peroxide was spectrophotometrically followed at 30 °C for 5 min by measuring the absorbance at 555 nm. The enzyme activity was calculated using the value obtained by subtracting the value of 555 nm

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doi:10.1016/j.jbiosc.2008.10.015

TABLE 1. Effects of carbon source on AOD production

Carbon source	Cell growth (g/dl of broth)	AOD activity (m unit/dl of broth)	Protein extracted (mg/dl of broth)	Specific activity (m unit/mg of protein)
Methanol	0.17	295	16.8	17.5
Ethanol	0.23	247	27.4	9.0
Ethylene glycol	0.17	343	24.2	14.2
Glycerol	1.30	387	167	2.3
Glucose	1.20	394	158	2.5
Malic acid	0.17	270	26.2	10.3
Citric acid	0.20	330	28.6	11.5
Cholesterol	0.23	89	7.0	12.8

P. purpurescens AIU 063 was incubated at 30 °C for 2 d in the medium containing 0.2% indicated carbon source. The cell-free extract was prepared by disrupting 50 mg of wet mycelia with glass beads. AOD activity on ethanol was assay under standard assay conditions using the cell-free extract. Cell growth indicates wet weight of mycelia.

without enzyme from that with enzyme, and a molar absorption coefficient of 16,500 M^{-1} cm^{-1} for the dye formed. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of hydrogen peroxide per min under the above conditions.

Purification of enzyme The isolated strain was incubated at 30 °C for 2 d in the methanol medium, and mycelia were obtained by filtration. AOD was purified from the mycelia (11.6 g of wet weight obtained from 6 l of culture broth) as follows. The cell-free extract (70 ml) was prepared by disrupting the mycelia below 5 °C for 6 min with glass beads using a Multi-beads shocker, and then applied to a DEAE-Toyopearl column (2×16 cm). The adsorbed enzyme was eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 7.0, and 0.1 M NaCl. The eluate was then applied to a Phenyl-Toyopearl column (1.6×10 cm), and the adsorbed enzyme was eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 7.0, containing 1.5 M ammonium sulfate and 0.8 M ammonium sulfate. The active fractions were then applied to a Hydroxyapatite column (1×13 cm), and the adsorbed enzyme was eluted by a linear gradient with 50 and 300 mM potassium phosphate buffer, pH 7.0. The eluate was collected, and its purity was analyzed.

Other analytical methods Protein concentration was measured with a Protein Quantification Kit (Dojindo Laboratories, Tokyo).

SDS-PAGE was performed according to the method of Laemmli (14), and proteins were stained with Coomassie Brilliant Blue R-250. Molecular mass was estimated by gel filtration on a TSK gel G3000SW_{XL} column and by SDS-PAGE using molecular marker standards of Bio Rad Japan (Tokyo).

The isoelectric point was determined with an isoelectric focusing apparatus (Nippon Eido, Tokyo) under conditions of 1% Ampholine, pH 3.5–10, with a sucrose gradient at 400 V for 2 d at 4 °C. One-milliliter fractions were collected, and the pH was measured at 4 °C.

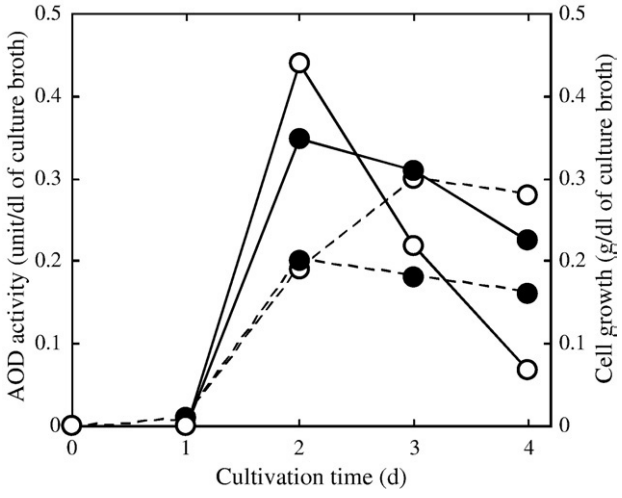

FIG. 1. Time course of AOD production by *P. purpurescens* AIU 063. *P. purpurescens* AIU 063 was incubated at 30 °C in the medium containing 0.2% methanol (closed circles) or 0.2% ethylene glycol (open circles) as a carbon source with shaking (120 strokes/min). Solid lines, AOD activity; dashed lines, cell growth.

TABLE 2. Summary of purification of AOD from *P. purpurescens* AIU 063

Step	Activity (unit)	Protein (mg)	Specific activity (unit/mg of protein)	Recovery (%)	Purification (fold)
Cell-free extract	10.1	1115	0.009	100	1
DEAE-Toyopearl	9.5	5.5	1.74	94	192
Phenyl-Toyopearl	5.9	2.8	2.10	58	232
Hydroxyapatite	3.7	1.27	2.91	36	321

Enzyme activity was assayed under standard assay conditions. Specific activity was expressed as units per milligram of protein.

The amino acid sequence of the intact enzyme was determined using an Applied Biosystems gas-phase protein sequencer equipped with an on-line reverse-phase chromatography system for identification of PTH-amino acids.

RESULTS

Identification of isolated strain The selected strain grew well at 25 °C, slowly at 5 °C, but not at 37 °C on a CYA plate, and the colonies cultured at 25 °C for 7 d were 48–50 mm in diameter. The conidiophores arose from basal hyphae and aerial hyphae, and the surface was smooth or slightly rough. They were 2.4–3.2×56–240 μm in length, and the terminal apex formed a bulbous vesicle 4.0–7.6 μm in diameter. Penicillus was monoverticillate and phialides were 3.2–3.6×8.0–11.2 μm. Conidia were spherical and rough, with a diameter range of 3.2–3.6 μm, and the color was light green to yellow-green. The colonies grown on a MEA plate and a G25N plate at 25 °C for 7 d were 44–47 mm and 18–20 mm, respectively. These results agreed well with the morphological characteristics of *P. purpurescens* classified by Pitt (13), although the colony reverse was light orange yellow. It was therefore concluded that the isolated strain belonged to the *P. purpurescens* group, and it was named *P. purpurescens* AIU 063.

Enzyme production The isolated strain, *P. purpurescens* AIU 063, was incubated at 30 °C for 2 d in the methanol medium but with the methanol replaced by other carbon sources. The strain grew well in all media tested, and AOD activity was also obtained from mycelia grown in all media tested. However, the level of AOD activity in relation to protein in cell-free extract from methanol medium and ethylene glycol medium was higher than those from the other carbon sources (Table 1). This isolated strain was then incubated in the medium containing methanol or ethylene glycol, and optimum cultivation time was investigated. The AOD activity reached maximum at 2 d of cultivation in both media. The maximum AOD amount of ethylene glycol medium was higher than that of the methanol

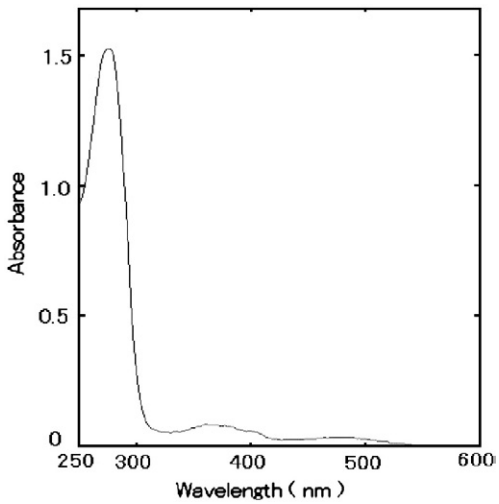

FIG. 2. Absorption spectrum of purified enzyme solution.