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A new enzymatic method for glycolaldehyde production from ethylene glycol

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Abstract

A new enzymatic method for glycolaldehyde production from ethylene glycol was investigated using alcohol oxidase from *Pichia pastoris* or glycerol oxidase from *Aspergillus japonicus*. Both alcohol and glycerol oxidases oxidize ethylene glycol to glyoxal via glycolaldehyde, but glycolaldehyde was remarkably accumulated using a high concentration of ethylene glycol. The glycolaldehyde formation was also affected by buffer species and reaction pH. Under the optimum conditions, 0.92 or 0.97 M glycolaldehyde was formed from 1.0 M ethylene glycol using alcohol oxidase or glycerol oxidase. This enzymatic method was superior to the chemical method in terms of conversion yields and selectivity of glycolaldehyde.

Keywords: Glycolaldehyde production; Ethylene glycol oxidation; Alcohol oxidase; Glycerol oxidase

1. Introduction

Glycolaldehyde is useful as a raw material to synthesize D,L-serine, medicines or agrochemicals. Some chemical methods of producing glycolaldehyde from ethylene glycol have been demonstrated by the dehydrogenation of ethylene glycol using a combination of metals such as copper, zinc, silver, or gold as a catalyst [1–3]. However, such methods have some drawbacks such as low conversion yields of glycolaldehyde, formation of a high concentration of by-products, and high reaction temperature requirement.

Recently, from our studies of ethylene glycol oxidation by alcohol oxidases (alcohol: oxygen oxidoreductase, EC 1.1.3.13) from methanol yeasts such as *Candida* sp. [4] or *Pichia pastoris* [5], or by glycerol oxidase (glycerol: oxygen 1-oxidoreductase) from *Aspergillus japonicus* [6], we have found that these enzymes oxidize ethylene glycol to glyoxal via glycolaldehyde, and revealed some characteristics of the reactions [7,8]. We have also demonstrated an attractive new enzymatic method for production of glyoxal from ethylene glycol using these enzymes [7,8]. During further investigations of the ethylene glycol oxidation based on the view that oxidation of glycolaldehyde to glyoxal may be a rate-limiting step in the oxidation of a high concentration of ethylene glycol [7,8], we found that a high concentration of glycolaldehyde may be remarkably accumulated by optimizing the reaction conditions such as ethylene glycol concentration, buffer species and reaction pH. The present paper describes the optimization of the reaction condi-

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tions for glycolaldehyde production using alcohol oxidase or glycerol oxidase and provides an evaluation of a new enzymatic method for glycolaldehyde production.

2. Materials and methods

2.1. Chemicals

Ethylene glycol, glycolaldehyde dimer, 40% glyoxal solution, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and Good buffers including *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) were purchased from Wako Pure Chemicals (Osaka, Japan). Catalase from beef liver, alcohol oxidase from *Pichia pastoris* and glycerol oxidase from *Aspergillus japonicus* were from Boehringer Mannheim (Germany), Moldowan Labs. (Philomath, USA) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively, and used without further purification. All chemicals used were the highest grade products available from commercial sources.

2.2. Assay of enzyme activity and kinetic parameters

Enzyme activities of alcohol oxidase and glycerol oxidase were measured by following H_2O_2 formation at 25 and 37°C with the methods of Tani et al. [9], and of Uwajima and Terada [10], respectively, except that the reaction volume was changed from 3 to 1 ml. The apparent kinetic parameters were calculated from initial velocity measurements by following H_2O_2 formation at 25°C.

2.3. Standard reaction conditions for glycolaldehyde formation

One molar ethylene glycol dissolved in 0.2 M TES–NaOH, pH 7.0 (0.9 ml), containing 2600 units of catalase was incubated at 20°C with shaking (140 rpm, 3 cm). The reaction was started by adding 0.1 ml of alcohol oxidase or glycerol oxidase.

2.4. Determination of glycolaldehyde and glyoxal

MBTH derivatives 1 and 2 of glycolaldehyde or glyoxal were prepared by the reaction of their carbonyl bonds with MBTH [11] as described previously [7], and the concentrations of glycolaldehyde and glyoxal were photometrically assayed using the MBTH derivatives 1 or 2 [7]. Glycolaldehyde and glyoxal were also identified by a reversed-phase high-performance liquid chromatography with an eluent of 20 mM sodium dihydrogenphosphate and acetonitrile under the same conditions as described previously [7].

3. Results

3.1. Effects of ethylene glycol concentrations on glycolaldehyde formation

Effects of ethylene glycol concentrations on the glycolaldehyde formation were investigated under the standard reaction conditions for glycolaldehyde formation, except that 0.1 to 10 M ethylene glycol was incubated with 10 units of alcohol oxidase or 6 units of glycerol oxidase per ml of the reaction mixture for 6 h. The glycolaldehyde concentration formed for 6 h of incubation was much higher than the glyoxal concentration at more than 0.5 M ethylene glycol. In the case of alcohol oxidase, the glycolaldehyde concentration formed for 6 h of incubation increased up to 4 M ethylene glycol and gradually decreased at more than 6 M ethylene glycol, whereas the glyoxal formation was similar between 0.1 M and 2 M ethylene glycol, and decreased against increasing ethylene glycol concentration (Fig. 1). In the case of glycerol oxidase, the glycolaldehyde concentration formed for 6 h of incubation increased up to 2 M ethylene glycol and decreased at more than

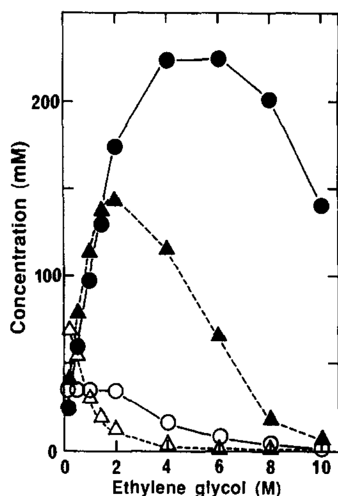

Fig. 1. Effects of ethylene glycol concentrations on glycolaldehyde formation. The reaction was done under the standard reaction conditions for glycolaldehyde formation, except that 0.1 to 10 M ethylene glycol was incubated with 10 units of alcohol oxidase (circles + solid line) or 6 units of glycerol oxidase (triangles + broken line) per ml of the reaction mixture for 6 h. Closed symbols: glycolaldehyde; open symbols: glyoxal.

4 M ethylene glycol, whereas the glyoxal formation decreased against the ethylene glycol concentration (Fig. 1). Since these results indicate that a high concentration of ethylene glycol or glycolaldehyde would cause an inhibition, and an inhibition by glycolaldehyde would be triggered at a lower concentration, the inhibition constant for the substrate action as inhibitor of ethylene glycol or glycolaldehyde was measured by following H_2O_2 formation at 25°C. The inhibition constant of ethylene glycol for ethylene glycol oxidation or that of glycolaldehyde for glycolaldehyde oxidation by alcohol oxidase could not be calculated, because the reaction velocity of ethylene glycol and glycolaldehyde oxidations did not decrease to 6 M and 0.8 M, respectively (data not shown). The inhibition constant of ethylene glycol for ethylene glycol oxidation by glycerol oxidase was approximately 950 mM, which is larger than that of glycolaldehyde for glycolaldehyde oxidation [8]. Thus, formation of glycolaldehyde and glyoxal by alcohol oxidase or glycerol oxidase might be affected by not only K_m and V_{max} values but also substrate inhibition. These results also indicate that glycerol oxidase may be better than alcohol oxidase to yield a high concentration of glycolaldehyde solution containing virtually no glyoxal, when 1 to 4 M ethylene glycol is used, because the ratios of the glyoxal to glycolaldehyde concentration formed by glycerol oxidase were lower than those formed by the alcohol oxidase; however, alcohol oxidase may be more useful than glycerol oxidase at more than 4 M ethylene glycol, because glycolaldehyde formation by glycerol oxidase was strongly inhibited by a high concentration of ethylene glycol.

3.2. Effects of enzyme concentrations on glycolaldehyde formation

Effects of enzyme concentrations on the glycolaldehyde formation were investigated under the standard reaction conditions for glycolaldehyde formation using 22 to 177 units of alcohol oxidase or 7.5 to 68 units of glycerol oxidase per ml of the reaction mixture. As shown in Fig. 2, the glycolaldehyde formation by both enzymes was increased by increasing the amounts of enzymes, but did not quantitatively parallel the enzyme amounts. In addition, the ratios of the glyoxal to glycolaldehyde concentration gradually increased by addition of a large amount of

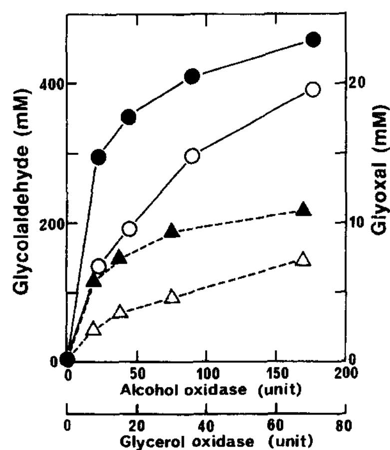

Fig. 2. Effects of enzyme concentrations on glycolaldehyde formation. The reaction was done under the standard reaction conditions for glycolaldehyde formation, except that 10 M ethylene glycol was incubated with 22 to 177 units of alcohol oxidase (circles + solid line) per ml of the reaction mixture for 12 h, and 6 M ethylene glycol was incubated with 7.5 to 68 units of glycerol oxidase (triangles + broken line) per ml of the reaction mixture for 10 h. Closed symbols: glycolaldehyde; open symbols: glyoxal.

Table 1
Effects of buffer species on glycolaldehyde formation

	Buffer	pH	Glyoxal (mM)	Glycolaldehyde (mM)	Glyoxal/glycolaldehyde (%)
(A)	TES–NaOH	7.0	28.6	212	13.5
	BES–NaOH	7.0	14.4	173	8.3
	Tris–HCl	7.0	16.5	182	9.1
	TES–NaOH	8.5	25.6	212	12.1
	AMPD–HCl	8.5	19.3	222	8.7
	Tris–HCl	8.5	9.3	189	4.9
(B)	TES–NaOH	7.0	9.7	180	5.4
	PIPES–NaOH	7.0	5.0	116	4.3
	Potassium phosphate buffer	7.0	2.5	86	2.9
	Tris–HCl	7.0	1.0	78	1.3
	TES–NaOH	8.5	8.4	186	4.5
	Potassium phosphate buffer	8.5	3.3	104	3.2
	AMPD–HCl	8.5	6.5	214	3.1
	Tris–HCl	8.5	5.4	242	2.2

Four molar ethylene glycol was incubated with 50 units of alcohol oxidase (A) or 30 units of glycerol oxidase (B) in 0.15 M buffers, pH 7.0 or 8.5, at 20°C for 4 h. TES: *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BES: *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; PIPES: piperazine-1,4-bis(2-ethanesulfonic acid); AMDP: 2-amino-2-methyl-1,3-propanediol.

enzyme. These results suggest that the addition of a large amount of enzyme may not be effective to yield a high concentration of glycolaldehyde solution containing virtually no glyoxal.

3.3. *Effects of buffer species on glycolaldehyde formation*

Effects of buffer species on the formation of glycolaldehyde and glyoxal were compared with TES–NaOH buffer under the standard reaction conditions for glycolaldehyde formation, except that 4 M ethylene glycol was incubated with 50 units of alcohol oxidase or 32 units of glycerol oxidase per ml of the reaction mixture in 13 kinds of buffers, pH 7.0, or 16 kinds of buffers, pH 8.5. In the case of alcohol oxidase, the glycolaldehyde concentration formed for 4 h of incubation did not vary much among all buffers tested (data not shown), but the glyoxal concentration in BES–NaOH, pH 7.0, Tris–HCl, pH 7.0 and 8.5, or 2-amino-2-methyl-1,3-propanediol (AMPD)–HCl buffer, pH 8.5, was lower than in the TES–NaOH buffer (Table 1). When glycerol oxidase was incubated at pH 7.0, the ratios of the glyoxal to glycolaldehyde concentration in PIPES–NaOH, Tris–HCl or potassium phosphate buffer were lower than in the TES–NaOH buffer, and the glycolaldehyde concentration formed in these three buffers was also lower than in the TES–NaOH buffer (Table 1). Among 16 kinds of buffers, pH 8.5, the glycolaldehyde concentration formed in both Tris–HCl and AMPD–HCl buffers, pH 8.5, was higher than in the TES–NaOH buffer, pH 7.0 or 8.5, and the ratios of the glyoxal to glycolaldehyde concentration in both buffers were smaller than in the TES–NaOH buffer. The ratio of the glyoxal to glycolaldehyde concentration in potassium phosphate buffer was also smaller than in the TES–NaOH buffer, but not smaller than in either Tris–HCl or AMPD–HCl buffer (Table 1). The ratios of the glyoxal to glycolaldehyde concentration in other buffers were not smaller than that in the TES–NaOH buffer (data not shown). Therefore, both Tris–HCl and AMPD–HCl buffers, pH 8.5, might be better than TES–NaOH or other buffers tested for production of a high concentration of glycolaldehyde containing virtually no glyoxal. Since the ratios of the glyoxal to glycolaldehyde concentration by glycerol oxidase were smaller than by the alcohol oxidase, glycerol oxidase might be better than alcohol oxidase to yield a glycolaldehyde solution containing virtually no glyoxal.

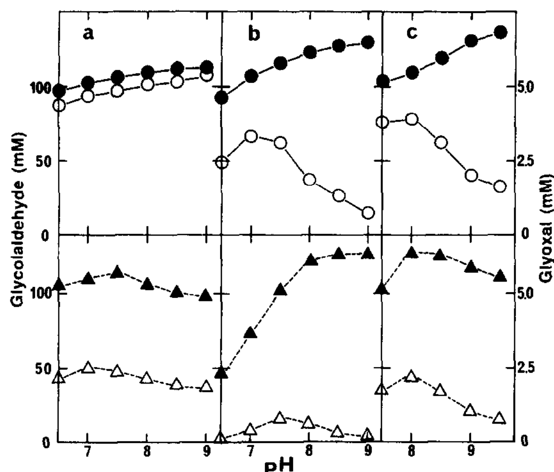

Fig. 3. Effects of reaction pH on glycolaldehyde formation. Four molar ethylene glycol was incubated with 50 units of alcohol oxidase (circles + solid line) or 32 units of glycerol oxidase (triangles + broken line) per ml of the reaction mixture for 2 h under the standard conditions for glycolaldehyde formation, except that 0.15 M TES–NaOH (a), Tris–HCl (b), or AMPD–HCl (c) buffer was used. Closed symbols: glycolaldehyde; open symbols: glyoxal. TES: *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; AMDP: 2-amino-2-methyl-1,3-propanediol

3.4. Effects of pH on glycolaldehyde formation

Since the glycolaldehyde formation was affected by buffer species and pH, the effects of pH on the oxidation of ethylene glycol and glycolaldehyde were investigated in detail using 0.15 M Tris–HCl, AMPD–HCl or TES–NaOH buffer. It was revealed that glycolaldehyde and glyoxal formations might reflect the difference of velocity of ethylene glycol and glycolaldehyde oxidations in buffer species and pH, because K_m values for ethylene glycol and glycolaldehyde of both enzymes were similar to those reported previously [7,8], and did not vary with the buffer species and pH (data not shown). In TES–NaOH buffer from pH 7.0 to 9.0, the glycolaldehyde concentration formed for 2 h of incubation was parallel to the glyoxal concentration (Fig. 3). The ratios of the glyoxal to glycolaldehyde concentration were therefore similar between pH 7.0 and 9.0. In Tris–HCl or AMPD–HCl buffer, however, optimum pH of the glycolaldehyde formation was shown in the high alkaline pH region, whereas the glyoxal formation decreased in the high alkaline pH region, and the ratios of the glyoxal to glycolaldehyde concentration in Tris–HCl buffer were lower than in the AMPD–HCl buffer (Fig. 3). Therefore, the oxidation of ethylene glycol in alkaline pH of Tris–HCl buffer might be better than in AMPD–HCl and all other buffers tested to yield a high concentration of glycolaldehyde containing virtually no glyoxal. In addition, it is also important that the reaction pH is maintained in the alkaline pH region, because the reaction pH gradually moved to the acidic region by the formation of a high concentration of glycolaldehyde.

3.5. Effects of temperature on glycolaldehyde formation

Effects of temperature on the formation of glycolaldehyde and glyoxal were investigated under the standard reaction conditions for glycolaldehyde formation, except that 4 M ethylene glycol was incubated with 50 units of alcohol oxidase per ml of the reaction mixture in 0.15 M Tris–HCl, pH 8.5, from 10 to 30°C for 4 h. The glycolaldehyde concentration formed at 10 and 20°C was approximately 82.9 and 99.4% of that at 30°C, respectively, and the ratios of the glyoxal to glycolaldehyde concentration formed at 10, 20 and 30°C were 2.4, 3.3 and 5.4%, respectively (data not shown). In the case of glycerol oxidase, similar results were obtained; the glycolaldehyde concentration formed at 10 and 20°C for 4 h of incubation was approximately 90.3 and 95.3% of that at 30°C, respectively, and the ratios of the glyoxal to glycolaldehyde concentration formed at 10, 20 and 30°C were 1.5, 1.6 and 2.0%, respectively.

When alcohol oxidase was incubated with 2 M ethylene glycol in 0.8 M Tris–HCl, pH 9.0, at 5°C for 36 h, 35% of the activity remained, but no remaining activities were shown by incubating at 20°C for 36 h. In the case of glycerol oxidase, 23% of the enzyme activity remained by incubating at 5°C for 36 h, but no activity was shown at 20°C (data not shown). These results indicate that the low-temperature reaction might be better than a high-temperature reaction to obtain a high

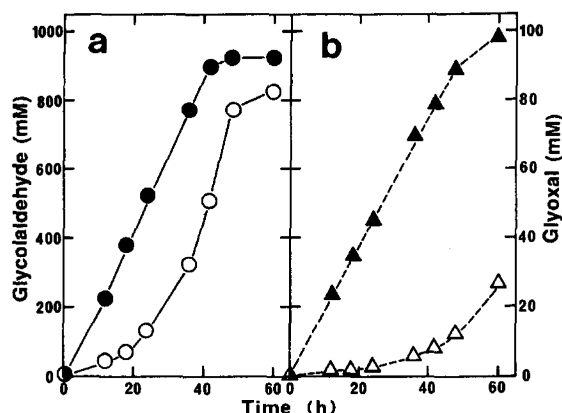

Fig. 4. Formation of glycolaldehyde under optimized conditions. One molar ethylene glycol was incubated with 100 units of alcohol oxidase (a) or 50 units of glycerol oxidase (b) per ml of the reaction mixture in 0.8 M Tris–HCl, pH 9.0, containing 2600 units of catalase (total 1 ml) at 5°C while maintaining the pH between 8.5 and 9.0, and shaking. Closed symbols: glycolaldehyde; open symbols: glyoxal.

glycolaldehyde concentration by long incubation.

On the basis of these results, it was concluded that the optimum conditions for the glycolaldehyde production were that more than one molar ethylene glycol should be incubated with 100 units of alcohol oxidase or 50 units of glycerol oxidase in 0.8 M Tris–HCl, pH 9.0, containing 2600 units of catalase (total 1 ml) at 5°C while maintaining the pH between 8.5 and 9.0, and shaking.

3.6. Formation of glycolaldehyde under optimized conditions

The production of glycolaldehyde and glyoxal by alcohol oxidase or glycerol oxidase under optimized conditions was shown in Fig. 4. Both enzymes were able to completely oxidize 1 M ethylene glycol for 48 or 60 h of incubation; approximately 920 mM glycolaldehyde was formed by alcohol oxidase for 48 h of incubation, and 8.4% glyoxal of glycolaldehyde concentration was also formed. In the case of glycerol oxidase, approximately 970 mM glycolaldehyde and 27 mM glyoxal were formed for 60 h of incubation (the ratio of the glyoxal to glycolaldehyde concentration was 2.8%). The conversion yield and selectivity for glycolaldehyde by both enzymes were thus higher than with chemical methods. The ratio of the glyoxal to glycolaldehyde concentration could be further decreased by incubating with a higher concentration of ethylene glycol. For example, when 6 M ethylene glycol was incubated with 100 units of alcohol oxidase for 90 h under the optimized conditions, approximately 1.60 M glycolaldehyde was formed and the ratio of the glyoxal to glycolaldehyde concentration decreased to 1.5%.

4. Discussion

We demonstrated the optimization for glycoaldehyde formation using alcohol oxidase from *Pichia pastoris* or glycerol oxidase from *Aspergillus japonicus*, and evaluated it as a new enzymatic method for glycolaldehyde production. Since alcohol oxidase and glycerol oxidase oxidized both ethylene glycol and glycolaldehyde [7,8], it was presumed that increasing the difference in the velocity between ethylene glycol oxidation and glycolaldehyde oxidation may be required to obtain a high concentration of glycolaldehyde solution containing virtually no glyoxal. At first, investigation of the substrate concentration on the oxidation of ethylene glycol and glycolaldehyde indicated that a high concentration of ethylene glycol serves to increase the velocity of glycolaldehyde formation, and the oxidation of glycolaldehyde to glyoxal may be a rate-limiting step in the oxidation of a high concentration of ethylene glycol, because K_m and V_{max} values of alcohol oxidase for ethylene glycol were at least 10 and 2 times larger than those for glycolaldehyde, respectively [7], and these values of glycerol oxidase for ethylene glycol were also approximately 4 and 1.4 times larger than those for glycolaldehyde, respectively [8]. Investigation of the substrate concentration further indicated that ethylene glycol and glycolaldehyde also act as inhibitors in a high concentration, and the glycolaldehyde oxidation may be strongly suppressed by a high concentration of glycolaldehyde formed from ethylene glycol; in the case of alcohol oxidase, glyoxal formation decreased at more than 2 M ethylene glycol, while glycolaldehyde

formation gradually decreased at more than 6 M ethylene glycol. The inhibition constant of glycolaldehyde for glycolaldehyde oxidation by glycerol oxidase was 6 times smaller than that of ethylene glycol for ethylene glycol oxidation. Thus, it was revealed that since a high concentration of ethylene glycol contributes not only to increase the velocity of glycolaldehyde formation, but also to suppress the further oxidation of glycolaldehyde to glyoxal, a high concentration of ethylene glycol is effective to produce a high concentration of glycolaldehyde containing virtually no glyoxal. The buffer species and pH also play an effective role to increase the glycolaldehyde production. The glycolaldehyde formation in Tris–HCl buffer, pH 8.5, was higher than in other buffers, pH 8.5; moreover, optimum pH of the glycolaldehyde formation was shown in the high alkaline pH region of Tris–HCl buffer, whereas the glyoxal formation decreased in the high alkaline pH region. Thus, the difference of optimum pH between ethylene glycol oxidation and glycolaldehyde oxidation also serves to increase the glycolaldehyde production. Therefore, it was concluded that incubation of a high concentration of ethylene glycol in the high alkaline pH region of Tris–HCl buffer is important to produce a high concentration of glycolaldehyde containing virtually no glyoxal. Addition of catalase was also effective to increase the glycolaldehyde formation, because hydrogen peroxide generated by the ethylene glycol oxidation caused a decrease in glycolaldehyde formation as described previously [7]. A low-temperature reaction was important for long incubation, because both enzymes were not stable in high temperature. On the basis of these results, the optimum conditions for glycolaldehyde production were decided as described in a text, and more than 90% of the glycolaldehyde could be produced from 1 M ethylene glycol under the optimum conditions. The new enzymatic method for glycolaldehyde formation presented here has the following advantages compared to the enzymatic approach of glyoxal formation [7,8]: (a) a high concentration of ethylene glycol can be used; (b) the velocity of glycolaldehyde formation is much higher than that of glyoxal formation; and (c) a much higher concentration of glycolaldehyde can be accumulated in the reaction mixture.

The chemical method shows a high selectivity of glycolaldehyde with a low ratio of ethylene glycol oxidation. However, the selectivity of glycolaldehyde became lower due to the formation of a high concentration of by-products, when ethylene glycol was highly oxidized by the chemical method. For example, 80–90% of selectivity of glycolaldehyde was obtained by approximately 20% oxidation of ethylene glycol, but the selectivity of glycolaldehyde decreased to 60% by approximately 70% of ethylene glycol oxidation [1–3]. Since this proposed method is superior to the chemical method in terms of conversion yield and selectivity for glycolaldehyde, this enzymatic production method for glycolaldehyde might be better suited to the manufacture of glycolaldehyde.

Acknowledgements

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