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Relationship between Nisin Z Fermentative Production and Aeration Condition Using *Lactococcus lactis* IO-1

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Lactococcus lactis IO-1 produces a peptide antibiotic, nisin Z by using glucose as well as xylose as a carbon source. To investigate the relationship between nisin Z production and aeration condition for the fermentation, fermentation was run in flask and jar fermenter with xylose and glucose. Optimal aeration condition for nisin Z production was not always that for cell growth and lactate production. The influence of aeration on nisin Z production was observed highly severe in flask-based fermentation because of uncontrolled pH condition even in the presence of $CaCO_3$. Degradation of nisin Z produced was scarcely observed under the optimized conditions for aeration, which might give less expression of the activities of the degrading enzymes.

INTRODUCTION

Lactic acid bacteria are believed to be facultatively anaerobic or microaerophilic. These bacteria have no cytochrome system and therefore produce ATP through substrate-level-phosphorylation. Oxygen sensitivity of lactic acid bacteria is variable and strain-dependent. Therefore, oxygen retarded the growth of some species (Archibald and Fridovich, 1981) and in certain cases stimulated that (Smart and Thomas, 1987; Cogan *et al.*, 1989). A number of lactic acid bacteria utilize molecular oxygen and hydrogen peroxide to generate NAD^+, by the action of NADH oxidase and NADH peroxidase (Codon, 1987). NADH oxidases catalyze one, two, and four electron reductions of oxygen to form superoxide, H_2O_2, and H_2O, respectively. Superoxide anion, an intermediate, is dismuted to H_2O_2, spontaneously or by superoxide dismutase and high intracellular Mn^{2+} (Archibald and Fridovich, 1981; Codon, 1987). H_2O_2 is subsequently converted to H_2O by NADH peroxidase.

In lactococci, the effect of oxygen is strain-dependent. Smart and Thomas (1987) reported under an anaerobic condition with excess substrate, metabolism of glucose and lactose by *Streptococcus lactis* ML8 led to a homolactic fermentation. On the other hand, the major product was shifted to acetate under an aerobic condition with limited substrate. The shift toward acetate production is related to the induction of various enzymes to oxidize NADH under an aerobic condition. On the contrary, Cogan *et al.* (1989) reported little effect of aeration on metabolites produced from lactose by *Streptococcus lactis* 712. The microorganism remained homolactic even under an aerobic condition. However, with glucose and galactose as a carbon source, the strain

produced mainly acetate and acetoin.

In aerobically grown cells, levels of NADH oxidase, NADH peroxidase and pyruvate dehydrogenase were increased and lactate dehydrogenase was decreased (Codon, 1987). In addition, pyruvate-formate lyase was likely to be inactivated. The production of acetate instead of lactate under aerobic conditions leads to the generation of ATP by substrate-level-phosphorylation via acetate kinase reaction. De Vuyst *et al.* (1996) reported on the induction of bacteriocin production by *Lactobacillus amylovorus* under different physiological conditions including aeration. Higher air saturation level enhanced specific production rate of amylovorin. However, few results are available in the literature for systematic research on the effect of aeration condition on bacteriocin production.

Lactococcus lactis IO-1, a L-lactic acid homofermentative isolated in our laboratory (Ishizaki *et al.*, 1990), produces a novel bacteriocin which was identified as nisin Z, a natural nisin variant (Matsusaki *et al.*, 1996). In our previous paper (Chinachoti *et al.*, 1997), xylose as a carbon source was found to be utilized for the efficient fermentative production of lactate as well as nisin Z. In the present paper, differences in nisin Z production by strain IO-1 under several aerobic conditions were noticed in the course of optimization.

MATERIALS AND METHODS

Microorganism and media

A nisin-producing strain, *Lactococcus lactis* IO-1 was stored at −80 °C in 15% glycerol. The culture was subcultured twice in TGC medium without glucose (Difco Laboratories, Detroit, MI, USA) and was kept at 4 °C as a stock culture. The stock culture was refreshed in TGC medium at 37 °C for 18 h. The refreshed culture was then precultured in CM medium which contained 0.5% yeast extract (Difco Laboratories, Detroit, MI, USA), 0.5% polypeptone (Nihon Seiyaku Co., Ltd., Tokyo, Japan) and 0.5% NaCl with 5% inoculation. The medium was added with 1% xylose for xylose fermentation or with 1% glucose for glucose fermentation. The preculture was incubated at 30 °C and 100 strokes/min for 3 h.

Fermention conditions

1) Fermentation in flask

The bacteria were cultivated in 100 ml CM medium with 4% xylose or 3% glucose with an inoculation volume of 5%. The Erlenmeyer flasks were reciprocated at 100 strokes/min. Fermentation conditions in stirred-flask were the same as in reciprocated-flask except the flask culture was stirred at the indicated speed. $CaCO_3$ at 1.5% was added to prevent rapid decrease in pH.

2) Fermentation in jar fermenter

One liter jar fermenter containing 300 ml of CM medium supplemented with 0.1 M $CaCl_2$ and 4% xylose or 3.6% glucose was inoculated with 5% of the preculture. The fermentation was done at 30 °C and the indicated stirring speed and with or without air flow. pH of the fermentation broth was controlled at 6.0 for xylose and at 5.5 for glucose with 3 N NaOH.

Analytical methods

Dry cell weight was obtained by conversion from optical absorbance at 562 nm with spectrophotometer (Uvidec 320, Japan Spectroscopic, Tokyo, Japan). Dissolved oxygen (DO) was observed by DO meter (Mini recorder SJ-3462, Atto Co., Ltd., Tokyo, Japan). Glucose, xylose, lactate and acetate were determined by high performance liquid chromatography (HPLC) with Aminex ion exclusion column HPX-87H (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Nisin Z was determined by reversed-phase HPLC with Shodex Asahipak ODP 50–6E column (Showa Denko Co., Ltd., Tokyo, Japan) (Matsusaki *et al.*, 1996). One unit of nisin Z activity is defined as an arbitary unit (AU) of activity that is equivalent to the activity of 1 μg of commercial nisin (ICN Biomedicals Inc., Costa Mesa, CA, USA; activity, 1000 IU/mg-solid; nisin content, 2.5%).

RESULTS

Xylose fermentation

1) Effect of agitating condition in flask fermentation

The comparison between nisin Z production in reciprocated and stirred-flask

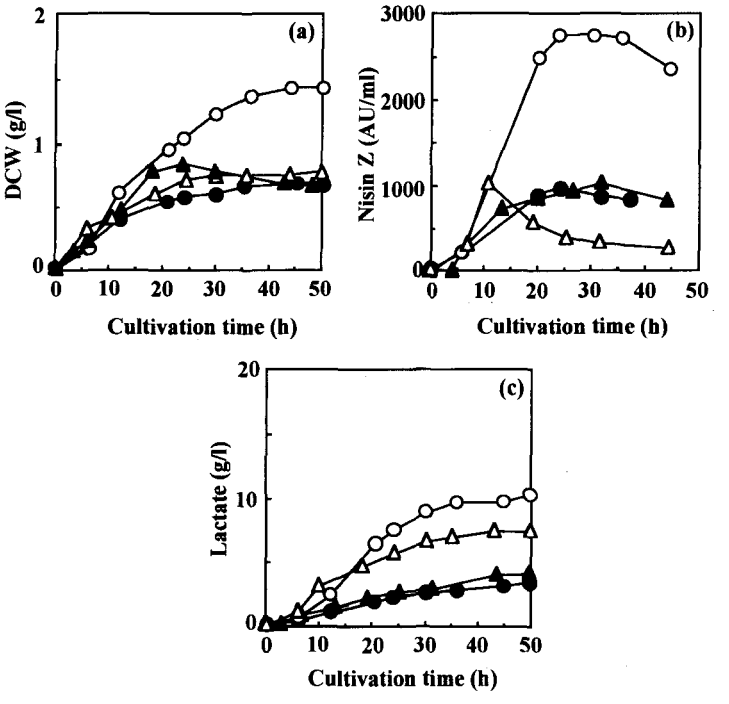


Fig. 1. Effect of agitation conditions on nisin Z production by *Lactococcus lactis* IO-1 in flask fermentation. The cultivation was carried out in 100 ml of CM medium with 4% xylose and 2% $CaCO_3$. (a) Cell growth, (b) nisin Z production and (c) lactate production.

Symbols: ● at 100 strokes/min and 30 °C, ○ at 320 rpm and 30 °C,
▲ at 100 strokes/min and 37 °C, △ at 320 rpm and 37 °C

fermentation was studied. The results are shown in Fig. 1. At 37 °C, growth of IO-1 strain was hardly affected by both agitating conditions. In stirred-flask fermentation, nisin Z activity increased and dropped sharply and lactate was produced 2 times higher than in reciprocated-flask.

At 30 °C, stirred-flask fermentation gave an unexpectedly favorable production of nisin Z, which has never observed in xylose fermentation (Fig. 1b). On the other hand, reciprocated-flask fermentation showed the usual nisin Z production as reported previously (Chinachoti *et al.*, 1997). Cell growth and lactate production in stirred-flask were also superior to those in reciprocated-flask (Fig. 1a, c).

2) Effect of agitation speed in jar fermenter

In pH-controlled fermentation at 30 °C, the agitation speed was varied at 320, 540 and 760 rpm. The results are shown in Fig. 2. When the agitation speed was increased from 320 rpm to 540 rpm, no effect on cell growth was observed. Specific production rate of nisin Z increased from 0.07 h^{-1} to 0.16 h^{-1}. The highest amount of nisin Z was 3200 AU/ml which was obtained at 18 h of incubation at 540 rpm (Fig. 2b). Long lag-phase accompanied with low nisin Z production was observed at 760 rpm (Fig. 2c). The increase in agitation speed resulted in the decrease in lactate produced. Dissolved oxygen decreased rapidly in the lag-phase of cell growth and became nearly zero along with cell propagation at all the agitation speeds.

The fermentation was done at 540 rpm with air flow at the rate of 10 ml/min and 40 ml/min. The results were compared with the control fermentation at 540 rpm without air flow (Fig. 2b). The air flow resulted in the decrease in nisin Z production (Fig. 3).

Glucose fermentation

1) Effect of agitation speed in flask fermentation

The agitation speed was varied from 120 to 410 rpm and the results are shown in Fig. 4. Glucose consumption rate increased with the increase in agitation speed. At low agitation speed, low cell yield was observed and thus accompanied with low lactate and nisin Z production. Cell growth was hardly affected at the agitation speed over 320 rpm.

Table 1. Comparison of nisin Z and lactate productivities at different agitation speeds in flask-based cultivation

Agitation speed(rpm)	Maximum nisin Z activity (AU/ml)(Time)[a]	Yield of nisin Z ($\times 10^4$ AU/g)		Maximum lactate produced(g/l)(Time)[a]	Yield of lactate (g/g)	
		$Y_{Np/x}$[b]	$Y_{Np/s}$[c]		$Y_{Lp/x}$[d]	$Y_{Lp/s}$[e]
120	1680 (15 h)	174	9.31	21.9 (21 h)	27.0	0.982
210	3720 (12 h)	310	16.7	24.3 (15 h)	30.3	1.03
320	4040 (15 h)	326	16.7	28.8 (18 h)	21.5	1.18
340	4270 (21 h)	341	15.4	28.2 (21 h)	22.5	1.02
410	4180 (12 h)	292	15.2	26.4 (15 h)	19.4	1.11

[a] Cultivation time when maximum nisin Z activity or lactate were obtained.
[b] Yield of nisin Z production per cell mass when the nisin Z activity reached a maximum level.
[c] Yield of nisin Z production per mass of glucose consumed when the nisin Z activity reached a maximum level.
[d] Yield of lactate per cell mass when lactate reached a maximum level.
[e] Yield of lactate per mass of glucose when lactate reached a maximum level.

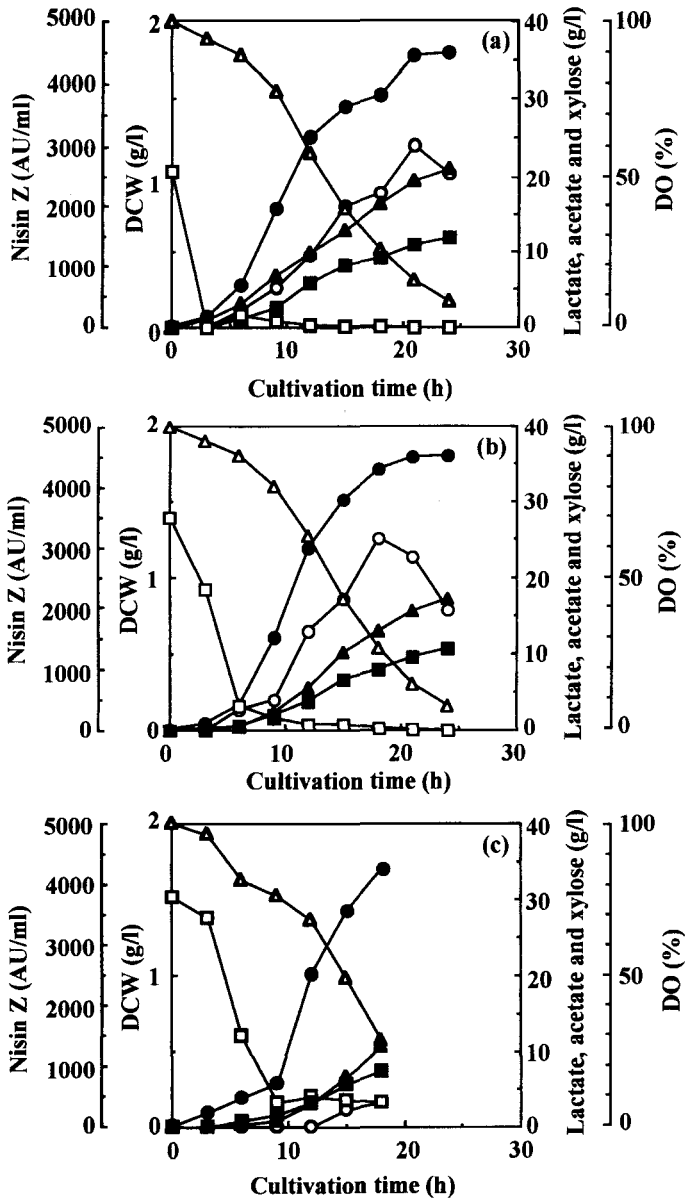


Fig. 2. Effect of agitation speeds on nisin Z production by *Lactococcus lactis* IO-1 in pH-controlled fermentation. The cultivation was carried out at 30 °C in 1-L jar fermenter containing 300 ml of CM medium with 4% xylose and 0.1M $CaCl_2$. pH was maintained at 6.0 with 3 N NaOH. (a) 320 rpm, (b) 540 rpm and (c) 760 rpm.

Symbols: ● Dry cell weight (DCW), ○ nisin Z, ▲ lactate, △ xylose, ■ acetate, □ dissolved oxygen (DO)

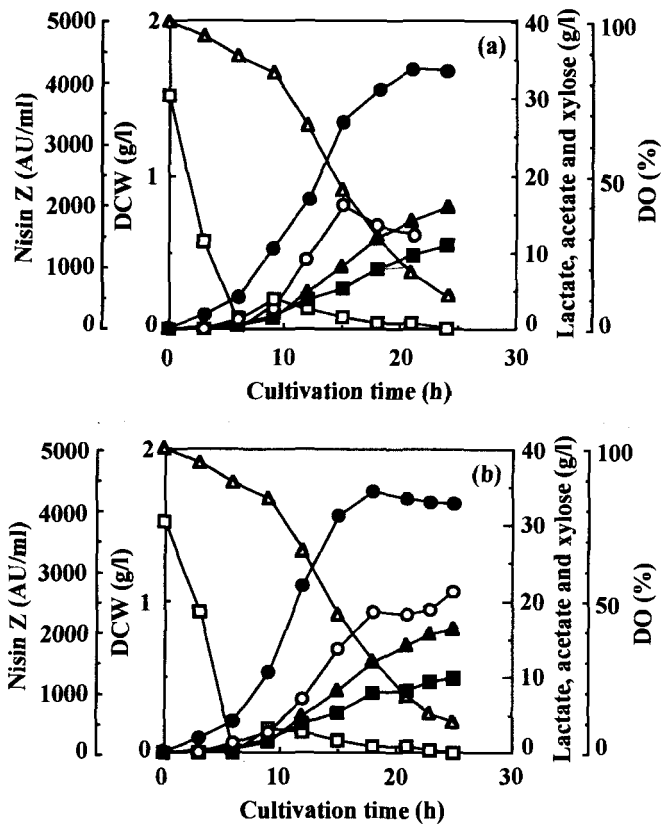


Fig. 3. Effect of air flow on nisin Z production by *Lactococcus lactis* 10-1 in pH-controlled fermentation. The cultivation was carried out at 540 rpm in the same manner as described in Fig. 2 except with air flow at (a) 10 ml/min and (b) 40 ml/min.

Symbols: ● Dry cell weight (DCW), ○ nisin Z, ▲ lactate, △ xylose, ■ acetate, □ dissolved oxygen (DO)

The cell growth at 320 rpm was almost equal to that at 340 rpm but nisin Z from the latter was higher than the former (Fig. 4c, d). Maximum nisin Z activity was increased with the increase in agitation speed. It reached the highest value at 340 rpm and was decreased at 410 rpm (Table 1). Yield of nisin Z was decreased with the increase in agitation speed after it reached the maximum value. Lactate was produced in the same pattern as nisin Z. Maximum lactate produced was observed at 320 rpm.

2) Effect of agitation speed in jar fermentation

The experiment was done with various agitation speeds from 120 to 1000 rpm. In

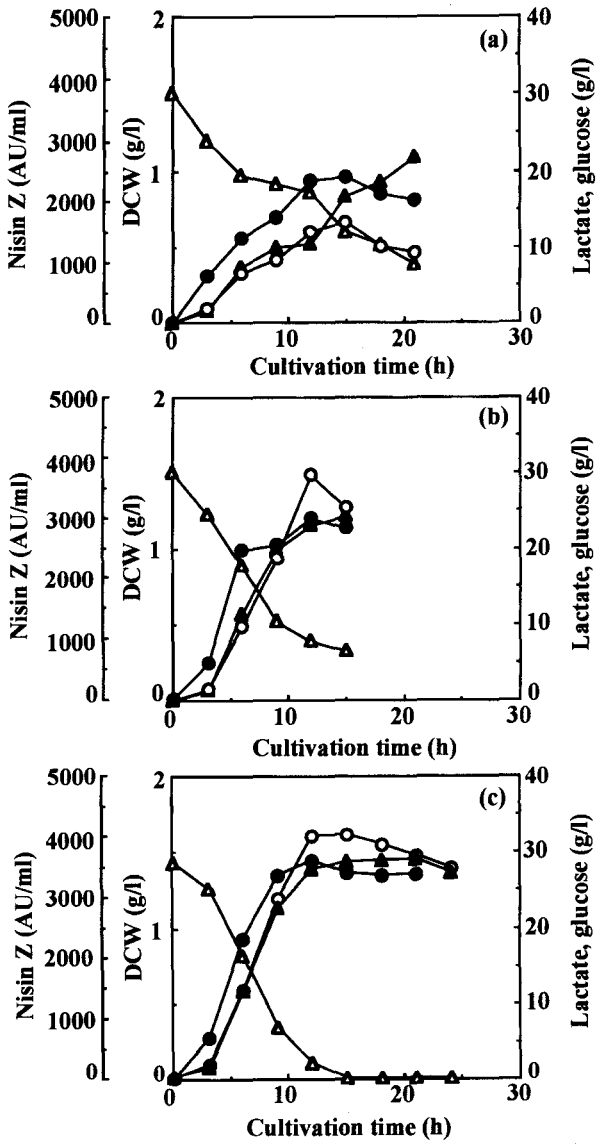


Fig. 4. Effect of agitation speeds on nisin Z production by *Lactococcus lactis* IO-1 in flask fermentation. The cultivation was carried out at 30 °C in 100 ml of CM medium with 3% glucose and 1.5% $CaCO_3$. (a) 120 rpm, (b) 210 rpm, (c) 320 rpm, (d) 340 rpm and (e) 410 rpm.

Symbols: ● Dry cell weight (DCW), ○ nisin Z, ▲ lactate, △ glucose

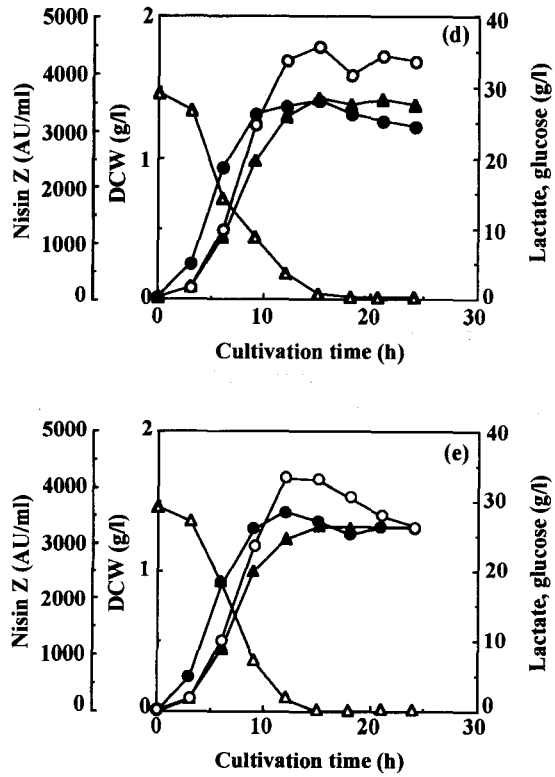


Table 2. Comparison of nisin Z and lactate productivities at different agitation speeds in pH-controlled jar fermentation

Agitation speed(rpm)	Maximum nisin Z activity (AU/ml)(Time)[a]	Yield of nisin Z ($\times 10^4$ AU/g)		Maximum lactate produced(g/l)(Time)[a]	Yield of lactate (g/g)	
		$Y_{Np/x}$[b]	$Y_{Np/s}$[c]		$Y_{Lp/x}$[d]	$Y_{Lp/s}$[e]
100	3380 (15 h)	224	9.97	26.7 (21 h)	19.8	0.789
200	3720 (18 h)	255	10.3	28.7 (15 h)	18.1	0.821
320	3940 (18 h)	274	11.2	28.7 (18 h)	19.9	0.812
540	3590 (15 h)	220	10.0	29.7 (15 h)	18.2	0.828
800	3390 (15 h)	225	9.74	29.7 (21 h)	21.6	0.850
1000	3410 (15 h)	218	9.67	28.6 (18 h)	18.8	0.813

[a] Cultivation time when maximum nisin Z activity or lactate were obtained.
[b] Yield of nisin Z production per cell mass when the nisin Z activity reached a maximum level.
[c] Yield of nisin Z production per mass of glucose consumed when the nisin Z activity reached a maximum level.
[d] Yield of lactate per cell mass when lactate reached a maximum level.
[e] Yield of lactate per mass of glucose when lactate reached a maximum level.

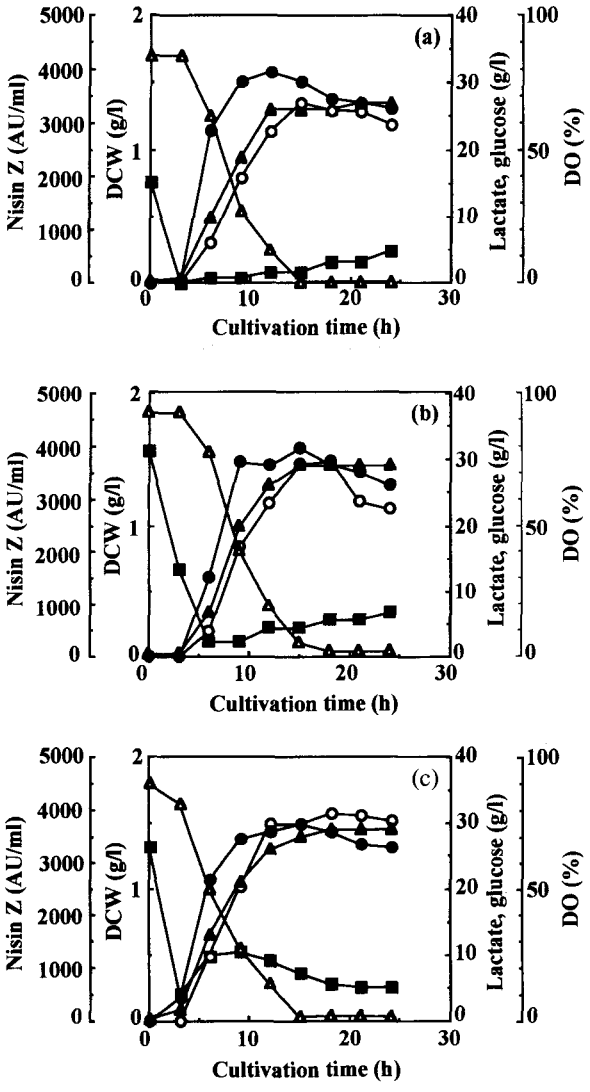
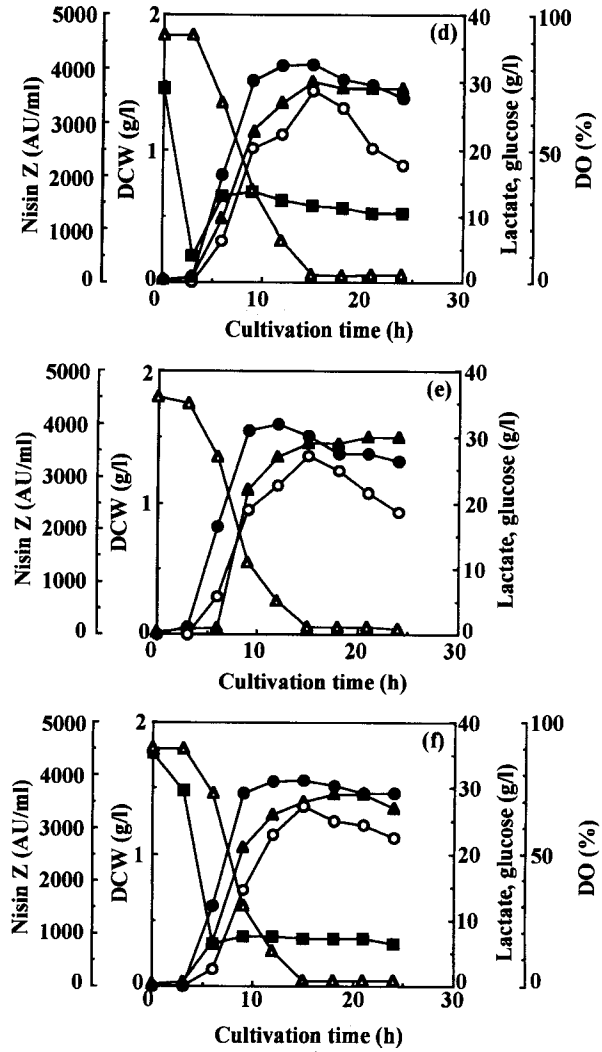


Fig. 5. Effect of agitation speeds on nisin Z production of *Lactococcus lactis* IO-1 in pH-controlled fermentation. The cultivation was carried out at 30 °C in 1-L jar fermenter containing 300 ml of CM medium with 3.6% glucose and 0.1 M $CaCl_2$. pH was maintained at 5.5 with 3 N NaOH. (a) 100 rpm, (b) 200 rpm, (c) 320 rpm, (d) 540 rpm, (e) 800 rpm and (f) 1000 rpm.

Symbols: ● Dry cell weight (DCW), ○ nisin Z, ▲ lactate, △ glucose, ■ dissolved oxygen (DO)



glucose fermentation, the increase in agitation speed has no significant effect on cell growth, lactate production and glucose consumption (Fig. 5). On the other hand, nisin Z production gradually increased with the increase in agitation speed up to 320 rpm. Higher agitation speed than 320 rpm gave low nisin Z production. The increase in nisin Z activity was in the same pattern as in flask fermentation (Tables 1 and 2). Among all of the agitation speeds tested, no degradation of nisin Z produced was observed at 320 rpm. Under this condition, nisin Z activity was remained in high level even after the cells entered stationary phase. Yield of nisin Z was in the same pattern as maximum nisin Z activity, whereas maximum lactate and its yield were not affected so much by the agitation speed.

DISCUSSION

To investigate the effect of aeration on cell growth and nisin Z production, fermentations were performed with xylose and glucose at different aeration conditions.

In flask-based xylose fermentation, the effect of aeration on growth and productivity was clearly seen at 30°C (Fig. 1). The higher cell growth was observed in stirred-flask fermentation which should contain more dissolved oxygen than reciprocated-flask fermentation. In consequence lactate was produced in high level. Furthermore, nisin Z production was observed in an unexpectedly favorable level which was as high as that from glucose fermentation. Lucey and Condon (1986) reported that *Leuconostoc mesenteroides* grew more rapidly under an aerobic than anaerobic condition. This is due to the stimulated synthesis of NADH oxidase which allows O_2 to act as a terminal electron acceptor. As a consequence, acetyl phosphate is not wasted in the formation of the alternative electron acceptors, acetyl-CoA and acetaldehyde. Therefore, acetyl phosphate is conserved for ATP synthesis. The efficient synthesis of ATP is responsible for the faster growth rates under an aerobic condition. *L. lactis* IO-1 has a moderate activity of NADH oxidase which is expressed much more in an aerobic cultivation (unpublished result).

In pH-controlled fermentation with xylose, the increase in agitation speed from 320 rpm to 540 rpm hardly affected cell growth (Fig. 2a, b), and further increase in agitation speed led to long lag phase (Fig. 2c). Lactate and acetate were decreased when the agitation speed was increased. Nisin Z productivity increased when the agitation speed was increased from 320 rpm to 540 rpm. However, nisin Z production was very low at 760 rpm.

In the presence of oxygen, NADH oxidase and NADH peroxidase, which might be induced by oxygen level, oxidize NADH in the reduction of oxygen to H_2O_2 or H_2O. This enzyme system would compete with lactate dehydrogenase in the oxidation of NADH to NAD^+. This competition resulted in the decrease of lactic acid production (Jung, 1991).

In flask-based glucose fermentation, the effect of aeration on *L. lactis* IO-1 was in the same pattern as in xylose fermentation. Cell growth increased with an increase in agitation speed from 120 rpm to 320 rpm (Fig. 4a, b, c). The agitation speed at 340 rpm and 410 rpm hardly affected the growth (Fig. 4d, e). The increase in agitation speed from 120 rpm to 320 rpm enhanced lactate production. However, lactate production was not stimulated at the agitation speed of 340 rpm and 410 rpm. Nisin Z activity was found to reach the highest level at 340 rpm among the agitation speeds tested. Furthermore, the resulting nisin Z was hardly diminished under the condition after the maximum was obtained.

In pH-controlled fermentation with glucose, cell growth and lactate were hardly affected by all the agitation speeds tested. The highest nisin Z production was achieved by the cultivation at 320 rpm (Fig. 5c). The higher agitation speed than 320 rpm caused the decrease in nisin Z production (Fig. 5d, e, f). The decrease in nisin Z level in the stationary phase of cell growth was not observed in the cultivation at 320 rpm. The influence of aeration on nisin Z production was much clear in the flask-based fermentation compared to the jar fermentation, because pH was uncontrolled in the former cultivation even in the presence of $CaCO_3$. De Vuyst *et al.* (1996) suggested that

under uncontrolled pH conditions bacteriocin production is liable to be affected by growth factors, such as temperature, aeration and nutritional source.

At the optimized conditions for aeration, nisin Z produced was hardly decreased after the maximum reached. These results suggest that the optimal aeration conditions obtained here could affect the nisin Z decomposition and consequently give less expression of the activities of the degrading enzymes. The possibility of physico-chemical inactivation and decomposition of nisin Z can be ruled out by the following fact. Nisin Z was not degraded without any cells under the same aeration conditions as described above (data not shown).

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