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Vanillin Production from Ferulic Acid by the Alkalophilic Bacterium, *Micrococcus* sp. TA1

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The production of vanillin, important as a food-additive, from ferulic acid by the newly isolated *Micrococcus* sp. TA1, which is an alkalophilic bacterium (pH for optimal growth was 10), with a batch cultivation and a continuous cultivation was examined. The optimal vanillin yield (ca. 3.5 %) per amount of ferulic acid consumed under the indicated experimental conditions with continuous cultivation was higher than that (ca. 2.2 %) with batch cultivation. Although the vanillin yield was low, it was confirmed that the production of vanillin from a high feeding concentration of ferulic acid was possible for a long cultivation period with a continuous cultivation apparatus. Investigation of the production of vanillin from ferulic acid with continuous cultivation has not yet been reported.

Introduction

Lignin, which is contained in large amounts in waste lignocellulose must be recycled effectively. However, since lignin is highly resistant to biodegradation, an effective utilization method with chemical and fermentative raw materials has not been established. Although lignin is degradable in alkaline conditions[1,2], because the liquid obtained is a mixture of a small amount of many kinds of aromatic compounds, the problem is how to separate and purify the useful components in the mixture. However, since the mixture contains some materials which can be assimilated and metabolized, the bioconversion of such materials to somewhat valuable materials has been examined in various fields.

On the other hand, rice is a staple food in Japan and other countries in Asia. The quantity of rice produced worldwide is estimated to be approximately 5×10^8 tons per year. While a large amount of rice straw is treated as agrowaste and rice bran is used to produce rice oil, the liquid used to extract the oil from the bran is considered industrial waste. The waste liquid cannot be reused at all. Since considerable amounts of aromatic compounds, especially ferulic acid, are contained in the waste liquid[3], the development of an effective recycling process for those compounds is very important. However, the pH of the waste liquid is usually 10 to 12, making the microbial conversion of aromatic compounds in the liquid difficult.

We have been investigating the development of an effective bioconversion process for aromatic compounds obtained by lignin degradation and those in the waste liquid after the extraction of rice oil from rice bran to additional highly valuable materials. In general, some aromatic compounds, especially ferulic acid, have a high solubility in alkaline solution. Therefore, we isolated a species of alkalophilic bacteria from the alkaline soils near an alkaline hot spring. Bioconversion in a high concentration of aromatic compounds by alkalophilic bacteria should be possible. At present, we expect the development of some effective uses of ferulic acid contained in the waste liquid after the extraction of rice oil to be forthcoming. On the other hand, vanillin is an aromatic flavor for the food industry; however, natural vanillin obtained from plants is very expensive, despite being desirable as a food-additive. In the US and Europe, vanillin obtained from living cells, including food-grade microorganisms and their enzymes is defined as natural flavor [4]. Therefore, the production of vanillin via biotechnological processes may offer a viable alternative to natural and chemical

resources. In this study, the bioconversion of ferulic acid to vanillin by an isolated alkalophilic bacterium was investigated under batch culture or continuous culture conditions.

2. Experimental

2.1. Isolation of lignin model compound-assimilative alkalophilic bacteria

The alkaline soil near an alkaline hot spring was added in the following culture medium: $(NH_4)_2SO_4$, 1.0 g/l; K_2HPO_4, 1.0 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; $CaCl_2 \cdot 2H_2O$, 0.1 g/l; $FeCl_3 \cdot 6H_2O$, 0.02 g/l; Yeast extract, 1.0 g/l; Vanillin, 2.0 g/l. This culture liquid was adjusted to alkaline pH with sodium carbonate. The enrichment culture was repeated using vanillin as the sole carbon source. Some colonies that could assimilate vanillin were obtained by cultivation in a Petri dish. The single colony of the highest assimilation activity was selected.

2.2. Production of vanillin from ferulic acid by the isolated *Micrococcus* sp. TA1 with batch cultivation

The above culture medium containing ferulic acid in place of vanillin was added to water. The culture liquid was autoclaved, and it was adjusted to pH10 with sodium carbonate, aseptically. After *Micrococcus* sp. TA1 was inoculated into the culture liquid (40 ml), the cultivation was carried out for ca. 2 days at 28 ℃ with shaking. The cells obtained aseptically by centrifugation were inoculated in the same fresh culture liquid (100 ml) to be the initial turbidity of 0.2. The main cultivation was initiated at 28 ℃ with shaking (105 strokes/min). The sample liquid (ca. 1 ml) was taken at the beginning of the cultivation and then at arbitrary intervals. The initial concentration of ferulic acid was changed. The influence of the addition of glucose or inositol was also examined. The cell turbidity and pH of the culture liquid were measured respectively by the absorbance at a wavelength of 610 nm and a pH meter. The high pressure liquid chromatograph (HPLC; column: TSK-GEL, detector: UV-8020 (λ = 254 nm), Tosoh) was used to measure the concentration of ferulic acid and vanillin in the culture liquid after removal of the cells.

2.3. Continuous production of vanillin from ferulic acid by the isolated *Micrococcus* sp. TA1

A commercial jar fermenter (Jar Fermenter TFL5, Chiyoda) was used as the main culture tank (working volume; 2000 ml). Air was supplied at the rate of 0.18 Nm^3/h from the bottom of the tank, the air and the cells were dispersed by agitation paddles (400 rpm) attached to the tank and the air was exhausted from an air outlet of top of the tank. On the other hand, the culture liquid, containing 1.5～6.9 g/l ferulic acid in the culture liquid reservoir (3500 ml), was supplied to the tank through a microtube pump at the rate of 60 ml/h. The supply rate of the culture liquid was controlled by the microtube pump. The components of the culture liquid was the same as the case of the batch cultivation described above. The culture liquid supplied was taken out through the other microtube pump at the rate of 60 ml/h and recovered to a fraction collector. The initial cell turbidity in the culture tank was 0.2. The initial concentration of ferulic acid in the culture liquid in the culture tank was the same as that in the culture liquid reservoir. The culture temperature was maintained at 30 ℃. The sample liquid (5 ml) was taken at the beginning of the cultivation and then at arbitrary intervals. The cell turbidity of the culture liquid was measured by the absorbance at a wavelength of 610 nm. The high pressure liquid chromatograph (HPLC; column: TSK-GEL, detector: UV-8020 (λ = 254 nm), Tosoh) was used to measure the concentration of ferulic acid and vanillin in the culture liquid after removal of the cells.

3. Results and Discussion

3.1. Identification of the isolated bacterium

The optimal temperature of growth of the isolated bacterium was 28°C. The cells were spherical, Gram-positive, non-sporular and had no flagella. The

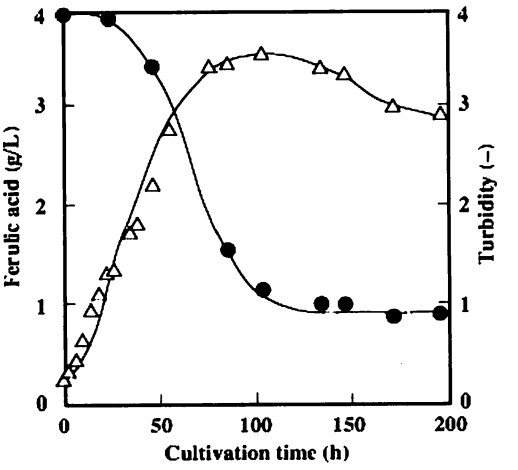

Fig. 1. Vanillin production from ferulic acid by *Micrococcus* sp. TA1 with batch cultivation. △, Cell turbidity(–); ●, Concentration of ferulic acid (g/l). The initial concentration of ferulic acid and the initial cell turbidity were 4.0 g/l and 0.2, respectively.

catalase activity was positive, and the oxidase activity was negative. From these characteristics and the gene analysis of 16S ribosomal RNA (data not shown), the isolated bacterium was identified as *Micrococcus* sp. TA1.

3.2. Production of vanillin from ferulic acid with batch cultivation

Figure 1 shows the production of vanillin from ferulic acid (initial concentration; 4.0 g/l) with batch cultivation. With the growth of the cells, ferulic acid was consumed, but vanillin was not produced. With a decrease in the growth activity of the cells, the consumption of ferulic acid ceased. This tendency was observed when the initial concentration of ferulic acid was 8.0 g/l (data not shown). Figure 2 shows the production of vanillin from ferulic acid (initial concentration; 12.0 g/l) by the cells with a batch cultivation. With the growth of the cells, ferulic acid was consumed. However, the production of vanillin began after ca. 80 h of cultivation, reached a maximum of ca. 130 mg/l at ca. 120 h of cultivation and decreased as the cultivation time increased. The maximum yield of vanillin per amount of ferulic acid consumed was ca. 2.2 %. After the logarithmic phase of cell growth, the cell concentration increased. This phenomenon seemed to depend on the change of the culture liquid to brown during the long cultivation period. In fact, the growth of the cells was slow after the logarithmic phase. In the case of the initial concentration of ferulic acid being16.0 g/l, the same tendency was observed, and the maximum concentration of vanillin was also almost the same. This suggests that the optimal initial concentration of ferulic acid for vanillin production was nearer to 12 g/l. The addition of glucose (2.0 or 4.0 g/l) or inositol (2.0 g/l) scarcely increased the concentration of vanillin (data not shown). That is, the maximum concentration of vanillin under the indicated culture conditions was ca. 130 mg/l. Obviously the productivity of vanillin from ferulic acid by *Micrococcus* sp. TA1 appeared to be extremely low. On the other hand, the production of vanillin from ferulic acid by various microorganisms has been reported[5] ~ [9]. In some of these results, the productivity of vanillin from ferulic acid by only one kind of microorganism appeared to be considerably high. However, in general, the production of a high yield of vanillin from ferulic acid by only one kind of microorganism seemed to be very difficult. The production of vanillin from ferulic acid by only one kind of microorganism with continuous cultivation has not been reported.

3.3. Production of vanillin from ferulic acid with continuous cultivation

Figure 3 shows the continuous production of vanillin under the directed cultivation conditions. The initial concentration of ferulic acid in the culture tank and the feeding concentration to the tank were both 6.9 g/l. The feeding rate was 60 ml/h. The working volume in the tank was 2000 ml as described above. The cell turbidity in the tank reached ca. 1.0 at a steady state after ca. 50 h of

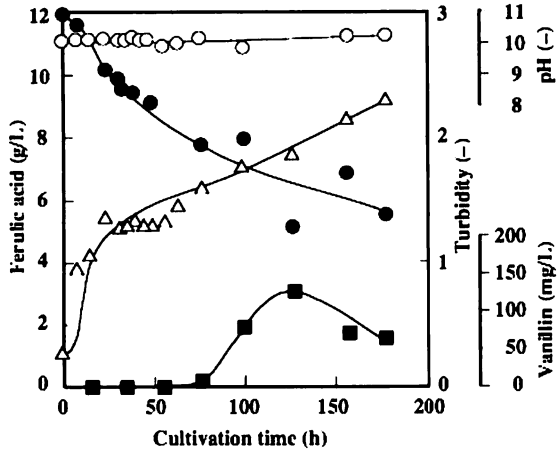

Fig. 2. Vanillin production from ferulic acid by *Micrococcus* sp. TA1 with batch cultivation. △, Cell turbidity (–); ●, Concentration of ferulic acid (g/l). ■, Concentration of vanillin (mg/l); ○, pH (–). The initial concentration of ferulic acid and the initial cell turbidity were 12.0 g/l and 0.2, respectively.

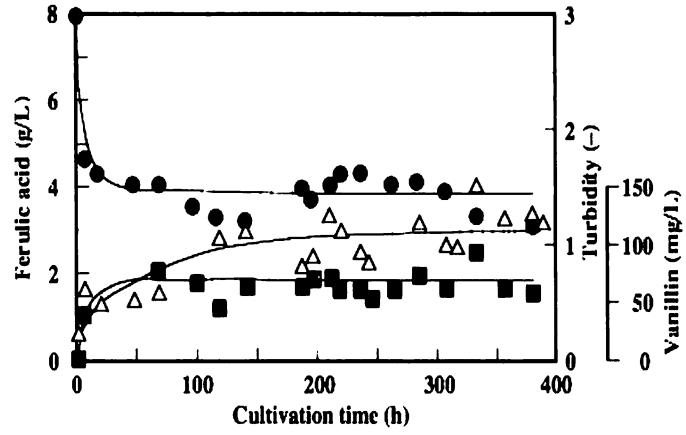

Fig. 3. Vanillin production from ferulic acid by *Micrococcus* sp. TA1 with continuous cultivation. △, Cell turbidity (–); ●, Concentration of ferulic acid in the tank (g/l); ■, Concentration of vanillin produced in the tank (mg/l). The initial concentration of ferulic acid in the tank was 6.9 g/l. The feeding rate of ferulic acid (6.9 g/l) was 60 ml/h. The aeration rate to the tank was 0.18 Nm^3/h.

cultivation. After ca. 50 h of cultivation, the consumption of ferulic acid and the production of vanillin reached a steady state, and the steady state continued up to at least ca. 400 h of cultivation. The consumption of ferulic acid and the production of vanillin under steady state conditions were ca. 1.6 g and ca. 0.056 g, respectively, that is, the vanillin yield per amount of ferulic acid consumed was ca. 3.5 %. The vanillin yield with an initial ferulic acid concentration of 3.6 g/l and 1.5 g/l was ca. 0.57 % and 0 %, respectively. A higher vanillin yield was achieved with a higher initial concentration of ferulic acid; however, the consumption ratio of ferulic acid could not be increased. In the case that the initial ferulic acid concentration in the tank was 3.6 g/l, the cell turbidity under steady state conditions as described above. In the former case, the average concentration of ferulic acid in the tank in the steady reached ca. 2.5. However, in the case that the initial concentration was 6.9 g/l, the cell turbidity was ca. 1.0, state was ca. 1.8 g/l and in the latter case, it was ca. 4.0 g/l. In the latter case, the high concentration of ferulic acid probably decreased the cell growth activity over the long period of cultivation. Therefore, a high concentration of ferulic acid was kept in the tank, and a high vanillin yield was not obtained. Considering the results of the batch cultivation, it may be effective to the increase of the vanillin yield to increase the initial cell turbidity in the culture tank in order to increase the consumption rate of ferulic acid with a continuous or a fed-batch cultivation.

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