Nisin Production by Immobilized Microbial Cell Culture during Batch and Fed-Batch Fermentations with Various pH Profiles

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ABSTRACT

In this study, nisin production has been enhanced by using batch and fed-batch fermentation with calcium-alginate immobilized cell culture. Due to the inhibitory effects of original phosphate rich growth medium on the immobilizing beads, an altered complex growth medium for nisin production was used. Various pH profiles were evaluated for both batch and fed-batch fermentations. For batch fermentations, a 2.1 fold higher nisin activity was obtained by allowing the pH to drop freely after 4 hrs of fermentation at constant pH. A periodic pH profile exhibited a detrimental effect on nisin production during batch fermentations. For fed-batch fermentations, a 2.9 fold higher nisin activity was obtained by allowing the pH to remain at pH of 6.8. Approximately the same maximum concentration, 3300 IU/ml, of nisin was observed when the best pH profile for batch and fed batch experiments were compared. The results also showed that immobilized cell culture can be used in order to improve nisin fermentation for both batch and fed-batch fermentation.

Keywords: Nisin, *Lactococcus lactis*, immobilization, pH profile, bacteriocin, batch fermentation, USA

1. INTRODUCTION

Bacteriocins are a class of antimicrobial agents produced by lactic acid producing bacteria (LAB). Among all of the bacteriocins known today, nisin is the only bacteriocin that has been given a generally regarded as safe (GRAS) status by the United States Food and Drug Administration (FDA, 2001). Today, over 50 countries use nisin as a food preservative.

Nisin inactivates Gram positive bacteria by creating small pores in the cytoplasmic membrane (Breukink, 2005). This action causes cell leakage and a hindrance in the bacteria's ability to produce energy. Nisin has been proven to inhibit the growth of *Bacillus*, *Bifidobacrium*, *Brochothrix*, *Clostridium*, *Corynebacterium*, *Enterobacter*, numerous *Lactobacillus*, *Listeria*, *Micrococcus*, *Pediococcus*, *Staphylococcus*, and some *Actinomyceae* (Millette *et al.*, 2004). In

conjunction with chelating agents, such as EDTA, nisin is able to destroy the cytoplasmic membrane of some Gram negative bacteria as well (Millette *et al.*, 2004).

Nisin is produced commercially by suspension cell fermentation within pH-control and a milk-based fermentation medium (De Vuyst and Vandamme, 1992). Unfortunately, nisin production levels are low; approximately 2000 IU/ml (De Vuyst and Vandamme, 1992; Matsusaki *et al*., 1996). Cell density in the bioreactor, carbon source, and pH control profile during fermentation all play a vital role in the activity and yield of nisin (De Vuyst and Vandamme, 1994; Parente and Ricciardi, 1999).

Hirsch and Grinsted (1951) found a phenotypic correlation between the use of sucrose, as a carbon source, in LAB fermentation and nisin production. In order to produce the greatest amount of nisin, De Vuyst and Vandamme (1992) determined an optimum concentration of sucrose that needs to be provided to *L. lactis* NIZO 22186. They observed the following: (1) the highest specific nisin productivity (19.1 mg nisin/g cells dry weight (CDW)) takes place when a low initial concentration (10 g/L) of sucrose was provided to the reactor; (2) the greatest activity of nisin (maximum of 3267 IU nisin/ml medium) was found at higher initial concentrations of sucrose (up to 40 g/L of sucrose); and (3) the biomass and nisin titres decreased at concentrations of sucrose above 40 g/L. At 50 g/L and 70 g/L of sucrose the maximum nisin activity was 2937 IU nisin/ml and 1596 IU nisin/ml, respectively. Therefore, it was suggested that the optimum initial sucrose concentration for nisin production was 40 g/L. A strong link between nisin activity and sucrose concentration exists. A regimen of sucrose additions during fermentation may provide favorable results for nisin activity.

Numerous studies have shown that the biosynthesis of nisin is optimum at a pH between 5.5 to 6.0, whereas the optimum pH for growth of LAB is between 6.0 and 6.8 (Parente and Ricciardi, 1999; 1994). The dichotomy in optimum conditions for LAB growth and nisin production suggests that an optimum pH value, range, or procedure is required to yield the highest amount of nisin.

A study performed by Cabo *et al.* (2001) explains the relationship between pH and nisin production by (*L. lactis* subsp. *lactis* IIM lb. 1.13). The important observations identified in this study are: (1) the production of nisin is not enhanced by fermenting *L. lactis* at a low buffered pH value of 4.5; (2) the production of nisin is not enhanced by fermenting *L. lactis* at the bacteria's optimum pH value (pH 6.0 – 6.8); (3) the maximum production of nisin is enhanced by approximately 10 AU/ml due to a drop in pH from high pH to low pH; (4) the production of nisin is most greatly enhanced when the culture is subjected to the "steepest pH gradient within the pH range of production" (Cabo *et al.* 2001). 28 AU/ml was the maximum value produced by this best performing pH profile; (5) the production of biomass was greatest when the pH was buffered at the bacteria's optimum growth pH, approximately 0.75 g/L; (6) the greatest amount of carbohydrate consumption occurred when the fermentation medium was buffered at the bacteria's optimum growth pH. The study also compared the effect of two different pH profiles on the production of nisin. A natural acidification pH profile and a repeatedly re-alkalized pH profile were observed. The repeatedly re-alkalized pH profile was shown to produce more nisin then the fermentation that used no pH control.

2. MATERIALS AND METHODS

2.1 Microorganisms and Medium

Lactococcus lactis subsp. *lactis* (NIZO 22186) was obtained from the Netherlands Institute of Dairy Research (Ede, The Netherlands) and used in this study as the nisin Z-producing strain. *Lactobacillus sakei* (ATCC 15521) was obtained from American Type Culture Collection (Manassas, VA) and used as the nisin sensitive indicator organism. *L. lactis* and *L. sakei* were grown for 20 h at 30°C in a complex medium (CM) and Lactobacillus MRS broth (Difco Laboratories, Detroit, MI), respectively. CM consisted of 40 g of sucrose; 10 g of yeast extract (Ardamine Z, Sensient Bionutrient, Indianapolis, IN); 10 g of peptone (Amber Ferm 401G, Universal Flavors, Milwaukee, WI); 2 g of NaCl; 10 g KH_2PO_4; and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized (DI) water. The initial pH of the CM was adjusted to 6.8 using 4N NaOH. Working cultures were stored at 4°C and subcultured every 2 weeks. Stock cultures were prepared in a 20% glycerol solution and stored at -80°C.

2.2 Preparation of Inoculum

CM described above was used to prepare *L. lactis* inoculum. Peptone and yeast extract and the sucrose and salts were dissolved in DI water in separate containers and autoclaved at 121.1°C for 20 min. After cooling, they were combined aseptically. For cell immobilization, eight one-liter centrifuge bottles, containing 800 ml of the complex medium in each, were prepared for each fermentation run. Each bottle was inoculated with 1% *L. lactis* suspension cells and incubated at 30°C for about 20 hours.

2.2.1 Suspended-Cell

As a control, suspended cell fermentation was also performed. A 1% overnight grown inoculum of *L. Lactis* was added to the fermentation medium at time zero. The fermentation was run for 24 hours. Samples were taken every 2 hours for the first 12 hours. A final sample was taken at 24 hours. Suspended-cell fermentations were completed for batch and fed-batch fermentations.

2.2.2 Cell Immobilization

The method for cell immobilization was adapted from Rickert *et al.* (1998). After incubation for 20 h at 30°C, the broth was centrifuged at 3800 x *g* for 20 min at 4ºC. The supernatant liquid was decanted and replaced with 0.85% sterile sodium chloride solution. The cells were resuspended and collected in even volumes into two one-liter centrifuge bottles. The bottles containing the culture were centrifuged again at 3800 x *g* for 25 min at 4ºC. The supernatant liquid was decanted and a 1:1 ratio of 0.85% sterile sodium chloride solution:biomass was prepared. Next, 2.5% w/v alginate solution was prepared, sterilized, and cooled. Alginate was added to the saline/biomass mixture in a ratio of 1:1:6, saline: cell volume: alginate. The solution was mixed well and poured into a sterile 50 ml syringe containing a 16G1½ needle. The cells were aseptically added drop-wise to 1 L of sterile 0.1 M calcium chloride solution. After the alginate beads were formed, the calcium chloride solution was aseptically decanted. In order to strengthen the beads, one liter of sterile 0.05 M $CaCl_2$ solution was aseptically added to the flask. The beads remained in the 0.05 M $CaCl_2$ solution for at least 12 hours but no longer then

24 hours. The $CaCl_2$ solution was than aseptically decanted off the beads. The beads were washed with approximately 500 ml of 0.85% sterile NaCl solution. The wash solution was decanted and the beads were immediately transferred to the sterile bioreactor vessel. Any remaining saline wash solution was aseptically drawn out of the reactor before the prepared CM was transferred into the reactor.

2.3 Bioreactor

A bench-top reactor (Cellogen, New Brunswick, Edison, NJ) equipped with pH, temperature, and agitation control was used. The 1.5 L reactor vessel containing DI water was autoclaved for 45 min at 121.1ºC. The DI water was pumped out of the reactor prior to the addition of the medium and inoculum. pH was maintained at various pH profiles with 4N NaOH during fermentation. Temperature was maintained at 30°C. No aeration was employed due to the facultative nature of *L. lactis*. Complex medium was modified by using 1 g/l of KH_2PO_4 and 5 g/l of NaCl in order to improve the stability of ca-alginate beads during fermentation.

2.4 Batch Fermentation with Various pH Profiles

During fermentation, the following pH profiles were evaluated (Fig. 1); i) pH Profile B-const24 (constant pH): pH was held constant at 6.8 throughout the entire fermentation; ii) pH Profile B-const6 (constant pH for first 6 hours): pH was held constant at 6.8 for the first 6 hours. After 6 hours, the pH of the broth was uncontrolled; iii) pH Profile B-periodic (Periodic pH control)**:** The pH was held constant for the first 4 hours at 6.8. The fermentation was allowed to auto-acidify every two hours before being readjusted back to pH 6.8; iv) pH Profile B-const4 (constant pH control for first 4 hours): pH was held constant at 6.8 for the first 4 hours. After 4 hours the broth was allowed to auto-acidify by the growing cells.

During fermentation, samples were aseptically drawn from the bioreactor every 2 hours for 12 hours and once at 24 hours, and an analysis of nisin, lactic acid, and sucrose on each sample was preformed. The trial for each pH profile was replicated.

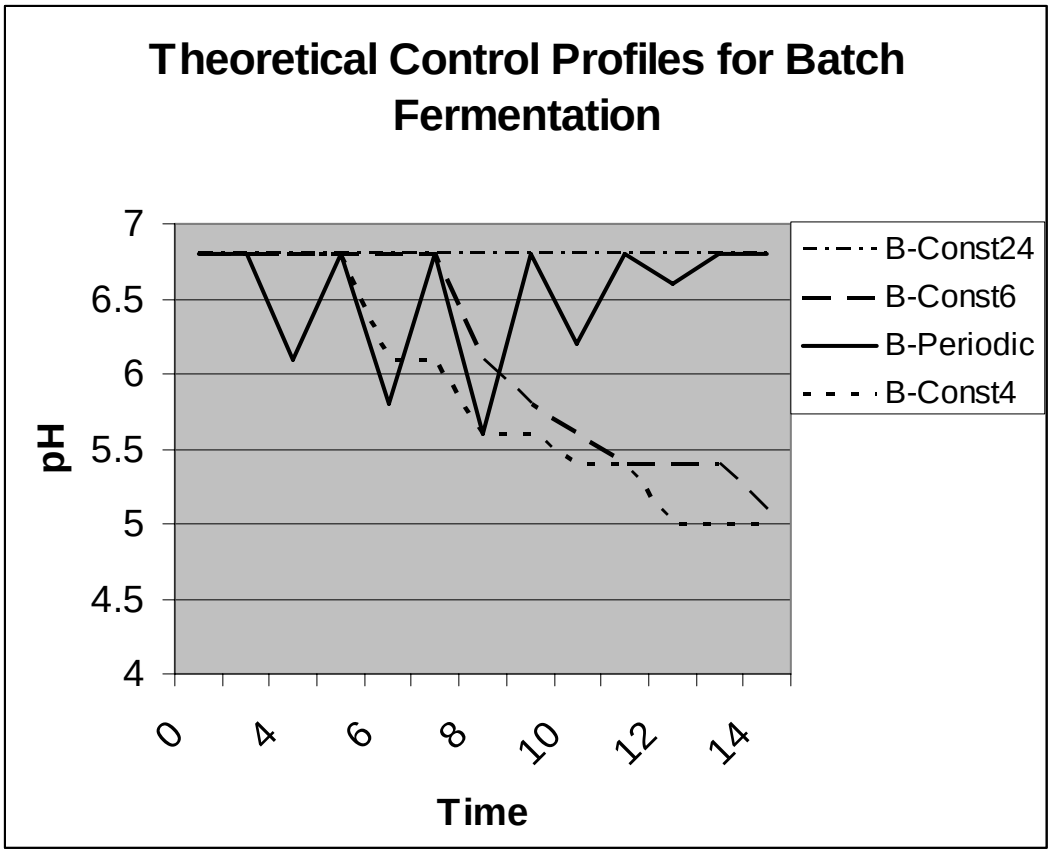

Figure 1. Graphical representation of theoretical pH control profiles for batch fermentation.

2.5 Fed-Batch Fermentation with Various pH Profiles

All fed-batch experiments used the same sucrose addition method. A 50% sterile sucrose solution was pumped into the bioreactor at 2 ml/min for 10 min at time 0 and 2 hours. For the next 10 hours: A sample was taken immediately prior to a 20 min addition of 50% sucrose at a flow rate of 2 ml/min. A second sample was taken immediately following the addition of sucrose. Samples were taken in 2 hour intervals.

Three different pH profiles were evaluated to determine which pH profile increased nisin production the most during semi-continuous fed-batch fermentation (Fig. 2); i) pH Profile FB-const24 (constant pH control with semi-continuous sucrose Addition): pH was held constant at 6.8 throughout the entire fermentation; ii) pH Profile FB-periodic (Periodic pH control with semi-continuous sucrose addition): Fermentation was allowed to auto-acidify every two hours before being readjusted back to pH 6.8; iii) pH Profile FB-const4 (Partial pH control with semi-continuous sucrose addition): pH was held constant at 6.8 for the first 4 hours. After 4 hours the broth was allowed to auto-acidify by the growing cells.

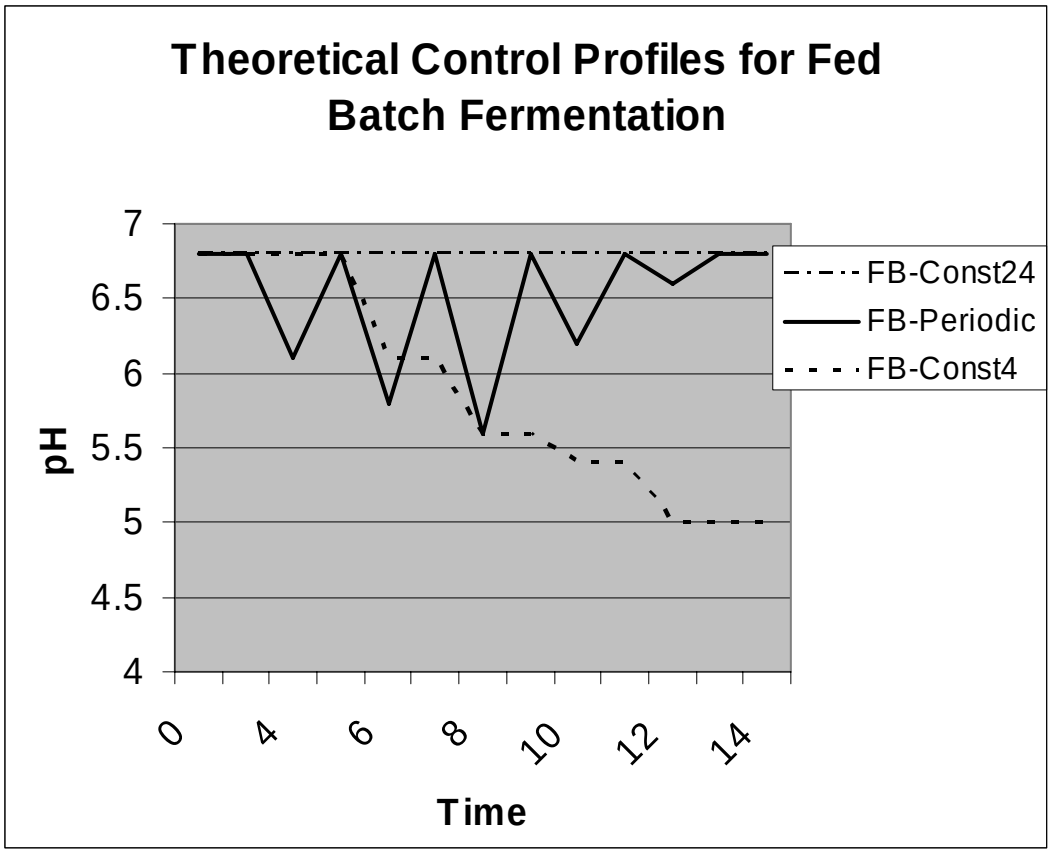

Figure 2. Graphical representation of theoretical pH control profiles for fed-batch fermentation.

2.6 Analysis

Nisin production and sucrose consumption were the tested variables in all the fermentation experiments. A nisin analysis was preformed according to Pongtharangkul and Demirci (2004). The sample was heated for 5 min. in a 100°C water bath. After heating, the sample was centrifuged for 15 min. at 4°C and 3800 x *g*. A 1 ml aliquot was aseptically extracted from the sample and frozen at -20°C until analyzed by bioassay. Samples were diluted 40 times using a sterile 0.1% Tween 80 solution with pH adjusted to 3.0 by using concentrated HCl. The Dinitrosalicylic acid (DNSA) method was used to determine the concentration of sucrose (Miller, 1959). The samples were centrifuged for 15 min at 4°C and 3800 x *g*. The supernatant was aseptically transferred to a clean and sterile vial and kept at 4°C until analysis.

3. RESULTS

The complex media formulation used in the study of Pongtharangkul and Demirci (2006a) was adapted initially, which included 40 g/L of sucrose, 2 g/L of NaCl, 0.2 g/L of potassium sulfate, 10 g/L of yeast extract, and 10 g/L of peptone. However, KH_2PO_4 and KCl concentrations were varied in this study (Table 1).

Maximum nisin concentration, nisin production rate, and bead stability in the media were the factors that determined which complex medium was optimum to use for this study (Data not shown). Ultimately, Complex Medium B was chosen based on its overall ability to promote nisin production and bead stability.

Table 1. Summarized media compositions.

Complex Medium*	**KH_2PO_4 (g/L)**	**KCl (g/L)**
A	1.0	0.0
B	1.0	5.0
C	0.0	0.0

* All complex media contain the following: 40 g/L sucrose, 2 g/L NaCl, 0.20 g/L potassium sulfate, 10 g/L yeast extract, 10 g/L peptone.

Batch fermentations were performed using different pH profiles. Nisin concentrations were tested at various times throughout the 24 hour fermentation (Fig. 3). pH profile B-const4 showed dramatic improvement in maximum nisin production. Maximum nisin concentrations for this pH profile reached 3379 IU/ml. Conversely, pH profile B-periodic performed the worst, for which maximum nisin concentrations only reached 970 IU/ml.

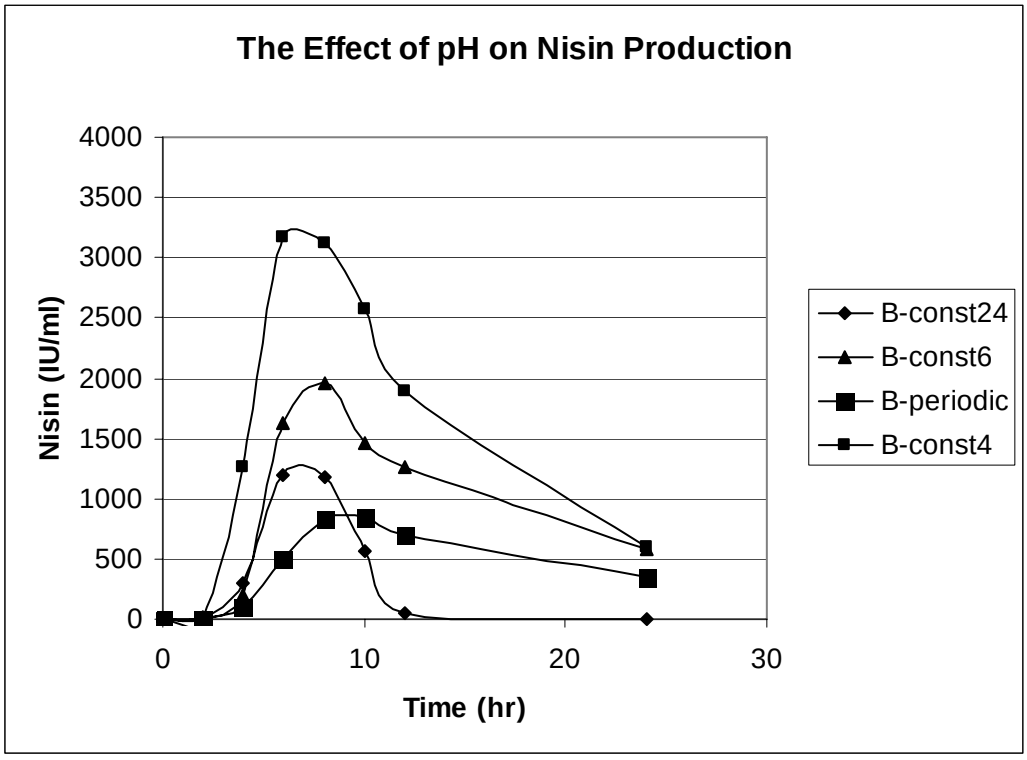

Figure 3. Nisin activity data for different pH profile controls during batch fermentation.

Data recorded for all batch fermentations are provided in Table 2. All fermentations began at a neutral pH. Autoacidification by *L. lactis* was counteracted by 4 N NaOH. Experiments using pH profiles B-const24, B-const6, and Bconst-4 exhibited apparent lag, exponential, and degradation phases in the production cycle of nisin. For all batch fermentations, sucrose concentrations reduced to zero between 7 and 9 hours. Profile B-periodic exhibited the longest time needed to consume sucrose, while Profile B-const4 exhibited the shortest time. For all batch fermentations, the time needed to observe maximum nisin levels fell between 7 and 9 hours. Similar to sucrose consumption times, Profile B-periodic exhibited the longest time needed to observe maximum nisin concentrations, while Profile B-const4 and B-const24 exhibited the shortest time.

Table 2. Summary of fermentation parameters during batch fermentations.

Fermentation Parameters	Profile B-const24	Profile B-const6	Profile B-periodic	Profile B-const4
Time required to consume sucrose (hr)	8 ± 0	8 ± 0	9 ± 1	7 ± 1
Maximum nisin (IU/ml)	1605^{AB} ± 164	1958^{AB} ± 69	970^{C} ± 86	3379 ± 5.2
Time of Max. nisin (hr)	7 ± 1	8 ± 0	9 ± 1	7 ± 1
Max. nisin productivity rate (IU/ml/h)	573^{ABC} ± 10	718^{ABC} ± 14	244^{AC} ± 48	984^{B} ± 164

*Means are significantly different ($p < 0.05$) if followed by a different capital letter for each fermentation parameter measured.

For fed-batch fermentations, sucrose was provided to the fermentation broth at an average rate of 10 g/h of 50% sucrose for the first 2 hours of the fermentation, and an average of 5 g/h of 50% sucrose between 2 and 12 hours of the fermentation. This sucrose feeding regiment was based upon the sucrose consumption rates of *L. lactis* during batch fermentations (Fig. 4). Maximum nisin concentrations ranged from 534 IU/ml for Profile FB-const4 to 3260 IU/ml for Profile FB-const24.

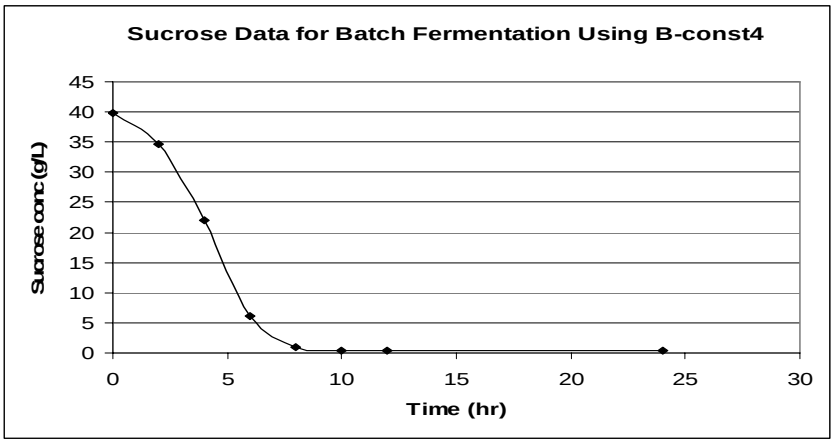

Figure 4. Sucrose consumption during batch fermentation.

Data recorded for all fed-batch fermentations are provided in Table 3. pH profile FB-const24-immobilized cells yielded the most nisin because the *L. lactis* were able to grow and consume sucrose for a longer period of time. This allowed the cells to continue to produce nisin for a longer period of time. The autoacidification of the broth by *L. lactis* caused a detrimental effect on nisin production for pH profiles FB-periodic, FB-const4, and FB-const24-suspended cell. The reduced growth rate of the *L. lactis* in these profiles caused an accumulation of sucrose in the broth (data not shown).

Table 3. Summary of fermentation parameters for fed-batch fermentations.

Fermentation Parameters	**Profile FB-const24 with Suspended Cells**	**Profile FB-const24 with Immobilized Cells**	**Profile FB-periodic**	**Profile FB-const4**
Maximum sucrose consumption rate (g-sucrose/h)	3.66 ± 0.7	8.38 ± 0.17	4.69 ± 0.25	3.27 ± 0.08
Maximum nisin (IU/ml)	1102 ± 222^{A}	3260 ± 209^{BC}	1271 ± 21^{A}	534 ± 47^{AC}
Time of Max. nisin (hr)	12 ± 0	8 ± 0	8 ± 0	12 ± 0
Max. nisin productivity rate (IU/ml/h)	234 ± 6^{C}	787 ± 62^{C}	194 ± 35^{C}	161 ± 10^{D}

* Means are significantly different ($p < 0.05$) if followed by a different capital letter for each fermentation parameter measured.

4. DISCUSSION

Medium formulation plays an important role for nisin production. Studies of Hirsch and Grinsted (1951) and De Vuyst and Vandamme (1992) suggested that there is a phenotypic correlation between the use of sucrose and nisin production in LAB. At high sucrose concentrations, the activity of nisin is the greatest. A sucrose concentration of 40 g/L has been found to be optimum for producing nisin during batch fermentation. Cotton seed meal, yeast extract, and peptones as nitrogen sources were the best organic nitrogen sources that aided in producing nisin (De Vuyst and Vandamme, 1993). Phosphates are proven to aid in nisin production due to their buffering

abilities. Concentrations of phosphates below 6% were proven to be beneficial to nisin production (Egorov et al., 1971; De Vuyst and Vandamme, 1993). Also, pH affects the growth of LAB and, therefore, the production of nisin (Parente and Ricciardi, 1999; 1994; Cabo et al., 2001; Pongtharangkul and Demirci, 2006a). A change in pH also changes nisin's affinity for adherence to surfaces (Biswas et al, 1991). Therefore, this study evaluated high cell density cultures, pH affects, and fed-batch systems for the production of nisin.

4.1 Batch Fermentation with Various pH Profiles

pH profiles used during fermentation played an important role on the observed nisin production (Fig. 3). Profile B-const4 performed the best with a maximum nisin activity of 3379 ± 5.2 IU/ml (Table 2). This is a 110% increase in the maximum nisin activity when compared to profile B-const24. The maximum nisin activity of Profile B-const4 was 3% greater than the value Devuyst and Vandamme (1992) determined 3267 IU/ml. Profile B-const4 preformed better because the rapid reduction of pH hindered the ability for nisin to adsorb onto the producer cell. In addition, protease-nisin interactions may have been impeded by lowering the pH. These suspected interactions would result in the observed increased nisin activity in the fermentation broth. Pongtharangkul and Demirci (2006a) achieved similar results using passively immobilized batch fermentation (biofilm) with the same pH profile. A maximum nisin activity of 3553 IU/ml was observed in that study as well (Pongtharangkul and Demirci, 2006a).

Profile B-const6 demonstrated the next highest maximum nisin activity. The maximum nisin activity for this profile was 1958 ± 69 IU/ml. This was a 22% increase in maximum activity when compared to a constant pH profile (profile B-const24). This observation supports the Protease-nisin interaction and nisin cell binding theory. The more time a pH profile allows for these interactions to occur, the more the nisin curve will mimic profile B-const24 (Fig. 3). For example, a predicted maximum nisin range for a pH profile that is kept constant for 8 hours is expected to be between 1605 IU/ml and 1958 IU/ml.

In addition to improving maximum nisin activity levels, profile B-const4 improved the rate of nisin production of the immobilized cell system without changing the time required to reach the maximum nisin activity (Table 2). Profile B-const4 showed a 1.7 fold increase in the production rate of nisin when compared to profile B-const24, the constant pH profile (984 ± 164 IU/ml and 573 ± 10 IU/ml, respectively).

The worst performing pH profile was profile B-periodic, max nisin activity of 970 ± 86 IU/ml. Pongtharankul and Demirci (2006a) achieved similar results using a passively immobilized batch fermentation with a similar pH profile. The periodic re-alkalization pH profile began at 0 h and was readjusted every 2 hours. A maximum nisin activity of 593 IU/ml was observed in that study (Pongtharangkul and Demirci, 2006a). Conversely, Cabo *et al.* (2001), achieved a 4-fold increase in nisin production when a suspended cell batch fermentation was used with a similar pH profile. The periodic re-alkalization profile began at 0 h and was readjusted every 3 hours. The profile used in this study observed lower nisin concentrations because the pH stress was applied to the bacteria before the exponential growth phase. This result was similar to the study performed by Pongtharankul and Demirci (2006a). This suggests that stressing *L. lactis* NIZO 22186 cells during the lag phase results in unfavorable nisin production conditions and a less

robust cellular growth phase. When compared to profile B-const24, this result resulted in a 2.3 fold reduction in the maximum nisin production rate and increased the time required to reach the maximum nisin concentration (244 ± 48 IU/ml/h and 9 hr and 573 ± 10 IU/ml/h and 7 h, respectively).

It should be noted that the pH profile in this study began stressing the producer cells 2 hours later than the study by Pongtharankul and Demirci (2006a), resulting in a 60% increase in maximum nisin activity. This confirms the suspicion that the success of a re-alkalization procedure is dependent upon when the re-alkalization procedure begins, the interval between re-alkalizations, and the time needed for the bacteria to acidify the fermentation broth (Pongtharankul and Demirci, 2006a). In attempt to determine maximum nisin activity per batch, sucrose additions were made to ensure nutrient limitations were not causing a halt in nisin production.

4.2 Fed-Batch Fermentation with Various pH Profiles

Cabo *et al.* (2001) and Pongtharangkul and Demirci (2006b) also investigated the effects of a pH profile on nisin production in fed-batch fermentations. They determined that pH also plays an important role in the production of nisin during fed-batch fermentations. This study assessed three pH profiles similar to those used in Pongtharangkul and Demirci (2006b).

Cabo *et al.* (2001) tested pH profiles using a suspended cell culture under different fed-batch fermentation conditions. The study used a continuous sucrose feeding regiment that provided 1.15 g/L/h to the reactor (Cabo *et al*., 2001). The criteria used to determine the sucrose addition amount was based upon sucrose consumption rates of *L. lactis* (IIM Lb. 1.13) during previous batch fermentations (Cabo *et al.*, 2001).

The determination of sucrose feeding rates for this study was determined by analyzing the sucrose consumption rate of the immobilized *L. lactis* under the optimized pH batch conditions (profile B-const4). Figure 4 is the average sucrose consumption profile of the batch fermentations when profile B-const4 was used. The consumption of sucrose between 0 and 4 hours averaged 4.4 g/L/h. The maximum rate of sucrose consumption was 8 g/L/h.

This study used a sucrose feeding regiment that provided an average of 5 g/L/h of 50% sucrose for the first 2 hours of the fermentation, and an average of 10 g/h of 50% sucrose between 2 – 12 hours of the fermentation. The feeding occurred every two hours for a time period that provided the correct amount of sucrose desired for a two hour period. In an attempt to not shock the cells with a spike of sucrose, the feeding time was deliberately prolonged. The feeding time for each sucrose addition was 10 minutes for the first two hours, and 20 minutes from 2 – 12 h.

4.3 Evaluation the pH Profiles for Fed-Batch Fermentation

Profile FB-const24 (constant pH) demonstrated the highest nisin activity, with an average maximum nisin activity of 3260 ± 290 IU/ml (Fig. 5 and Table 3). This is a 2-fold increase in maximum nisin activity when compared to the constant pH profile batch fermentation (profile B-const24). Profile FB-const4 performed the worst, with an average maximum nisin activity of 534

± 47. This is a 33% reduction in maximum nisin activity when compared to a constant pH profile batch fermentation (profile B-const24).

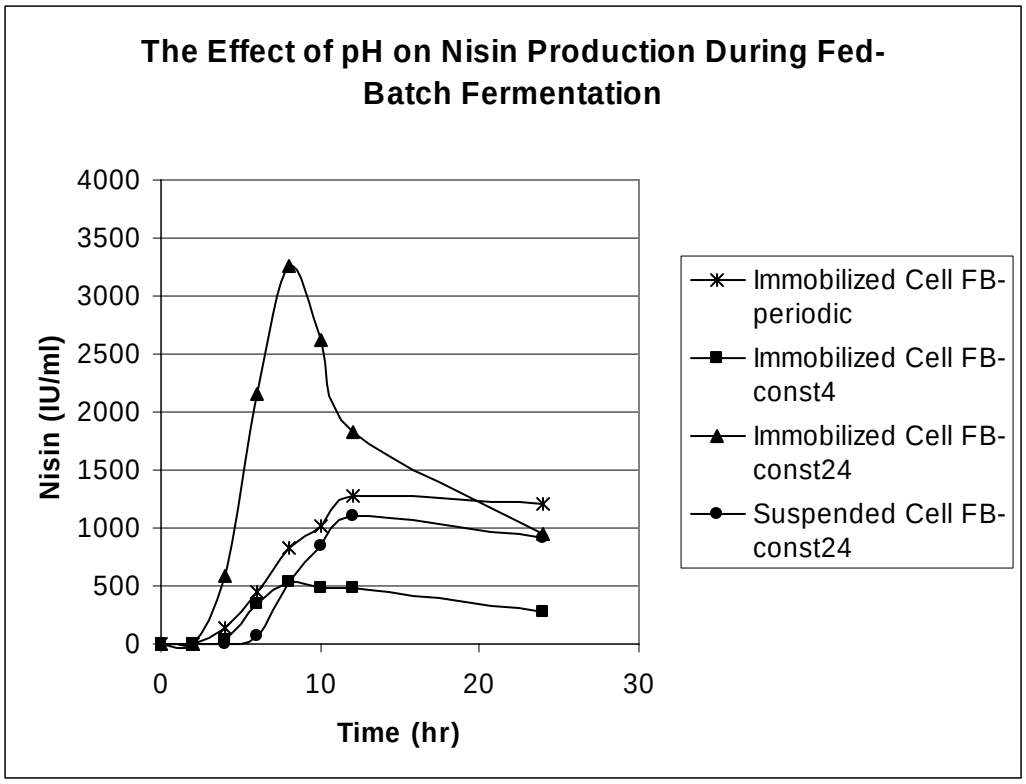

Figure 5. The effect of pH on nisin production during fed-batch fermentation.

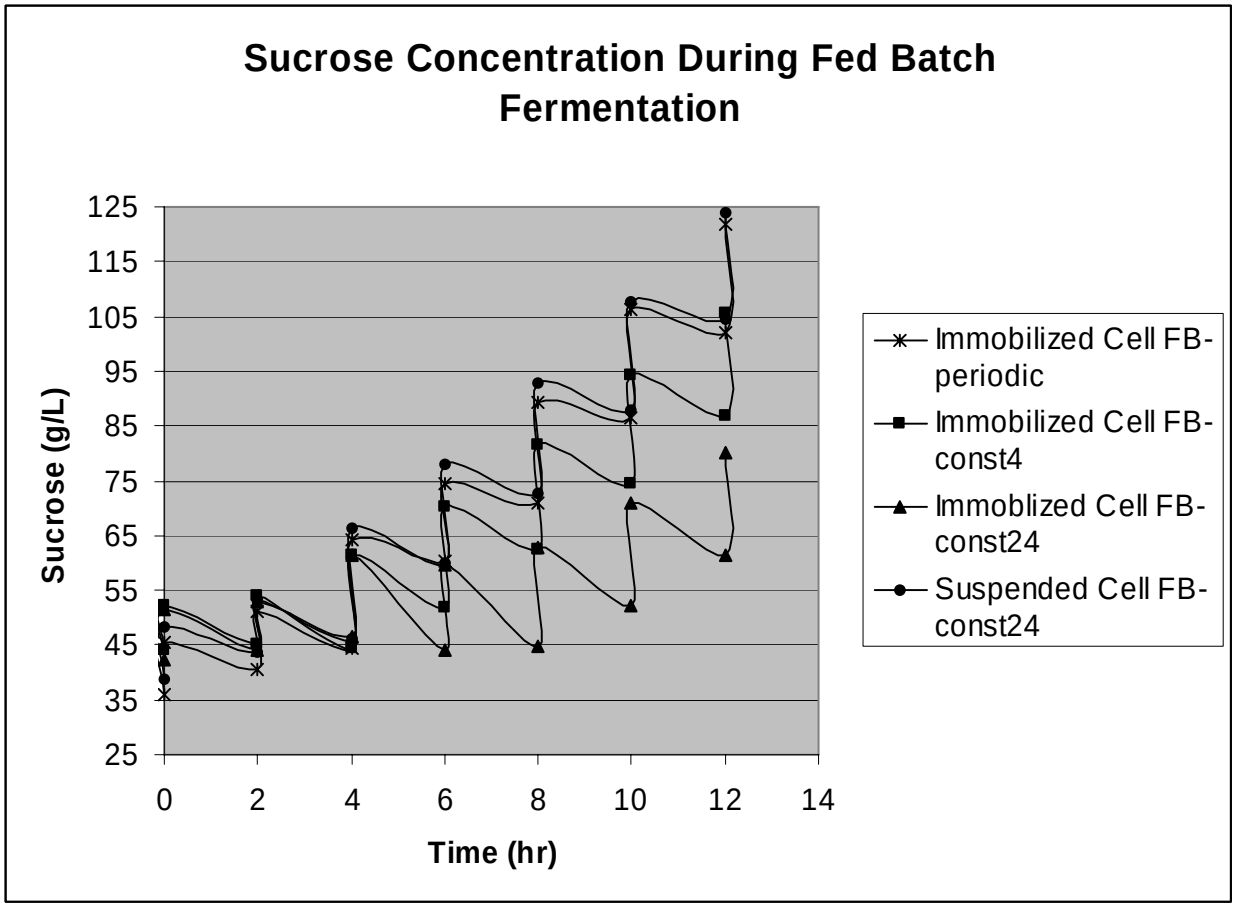

Figure 6. Sucrose concentrations during fed batch fermentations

Pongtharangkul and Demirci (2006b) observed similar results when using fed-batch fermentation with a biofilm culture of *L. lactis* NIZO 22186. They observed a maximum nisin concentration of 3,841 IU/ml at the constant pH profile (Pongtharangkul and Demirci, 2006b). Similarly, this study observed the highest activity of nisin when a constant pH profile was used. The observed maximum nisin activity of the study performed by Pongtharangkul and Demirci (2006b) that used the same auto-acidification pH profile was 2,341 IU/ml (Pongtharangkul and Demirci, 2006b). The differences in sucrose addition methods did not seem to change which profile worked the best, however, this may explain variations that occurred in the data pertaining to other fermentations that were unable to consume sucrose as quickly (Figure 6), i.e. profile FB-periodic and profile FB-const4. The large discrepancy in nisin activity for the auto-acidification pH profile (profile FB-const4) between this study and that of Pongtharangkul and Demirci (2006 b) can be attributed to differences in medium formulation, like KH_2PO_4 concentration.

In addition to maximum nisin activity, maximum sucrose consumption, time for fermentation to reach maximum nisin activity, and maximum nisin productivity rate were also determined (Table 3). While these parameters are important to note, they tend to be dependent upon one another.

In general, the fermentation profile that produces the greatest amount of nisin tends to have a high maximum nisin production rate, consumes sucrose at a fast rate, and produces a maximum nisin activity in a short time. When compared to other immobilized fed-batch fermentations, profile FB-const24 was able to consume sucrose at the fastest rate (maximum rate of 8.38 ± 0.17 g-sucrose/h), produced the maximum nisin activity in a short time (8 hr), and had the highest productivity rate (787 ± 62 IU/ml/h).

4.4 Comparison of Immobilized Fermentation and Suspended Cell Fermentation at various pH Profiles

Table 3 shows the fermentation parameters between the immobilized cell fed-batch fermentations and the suspended cell batch-fermentation that used a constant pH profile (profile FB-const24). The maximum nisin activity produced by the best immobilized cell fed-batch fermentation profile increased the maximum nisin activity by 2.9 fold over the suspended cell fed-batch fermentation (3,260 ± 209 IU/ml and 1,102 ± 222 IU/ml, respectively), which shows that the measurable activity of nisin depends on the pH profile used during the fermentation. In addition, the measurable activity of nisin increases with the use of immobilized cell cultures.

The other measured parameters also suggest that immobilized cell cultures are better for producing nisin than suspended cell cultures. The maximum productivity of nisin was approximately 3 times greater with the immobilized cell culture than with the suspended cell culture (787 ± 62 IU/ml/h vs 234 ± 6 IU/ml/h). The time to reach the maximum nisin activity for the immobilized cell culture was 4 hours shorter than the time required to reach the maximum nisin activity for the suspended cell culture (8 ± 0 and 12 ± 0, respectively). Lastly, the maximum sucrose consumption rate for the immobilized cell culture was greater than the maximum sucrose consumption rate for the suspended cell culture (8.38± 0.17 g-sucrose/h and 3.66 ± 0.7 g-sucrose/h). All these data clearly demonstrated that immobilized cell fermentation enhances nisin fermentation.

4.5 Suspected Causes for the Relationship Between pH Profiles and Measured Nisin Activity

This study and the studies of Cabo *et al.* (2001), Pongtharangkul and Demirci (2006a,b) and others have observed a strong relationship between measured nisin activity and pH profiles used during fermentation. It has been shown that an acidification of the fermentation broth during the growth phase of LAB increases the measurable nisin activity within the broth. Possible reasons for an increase in measurable nisin activity, when pH was reduced at early times, are due to decreased functionality of proteases, desorption of nisin from producer cells, and an overall better environment for the stability of nisin.

The increase in measurable nisin is due to the loss of attraction that nisin has with cells. The pI of nisin is 8.8 (estimated using a program ‘Compute pI/MW’ obtained from http://aegis.ateneo.net/nrojas/abi/tools.htm). A reduction in pH from 6.8 increases the number of

positively charged nisin molecules. In addition, a reduction of pH reduces the overall negative charge that cells generally maintain. Thus, in a low pH environment, the attraction between the nisin molecule and cell is reduced. Therefore, a low pH would not only increase nisin's stability, but also reduce nisin's affinity for attachment to cells. Further research must be performed, however, to confirm this conjecture.

5. CONCLUSION

Different pH control profiles applied during fermentation exhibited significant effects on nisin production in immobilized cell fermentation. By allowing pH to drop freely after 4 hr of fermentation, a 2.1-fold (profile B-const6) higher nisin activity was obtained. Presumably, this observation was a result of less nisin adsorption on producer cells and/or lower activity of nisin-degrading proteases at low pH. Conversely, a periodic pH profile (profile B-periodic) exhibited a significant reduction in nisin production in the immobilized cell reactor. Studies found in literature both support and refute the results presented in this study. The study of Pongtharangkul and Demirci (2006a,b) most closely represents the parameters used in this study and supports these results. However, study of Cabo et al. (2001) less related to this study, refutes these findings.

Different pH control profiles applied during fed-batch fermentation exhibited significant effects on nisin production in immobilized cell fed-batch fermentations. By allowing the pH to remain constant throughout the fermentation, a 1.96-fold (profile FB-const24 immobilized) higher nisin activity was obtained when compared to a suspended cell fed-batch fermentation. The constant pH profile preformed best because it was most capable at consuming the added sucrose. Other pH profiles, such as the periodic and partially controlled pH profiles, did not produce nearly as much nisin as they had in the batch fermentations. The inability of the cells to consume the extra supply of sucrose may have caused a reduction in cellular growth; thereby, a reduction of total nisin activity would have been observed. Figure 6 supports this hypothesis. The sucrose concentration was approximately twice the optimal concentration by the time the maximum nisin activity was observed in the batch experiments. Overall, the batch profile that held the pH constant for 6 hours (B-const6) and the fed batch profile that held the pH constant for 24 hours enhanced the production of nisin in the batch and fed-batch systems respectively.

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