Nisin Fermentation by *Lactoccocus lactis* subsp. *lactis* Using Plastic Composite Supports in Biofilm Reactors

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ABSTRACT

Novel plastic composite supports (PCS), composed of equal parts polypropylene and agricultural products, were evaluated as supports for biofilm production in the bioreactor. Various reduced nitrogen content media (2.5 – 10 g/L yeast extract and 2.5 – 10 g/L peptone) were tested with equal parts of PCS rings and discs during 48-hr repeated-batch fermentations for nisin production and compared to a suspended-cell culture during pH controlled (pH 6.8) and pH uncontrolled fermentations. Also, fed-batch fermentation with glucose addition was attempted to prevent glucose depletion during fermentation. Results demonstrated that there was no significant increase in nisin production or productivity rates using PCS with any reduced content media over the suspended-cell control during either pH condition. Attempts to increase glucose concentration in the fed-batch fermentations slightly improved nisin production. The metabolism of nisin production and possible nisin absorption by *L. lactis* cells can be limiting factors which shadow the capability of PCS. It is very clear that nisin starts to be degraded/absorbed after reaching a maximum level even for suspension-cell culture fermentation. Therefore, further research is needed to eliminate or minimize nisin degradation/absorbtion.

Keywords: Nisin fermentation, Biofilm, Plastic composite supports, *Lactococcus lactis* subsp. *lactis*.

INTRODUCTION

Foodborne pathogens can be a health hazard in many types of foods including raw poultry and beef, dairy products, fruits, vegetables, and processed foods. Certain antimicrobial agents, such as nisin, added to foods during processing eliminate pathogenic and spoilage microorganisms in foods such as canned foods, processed cheeses, meats, and alcoholic beverages (Delves-Broughton, 1990). Nisin, a GRAS (generally recognized as safe) compound, is the only approved bacteriocin for use in foods in the U.S. Nisin is primarily effective against Gram-positive microorganisms, such as *Micrococcus, Streptococcus, Lactobacillus,* and *Staphylococcus* species, and inhibits the germination of bacterial endospores, such as *Bacillus* and *Clostridium* species (Delves-Broughton, 1990). Nisin is commercially made by the fermentation of *Lactococcus lactis* subsp. *lactis* in pH controlled batch fermentations using a milk-based fermentation medium (Qiao et al., 1997; Vandamme, 1984). However, De Vuyst and Vandamme (1994) stated that conventional bacteriocin fermentation inefficient due to limited production rates and final nisin concentration achieved by fermentation. Some of the factors that make commercial nisin production costly are slow nisin production rate and expensive fermentation medium requirement.

It is a well-known fact that increased biomass cell concentration increased product production. One method used to increase cell concentration is immobilizing cells in microcapsules, such as calcium alginate beads. The microorganisms are entrapped inside of the beads, while nutrients and products can migrate through the semi-permeable membrane of the bead (Qiao et al., 1997). It was demonstrated that the beads produced bacteriocin concentrations

in the reactor outflow at least equal to traditional suspended-cell fermentations (Wan et al., 1995). Another method to increase biomass cell concentration during fermentation is the formation of a biofilm within the fermentor. A biofilm is a natural type of cell immobilization that involves the attachment of microorganisms to fixed supports (Characklis, 1990). Biofilms are used in numerous wastewater facilities in trickling filters (Characklis, 1990) and have been used in other applications such as ethanol, acetic acid production, polysaccharides, as well as for metal ore leaching (Bryers, 1990). Biofilms have been found to be successful in several fermentation studies to consistently produce higher biomass concentrations and increased product concentration as well as production rates (Demirci and Pometto, 1995; Demirci et al., 1997; Ho et al., 1997a; Ho et al., 1997a; Ho et al., 1997c).

To be used as a solid support in the biofilm reactor, plastic composite supports (PCS) are extruded nutrient supports containing various combinations of polypropylene and agricultural products. The polypropylene acts as a matrix to integrate the mixture of agricultural materials. The agricultural products (soybean hulls, yeast extract, bovine albumin, and soybean flour) serve to generate a porous structure on the PCS, and to provide cells with essential nutrients to sustain cell growth. Studies done by Ho et al. (1997b) showed that PCS leaches nutrients slowly, and consequently, lowers the nutrient requirement of the fermentation broth. Biofilm reactors with PCS have been shown to improve lactic acid and ethanol productions in continuous and fed-batch fermentations (Demirci and Pometto, 1995; Demirci et al, 1997). PCS, as a solid support, could provide biofilm formation during nisin fermentation, thus increasing nisin production in the bioreactor. Therefore, it was anticipated that biofilm reactors with PCS could be used to improve the production of nisin with less expensive medium. Thus, this study was undertaken to evaluate the production of nisin using bench-scale repeated-batch fermentations of *L. lactis* in biofilm reactors with PCS.

MATERIALS AND METHODS

Microorganisms and media.

Lactococcus lactis subsp. *lactis* (NIZO 22186) was grown in a medium containing 20 g of glucose (Staley, Decatur, IL), 10 g of peptone (Difco, Sparks, MD), 10 g of yeast extract (Difco, Sparks, MD), 10 g of KH_2PO_4, 2 g of NaCl, and 0.2 g of $MgSO_4 \cdot 7H_20$, per liter of deionized water, adjusted to pH 6.8 with 4N NaOH, and stored at 4˚C. Monthly subculturing was performed to maintain viability.

For the fermentation medium, the glucose concentration was increased to 80 g/L and less expensive, industrial grade yeast extract (Ardamine Z, Sensient Bionutrients, Indianapolis, IN) and peptone (Amberferm 4015AG, Sensient Bionutrients, Indianapolis, IN) were substituted to reduce fermentation medium costs. During fermentation, three nitrogen source (yeast extract and peptone) concentrations were evaluated and denoted as 1X (10 g yeast extract and 10 g peptone), 0.5X (5 g yeast extract and 5 g peptone), and 0.25X (2.5 g yeast extract and 2.5 g peptone) per liter deionized water.

Micrococcus luteus (ATCC 10240) obtained from American Type Culture Collection (Rockvile, MD) was used as the indicator microorganism in the bioassay agar when analyzing the fermentation samples for nisin. *M. luteus* was grown in a *Micrococcus luteus* (ML) medium

containing 1 g of glucose (Staley, Decatur, IL), 10 g of peptone (Difco, Sparks, MD), 1.5 g of yeast extract (Difco, Sparks, MD), and 3 g of NaCl per liter of deionized water. *M. luteus* was maintained on ML agar slants composed of ML medium plus 20 g of Bacto-agar (Difco, Sparks, MD) per liter of deionized water. The slants were kept at 4ºC and subcultured bi-weekly. When fresh cultures of *M. luteus* were needed for bioassay agar, a loopful of *M. luteus* from the slant was transferred to the ML medium, and incubated for 24 hr in a shaker water bath (Presicion Shaker Bath Model 25, Winchester, VA) at 30ºC with 200 rpm agitation.

Plastic composite supports.

Plastic composite supports (PCS) were used in biofilm reactors, which consisted of 50% (w/w) polypropylene (Quantum USI Division, Cincinnati, OH), 35% (w/w) soybean hulls (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (w/w) yeast extract (Ardamine Z, Champlain Industries), 5% (w/w) defatted soybean flour (Archer Daniels Midland, Decatur, IL), and 5% (w/w) bovine albumin (American Protein Corp., Ames, IA) (Ho et al., 1997c). Two types of PCS were manufactured; discs (3.2 mm I.D., 12.7 mm O.D.) and rings (9.5 mm I.D., 12.7 mm O.D.), by methods described by Demirci et al. (1997) by the means of high-temperature extrusion in a Brabender PL2000 co-rotating twin-screw extruder (Model CTSE-V, C.W. Brabender Instruments, Inc., South Hackensack, NJ). The PCS pipes were prepared at Iowa State University and sent to our laboratory. The PCS pipes were then cut into approximately 3-mm slices with a band saw in the machine shop.

Repeated-batch test-tube fermentations.

To confirm the formation of biofilm on PCS for *L. lactis*, duplicate test-tube repeated-batch fermentations were conducted and compared to a control using no PCS. In the test-tubes, approximately 3 g of the sliced PCS discs were placed into 25 x 150 mm screw-cap culture-tubes and autoclaved dry for 30 minutes. After cooling to room temperature, 10 ml of sterile *L. lactis* medium was added to the PCS in each test-tube, followed by incubation at 30°C for 24 hr. The tubes were aseptically decanted to eliminate excess particles. Fresh sterile medium (10 ml) was added to both sets of tubes followed by the inoculation of 0.1 ml of an overnight culture of *L. lactis*. The tubes were incubated at 30°C for 24 hr. The tubes were aseptically decanted and filled with 10 ml of fresh medium to make a total of 5 medium transfers. At the end of the repeated-batch fermentations involving PCS, 4 discs were removed and analyzed for biofilm formation by the stripping sand method as described earlier by (Demirci et al., 1997; Ho et al., 1997c).

Repeated-batch fermentations.

Celligen Cell Culture fermenter (New Brunswick Scientific, Edison, NJ) equipped 1.3 L vessel with pH, temperature, and agitation controls was used for bench-scale fermentations. A customized stainless-steel perforated disk was fabricated to support and keep the PCS from the bottom of the reactor to prevent disruption of agitator. After the fabricated disk was in place, equal amounts of PCS discs and rings (50 g of PCS discs: 50 g of PCS rings, bulk volume 430 cm^3) were added in the reactor vessel. The total external surface area supplied by the equal parts of discs and rings amounted to approximately 2,400 cm^2. The reactor vessel, PCS, and all medium lines were sterilized as described by Ho et al. (1997a). Various concentrations of media

to be tested (working volume 800 ml) were prepared (glucose and nitrogenous components with mineral salts were sterilized separately) and added to the reactor aseptically. After inoculation with an overnight culture of *L. lactis* (1% v/v), a total of 4 or 5 repeated fermentations were performed in order to form a visible biofilm on the PCS. Once the visible biofilm was formed, sampling of each of the different fermentation runs commenced. Fermentation broth was agitated at 75 rpm without aeration. The bioreactor was kept at a constant temperature of 30˚C during fermentation. If pH was controlled during fementation, 4N NaOH was used to control at pH 6.8. Each condition was replicated.

Duplicate fermentation runs using PCS and nitrogen concentrations of 1X, 0.5X, and 0.25X were conducted at pH 6.8 and uncontrolled pH. Samples (total of 11) were drawn periodically during the 48-hr fermentations and analyzed for nisin activity, optical cell density (OD), and glucose and lactic acid concentrations. The samples collected were kept at 4˚C until they were analyzed. Suspended-cell fermentations with 1X medium were performed under the identical conditions to serve as a control. The full-strength nitrogen content (1X) was chosen as a control since it represents the best possible suspended-cell nisin producing conditions.

Fed-batch fermentations.

Since glucose was being depleted after about 24-hr of fermentation, fed-batch fermentation was performed by the addition of glucose before the depletion occurred. The glucose spiking was performed to explain if the nisin degradation after reaching a maximum was due to glucose depletion in the bioreactor or due to the production of proteases that degraded nisin. Before glucose was depleted in the 48-hr fermentation, 100 ml of medium was aseptically removed from the bioreactor and an equal amount of 55% sterile glucose solution was added to bring the bioreactor up to 86 g/L glucose concentration. During fermentation, nisin activity, glucose consumption, lactic acid production, and optical cell density were analyzed.

Nisin inhibition by PCS utilization.

To test whether the PCS used in the biofilm fermentations inhibited the production of nisin and/or contributed to the nisin degradation after 48-hr of fermentation, a known amount of nisin was added to uninoculated fermentation medium containing PCS with the identical PCS:working volume ratio used in the bench-scale fermentations. Briefly, 2.5 g of PCS discs were added to duplicate 50 ml-test tubes. Duplicate test tubes without PCS were also prepared which served as the control. All the test tubes were autoclaved for 20 minutes. After cooling, 20 ml of sterile fermentation medium was aseptically pipetted into each of the test tubes. One ml of a standard stock solution (1:9 ratio of 1X fermentation medium to 0.02 M HCl) containing 10,000 IU/ml nisin (Sigma Chemical Co., St. Louis, MO) was added to each test tube to give a final nisin activity of 500 IU/ml. An initial 1 ml sample was taken from each test tube and stored at 4°C until nisin determination. The tubes were then incubated for 48 hr at 30°C to simulate the length of the bench-scale fermentations. After the incubation period, another one ml was sampled from each tube and assayed for nisin activity to determine nisin inhibition or degradation caused by PCS.

Analysis.

Nisin activity.

Nisin bioassay agar (NBA) was prepared according to the methods of Tramer and Fowler (1964) with modifications by Wolf and Gibbons (1996). NBA consisted of 1 g of glucose (Staley, Decatur, IL), 10 g of peptone (Difco, Sparks, MD), 1.5 g of yeast extract (Difco, Sparks, MD), 3 g of NaCl, and 7.5 g of Bacto-agar (Difco, Sparks, MD) per liter of deionized water. The agar medium was brought to boil and 20 ml of 1:1 ratio of Tween 20 and deionized water was added per liter agar before sterilization.

After autoclaving, the bioassay agar was cooled to 45ºC in a water bath. Then, 1% (v/v) of a 24-h grown culture of *M. luteus* was inoculated into the bioassay agar. To ensure that an equivalent biomass of *M. luteus* was inoculated into the bioassay agar each time, optical cell density (OD) at 600 nm of *M. luteus* was measured and adjusted to 1.7 prior to inoculation into the bioassay agar.

The agar medium (25 – 30 ml) was aseptically poured into sterile petri plates and allowed to solidify for about 2 hr. Six holes were bored into each agar plate using a sterilized 7-mm outer diameter stainless steel borer with slight suction. Aliquots of each sample for nisin determination (0.1 ml) were added in triplicate into each hole and incubated at 30ºC for 24 hr. Diameters of the zones of inhibition (mm) were measured using a digital caliper (Mitutoyo ABS Digimatic Caliper, Series 500, Singapore). The average inhibition diameter was plotted against the log of the corresponding known nisin activity in standard solutions to create the standard curve. Nisin activities in fermentation samples were interpolated by using the standard curve by using fresh bioassay agar. A new nisin standard curve was prepared for each fermentation batch analysis in order to accurately correlate known nisin activities to inhibition zone diameters obtained from nisin in the fermentation samples tested.

Nisin standard curve was prepared according procedures developed by Wolf and Gibbons (1996). Fifty ml of 500 IU/ml of nisin (Sigma Chemical Co., St. Louis, MO) was prepared by using 9:1 sterile 0.02N HCl acid:sterile fermentation medium in a pre-sterilized flask. Fermentation samples were analyzed for nisin by centrifuging at 3,300 x *g* (Sorvall Super T21, Newton, CT) for 30 minutes at 10˚C. The supernatant was diluted ten-fold by the same HCl:medium solution. For the pH controlled fermentation runs, a fifty-fold dilution was needed since zone sizes on the bioassay agar overlapped.

After the dilutions were made for the nisin standards or the fermentation samples, the microcentrifuge tubes were placed into a boiling water bath for 5 minutes to release the nisin from proteins and centrifuged for 10 minutes (Mini centrifuge model C-1200, National Labnet Co., Woodbridge, NJ). Then, the samples were kept at 4˚C until analysis.

Analysis of biofilm.

The stripping sand method was used to estimate the biofilm formation on the PCS as described in earlier studies (Demirci et al., 1997; Ho et al., 1997c). After five repeated-batches of fermentation, four PCS discs were washed in 100 ml of sterile 0.1% (w/v) peptone water by inverting the bottle 10 times. The discs were then aseptically transferred into a sterile 25 x 150 mm screw-cap culture-tube containing 5 g of sterile sand and 9 ml of sterile 0.1% (w/v) peptone water. The tubes were vortexed 3 times in 30-second intervals. The suspended cell solutions were serially diluted and the 10^{-2} through 10^{-6} dilutions were spread plated on *L. lactis* growth

agar (with 20 g/L Bacto-agar) and incubated aerobically at 30°C for 24 hr to obtain colony-forming units (CFU) per ml of biofilm broth. The PCS were then dried and weighed. The biofilm accumulated on the PCS was reported as CFU/g of PCS.

Optical cell density.

The optical cell density (OD) for suspended cell culture was measured using a spectrophotometer (Spectronic 20D, Milton Roy Company, Rochester, NY) at 600 nm.

Glucose and lactic acid.

Glucose and lactic acid concentrations were determined by means of Waters High Pressure Liquid Chromatographer (Waters, Franklin, MA) equipped with a refractive index detector, column heater and auto-sampler. Separation of glucose and lactic acid from other broth ingredients was accomplished by a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Divison, Richmond, CA) using 0.012N sulfuric acid as the mobile phase. The mobile phase operated at a flow rate of 0.8 ml/min, while the column was kept at 65°C. The injection volume was 20 μl.

Statistical Analysis.

Each experiment described was duplicated unless otherwise stated. The data were examined by analysis of variance (ANOVA) using the MINITAB Statistical Software package (Release 13.30) (State College, PA). Graphs of the fermentations were plotted using SigmaPlot (version 5.0) (SPSS Science, Chicago, IL).

RESULTS AND DISCUSSION

Biofilm formation.

At the end of a series of five repeated-batch fermentations, the cells attached to the PCS were analyzed by the stripping sand method and quantified in CFU/g. PCS resulted in ~10^9 CFU/g in all tested media; 1X Difco control, 1X, 0.5X, and 0.25X Sensient Bionutrients (SB), which clearly demonstrated the biofilm formation on PCS. No significant differences ($p > 0.05$) were found between the decreased nitrogen medium used and CFU/g PCS when compared to 1X control.

Repeated-batch bench-scale fermentations.

pH controlled fermentations. Generally for all the 1X, 0.5X, and 0.25X media, fermentations at a pH 6.8, nisin quickly reaches a maximum point then degrades gradually (Figure 1), agreeing with results found for pH controlled fermentations by De Vuyst and Vandamme (1992). This pattern is evident in all the media tested and in the 1X control suspended-cell fermentations. No significant difference ($p > 0.05$) was found in nisin production or nisin productivity rates between the 1X biofilm (948 IU/ml, 222 IU/ml/hr) and the 1X suspended-cell control (934 IU/ml, 212 IU/ml/hr) in pH controlled fermentations (Table 1). Furthermore, both the 0.5X (577 IU/ml, 71 IU/ml/hr) and 0.25X biofilm fermentations (214 IU/ml, 19 IU/ml/hr) produced significantly less nisin and slower productivity rates ($p < 0.05$) than the 1X suspended-cell

control. Two reasons can be speculated for not getting higher nisin production in biofilm reactors; increase in protease production, which degrades the produced nisin, or absorption rate of nisin in the cells. Also, the maximum nisin produced and nisin productivity rates seemed to decrease linearly with decreasing nitrogen media in the biofilm fermentation runs; truly understandable since the nitrogen provides essential nutrients to *L. lactis*.

Table 1. Fermentation parameters from duplicate bench-scale *L. lactis* (NIZO 22186) fermentations using a reduced nitrogen content medium and 80 g/L initial glucose at a controlled pH of 6.8*.

Fermentation Parameter	Suspended-cell[a] Fermentations	Biofilm[b] Fermentations		
Measured	1X	1X	0.5X	0.25X
Final Suspended Optical Cell Density[c]	3.4 ± 0.1	3.1 ± 0.7	2.3 ± 0.2	1.5 ± 0.8
Final Lactic Acid Produced (g/L)	50.6 ± 4.9	50.6 ± 5.4	49.0 ± 1.1	45.5 ± 2.8
Lactic Acid Yield (%)[d]	63.0 ± 6.2	63.0 ± 6.7	61.0 ± 1.4	62.0 ± 0.3
Max Lactic Acid Productivity Rate (g/L/hr)	4.0 ± 0.4	4.0 ± 0.1	3.3 ± 0.1	2.3 ± 0.3
Maximum Nisin (IU/ml)	934^{A} ± 2.7	948^{A} ± 106.4	577^{B} ± 19.6	214^{C} ± 49.7
Time of Max Nisin (hr)	14.0 ± 0.7	14.0 ± 0.7	19.0 ± 7.1	19.0 ± 7.1
Final Nisin (IU/ml)[e]	290 ± 96.7	298 ± 180.1	139 ± 56.2	113 ± 26.6
Max Nisin Productivity Rate (IU/ml/hr)	212^{E} ± 0.6	222^{E} ± 14.0	71^{F} ± 34.3	19^{G} ± 3.4

*Means are significantly different ($p < 0.05$) if followed by a different capital letter
[a]Without PCS supports.
[b] With PCS supports.
[c]Optical cell density of spent fermentation medium at 600 nm.
[d]Percentage yield was calculated by dividing the final lactic acid concentration (g/L) by the glucose consumed (g/L).
[e]Final nisin produced at the end of 48-hr fermentation.

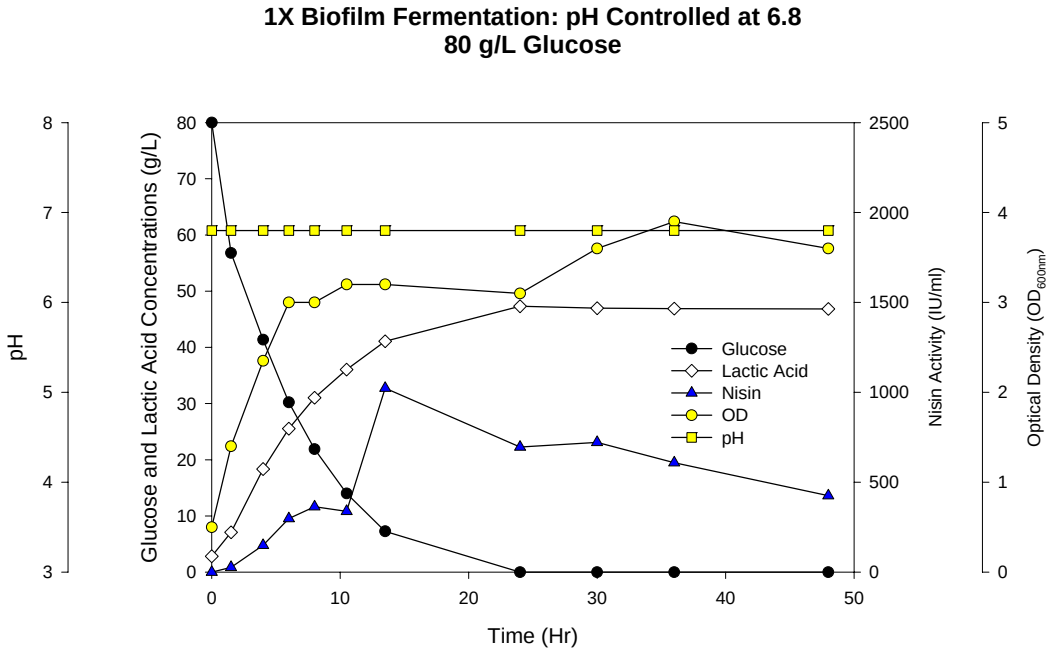

Figure 1. Typical graph of pH 6.8 1X biofilm fermentation of *L. lactis* (NIZO 22186) using plastic composite supports.

pH uncontrolled fermentations. When compared to pH controlled fermentations at 6.8, the maximum nisin concentrations obtained during pH uncontrolled fermentations runs were not as high as pH controlled fermentations. Generally for all the 1X, 0.5X, and 0.25X media fermentations at an uncontrolled pH, nisin concentration did not reduce after reaching the maximum level (Figure 2). This pattern is also evident in all the media tested using the PCS and also in the 1X control. Not controlling the pH during both the biofilm and suspended-cell fermentations yielded far less nisin than keeping the pH constant at 6.8. No significant difference ($p > 0.05$) was found in nisin production between both the 1X and 0.5X biofilm runs (510 and 309 IU/ml, respectively) compared to the 1X suspended-cell control (475 IU/ml) (Table 2). Significantly less ($p < 0.05$) nisin activity was found in 0.25X biofilm fermentations (210 IU/ml) compared to the 1X suspended-cell control. However, final nisin activities were higher for all PCS biofilm fermentations (468, 230, 210 IU/ml for 1X, 0.5X, and 0.25X, respectively) compared to 1X suspended-cell control (114 IU/ml) (Table 2). As for the nisin productivity rates, the 1X, 0.5X, and 0.25X biofilm runs (approximately 59, 58 and 52 IU/ml/hr respectively) were not significantly different ($p > 0.05$) amongst each other, but were all significantly less ($p < 0.05$) than the 1X suspended-cell control (approximately 216 IU/ml/hr).

Although nisin is more stable and does not absorb as well to the producer cells at lower pH values, far less nisin is produced during pH uncontrolled fermentation runs compared to pH controlled fermentation runs using all media tested. Since all reduced nitrogen media biofilm runs yielded approximately the same productivity rate, it was questionable whether or not the

PCS itself contributed to the extreme decrease in productivity rates compared to the suspended-cell control during the pH uncontrolled runs. It is clear that the combination of utilizing biofilm fermentation using PCS with reduced nitrogen media and not controlling pH during fermentation did not produce maximal nisin production and greater nisin productivity rates. However, utilizing the PCS for biofilm did decrease the difference between the maximum nisin and the final nisin after 48 hr when compared to the suspended-cell control.

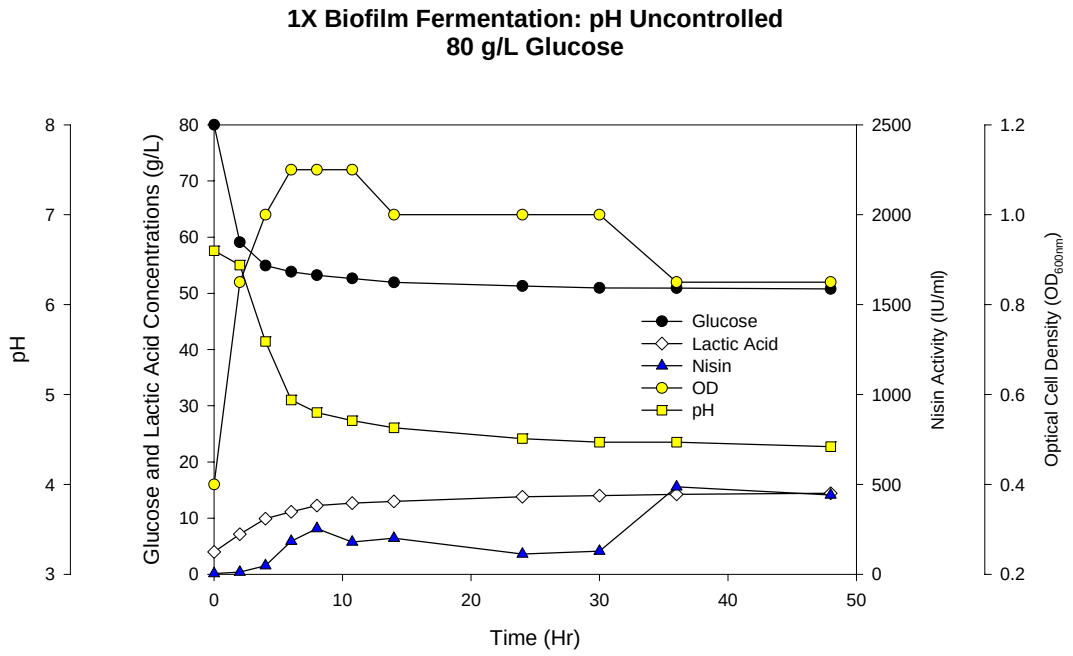

Figure 2. Typical graph of pH uncontrolled 1X biofilm fermentation of *L. lactis* (NIZO 22186) using plastic composite supports.

Fed-batch fermentation. During bench-scale pH controlled fermentations at pH 6.8, glucose depleted after 24 hr, but the nisin activity began to decrease after 14 hr. The maximum nisin for the 1X biofilm and 1X control runs occurred after 14 hr, while the maximum nisin for the 0.5X and 0.25X biofilm runs occurred after approximately 19 hr.

Table 3 shows the effect of the addition of the concentrated glucose solution on nisin production. The glucose did not reach depletion during the suspended-cell and biofilm runs; therefore, an assessment of maximum nisin production behavior could be seen. Approximately similar nisin concentrations could be seen in the biofilm fermentation (874 IU/ml) compared to suspended-cell fermentation (923 IU/ml).

Table 2. Fermentation parameters from duplicate bench-scale *L. lactis* (NIZO 22186) fermentations using a reduced nitrogen content medium and 80 g/L initial glucose at uncontrolled pH*.

Fermentation Parameter Measured	Suspended-cell[a] Fermentations	Biofilm[b] Fermentations		
	1X	1X	0.5X	0.25X
Final Suspended Optical Cell Density[c]	2.0 ± 0	0.8 ± 0.1	0.5 ± 0.4	0.4 ± 0.3
Final Lactic Acid Produced (g/L)	10.7 ± 0.3	15.3 ± 1.3	13.9 ± 1.3	10.1 ± 5.3
Lactic Acid Yield (%)[d]	58 ± 0.2	61 ± 17.1	53 ± 2.1	42 ± 15.9
Max Lactic Acid Productivity Rate (g/L/hr)	1.2 ± 0.1	1.8 ± 0.7	2.2 ± 1.3	1.0 ± 0.1
Maximum Nisin (IU/ml)	475^{A} ± 93.7	510^{A} ± 32.1	309^{A} ± 75.6	210^{B} ± 69.8
Time of Max Nisin (hr)	6.0 ± 0	36.0 ± 0	23.3 ± 13.1	48.0 ± 0
Final Nisin (IU/ml)[e]	114 ± 9.8	468 ± 37.7	230 ± 37.2	210 ± 69.8
Max Nisin Productivity Rate (IU/ml/hr)	216^{C} ± 34.0	59^{D} ± 10.6	58^{D} ± 56.2	52^{D} ± 0.5

*Means are significantly different ($p < 0.05$) if followed by a different capital letter
[a]Without PCS supports.
[b] With PCS supports.
[c]Optical cell density of fermentation medium at 600 nm.
[d]Percentage yield was calculated by dividing the final lactic acid concentration (g/L) by the glucose consumed (g/L).
[e]Final nisin after 48-hr fermentation.

Since glucose did not reach depletion during the suspended-cell and biofilm runs, an assessment of maximum nisin production behavior could be seen. Even though glucose was still available in the bioreactors as an available carbon source, nisin still degraded from 923 IU/ml to 172 IU/ml in the suspended-cell fermentation and from 874 IU/ml to 203 IU/ml in the biofilm fermentation.

Nisin adsorption by PCS. Since utilization of PCS proved successful in other applications such as lactic acid and ethanol productions, it was surprising that biofilm fermentation did not improve nisin production over the suspended-cell fermentations. It could be a possibility that nisin might be absorbed or attached by PCS used in the biofilm fermentations, and contributed to the decrease in nisin activity after 48-hr of fermentation. To test possible nisin adsorption on

PCS, a known activity of nisin was added to sterile fermentation medium and assayed for nisin before and after incubation at 30°C for 48 hr. The initial and final nisin activities with and

Fermentation Parameter Measured	Suspended-cell[a] Fermentations 1X	Biofilm[b] Fermentations 1X
Final Suspended Optical Cell Density[c]	1.5	3.0
Final Lactic Acid Produced (g/L)	63.3	68.6
Lactic Acid Yield (%)[d]	49	51
Max Lactic Acid Productivity Rate (g/L/hr)	3.9	4.0
Maximum Nisin (IU/ml)	923	874
Time of Max Nisin (hr)	24.0	24.0
Final Nisin (IU/ml)[e]	172	203
Max Nisin Productivity Rate (IU/ml/hr)	81	110

Table 3. Fermentation parameters from single bench-scale pH controlled fermentations using 80 g/L initial glucose spiked with 55% glucose at 14 hr.

[a]Without PCS supports.
[b] With PCS supports.
[c]Optical cell density of spent fermentation medium at 600 nm.
[d]Percentage yield was calculated by dividing the final lactic acid concentration (g/L) by the glucose consumed (g/L).
[e]Final nisin produced at the end of 48-hr fermentation.

without PCS demonstrated no significant differences ($p<0.05$). These results indicated that PCS did not contribute to any significant decrease in nisin activity. Since *L. lactis* cells were not present during this nisin adsorption test, the presence of *L. lactis* cells and consequent protease production during the bench-scale fermentations might have contributed to nisin degradation. The production of these proteases has been offered as an explanatin to nisin degradation in fermentation medium (Parente and Ricciardi, 1999). Since it was shown that PCS itself did not contribute to nisin absorption, other factors involving PCS orientation must have contributed to the inability of the PCS biofilm reactor to produce higher nisin concentrations than suspended-cell fermentations. A probable reason for the lack of improved nisin activity and production rates is the composition of PCS. The PCS used in this study was not specially formulated for nisin production by *L. lactis*, but was formulated for *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) used in previous studies (Ho et al., 1997a; Ho et al., 1997a; Ho et al., 1997c). Since the PCS was specially formulated for *L. casei* and exhibited variable lactic acid concentrations depending entirely on agricultural product composition, it is possible that the composition of agricultural products in the PCS did not provide sufficient type or quantity of nutrients *L. lactis* cells needed for optimal nisin production.

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The metabolism of nisin production and possible nisin absorption by *L. lactis* cells can be limiting factors which limit the capability of PCS. It is clear that nisin concentration starts to decrease after reaching a maximum level (Figure 1) even for suspension-cell culture fermentation. The possible degradation or absorption into the producer cell is reducing the nisin level in the fermentation broth, and prevents biofilm to demonstrate higher nisin levels. Therefore, it is essential to first determine what causes the reduction of nisin, and then to apply the biofilm reactor concept to maximize the nisin levels.

CONCLUSIONS

There was no increase in nisin activity or productivity rates using the PCS with any medium tested over the 1X suspended-cell control during pH controlled or uncontrolled conditions. The 1X biofilm, performing the best out of the biofilm media tested, yielded approximately the same results as the 1X suspended-cell during pH controlled fermentations. The 0.5X and 0.25X biofilm fermentations produced a linear decrease in nisin and nisin productivity rates in both the pH conditions tested. The pH uncontrolled fermentations produced even less nisin than the pH controlled runs and also exhibited a linear decrease in nisin production and nisin productivity rates in the 1X, 0.5X, and 0.25X biofilm fermentations. The addition of glucose did not improve nisin production or nisin productivity rates and nisin degradation still occurred, leading to the hypotheses that protease production degrades nisin produced during the fermentations or adsorbing of the nisin by the bacterial cells. Therefore, further research is needed to eliminate or minimize nisin degradation/absorbtion to be able to see advantage of biofilm reactors for enhanced nisin production.

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