J Ind Microbiol Biotechnol (2007) 34:657–663
DOI 10.1007/s10295-007-0238-0

ORIGINAL PAPER

Over-expression of a hydroxypyruvate reductase in *Methylobacterium* sp. MB200 enhances glyoxylate accumulation

Pei-Hong Shen · Bo Wu

Received: 27 February 2007 / Accepted: 26 June 2007 / Published online: 25 July 2007

Abstract *Methylobacterium* sp. MB200 capable of producing glyoxylate from methanol was obtained by enrichment culture using a medium containing methanol as the sole carbon source. A *hpr* gene that encodes a hydroxypyruvate reductase (HPR) was cloned from this strain and was ligated into the vector pLAFR3 to obtain the recombinant plasmid pLAFRh, which was transferred into *M*. sp. MB200 to generate an recombinant strain MB201. Homologous expression of *hpr* under the control of the lacZ promoter led to the enhanced glyoxylate accumulation in cultures of *Methylobacterium* sp MB201. The yield of glyoxylate reached 14.38 mg/mL, representing nearly a two-fold increase when compared with the wild-type strain.

Keywords *Methylobacterium* · *hpr* · Hydroxypyruvate reductase · Glyoxylate · Metabolic engineering · Strain improvement

Introduction

Methylotrophic bacteria are a group of microorganisms that are able to use compounds containing one-carbon as well as multi-carbons as energy and carbon sources. According to the range of carbon compounds utilized, the methylobacteria can be divided into three subgroups: the obligate methylotrophs that grow only on single-carbon (C1) compounds; the restricted facultative methylotrophs that use not only C1 compounds but also a limited range of more complex organic compounds; and the typical facultative methylotrophs that can grow not only on C1 compounds but also on a wider range of multi-carbon compounds [3, 5]. Most methylotrophic bacteria use a serine cycle to metabolize C1 compounds to supply the energy and intermediates for their growth. In the industry, these bacteria have been studied extensively for the production of amino acids, industrial enzymes and cofactors, polyhydroxyalkanoates (PHAs), polysaccharides, and carotenoids from C1 compounds [21, 22, 27, 29]. *Methylobacterium* strains are facultative methylotrophs that have been isolated and studied in different countries [8, 9, 17]. Especially, the genetics and metabolic pathways of *Methylobacterium extorquens* AM1 have been studied in detail by Lidstorm and coworkers. They, along with others, have developed ways that allow manipulation of *Methylobacterium* genes in vivo, which facilitate the metabolic engineering of the metabolic pathways. They identified *hpr*, a gene encodes a hydroxypyruvate reductase in *M. extorquens* AM1 [2]. It is a key genetic component of the serine cycle (Fig. 1). HPR catalyzes the reversible reaction of hydroxypyruvate to D-glycerate. The enzyme has also been purified and characterized from several organisms including humans [15–18].

Glyoxylate is an essential intermediate in the serine cycle. This compound is commercially important in the manufacture of perfume and as an intermediate in drug and pesticide manufacture [31]. Because of the expanded applications and market in recent years, glyoxylate has been a kind of commodity in short supply. Enzymatic and chemical means of glyoxylate production have been extensively investigated [4, 11, 13, 19]. It is currently manufactured by the nitric acid oxidation of glyoxal or via a three-step reaction involving ozonolysis of dimethyl maleate, hydrogenation of the resulting hydroperoxide intermediate, and the

P.-H. Shen · B. Wu (✉)
The Key Laboratory of Ministry of Education
for Microbial and Plant Genetic Engineering,
The College of Life Science and Technology,
Guangxi University, 100 Daxue Road, Nanning,
Guangxi 530005, China
e-mail: wubo@public.nn.gx.cn; wubogxu@yahoo.com.cn

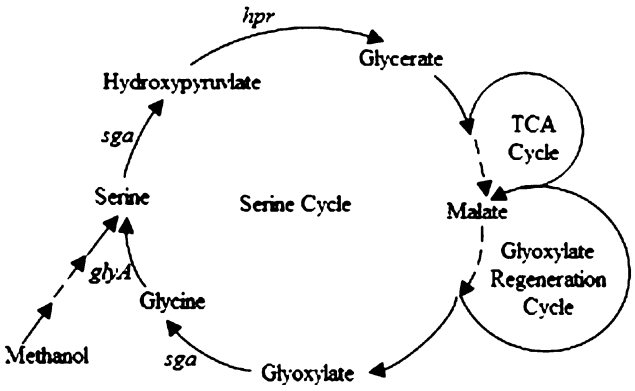

Fig. 1 The serine cycle in methylotrophic bacteria

hydrolysis of the resulting methyloglyoxylate hemiacetal [31]. The enzymatic method for producing glyoxylate has also been investigated. It was mainly through spinach glycolate oxidase (GO) catalyzing the oxidation of glycolate [14, 20, 25]. But, to date, the feasibility of microbial synthesis of glyoxylate has not been demonstrated.

An important strategy to develop a method to synthesize commercial chemicals and materials by bacteria is to deregulate and increase the levels of gene expression, especially to increase the copy number of genes, by genetic engineering. In this report, a *hpr* gene was over-expressed under the control of lacZ promoter in the $pLAFR_3$ vector in *M*. sp. MB200 to produce glyoxylate.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *M*. sp. MB200 was grown at 32 °C in an ammonium mineral salt medium supplemented with 1.2% methanol as the sole carbon source [7]. *Escherichia coli* strains DH5α and Tuner (DE3) were cultivated in LB medium at 32 and 37 °C, respectively. Antibiotics were added to the medium at the following final concentrations: 50 μg/mL ampicillin, 25 μg/mL kanamycin, 200 μg/mL carbencillin, 50 μg/mL choramphenicol, 50 μg/mL nalidixic acid, and 75 μg/mL tetracycline.

Screening for glyoxylate-producing strains

The basal isolation medium (MM), pH 7.0, consisting of 0.3% $(NH_4)_2HPO_4$, 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, and 0.0005% $MnSO_4{\cdot}6H_2O$ was supplemented with thiamine·HCl (1 μg/100 mL), riboflavin (2 μg/100 mL), calcium pantothenate (2 μg/100 mL), pyridoxine·HCl (2 μg/100 mL), biotin (0.1 μg/100 mL), and aminobenzoic acid (1 μg/100 mL). Soil samples were suspended in the mineral, salt medium containing methanol as the sole carbon source and incubated at 32 °C for 3 days. An aliquot of the resulting culture was inoculated into fresh medium and incubated as before; this was repeated two more times. An aliquot of the final culture suspension was transferred to the same medium supplemented with agar (1.6%) and incubated at 32 °C. Representative colonies were obtained for further physiological, nutritional, and biochemical testing using standard methodologies according to the general procedure of Bergey's Manual of Systematic Bacteriology and 16S rDNA sequence analysis.

Identification of glyoxylate

Cells were harvested and washed once with phosphate buffer (pH 7.0), suspended in the same solution to the concentration of 200 mg wet cells per mL, and lysed by sonication. The cell-free supernatant obtained by centrifugation at

Table 1 Plasmids and strains used in this study

Strain/Plasmid	Relevant characteristics	Source
Bacteria strains		
Methylobacterium sp. MB200	Wild type strain, Nm^r	Laboratory stock
Methylobacterium sp. MB201	MB200 containing pLAFR3-hpr	This study
Escherichia coli		
DH5α	SupE44,Δ*lacU*169(ψ80*lacZ*ΔM15)hsdR17 RecA1 endA1 gyrA96 thi-1 relA1	[23]
Tuner(DE3)	Expression strain, $Carb^r$, Amp^r	Novagen
Plasmids		
pGEM–T easy	T-A cloning vector, Amp^r	Promega
pET-blue	Expression vector, Cm^r	Novagen
pRK2013	Helper plasmid, Mob^+, ColE1, Km^r, Tra^+	[6]
pLAFR3	Broad-host range cloning vector, Mob^+, Tra^-, cosmid, Tc^r	[28]
pLAFR	pLAFR3 containing *hpr* gene	This study

14,000*g* was used for glyoxylate analysis on a Waters 2695 high performance liquid chromatography system (Waters, Milford, MA, USA) equipped with an Inertsil ODS-3 column (4.6 × 150 mm, 5 μm; GL Sciences, Torrance, CA, USA). The column was eluted with 0.04 M $(NH_4)_2HPO_4$ (pH 2.7) at a flow rate of 1.0 mL/min and the eluents were detected by UV at a wavelength of 220 nm.

DNA manipulation

Chromosomal DNA and plasmid were isolated using a commercially available kit (Qiagen, Valenica, CA, USA). DNA ligation and digestion were performed according to the suppliers' instructions. Other DNA manipulations were done by standard procedures [1].

hpr cloning and expression

M. sp. MB200 *hpr* was amplified by polymerase chain reaction (PCR) using the genomic DNA of *M*. sp. MB200 as the template. Primers ph1/ph2 (5′-GGCAGGGGTT TTGTG AGC-3′/5′-CTCTGGCCGGTTGTTCAT-3′) were designed according to the *hpr* gene sequence of *M. extorquens* AM1 (Genbank accession number is L27235.1). The PCR conditions were one cycle of 95 °C for 2 min; 30 cycles each of 94 °C for 40 s, 53 °C for 40 s, and 72 °C for 60 s; and one cycle of 72 °C for 3 min. The PCR products were purified by using a DNA purification kit and then cloned into the pGEM T-easy vector (named pGEM-T:hpr). The sequence of the cloned DNA fragment was determined by DNA sequencing on an ABI 377 sequencer (AME Bioscience, Toroed, Norway).

A 945-bp DNA fragment containing the coding region of *M*. sp. MB200 *hpr* was amplified by PCR using pGEM-T:hpr as the template and primers P1/P2[(5′-ATGGATCC GATGACAAAGAAAGTGG-3′/5′-ATAAGCTTGCCTCG ACGACGTTCTG-3′) (*Hind*III and *Bam*HI sites are underlined)], which were designed according to the coding sequence of *M*. sp. MB200 *hpr*. The PCR conditions were one cycle of 95 °C for 2 min; 30 cycles each of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 50 s; and one cycle of 72 °C for 3 min. The PCR products were cloned into pGEM-T:hpr. After confirmation by sequencing, the 945-bp DNA fragment was excised from the vector using *Hind*III and *Bam*HI and cloned into the expression vector pET-blue. The resulting plasmid (pET:ohpr) was introduced into *E. coli* Tuner (DE3) to generate the expression host strain Tuner-(pET:ohpr).

Tuner-(pET:ohpr) was induced by isopropyl *β*-D-1-thiogalactopyranoside to express the main proteins. Cells were harvested by centrifugation and lysed by sonication with a JY92-II ultrasonic cell disrupter (Ningbo Kesheng Instrument Factory, Ningbo, China) operating at a power level of 300–400 W, in 30 cycles of 10 s bursts separated by 30 s intervals. After centrifugation at 14,000*g*, the cell-free supernatant was transferred to another tube and stored at 4°C until analyzed.

HPR was purified using a Ni-NTA column (Novagen, Madison, WI, USA) according to the manufacturer's instructions, and the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stored at 4 °C.

Triparental mating to construct *Methylobacterium* sp. MB201 strain

The *hpr* gene digested by *Bam*HI and *Hind*III was ligated into the pLAFR3 vector to obtain the recombinant plasmid designated as pLAFRh, which was transferred into *E. coli* DH5α. *M*. sp MB200 was cultured in MM with nalidixic acid at 32 °C for 48 h. *E. coli* strain HB101 containing plasmid pRK2013 and DH5α containing pLAFRh were both cultivated in LB with kanamycin (for HB101) and tetracycline (for DH5α) at 37 °C overnight. Volumes of the cultures (200 μL of *M*. sp MB200 and 50 μL of HB101 and DH5α) were co-cultured on a MM plus 20% LB plate and grown at 32 °C for 2 days. Representative colonies of each organism were spread onto tetracycline- and nalidixic acid-amended MM and incubated for 3–4 days at 32 °C. pLAFRh was conjugated from *E. coli* DH5α into *M*. sp. MB200 to generate the recombinant strain designated as *M*. sp. MB201.

Enzyme assay

HPR activity was assayed in 1 mL standard assay mixture containing 50 mM MOPS buffer (pH 5.5), 0.3 mM NADPH, 3.5 mM lithium hydroxypyruvate, and appropriate amount of enzyme. The reaction was started by the addition of the enzyme solution to the reaction mixture. The decrease of absorbance at 340 nm, which indicated the consumption of NADPH, was followed with time. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the oxidation of 1 μmol NADPH per min under the optimum conditions.

Activity measurements for *M*. sp. MB200 and *M*. sp. MB201 HPR were determined as described above using cells that had been collected from culture, cultivated for 3 days, washed once with 50 mM MOPS buffer (pH 5.5), and lysed by sonication as detailed above.

Production of glyoxylate

One loopful of the recombinant strain *M*. sp. MB201 and the wild strain *M*. sp. MB200 were inoculated into a test tube containing 10 mL medium with 1.2% methanol and 0.1% glycine. Cultures were incubated at 32 °C for 2 days

with reciprocal shaking (250 strokes/min). An aliquot (0.2 mL) of this seed culture was transferred to a triangular flask (the volume was 250 mL) with 50 mL fresh medium and incubated at 32 °C with reciprocal shaking (250 strokes/min). After cultivation for 12 h, the absorbance of the cultures at 600 nm was determined. Cells were harvested from the culture by centrifugation every 3 h, washed once with phosphate buffer (pH 7.0), and suspended in the same solution to the concentration of 200 mg wet cells per mL. The final suspension was lysed by sonication as described above, and the cell-free supernatant obtained following centrifugation was stored at −70 °C until analyzed.

Results

Screening and taxonomic studies of a glyoxylate-producing strain

A total of 20 strains grew well on agar with methanol as the sole carbon source. Among them, the strain MB200 tolerated methanol the best at high methanol levels (up to 3.0%) and displayed the highest absorbance at 610 nm. Its cell-extraction had a high peak at the same retention time as the authentic glyoxylate in HPLC (Fig. 2). Therefore, the strain MB200 was used in the following investigations.

MB200 colonies were circular and smooth. The organism was found to be a Gram-negative non-spore forming rod, formed pink colonies, and exhibited fastest growth at about 32 °C. 16S rDNA obtained from the organism was 99% identical to the 16S rDNA of *M. extorquens* AM1 (Accession no. M29027.1). MB200 grew on media containing methanol and other multi-carbon compounds as the carbon source. According to these characteristics, the strain should be a member of the genus *Methylobacterium*.

MB200 hpr gene sequence analysis

Sequence analysis showed that the PCR amplified 1.1 kb DNA fragment containing *hpr* gene had 98% identity to the *hpr* region of *M. extorquens* AM1. Furthermore, ORF prediction results showed that the 1140-bp fragment contained a 945-bp ORF showing 98% identity to the *hpr* of *M. extorquens* AM1 at the nucleotide level and 99% identity at the amino acid level (Fig. 3). Three amino acid residues at positions 50, 236 and 305 were different among these two sequences, I50, V236, S305 in MB200, while V50, A236, A305 in AM1.Conserved domains analysis through NCBI showed that these residues have no much effect on the HPR function or structure.

Expression of HPR of M. sp. MB200

To determine if the product of *M*.sp MB200 *hpr* was a hydroxypyruvate reductase, the ORF containing *hpr* was ligated into the vector pET-blue to generate the expression vector pET:ohpr. The recombinant plasmid pET:ohpr was transformed into expression host strain Tuner(DE3) to generate the expression strain Tuner (pET:ohpr). Finally, a His-tagged protein was purified from Tuner (pET:ohpr). SDS-PAGE analysis indicated that the purified protein had a molecular weight of 34 kDa (Fig. 4). HPR activity of the purified protein from *E. coli* Tuner (pET:ohpr) was determined and found to be approximately 901 units per milligram of purified protein.

Construction of the recombinant strain MB201

The *hpr* gene was cloned into pLAFR3 and the resulting plasmid (pLAFRh) was transferred into *E. coli* DH5α, which was used as a donor in triparental mating with *M.* sp. MB200. Tc^r transconjugant MB201 was selected for further characterization and study of HPR activity. HPR activities of the wild strain MB200 and the recombinant strain MB201 cultivated with two carbon sources (methanol and succinate) were determined. The results demonstrated that the use of succinate invariably produced HPR activity in both strains. In the presence of methanol, the HPR activity in cell-free extracts of MB200 was about 107 units per milligram, while in cell-free extracts of MB201 it was about 226 units, which represented an approximately twofold increase over the activity displayed by MB200.

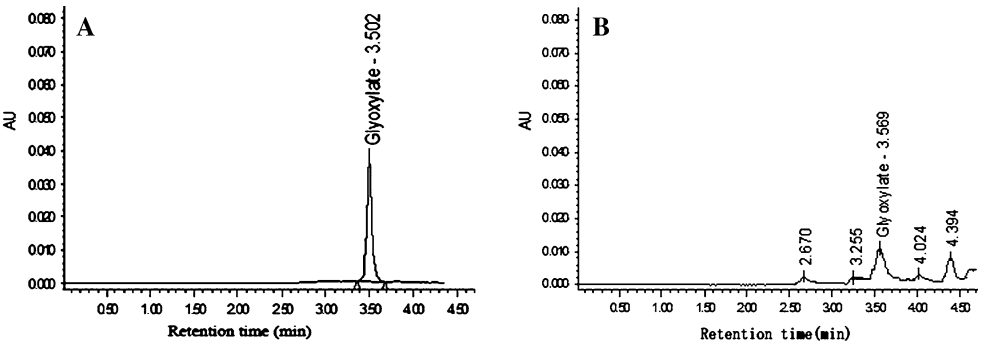

Fig. 2 HPLC chromatograms of the standard glyoxylate **a** and glyoxylate produced by MB200 **b**. The sample was separated on an Inertsil ODS-3 column, eluted with 0.04 M $(NH_4)_2HPO_4$ (pH2.7) at a flow rate of 1.0 mL/min, and detected at a wavelength of 220 nm

```
M. extorquence  MTKKVVFLDRESLDATVREFNFPHEYKEYESTWTPEEIVERLQGAEIAMINKVPMRADTL
M. MB200        MTKKVVFLDRESLDATVREFNFPHEYKEYESTWTPEEIVERLQGAEIAMVNKVPMRADTL
                ************************************************:**********
M. extorquence  KQLPDLKLIAVAATGTDVVDKAAAKAQGITVVNIRNYAFNTVPEHVVGLMFALRRAIVPY
M. MB200        KQLPDLKLIAVAATGTDVVDKAAAKAQGITVVNIRNYAFNTVPEHVVGLMFALRRAIVPY
                ************************************************************
M. extorquence  ANSVRRGDWNKSKQFCYFDYPIYDIAGSTLGIIGYGALGKSIAKRAEALGMKVLAFDVFP
M. MB200        ANSVRRGDWNKSKQFCYFDYPIYDIAGSTLGIIGYGALGKSIAKRAEALGMKVLAFDVFP
                ************************************************************
M. extorquence  QDGLVDLETILTQSDVITLHVPLTPDTKNMIGAEQLKKMKRSAILINTARGGLVDEAALL
M. MB200        QDGLVDLETILTQSDVITLHVPLTPDTKNMIGAEQLKKMKRSAILINTARGGLVDEVALL
                ************************************************.***********
M. extorquence  QALKDGTIGGAGFDVVAQEPPKDGNILCDADLPNLIVTPHVAWASKEAMQILADQLVDNV
M. MB200        QALKDGTIGGAGFDVVAQEPPKDGNILCDADLPNLIVTPHVAWASKEAMQILADQLVDNV
                ************************************************************
M. extorquence  EAFVAGKPQNVVEA
M. MB200        EAFVSGKPQNVVEA
                ****:*********
```

Fig. 3 Alignment of HPR protein sequences from *M. extorquens* AM1 and *M.* sp. MB200. The alignment was performed with Clustalw software. The *shaded characters* indicate substitution

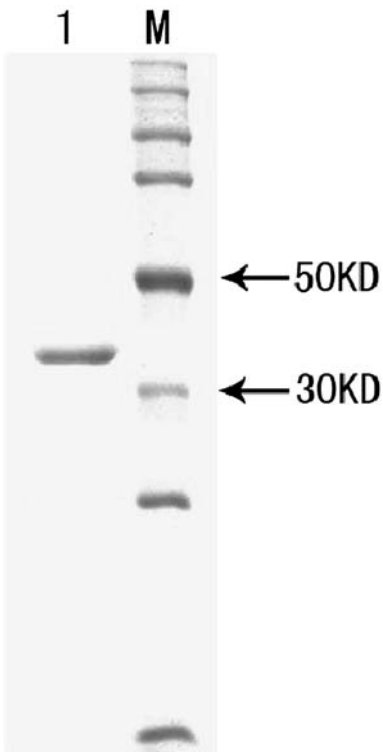

Fig. 4 SDS-polyacrylamide gel electrophoresis of the purified hydroxypyruvate reductase *Lanes* M and 1 indicate size marker and the purified hydroxypyruvate reductase, respectively. The *numbers in the right* indicate the molecular masses of the marker proteins

Time course of glyoxylate accumulation

Figure 5 shows a typical time course of glyoxylate accumulation by strain MB200 and MB201. Compared with the parent strain MB200, the recombinant strain MB201 showed the same culture-phase but higher yield of glyoxylate. The highest content of glyoxylate in both MB200 and MB201 was produced in the logarithm phase. After cultivation for 33 hours, MB201 accumulated 14.38 mg/ml glyoxylate, representing nearly a twofold increase in the yield of the wild-type strain.

Discussion

Glyoxylate, an essential compound in the chemical industry, is currently manufactured mainly by chemical synthesis. But, yields are low due to the formation of by-products. To overcome this shortcoming, enzymatic methods that use glycerol oxidase from *Aspergillus japonicus* [10] and spinach [16] have been developed. However, these methods have some drawbacks owing to the complex reaction and undesirable properties of the enzymes.

Methylotrophic bacteria have been exploited for the conversion of methanol for a long time. Such metabolic engineering allows the production of amino acids, industrial enzymes and cofactors, PHAs, and carotenoids. Especially in methylotrophic bacteria that possess the serine cycle, serine, glyoxylate, and glycine are produced from methanol [12, 21, 22, 27, 30]. HPR has been demonstrated to play a central role in the metabolism of C1 compounds. It has been identified and characterized in bacteria, plants and mammals [15, 16, 24]. In the present study, *M.* sp. MB200 *hpr* was cloned and sequenced. Three amino acid residues at position 50, 236 and 305 were different among MB200 and AM1 HPR sequences, I50, V236, S305 in MB200,

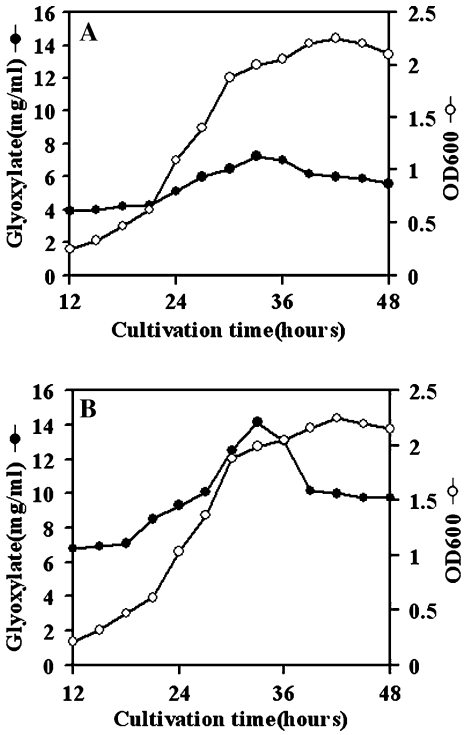

Fig. 5 Intracelluar levels of glyoxylate in *M*. sp. MB200 **a** and MB200 **b** during the incubation period. Strains were grown at 32 °C for 48 h in a 250 mL flask containing 50 mL of the respective medium. Cells were harvested and suspended in the phosphate buffer (pH 7.0) to the concentration of 200 mg wet cells per mL. The concentration of glyoxylate in lysed cells was determined by HPLC

while V50, A236, A305 in AM1. But conserved domains analysis performed using NCBI resources showed that these residues should have no much effect on the HPR function or structure. The gene product (HPR) was purified, and the enzyme activity was determined.

No significant difference in cell growth between the wild-type *M*. sp MB200 and the recombinant *M*. sp MB201, which contained HPR expression plasmids, was observed. But the utilization of methanol and the yield of glyoxylate differed between the two strains. When the two strains were grown in the presence of the same concentration of methanol, the remaining methanol in *M*. sp. MB200 cultures always exceeded that in cultures of *M*. sp. MB201, suggesting that methanol was used more effectively by the MB201 strain. The HPR enzymatic activity and the yield of glyoxylate were higher in the MB201 strain.

The addition of glycine affected not only the production of glyoxylate but also the growth of cells. A glycine level of 0.1% increased the yield of glyoxylate since glycine is essential for the serine cycle. But the growth of culture was suppressed upon glycine addition, agreeing with earlier observations [26].

Glyoxylate metabolism in the cells is complex. To our knowledge, there is still no report about the metabolism of glyoxylate in microorganisms at the present day. In our study, glyoxylate was not detected in the medium; we speculate that it is mainly due to the fact that glyoxylate is a metabolism intermediate in cells. It will be metabolized by the glyoxylate cycle or through other pathways to other molecules when the content of glyoxylate is high. It is precisely as a result of this phenomenon, the output of glyoxylate was low, even though the yield of glyoxylate was improved by nearly twofold in our study. The future work will be to study the mechanism of glyoxylate metabolism and optimize the conditions of fermentation to improve the yield of glyoxylate.

The biosynthesis of glyoxylate by *M*. sp. MB200 bacteria with methanol as the starting material is a promising strategy, because it saves resources and it is a simpler method than chemical synthesis and enzymatic methods. We overexpressed HPR in the MB201 strain and achieved a twofold increase in the yield of glyoxylate. Further genetic and metabolic manipulations are however needed in order to increase the yield.

References

1. Ausuble FM, Brent R, Kingston RE (1999) Short protocols in molecular biology: fourth edition. Wiley, New York
2. Chistoserdova LS, Chen W, Lapidus A, Lidstrom ME (2003) Methylotrophy in *Methylobacterium* extorquens AM1 from a genomic point of view. J Bacteriol 185:2980–2987
3. Colby J B, Zatman LJ (1973) Trimethylamine metabolism in obligate and facultative methylotrophs. J Biochem 132:101–112
4. Coulter-Mackie MB (2006) 4-Hydroxyproline metabolims and glyoxylate production: a target for substrate depletion in primary hyperoxaluria?. Kidney Int 70:1891–1893
5. De VG, Kues U, Stahl U (1990) Physiology and genetics of methylotrophic bacteria. FEMS Microbiol Rev 6:57–101
6. Figurski D, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648–1652
7. Fulton GL, Nunn DN, Lidstrom ME (1984) Molecular cloning of a malyl coenzyme A lyase gene from *Pseudomonas* sp. strain AM1, a facultative methylotroph. J Bacteriol 160:718–723
8. Gallego V, Garcia MT, Ventosa A (2004) Methanotrophs and methylobacteria are found in woody plant tissues within a winter period. Mikrobiologiia 73:817–824
9. Gallego V, Garcia MT, Ventosa A (2005) *Methylobacterium isbiliense* sp. nov, isolated from the drinking water system of Sevilla, Spain. Int J Syst Evol Microbiol 55:2333–2337
10. Isobe K (1995) Oxidation of ethylene glycol and glycolic acid by glycerol oxidase. Biosci Biotechnol Biochem 59:576–581
11. Isobe K, Nishise H (1995) A new method for glycoldehyde production from ethylene glycol. J Mol Catal B: Enzymatic 1:37–34
12. Izumi Y, Yoshida T, Miyazaki SS, Mitsunaga T, Ohshiro T, Shimao M (1993) L-Serine production by a methylotroph and its related enzymes. J Appl Microbial Biotechnol 39:427–432
13. Janusz J, Tomasz B (2000) Glyoxylic acid derivatives in asymmetric synthesis. Pure Appl Chem 72:1589–1546
14. Jin J, Tan T, Wang H, Su G (2003) The expression of spinach glycolate oxidase (GO) in *E. coli* and the application of GO in the production of glyoxylic acid. Mol Biotechnol 25:207–214

15. John M, Utting, Leonard D (1975) Structural, kinetic and renaturation properties of an induced hydroxypyruvate reductase from *Pseudomonas acidovorans*. J Biol Chem 250:5233–5242
16. Leszek A, Kleczkowski D, Douglas D, Randall (1998) Purification and characterization of a novel NADPH(NADH)-dependent hydroxypyruvate reductase from spinach leaves. Biochem J 250:145–152
17. Martin FK, Zohre U (2003) Dichloromethane metabolism and C1 utilization genes in *Methylobacterium* strains. Microbiology 184:1915–1922
18. Michael PSB, Conners R, Rumsby G, Leo Brady R (2006) Structural basis of substrate specificity in human glyoxylate reductase/hydroxypyruvate reductase. J Mol Biol 360:178–189
19. Ochoa JR, Dediedo A, Santa-olalla J (1993) Electrosynthesis of glyoxylic acid using a continuously electrogenerated lead cathode. J Appl Electrochem 23:905–909
20. Payne MS, Petrillo KL, Gavagan JE, Wangner LW, Discosimo R, Anton DL (1995) High-level production of spinach glycolate oxidase in the methylotrophic yeast *Pichia pastoris*: engineering a biocatalyst. Gene 167:215–219
21. Piotr S, Karl-Heinz B, Bernd J (2004) Extraction of carotenoid produced during methanol waste biodegradation. Int Biodet Biodegrad 53:127–132
22. Rene V, Pieter DW (2003) *Methylobacterium* sp. isolated from a Finnish paper machine produces highly pyruvated galactan exopolysaccharide. Carbohydrate Res 385:1851–1859
23. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
24. Scott D, Cramer PMF, Kai L (1999) The gene encoding hydroxypyruvate reductase is mutated in patients with primary hyperoxaluria typrII. Hum Mol Gen 8:2063–2069
25. Seip JE, Fager SK, Gavagan JE, Anton DL, Dicosimo R (1994) Glyoxylic acid production using immobilized glycolate oxidase and catalase. Bioorg Med Chem 2:371–378
26. Silas GV, Mats A, Jens N (2005) Biosynthesis of glyoxylate from glycine in *Saccharomyces cerevisiae*. FEMS Yeast Res 5:703–709
27. Tairo H, Toyokazu Y, Yoshikazu I (1996) Efficient L-serine production from methanol and glycine by resting cells of *Methylobacterium* sp strain MN43. Biosci Biotech Biochem 60:1604–1607
28. Tang JL, Gough CL, Barber CE, Dow JM, Daniels MJ (1987) Molecular cloning of protease gene(s) from *Xanthomonas campestris* pv. *c*ampestris*:* expression in *Escherichia coli* and role in pathogenicity. Mol Gen Genet 210:443–448
29. Todd V, Michael M, Alex S (1999) A corrinoid-dependent catabolic pathway for growth of a *Methylobacterium* strain with chloromethane. Microbiology 96:4615–4620
30. Vorholt JA (2002) Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. Arch Microbiol 178:239–249
31. Xing-quan Chen, Tian-sheng Zhao, Yan-lai Chu, Jun Meng (2003) Methods for preparation of glyoxylic acid and market prospect. Appl Chem Ind 32:5–8