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SHORT COMMUNICATION

An amine: hydroxyacetone aminotransferase from *Moraxella lacunata* WZ34 for alaninol synthesis

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Abstract An amine:hydroxyacetone aminotransferase from an isolated soil bacterium, *Moraxella lacunata WZ34*, was employed to synthesize alaninol in the presence of hydroxyacetone and isopropylamine in this study. The optimal carbon and nitrogen sources were glycerol and beef extract, respectively. A wide range of amino donor specificity was detected with the aminotransferase, which exhibited a relative high activity (9.83 U mL^{-1}) in the presence of isopropylamine. The enzyme was the most active at pH 8.5, and showed relatively higher activity at alkaline than acidic pH. Maximum activity was achieved at 30 °C, and the enzyme had good thermal stability below 60 °C. Metal ions such as Mg^{2+} had positive effect (132.6%) on the enzyme, and (aminooxy)acetic acid, a typical aminotransferase inhibitor, significantly inhibited its activity. The enzyme activity was enhanced by the addition of 0.05 mM pyridoxal-5′-phosphate (PLP).

Keywords Aminotransferase · Alaninol · Hydroxyacetone · Characterization

Introduction

Alaninol is an important intermediate for the synthesis of ofloxacin, which is clinically used as an effective quinolone antimicrobial agent [1]. The formation of side products and the requirement for protection (and subsequently de-protection) of unstable functional groups of the reactants make chemical routes cumbersome. Biocatalysis, harboring intrinsic regio, chemo, and stereo-selectivity, offers one approach that may be considered to be the most efficient route. Additionally, the mild conditions under which most biocatalytic processes are operated reduce both running costs and the generation of waste streams detrimental to the environment [2, 3]. Nobuya screened and found a novel NAD^+-dependent amine dehydrogenase capable of producing alaninol by the reductive amination of hydroxyacetone [4]. However, it requires NADH as cofactor, which is expensive and hard to regenerate in the industrial use.

Aminotransferases are generally considered as excellent biocatalysts due to their broad substrate specificities and rapid conversion rates. They have been known to convert a carbonyl group to an amino group, most of all, keto acid to amino acid [5, 6]. Economy is a key advantage of transaminase-catalyzed reaction because these enzymes do not require nicotinamide cofactors. Consequently, transaminases have been widely applied in the large-scale synthesis of unnatural and nonproteinogenic amino acids, such as L-homophenylalanine, L-2-aminobutyrate, L-*tert*-leucine and so on [7, 8]. Besides, optically active amino acids and amines have been biosynthesized by transaminases as pharmaceuticals [9–11]. Recently, Shin and Kim employed a ω-transaminase from *Vibrio fluvialis* JS17 to convert acetophenone to (*S*)-α-methylbenzylamine using L-alanine as the amino donor [12]. Patel et al. prepared omapatrilat, a key intermediate of vasopeptidase inhibitor, in a relatively simple route mediated by an L-lysine ε-transaminase [13]. Among the transaminases, diamine-ketoglutaric transaminase, which belongs to the class of ω-transaminases and the family of subclass II of aminotransferases, was the first one reported to utilize a substrate bearing no carboxylic acid group [14]. However, there are few reports showing the ability of transaminase in transformation of keto alcohols

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to amino alcohols, which are extremely important class of compounds of considerable pharmaceutical interest [15, 16]. As transaminases are gaining increasing attention in production of high-value compounds, synthesis of amino alcohols by transaminases seem to be more promising in future.

To the best of our knowledge, there has been no report focusing on the synthesis of alaninol by transaminase. In this study, we isolated a strain, *Moraxella lacunata* WZ34, with transaminase activity and found it was capable of converting hydroxyacetone to alaninol in the presence of isopropylamine.

Materials and methods

Materials

Hydroxyacetone and alaninol were purchased from Fluka. Pyridoxal-5′-phosphate (PLP) were obtained from Sigma Chemical Co., (St. Louis, MO, USA). All other chemicals used were of analytical grade and commercially available.

Analytical methods

Identification of alaninol was carried out by TLC directly and HPLC after derivatisation with 2,4-dinitrofluorobenzene [17]. TLC was carried out on GF_{254} using developing solvent of ethyl acetate:methanol:triethylamine in a ratio of 25:70:5 (by vol), and alaninol was detected with 0.5% ninhydrin. HPLC (LC-10AS, Shimdazu, Japan) equipped with a 4.6 × 250 mm adsorbosphere C18 column (Alltech) was also used to determine the amount of alaninol. The parameters used for the detection of alaninol were a UV detector set at a wavelength of 362 nm, a flow rate of 1.0 mL min^{-1}, and a mobile phase of acetonitrile:water (30:70, by vol). Under these conditions, the retention time of alaninol was 16.2 min. Hydroxyacetone was analyzed by HPLC, which was performed with the above conditions except the wavelength of UV detector (280 nm).

Microbial screening

The following medium was used for the screening. S medium comprises 10 g glycerol, 5 g NaCl, 2 g KH_2PO_4, 2 g K_2HPO_4, 0.3 g $MgSO_4$, 0.1 g $CaCl_2$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.1 mg $ZnCl_2$, 0.02 mg H_3BO_3, 0.1 mg $CuSO_4$, 0.1 mg $CoCl_2$, 0.1 mg $NiSO_4$, 2.0 mg Na_2MoO_4 and 2 g alaninol in 1 L distilled water, pH 7.0. P medium was the same as S medium, except replacing 2 g alaninol with 10 g peptone.

One gram soil samples collected from different polluted sites around chemical and fertilizer plants were suspended in water for various dilution. Each 1 mL of suspension was added to 30 mL of S medium in a flask respectively, and then enrichment of the microorganisms was done by repeated dilution of the culture broth with fresh medium (50- to 100-fold). These procedures were repeated three times. After the enrichment, each culture was spread out on an S medium plate containing 2% agar, and then incubated at 30 °C. Microorganisms, which could grow on S medium plates, were isolated and the activities were examined. Washed cells of each microorganism were incubated in 5 mL of a reaction mixture (20 mM hydroxyacetone and 40 mM isopropylamine as substrates) for 24 h at 30 °C.

Preparation of the cell-free extract

Soil isolate WZ34 was cultured with shaking for 24 h at 30 °C. These pre-cultured cells (1.1×10^8 cells) were then added to 80 mL of the fresh medium, and the mixture was shaken for 36 h. The cells were harvested and washed with 100 mM KPB (pH 7.0), then resuspended in 30 mL of ice-cooled 50 mM potassium phosphate buffer (pH 7.2) and disrupted for 20 min with a sonicator (5 s pulse-on and 5 s pulse-off each cycle, 120 W). The lysate was separated from cell debris by centrifugation and used for transaminase reaction without further purification.

Enzyme activity assay

The standard reaction conditions for amination were as follows. One milliliter cell-free extract was added to 2 mL reaction mixture comprising 20 mM hydroxyacetone, 40 mM isopropylamine (pH 8.5), 1 mM Mg^{2+} and 0.05 mM PLP, and then incubated in a flask at 30 °C for 2 h with shaking (160 rpm). One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of alaninol per min under the above assay conditions.

Results and discussion

Microbial screening

To screen the microorganisms exhibiting aminotransferase activity for synthesizing alaninol with hydroxyacetone and isopropylamine, its reverse reaction was used with the minimal media containing 40 mM alaninol and 100 mM glycerol as the sole nitrogen and carbon source, respectively. Because the aminotransferase reaction is often quite

reversible, the microorganisms showing high activity for the synthesis of hydroxyacetone from alaninol may also exhibit good activity for the synthesis of alaninol from hydroxyacetone. Microorganisms bearing aminotransferase activity were screened out of 120 strains that were isolated from S medium plate. Among them, strain WZ34, with the maximum aminotransferase activity, was isolated, and selected as the best strain for further studies. It was classified as belonging to the genus *M. lacunata* by using standard procedures [18].

The time course of the reaction carried out by cell free extract from *M. lacunata* WZ34 was shown in Fig. 1. The enzyme displayed low activity when reacted after 8 h. It required much longer time to accomplish the reaction when compared with other aminotransferases [19, 20].

The conversion by amine: hydroxyacetone aminotransferase seemed to be low if used in large-scale industrial application of alaninol production. However, we succeeded in alaninol production by a new method, which requires no nicotinamide cofactors. Taylor et al. [21] demonstrated that the potential drawback of transaminase was the low conversion of reaction due to the reversible interconversion between products and substrates. In the case of alaninol transaminase, moreover, the low affinity of transaminase for keto alcohol may worsen such limit.

Recently, Yun et al. [15] succeeded in synthesis of trans-(1*R*,2*R*)-1-amino-2-indanol by ω-transaminase from *Escherichia coli* BL21, which harbored the ω-TA gene from *V. fluvialis* JS17. A transaminase, harboring the ability to aminate the L-erythrulose to produce 2-amino-1,3,4-butanetriol, was detected from *Pseudomonas aeruginosa* by Ingram et al. [16]. To our knowledge, the synthesis of alaninol by means of amination with an aminotransferase has not been previously reported.

Effects of cultivation conditions on the formation of alaninol aminotransferase

To screen the optimal carbon source for the high induction of alaninol transaminase, medium P was used by replacing 10 g L^{-1} glycerol with 10 g L^{-1} other carbon sources, such as glucose, sucrose, glycerol, maltose, citrate, lactose and pyruvate. The optimal carbon source was glycerol (data not shown). It was noteworthy that good carbon sources for cell growth, such as glucose and pyruvate, were not suitable for the production of alaninol transaminase.

Nitrogen source would significantly affect the induction level of transaminase because the enzyme participates in nitrogen metabolism. To screen the optimal nitrogen source for enzyme induction, medium P was used by replacing 10 g L^{-1} peptone with other nitrogen sources. The optimal nitrogen sources screened was beef extract, and peptone also performed considerable effect. However, most of the natural amino acids were not good nitrogen sources, and isopropylamine, the amino donor for transamination, exhibited no significant induction for enzyme production, suggesting that alaninol transaminase was a constitutive enzyme. This differs from the observations made with *Klebsiella pneumoniae* and *Bacillus thuringiensis* where primary amines performed as good inducers for the production of ω-transaminases [22]. When *M. lacunata* WZ34 was cultured using medium P, the activity of aminotransferase increased with the increase of cell density, and reached the maximum (9.27 U mL^{-1}) at 40 h, when the activity began to decrease sharply (Fig. 2).

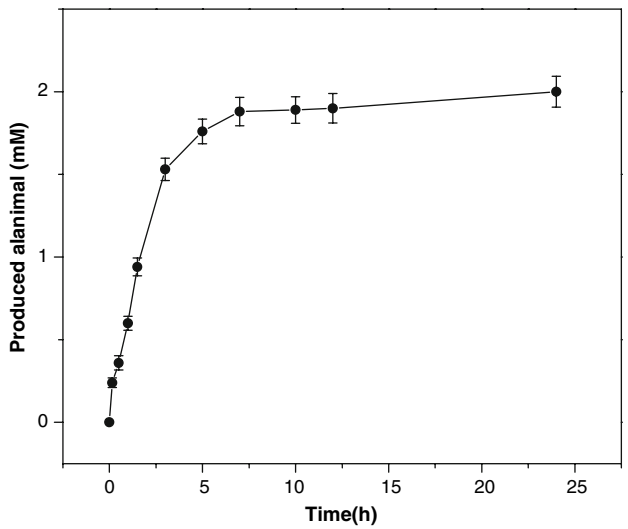

Fig. 1 Alaninol produced by cell free extract from *Moraxella lacunata* WZ34 from hydroxyacetone and isopropylamine. *Error bars* indicate standard deviation between replicates (n = 3)

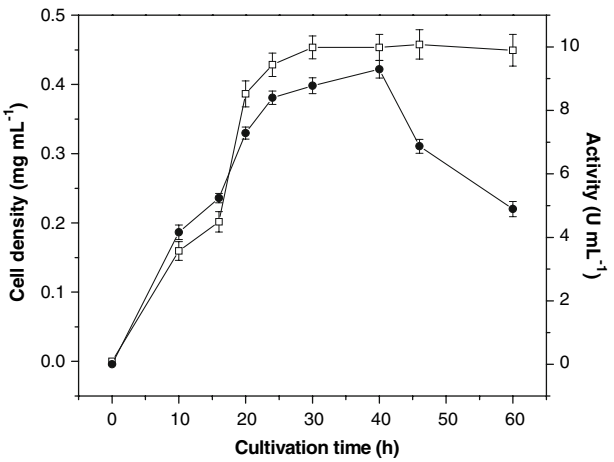

Fig. 2 The time-course of changes of cell density (*open square*) and activity of alaninol aminotransferase (*filled circle*). *Error bars* indicate standard deviation between replicates (n = 3)

Effect of amino donor on initial rate and reaction yield

A lot of microbial transaminases have showed substrate specificity and they are attractive biocatalysts with the potential of producing specific amino acids on the basis of various amino donors [19, 22]. In the synthesis of amino alcohol with aminotransferase, the choice of amino donor is also of great importance. Table 1 summarizes the amino donor specificity in terms of initial rate. Among the 11 amino donors, isopropylamine was the most effective one, and butylamine also showed considerable activity. It was surprising to see that the inorganic amines, such as NH_4Cl and $NH_3{\cdot}H_2O$ showed slightly lower activity than organic amine. The feasibility of transamination using inorganic amines as amine donors makes the enzyme greatly different from other aminotransferase [10], and would be of great importance for biotechnological applications. Glutamic acid and α-alanine showed similar activity to butylamine, which was also a good donor. β-Alanine, which was widely used as amino donor by previously reported ω-transaminases, also exhibited high activity with the alaninol aminotransferase. However, aspartic acid, serine and leucine performed lower activities than those mentioned above. Among them, urea displayed the lowest activity. No enzyme activity was observed in the absence of any amino donor. It can be seen from Table 1 that alaninol aminotransferase had a wide range of amino donor specificity in this study. The feature most distinct from other aminotransferases was a relatively higher activity using amines other than amino acids as amino donors. The transaminase family can be divided into four subgroups on the basis of their substrate specificities and sequence homology. α-Transaminases, which only attack α-amino group of α-amino acid, belong to subgroups I, III, and IV, whereas ω-transaminases, which can act on distal amino as well as α-amino group belong to subgroup II [23]. Therefore, the transaminase used in this study could be suggested as a type of ω-aminotransferase based upon the results of substrate specificity.

The reaction was also tested with hydroxyacetone concentrations ranging from 5 to 20 mM. Experiments at each initial substrate concentration all reached comparable levels of conversion (data not shown). This suggested that the reaction was equilibrium limited rather than substrate or product inhibited under these conditions. However, when the substrate increased to 80 mM, the activity reduced significantly to its 55% of that with 20 mM hydroxyacetone, indicating the occurrence of great substrate inhibition.

Table 1 Amino donor specificity in the synthesis of amino alcohol with aminotransferase from WZ34

Amino donor	Relative activity (%)	Amino donor	Relative activity (%)
Isopropylamine	100	β-Alanine	95.2 ± 8.4
Butylamine	97.3 ± 6.5	Aspartic acid	75.4 ± 7.2
Urea	20.6 ± 3.3	Glycine	37.7 ± 3.1
NH_4Cl	89.9 ± 6.7	Glutamic acid	96.2 ± 9.7
$NH_3{\cdot}H_2O$	90.3 ± 9.5	Serine	53.1 ± 3.6
α-Alanine	97.1 ± 8.9	Leucine	31.2 ± 3.7

Reaction yields are calculated based on the concentration of alaninol. Reaction condition: 20 mM hydroxyacetone; 40 mM amino donor; 1 mM Mg^{2+}; 0.05 mM PLP; 8 h reaction. The activity of isopropylamine (9.83 U mL^{-1}) was taken as 100%

Effects of pH and temperature

Enzyme activity was determined at 30 °C in the presence of 100 mM buffer. The pH-activity profile is shown in Fig. 3. The enzyme relative activity reached high at pH 8.0–9.0, and substantially decreased at pH values below 7.5 and above 10.0. The highest activity of alaninol aminotransferase was observed at pH 8.5. The pH optimum of the *M. lacunata* WZ34 aminotransferase was lower than the values of 9.0 of *E. coli* [24], 10.0 of *P. putida* [25], and 12 of *P. fluorescens* [26] for aminotransferase activity. But it was higher than the value 7.2–7.5 of *E. coli* [27] and 6.5 of *Prevotella bryantii* [28]. After treated with reaction systems which were different from pH values (pH 6–10), the residual activity was analyzed at pH 8.5 and temperature 30 °C. The enzyme showed relative higher activity at alkaline than acidic pH. The enzyme was stable in a wider range from pH 7.5 to 11. This differed from the enzyme from *P. bryantii*, which was active over a pH range 5.0–9.0 [28].

The effect of temperature on enzyme activity (Fig. 4) was determined at pH 8.5 where the enzyme was the most

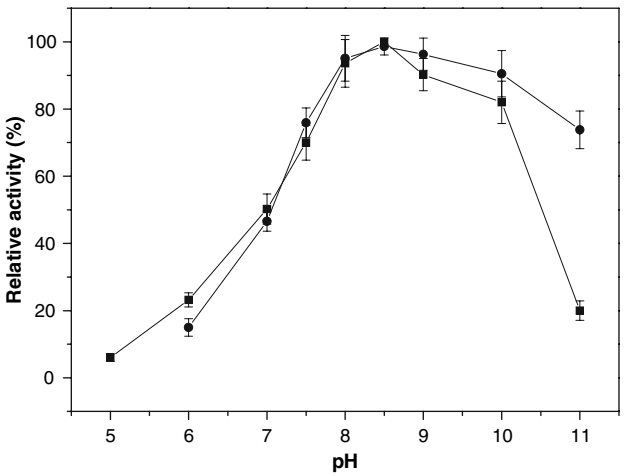

Fig. 3 The pH-activity (*filled square*) and pH-stability (*filled circle*) profile of alaninol aminotransferase

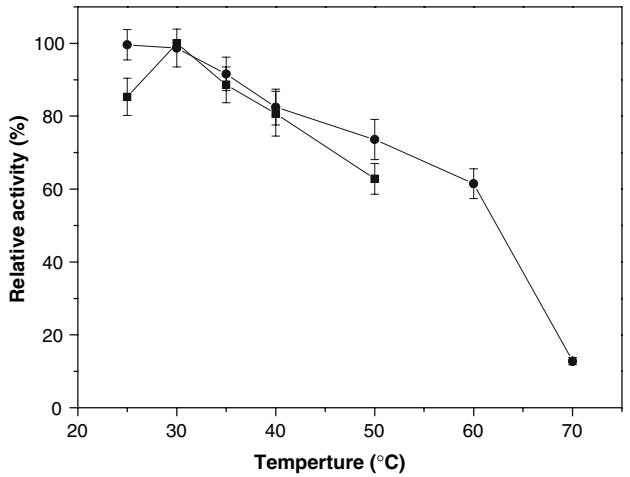

Fig. 4 Temperature-activity (*filled square*) and temperture-stability (*filled circle*) profile of alaninol aminotransferase

active. Starting from 25 °C, the activity increased with increasing temperature and it reached a maximum at 30 °C, but it gradually dropped down to 60% of the maximum activity at 50 °C. The optimum temperature is lower than most aminotransferases, such as 37 °C for *P. fluorescens* [26], 45 °C for *P. bryantii* [28], and 55 °C for *P. putida* [25]. In order to investigate the thermal stability of alaninol aminotransferase, the residual activities of enzyme after thermal treatment were measured. Figure 4 shows the residual activity of aminotransferase after heated at each tested temperature for 30 min. The enzyme was found to be stable up to 50 °C. Higher temperatures led to enzyme inactivation, but the activity reduced slowly when increased to 60 °C. Nevertheless, when temperature exceeded 60 °C, the activity declined dramatically and remained only 12.8% at 70 °C, similar with the reported properties of *P. bryantii* [28]. However, the enzyme was protected in the addition of PLP which make such aminotransferase activity remain 68% even at 70 °C. This observation was in agreement with 4-aminobutyrate: 2-oxoglutarate aminotransferase from *Streptomyees griseus* reported by Yonaha et al. [29]. Apoenzyme is usually more susceptible to irreversible inactivation than holoenzyme, and PLP tends to form a stable covalent intermediary complex with enzyme, so it is not surprising that PLP shows a strong stabilization effect on aminotransferase [30].

Effects of metal ions and inhibitor

The effect of selected metal ions was studied in phosphate buffer at 30 °C (Table 2). It is known that the metal ions are inhibitory to many aminotransferases [31]. However, the enzyme in this study was shown to be more active in the presence of some metal ions, such as Mn^{2+} and Ca^{2+}. The result showed that Mg^{2+} had the most positive effect (132.6%) on the aminotransferase.

Table 2 Effect of metal ions on aminotransferase

Metal ions	Relative activity (%)	Metal ions	Relative activity (%)
Control	100	Mn^{2+}	115.3 ± 8.9
Co^{2+}	89.3 ± 8.6	Al^{3+}	78.0 ± 7.3
Ca^{2+}	112.5 ± 10.3	Cu^{2+}	76.9 ± 6.8
Mg^{2+}	132.6 ± 11.2	Fe^{2+}	92.2 ± 6.5
Ba^{2+}	66.2 ± 7.5	EDTA	79.6 ± 5.4
Na^{+}	104.6 ± 9.4	(Aminooxy)acetic acid	5.0 ± 0.6
Li^{+}	105.7 ± 5.0		

Each concentration of the metal ions and inhibitor was 1 mM. The activity of transamination without any metal ions, corresponding to 7.56 U mL^{-1} was taken as control (100%)

The activity was substantially inhibited by (aminooxy)acetic acid, a typical aminotransferase inhibitor which could react with the active site of PLP. Similar complete inhibition was observed with that of the aminotransferase from *Pseudomonas stutzeri* [32]. Thus, the transaminase was further confirmed to be involved in the alaninol production.

Coenzyme requirement in the cell-free system

The use of aminotransferases to produce natural and unnatural amino acids is being more and more popular in recent years. It is economy because these enzymes do not require expensive nicotinamide cofactors. But coenzyme was usually necessary for the benefit of transferring amino groups [33, 34]. Transaminases are well known as pyridoxal-dependent enzymes which obey the ping-pong bi–bi mechanism [35]. All aminotransferases reported to date use the same coenzyme, PLP, to catalyze transamination reaction. Two forms of enzyme intermediates, transaminase-pyridoxal phosphate and transaminase-pyridoxamine phosphate, are involved in the reaction. Firstly the amino donor binds to the enzyme, after which the PLP coenzyme is aminated to pyridoxamine 5′-phosphate (PMP) and the respective keto product is released. Then the transamination is completed by transferring the amino group from enzyme-bound PMP to the acceptor. The aminotransferase in this study also required PLP to reach the maximum activity (Fig. 5). The rate of aminotransferase was only 0.267 nmol L^{-1} when worked in the absence of PLP, while reached 0.705 mmol h^{-1} with the addition of 0.05 mM PLP. Further increase of PLP concentration to 0.5 mM showed no obvious effect on the reaction rate. However, the reaction rate was almost maintained on the same level

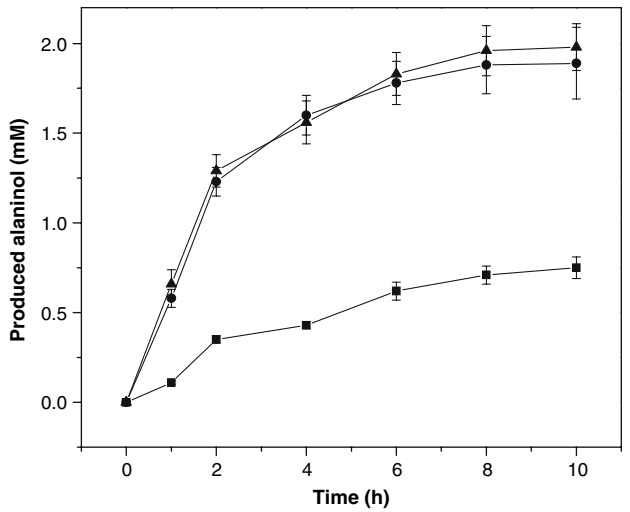

Fig. 5 Effect of PLP concentration on the activity of alaninol aminotransferase. Concentration of PLP in the reaction was as follow: absence (*filled square*), 0.05 mM (*filled circle*), 0.5 mM (*filled triangle*). *Error bars* indicate standard deviation between replicates ($n = 3$)

when PLP concentration increased to 0.5 mM, and the specific activity (0.67 U mg^{-1} protein) attained was lower than amine: pyruvate transaminase (1 U mg^{-1} protein) from *V. fluvialis* JS17 which synthesized acetophenone in the presence of (*S*)-a-methylbenzylamine [36].

Conclusion

To our knowledge, the synthesis of alaninol by means of amination with aminotransferase has not been reported previously. We have screened and isolated a bacterial with alaninol aminotransferase, which is capable of transforming the amino group to hydroxyacetone from isopropylamine in the presence of PLP and isopropylamine. Moreover, it has a wide range of amino donor specificity. We have established the reaction system to convert hydroxyacetone to alaninol in the presence of isopropylamine, which may differ from other aminotransferases previously described in the following aspects. Firstly, it can produce aminoalcohol using keto alcohol as amino-group acceptor. Secondly, it has wide range of donor specificity, and accepts amino donor from organic amines, inorganic amines, and amino acids. Thirdly, the reaction time is rather long compared with other aminotransferases. Thus, it is necessary to screen microorganism with high activity and improve the ability by genic method in the further study with respect to the low activity of this alaninol aminotransferase. Because aminoalcohols are of great significance for the pharmaceutical and fine-chemical industries, production of other economical aminoalcohols, e.g. serinol, is also important and valuable. Therefore, the findings of this study would be meaningful in aiding in the synthesis of any other interesting aminoalcohols which is currently in progress in our laboratory.

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