Journal of Oleo Science
Copyright ©2011 by Japan Oil Chemists' Society
J. Oleo Sci. **60**, (11) 585-590 (2011)

Effect of Glyceric Acid Calcium Salt on the Viability of Ethanol-Dosed Gastric Cells

Hiroshi Habe*†, Shun Sato†, Tokuma Fukuoka, Dai Kitamoto and Keiji Sakaki

Research Institute for Innovations in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST) (Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN)

† *The first two authors contributed equally to this work and should be considered co-first authors.*

Abstract: D-Glyceric acid (D-GA) calcium has been reported to accelerate ethanol oxidation *in vivo* in rats (Eriksson *et al.*, *Metabolism*, 56, 895-898 (2007)). However, no other reports have shown that D-GA can reduce the harmful effects of ethanol. In this study, the effects of D-, L-, and DL-GA calcium on ethanol-dosed gastric cell viability were investigated using human gastric carcinoma cells (Kato III) and normal rat gastric mucosa cells (RGM1). Addition of 2% and 3 % ethanol to Kato III and RGM1 cells, respectively, decreased their cell viability by approximately 20-50 % after 24 or 72 h of cultivation. In 2 % ethanol-dosed Kato III cells cultivated for 24 h, addition of 0.002-20 μg/mL D- and L-GA calcium did not affect cell viability. Similarly, addition of less than 20 μg/mL DL-GA calcium did not affect cell viability. However, when 20 μg/mL DL-GA calcium was added, cell viability increased by 35.7 % after 72 h of incubation, compared to the viability of control cells without ethanol or GA. Addition of 20 μg/mL DL-GA calcium to 3 % ethanol-dosed RGM1 cells cultivated for 24 or 72 h also increased cell viability up to those observed in control cells. These results suggest that a racemic mixture of GA may have the strongest effect on enhancing the viability of ethanol-exposed cells.

Key words: glyceric acid, ethanol, cell viability, gastric cells, glycerol use

1 INTRODUCTION

Glycerol is thought to be a promising and abundant carbon source for industrial microbiology because it can be obtained as a by-product of biodiesel fuel (BDF) or oleochemical production through the transesterification of vegetable oils and animal fats[1] Yields can be as high as 14% by weight. Glycerol production has increased significantly in several European countries due to generation of BDF, making glycerol an attractive ingredient in the production of various chemicals. Research on new applications of glycerol in both biotechnological[1] and chemical processes[2] is ongoing.

In earlier studies of the microbial production of D-glyceric acid (D-GA)[3-9] from glycerol, we found that D-GA could be mass-produced using biotechnological processes to yield > 100 g/L per batch[6]. D-GA was originally discovered in plants[10-12], and was reported to have several biological effects, including liver-stimulating and cholesterolytic activities[13], diuresis[11], and the acceleration of ethanol oxidation[14].

Research focusing on the ability of GA to minimize the toxic effects of alcohol is especially important because acetaldehyde, an ethanol metabolite, plays a role in many of the harmful effects associated with ethanol. Eriksson *et al.*[14] investigated whether D-GA calcium salt could accelerate ethanol and acetaldehyde metabolism *in vivo* in rats. They selected male rats with low and high alcohol preference (designated ANA and AA, respectively) and fed the rats with 1.2 g ethanol/kg with or without D-GA calcium administration (0.1 to 1.0 g/kg). Then, they measures blood concentrations of ethanol and acetaldehyde and liver free glycerol content during ethanol intoxication. Eriksson *et al.* found that glycerate treatment accelerated ethanol metabolism by approximately 25% and accelerated acetaldehyde oxidation in ANA. In addition, glycerate treatment elevated hepatic free glycerol levels by approximately 50% in AA[14]. On the basis of these results, the authors hypothesized that the reason for the accelerated oxidation of acetaldehyde by D-glycerate was that glyceric acid accelerates the rate-limiting step of aldehyde metabolism, reoxidation of nicotinamide adenine dinucleotide reduced-form (NADH) to its oxidized form (NAD^+). The authors

*Correspondence to: Hiroshi Habe, Research Institute for Innovation in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN
E-mail: hiroshi.habe@aist.go.jp

Accepted July 14, 2011 (received for review February 23, 2011)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

suggested that acetaldehyde oxidation to acetate by NAD^+-dependent aldehyde dehydrogenase (ALDH) could be coupled to the conversion of D-glycerate to glyceraldehyde, resulting in re-oxidation of NADH to NAD^+. However, no other positive effects of GA on *in vitro* and *in vivo* ethanol exposure have been reported.

We investigated whether the addition of GA calcium salt could enhance the viability of ethanol-exposed gastric cells. Because gastric cells are usually exposed to some form of stress, including alcohol, the enhancement of cellular proliferation or cell viability by medications is very important. Two types of gastric cells—Kato III (human gastric carcinoma) and RGM1 (normal rat gastric mucosa)—were used in this study. GA calcium had a positive effect on the viability of both cell types (ethanol-dosed, cultivated for 72 h).

2 EXPERIMENTAL PROCEDURES

2.1 Materials

All reagents and solvents were of the highest purity commercially available. D- and L-GA calcium salts were purchased from Sigma-Aldrich (St. Louis, MO).

Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/Ham's F-12 (Nacalai Tesque, Tokyo, Japan) were used for cell cultivation.

2.2 Preparation of glycerate samples

D- and L-GA calcium salts were dissolved in pure water to a final concentration of 20 mg/ml, and then filtered using Minisart 0.2-μm filters (Sartorius, Goettingen, Germany). Sample solutions of 50% D-GA/50% L-GA (DL-GA) calcium were prepared by mixing D- and L-GA calcium solutions at a 1:1 ratio.

2.3 Cell cultures

Kato III and RGM1 cells were obtained from Riken Cell Bank (Ibaraki, Japan). Kato III cells were cultivated in RPMI 16401 supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 1% penicillin-streptomycin solution (PSS; Nacalai Tesque). RGM1 cells were cultivated in DMEM/Ham's F-12 supplemented with 20% FBS and 1% PSS. All cells were cultivated in a humidified atmosphere at 37℃, 5% CO_2.

2.4 Cell viability assay

Cell viability was determined using Cell Count Reagent SF (Nacalai Tesque). After cultivation, the medium was removed, and 100 μL of the medium containing 10% SF reagent was added to each well. Cells were then incubated at 37℃ in 5% CO_2 for up to 3 h. During incubation, the absorbance of the cells at 450 nm was determined at intervals of 30 min by using a Precision microplate reader (Molecular Devices, Sunnyvale, CA).

The data are expressed in terms of cell viability (%), which was calculated according the following equation:

$$\text{Cell viability (\% of control)} = (A_{sT1} - A_{sT0})/(A_{cT1} - A_{cT0}) \times 100,$$

where A_{sT1} and A_{cT1} are the absorbance of the supplemented and control media after 1 (Kato III) or 3 h (RGM1) of cultivation, and A_{sT0} and A_{cT0} are the absorbance of the supplemented and control media after 30 min of incubation, respectively. The results presented are the means ± SD of 3 independent experiments.

2.5 Effect of ethanol on Kato III and RGM1 cell viability

Kato III and RGM1 cells were seeded in 96-well microplates (5×10^5 cells/well) and cultivated as described above. After 24 h, the medium was replaced with RPMI 16401 for Kato III cells and DMEM/Ham's F-12 for RGM1 cells (100 μL), both containing 10% FBS (20% for RGM1) and 1% PSS supplemented with 0.5−5% ethanol. The cells were then cultivated for another 24 or 72 h. Control cells were cultivated in the same media without ethanol.

Cell viability was determined as described above, except that in this experiment, A_c corresponded to the absorbance of cells cultivated without ethanol.

2.6 Effect of GA calcium on the viability of ethanol-dosed Kato III cells

Kato III cells were seeded in 96-well microplates (5×10^5 cells/well) and cultivated as described above. After 24 h, the medium was replaced with RPMI 16401 (100 μL) containing 10% FBS and 1% PSS supplemented with ethanol and GA, and the cells were cultivated for another 24 or 72 h. The final concentration of ethanol was 2%, and the final concentration of D- or L-GA calcium was 0.002, 0.02, 0.2, 2.0, or 20.0 μg/mL. Control cells were cultivated in the same medium without ethanol or GA calcium.

Cell viability was determined as described above, except that in this experiment, A_c was the absorbance of cells cultivated without either ethanol or GA calcium.

2.7 Effect of GA calcium on the viability of ethanol-dosed RGM1 cells

RGM1 cells were seeded in 96-well microplates (5×10^5 cells/well) and cultivated as described above. After 24 h, the medium was replaced with DMEM/Ham's F-12 (100 μL) containing 20% FBS and 1% PSS supplemented with ethanol and GA calcium. The cells were then cultivated for another 24 or 72 h. In this assay, the final concentration of ethanol was 3%, and the final concentration of D-GA, L-GA calcium, or their mixture was 0.002, 0.02, 0.2, 2.0, or 20.0 μg/mL. Control cells were cultivated in the same medium without ethanol or GA calcium.

Cell viability was determined as described above, except that in this experiment, A_c was the absorbance of cells cul-

tivated without ethanol or GA calcium.

2.8 Statistical analysis

The results are expressed as mean ± SD of values obtained from at least three independent experiments. Comparisons between specific groups were made using student's *t*-test.

3 RESULTS AND DISCUSSION

3.1 Effect of the ethanol concentration on the antiproliferative activity of Kato III cells

Before evaluating the effect of GA calcium on the viability of ethanol-dosed Kato III cells, we determined the ethanol concentration that decreased cell viability by approximately 50%. Kato III cells were incubated with 0.5, 1, 2, and 5% ethanol for 24 or 72 h and assayed for viability (Fig. 1). After 24 h of cultivation (black bars), cell viability gradually increased in the groups receiving treatment with up to 1% ethanol. When cells were incubated with ≥ 2% ethanol, there was a significant decrease in viability. After 72 h of cultivation (white bars), a gradual decrease in viability was observed up to 2% ethanol, and at 2% ethanol, the cell viability decreased by 45% compared to that of the controls. On the basis of these results, we used 2% ethanol as the dose in the following experiments with Kato III cells.

3.2 Effect of D-, L-, and DL-GA calcium on the viability of 2% ethanol-dosed Kato III cells

The effect of GA calcium was evaluated on the basis of the percent viability of Kato III cells incubated with or without 2% ethanol and 0.002 − 20 μg/mL GA calcium. Cell viability without ethanol or GA calcium was set as 100% (control). Figure 2 represents the cell viability assay results after 24 h of cultivation. Roughly the same results were obtained for all enantiomers (D-, L-, and DL-GA calcium). In the absence of ethanol (black bars), there was a minor decrease in cell viability with GA calcium, but we did not detect a significant relationship between GA

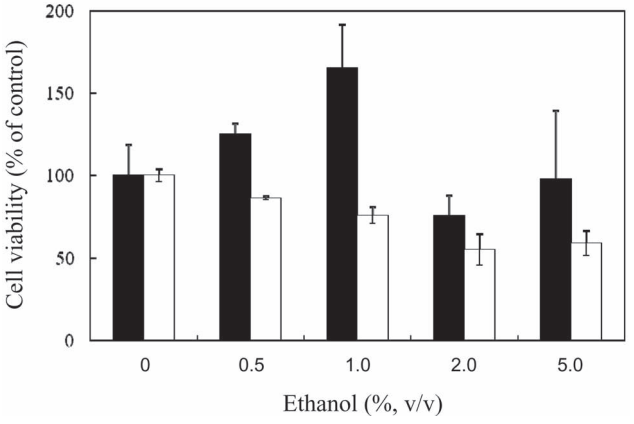

Fig. 1 Effect of ethanol on Kato III cell viability.
Kato III cells were cultivated for 24 (black bars) or 72 h (white bars) in a medium supplemented with 0.5–5 % ethanol. Control cells were cultivated without ethanol. The values are presented as the mean (± SD) percentage of the control from 3 independent experiments.

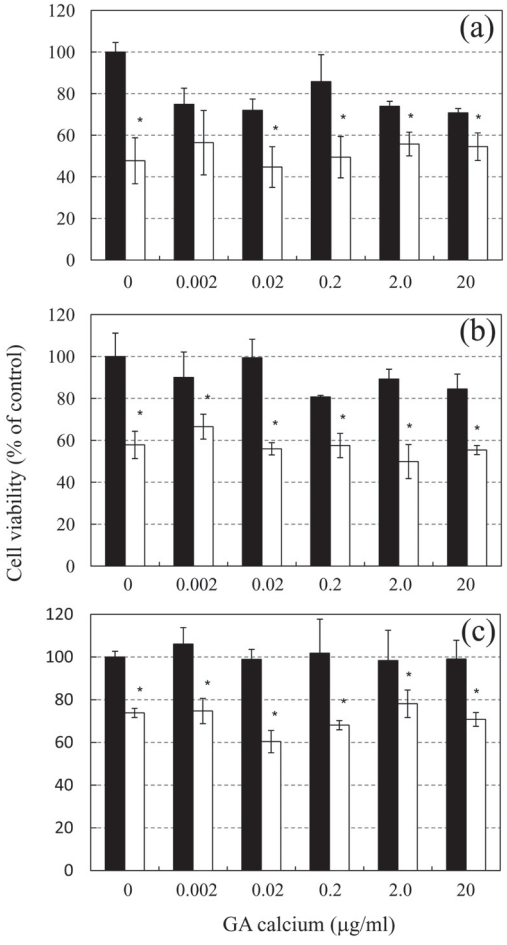

Fig. 2 Effect of D-GA (a), L-GA (b), and DL-GA (c) calcium on Kato III cell viability following 24 h of cultivation with or without 2 % ethanol (white and black bars, respectively).
Kato III cells and 2 % ethanol-dosed Kato III cells were cultivated for 24 h in medium supplemented with 0–20 μg/mL GA. Control cells were cultivated without ethanol or GA. The values are presented as the mean (± SD) percentage of the control from 3 independent experiments. Differences in cell viability with or without 2 % ethanol in each GA concentration group were compared by student's *t* test. * represents significant difference at $p < 0.05$.

calcium concentration and cell viability. We were also unable to detect any relationship between GA calcium concentration and cell viability after 24 h of incubation with 2% ethanol (white bars). These results indicate that GA calcium in the range of 0.002 − 20 μg/mL did not affect cell viability after 24 h of cultivation.

However, we found that the addition of GA calcium enhanced the recovery of cell viability after 72 h of cultivation (Fig. 3). Importantly, 20 μg/mL DL-GA calcium significantly increased cell viability compared to the viability of the control samples cultivated without ethanol or DL-GA and those cultivated with DL-GA but without ethanol (35.7% and 19.5%, respectively; $p < 0.05$). No statistically significant enantiospecific effects of D- and L-GA calcium were observed (Fig. 3). This result is intriguing as the D-enantiomer of GA is normally formed as a metabolite during fructose breakdown via D-glyceraldehyde. In previous reports, only D-GA was examined for biological activity, including the acceleration of ethanol oxidation[11−14].

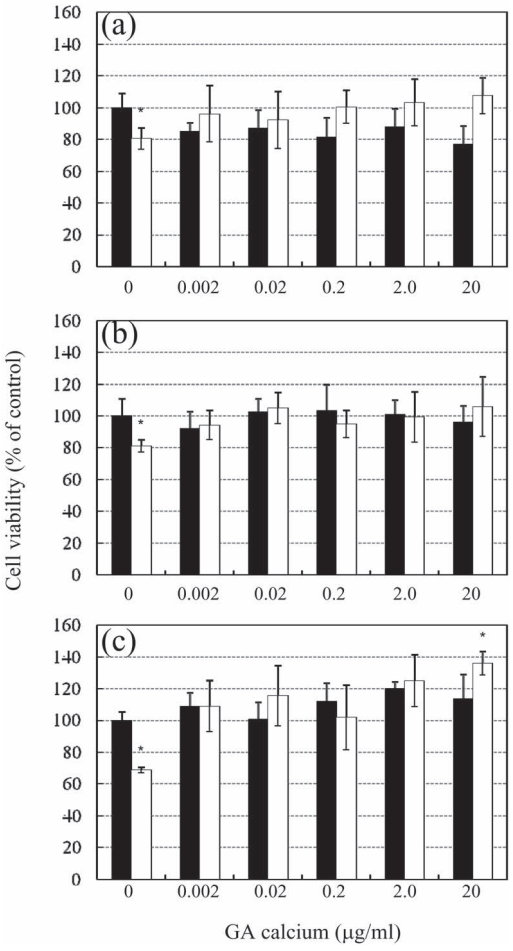

Fig. 3 Effect of D-GA (a), L-GA (b), and DL-GA (c) calcium on Kato III cell viability following 72 h of cultivation with or without 2 % ethanol (white and black bars, respectively).
Kato III cells and 2 % ethanol-dosed Kato III cells were cultivated for 72 h in medium supplemented with 0–20 μg/mL GA. Control cells were cultivated without ethanol or GA. The values are presented as the mean (± SD) percentage of the control from 3 independent experiments. Differences in cell viability with or without 2 % ethanol in each GA concentration group were compared by student's *t*-test. * represents significant difference at $p < 0.05$.

3.3 Effect of ethanol concentration on the antiproliferative activity against RGM1 cells

To further evaluate the effect of GA on cell viability in ethanol-dosed gastric cells, we used RGM1 cells because they differ from Kato III cells in both origin and condition. We investigated the inhibitory effect of ethanol on RGM1 cells by cultivating the cells with 1, 2, or 5% ethanol for 24 or 72 h (Fig. 4). We observed a difference in the extent of cell damage depending on the ethanol concentration when comparing the 24 and 72 h-cultured cells. After 24 h of incubation (black bar), there was only a 9% decrease in the number of viable cells at 2% ethanol, whereas at the same ethanol concentration, there was a 50% decrease in the number of viable cells after 72 h of incubation (white bar). This result suggests that 2% ethanol is suitable for cells cultured for 72 h, but the concentration is too low for cells cultured for 24 h. At 5% ethanol, 77% of the remaining cells were viable after 24 h of incubation, while only 4% of the cells were viable after 72 h (Fig. 4). We used linear regression to calculate that approximately 40% of cell viabil-

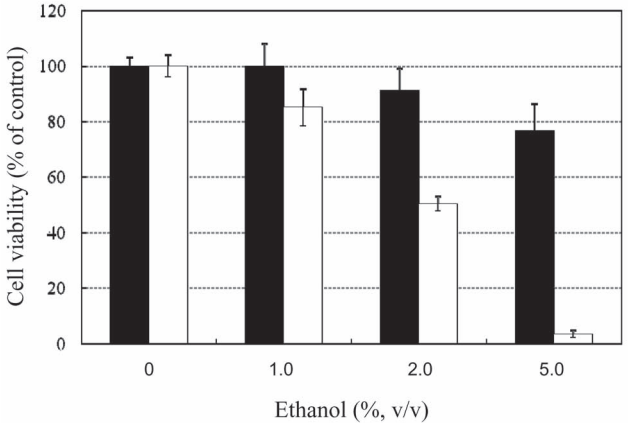

Fig. 4 Effect of ethanol on RGM1 cell viability.
RGM1 cells were cultivated for 24 (black bars) or 72 h (white bars) in medium supplemented with 1–5 % ethanol. Control cells were cultivated without ethanol. The values are presented as the mean (± SD) percentage of the control from 3 independent experiments.

ity should remain at 3% ethanol. Therefore, we decided to use 3% ethanol as the dose in the following experiments with RGM1 cells.

3.4 Effect of D-, L-, and DL-GA calcium on the viability of 3% ethanol-dosed RGM1 cells

Similar to Kato III cells, the effect of GA calcium was examined in terms of the percent viability of RGM1 cells cultivated with or without 3% ethanol and 0.002－20 μg/mL GA calcium. No enhancement of viability in ethanol-dosed RGM1 cells was observed with either D- or L-GA calcium after 24 h of cultivation (data not shown). The addition of DL-GA calcium, however, increased cell viability (Fig. 5a). While neither D- nor L-GA alone increased cell viability after 72 h of cultivation, DL-GA calcium did, but only in the presence of ethanol (Fig. 5b). As shown in Fig. 5, within the range of 0－0.02 μg/mL GA calcium, obvious differences ($p<0.05$) were observed in viability between cells cultivated with or without ethanol. However, with the addition of more than 0.2 μg/mL GA calcium, cell viability increased. At high doses of DL-GA, the increases in viability approached the level of controls.

DL-GA was effective for recovering viability in RGM1 cells, a finding consistent with our results for Kato III cells. This suggests a racemic mixture of GA may be the most efficient for recovering viability in both types of ethanol-damaged gastric cells. As DL-GA may promote cellular metabolic activity for ethanol, further investigation is warranted.

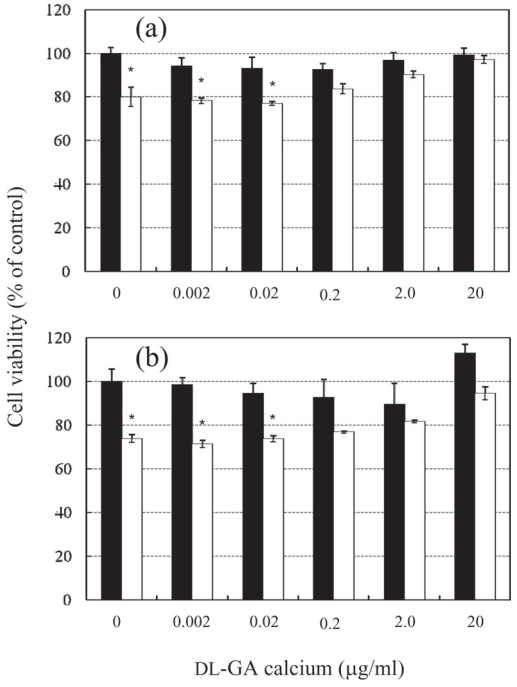

Fig. 5 Effect of DL-GA calcium on the viability of RGM1 cells cultivated for 24 (a) or 72 h (b) with or without 3 % ethanol (white and black bars, respectively).
RGM1 cells and 3 % ethanol-dosed RGM1 cells were cultivated for 24 or 72 h in medium supplemented with 0–20 μg/mL DL-GA. Control cells were cultivated without ethanol or GA. The values are presented as the mean (± SD) percentage of the control from 3 independent experiments. Differences in cell viability with or without 3 % ethanol in each concentration were compared by student's *t*-test. * represents significant difference at $p < 0.05$.

4 CONCLUSIONS

Our findings indicate that the addition of GA calcium salt to an ethanol-dosed gastric cell culture can enhance cell viability. Our study of 2 types of gastric cells suggests that a racemic mixture of GA, rather than D-GA, has the greatest effect on increasing the rate of cell viability. Thus far, investigations of the biological effects of GA have focused on D-GA[11－14], because D-GA is a natural plant phytochemical[12]. However, our results suggest that it is important to examine the biological effects of not only D-GA but also of L-GA and DL-GA, which may broaden the applications of GA.

ACKNOWLEDGEMENTS

This work was supported financially in part by the Japan-U.S. Cooperation Project for Research and Standardization of Clean Energy Technologies from the Ministry of Economy, Trade, and Industry of Japan.

References

1) da Silva, G. P.; Mack, M.; Contiero, J. Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnol. Adv.* **27**, 30-39 (2009).
2) Zhou, C.-H.; Beltramini, J. N.; Fan, Y.-X.; Lu, G. Q. Chemoselective catalytic conversion of glycerol as a biorenewable source to valuable commodity chemicals. *Chem. Soc. Rev.* **37**, 527-549 (2008).
3) Habe, H.; Fukuoka, T.; Kitamoto, D.; Sakaki, K. Biotransformation of glycerol to D-glyceric acid by *Acetobacter tropicalis*. *Appl. Microbiol. Biotechnol.* **81**, 1033-1039 (2009).
4) Habe, H.; Fukuoka, T.; Kitamoto, D.; Sakaki, K. Application of electrodialysis to glycerate recovery from a glycerol containing model solution and culture broth.

J. Biosci. Bioeng. **107**, 425-428(2009).
5) Habe, H.; Shimada, Y.; Fukuoka, T.; Kitamoto, D.; Itagaki, M.; Watanabe, K.; Yanagishita, H.; Sakaki, K. Production of glyceric acid by *Gluconobacter* sp. NBRC3259 using raw glycerol. *Biosci. Biotechnol. Biochem.* **73**, 1799-1805(2009).
6) Habe, H.; Shimada, Y.; Yakushi, T.; Hattori, H.; Ano, Y.; Fukuoka, T.; Kitamoto, D.; Itagaki, M.; Watanabe, K.; Yanagishita, H.; Matsushita, K.; Sakaki, K. Microbial production of glyceric acid, an organic acid that can be mass produced from glycerol. *Appl. Environ. Microbiol.* **75**, 7760-7766(2009).
7) Habe, H.; Fukuoka, T.; Morita, T.; Kitamoto, D.; Yakushi, T.; Matsushita, K.; Sakaki, K. Disruption of the membrane-bound alcohol dehydrogenase-encoding gene improved glycerol use and dihydroxyacetone productivity in *Gluconobacter oxydans*. *Biosci. Biotechnol. Biochem.* **74**, 1391-1395(2010).
8) Habe, H.; Shimada, Y.; Fukuoka, T.; Kitamoto, D.; Itagaki, M.; Watanabe, K.; Yanagishita, H.; Sakaki, K. Use of a *Gluconobacter frateurii* mutant to prevent dihydroxyacetone accumulation during glyceric acid production from glycerol. *Biosci. Biotechnol. Biochem.* **74**, 2330-2332(2010).
9) Habe, H.; Shimada, Y.; Fukuoka, T.; Kitamoto, D.; Itagaki, M.; Watanabe, K.; Yanagishita, H.; Sakaki, K. Two-stage electrodialytic concentration of glyceric acid from fermentation broth. *J. Biosci. Bioeng.* **110**, 690-695(2010).
10) Palmer, J. K. Occurrence of D-glyceric acid in tobacco leaves. *Science* **123**, 415(1956).
11) Morrison, R. I.; Dekock, P. C. Glyceric acid in broad bean(Vicia faba L.). *Nature* **184**, 819(1959).
12) Duke, J. A. *Handbook of Phytochemical Constituents of GRAS Herbs and Other Economic Plants*. CRC Press LLC. Boca Raton, FL(2001).
13) Handa, S. S.; Sharma, A.; Chakraborti, K. K. Natural products and plants as liver protecting drugs. *Fitoterapia* **57**, 307-351(1986).
14) Eriksson, C. J. P.; Saarenmaa, T. P. S.; Bykov, I. L.; Heino, P. U. Acceleration of ethanol and acetaldehyde oxidation by D-glycerate in rats. *Metabolism* **56**, 895-898 (2007).