Production of Benzaldehyde and Benzyl Alcohol by the Mushroom *Polyporus tuberaster* K2606

Tatsuya Kawabe* and Hideo Morita

Food Research Laboratories, Takara Shuzo Company, Ltd., Seta 3-4-1, Otsu, Shiga 520-21, Japan

The culture conditions of *Polyporus tuberaster* K2606 were investigated to find conditions with which much benzaldehyde and benzyl alcohol would be obtained. Strain K2606 reduced benzoic acid as well as L-phenylalanine to benzaldehyde and benzyl alcohol in high yield. The conversion rate of benzoic acid was about 60%. Two other metabolites of L-phenylalanine, 3-phenylpropionic acid and 3-phenylpyruvic acid, were reduced to benzaldehyde and benzyl alcohol as well. Veratryl alcohol, a secondary metabolite of L-phenylalanine, was not detected. Benzaldehyde produced by strain K2606 was reduced to benzyl alcohol, which was slowly converted again into benzaldehyde when culture with shaking continued. The maximum concentrations of benzaldehyde and benzyl alcohol produced by strain K2606 were 7.89 and 11.93 mM when L-phenylalanine was added to the culture medium at concentrations of 90 and 45 mM, respectively.

Keywords: *Mushroom; Polyporus tuberaster; biosynthesis; benzaldehyde; benzyl alcohol*

INTRODUCTION

Mushrooms have attracted attention as sources of natural flavors. Many compounds giving rise to flavors in mushrooms have been identified, such as 1-octen-3-ol (Tressl et al., 1982; Chen and Wu, 1984a; Mau et al., 1992), the best-known volatile compound of mushrooms; other aliphatic alcohols; lenthionin (Wada et al., 1967), a characteristic volatile sulfur compound of *Lentinus edodes*; terpenes (Hanssen, 1982, 1985); and lactones (Berger et al., 1986a). The pathways by which such compounds are synthesized have been studied, and strains that can produce the compounds in large amounts have been identified. In particular, there are a number of reports (Grove, 1981; Drawert et al., 1983; Chen and Wu, 1984b; Sastry et al., 1980; Berger et al., 1986b, 1987; Hanssen and Abraham, 1987) and reviews (Hadar and Dosoretz, 1991; Maga, 1981) about volatile compounds of mushrooms cultured in liquid medium. Organoleptic evaluation of 117 strains of mushrooms in liquid culture showed that *Polyporus tuberaster* K2606, which received the highest sensory score of the mushrooms tested, can produce benzaldehyde and benzyl alcohol in high yield from L-phenylalanine (Kawabe and Morita, 1993).

P. tuberaster is a white-rot fungus that grows in Japan, Europe, and North America. White-rot fungi can degrade lignin and lignocellulosic materials. Much work has been done on the biodegradation of lignin, but little is known about the relationship between its biodegradation and the generation of benzaldehyde and benzyl alcohol. Veratryl alcohol (3,4-dimethoxybenzyl alcohol), which is often used as the model substrate of enzymes that degrade lignin, is synthesized from L-phenylalanine (Shimada et al., 1981). Lignin peroxidase and veratryl alcohol peroxidase seem to have the same role in white-rot fungi and may be the same enzyme (Akamatsu et al., 1990).

In this study, the culture conditions that affect the production of benzaldehyde and benzyl alcohol by strain K2606 and the pathway by which these compounds are synthesized were investigated. The purpose was to increase the production of these two compounds.

MATERIALS AND METHODS

Materials. *P. tuberaster* K2606 was a stock culture of Takara Shuzo Food Research Laboratories. Authentic samples of volatile compounds, L-phenylalanine, and aromatic acids were purchased from Tokyo Chemical Industry Co., Ltd., Nacalai Tesque, Inc., and Wako Pure Chemical Industries, Ltd.

Cultivation of Strain K2606. The culture media used were PGY medium (0.2% polypeptone, 2% glucose, 0.2% yeast extract, 0.05% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) and modified Czapek–Dox medium (0–0.2% NH_4NO_3, 0.1% NaCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, and 3% glucose, pH 6.0). A 0.1-mL portion of a vitamin solution (2 mg of biotin, 2 mg of folic acid, 5 mg of thiamin, 5 mg of riboflavin, 10 mg of pyridoxine hydrochloride, 5 mg of nicotinic acid, 5 mg of D-calcium pantothenate, 5 mg of *p*-aminobenzoic acid, and 5 mg of thioctic acid in 1 L of distilled water) prepared according to a modification of the procedure of Kirk et al. (1978) was added to 150 mL of the latter medium. A 150-mL portion of one of the media was inoculated with 2 mL of the culture broth of strain K2606 and incubated at 25 °C in a 500-mL flask with baffles if shaken and without baffles if grown in static culture.

Analysis of Volatile Aromatic Compounds. A 1-mL portion of the culture broth of strain K2606 and 1 mL of ethyl ether were put into a 10-mL centrifuge tube and vigorously mixed for 1 min. The volatile compounds in the upper layer were analyzed by gas chromatography.

Gas Chromatography. A Shimadzu Model GC-9A gas chromatograph equipped with a flame ionization detector and connected to a Shimadzu C-R4A chromatointegrator apparatus was used. The liquid phase of the glass column (3 mm × 2.1 m) was 10% diethylene glycol succinate. Isothermal analysis was done at 170 °C, the temperature of the injector being 200 °C, and the flow rate of the carrier gas, N_2, being 30 mL/min. The injection volume of samples was 2 μL.

Gas Chromatography–Mass Spectrometry. A JEOL JMS-DX302 GC–MS system was used. The liquid phase of the glass column (3 mm × 2 m) was 5% diethylene glycol succinate and 1% H_3PO_4. The column oven temperature was 170 °C, and the flow rate of the carrier gas, He, was 20 mL/min. During MS, the ionization voltage was 70 eV and the ion source temperature was 150 °C.

RESULTS AND DISCUSSION

When cultured with shaking in PGY medium containing L-phenylalanine, strain K2606 produces much

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Table 1. Effects of NH_4NO_3 Concentration on Production of Benzaldehyde and Benzyl Alcohol by Strain K2606

	benzaldehyde		benzyl alcohol	
NH_4NO_3 added (%)	max concn (mM)	incubation time[a] (days)	max concn (mM)	incubation time[a] (days)
0	1.07	22	0.03	22
0.1	1.19	25	0.05	19
0.5	1.22	16	0.09	16
1.0	1.09	13	0.26	13
2.0	0.91	10	0.49	16

[a] Incubation was for 40 days. The day on which the concentration of the compound in question was maximum is given.

benzaldehyde and benzyl alcohol (Kawabe and Morita, 1993). In a discussion of the relationship between lignin metabolism and the secondary metabolism of L-phenylalanine of white-rot fungi when nitrogen is insufficient, Shimada (1992) speculated that the fungi obtain nitrogen in the form of ammonia from amino acids such as L-phenylalanine, generate secondary metabolites without nitrogen, and activate lignin peroxidase to attack lignin. In the exploration of the relationship between the nitrogen content of the medium and the speed at which L-phenylalanine is metabolized, strain K2606 was incubated at 25 °C in 150 mL of the modified Czapek–Dox medium containing 0–0.2% NH_4NO_3, 3 mM L-phenylalanine, and 0.1 mL of the vitamin solution, and the amounts of benzaldehyde and benzyl alcohol produced were measured. During the incubation time of 40 days, a 1-mL portion of the culture broth was sampled every 3 days starting on day 4 of culture. There was little relationship seen between the maximum yield of benzaldehyde and the concentration of NH_4NO_3, but the higher the concentration of NH_4NO_3, the greater was the yield of benzyl alcohol (Table 1): That is, under these experimental conditions, benzaldehyde and benzyl alcohol, which are secondary metabolites of L-phenylalanine, were not produced in greater amounts as the concentration of nitrogen got lower, as predicted by Shimada. Strain K2606 grew well regardless of the concentration of NH_4NO_3.

Strain K2606 did not produce veratryl alcohol from L-phenylalanine, and benzaldehyde was not produced in higher yield when strain K2606 was cultivated with shaking in PGY medium to which 2 mM veratryl alcohol was added compared with when the alcohol was not added. The maximum yield of benzaldehyde in the medium with the alcohol was 0.16 mM, which was almost the same of that in PGY medium containing no veratryl alcohol and much less than 0.85 mM, the maximum yield in PGY medium to which 1.26 mM L-phenylalanine was added (Figure 1). These results suggest that strain K2606 had no enzymes involved in reactions for the addition or elimination of methoxy groups in the metabolic pathway of L-phenylalanine. There was an unidentified peak in the gas chromatograph of the culture fluid of strain K2606 to which veratryl alcohol had been added. The peak was identified as that of veratraldehyde by comparison of its retention time and mass spectrum with those of an authentic sample; this finding suggests that strain K2606 can oxidize aromatic alcohols.

Next, compounds that might be the precursors of benzaldehyde and benzyl alcohol were investigated, together with the metabolites of L-phenylalanine. Of the metabolic pathways of L-phenylalanine in microorganisms, two are known in which the metabolism of the

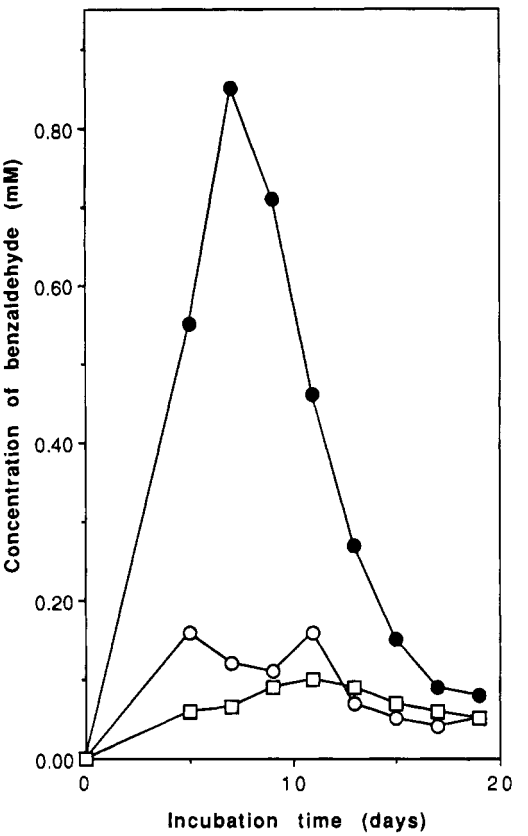

Figure 1. Production of benzaldehyde by strain K2606 in PGY medium containing 2 mM L-phenylalanine or veratryl alcohol: (●) in PGY medium containing L-phenylalanine; (○) in PGY medium containing veratryl alcohol; (□) in PGY medium (control).

amino acid proceeds without the addition of hydroxy groups to the aromatic ring after the elimination of an amino group from L-phenylalanine. In one pathway, the amino group of L-phenylalanine is eliminated by phenylalanine ammonia-lyase, and the *trans*-cinnamic acid generated is then converted into 3-phenylpropionic acid or benzoic acid; in the other pathway, the amino group is eliminated by transaminase or L-amino acid oxidase, and 3-phenylpyruvic acid is generated. Table 2 shows the maximum amounts of benzaldehyde and benzyl alcohol produced by strain K2606 in PGY medium to which 1 mM benzoic acid, 3-phenylpropionic acid, 3-phenylpyruvic acid, or L-phenylalanine was added. The speed of synthesis of benzaldehyde from benzoic acid was the fastest and its yield was greatest. These results showed that strain K2606 has an enzymic system to reduce benzoic acid into benzaldehyde and benzyl alcohol and suggest that phenylalanine ammonia-lyase might be the main enzyme in the metabolic pathway of L-phenylalanine. Chen and Wu (1983) earlier observed that the formation of benzaldehyde and benzyl alcohol could be increased if benzoic acid was mixed with fresh mushrooms. On the other hand, more benzaldehyde and benzyl alcohol were produced by strain K2606 in PGY medium containing 3-phenylpyruvic acid than in PGY medium without this acid, suggesting that strain K2606 had not only a pathway to metabolize L-phenylalanine via *trans*-cinnamic acid, 3-phenylpropionic acid, and benzoic acid but also a pathway for its metabolism via 3-phenylpyruvic acid. We expected to find phenylacetaldehyde and 2-phenylethanol, which are metabolites of 3-phenylpyruvic acid, in the culture fluid of strain K2606 to which 3-phe-

Table 2. Maximum Concentration of Aromatic Compounds Produced by Strain K2606 in a Medium Containing 1 mM Aromatic Acid or L-Phenylalanine

additive	benzaldehyde		benzyl alcohol	
	max concn (mM)	incubation time (days)	max concn (mM)	incubation time (days)
benzoic acid	0.63	4	0.54	8
3-phenylpropionic acid	0.32	10	0.43	15
3-phenylpyruvic acid	0.24	10	0.33	8
L-phenylalanine	0.32	5	0.40	6
none (control)	0.11	5	0.16	8

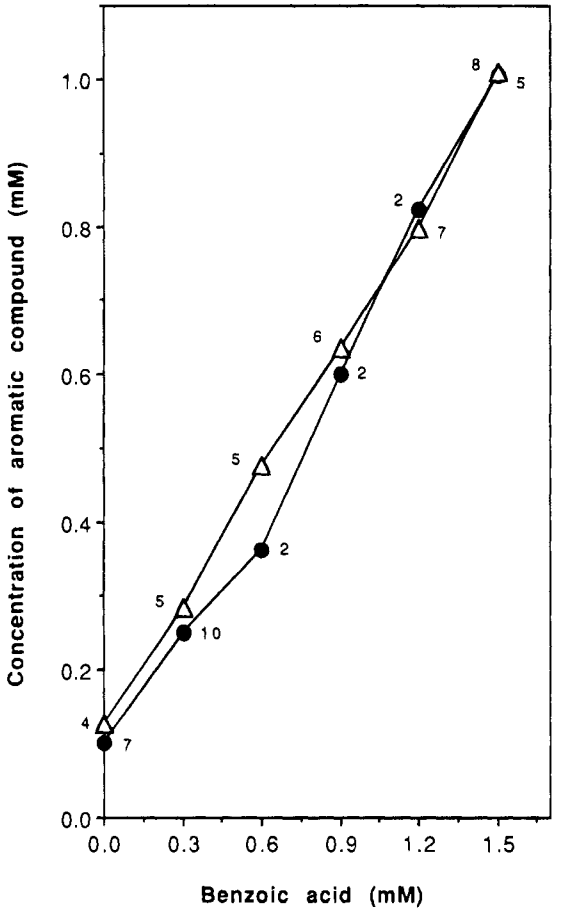

Figure 2. Production of benzaldehyde (●) and benzyl alcohol (△) by strain K2606 in PGY medium containing benzoic acid. Numerals indicate the incubation time (days).

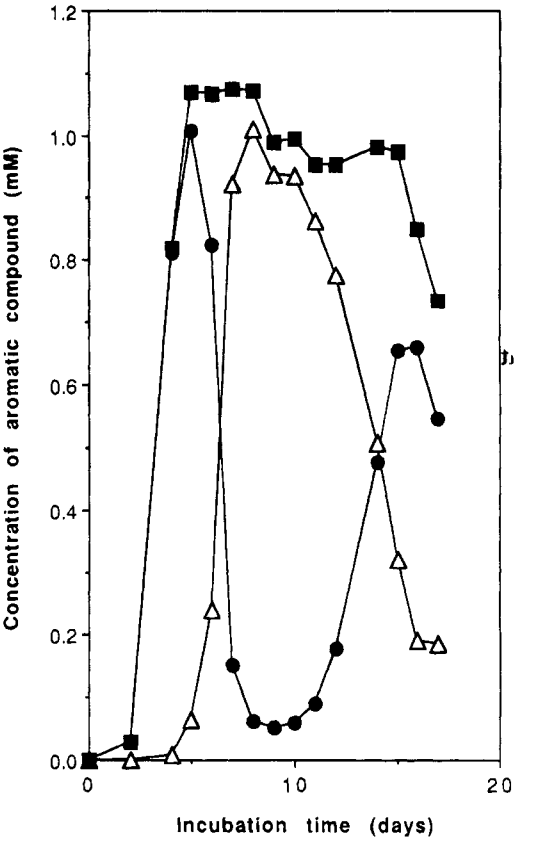

Figure 3. Production of benzaldehyde and benzyl alcohol by strain K2606 in PGY medium containing 1.5 mM benzoic acid: (●) benzaldehyde; (△) benzyl alcohol; (■) benzaldehyde plus benzyl alcohol.

nylpyruvic acid had been added, but only 2-phenylethanol was detected, in a trace amount.

To clarify the relationship between the amount of benzoic acid added to the PGY medium and the yields of benzaldehyde and benzyl alcohol, strain K2606 was cultured with shaking in PGY medium containing 0–1.8 mM benzoic acid, and 1 mL of the culture fluid was sampled every day and analyzed by gas chromatography. Benzoic acid is a food preservative, and strain K2606 could not grow at all in medium containing 1.8 mM benzoic acid. Its growth rate was slightly lower in the medium containing 1.5 mM benzoic acid than in the medium without benzoic acid, but the highest yield of both volatile compounds (both 1.01 mM) was obtained in this medium (Figure 2).

Next, changes with time in concentrations of benzaldehyde and benzyl alcohol in PGY medium to which benzoic acid was added to the final concentration of 1.5 mM were investigated. Both benzaldehyde and benzyl alcohol were metabolized immediately upon being formed. The concentration of benzaldehyde reached a maximum on day 5 and decreased gradually thereafter, but began to increase again on day 10. Benzyl alcohol reached a maximum on day 8 and decreased by half by day 14 (Figure 3). The highest yield of the sum of benzaldehyde and benzyl alcohol was 1.08 mM (0.15 mM benzaldehyde and 0.93 mM benzyl alcohol), and the highest conversion rate of benzoic acid into benzaldehyde and benzyl alcohol (calculated by subtraction of 0.15 mM, the highest yield of benzaldehyde plus benzyl alcohol in the medium containing no benzoic acid, from 1.08 mM, and division of the value by 1.5 mM) was 0.62. Calculated in the same way, the conversion rates in culture fluid to which 0.3, 0.6, 0.9, and 1.2 mM benzoic acid had been added were 0.60, 0.60, 0.58, and 0.58, suggesting that about 60% of the benzoic acid added to the medium could be reduced by strain K2606 without, or at least before, the addition of hydroxy groups. On the other hand, the maximum yield of the sum of benzaldehyde and benzyl alcohol produced by strain K2606 in the medium to which 0.63 mM L-phenylalanine was added was 0.49 mM (0.32 mM benzaldehyde on day 5 of culture in Table 2, at which time benzyl alcohol was 0.17 mM), and the calculated conversion rate of L-phenylalanine into benzaldehyde and benzyl alcohol was 0.54, suggesting that about 90% of L-phenylalanine was

Scheme 1. Proposed Production Pathway of Benzaldehyde and Benzyl Alcohol by Strain K2606: (1) Phenylalanine Ammonia-lyase; (2) Transaminase or L-Amino Acid Oxidase

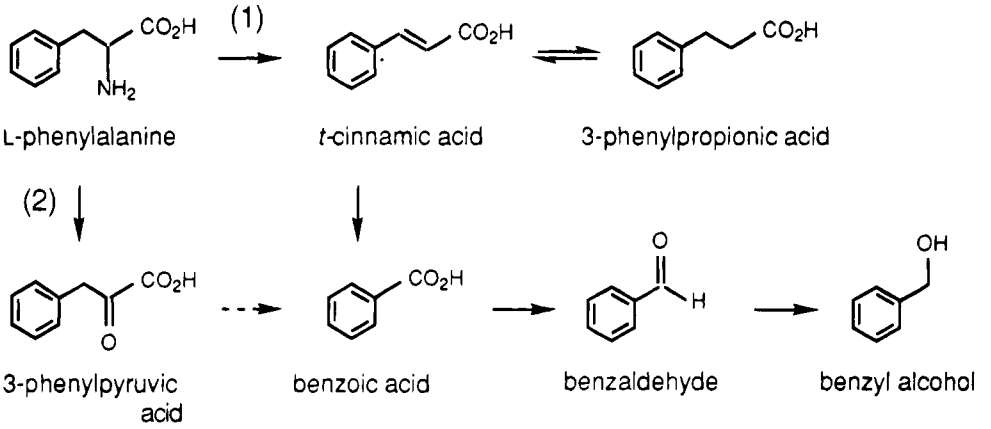

Table 3. Effects of L-Phenylalanine Concentration on Production of Benzaldehyde and Benzyl Alcohol by Strain K2606

	benzaldehyde		benzyl alcohol	
L-phenylalanine (mM)	max concn (mM)	incubation time (days)	max concn (mM)	incubation time (days)
0	0.10	10	0.12	6
15	1.78	30	8.68	18
30	2.73	21	10.60	18
45	2.32	42	11.93	24
60	4.85	45	9.79	21
75	6.84	42	9.28	24
90	7.89	36	9.21	24

metabolized via benzoic acid into benzaldehyde and benzyl alcohol. Considering this conversion rate, 90%, and the antimicrobial activity of benzoic acid, it seems best to culture strain K2606 in a medium to which the optimum amount of L-phenylalanine has been added.

Table 3 shows the maximum concentrations of benzaldehyde and benzyl alcohol produced by cultivation of strain K2606 with shaking in PGY medium to which 0–90 mM L-phenylalanine was added. The maximum concentration of benzaldehyde increased with increasing concentrations of L-phenylalanine and reached 7.89 mM when the amino acid was 90 mM. The maximum concentration of benzyl alcohol was 11.93 mM when L-phenylalanine was 45 mM, but there was not a great difference in the range of 15–90 mM L-phenylalanine. The growth of strain K2606 was poor when the concentration of the amino acid was more than 90 mM.

The changes with time in the concentrations of benzaldehyde and benzyl alcohol shown in Figure 3 indicate that benzoic acid is reduced into benzaldehyde, which is reduced in turn into benzyl alcohol; these steps were confirmed by the following experiment. Benzaldehyde or benzyl alcohol was added to autoclaved PGY medium to the concentration of 2 mM, and strain K2606 was cultured with shaking in these media. The strain grew rapidly after incubation for 2 days and reached the stationary phase by day 6. In the medium to which benzaldehyde was added, benzaldehyde decreased and benzyl alcohol was formed immediately after the cultivation started, but in the medium to which benzyl alcohol was added, the decrease in benzyl alcohol was very slight and benzaldehyde increased little (Figure 4). On the other hand, strain K2606 cultured without shaking in PGY medium with L-phenylalanine produced little benzaldehyde and some benzyl alcohol (Figure 5). These results indicate that the equilibrium of the enzymic system responsible for the reduction of benzaldehyde and the oxidation of benzyl alcohol tended toward the reduction of benzaldehyde when there was little oxygen. More benzyl alcohol might be obtained by incubation of strain K2606 with shaking at first, with the shaking stopped when benzyl alcohol reached its maximum.

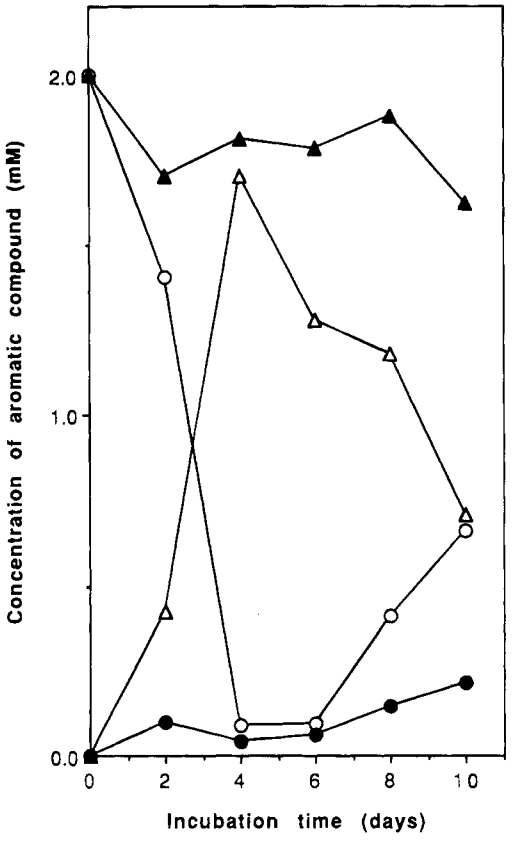

Figure 4. Production of benzaldehyde and benzyl alcohol by strain K2606 in PGY medium containing benzaldehyde or benzyl alcohol: (○) benzaldehyde in PGY medium containing benzaldehyde; (△) benzyl alcohol in PGY medium containing benzaldehyde; (●) benzaldehyde in PGY medium containing benzyl alcohol; (▲) benzyl alcohol in PGY medium containing benzyl alcohol.

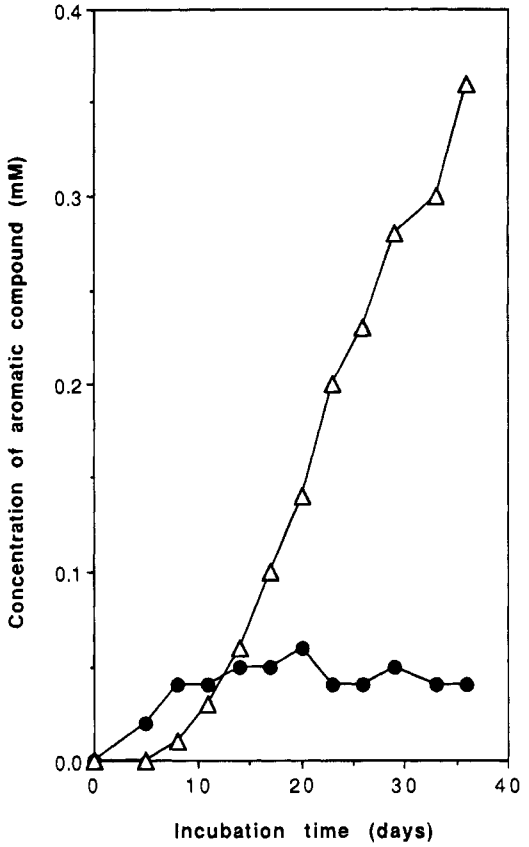

Figure 5. Production of benzaldehyde (●) and benzyl alcohol (△) during cultivation of strain K2606 without shaking.

On the basis of these results, a potential pathway scheme was proposed, which is presented in Scheme 1.

P. tuberaster K2606 could produce benzaldehyde from L-phenylalanine via aromatic acids, mainly benzoic acid, and subsequently reduce it to benzyl alcohol. The main direct precursor of benzaldehyde was benzoic acid, but the concentration of benzoic acid that could be added to the culture medium was limited because of its antimicrobial activity; the use of L-phenylalanine as the precursor gave more benzaldehyde and benzyl alcohol. Thus, it is possible to obtain large amounts of benzaldehyde and benzyl alcohol by control of the cultivation of *P. tuberaster* K2606.

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