International Journal of
Science and Engineering Investigations vol. 6, issue 69, October 2017
ISSN: 2251-8843

Effect of Adding Fatty Acid in Culture Medium on Cell Growth of Acid Tolerant Lactic Acid Bacterium

Kenji Tanaka[1], Kiko Sakai[2], Saki Goto[3], Hiromi Matsusaki[4]
[1,2]Department of Biological and Environmental Chemistry, Faculty of Humanity-Oriented Science and Engineering, Kindai University, Japan
[3.4]Department of Food and Health Sciences, Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, Japan
([1]tanaka@fuk.kindai.ac.jp, [2]1411110029u@ed.fuk.kindai.ac.jp, [3]g1675001@pu-kumamoto.ac.jp, [4]matusaki@pu-kumamoto.ac.jp)

Abstract- Tween 80 is important growth factor for lactic acid bacterium, especially for *Lactobacillus* spp. The composition of fatty acids of the lipid in cell membrane of highly acid-tolerant *Lactobacillus acetotolerans* HT was determined and the effect of tween 80 and 9 kinds of fatty acid on the cell growth was investigated. The strain HT grew after very long lag phase for about 300h in synthetic MRS medium without addition of fatty acid. Tween 80, stearic acid, oleic acid or *cis*-vaccenic acid stimulated the growth of strain HT. Particularly, the lag phase was drastically shortened by addition of 5mg/*l* of oleic acid or *cis*-vaccenic acid as well as 1000mg/*l* of tween 80. However, the addition of oleic or *cis*-vaccenic acid more than 50mg/*l* suppressed seriously the cell growth. Other kinds of unsaturated C18 (linoleic acid, conjugated linoleic acid, elaidic acid) and cycC19:0Δ^{11} (lactobacillic acid) were also effective for the enough growth of the strain HT. These results indicate that fatty acid is not essential for the growth of *Lb.acetotolerans* HT. However, fatty acid is very important to shorten the long lag phase of this bacterium and the effect greatly differs depending on kind of fatty acid and the concentration.

Keywords- *Lactobacillus, Fatty Acid, Acid Tolerance*

I. INTRODUCTION

Lactic acid bacteria are useful microorganisms used in food industry and the production of lactic acid and bacteriocins for instance nisin. Lactic acid bacteria contribute to human health with the intestine regulating function, protecting action on infectious disease, immunopotentiative action and carcinogen suppression action [1]. Lactic acid bacterium produce lactic acid from glucose and other sugars and lactic acid is widely used in various fields of industry. Polylactate, which is synthesized from lactic acid, is expected as biodegradable plastic [2]. In viewpoint of commercial use of lactic acid, it is important to produce this compound at high concentration in the fermentation process. However, that is very difficult because lactic acid is inhibitory to microorganisms including lactic acid bacteria. The neutralized salts like sodium lactate also inhibit the growth and fermentation of lactic acid bacteria [3]. We isolated a lactic acid bacterium grown in fermented-rice mash vinegar (acetic acid 6%, pH2.9) and named it as *Lactobacillus acetotolerans* HT. This bacterium is very tolerant to acidic condition, especially high acetic acid concentration and we succeeded to produce about 200g/*l* of DL-lactic acid by controlling the culture pH at 5.5, which was twice higher than those of other lactic acid bacteria [3]. The cell concentration of *Lb.acetotolerans* HT is also much higher than those of other lactic acid bacteria. For instance, the maximum cell concentration of the strain HT in test-tube culture with MRS broth reaches to about 20.0 in the OD_{600} of the culture liquid while that of *Lactococcus lactis* is only about 2.0 [3]. However, the growth of *Lb.acetotolerans* HT is slower than other lactic acid bacteria and it seems that a surfactant, tween 80 is essential for the growth. It is known that nutritional requirement of lactic acid bacteria is very complicate and many kinds of nutrients are necessary for their growth. In this study, we investigated the composition of fatty acids of the lipid in cell membrane of the strain HT and the effect of tween 80 and fatty acids on its cell growth.

II. MATERIALS AND METHOD

A. Microorganism

The lactic acid bacterium used in this study was isolated from fermented-rice mas rice vinegar (acetic acid concentration 6%, pH2.9) stored in the factory of Maruboshi Vinegar Co., Ltd., Japan. The bacterium was identified as *Lactobacillus acetotolerans* according to the homology of 16S rDNA and the results of other taxonomic tests, and it was named *Lb.acetotolerans* HT. The strain HT produces racemic DL-lactic acid and the growth is somewhat sensitive to oxygen. *Lb.acetotolerans* HT grows in liquid culture with test tube in atmosphere without shaking but in 300ml-flask culture with a shallow depth of the liquid medium (5ml) it does not grow at the oxygen concentration of the head space within the flask above 10v/v% [3]

B. Culture medium and culture condition

All the culture tests were carried out in test tube with a screw cap without shaking at 30°C. The culture medium used was commercially provided MRS broth (Difco™ Lactobacilli MRS Broth) which was composed of proteose peptone, 10 g; beef extract, 10 g; yeast extract, 5 g; D-glucose, 20 g; tween 80,

1.0 g; ammonium citrate 2.0 g; sodium acetate, 5.0 g; magnesium sulfate, 0.1 g; dipotassium phosphate 2.0 g; manganese sulfate 0.05 g per 1.0 *l* of distilled water. We also prepared MRS broth by adding each nutrient separately to distilled water and adjusting the pH to 6.3 (we call it "synthetic MRS medium" in this article). Preculture was carried out for 40h with 20ml of MRS broth. The cells were harvested from 0.5ml of the culture liquid then they were washed three times by repeating centrifugation and suspension to sterilized saline solution. The washed cells were inoculated to 20mL of the synthetic MRS medium for main culture.

C. Analyses

The cell growth in test-tube culture was monitored by measuring the optical density at 600nm of the culture liquid after diluted with a saline solution. Fatty acid composition was determined as follows. The cells were harvested in exponential growth phase by centrifugation and about 40mg of the wet pellet was put into a test tube with a screw cap. One milliliter of the mixture of sodium hydroxide and methanol was added into the test tube then it was heated 100°C for 30min for saponification of the lipid in the cells. Two milliliter of 1 M hydrochloric acid in methanol was then added into the test tube then it was heated 80°C for 10min for methyl esterification of fatty acids. To extract the methyl esters of fatty acids, 1.25ml of methyl *tert*-butyl ether and hexane solution was added then the mixture was shaken for 10min. The organic layer was used as the sample for gas chromatography to determine the methyl esters of fatty acids.

III. RESULTS

A. Effect of tween 80 on growth of Lb.acetotolerans HT

The effect of each component of MRS broth on the cell growth of *Lb.acetotolerans* HT was investigated. The strain HT was cultured for 120h in the synthetic media which were prepared by excluding one component from MRS broth except glucose.

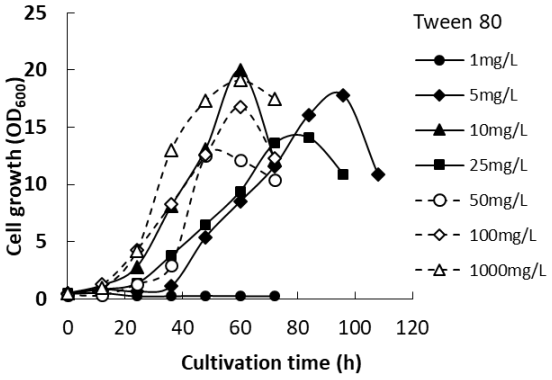

Figure 1. Growth curves of *Lb.acetotolerans* HT in synthetic MRS medium added with various concentrations of tween 80

As a result, there was no cell growth of the strain HT only in the medium of which tween 80 (1000mg/*l*) was excluded. MRS broth is widely used for cultivation of lactobacilli. It has been explained that tween 80, (*x*)-sorbitan mono-9-octadecenoate poly(oxy-1,2-ethanediyl) is added in MRS broth because oleic acid (*cis*-9-octadecenoic acid) is essential for the growth of many strains of *Lactobacillus* spp. [4]. Hence, the cell growth of the strain HT was investigated when concentration of tween 80 in the synthetic MRS medium was changed. The result is shown in Figure 1. The fastest cell growth was obtained when 1000mg/*l* of tween 80, which was the same concentration as the commercially provided MRS broth, was used. However, the decrease in concentration of tween 80 in the culture medium slowed down the cell growth and no cell growth was observed in the culture added with 1mg/*l* of tween 80.

B. Effect of fatty acids on growth of Lb.acetotolerans HT

Fatty acid composition of the lipid in cell membrane of *Lb.acetotolerans* HT was analyzed by gas chromatography. The fatty acids detected from the *Lb.acetotolerans* HT cells were myristic acid (C14:0) 3.3%, palmitic acid (C16:0) 39.1%, palmitoleic acid (C16:1$\triangle^9$) 3.7%, stearic acid (C18:0) 4.0%, oleic acid (C18:1$\triangle^9$) 17.8%, *cis*-vaccenic acid (C18:1$\triangle^{11}$) 11.8%, *cis*-9,10-methyleneoctadecanoic acid (cycC19:0$\triangle^9$) 9.7%, *cis*-11, 12-methyleneoctadecanoic acid (cycC19:0$\triangle^{11}$, lactobacillic acid) 9.7% and the slight amount of other fatty acids. Hence, the effect of the fatty acids with relative high content in the strain HT on the cell growth was investigated except in case of cycC19:0. Palmitic acid, stearic acid, oleic acid and *cis*-vaccenic acid were added respectively in the synthetic MRS medium at various concentrations then the cell growth in test-tube culture was monitored until 108h (Figure 2).

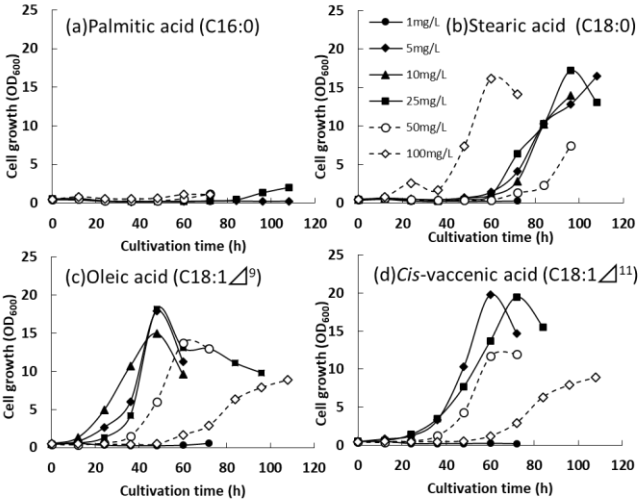

Figure 2. Growth curves of *Lb.acetotolerans* HT in synthetic MRS medium added with various concentrations of fatty acids

The addition of sight amount of C18 fatty acids promoted the growth of the strain HT while the growth promotion effect by C16:0 (palmitic acid) was in negligible levels. Particularly, addition of 5mg/*l* of unsaturated C18 fatty acids (oleic acid or

cis-vaccenic acid) gave the most vigorous cell growth which was comparable to that obtained by commercially provided MRS broth containing 1000mg/*l* of tween 80. However, the cell growth was suppressed at the unsaturated C18 fatty acid concentrations more than 50mg/*l*.

The effect of other kind of fatty acids was also investigated. Linoleic acid (C18:2$\Delta^{9,12}$), conjugated linoleic acid (C18:2$\Delta^{9,11}$), α-linolenic acid (C18:3 $\Delta^{9,12,15}$), lactobacillic acid (cycC19:0Δ^{11}) and elaidic acid (C18:1*trans*Δ^{9}) were added respectively at 25mg/*l* to the synthetic MRS medium without tween 80 (Figure 3). It was shown that the addition of these fatty acids except α-linolenic acid stimulated the cell growth of the strain HT. However, the growth in the cultures added with these four unsaturated fatty acids was slower than that of oleic acid and *cis*-vaccenic acid. There was no growth promotion effect by addition of α-linolenic acid.

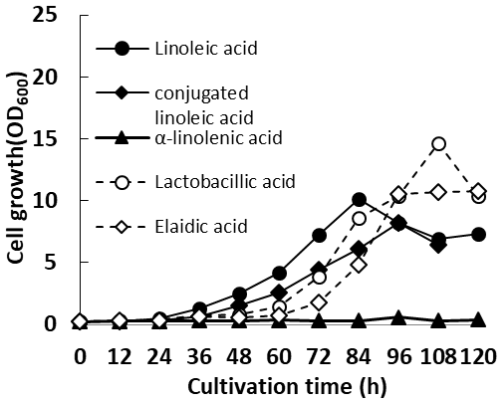

Figure 3. Growth curves of *Lb.acetotolerans* HT in synthetic MRS medium added with other fatty acids.

C. Effect of extending culture time on the growth of Lb.acetotolerans HT

As shown in Figure 2, there was no growth promotion effect on *Lb.acetotolerans* HT by addition of palmitic acid (C16:0) within 108h of cultivation but a slight increase in cell concentration was observed. Hence, the effect of palmitic acid was tested again with expanding the cultivation time (Figure 4). The cell growth without addition of any fatty acid was also investigated (in case of palmitic acid 0mg/*l* in Figure 4). As a result, cell growth was obviously observed after 144h, especially in the cultures added with 2, 5, 10 and 25mg/*l* of palmitic acid. Even in addition of 1mg/*l* palmitic acid, high cell concentration (the maximum OD_{600}, 9.52) was obtained. In the cultures added with the higher concentrations of palmitic acid (50 and 100mg/*l*), the cell growth was delayed or suppressed seriously. On the other hand, in the culture without addition of palmitic acid (0mg/*l*), the rise of cell growth was observed after 336h from the start of cultivation then the cell concentration increased to 8.6 in OD_{600}. The cells grown in the culture without palmitic acid was inoculated into MRS agar plates after diluted with sterilized saline solution and they were cultured in anaerobic condition. Some of the colonies formed in MRT agar were picked up and they were used for extraction of genome DNA. Then 16S rRNA gene was amplified by PCR with the extract as template DNA. The homology analysis for the PCR product from each bacterial colony showed that they were *Lb.acetotolerans*.

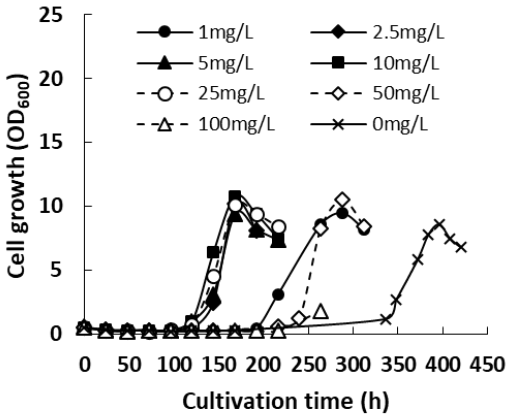

Figure 4. Growth curves of *Lb.acetotolerans* HT in synthetic MRS medium added with various concentrations of palmitic acid.

IV. DISCUSSION AND CONCLUSION

The composition of fatty acids in the cell membrane of *Lb.acetotolerans* HT were mainly myristic acid (C14:0) 3.28%, palmitic acid (C16:0) 39.1%, palmitoleic acid (C16:1Δ^{9}) 3.7%, stearic acid (C18:0) 4.0%, oleic acid (C18:1Δ^{9}) 17.8%, *cis*-vaccenic acid (C18:1 Δ^{11}) 11.8%, *cis*-9,10-methyleneoctadecanoic acid (cycC19:0Δ^{9}) 9.73%, *cis*-11, 12-methyleneoctadecanoic acid (cycC19:0Δ^{11}, lactobacillic acid) 9.7%. These fatty acids are widely detected in other lactic acid bacteria. When the ratios of C14:0, C16:0, C16:1, C18:0, C18:1 and cycC19:0 for the strain HT were respectively compared to those for other lactobacilli species [5, 6], there was no notable difference. However, it is not able to argue the ratios of oleic acid and *cis*-vaccenic acid for each because there are few reports that investigated the ratios of these two C18:1 fatty acid separately. Our results indicated that C18 fatty acids, especially unsaturated C18 are very effective for the rapid cell growth of *Lb.acetotolerans* HT. It is known that biosynthesis of straight-chain fatty acid from acetyl-CoA and NADPH by fatty acid synthases occurs until palmitic acid is produced. Palmitic acid is converted to stearic acid via a number of modifications. By the action of stearoyl-CoA 9-desaturase, stearic acid is converted to oleic acid. Therefore, it is supposed that the addition of C18 compounds to the culture medium will support to supply oleic acid and *cis*-vaccenic acid which are important components of the cell membrane in the strain HT. On the other hand, the requirement for oleic acid and *cis*-vaccenic acid was very slight and the increase of these fatty acids was rather inhibitory to the cell growth. It may be considered that the unsaturated C18 fatty acid have any catalytic role in the role.

There are also many reports for the relationship between oleic acid and the stress resistance in lactic acid bacteria and bifidobacteria, for instance of stability to freezing [7], survival of lactobacilli in gastric juice [8], bile resistance of *Lactobacillus* and *Bifidobacterium* [9], acid tolerance of *Bifidobacterium* [10]. We are now focusing on the effect of oleic acid on highly acid

REFERENCES

[1] Quinto, E.J., Jiménez, P., Caro, I., Tejero, J., Meteo, J. and Girbés, T. (2014). Probiotic Lactic Acid Bacteria: A Review, *Food and Nutrition Sciences*, 5(18), 1765-1775.

[2] Tanaka, K., Komiyama, A., Sonomoto, K., Ishizaki, A., Hall, S.J. and Stanbury P.F. (2002). Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. *Applied Microbiology and Biotechnology*, 60(1・2), 160-167.

[3] Tanaka, K., Tajiri, S., Sawada, R., Kawamoto, Y., Matsubara, T., Hosihino, H. and Matsusaki, H. (2015). Acid-tolerant lactic acid bacterium isolated from rice vinegar. *International Journal of Research in Applied,Natural and Social Sciences*, 3(10), 29-36.

[4] William, W.L., Harry, B.P, and Esmond, S.E. (1947). Oleic acid and related compounds as growth factors for lactic acid bacteria. *Journal of Biological chemistry*, 170, 619-630.

[5] Rizzo, A.F., Korkeala, A. and Mononen, I. (1987). Gas chromatography analysis of cellular fatty acids and neutral monosaccharides in the identification of Lactobacilli. *Applied Environmental and Microbiology*, 53(12), 2883-2888.

[6] Fernández Murga, M.L., Cabrera, G. M., Font De Valdez, G., Disalvo, A. and Seldes, A. M. (2000). Influence of growth temperature on cryotolerance and lipid composition of *Lactobacillus acidophilus*. *Journal Applied Microbiology*, 88, 342-348.

[7] Goldberg, I and Eschar, L. (1977). Stability of Lactic Acid Bacteria to Freezing as Related to Their Fatty Acid Composition. *Applied Environmental and Microbiology*, 33(3), 489-96.

[8] Corcoran, B.M., Stanton, C., Fitzgerald, G.F. and Ross, R.P. (2007). Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. *Microbiology*, 153, 291-299.

[9] Ruiz, L., Margolles, A. and Sánchez, B. (2013). Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Frontiers in Microbiology*, 4, 3-8.

[10] Yang, X., Hang, X., Zhang, M., Liu, X. and Yang, H. (2015). Relationship between acid tolerance and cell membrane in *Bifidobacterium*, revealed by comparative analysis of acid-resistant derivatives and their parental strains grown in medium with and without Tween 80. *Applied Microbiology and Biotechnology*, 99(12), 5227-5236.