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Higher Biomass Production of *Lactobacillus bulgaricus* NLS-4 by Improvement of Cultural Conditions

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배양조건 개선에 의한 *Lactobacillus bulgaricus* NLS-4의 균체 생산성 향상

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Abstract

Some of the cultural conditions were improved in order to obtain the higher biomass of *Lactobacillus bulgaricus* NLS-4 which has the higher lactic acid producing activity as well. Among eight media including 11% non-fat milk medium as a control, the TIP medium was selected. By a batch experiment, the maximum cell concentration could be increased to 1.0×10^9 cells per m*l* when the organism was grown at 38°C for 18 hours with agitation speed of 200 rpm and under the constant level of pH 6.5 controlled with 1 N KOH solution in the selected medium. The cell concentration was further increased to 2.3×10^9 cells per me in the steady state of continuous culture at the dilution rate of 0.17 hr^{-1} for 18 hours.

Introduction

In dairy industry, the effective preservation of the starter bacteria is one of the most important and practical problems. Therefore, interests in forzen or freeze-dried cultures have increased during the recent years and a lot of extensive investigations have been carried out[1~4]. However, the low viability of cells caused by various steps of treezing and drying is the most striking defect in fhese methods. As the viability of cells against freezing and/or drying depends upon the cell concentration before the treatments[5], the method for the increase of cell concentration should be taken into account.

In the present study, some of the cultural conditions were improved in order to obtain the higher biomass having the higher lactic acid producing activity of *Lactobacillus bulgaricus* NLS-4 which is one of the mixed strains of yogurt and has a significant role in flavor development of swiss cheese[6].

Methods and Materials

Bacterial culture. *Lactobacillus bulgaricus* NLS-4, originated from W.E. Sandine of the Oregon State University, Corvallis, was used throughout the study. A frozen stock culture was made in 11% (w/v) non-fat-milk (NFM) medium with addition of 10% (v/v) glycerol. The culture was transferred at least three times to fresh media and incubated at 38°C for 18 hours before being used experimentally.

Mgdia. Seven types of different semior synthetic media reported by a number of investigators [7~15] were used for the selection of the most suitable medium for the growth of *L. bulgaricus* NLS-4. As a control, the conventional 11% NFM medium was also used.

Cultivation method. A New Brunswick Bio Flo Model C 30 Chemostat was used. The working volume of growth vessel in batch experiment was 500 m*l* and pH was controlled automatically at the desired level with some alkaline solutions by use of a New Brunswick Model pH-40 automatic pH controller.

When continuous culture was applied, the volume of medium in the growth vessel was kept constant of 370 m*l* by means of an overflow. The medium was allowed to flow at least three times of working volume before the establishment of a steady state.

Growth determination. Colony counts were made on the Elliker's lactic agar[7]. All samples were plated out in triplicate and incubated at 38°C for 72 hrs after which all visible colony forming units (CFU) were counted. Cell mass in broth media was determined turbidometrically at 660 nm in terms of optical density unit (O. D. U.) with spectronic 20 (Bausch & Lomb). O. D. U. was defined as followings; O. D. U. =optical density reading x dilution ratio of sample.

Measurement of lactic acid producing activity. Activity was expressed as % lactic acid by titration with 0. 1 N NaOH solution which was previously standardized by the method in A. O. A. C. [16] until pH 8. 3. % lactic acid was determined from the equaton;

$$\% \text{ lactic acid} = \frac{m\text{l of } 0.1\text{N NaOH} \times \text{m. e. lactic acid}}{\text{weight of sample in gram}} \times 100$$

where molecular equivalent of lactid is 0. 009.

Results

Selection of medium. Seven different types of broth media ever reported were used with the comparison of the conventional 11% NFM medium in order to select the most effective medium for the growth and lactic acid producing activity of *L. bulgaricus* NLS-4. As shown in Table 1, the TIP medium which consisted of 4% tryptone, 1. 4% yeast extract, 9% lactose and 0. 014% $MnSO_4$. $4H_2O$ was proved to be best among all of the media tested. From the reason, the TIP medium was exclusively used throughout the study.

However, all broth media showed the similar patterns of growth and activity. Fig. 1 depicts the growth curve, the change of pH and lactic acid production of *L. bulgaricus* NLS-4 grown at 38°C in the TIP medium as a representative pattern.

Determination of optimum temperature. To determine the optimal growth temperature in the TIP medium, generation time of the organism in log phase grown at various temperature was calculated from the following equations;

$$K = 2.303(\log_{10}X_2 - \log_{10}X_1)/(t_2 - t_1),$$

$$g = 0.693/K$$

where t_1 and t_2 are the times at which the corresponding O. D. U. values, X_1 and X_2 can be obtained. K is the specific growth rate and g, the generation time (hr).

At 38°C, the organism revealed the shortest generation time of 85 min. indicating that *L. bulgaricus* NLS-4 can reproduce more rapidly at this temperature than at 45°C during the same time interval (Fig. 2). Fig. 2 also represents that 38°C was optimal for the production of lactic acid by this organism.

Effect of agitation and aeration. Relatively slow agitation speed of 200 rpm could enhance the both of cell growth and lactic acid production (Fig. 3). However, as shown in Fig. 4, no distinct effect of aeration on the positive result of growth and activity could be observed. The air flow rate of 0. 5 vvm rather showed the inhibitory effect on growth and activity. From the results, it seems likely that the maintenance of microaerophilic condition without aeration is preferable to *L. bulgaricus* NLS-4.

Effect of pH on the cell growth. The level of pH in the culture medium was controlled automatically at various level with some alkaline sol-

utions. Table 2 shows that the controlled pH at 6.5 with 1N KOH solution was the most suitable for the growth of cells. In this case, the cell population obtained was 40% higher than that of a culture grown without the pH control.

Biomass production in continuous culture. Continuous culture was carried out in order to compare the biomass production with that of batch culture. All optimum growth variables determined were kept constant as same as in batch culture. Maximum cell concentration of 7.5×10^8 cells per ml was obtained in the steady state at $D=0.17$ hr^{-1} (Table 3). This means that the total biomass obtained in continuous culture for 18 hours could be at least 2.3×10^9 cells per ml which was 2.3 times higher than that in batch culture for the same time.

Discussion

A number of investigators have tried to prepare some semi or synthetic media[7~15] in order to obtain the high concentration of lactic starter cells having the high lactic acid producing activity. We have chosen seven broth media among them and the conventional 11% NFM medium for the comparison.

The common constituents of seven media are lactose as energy source and yeast extract as source of nitrogen and growth factors. Besides these two, many media widely contain glucose and tryptone while the other constituents are so versatile. Only the TIP medium of the first choice in our experiment which was originally formulated by Bergére [10] and afterwords proved to be effective for the mass culture of some mesophilic streptococci by Pettersson[9] contains $MnSO_4.4H_2O$ uncommonly to the other media. It is very that the Mn^{++} ion promotes cell growth. This fact may be supported by De Man et al.[17] that growth of some starter bacteria including betacocci could be stimulated by addition of Mn^{++} ion into the medium.

The species *L. bulgaricus* is thermophilic and its optimum temperature in milk is known as 40°C to 45°C[18], but as shown in Fig. 2, the organism could grow better at 38°C rather than above 40°C

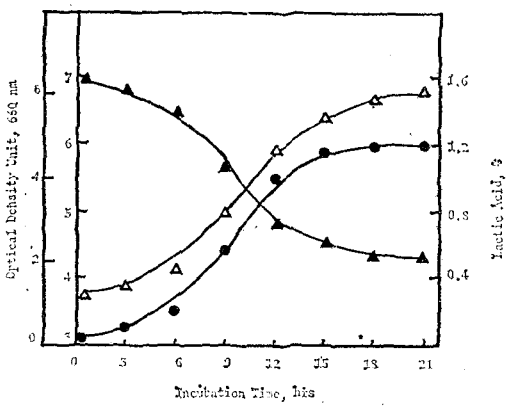

Fig. 1. Growth, Change of pH, and Lactic Acid Production by *L. bulgaricus* NLS-4 in the TIP Medium during Static Culture at 38°C.
Symbols; ● : O.D.U, ▲ : pH, △ : lactic acid

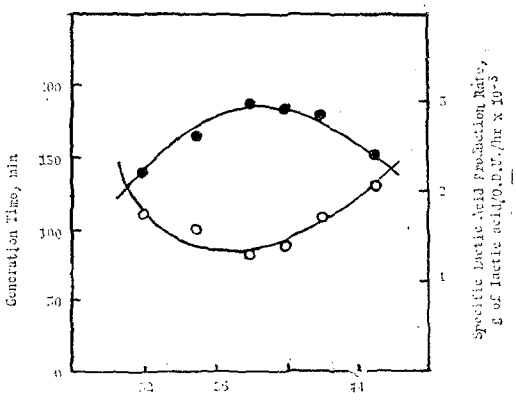

Fig. 2. Effect of Temperature on the Generation Time and Specific Lactic Acid Production Rate of *L. bulgaricus* NLS-4 in the TIP Medium during Static Culture.
Symbols; ○ : generation time, ● : specific lactic acid production rate.

in TIP medium. The phenomenon is very similar with the results of Lee et al.[14] which show the optimum temperature of streptcocci cultured in a semi-synthetic medium was lower about 2 to 3°C than in than in milk medium.

Usually to the lactic starter culture has been applied a static cultivation method. However, it was found that the appropriate agitation speed of

Table 1. The Growth and Lactic Acid Producing Activity of *L. bulgaricus* NLS-4 in 8 Different Media[a].

Media	CFU[b](per ml)	Initial pH	Final pH	△pH[c]	Reference
LB	2.1×10^8	6.8	4.24	2.56	7
M17	5.3×10^8	7.2	5.78	1.42	8
TIP	6.0×10^8	7.0	4.32	2.68	9, 10, 11
TJ	2.2×10^8	5.5	3.85	1.65	12
IMAI	4.7×10^8	6.8	4.13	2.67	13
LEE	4.5×10^7	6.5	5.72	0.78	14
LAB	3.8×10^8	6.6	4.51	2.09	15
NFM	5.2×10^5	6.2	3.70	2.50	—

a. Cells were incubated at 45°C for 10 hrs in flask after three successive transfer into each medium to give an enough adaptation time.

b. Colony forming units were counted after the incubation at 38°C for 72 hrs on Elliker's lactic agar medium.

c. Amount of pH drop for 18 hrs.

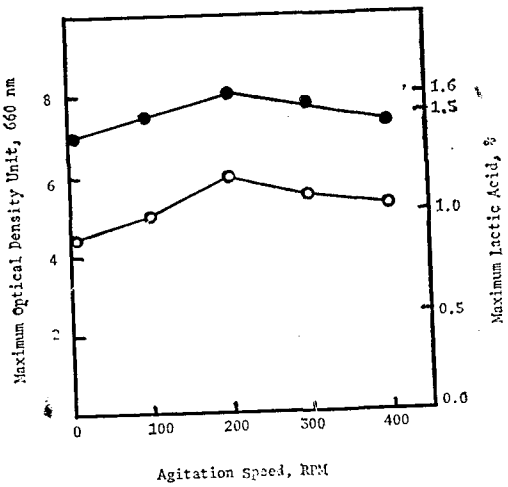

Fig. 3. Effect of Agitation Speed on the Maximum Growth and Lactic Acid Production of *L. bulgaricus* NLS-4 Grown in the TIP Medium at 38°C for 18 hrs without Aeration.
Symbols ; ○ : O. D. U, ● : % lactic acid

200 rpm enhanced growth and activity of *L. bulgaricus* NLS-4 (Fig. 3) indicating that agitation might cause an increase in the rate of nutrient transport into the cells[19]. On the other hand, the decrease of growth and activity with an increase rate of air flow appears to be caused by the oxygen toxicity. This might arise from the formation of toxic amounts of hydrogen peroxide and of the unfavorable redox potentials of the environment[20].

The cessation of growth and metabolism after 18 hours as shown in Fig. 1 have two possible reasons of nutrient exhaustion and of the unfavorable growth conditions caused by pH decrease. Rogers et al.[19] postulated that the concentration of undissociated lactic acid is the principal factor responsible for the limitation of growth and metabolism. In practice, Friedman et al.[21] could obtain the higher cell population of *L. delbrueki* by use of a dialysis culture system in which lactic acid produced was removed simultaneously from

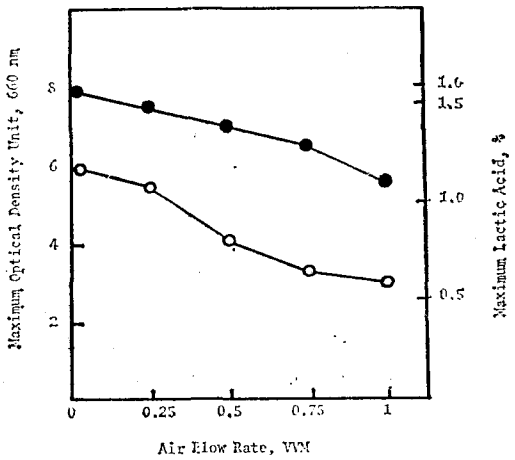

Fig. 4. Effect of Ar Flow Rate on the Maximum Growth and Lactic Acid Production of *L. bulgaricus* NLS-4 Grown in the TIP Medium at 38°C for 18 hrs with Agitation Speed of 200 rpm.
Symbols ; ○ : ODU, ● : % lactic acid

Table 2. Effect of Some Alkaline Solutions and pH Level on the Growth of *L. bulgaricus* NLS-4 in TIP Medium[a].

Alkaline solutions	pH level	CFU (per m*l*)
No pH control		7.1×10^8
1 N KOH	6.0	8.2×10^8
	6.5	1.0×10^9
	7.0	9.5×10^8
1 N NaOH	6.5	9.1×10^8
	7.5	5.4×10^8
1 N NH_4OH	6.5	7.6×10^8

a. Incubated at 38°C with Agitation Speed of 200 rpm for 18 hrs.

the culture medium by dialysis.

In order to remove lactic acid, various neutralizers such as NaOH[22,23], NH_4OH[9,22,23], KOH[24], Na_2CO_3[11,25] have been used. In our experiment, automatic pH control at the level of 6.5 with 1 N KOH solution showed the best result for the biomass production of *L. bulgaricus* NLS-4.

After the improvement of growth condition, we could obtain the final cell concentration of 1.0×10^9 cells per m*l* in batch culture (Table 2). The is more than 66% increase of biomass than that which could be obtained from a static culture in the TIP medium as shown in Table 1. Comparing with the control grown in 11% NFM medium, this is about 500 times higher population.

By use of continuous culture, the cell population was further increased to 2.3×10^9 cells per m*l* for 18 hrs. This indicates that the efficiency is much better in continuous culture than that of batch culture for the biomass production of *L. bulgaricus* NLS-4.

Table 3. Steady-state Biomass Production in Continuous Culture[a].

Dilution rate (hr^{-1})	CFU (per m*l*)	Productivity (CFU per m*l* per hr)
0.05	6.6×10^6	3.3×10^5
0.10	4.1×10^8	4.1×10^7
0.17	7.5×10^8	1.3×10^8
0.20	3.3×10^8	6.6×10^7
0.25	3.2×10^8	8.0×10^7
0.40	1.0×10^8	4.0×10^7
0.50	8.4×10^7	4.2×10^6

a. Incubated at 38°C with agitation speed of 200 rpm under the constant level of pH 6.5 controlled with 1 N KOH solution in TIP medium.

요 약

높은 유산생성 능력을 가진 *Lactobacillus bulgaricus* NLS-4 주를 대량 배양하기 위하여 배양조건을 개선하였다. 이제까지 보고된 많은 배지중에서 7개를 선정하고 흔히 사용되고 있는 11% 탈지유 배지를 대조군으로 하여 사용 균주의 증식과 유산 생성능을 비교한 결과 TIP 배지를 최종 선정하였다. 이 배지를 이용하여 38°C에서 통기없이 200 rpm의 속도로 교반해 주면서 1 N KOH용액을 사용하여 pH를 6.5로 자동 조절해준 결과 18시간 동안의 회분배양에서 얻은 균체수는 생균수로 m*l* 당 1.0×10^9이었으며 이를 다시 연속 배양한 결과 희석 배율 $D=0.17hr^{-1}$에서 m*l* 당 총 2.3×10^9의 균체를 얻을 수 있었다.

References

1. Reddy, M. S., E. R. Vedamuth, C. J. Waeham and G. W. Reinbold: J. Dairy Sci. **57**, 124 (1974)
2. Kawashima, T., T. Kodama and M. Maneo: Jap. J. Zootech. Sci. **34**, 218 (1963)
3. Speckman, C. A., W. E. Sandine and P. R. Elliker: J. Dairy Sci. **57**, 165 (1974)
4. Stadhouders, J., G. Hup and L. A. Jansen: Neth. Milk Dairy J. **25**, 229 (1971)
5. Heckly, R. J.: Adv. Appl. Microbiol. **13**, 1 (1961)
6. Biede, S. L., G. W. Reinbold and E. G. Hammond: J. Dairy Sci. **59**, 854 (1976)
7. Elliker, P. R., A. W. Anderson and G. Hannesson: J. Dairy Sci. **39**, 1611 (1956)
8. Terzaghi, B. E. and W. E. Sandine: Appl. Microbiol. **29**, 807 (1975)
9. Pettersson, H. E: Appl. Microbiol: **29**, 437 (1975)
10. Bergére, J. L.: Le lait **48**, 1 (168)
11. Stadhouders, J., G. Hup and L. A. Jansen: Neth. Milk Dairy J. **23**, 182 (1969)

12. Goldberg, I., and L. Eschar: Appl. Environ. Microbiol. **33**, 489 (1977)
13. Imai, M. and M. Kato: J. Agr. Chem. **49**, 93 (1975)
14. Lee, D. A. and E. B. Collins: J. Dairy Sci. **59**, 405 (1976)
15. Davis, J. G., T. R. Ashton, M. McCaskill: Dairy Ind. **10**, 569 (1971)
16. Horowitz, W. : Editor, Official methods of analysis of the Association of Official Analytical Chemists. 11th edn., A. O. A. C. Washington, P. 746 (1970)
17. De man, J. C. and Th. E. Galesloot: Neth. Milk Dairy J. **16**, 1 (1962)
18. Buchanan, R. E., and N. E. Gibbons: Bergey's manual of determinative bacteriology 8th edn. The Williams & Wilkins Company, Baltimore (1974)
19. Rogers, L. A., and E. O. Wittier: J. Bacteriol. **16**, 211 (1928)
20. Keen, A. R. : J. Dairy Res. **39**, 141 (1972)
21. Friedman, M. R. and E. L. Garden: Jr. Biotech. Bioeng. **12**, 961 (1970)
22. Peebles, M. M., S. E. Gilliland, M. L. Speck: Appl. Microbiol. **17**, 805 (1969)
23. Gilliland, S. E., E. D. Anna, and M. L. Speck: Appl. Microbiol. **19**, 890 (1970)
24. Christensen, V. W. : U. S. Patent 3, 592, 740 (1971)
25. Gilliland, S. E. : J. Dairy Sci. **54**, 1129 (1971)