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A biocatalyst for pyruvate preparation from DL-lactate: lactate oxidase in a *Pseudomonas* sp.

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Abstract

For the purpose of producing pyruvate from DL-lactate by enzymatic methods, four microorganism strains that produce lactate oxidase (LOD) were screened and isolated from many soil samples. Among them, strain SM-6, which showed high potential for pyruvate production, was chosen for further research. Physiological studies and 16S rDNA relationship reveal that SM-6 belongs to *Pseudomonas putida*. The optimized pH and temperature of the enzyme-catalyzed reaction were pH 7.2, and 39 °C, respectively. Low-concentration EDTA (1 mM) could improve the stability of pyruvate and conversion ratio of lactate oxidase. V_{max} and K_m value for DL-lactate were 2.46 μmol/(min mg) protein and 9.53 mM, respectively. On preparation scale, cell-free extract from SM-6, containing 300 mg/l of crude enzyme (4037 U/ml lactate oxidase), could convert 66% of 116 mM of DL-lactate into 76.6 mM pyruvate in 18 h, and 82% of substrate was transformed after 48 h, giving 95.0 mM (10.5 mg/ml) of pyruvate. The ratio of product to biocatalyst was 34.8:1 (g/g).

Keywords: Pyruvate; Lactate oxidase; DL-Lactate; Biocatalyst; *Pseudomonas*

1. Introduction

Pyruvate is an important intermediate in the in vivo metabolism and a starting material for synthesizing various physiologically active substances. For example, L-tryptophan is obtained by enzymatic reaction of tryptophanase on indole, pyruvate, and ammonia [1].

Pyruvate can be formed and converted by many reactions. In the last 10 years, there have been a lot of patents dealing with the preparation of pyruvate. Chemical as well as biochemical procedures were described. Compared with other small non-chiral building blocks, pyruvate is relatively expensive. But pyruvate formation by fermentation usually does not lead to high volumeric yield. Also the yield as much and the concentrations reached are often not very high. In addition, being a quite active intermediate, pyruvate is difficult to accumulate in cells. This can be seen from a paper in which pyruvate is produced from gluconate [2].

Lactate can be converted to pyruvate by extracellular biocatalytic processes. What should be emphasized is that racemic as well as stereochemically pure forms of lactate are much cheaper than pyruvate. In many living organism, lactate can be converted to pyruvate by lactate dehydrogense (LDH) which acts in reverse reaction

$$\text{lactate} + \text{NAD}^+ \leftrightarrow \text{pyruvate} + \text{NADH} + \text{H}^+ \qquad (1)$$

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however, on preparative scale, this enzyme can not be used to form pyruvate because stoichiometric amounts of expensive pyridine nucleotides (NAD^+) are required for the oxidative reaction. Some enzymatic methods are available to regenerate NAD^+, but such systems are complicated since two substrates, two products, and the coenzyme NAD^+ are required for the recycling reaction.

Lactate oxidase (LOD) catalyzes the direct formation of pyruvate from lactate without requiring NAD^+ as a cofactor. Thus, lactate oxidase would be very useful for the formation of pyruvate on a preparative scale. Lactate oxidase from *Aerococcus viridans* catalyzes the following reactions:

$$\text{lactate} + O_2 \leftrightarrow \text{pyruvate} + H_2O_2 \quad (2)$$

The enzyme, which is a flavoprotein and belongs to the 2-hydroxy acid oxidase family, has been reported in several microbial species. Some *Pediococcus* have been found to produce lactate oxidase previously [3]. The *A. viridans* LOD has been purified and characterized, and the LOD genes of *Escherichia coli* and *A. viridans* have also been cloned and sequenced [4,5]. Recently, alteration of substrate specificity and improvement of thermal stability of the *A. viridans* LOD have been achieved using the protein engineering techniques [6,7]. But unfortunately, most of these studies dealt with lactate determination in dairy and in clinical analysis, whereas pyruvate preparation was seldom involved.

Generally most aerobic bacteria contain oxidases. In aerobic condition, if DL-lactate is used as sole carbon source, bacterial strains with high activity of LOD may be screened from lactate medium. In the work presented here, a new lactate oxidase producer was screened and isolated from various soil samples. Crude enzyme was prepared from the bacterial cells and further characterized for pyruvate preparation. Kinetics of the biocatalyst was also preliminarily studied in this paper.

2. Materials and methods

2.1. Samples

Natural samples: soil samples from Jining region, Jinan suburb, Linyi region and Taian region, Shandong Province, China were used as sources of microorganisms.

2.2. Media

Medium 1 consisted of 10.0 g sodium DL-lactate 1.1 g $KH_2PO_4{\cdot}3H_2O$, 0.9 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $MgSO_4{\cdot}7H_2O$, 1 g yeast extract, 5 mg $CaCl_2$, 1.2 ml trace elements, 1000 ml distilled H_2O; pH 7.0, adjusted with 1 M KOH or 1 M HCl. Trace elements solution consisted of: 50 g Na_2EDTA, 20 g $ZnSO_4{\cdot}7H_2O$, 5.5 g $CaCl_2$, 5 g $MnCl_2{\cdot}4H_2O$, 1.0 g $(NH_4)_2Mo_7O_{24}{\cdot}4H_2O$, 5.0 g $FeSO_4{\cdot}7H_2O$, 1.5 g $CuSO_4{\cdot}5H_2O$, 1.61 g $CoCl_2{\cdot}6H_2O$, 1000 ml distilled H_2O. Medium 2 consisted of 10.0 g sodium DL-lactate, 0.9 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $MgSO_4{\cdot}7H_2O$, 1 g yeast extract, 5 mg $CaCl_2$, 1.2 ml trace elements, 1000 ml distilled H_2O; pH 7.0. For slants and plates we developed medium 1 by supplementing with 2% (w/v) agar. The medium was autoclaved at 120 °C for 25 min.

2.3. Screening procedure

2.3.1. Enrichment cultivation

Natural samples were cultivated in 100 ml medium 1 (500 ml conical flask). Cultivation was carried out at 30 °C with shaking at 150 rpm. After 24 h, the culture with appropriate dilution was used to inoculate plates, which subsequently were used for the isolation of typical single colonies. Single colonies screened were cultured for 48 h on the plates and used to inoculate liquid mediums 1 and 2 (50 ml in 300 ml conical flasks). After 24–48 h of cultivation, the cells were harvested by centrifugation (10,200 × *g*, 15 min, 4 °C).

2.3.2. Crude enzyme preparation

The harvested cells were washed twice with 20 ml 67 mM phosphate buffer (pH 7.4) or 0.01 M imidazole/HCl buffer (pH 7.0) followed by resuspension in the same buffer. The next step involved disruption of the cells using ultrasonic oscillator (Sonic & Material Inc., Danbury CT, USA) for 5 min at 0 °C and a minimum power of 100 W. After disruption, the cell extracts were further centrifuged (14,700 × *g*, 15 min, 4 °C), the supernatant was collected and used as an enzyme source.

2.4. Cultivation

The inoculum (50 ml in 300 ml conical flasks) was grown for 12 h. Batch fermentation was carried out in two 1000 ml conical flasks with 300 ml each medium 2. The cultivation temperature was 30 °C. The rotary speed was set at 180 rpm. The fermentation was carried out for 48 h.

2.5. Assay of lactate oxidase activity

A reaction mixture (1 ml), consisting of 33 mM phosphate buffer (pH 7.4) and 10 mM DL-lactate, was preheated at 37 °C for 5 min. Then 0.10 ml enzyme solution was added and well mixed. After incubation for 10 min at 37 °C, 0.5 ml of 1 mM 2,4-dinitrophenylhydrazine was added and the mixture was further incubated at 37 °C for another 20 min. The reaction was terminated by addition of 5 ml of 0.4 M NaOH. The appearance of brown color was measured at 520 nm by spectrophotometry. One unit of activity was defined as the amount of enzyme that causes the formation of 1.0 nmol of pyruvate per minute under test condition.

2.6. Assay of lactate, pyruvate, biomass and protein

The lactate concentration was measured by SBA-40C lactate analyzer (The Academy of Science in Shandong Province, PR China). Pyruvate estimations were carried out spectrophotometrically as the 2,4-dinitrophenylhydrazone derivative [8].

The concentration of pyruvate and lactate was also determined by P/ACE^{TM} MDQ capillary electrophoresis (Beckman Coulter Co. Ltd., USA). Fused silica capillaries (Yongnian Co., Hebei, PR China) of 50 μm (i.d.), 370 μm (o.d.) of total length 60 cm were used. The detection window was 10 cm from the end of the column. Detection was carried out at 195 nm by the direct method. The electrolyte consisted of 20 mM sodium tetraborate (pH 8.0). Separation voltage: 20 kV; positive; injection time: 5 s; injection pressure: 0.17 psi; temperature: 25 °C.

Protein was assayed by method of Bradford test [9] with bovine serum albumin (Sigma, USA) as the standard. Off-line measurement of cell concentration was carried out using a spectrophotometer (UV-340, Beckman Co. Ltd., USA) at 660 nm with appropriate dilution. The value of optical density was converted to dry cell weight (DCW) using the calibration equation such that 1 OD_{660} = 0.30 g DCW/l.

2.7. Dialysis

The cell-free extract was placed into a dialyzing parchment and dialyzed in the ice chest against 67 mM phosphate buffer (pH 7.4) for 48 h.

2.8. DNA extraction, amplification, sequencing and phylogenetic analysis

DNA was extracted following a modified protocol using cetyltrimethylammonium bromide (CTAB) [10]. SM-6 strain was cultivated to logarithmic phase. A 1000 μl aliquot of the culture sample was centrifuged for 5 min, the supernatant removed, and the pellet resuspended in 500 μl of TE buffer (10 mM Tris–HCl and 1 mM EDTA) and an equal volume of extraction buffer (100 mM EDTA, 0.5% SDS, 0.25 mg/ml proteinase K). The sample was incubated at 37 °C for 1 h. The sample was spun briefly to separate the cell lysate. NaCl (0.8 M final concentration) and a CTAB/NaCl solution (1% CTAB and 0.7 M NaCl final concentration) were added. The sample was incubated at 65 °C for 30 min. The DNA was first extracted with chloroform:isoamyl alcohol (24:1) and then with phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated with an equal volume of 100% isopropanol overnight at −20 °C.

Primers used to amplify 16S rDNA were the bacterial specific primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TAC GGC TAC CTT GTT ACG ACT T-3′). Conditions for PCR were an initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C, 1 min; 50 °C, 1 min; 72 °C, 3 min; and a final 10 min at 72 °C. The PCR reaction (2 μl × 50 μl) contained: 10 mM Tris–HCl (pH 8.0), 2.5 mM $MgCl_2$, 150 μM each of dATP, dCTP, dGTP and dTTP, 100 pmol; each of primers 1 and 2, 2.5 U of Taq DNA polymerase (Sangon, Shanghai, PR China), and 1 μl cell lysate (see above). Amplification products were confirmed by visualization on 1.4% agarose gels.

The PCR product was purified with the Wizard Prep kit (Sangon, Shanghai, PR China) following the manufacturer's instruction. Both strands of the 16S rDNA were fully sequenced by Sangon Co. Ltd.

Sequence was submitted to basic logical alignment search tool (BLAST) programs linked with www.biosino.org website. Analysis of the sequences was performed by alignment of the partial isolate sequences to the EMBL database of 16S rDNA sequences.

3. Results and discussion

3.1. Screening and isolation of strains

A total of 64 different strains of microorganism were capable of growing in the medium containing lactate as carbon source. All the strains were inoculated into liquid medium, respectively for further screening. After enrichment culture and cell disruption, cell-free extracts were dialyzed to remove small molecules including NAD^+, which minimum the influence of lactate dehydrogenase. Four strains showing high LOD activities were screened from 64 strains of microorganism, as shown in Table 1 (four strains described in this paper are deposited in the Culture Collection of State Key Laboratory of Microbial Technology, Shandong University. The strain acquisition numbers are LOD-SM-6, LOD-SM-10, LOD-SM-29 and LOD-SM-51, respectively).

Comparing the four strains on pyruvate conversion ratio, as shown in Fig. 1, cell-free extract from SM-6 is a potential biocatalyst for pyruvate preparation. Within 3 h, cell-free extract from SM-6, containing 70 mg/l of protein, can convert DL-lactate (5.54 mM) to pyruvate with yield of 92.0%, which corresponds to a space time yield of 40.8 mM per day or about 4.49 g/l per day (sodium pyruvate). After 5 h, it can transform >99% DL-lactate (5.54 mM) into pyruvate, while cell-free extracts from other three strains did not accumulate pyruvate as much as DL-lactate decreased. According to the dialysis results (Table 1), a majority of LOD activity still remained after dialysis. So, it was confirmed that the formation of pyruvate from lactate was catalyzed mainly by lactate oxidase rather than lactate dehydrogenase in cell-free extract of SM-6. Therefore, strain SM-6 was chosen as the source of the enzyme for further investigation.

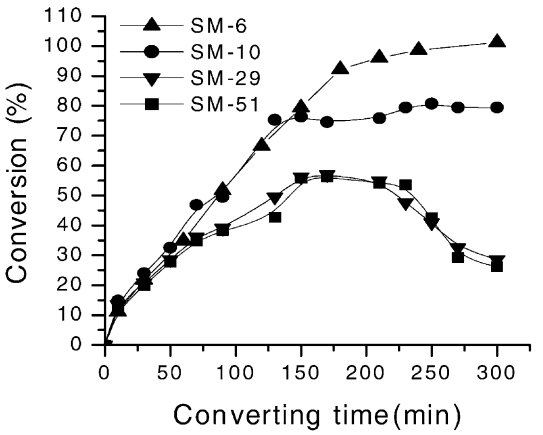

Fig. 1. Comparison of conversion ratio between four kinds of cell-free extracts. Reaction were carried with 5.54 mM initial sodium DL-lactate concentration, 70 mg/l of crude enzyme in 33 mM phosphate buffer (pH 7.4) at 37 °C.

SM-10 and SM-6 could transform more than 50% of DL-lactate into pyruvate (Fig. 1), which proves that the enzymes from these two strains do not have stereospecificities for L-lactate, and shows that they are different from the L-lactate oxidase from *Pediococcus* [3] and *Aerococcus* [5,6], glycolate oxidase from spinach [11].

Table 1
Comparison of screened strains in LOD activity

	Strain number			
	SM-10	SM-6	SM-51	SM-29
Medium pH after cultivation	8.2	9.0	8.5	8.5
Biomass ($OD_{660\,nm}$)	5.12	3.44	5.36	4.8
Residual lactate in culture (g/l)	0.5	0	0	0
Activity of cell-free extract (U/ml)	774.47	941.92	337.59	595.74
Activity of cell-free extract after dialysis (U/ml)	425.53	686.85	216.31	323.40

3.2. Identification of SM-6

Strain SM-6 was identified taxonomically based on the following characteristics: straight rods, measuring 0.7 μm × (2.5–3.0) μm; gram stain negative; non-spore forming; motile by one polar flagella; aerobic; optimum growth temperature, 30 °C; growth of small yellow colonies ~1–2 mm in diameter on peptone or similar media after 24 h incubation; fluorescent, diffusible pigments; catalase positive; oxidase positive; denitrification negative but can reduce nitrate to nitrite; gelatin hydrolysis negative; starch hydrolysis negative; lipid hydrolysis negative; methyl red negative; urease negative; Voges-Proskauer negative; acid production from: glucose positive, lactose negative; other carbon source: 2-ketogluconate positive, β-alanine positive, L-arginine positive, citrate positive; protein reaction: indole production positive. According to Bergey's *Manual of Systematic Bacteriology* (9th ed.) [12], the strain was identified as *Pseudomonas putida*.

The sequence analysis 16S rDNA fragment amplified from total DNA of SM-6 strains by PCR showed that SM-6 shares 96% 16S rDNA sequence homology with *P. putida* biotype A. This result showed coherence with the standard strain identification above.

3.3. Optimization of the enzymatic catalysis condition for pyruvate production

3.3.1. Effect of temperature and pH on the activity of LOD

Enzyme activity was assayed in citric acid–NaOH (50 mM), phosphate (33 mM), Tris–HCl (50 mM), and tetraborate–NaOH (50 mM) buffer systems at pH 3.5–5.8, 5.8–8.0, 8.0–8.9 and 9.1–12.0, respectively. As shown in Fig. 2a and b, the enzyme exhibited maximal activity at pH 7.2 and the optimum temperature for LOD activity is 39 °C.

3.3.2. Effect of substrate concentration on catalytic reaction

The effects of substrate concentrations on the initial velocity of the enzyme reaction were examined at 39 °C, pH 7.2, using sodium DL-lactate as substrate with crude protein concentration at 70 mg/l. Lactate concentration varied from 2.8–28 mM. The Michaelis apparent constant (K_m) for DL-lactate was

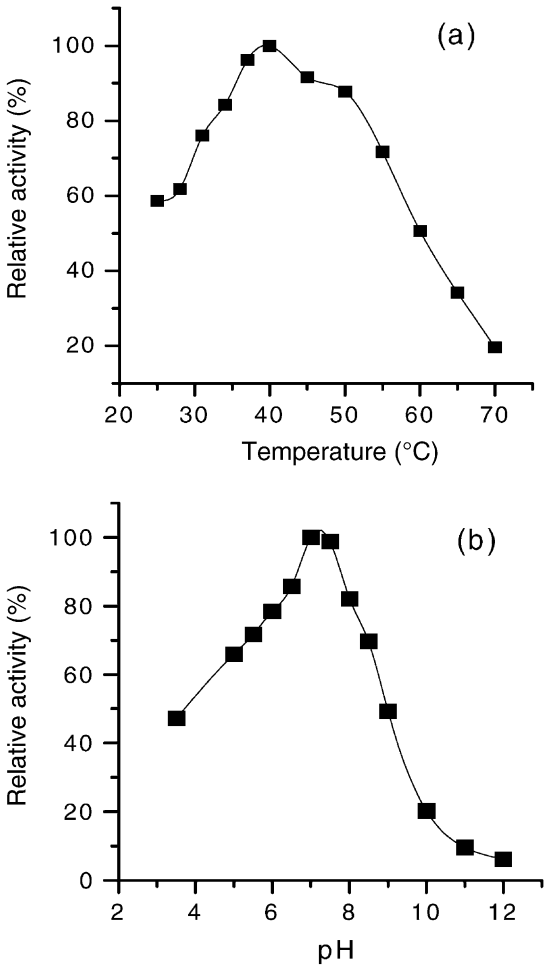

Fig. 2. (a) Effect of temperature on activity of enzyme in 33 mM phosphate buffer pH 7.4 solutions. The 100% of relative activity corresponds to 976.4 U/ml enzyme activity. Enzyme activity was assayed according to 2.5. procedure. (b) Effect of pH on activity of enzyme at 37 °C. The 100% of relative activity corresponds to 985.7 U/ml enzyme activity. Enzyme activity was assayed according to 2.5 procedure.

9.53 mM ($-1/K_m = -0.105\,mM^{-1}$), and V_{max} value was 2.46 μmol/(min mg) protein, which were calculated with lineweaver-Burk plot of the enzyme reaction. Higher concentrations than 16.6 mM ($1/s = 0.06\,mM^{-1}$) of DL-lactate caused the substrate inhibition of the enzyme activity.

3.3.3. Effect of EDTA on the pyruvate preparation

When initial substrate concentration reaches 65 mM, the forming pyruvate decreased rapidly after

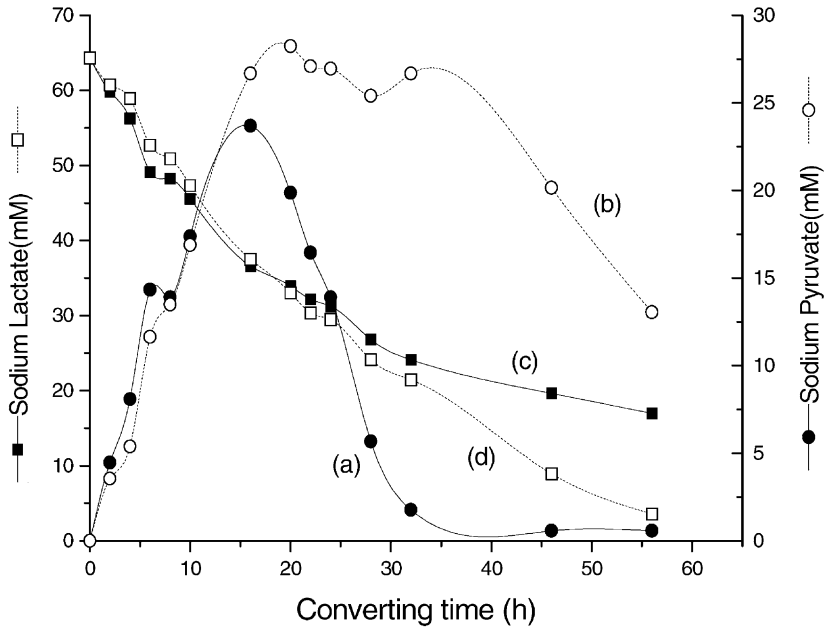

Fig. 3. Influence of 1 mM EDTA on the stability of forming pyruvate with SM-6 cell-free enzyme (70 mg/l) in 33 mM phosphate buffer pH 7.2 solutions containing 65 mM DL-lactate at 39 °C. Dash and solid lines represent converting curves with 1 mM EDTA and converting curve without EDTA, respectively. Closed and open squares represent sodium DL-lactate concentrations; closed and open circles represent sodium pyruvate concentrations.

16 h (Fig. 3, curve (a)). It was difficult to obtain ideal conversion under this condition. A possible reason for it was that the cell-free extract from SM-6 contains some other enzymes. Some of them can be helpful to the biocatalysis. For example, catalase can destroy hydrogen peroxide and limits the non-enzymatic oxidation of pyruvate to acetate, carbonate [13]. But most of them can just degrade pyruvate. For instance, pyruvate dehydrogenase, pyruvate:formate-lyase [14]. Therefore, it was necessary to block these pyruvate degrading enzymes. Based on the activation mechanism of these enzymes, the bivalent ions (Zn^{2+}, Fe^{2+}, Mg^{2+}) necessary for these enzyme reactions could be complexed with low concentration of EDTA. Curve (b) results in Fig. 3 indeed show the stabilizing effect of 1 mM EDTA for pyruvate formed from lactate by SM-6 cell-free extract. So, it was confirmed as an employable method for the preparation of pyruvate from DL-lactate. In addition, as shown in the curves (c) and (d) in Fig. 3, 1 mM of EDTA can do some help to release substrate inhibition to some extent. If crude enzyme concentration higher than 1 g/l were applied, the concentration of EDTA had to be enlarged to 2–5 mM (details not shown).

3.4. *Preparative production of pyruvate from* DL-*lactate under optimal conditions*

The preparative production of pyruvate from DL-lactate was carried out under the optimal conditions: crude enzyme concentration, 300 mg/l; 33 mM phosphate buffer, pH 7.2; temperature, 39 °C; shaking speed, 110 rpm; EDTA concentration, 1.0 mM; initial DL-lactate concentration, 116 mM. As shown in Fig. 4, 66% of sodium DL-lactate was transformed by 300 mg/l crude enzyme (4037 U/ml LOD) in 18 h with production of 76.6 mM sodium pyruvate (8.4 mg/ml). After 48 h, while sodium DL-lactate was completely consumed, 82% of substrate was transformed, resulting in 95.0 mM (10.5 mg/ml) of pyruvate production. The ratio of product to biocatalyst was 34.8:1 (g/g).

Pyruvate, an important and useful organic acid, is widely used in the industry of chemicals and drugs as well as agrochemicals. Pyruvate production by

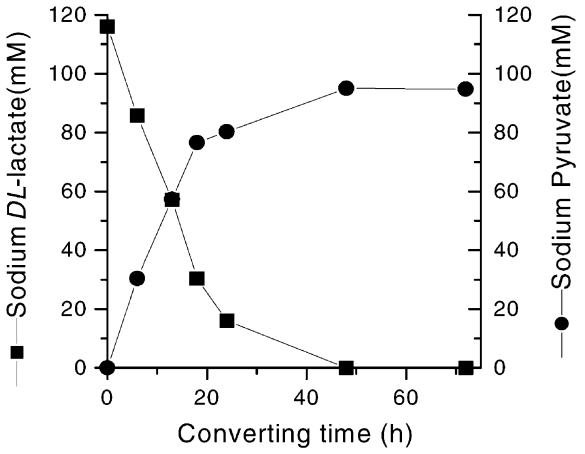

Fig. 4. Preparative production of pyruvate from DL-lactate. Reaction were carried with 300 mg/l of crude enzyme in 33 mM phosphate buffer (pH 7.2) at 39 °C for 72 h.

enzymatic method is a fairly competitive and attractive process because of its novelty and pollution-free quality. Lactate oxidase plays an important role in such kind of biotransformation. It catalyzes the direct formation of pyruvate from lactate without requirement of NAD^+ or $NADP^+$ as a cofactor, which makes its prospect of application broad. The results described above indicated that the biocatalyst actually had been found in several strains. Among them, *P. putida* SM-6, which does not have stereospecificity for L-lactate, and has been confirmed as a new bacterial resource for LOD other than L-lactate oxidase from *Pediococcus* and *Aerococcus*, showed potential for pyruvate preparation. The 10.5 g/l of pyruvate could be produced by 300 mg/l crude enzyme. The ratio of product to catalyst reached to 34.8:1 (g/g).

To obtain high accumulation of pyruvate, pH and temperature of the transforming reaction were important. Low concentration of EDTA can effectively stabilize the forming of pyruvate and improve conversion ratio (Fig. 3). The Michaelis apparent constant (K_m) for the crude enzyme was 9.53 mM, and V_{max} value was 2.46 μmol/(min mg) protein, while substrate varied from 2.8–28 mM. Those were very useful data for further studying on enzyme mechanism.

Pyruvate was effectively produced in SM-6 biocatalyst system. This novel catalyzed process is a promising one in that it uses cheap starting material DL-lactate and brings high yield that is hardly achieved by fermentation methods. The application of the biocatalyst is now under further investigation.

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