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Alcohol oxidase from the yeast *Pichia pastoris*—a potential catalyst for organic synthesis

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Abstract

Alcohol oxidase (AO) from the methylotropic yeast *Pichia pastoris* was isolated and investigated. Wide substrate specificity is characteristic for this enzyme. Unbranched primary alcohols are effectively oxidized by AO to aldehydes, including propargyl alcohol, 2-chloroethanol, 2-cyanoethanol, leading to important synthetic intermediates. AO was immobilized by covalent linking to macroporous cellulose activated by glutaraldehyde, yield of immobilization 80%. Presence of two izoenzymes of AO was suggested from the pH activity dependence.
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1. Introduction

Methylotropic yeasts of the genera *Pichia*, *Candida*, *Hansenula* and *Torulopsis* can grow by using methanol as the sole source of carbon [1,2]. In such conditions they produce high level of AO, which is the first key enzyme for methanol metabolism. Not only methanol but also numerous higher primary alcohols are oxidized into aldehydes by AO. It makes this enzyme a potential catalyst for organic synthesis [3–5]. The most wide substrate specificity is found for AO from the yeast *Pichia*. We present here the results of investigation of AO from a *Pichia pastoris* strain, showing real possibility of its use in organic synthesis.

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2. Experimental

2.1. Cultivation of the yeast

The strain *P. pastoris wt* was obtained from the yeast collection of the Eucariot Gene Engineering Laboratory of the Institute of Biotechnology, Vilnius. The medium used for growth of the yeast was selected according to [1].

2.2. Isolation of the enzyme

The yeast cells were disrupted by treatment with glass beads. After centrifugation, alcohol oxidase (AO) was precipitated from the supernatant by 1.5 volumes of isopropanol and re-dissolved in 0.1 M phosphate buffer, pH 7.3.

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2.3. Immobilization

AO was immobilized by glutaraldehyde method on macroporous cellulose carrier Granocel-2000 modified by epichlorohydrin and polyamine.

2.4. Analytical methods

The activity of native AO was assayed in 2 cm^3 of the reaction mixture, containing 3% methanol in 0.1 M phosphate buffer, pH 7.3, at 303 K. The reaction was stopped after 15 min by addition of 0.14 cm^3 of 4N HCl. The amount of formaldehyde produced was measured by the Hantzsh reaction [6]. One unit of AO activity was defined as the amount of enzyme that causes the oxidation of 1 μmol of methanol at 303 K and pH 7.3. The activity of the immobilized enzyme was measured by the same method after intensive mixing of 10–20 mg of wet immobilized preparation, in 6 cm^3 of phosphate buffer, pH 7.3, at 303 K, for 15 min.

Substrate specificity of the enzyme was investigated by means of Clark electrode from the rate of oxygen consumption in 0.5 M alcohol solution, pH 7.3.

Native polyacrylamide gel electrophoresis was carried out according to Laemmli's gel system without SDS. AO activity was identified on the gels by 2-hydrazinobenzothiazole reagent [7].

3. Results and discussion

Growth of *P. pastoris wt* strain in cultivation media containing only methanol as a carbon source was very slow. Much better results were obtained by using two-stage process: at 303 K with shaking for 48–72 h in glycerol containing media and for 48–72 h in methanol containing media. The amount of biomass 18–20 g and total AO activity 1200–1500 U/dm^3 of cultivation media was normally reached.

Substrate specificity data are presented in the Table 1. Both our results and previously published data show that numerous unbranched primary alcohols are effectively oxidized by the AO from the yeast *P. pastoris*, including several ones (propargyl alcohol, 2-chloroethanol, 2-cyanoethanol) that may give aldehydes–reagents for the synthesis of important heterocycles. It seems that decrease in oxidation rate

Table 1
Substrate specificity of alcohol oxidases (AO) from the yeast *Pichia*

Substrate	Relative rate of oxidation (%)		
	P. pastoris, this work	*P. pastoris*, [8]	*Pichia* sp., [9]
CH_3OH	100	100	100
CH_3CH_2OH	83	82	92
$CH_3CH_2CH_2OH$	–	43	74
$CH_3CH_2CH_2CH_2OH$	67	20	52
$CH_3(CH_2)_4CH_2OH$	–	–	4
$(CH_3)_2CHCH_2OH$	21	1.2	2
$CH_3CHOHCH_3$	–	2	0
CH_2=CH–CH_2OH	81	–	–
CH≡C–CH_2OH	90	–	–
Cl–CH_2CH_2OH	66	–	70
Cl–$CH_2CH_2CH_2OH$	–	–	22
H_2N–CH_2CH_2OH	–	–	0
HS–CH_2CH_2OH	–	–	25
CH_3O–CH_2CH_2OH	40	–	15
NC–CH_2CH_2OH	30	–	–

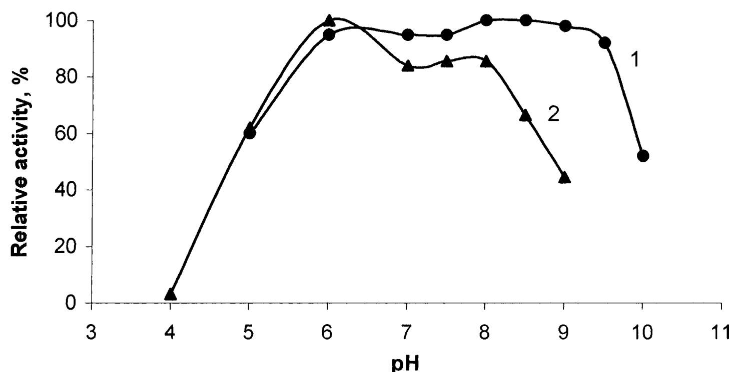

Fig. 1. Activity of AO at different pH values (1: native AO, 2: immobilized AO).

with increasing chain length is primarily caused by lower solubility in reaction media. By using a biphasic reaction medium water–hexane aliphatic alcohols C_6–C_{11} and some aromatic alcohols (benzyl alcohol, phenethyl alcohol) were successfully oxidized [4].

AO from the yeast *Pichia* has free thiol groups essential for its catalytic activity [8]. It may make covalent immobilization difficult. We found that immobilization by glutaraldehyde method is possible. AO was indeed rapidly inactivated by free glutaraldehyde, but not by the glutaraldehyde activated carrier (macroporous cellulose). The yield of immobilization was up to 80%. By oxidizing *n*-butanol and 2-methoxyethanol it was demonstrated that repeated use of immobilized enzyme is possible. No apparent loss of activity was noticed after seven to eight cycles of oxidation.

Both native and immobilized AO had wide pH interval of activity and two maxima could be distinguished (see Fig. 1). It means that two izoenzymes may be present. Some published data support such a conclusion [3,5]. But we could not demonstrate the presence of the izoenzymes by electrophoresis in non-denaturing conditions. Only one band with AO activity was present.

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