Appl Biochem Biotechnol (2009) 152:372–382
DOI 10.1007/s12010-008-8335-8

Variable Volume Fed-Batch Fermentation for Nisin Production by *Lactococcus lactis* subsp. *lactis W28*

Zhaoliang Wu · Lin Wang · Yingjun Jing · Xueliang Li · Yanli Zhao

Received: 5 January 2008 / Accepted: 29 July 2008 /
Published online: 19 August 2008

Abstract A feeding technology that was suitable for improving the nisin production by *Lactococcus lactis* subsp. *lactis W28* was established. The effects of initial sucrose concentration (ISC) in the fermentation broth, feeding time, and feeding rate on the fermentation were studied. It was observed that a fed-batch culture (ISC=10 g l^{-1}) with 100 ml sucrose solution (190 g l^{-1}) being evenly fed (9–10 ml h^{-1}) into the fermenter after 3-h fermentation gave the best performance in terms of biomass and nisin yield. Under these conditions, the total biomass and the total nisin yield were approximately 23% and 51% higher than those in batch fermentation, respectively. When the sucrose concentration was controlled at 5–10 g l^{-1} in variable volume intermittent fed-batch fermentation (VVIF) with ISC=10 g l^{-1}, the total biomass and the total nisin yield were 29% and 60% above those in batch fermentation, respectively. The VVIF proved to be effective to eliminate the substrate inhibition by maintaining sucrose at appropriate levels. It is also easy to be scaled up, since various parameters involved in industrial production were taken into account.

Keywords Nisin · Fed-batch · Fermentation · Lactic acid bacteria · Biotechnology

Introduction

Nisin is an antimicrobial peptide produced by some strains of *Lactococcus lactis*. It is effective against a wide range of Gram-positive bacteria, including *Listeria monocytogenes* and most heat-resistant spores in foods and beverages [1]. Nisin has been accepted as an innocuous, safe, and natural preservative in more than 50 countries [2], thus has a good prospect in the food industry.

Commercial nisin is produced mainly by liquid fermentation, and surely it is of great significance to improve the productivity. The fed-batch fermentation (FF) is a good way to

Z. Wu · L. Wang (✉) · Y. Jing · X. Li · Y. Zhao
Department of Bioengineering, Hebei University of Technology, Tianjin 300132, China
e-mail: wanglin810325@163.com

Z. Wu
e-mail: zhaoliangwu@163.com

increase the output of bacteriocin [3]. As a transition culture method between batch fermentation and continuous fermentation, FF can eliminate the substrate inhibition, reduce the production of metabolic outgrowth, and favor the stability of bacterial metabolism like continuous fermentation. However, it does not need stringent aseptic conditions; neither the aging issues of strains would be involved. Furthermore, this technology is especially suitable for certain high-density culture and fermentation process in which the final product concentration needs to be improved.

Batch fermentation of nisin has been well studied, while reports on its FF were relatively scarce. De Vuyst and Vandamme [4] found that carbon source had an important influence on nisin biosynthesis due to the genetic link between sucrose metabolism and nisin production, and the regulation of carbon metabolism appeared to be a major control mechanism for nisin biosynthesis. Lv et al. [5–7] investigated the effects of feeding of carbon source and nitrogen source on the biosynthesis of nisin and established a pH feedback-controlled fed-batch fermentation technique for nisin production. These studies focused more on the effects of the limitation of carbon and nitrogen sources on nisin biosynthesis, while variations in broth volume caused by feeding were generally ignored despite its importance in industrial production. Systematic studies on the ISC, feeding time, and feeding rate, which are also important parameters that have to be taken into account in commercial production, were also devoid. In this paper, the variable volume constant feeding fed-batch fermentation (VVCF) with sucrose as the limited substrate was investigated. The ISC, feeding time, as well as feeding rate were studied to optimize the nisin production. Based on the results of VVCF, the variable volume intermittent fed-batch fermentation (VVIF) was carried out to reinforce the effect of feeding and further enhance the nisin production.

Materials and Methods

Bacterial Strains and Media

The nisin-producing strain (*Lactococcus lactis* subsp. *lactis W28*) was provided by Tianjin Kangyi Bioengineering Co., Ltd., China. The fermentation medium consisted of 50 g corn syrup (liquid), 40 g sucrose, 20 g KH_2PO_4, 10 g peptone, 10 g yeast extract, 2 g NaCl, and 0.2 g $MgSO_4 \cdot 7H_2O$ per liter of distilled water. The initial pH of medium was adjusted to 7.2 by 1 mol l^{-1} NaOH. Prior to fermentation, the inoculums were propagated twice at 30 °C for 8 h in the seed medium (initial pH 6.9), which was composed of 20 g KH_2PO_4, 15 g sucrose, 15 g peptone, 15 g yeast extract, 2 g NaCl, and 0.2 g $MgSO_4 \cdot 7H_2O$ per liter of distilled water. *Micrococcus flavus* NCIB 8166, purchased from Microbial Culture Collection Center by Chinese Academy of Sciences, was used as the indicator strain in the nisin bioactivity assay. It was grown in the SI medium (initial pH 7.2), which consisted of 10 g agar, 10 g tween-20, 8 g tryptone, 5 g glucose, 5 g NaCl, 3 g yeast extract, and 2 g Na_2HPO_4 per liter of distilled water. This medium was also used in the bioassay of nisin, as will be described later in "Analytical Methods". All media were autoclaved at 121 °C for 20 min and stored at 4 °C before use.

Batch and Fed-Batch Fermentation

Batch and fed-batch fermentation were carried out in a 1-l jar fermenter equipped with a digital pH controller and a blender. The fermenter contained 500 ml fermentation medium for primary culture and was inoculated with 8-h culture of *L. lactis* (6%, *v*/*v*). The cells were cultured at

30 °C. A slow agitation (100 rpm) was maintained to keep the fermentation broth homogeneous. When the pH dropped to 6.25, 2.5 mol l^{-1} NaOH was added automatically into the medium to maintain a constant pH. Samples were withdrawn aseptically from the broth at regular time intervals and analyzed for biomass and nisin titer. For VVCF, sucrose solution (190 g l^{-1}) was added to the broth at invariable rates (8, 9, 10, and 12.5 ml h^{-1}) after 3-h (determined by pre-experiments) fermentation. For VVIF, the sucrose solution (190 g l^{-1}) was added in batches to keep the concentration of sucrose in an appropriate range (5–10 g l^{-1} in the main experiments, determined by pre-experiments). In FF, the final total sucrose concentration was 40 g l^{-1}, the total volume of sucrose solution added to the broth by feeding was 100 ml, and the feeding rate was actually the sucrose solution adding rate. Therefore, the added sucrose solution concentration can be calculated by this equation: $C_f V_f + C_i V_i = C_t V_t$, where C_f was the added sucrose solution concentration, V_f (100 ml) was the volume of added sucrose solution, C_i was the ISC in FF, V_i (500 ml) was the volume of broth for primary cultivation, C_t (40 g l^{-1}) was the final total sucrose concentration in FF, and V_t (600 ml) was the final volume of broth in FF. Decreases in broth volume caused by sampling were counteracted by continuously adding of NaOH, which was used to keep the pH constant during the whole fermentation process. It should be noted that due to feeding, the final volume of the fermentation broth in FF was 100 ml larger than that in batch culture. The increased volume is very important in industrial-scale production, so it has to been taken into account.

Analytical Methods

Biomass was estimated by optical density at 600 nm. The values were converted to cell dry weight (CDW) from a predetermined standard curve. The concentration of sucrose and lactic acid in the broth were measured using the Roe method and a rapid colorimetric method [8], respectively.

Nisin titer was determined by a modified agar diffusion assay [9]. Briefly, sterilized SI medium was melted in a water bath (95 °C) and then tempered to 50 °C. A 24-h grown culture of the indicator species was suspended and adjusted in normal saline solution to give an OD_{600}=0.45. Of this suspension, 48 μl was diluted in 6 ml normal saline solution and then inoculated into the 26 ml bioassay agar. The agar medium was aseptically poured into sterile Petri plates (Φ=90 mm) and allowed to solidify for 2 h at room temperature. Eight holes were bored into each agar plate using a sterilized stainless steel borer (outer diameter=7 mm).

A standard nisin solution was prepared by dissolving 0.01 g of commercial nisin (10^6 IU g^{-1}, Sigma) in 5 ml of 0.02 mol l^{-1} HCl. All samples for nisin determination were adjusted to pH 2.0 with a few drops of 10 mol l^{-1} HCl and heated in boiling water bath for 5 min and then cooled to room temperature. Centrifugation at 15,000×g for 10 min were applied to remove undigested substrates and heat-killed cells. Three hundred-fold and 600-fold dilutions of the supernatant, as well as the standard nisin solution, were made using 0.02 mol l^{-1} HCl. In each agar plate, 140 μl of both the 300-fold and the 600-fold standard nisin solution were added into separated holes. The rest of the holes were injected with aliquots of the samples, and the plate was then incubated at 37 °C for 24 h. Diameters of the inhibition zones were measured and nisin activity expressed as international unit (IU) per milliliter was calculated from:

$$\lg \frac{Cx}{Cs} = \frac{(\Phi xh + \Phi xl) - (\Phi sh + \Phi sl)}{(\Phi xh + \Phi sh) - (\Phi xl + \Phi sl)} \times \lg 2$$

where Cx is the titer of the sample in question (IU ml^{-1}) and Cs is that of the standard solution (2,000 IU ml^{-1} here); Φxh is the inhibition zone diameter generated by the 300-fold dilution of

the sample (mm) and Φxl by the 600-fold dilution (mm). Similarly, Φsh is the inhibition zone diameter generated by the 300-fold standard nisin solution in the same plate (mm) and Φsl by the 600-fold dilution (mm). Measurement of each sample was performed in triplicate using three different plates.

All experiments described above were carried out in triplicate, and the data were examined by two-way analysis of variance (ANOVA) using the built-in data analysis tools of Microsoft® office Excel Version 2003.

Results

Determination of the Feeding Time

The time course of nisin production in batch fermentation is shown in Fig. 1. In the lag phase of growth (0–3 h), the consumption of sucrose, production of nisin, and lactic acid were little. In exponential phase, the biomass increased quickly and reached a maximum of 4.10 g l^{-1} at 14 h. At the same time, the nisin titer increased with cell growth and reached a maximum of 3,389 IU ml^{-1}, and the sucrose was completely consumed. As shown in Fig. 1, the sucrose concentration decreased sharply after 3 h. To prevent the nisin biosynthesis from being restricted by the lack of carbon source, sucrose solution should be added to the broth at this time.

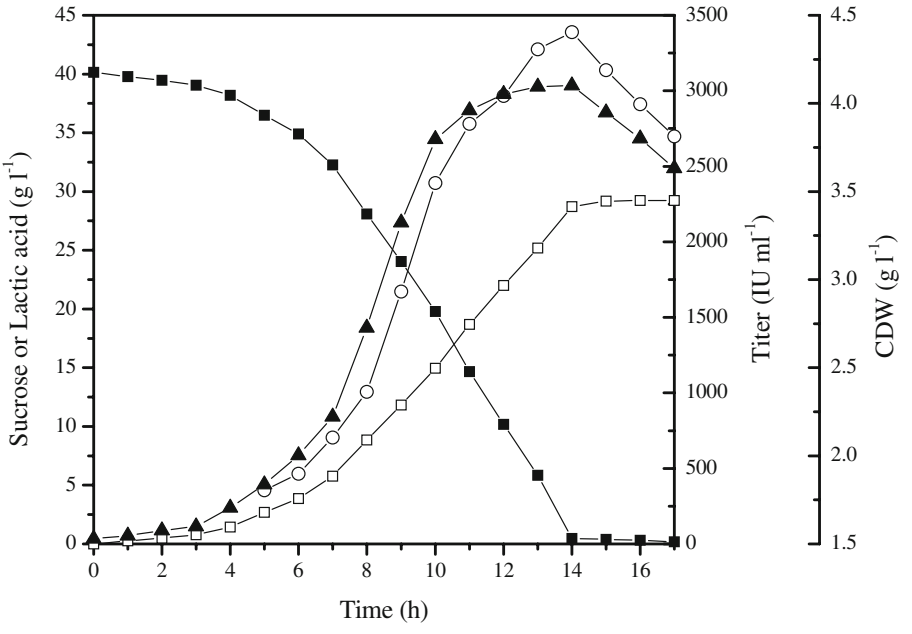

Fig. 1 Nisin production in batch fermentation with the ISC of 40 g l^{-1}. Sucrose concentration (*filled square*), lactic acid (*open square*), nisin titer (*circle*), CDW (*triangle*)

Determination of the ISC

Cell growths at different initial sucrose concentrations were measured, and the results are presented in Fig. 2. It can be seen that the lag phase was prolonged with the increase of ISC. When sucrose was initially at 10 and 40 g l^{-1}, the exponential phase started at about 1 and 3 h, respectively. ANOVA results [F=290.0439>$F_{0.95}$(2,4)=2.5252] indicated that cell growth rate was significantly influenced by ISC. At an ISC of 10 g l^{-1}, the cell growth rate was the highest, giving a 0.44 g l^{-1} increase in biomass yield at 3 h. Therefore, 10 g l^{-1} was used as the optimum ISC for the FF.

Constant Feeding Fed-Batch Fermentation

The cell growth curves in constant feeding fed-batch fermentations with different feeding rates are shown in Fig. 3. The growth rate during the exponential phase was increased with the decrease of the feeding rate. When sucrose feeding rate was 8 ml h^{-1}, the cell growth rate was the fastest at the beginning of feeding, but after 7-h fermentation, the cell growth was decelerated. The CDW (3.79 g l^{-1}, 8 ml h^{-1}) began to be less than that in batch fermentation (3.80 g l^{-1}) from 10 h. The cell growth trends in fed-batch fermentations with different feeding rates (12.5, 10, and 9 ml h^{-1}) were similar to that of the batch culture. When the sucrose was added at the rate of 9 ml h^{-1}, the maximum CDW of 4.24 g l^{-1} was obtained at 15 h, which was higher than that in other fed-batch cultures. Two-factor ANOVA with replication was performed with incubation time and feeding rate as the independent factors. Result for feeding rate [F=3.9494>$F_{0.95}$(2,4)=2.5066] indicated that the effect of feeding rate on cell growth is statically significant.

The residual sucrose concentrations in fed-batch fermentations with different feeding rates were compared, and the results are shown in Fig. 4. In batch fermentation, very little sucrose was consumed in the lag phase; therefore, the concentration was nearly invariable. However, the sucrose concentration decreased drastically during the exponential phase, and it was

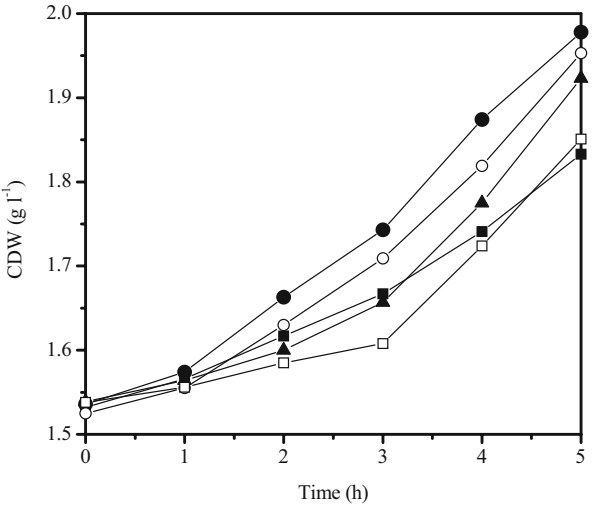

Fig. 2 Effects of different sucrose concentrations on cell growth. The ISC: 5 g l^{-1} (*filled square*), 10 g l^{-1} (*filled circle*), 15 g l^{-1} (*open circle*), 20 g l^{-1} (*triangle*), 40 g l^{-1} (*open square*)

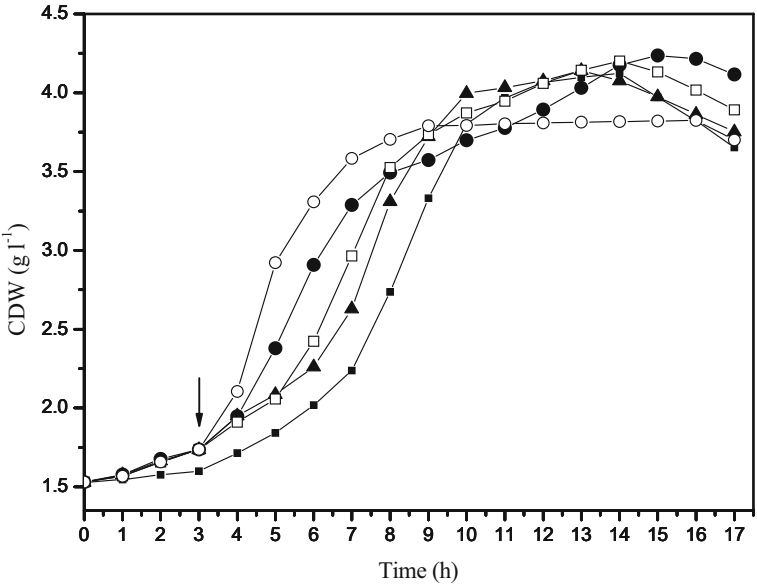

Fig. 3 Comparison of cell growth in constant feeding fed-batch fermentations with different feeding rates. Feeding rate: 8 ml h^{-1} (*open circle*), 9 ml h^{-1} (*filled circle*), 10 ml h^{-1} (*open square*), 12.5 ml h^{-1} (*triangle*), batch fermentation (*filled square*)

consumed completely at 14 h. In the fed-batch fermentations with different feeding rates, the residual sucrose curves were similar. Sucrose in the broth was consumed little in the lag phase and increased gradually 3 h later. Then, the sucrose concentration decreased inchmeal due to the sucrose consumption rate faster than its feeding rate. When the feeding rate was 8 ml h^{-1}, the sucrose concentration was very low (0–10 g l^{-1}) in the broth. This resulted in the nutritional limitation, which restricted the cell growth. However, when sucrose was fed at the rates of 12.5, 10, or 9 ml h^{-1}, its concentrations were controlled in smaller bounds. Especially when the rate was 9 ml h^{-1}, the sucrose was controlled in appropriate bounds, the exponential phase was prolonged, and the final biomass was improved (Fig. 3).

The time courses of nisin production in fed-batch fermentations with different feeding rates are shown in Fig. 5. ANOVA results showed that when feeding rate was 9, 10, and 12.5 ml h^{-1} [F=35.2434, 31.9909, and 18.6701, respectively, while $F_{0.95}(2,1)$=4.7472], the maximum nisin titers obtained in fed-batch fermentations were all significantly higher than that in batch culture (3,406 IU ml^{-1}). The only exception was when feeding rate was 8 ml h^{-1}. The nisin titer seemed to be lower than in batch culture; however, ANOVA result [F= $0.3744<F_{0.95}(2,1)=4.7472$] indicated that when $p<0.05$, this difference had no statistical significance. It is interesting that this instability may be because insufficient sucrose restricted cell growth as well as the biosynthesis of nisin; however, we will not go any further on this topic in the present study.

Nisin yields based on biomass, which may be more reasonable indexes to evaluate the fermentation process at different feeding rates, were summarized in Table 1. As there is hardly any difference in nisin yield between feeding rates of 9 and 10 ml h^{-1}, the former was chosen objectively for the subsequent experiments. An optimal culture condition of *L. lactis* in constant feeding fed-batch fermentation could be that 100 ml concentrated sucrose solution of 190 g l^{-1} was added to the broth with ISC of 10 g l^{-1} at the rate of 9–10 ml h^{-1} starting from 3 h of fermentation.

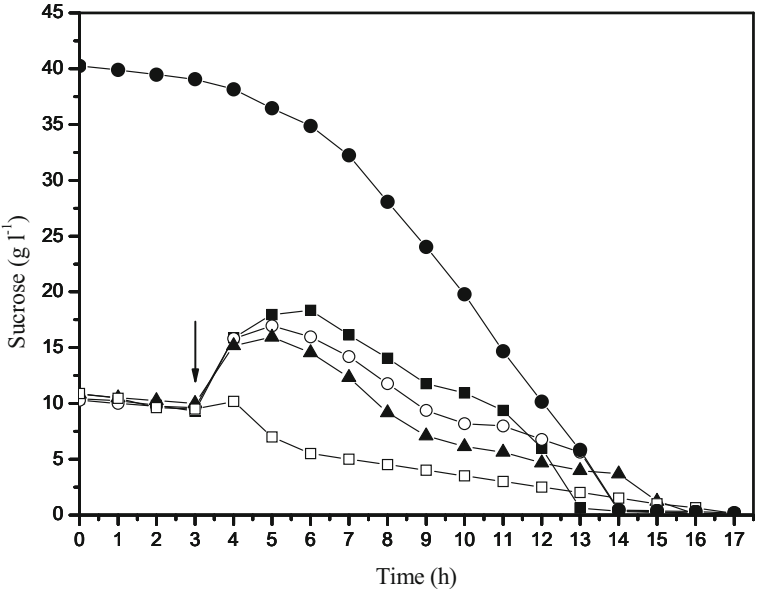

Fig. 4 Comparison of residual sucrose in constant feeding fed-batch fermentations with different feeding rates. Feeding rate: 8 ml h^{-1} (*open square*), 9 ml h^{-1} (*triangle*), 10 ml h^{-1} (*open circle*), 12.5 ml h^{-1} (*filled square*), batch fermentation (*filled circle*)

Intermittent Feeding Fed-Batch Fermentation

The results of VVCF clearly indicated that feeding was an effective way in improving the nisin production. Although VVCF could control the sucrose concentration within bounds, the feeding could only be described as blind, as the demand of carbon source of the producer was not considered; therefore, VVIF was attempted. In the VVCF (feeding rate was 8 ml h^{-1}), the sucrose concentration was controlled between 5 and 10 g l^{-1} during 3–7 h, and the cell growth rate was the fastest. While after 7 h of fermentation, the cell growth rate decreased. In the VVIF (ISC=10 g l^{-1}), samples were drawn from the broth every hour to monitor the sucrose concentration, and this was taken as an indicator of the carbon source demand. When the residual sucrose concentration was close to 5 g l^{-1}, 14.28 ml sucrose solution (190 g l^{-1}) was added into the broth immediately until the 100 ml concentrated sucrose solution was fed completely. Thus, the sucrose concentration could be maintained between 5 and 10 g l^{-1}.

As shown in Fig. 6, although some fluctuations of sucrose concentration in the VVIF were observed, the sucrose concentration was well controlled within the range of 5–10 g l^{-1} approximately. The maximum nisin titers in both batch and intermittent feeding fed-batch culture appeared at 14 h (Fig. 6). However, in VVCF with a feeding rate of 9 ml h^{-1}, the maximum titer was at 15 h (Fig. 5). Those might be because the sucrose was completely consumed at 14 h in BF and VVIF, but at 15 h in VVCF. It reflected that the VVIF had a shorter fermentation cycle than that of constant feeding culture. The titer and biomass (4,490 IU ml^{-1}, 4.42 g l^{-1}) obtained in intermittent feeding fed-batch culture were higher than those (3,375 IU ml^{-1}, 4.12 g l^{-1}) in batch fermentation. The significance of the differences between those values were justified by ANOVA. As for nisin titer, $F=149.8005>F_{0.95}(2,1)=4.4139$, for biomass, $F=51.6561>F_{0.95}(2,1)=4.3248$. Moreover, since the final volume of

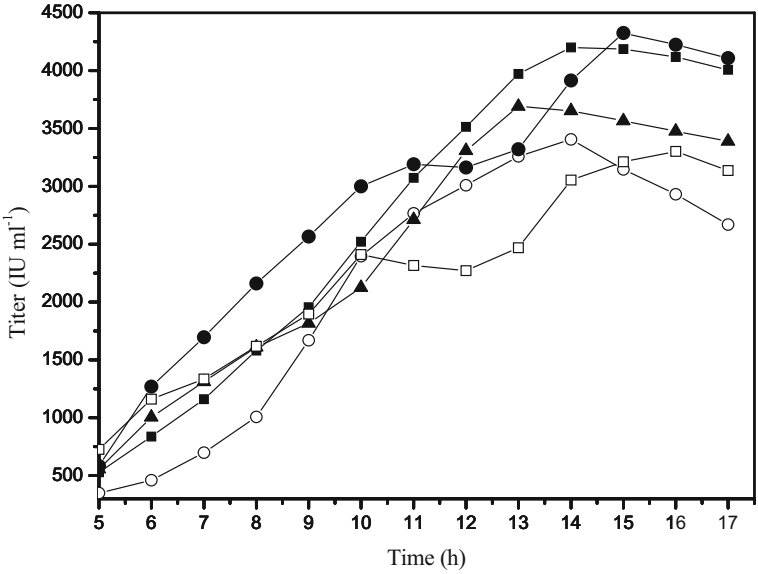

Fig. 5 Comparison of nisin biosynthesis in constant feeding fed-batch fermentations with different feeding rates. Feeding rate: 8 ml h^{-1} (*open square*), 9 ml h^{-1} (*filled circle*), 10 ml h^{-1} (*filled square*), 12.5 ml h^{-1} (*triangle*), batch fermentation (*open circle*)

FF was 20% higher than the batch culture, the final total nisin production and total biomass were 60% and 29% above those of the batch fermentation, respectively.

Discussion

Lv et al. [6] and Papagianni et al. [10] found that the biomass was not apparently increased compared with that in batch culture and attributed that to the inhibition of lactic acid accumulated in the fermentation. In this work, the biomass was also increased indistinctively, but the total biomass was 1.29 times as high as that in batch fermentation. This might be because the increase of the volume led to the increase of total biomass.

The investigation of Van't Hul and Gibbons [11] and Cheigh et al. [12] showed that in both batch and fed-batch fermentations, a typical exponential growth phase was observed with simultaneous production of lactic acid followed by an increase in nisin concentration. However, lactate progressively inhibited cell growth [13, 14]. Although there was a direct

Table 1 Comparison of batch fermentation and constant feeding fed-batch fermentations.

Feeding rate (ml h^{-1})	Residual sucrose concentration (g l^{-1})	Maximum titer (IU ml^{-1})	Total nisin yield (×10^6 IU)	Maximum biomass (g l^{-1})	Total biomass (g)	$Y_{N/X}$ (mg g^{-1})
Batch culture	0–40.0	3,406	1.703	4.12	2.06	20.67
8	0–10.9	3,302	1.981	3.83	2.30	21.53
9	0–16.0	4,324	2.594	4.24	2.54	25.53
10	0–17.0	4,201	2.521	4.20	2.52	25.01
12.5	0–18.4	3,691	2.215	4.14	2.48	22.33

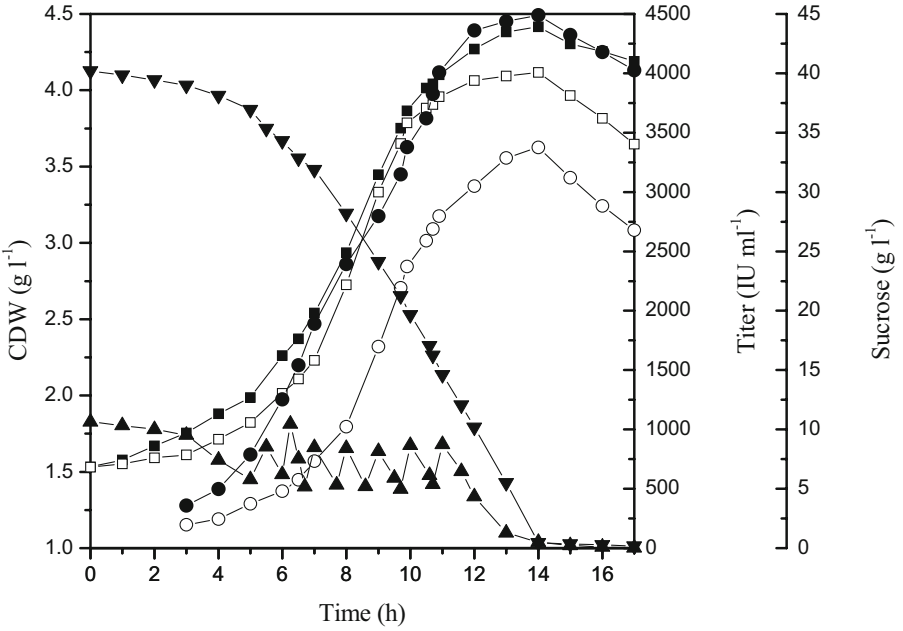

Fig. 6 Comparison of batch fermentation and intermittent feeding fed-batch fermentation. Sucrose concentration (*inverted triangle*), CDW (*open square*), nisin titer in the batch fermentation (*open circle*). Sucrose concentration (*triangle*), CDW (*filled square*), nisin titer in the intermittent feeding fed-batch fermentation (*filled circle*)

relationship between growth and nisin production, it was not known whether nisin production was directly affected by lactate [13].

Callewaert and De Vuyst [3] reported that during the batch fermentation of *L. amylovorus* DCE 471, a rapid decline in bacteriocin activity was observed after the growth-associated bacteriocin production phase. Semblable fermentation patterns were obtained in the works of Papagianni et al. [10], Cheigh et al. [12], and Parente et al. [15]. In this paper, a similar result was also obtained (Fig. 1). The cell growth and nisin titer reached their peak values at 14 h when sucrose was consumed completely, and then both of the values dropped sharply. This may be due to the proteolytic inactivation, protein aggregation [14, 16, 17], and the adsorption of nisin molecules to the cell surface of the producer cells [4, 18].

According to the studies of De Vuyst and Vandamme [4], De Vuyst et al. [16], and Moortvedt-Abildgaard et al. [19], nisin production was correlated with bacterial growth, implying that the volumetric bacteriocin production was dependent on the total biomass formation, and this was consistent with our study (Figs. 3 and 5). The exponential phase of the fed-batch fermentation with feeding at 9 ml h^{-1} was significantly longer than that at 8 ml h^{-1}. The maximum nisin titer under those conditions were 4,324 and 3,302 IU ml^{-1}, respectively. This might be due to the increase in nisin production mainly in the exponential phase. Thus, a greater biomass and a longer exponential growth phase favor the accumulation of nisin.

It was obvious that high sucrose concentration would inhibit cell growth and nisin biosynthesis. However, a low sucrose level could not provide sufficient nutrition; hence, cell growth and nisin production might be restricted (Figs. 3 and 5). Therefore, an appropriate sucrose concentration level is propitious to the increase of biomass and the

extension of exponential phase, which will stimulate the biosynthesis of nisin. That may be due to the effect of the stress situation and the sucrose regulation.

Stress situations might shift the metabolism towards nisin biosynthesis as an evolutionary protection mechanism [20]. Nisin has an antibacterial activity against species closely related to the producer, and more than 60% of the carbon source in the medium is converted to lactic acid, so nisin may be a competitive and non-main production. The more serious a stress situation is, the more nisin may be produced. Furthermore, some studies indicated that the biomass of amylovorin was improved under the non-optimum temperature [21]; the slowly metabolized nitrogen source, such as cotton-seed, blood meal, or fish meal, favored nisin biosynthesis [22]; the deficiency of nitrogen caused a stress situation, which could stimulate the nisin biosynthesis [23]; and the low residual sucrose concentration stimulated the nisin biosynthesis [5]. Therefore, some unfavorable factors are conducive to nisin synthesis. This is probably because nutrition limitation causes a stress effect, which will stimulate the expression of nisin, and at the same time, the medium can be used more efficiently.

Sucrose has a regulating effect on nisin biosynthesis. The research of De Vuyst and Vandamme [4] indicated that the regulation of carbon metabolism appeared to be a major control mechanism for nisin biosynthesis; this might be because a genetic correlation exists between sucrose metabolism and nisin production [24]. The microbial metabolic regulation was mainly performed through the production and activity of various enzymes, and nisin was the activated part of the pre-nisin which was modified by enzymes. De Vuyst [25] considered that the nisin production was related to the expression, activity, and the resistance to nisin of the mature enzyme. Thus, controlling the sucrose concentration at the appropriate level by feeding, the expressions of the pre-nisin modifying enzymes are stimulated, and the production of nisin is improved greatly. In a word, fed-batch fermentation can eliminate the excessive sucrose inhibition, avoid nutritional limitation, provide the appropriate substrate concentration for cells, and increase the nisin production prominently. Therefore, it is a good technology of nisin production.

Conclusions

The variable volume fed-batch fermentation scheme was successfully applied to nisin production. In the proposed technique, the final broth volume was 20% higher than its initial value due to feeding, which may be of great significance for the large-scale production. In the present work, the increase in broth volume was taken into account, and the total biomass and total nisin yield were used to evaluate the performance of the process. It was found that in VVIF, the total biomass and the total nisin yield were 29% and 60% above those in batch culture, respectively. It can be seen that the VVIF can be used for industrial production of nisin.

Acknowledgments We thank the Tianjin Kangyi Bioengineering Co., Ltd. of China for providing us with the nisin-producing strain.

References

1. Goulhen, F., Meghrous, J., & Lacroix, C. (1999). *Journal of Applied Microbiology*, *86*, 399–406. doi:10.1046/j.1365-2672.1999.00675.x.
2. Shimizu, H., Mizuguchi, T., Tanaka, E., & Shioya, S. (1999). *Applied and Environmental Microbiology*, *65*, 3134–3141.

3. Callewaert, R., & De Vuyst, L. (2000). *Applied and Environmental Microbiology*, *66*, 606–613. doi:10.1128/AEM.66.2.606-613.2000.
4. De Vuyst, L., & Vandamme, E. J. (1992). *Journal of General Microbiology*, *138*, 571–578.
5. Lv, W., Cong, W., & Cai, Z. (2004). *Biotechnology Letters*, *26*, 235–238. doi:10.1023/B:BILE.0000013721.78288.1d.
6. Lv, W., Cong, W., & Cai, Z. (2004). *Biotechnology Letters*, *26*, 1713–1716. doi:10.1007/s10529-004-3742-6.
7. Lv, W., Cong, W., & Cai, Z. (2005). *Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire: 1986)*, *80*, 511–514. doi:10.1002/jctb.1230.
8. Steinsholt, K., & Calbert, H. E. (1960). *Milchwissenschaft. Milk Science International*, *15*, 7–11.
9. Xin, Y., Wu, Z., & Yu, G. (2005). *Journal of Hebei University of Technology*, *34*, 80–84.
10. Papagianni, M., Avramidis, N., & Filiousis, G. (2007). *Enzyme and Microbial Technology*, *40*, 1557–1563. doi:10.1016/j.enzmictec.2006.10.035.
11. Van't Hul, J. S., & Gibbons, W. R. (1997). *World Journal of Microbiology & Biotechnology*, *13*, 527–532. doi:10.1023/A:1018509207939.
12. Cheigh, C. I., Choi, H. J., Park, H., Kim, S. B., Kook, M. C., Kim, T. S., et al. (2002). *Journal of Biotechnology*, *95*, 225–235. doi:10.1016/S0168-1656(02)00010-X.
13. Yu, P. L., Dunn, N. W., & Kim, W. S. (2002). *Biotechnology Letters*, *24*, 59–64. doi:10.1023/A:1013865502420.
14. Parente, E., Brienza, C., Ricciardi, A., & Addario, G. (1997). *Journal of Industrial Microbiology & Biotechnology*, *18*, 62–67. doi:10.1038/sj.jim.2900368.
15. Parente, E., Ricciardi, A., & Addario, G. (1994). *Applied Microbiology and Biotechnology*, *41*, 388–394.
16. De Vuyst, L., Callewaert, R., & Pot, B. (1996). *Systematic and Applied Microbiology*, *19*, 9–20.
17. Parente, E., & Ricciardi, A. (1994). *Letters in Applied Microbiology*, *19*, 12–15. doi:10.1111/j.1472-765X.1994.tb00891.x.
18. Joerger, M. C., & Klaenhammer, T. R. (1986). *Journal of Bacteriology*, *167*, 439–446.
19. Moortvedt-Abildgaard, C. I., Nissen-Meyer, J., Jelle, B., Grenov, B., Skaugen, M., & Nes, I. F. (1995). *Applied and Environmental Microbiology*, *61*, 175–179.
20. Hurst, A. (1981). *Advances in Applied Microbiology*, *27*, 85–123. doi:10.1016/S0065-2164(08)70342-3.
21. Lejune, R., Callewaert, R., Crabbe, K., & De Vuyst, L. (1998). *The Journal of Applied Bacteriology*, *84*, 159–168.
22. De Vuyst, L., & Vandamme, E. J. (1993). *Applied Microbiology and Biotechnology*, *40*, 17–22. doi:10.1007/BF00170422.
23. De Vuyst, L., Callewaert, R., & Crabbe, K. (1996). *Microbiology*, *142*, 817–827.
24. James, L. S., & Larry, L. M. (1986). *Applied and Environmental Microbiology*, *51*, 57–64.
25. De Vuyst, L. (1994). *Biotechnology Letters*, *16*, 287–292. doi:10.1007/BF00134627.