Efficient L-Serine Production from Methanol and Glycine by Resting Cells of Methylobacterium sp. Strain MN43

Tairo HAGISHITA, Toyokazu YOSHIDA, Yoshikazu IZUMI,* and Toshio MITSUNAGA

Department of Food and Nutrition, Faculty of Agriculture, Kinki University, Nara 631, Japan
*Department of Biotechnology, Faculty of Engineering, Tottori University, Tottori 680, Japan

Received February 9, 1996

Resting cells of methanol-utilizing microorganisms isolated from soils were examined for L-serine production under conditions in which L-serine-degradation was suppressed. Strain MN43, a facultative methylotrophic bacterium identified as a Methylobacterium sp., was selected for further studies. Under the optimal conditions, 65 mg/ml L-serine was produced by this bacterium from 50 mg/ml glycine and 104 mg/ml methanol in 5 days, with a molar conversion ratio from glycine to L-serine of 93%. This production is the highest so far reported for microbes producing L-serine.

Key words: L-serine production; methylotroph; Methylobacterium; serine pathway; enzymatic production

There have been several reports on L-serine production from glycine and methanol by methylotrophic bacteria with the serine pathway, such as Pseudomonas sp.,1) Hyphomicrobiurn methylivororum,2,3) Hyphomicrobiurn sp. strain NCIB10099,4,5) and Methylobacterium extorquens.6) The enzymes involved in L-serine synthesis are methanol dehydrogenase and serine hydroxymethyltransferase. The former catalyzes the oxidation of methanol to formaldehyde, and the latter converts formaldehyde and glycine to L-serine by an aldolistic reaction of serine hydroxymethyltransferase. Enzymatic L-serine production by resting cell systems is possible with H. methylivororum and Hyphomicrobiurn sp. strain NCIB10099,3) and suppression of L-serine degradation is important for efficient L-serine production.4,5) With cells of Hyphomicrobiurn sp. strain NCIB10099, 53 mg/ml L-serine is produced from 100 mg/ml glycine when L-serine degradation is suppressed by maintenance of the methanol concentration at 14.1-29.6 mg/ml methanol, under appropriate aerobic conditions.5) However, the conversion ratio of glycine to L-serine was only 38%. In this study, screening was done under conditions that suppressed L-serine degradation, and one methylotroph, Methylobacterium sp. strain MN43, that produced more L-serine was found.

Materials and Methods

Materials. All chemicals were of reagent grade and were obtained from commercial sources.

Organisms. Hyphomicrobiurn sp. strain NCIB10099 was previously reported as a serine-producing methylotroph.4,6) H. methylivororum GM2 was a glycine-resistant mutant derived from H. methylivororum KM146.21

Isolation of methanol-utilizing microorganisms. Medium II used for the isolation and cultivation of methanol-utilizing microorganisms was described previously.2) Soil samples were put in 5 ml of medium II containing 1% methanol, and the culture was shaken at 28°C for 2-4 days. Colonies of the strains were isolated by being streaked on a plate of the same medium. After 5-10 days, the colonies formed were used to inoculate a plate of medium II containing 1% methanol, and the plate was cultivated at 28°C for 3 or 4 days. Strains that grew rapidly on the plate were stored on agar slants of medium II containing 1% methanol before use.

Culture conditions. Methanol-utilizing microorganisms were inoculated in 5 ml of medium II containing 1% methanol, and the culture was incubated at 28°C for 2 days with reciprocal shaking (250 strokes/min). The culture was transferred to 100 ml of the same medium in a 500-ml shaking flask, which was incubated at 28°C for 2 days with reciprocal shaking (160 strokes/min).

Preparation of resting cells. Cells were harvested from the culture broth by centrifugation at 8000 × g for 10 min, washed once with 0.15 M NaCl, and suspended in the same solution. This cell suspension was used for L-serine production.

L-Serine production. The standard reaction mixture contained 25 mg of cells (dry weight), 50 μmol of Tris–HCl buffer (pH 9.0), 56 mg of methanol, and 100 mg of glycine in a final volume of 1 ml in a 16.5-mm-diameter test tube with a silicon gum plug (Silo-sea, Nichiden Rika, Kobe). The mixture was kept at 28°C for 3 days with reciprocal shaking (250 strokes/min), and 16 mg of methanol was added each day. Then, the mixture was centrifuged at 8000 × g for 10 min and the supernatant was assayed for L-serine.

Analysis. Growth of cells was measured in terms of the optical density at 610 nm of the culture broth. L-Serine and glycine were assayed by HPLC (Shimadzu LC-6A) on a column of Wakoosil SC18 (4.6 × 150 mm, Wako Pure Chemical Industries, Ltd., Tokyo). Elution was done with a mixture of 1.0 mM KH2PO4, 0.5 mM CuSO4, and 7.5 mM 1-heptanesulfonic acid. The flow rate of the buffer was 1.0 ml/min, and L-serine and glycine were detected at 230 nm as chelate complexes with copper ions. The methanol concentrations were measured by gas-liquid chromatography with a Porapak Q column (Nihon Millipore Ltd.) and a flame ionization detector.

Results

Screening of methanol-utilizing microorganisms for L-serine production

About 200 methanol-utilizing microorganisms were isolated from soils by enrichment culture on medium II containing 1% methanol. Sixty-eight of these strains grew rapidly, and 32 strains produced > 1 mg/ml L-serine within 2 days under conditions where L-serine degradation was suppressed. Strains MN37 and MN43 produced 20 and 36 mg/ml L-serine, respectively, in 2 days, so strain MN43 was used in later experiments.

* To whom correspondence should be addressed.
Identification of strain MN43

Taxonomical characteristics of strain MN43 were investigated (Table 1). This strain utilized ethanol, methylvamine, formate, acetate, citrate, succinate, malate, fumarate, glucose, fructose, and L-glutamate as well as methanol as the carbon source. We concluded from our results and data provided by the National Collection of Industrial and Marine Bacteria, Aberdeen, U.K., that this strain belongs to the genus Methylobacterium, but that it is not any of the eight known species.

Comparison of L-serine production by Methylobacterium sp. strain MN43, Hyphomicrobium sp. strain NCIB10099, and H. methyllycorum GM2

We previously established that L-serine is produced by resting cells of H. methyllycorum GM2 and Hyphomicrobium sp. strain NCIB10099. The L-serine production of Methylobacterium sp. strain MN43 was compared with that of the two Hyphomicrobium strains. L-Serine production was brought about in different glycine concentrations in the standard reaction mixture (Fig. 1). At 25 mg/ml glycine or less, Methylobacterium sp. strain MN43 produced L-serine with a high conversion ratio (>90%), but the ratio with the two Hyphomicrobium strains was lower (<60%).

Conditions for L-serine production by resting cells of Methylobacterium sp. strain MN43

The effects of the initial concentrations of methanol and glycine and of the cell density on L-serine production by resting cells of Methylobacterium sp. strain MN43 were investigated (Fig. 2). The L-serine production was highest when the reaction mixture contained 32-56 mg/ml methanol, 50-80 mg/ml glycine, and 20 mg/ml cells (dry weight). A glycine concentration of 50 mg/ml gave the highest molar conversion ratio of glycine to L-serine.

The effects of various buffers at different pHs were examined. L-Serine was produced with buffer of Tris–HCl (pH 7.0-9.5), potassium phosphate (pH 6.0-8.0), H3BO3–KCI–NaOH (pH 9.0), and Na2HPO4–NaOH (pH 11.0). Of these, results were best with Tris–HCl (pH 9.0). The optimum concentration of Tris–HCl (pH 9.0) buffer was 50 mm.

Effects of culture conditions on L-serine production

The effects of the initial pH, the nitrogen source, and the initial methanol concentration in culture medium on growth and L-serine production were examined. When the initial pH of medium II containing 1% methanol was 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, 36, 22, 44, 32, 29, 25, and 19 mg/ml L-serine was produced. The number of cells and L-serine produced were greatest at pH 7.0.

Of nitrogen sources tested, sodium nitrate allowed the highest production, 51.6 mg/ml (Table II). L-Glutamate and malt extract inhibited L-serine production.

The effect of the initial methanol concentration in the culture medium on L-serine production was investigated. After Methylobacterium sp. strain MN43 was cultured in medium II containing 0.1–2.0% methanol for 2 days, the amount of methanol remaining in the medium was assayed, and the harvested cells were added to a reaction mixture. When cells cultured in medium II containing 0.5% methanol were used, activity was high for all 3 days of the reaction (Table III). Methanol was not detected in this medium after cultivation, suggesting that methanol reduced L-serine production. With medium II containing 1% methanol, the effect of cultivation time was examined. When the bacterium was cultivated for at least 3 days, methanol

Table 1. Taxonomical Properties of Strain MN43

<table>
<thead>
<tr>
<th>Morphological characteristics</th>
<th>Oxidase reaction</th>
<th>Temperature for growth</th>
<th>pH for growth</th>
<th>Carbon source for growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape and size of cells: rods (0.8 x 3.0 µm)</td>
<td>Temperature about 28°C</td>
<td>to 37°C, optimum</td>
<td>6 to 9, optimum pH about 7</td>
<td>Methanol, ethanol, methylamine, formate, acetate, citrate, succinate, malate, fumarate, glucose, fructose, and L-glutamate</td>
</tr>
</tbody>
</table>

The following compounds were not utilized: sucrose, N,N-dimethylformamide, D-lactose, tartarate, and tetramethylammonium

Nitrogen source for growth: ammonium salts, nitrate, L-glutamate, yeast extract, and malt extract

Fig. 1. Comparison of L-Serine Production by Methylobacterium sp. Strain MN43, Hyphomicrobium sp. Strain NCIB10099, and H. methyllycorum GM2.

L-Serine production was brought about under the standard conditions with shaking for 2 days except that the glycine concentrations were as shown: ○, Methylobacterium sp. MN43; △, Hyphomicrobium sp. NCIB10099; ■, H. methyllycorum GM2.

Fig. 2. Effects of Initial Methanol, and Glycine Concentrations and of Cell Density on L-Serine Production by Resting Cells of Methylobacterium sp. Strain MN43.

The reactions were done under standard conditions for 3 days except that the initial methanol concentration (A), glycine concentration (B), and cell density (C) were as indicated. The effect of the initial methanol concentration was investigated without methanol being added later. Reaction time: ▲, 1 day; ■, 2 days; ○, 3 days.
Table II. Effect of Nitrogen Source on L-Serine Production

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Growth (OD_{610})</th>
<th>L-Serine (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH_4)_2HPO_4</td>
<td>2.8</td>
<td>34.6</td>
</tr>
<tr>
<td>(NH_2)SO_4</td>
<td>1.5</td>
<td>49.0</td>
</tr>
<tr>
<td>NH_4Cl</td>
<td>2.0</td>
<td>38.7</td>
</tr>
<tr>
<td>NaNO_3</td>
<td>2.9</td>
<td>51.6</td>
</tr>
<tr>
<td>NH_NO_3</td>
<td>2.8</td>
<td>43.5</td>
</tr>
<tr>
<td>t-Glutamate</td>
<td>1.6</td>
<td>25.6</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.4</td>
<td>36.5</td>
</tr>
<tr>
<td>Malt extract</td>
<td>1.9</td>
<td>18.8</td>
</tr>
</tbody>
</table>

The bacterium was cultivated for 2 days at 28°C in medium II containing 1% methanol with the addition of one of the indicated nitrogen sources instead of (NH_4)_2HPO_4. The amounts of L-serine produced were measured after 3 days.

Table III. Effect of Initial Methanol Concentrations in the Culture Medium on L-Serine Production

<table>
<thead>
<tr>
<th>Initial methanol (%)</th>
<th>Methanol remaining (%)</th>
<th>Growth (OD_{610})</th>
<th>L-Serine (mg/ml) after incubation of 1 day</th>
<th>2 days</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0</td>
<td>0.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>0.3</td>
<td>2.7</td>
<td>17.6</td>
<td>30.4</td>
<td>38.9</td>
</tr>
<tr>
<td>1.0</td>
<td>0.3</td>
<td>3.2</td>
<td>14.5</td>
<td>25.4</td>
<td>25.1</td>
</tr>
<tr>
<td>1.5</td>
<td>1.1</td>
<td>4.4</td>
<td>11.8</td>
<td>22.9</td>
<td>23.9</td>
</tr>
<tr>
<td>2.0</td>
<td>1.6</td>
<td>2.2</td>
<td>11.3</td>
<td>21.7</td>
<td>26.0</td>
</tr>
</tbody>
</table>

Cultivation was for 2 days at 28°C in medium II containing the indicated concentrations of methanol. Then the methanol remaining was measured. L-Serine production was under the standard conditions.

Fig. 3. Effect of Cultivation Time on L-Serine Production. Methylobacterium sp. strain MN43 was cultivated in medium II containing 1% methanol for (C) 2 days, (●) 3 days, (□) 4 days, or (▲) 5 days.

added in the medium was completely consumed, and cells cultivated for 3–5 days maintained high activity, although the cells harvested after 2 days of cultivation had lost L-serine-producing activity after 2 days of reaction (Fig. 3).

Time course of L-serine production by resting cells of Methylobacterium sp. strain MN43 under the optimal conditions

When organism was cultivated for 3 days in medium containing 0.3% sodium nitrate as the nitrogen source and 1% methanol as the carbon source under the optimal conditions, 65 mg of L-serine was produced from consumed 46 mg of glycine in 5 days of reaction, giving a molar conversion ratio of 93% (Fig. 4).

Discussion

In our previous study of L-serine production by Hyphomicrobium strains, all the strains that produced L-serine degraded the L-serine, and suppression of this activity by maintenance of the methanol concentration at 14.1–29.6 mg/ml increased L-serine production. In the present study, under conditions that suppressed L-serine degradation, Methylobacterium sp. strain MN43 was found to produce much L-serine. The strain also degraded L-serine rapidly (32 mg/ml L-serine was degraded in 2 days of reaction), which rate was as rapid as that of the Hyphomicrobium strains, so L-serine production should be evaluated under such conditions. The level of L-serine production (65 mg/ml), with a molar conversion ratio from glycine to L-serine of 93%, was the highest so far reported.

There was a difference between Methylobacterium sp. strain MN43 and the Hyphomicrobium strains in the molar conversion ratio in L-serine production, especially when glycine was 25 mg/ml or less. In a preliminary experiment in which we used 100 mg/ml glycine (data not shown), 71 mg/ml L-serine was produced in 5 days of reaction under the optimal conditions. However, the molar conversion ratio was only 51%. An excess of glycine was inefficient for the enhancement of L-serine production.

Cells harvested from a culture broth containing methanol at harvest produced little L-serine. Under the conditions we selected, 3.38 mol of methanol was consumed while 0.62 mol of L-serine was produced. This suggests that cells that can oxidized large amounts of methanol produce little L-serine.

References
t-Serine Production by a Methylo bacter

3) H. Yamada, S. S. Miyazaki, and Y. Izumi, Agric. Biol. Chem., 50,
17-21 (1986).
4) T. Yoshida, T. Mitsunaga, and Y. Izumi, J. Fer ment. Bioeng., 75,
405-408 (1993).
6) P. Sirrote, T. Yamane, and S. Shimizu, J. Fer ment. Technol., 66,