THE BULLETIN OF OKAYAMA UNIVERSITY OF SCIENCE No. 41 A pp. 163-168 (2005)

Efficient Conversion of Some Aromatic Compounds by a Neutral Bacterium and an Alkalophilic Bacterium

Ryoji MITSUI, Chihiro KOBAYASHI*, Takuo TSUNO* and Mitsuo TANAKA

Department of Biochemistry, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama city, Okayama 700-0005, Japan

* *Tsuno Rice Fine Chemicals Co. Ltd., 2283 Chonomachi, Katsuragi-cho, Ito-gun, Wakayama 649-7194, Japan*

(Received July 26, 2005; accepted November 7, 2005)

Some aromatic compounds such as ferullic acid and vanillin have a high solubility in an alkaline solution. Therefore, to carry out an efficient conversion reaction at high concentrations of aromatic compounds in the alkaline solution, *Micrococcus* sp. TA1, an alkalophilic bacterium isolated from soil at alkaline hot springs, was used. *Micrococcus* sp. TA1 and *Burkholderia cepacia* TM1, which have a high level of activity over alkaline and neutral pH ranges, respectively, were used to investigate the conversion activity of some aromatic compounds and the characteristics of cell growth. *B. cepacia* TM1 converted some aromatic compounds to the corresponding aromatic carboxylic acids and these acids were accumulated out of the cells. The acid was recovered at high yield by using a cell-holding reactor into which an aromatic aldehyde was supplied continuously with a neutral pH solution. *Micro.* sp. TA1 did not grow at a neutral pH and showed good growth activity over an alkaline pH range as a kind of alkalophilic bacteria, when the carbon source was ferulic acid or vanillin. None of the metabolites was detected in the culture liquid, though the substrate was consumed quickly. However, *Micro.* sp. TA1 may be useful for the treatment of alkaline waste liquid such as the liquid used to extract the oil from rice bran.

1. Introduction

Rice is a staple food in Japan and other countries in Asia. The quantity of rice produced worldwide is estimated to be approximately $5x10^8$ tons per year. While a large amount of rice straw is treated as agrowastes and rice bran is used to produce rice oil, the liquid used to extract the oil from the bran is considered industrial wastes. The waste liquid can not be reused at all. Since considerable amounts of aromatic compounds are contained in the waste liquid, 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.

Recently, we isolated bacteria which could convert aromatic compounds over neutral pH range (*Burkholderia cepacia* TM1) and alkaline pH range (*Micrococcus* sp. TA1). The bacterial characteristics of cell growth and activity to convert aromatic compounds were investigated.

2. Experimental

2-1 A cell-holding reactor system[1,2)]

Figure 1 shows the outline of a cell-holding reactor system for the continuous oxidation of an aromatic aldehyde to the corresponding aromatic carboxylic acid [1)]. A device for ultrafiltration (Amicon, USA) was used as the reactor ⑩. An ultra-

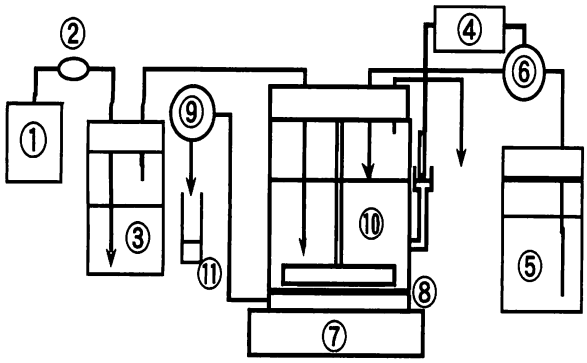

Fig. 1. Outline of a cell-holding reactor system for the continuous oxidation of aromatic aldehyde. ①, air compressor; ②, air filter; ③, water reservoir; ④, liquid level controller; ⑤, substrate reservoir; ⑥, microtube pump; ⑦, stirrer; ⑧, ultramembrane filter; ⑨, microtube pump; ⑩, reactor; ⑪, product collector

membrane filter PM 10 (Amicon) ⑧ was used to retain *B. cepacia* TM1 in the reactor. The working volume in the reactor was held constant by connecting the liquid surface controller ④ and the microtube pump ⑥ to feed the aromatic aldehyde to the reactor from the substrate reservoir ⑤. Air passing through the air filter ② from the air compressor ① was aseptically fed to the solution in the reactor as damp air via the water reservoir ③, and exhausted from the top of the reactor. The solution in the reactor was agitated by the stirrer ⑦. When the reaction was initiated, the solution containing thearomatic aldehyde was fed to the reactor through the microtube pump ⑥ and the reaction solution was effused from the reactor with a constant flow rate by the microtube pump ⑨. The effused reaction solution was recovered into the product collector ⑪ during the reaction. The average residence time of the reaction solution in the reactor was appropriately adjusted using the microtube pump ⑨.

2-2 Reaction conditions and analytical methods

Tanaka and Hirokane [2] reported that the oxidation of aromatic aldehydes by *B. cepacia* TM1 occurred in distilled water lacking nutritive compounds other than an aromatic aldehyde. On the basis of those findings, the continuous oxidation of aromatic aldehydes was performed as follows [1]. The reaction temperature was 28℃, and the reaction solution was 0.2 M or 0.05 M phosphate buffer (pH7.2) containing either vanillin, *p*-hydroxybenzaldehyde or syringaldehyde. The feeding concentration to the reactor was 2.0 or 20.0 g/l for vanillin, 2.0 or 20.0 g/l for *p*-hydroxybenzaldehyde and 4.0 g/l for syringaldehyde. All reaction solutions were autoclaved and adjusted to an initial pH of 7.2, and active cells obtained by several rounds of pre-culture were aseptically added to the reactor. The initial turbidity of the cells was changed appropriately depending on the type and concentration of the aromatic aldehyde used as the substrate and was 0.4, 10, 30 or 55 as an absorbance value at a wavelength of 540 nm. The initial concentration of aromatic aldehyde in the reactor was 0 g/l. The working volume in the reactor was 35 or 150 ml of the reaction solution containing the cells. The air flow rate was 40 ml/min when the initial turbidity of the cells was 0.4, and in other cases, 100 ml/min. When the reaction was initiated, the reaction solution containing an aromatic aldehyde was fed to the reactor. The reaction solution was effused from the reactor with a constant flow rate and collected during the reaction. The pH and the concentration of the aromatic aldehyde and products in the reaction solution recovered at the outlet of the reactor at a constant time interval were measured, respectively, using a pH meter and HPLC. The analytical conditions for HPLC were as follows: column ; ODS80TM (4.6 x 25 cm), eluent; [water : acetic acid = 99 : 1] : methanol = 4 : 1, flow rate; 0.9 ml/min, detection; 254 nm. The cumulative quantity of the aromatic aldehyde and products in all collected

reaction solutions were also measured as described above. A calibration curve was obtained using each substrate as a standard. The molar yield was estimated as the ratio of the moles of the aromatic carboxylic acid produced to those of the aromatic aldehyde fed, during the steady state of the reaction. All reagents used were of guaranteed grade.

2-3 Isolation of alkalophilic bacteria capable of assimilating lignin model compounds

B. cepacia TM1 active over a neutral initial pH range was isolated according to the method of Tanaka and Hirokane[1]. Alkalophilic bacteria capable of assimilating lignin model compounds were isolated by repeating enrichment culture with an alkaline solution containing vanillin as a carbon source. The alkaline medium was composed of components listed in **Table 1**.

Table 1. Components of the alkaline basal 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

pH was adjusted with Na_2CO_3

The initial pH of the medium was adjusted with sodium carbonate. Finally a kind of bacterium, which showed good growth activity in an alkaline medium, was isolated.

Incidentally, the culture or reaction temperature of all the subsequent experiments was 28℃.

3. Results and Discussion

3-1 Identification of bacteria isolated

From the morphological characteristics and physiological properties, the bacterium having strong growth activity in a neutral medium and in an alkaline medium was identified as *Burkholderia cepacia* and *Micrococcus* sp., respectively. The species of *Micrococcus* could not be identified, because it was analogous to *luteus*, but did not fit on the basis of the gene analysis of 16S-RNA.

3-2 Oxidation of aromatic aldehyde by *B. cepacia* TM1

3-2-1 Metabolism of vanillin in batch culture by *B. cepacia* TM1

B. cepacia TM1 grew well in a neutral pH medium containing vanillin as a carbon source. The culture liquid taken out at an appropriate time was analyzed by HPLC. The results obtained are shown in **Figure 2**. Vanillin (2 g/l) was consumed completely in about 24 h. During the period of consumption, vanillic acid was produced in high yield in the medium.

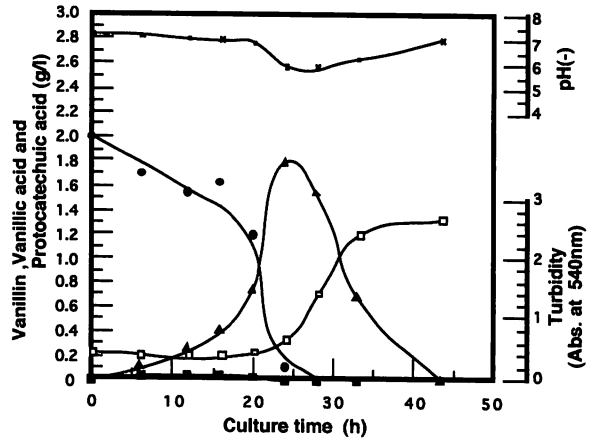

Fig. 2. Time course of vanillin oxidation by *B. cepacia* TM1 cells in a batch culture. x, pH of the culture liquid; □, optical density of cells; ○, vanillin; ▲, vanillic acid

3-2-2 Continuous oxidation in a cell-holding reactor

On the basis of the above result, a continuous oxidation of vanillin to vanillic acid by *B. cepacia* TM1 held in the reactor ⑩ using the apparatus shown in Fig.1 was carried out with only vanillin as a substrate

as described in 2. Experimental. The reaction solution passed through the ultra-membrane filter was recovered at an appropriate time and products and residuals analyzed by HPLC. The optimal continuous oxidation of vanillin by *B. cepacia* TM1 rest cells in the cell-holding reactor (see Fig. 1) was carried out. Vanillin was dissolved in 0.2 M phosphate buffer (pH 7.2). The operation conditions were as follows: working volume: 150 ml, cell concentration in the reactor: 30 at 540 nm wavelength, concentration of vanillin at the inlet of the reactor: 20.0 g/l, feeding rate: 0.110 g/l and average residence time: 27.3 h. Vanillic acid was found to be produced in very high yield over 800 h or more. The vanillin supplied as a substrate into the reactor was almost completely consumed in the residence time. The molar yield of vanillic acid produced from the vanillin supplied was 95% (0.770 g/l/h) in Fig.3.

3-2-3 Continuous oxidation of aromatic aldehyde to the corresponding acid

In the nutritive medium containing a *p*-hydroxybenzaldehyde or syringaldehyde other than vanillin as a carbon source, *B. cepacia* TM produced the corresponding acid in the medium of a batch culture (data not shown). On the basis of the results, a continuous oxidation of those aldehydes was performed as described for vanillin as a substrate and the corresponding acid, that is *p*-hydroxybenzoic acid or syringic acid, was obtained. Although the reaction rate was different depending on the substrate, a high molar yield was obtained for all substrates. In the case of the optimal operation conditions, the molar yield of *p*-hydroxybenzoic acid or syringic acid produced from *p*-hydroxy-benzaldehyde or syringaldehyde supplied was 80% (0.350 g/l/h, period of steady state: 450 h) or 96 % (0.169 g/l/h, period of steady state: 160 h), respectively. The metabolic rate for ferulic acid, which was abundant in the waste liquid obtained by the extraction of rice oil from bran, by *B. cepacia* TM1 was extremely high (data not shown). None of the metabolic products was detected in the culture medium. The production process of a useful substance such as vanillin from ferulic acid using a metabolic system of microbial cells should be developed.

3-3 Conversion of lignin model compounds by *Micro.* sp. TA1 under alkaline conditions

3-3-1 Vanillin degradation by *Micro.* sp. TA1

Micro. sp. TA1 grew well with vanillin as a carbon source at an initial pH of 10. According to the procedure used in the case of *B. cepacia* TM1, the culture liquid removed at an appropriate time was analyzed by HPLC. **Figure 3** shows the results obtained. *Micro.* sp. TA1 also could completely consume vanillin in the culture time of 24 h. However, vanillic

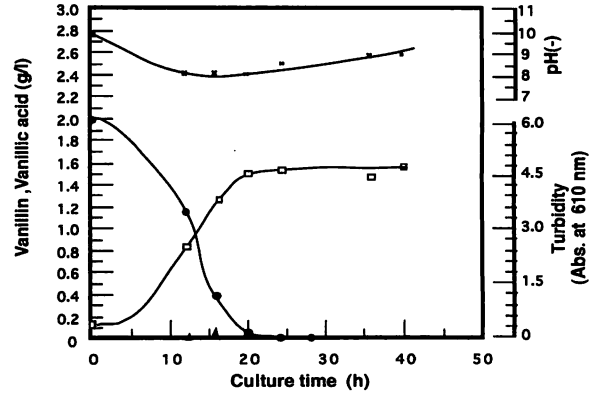

Fig. 3. Time course of vanillin metabolism by *Micro.* sp. TA1 cells in a batch culture with an initial pH of 10. x, pH of the culture liquid; □, optical density of cells; ●, vanillin; ▲, vanillic acid.

acid could not be detected in the culture liquid during the culture period, different from the case of *B. cepacia* TM1 active in a neutral pH range. That is, none of the products was detected in the culture liquid.

3-3-2 Influence of initial pH on vanillin conversion by *Micro.* sp. TA1

The cultivation of *Micro.* sp. TA1 was performed at pH8.5 or 7.0 as the case of pH10. **Figures 4** and **5** show the results obtained at pH8.5 and 7.0, respectively. When the initial pH of the culture medium was neutral, the cell growth and vanillin consumption were extremely low compared with the case for the alkaline conditions. These findings suggest that *Micro.* sp. TA1 is a kind of alkalophilic bacteria.

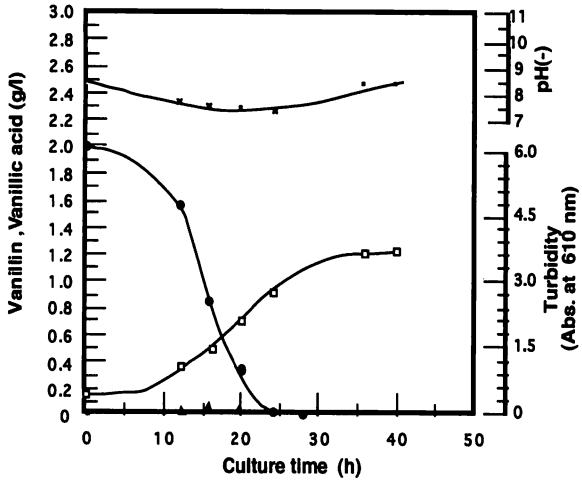

Fig. 4. Time course of vanillin metabolism by *Micro.* sp. TA1 cells in a batch culture with an initial pH of 8.5. x, pH of the culture liquid; □, optical density of cells; ●, vanillin; ▲, vanillic acid

3-3-3 Metabolism of ferulic acid by *Micro.* sp. TA1

Ferulic acid is contained in large amounts in rice bran. The development of a method to convert ferulic acid to a more useful substance has been studied[3,4]. For example, the production of vanillin from ferulic acid by enzymes or microorganisms has been investigated. However, the presence of microorganisms capable of metabolizing ferulic acid under alkaline conditions has not been reported. Therefore, the conversion of ferulic acid under alkaline conditions by *Micro.* sp. TA1 was investigated. **Figure 6** shows the results obtained. Ferulic acid was metabolized at almost the same rate as vanillin (see Fig. 3). However, no metabolic products were detected in the

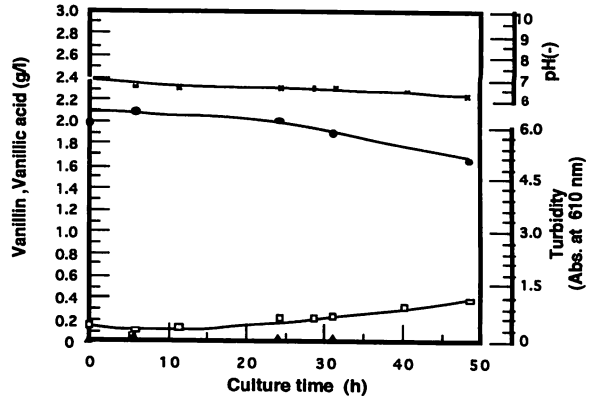

Fig. 5. Time course of vanillin metabolism by *Micro.* sp. TA1 cells in a batch culture with an initial pH of 7. x, pH of the culture liquid; □, optical density of cells; ●, vanillin; ▲, vanillic acid

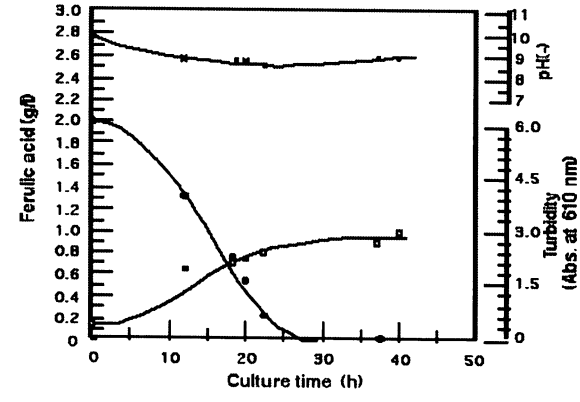

Fig. 6. Time course of ferulic acid metabolism by *Micro.* sp. TA1 cells in a batch culture with an initial pH of 10. x, pH of the culture liquid; □, optical density of cells; ●,ferulic acid

culture liquid. The influence of the initial pH on the conversion of ferulic acid by *Micro.* sp. TA1 was also investigated, and was very similar to the case for vanillin (data not shown).

4. Conclusions

B. cepacia TM1 converted some aromaric compounds to the corresponding aromatic carboxylic acids and these acids were accumulated out of the cells. The acid was recovered at high yield by using a cell-holding reactor into which an aromatic aldehyde was supplied continuously with a neutral pH solution. *Micro.* sp. TA1 did not grow at a neutral pH and showed good

growth activity over an alkaline pH range as a kind of alkalophilic bacteria, when the carbon source was ferulic acid or vanillin. None of the metabolites was detected in the culture liquid, though the substrate was consumed quickly. However, since *Micro.* sp. TA1 had the high metabolic activity for above two compounds, in the future, an effect treatment method of the industrial waste liquid such as that after extraction of the oil from rice bran, must be developed by investigating the metabolic activity of *Micro.* sp. TA1 for the other compounds in the liquid, and the production of more useful substances from those compounds must be tried.

References

(1) Tanaka, M., Hirokane, Y., Mitsui, R. and Tsuno, T., *J. Biosci. Bioeng.*, **91**, 267-271 (2001).
(2) Tanaka, M. and Hirokane, Y., *J. Biosci. Bioeng.*, **90**, 341-343 (2000).
(3) Civolani, C., Barghini, P., Roncetti, A. R., Ruzzi, M. and Schiesser, A., *Appl. Environ. Microbiol.*, **66**, 2311-2317 (2000).
(4) Lesage-Meessen, L., Delattre, M., Haon, M., Thibault, J.-F., Ceccaldi, B. C., Brunerie, P. and Asther, M., *J. Biotechnol.*, **50**, 107-113 (1996).