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Culture Medium Optimization for Acetic Acid Production by a Persimmon Vinegar–Derived Bacterium

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Abstract

A new acetic acid–producing microorganism, *Acetobacter* sp. RKY4, was isolated from Korean traditional persimmon vinegar, and we optimized the culture medium for acetic acid production from ethanol using the newly isolated *Acetobacter* sp. RKY4. The optimized culture medium for acetic acid production using this microorganism was found to be 40 g/L ethanol, 10 g/L glycerol, 10 g/L corn steep liquor, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and 1.0 g/L $(NH_4)H_2PO_4$. *Acetobacter* sp. RKY4 produced 47.1 g/L of acetic acid after 48 h of fermentation in a 250 mL Erlenmeyer flask containing 50 mL of the optimized medium.

Index Entries: *Acetobacter* sp. RKY4; acetic acid; ethanol; persimmon vinegar.

Introduction

Acetic acid is a represented C_2 organic chemical and an important feedstock for various chemicals such as vinyl acetate polymer, cellulose acetate, terephthalic acid, dimethyl terephthalate, acetic acid ester, acetic anhydride, and calcium magnesium acetate *(1)*. In the food industry, there has been much interest in conventional vinegar from natural resources, for which the biological production of acetic acid by microorganism has been extensively studied. The recently potential increases of acetic acid consumption have been related to the production of environmentally friendly de-icers, calcium magnesium acetate (CMA) as a non-corrosive road de-icer. Acetic acid is the main component of CMA and its demand has gradually increased *(2)*. Acetic acid can be biologically obtained from cheap and abundant biomass as a raw material. The advantage of the biological production of acetic acid is that the renewable resources can be used for its production, which causes no environmental pollution (2–4).

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There are two microbiological approaches for the biological production of acetic acid. First, through a two-step process, glucose is fermented into ethanol by yeast and the resulting ethanol is finally converted into acetic acid by *Acetobacter (5)*. The high concentrations of acetic acid can be produced via the yeast–*Acetobacter* process, but the oxygen requirement for *Acetobacter* cultivation makes the process energy intensive. Second, *Clostridium thermoaceticum* is used for direct conversion of glucose derived from several carbohydrates (e.g., corn, sweet sorghum, sugarcane) into acetic acid *(6)*. This thermopile process (i.e., 55–60ºC) requires only a single bioreactor, but it is also energy intensive, which results in low concentration of acetic acid *(3, 5)*. In addition, the productivity of acetic acid by *Clostridium* strains is generally low due to product inhibition. *Clostridium* strains produce several organic acids as by-products, which results in low yield of acetic acid based on consumed substrate, and they require a high temperature for their cultivation (7–8). Therefore, it is important to develop the process for the production of acetic acid using a single bioreactor and the acetic acid–producing microorganisms with high productivity at low culture temperature. In this work, we isolated an acetic acid–tolerant bacterium from Korean traditional persimmon vinegar and optimized the culture medium for acetic acid production using this newly isolated organism.

Materials and Methods

Microorganism

A new acetic acid–producing bacterium, a strain RKY4, was isolated from Korean traditional persimmon vinegar. The strain RKY4 is Gram-negative and oxidase-positive rod bacterium with the size of approx 1.8 μm in length and 0.6 μm in width (Fig. 1). It was dubbed *Acetobacter* sp. RKY4 because it belonged to the genus *Acetobacter* based on the partial 16S rDNA sequence analysis (unpublished data). *Acetobacter* sp. RKY4 was used for the optimization of culture medium for acetic acid production throughout this study. Stock cultures were maintained at -20ºC in 5 mL vials containing 1 mL of Lactobacilli deMan Rogosa Sharpe (MRS) medium (Difco, Detroit, MI, USA) and 50% (v/v) glycerol until use. Lactobacilli MRS medium was composed of the following components per liter: 20 g glucose, 10 g peptone, 10 g beef extract, 5 g yeast extract, 1 g Tween 80, 5 g ammonium citrate, 5 g sodium acetate, 0.1 g $MgSO_4$, 0.1 g $MnSO_4$, and 2 g K_2HPO_4.

Medium and Culture Condition

For inoculum preparation, the cells from stock cultures were transferred to 50 mL of MRS broth in a 250 mL Erlenmeyer flask, and then this was incubated at 30ºC for 48 h in a shaking incubator (KMC-8480SF, Vision Scientific, Daejon, Korea) set to 200 rpm, which was used for the inoculum for all experiments. The basal medium for main cultivation contained the

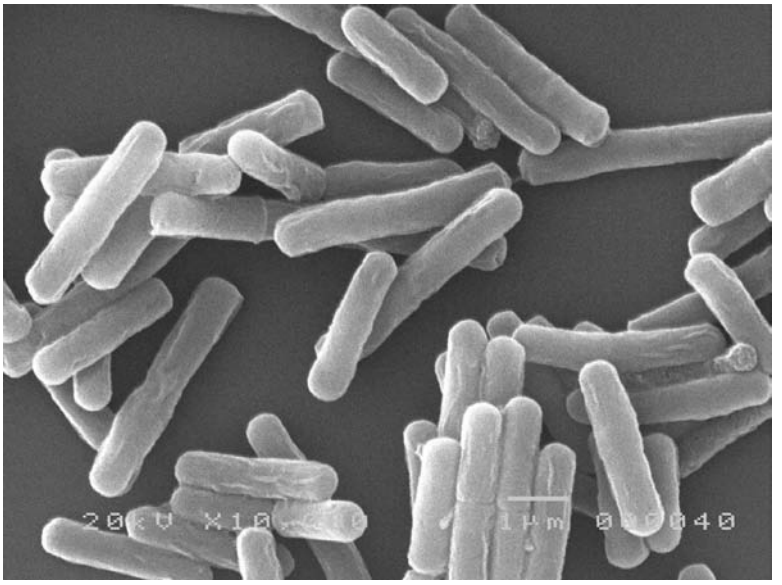

Fig. 1. Scanning electron micrograph of *Acetobacter* sp. RKY4. The cells were fixed with 2% (w/v) glutaraldehyde, dehydrated with graded ethanol, dried at critical point, coated with gold, and then examined by JSM-5400 scanning electron microscope (Jeol, Tokyo, Japan).

following components per liter: 30 g ethanol, 10 g glycerol, 10 g corn steep liquor (CSL), 2.0 g K_2HPO_4, 0.5 g $MgSO_4 \bullet 7H_2O$, and 10 g $CaCO_3$. The initial pH of the fermentation medium was adjusted to 6.5. The main cultivation for acetic acid production was conducted in a shaking incubator set to 30ºC and 200 rpm for 48 h. To find the optimum medium compositions for acetic acid production, each component of the fermentation medium was replaced with different sources.

Analytical Method

Acetic acid concentration was quantified by a Waters high-performance liquid chromatography (HPLC) system (Millipore, Milford, MA, USA) equipped with a Waters 486 tunable absorbance detector set to 210 nm. An Aminex HPX-87H ion-exclusion column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) was eluted with 5 m*M* H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min, while the column temperature was maintained at 40ºC.

Results and Discussion

Effect of Carbon Sources

To investigate the effect of carbon sources on acetic acid formation, *Acetobacter* sp. RKY4 was cultivated in the cultivation medium containing 10 g/L of various carbon sources (e.g., galactose, maltose, glucose, sucrose,

fructose, lactose, starch, and glycerol). After 48 h of incubation at 30ºC and 200 rpm, *Acetobacter* sp. RKY4 showed different characteristics on the utilization of each carbon source (data not shown). Glucose or glycerol being used as a carbon source, *Acetobacter* sp. RKY4 produced above 20 g/L acetic acid. However, *Acetobacter* sp. RKY4 rarely utilized galactose, sucrose, and lactose, which resulted in a low concentration of acetic acid in the broth. *Acetobacter* species oxidize ethanol with alcohol dehydrogenase to yield acetic acid and reducing power in the form of $PQQH_2$ *(9)*. Some carbohydrates are oxidized to CO_2 exclusively through the pentose phosphate pathway. Whereas pyruvate is oxidized to acetyl-CoA and CO_2 in most aerobes, the acetic acid bacteria decarboxylate it non-oxidatively to acetaldehyde, which is the precursor of acetic acid from ethanol *(10)*. Therefore, *Acetobacter* sp. RKY4 might not only convert some moieties of glucose and glycerol to acetic acid but also use some moieties of them for energy source and cell maintenance.

To investigate the effect of glucose and glycerol concentrations on acetic acid formation, *Acetobacter* sp. RKY4 was cultivated in the main cultivation medium containing various concentrations of glucose or glycerol as a carbon source. As shown in Table 1, the produced acetic acid concentration increased with increase in glucose or glycerol concentration up to 10 g/L. When 10 g/L of glucose or glycerol was used as a carbon source for acetic acid formation, *Acetobacter* sp. RKY4 produced 33.6 g/L or 35.6 g/L of acetic acid, respectively. However, beyond 10 g/L of glucose or glycerol, acetic acid concentration and yield decreased gradually, which might be caused by substrate inhibition. The maximum acetic acid yields based on consumed ethanol were 86% for glucose and 91% for glycerol. When the fermentation medium contained 30 g/L or higher glucose, *Acetobacter* sp. RKY4 produced small amounts of lactic and propionic acids as by-products. We selected glycerol as an optimum carbon source for acetic acid formation because *Acetobacter* sp. RKY4 could produce more acetic acid using glycerol than using glucose, as shown in Table 1.

Effect of Ethanol Concentrations

The bacterial tolerance to acetic acid can be increased by increase in ethanol concentration in the cultivation medium *(11–13)*. However, the cell growth of the acetic acid bacteria would be inhibited significantly if the medium contains more than 50 g/L ethanol *(14)*. Therefore, we investigated the effect of ethanol concentrations as an oxidizable substrate on acetic acid formation. For that purpose, *Acetobacter* sp. RKY4 was cultivated in the main cultivation medium containing 0–50 g/L ethanol and 10 g/L glycerol. As shown in Fig. 2, the produced acetic acid concentration increased with increase in ethanol concentration up to 40 g/L, but decreased beyond this value. The maximum concentration of acetic acid was obtained at 45.5 g/L from the medium containing 40 g/L ethanol.

Table 1
Effect of Glucose and Glycerol Concentrations on Acetic Acid Formation and Yield

Glucose (g/L)	Acetic acid (g/L)	$Y_{Ac/Et}$ [a]	Glycerol (g/L)	Acetic acid (g/L)	$Y_{Ac/Et}$
0	24.7	0.63	0	29.4	0.75
2.5	26.5	0.68	2.5	30.2	0.77
5.0	26.4	0.68	5.0	34.4	0.88
7.5	31.6	0.81	7.5	35.6	0.91
10	33.6	0.86	10	35.6	0.91
15	33.0	0.84	15	35.0	0.89
20	29.8	0.76	20	31.5	0.81
30	27.0	0.70	30	24.8	0.63

Medium composition (g/L): ethanol 30, CSL 10, K_2HPO_4 2, $MgSO_4 \bullet 7H_2O$ 0.5, and $CaCO_3$ 10.

[a] $Y_{Ac/Et}$: moles of the produced acetic acid/moles of the consumed ethanol.

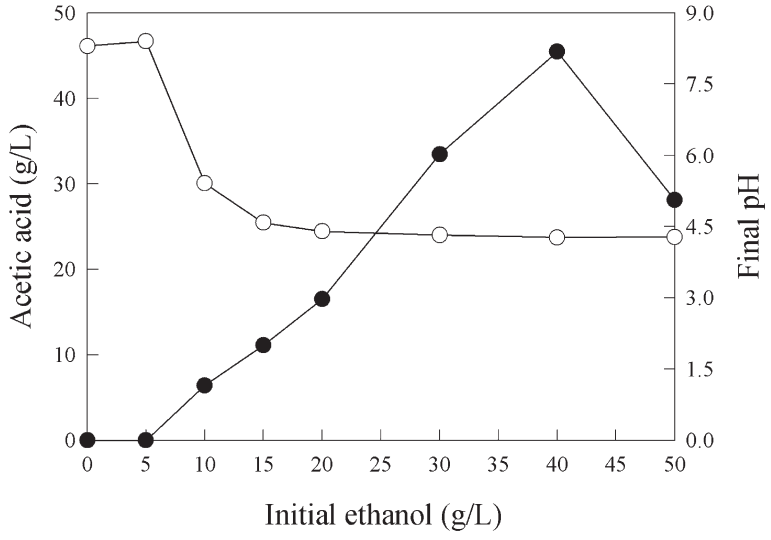

Fig. 2. Effect of ethanol concentrations on acetic acid formation and final pH. The cultivation medium was composed of 10 g/L glycerol, 10 g/L CSL, 2 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and 10 g/L $CaCO_3$. Symbols: ●, acetic acid and ○, final pH.

Effect of Nitrogen Sources

To investigate the effect of nitrogen sources on acetic acid formation by batch culture of *Acetobacter* sp. RKY4, 10 g/L of several nitrogen sources (e.g. yeast extract, peptone, CSL, malt extract, beef extract, NH_4Cl, $(NH_4)_2SO_4$, and urea) were tested. The complex nitrogen sources such as yeast extract, peptone, CSL, and beef extract were superior to the

inorganic nitrogen sources such as NH_4Cl, $(NH_4)_2SO_4$, and urea (data not shown). Among those nitrogen sources tested, yeast extract was previously considered as one of the effective sources for acetic acid formation *(15)*, which was well agreed with our results. Although *Acetobacter* sp. RKY4 produced above 42 g/L acetic acid in the medium containing yeast extract, peptone, or CSL, it produced only 12 g/L acetic acid in the medium containing malt extract and NH_4Cl. The addition of inorganic nitrogen sources such as $(NH_4)_2SO_4$ and urea gave little stimulation in acetic acid formation. These results suggest that *Acetobacter* sp. RKY4 should be a fastidious microorganism like most acetic acid bacteria (9, 10).

To investigate the effect of nitrogen sources concentrations on acetic acid formation, *Acetobacter* sp. RKY4 was cultivated with various concentrations of three nitrogen sources, yeast extract, peptone, and CSL. As shown in Fig. 3, acetic acid concentration produced by *Acetobacter* sp. RKY4 increased linearly with increases in those concentrations up to 10 g/L, but then remained constant or decreased beyond this value. The maximum acetic acid concentration was given at 47.4 g/L in the medium containing 10 g/L yeast extract. The molar yield of acetic acid based on the consumed ethanol was 91% for yeast extract, 87% for peptone, and 86% for CSL. Because the purchase cost of technical grade yeast extract and CSL is 3.0 US$/kg and 0.4 US$/kg *(16, 17)*, respectively, CSL seems to be an effective nitrogen source for acetic acid formation according to the consideration of its economical feasibility.

Effect of $MgSO_4 \bullet 7H_2O$ Concentrations

Some acetic acid bacteria require a mineral source as growth factor *(9)*. Therefore, to evaluate the influence of $MgSO_4 \bullet 7H_2O$ concentrations on acetic acid formation by *Acetobacter* sp. RKY4, the cultivations supplemented with 0–2.0 g/L $MgSO_4 \bullet 7H_2O$ were performed in a 250 mL Erlenmeyer flask containing 50 mL of the main cultivation medium. As shown in Fig. 4, acetic acid concentration increased with increases in $MgSO_4 \bullet 7H_2O$ concentration up to 0.5 g/L, in which the molar yield of acetic acid based on the consumed ethanol was 87%, but then decreased beyond this value. Therefore, *Acetobacter* sp. RKY4 may require a mineral source such as magnesium for efficient production of acetic acid, because some acetic acid bacteria in mineral media induce the key enzymes of the glyoxylate cycle during growth on ethanol *(9)*.

Effect of Phosphate Sources

To investigate the effect of phosphate sources on acetic acid formation by batch culture of *Acetobacter* sp. RKY4, several phosphate sources of 2 g/L were tested. The monobasic phosphate sources such as KH_2PO_4, NaH_2PO_4, and $NH_4H_2PO_4$ showed better results in terms of acetic acid formation than di-basic phosphate sources such as K_2HPO_4, Na_2HPO_4,

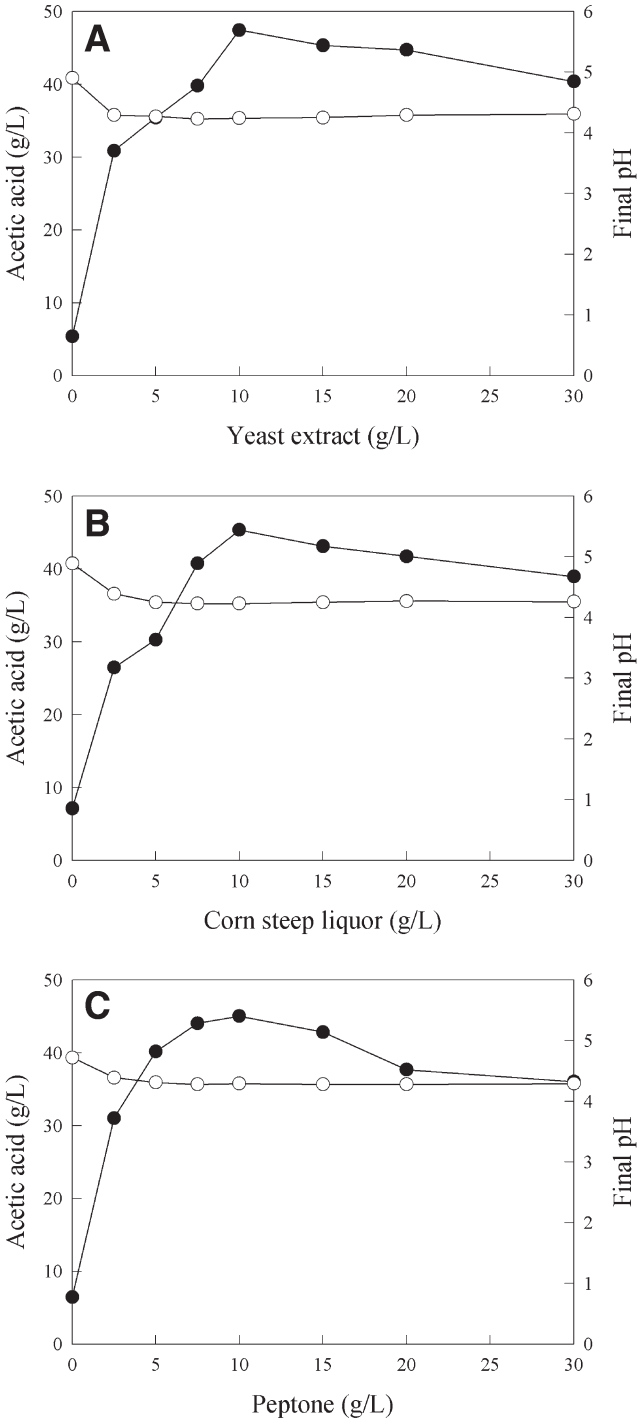

Fig. 3. Effect of the concentrations of yeast extract **(A)**, CSL **(B)**, and peptone **(C)** on acetic acid formation and final pH. The cultivation medium was composed of 10 g/L glycerol, 30 g/L ethanol, 2 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and 10 g/L $CaCO_3$. Symbols: ●, acetic acid and ○, final pH.

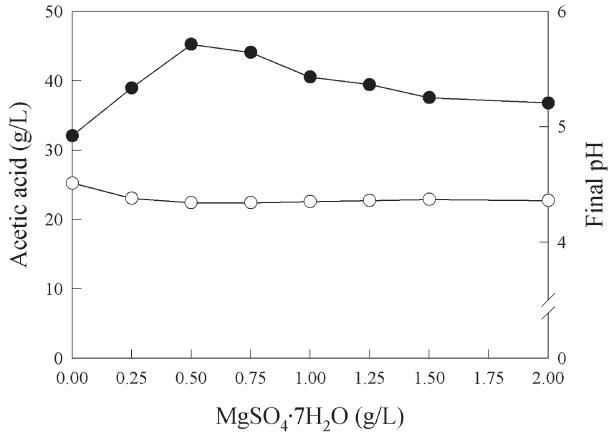

Fig. 4. Effect of $MgSO_4 \bullet 7H_2O$ concentrations on acetic acid formation and final pH. The cultivation medium was composed of 10 g/L glycerol, 30 g/L ethanol, 10 g/L CSL, 2 g/L K_2HPO_4, and 10 g/L $CaCO_3$. Symbols: ●, acetic acid and ○, final pH.

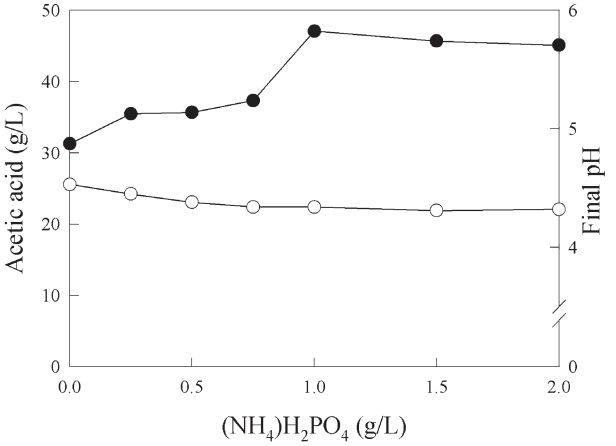

Fig. 5. Effect of $(NH_4)H_2PO_4$ concentrations on acetic acid formation and final pH. The cultivation medium was composed of 10 g/L glycerol, 30 g/L ethanol, 10 g/L CSL, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and 10 g/L $CaCO_3$. Symbols: ●, acetic acid and ○, final pH.

and $(NH_4)_2HPO_4$ (data not shown). Although *Acetobacter* sp. RKY4 produced above 39 g/L acetic acid in the medium containing 2 g/L monobasic phosphates, it produced below 39 g/L acetic acid in the medium containing 2 g/L di-basic phosphates. These results might be caused by the acidophilicity of acetic acid bacteria. Many of acetic acid bacteria are markedly acidophilic with an optimum between pH 5 and 6 *(10)*. Therefore, we selected $(NH_4)H_2PO_4$ as the best phosphate source for acetic acid formation, and investigated the effect of its concentrations on acetic

acid formation. Figure 5 shows the results of 250 mL Erlenmeyer flask experiments containing 0–2 g/L $(NH_4)H_2PO_4$. Acetic acid concentration produced by *Acetobacter* sp. RKY4 increased with increases in $(NH_4)H_2PO_4$ concentration up to 1 g/L, but then decreased beyond this value. The maximum acetic acid concentration and molar yield based on the consumed ethanol were 47.1 g/L and 91%, respectively. Consequently, the optimum medium for acetic acid formation by batch culture of *Acetobacter* sp. RKY4 was found to be 10 g/L glycerol, 40 g/L ethanol, 10 g/L CSL, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and 1 g/L $(NH_4)H_2PO_4$.

Conclusion

We isolated a novel acetic acid bacterium, *Acetobacter* sp. RKY4, from Korean traditional persimmon vinegar, and optimized the culture medium for acetic acid formation using this microorganism. When *Acetobacter* sp. RKY4 was cultivated in the optimized medium that was 10 g/L glycerol, 40 g/L ethanol, 10 g/L CSL, 0.5 g/L $MgSO_4 \bullet 7H_2O$, and 1 g/L $(NH_4)H_2PO_4$, it produced 47.1 g/L acetic acid with 91% molar yield. However, it must be necessary to optimize the culture conditions such as culture temperature and pH for more efficient production of acetic acid. Therefore, we focus our further studies on the optimization of the culture conditions (e.g., temperature, pH, aeration rate) in laboratory-scale and pilot-scale bioreactors.

References

1. Kim, J. S., Kim, H. J., Oh, K. K., and Kim, Y. S. (2002) *J. Ind. Eng. Chem.* **8**, 519–523.
2. Girard, A. S. (1992) *Resour. Conserv. Recycl.* **7**, 161–170.
3. Freer, S. N. (2002) *World J. Microbiol. Biotechnol.* **18**, 271–275.
4. Woo, C. H., Park, C. H., and Yoon, H. H. (2000) *Kor. J. Biotechnol. Bioeng.* **15**, 458–463.
5. Tetsuya, K. and Kondo, M. (1996) *J. Ferment. Bioeng.* **81**, 42–46.
6. Ravinder, T., Swamy, M. V., Seenayya, G., and Reddy, G. (2001) *Bioresour. Technol.* **80**, 171–177.
7. Du, W. J. and Lambrechts, M. G. (2002) *Int. J. Food Microbiol.* **74**, 57–64.
8. Talabardon, M., Hwitzguebel, J. P., and Peringer, P. (2000) *Biotechnol. Prog.* **16**, 1008–1017.
9. Gottschalk, G. (1986) *Bacterial Metabolism*, 2nd ed. Springer-Verlag, NY.
10. Stainer, R. Y., Ingraham, J. L, Wheelis, M. L., and Painter, P. R. (1986) *The Microbial World*, 5th ed. Prentice-Hall, NJ.
11. Krisch, J. and Szajani, B. (1997) *Biotechnol. Lett.* **19**, 525–528.
12. Arneborg, N., Moos, M. K., and Jakbsen, M. (1997) *Biotechnol. Lett.* **19**, 931–933.
13. Lee, O. S., Jang, S. Y., and Jeong, Y. J. (2003) *J. Kor. Soc. Food Sci. Nutr.* **32**, 181–184.
14. Yoo, Y. J. Park, K. M., Ryu, Y. W., and Choi C. U. (1990) *Kor. J. Biotechnol. Bioeng.* **5**, 167–173.
15. Suárez, D. C., Liria, C. W., and Kilikian, B. V. (1998) *World J. Microbiol. Biotechnol.* **14**, 331–335.
16. Butos, G., Moldes, A. B., Alonso, J. C., and Vázquez, M. (2004) *Food Microbiol.* **21**, 143–148.
17. Harrison, R. C., Todd, P., Rudge, S. R., and Petrides, D. P. (2003) *Bioseparations Science and Engineering*. Oxford University Press, NY.