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Stimulation of Nisin Production From Whey by a Mixed Culture of *Lactococcus lactis* and *Saccharomyces cerevisiae*

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Abstract

The production of nisin, a natural food preservative, by *Lactococcus lactis* subsp. *lactis* (ATCC 11454) is associated with the simultaneous formation of lactic acid during fermentation in a whey-based medium. As a result of the low concentration and high separation cost of lactic acid, recovering lactic acid as a product may not be economical, but its removal from the fermentation broth is important because the accumulation of lactic acid inhibits nisin biosynthesis. In this study, lactic acid removal was accomplished by biological means. A mixed culture of *L. lactis* and *Saccharomyces cerevisiae* was established in order to stimulate the production of nisin via the *in situ* consumption of lactic acid by the yeast strain, which is capable of utilizing lactic acid as carbon source. The *S. cerevisiae* in the mixed culture did not compete with the nisin-producing bacteria because the yeast does not utilize lactose, the major carbohydrate in whey for bacterial growth and nisin production. The results showed that lactic acid produced by the bacteria was almost totally utilized by the yeast and the pH of the mixed culture could be maintained at around 6.0. Nisin production by the mixed culture system reached 150.3 mg/L, which was 0.85 times higher than that by a pure culture of *L. lactis*.

Index Entries: Nisin; whey; mixed culture; fermentation.

Introduction

Cheese whey is a byproduct of the dairy industry obtained by separating the coagulum from whole milk, cream, or skim milk and represents about 85–90% of the milk volume whereas retaining 55% of the milk nutrients *(1)*. About 30×10^6 t of liquid whey is produced annually in the United States alone with just a small portion being actively utilized to produce whey protein *(2)*. Whey permeate is the liquid that passes through the ultra-filtration membrane by which the whey protein is retained *(1)*. The majority of the nutrition in whey and whey permeate, which includes lactose, soluble proteins, lipids, and mineral salts, is not fully utilized because

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of the low value and limited market of whey products. Because of its low concentration of milk constituents (e.g., lactose content is only 4.5–5%, [w/v]), whey and whey permeate are commonly considered wastes. Environmentally friendly and economic disposal of these byproducts is a great challenge to the dairy industry (3). Biological processing of these cheese byproducts for production of value-added products is considered one of the most profitable utilization alternatives, with generation of high-value coproducts from this currently available waste stream being an example of "biorefinery" in action (4).

Nisin is an antimicrobial peptide produced by certain *Lactococcus* bacteria (5). The peptide has strong antimicrobial activity against almost all Gram-positive bacteria and their spores, especially several food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and psychrotrophic enterotoxigenic *Bacillus cereus* (6–8). Therefore, nisin has been accepted as a safe and natural preservative in more than 50 countries and is widely used as an antimicrobial agent in the food industry (9). The US Food and Drug Administration views nisin derived from *Lactococcus lactis* subsp. *lactis* to be a GRAS (generally recognized as safe) substance for use as an antimicrobial agent (10). Direct addition of nisin to various types of foods, such as cheese, margarine, flavored milk, canned foods, and so on, is permitted (11). In addition, nisin is also being considered for use in health and cosmetic products (12).

Several studies (13,14) have indicated that cheese whey could be used as feedstock for the production of nisin, assuming supplementation of some essential nutrients within the fermentation process. Biosynthesis of nisin is coupled with the growth of lactic acid bacteria, and a significant amount of lactic acid is simultaneously formed alongside the nisin biosynthesis. Lactic acid is an important chemical for food processing. It can also be used as a raw material in the production of the biodegradable polymer poly(lactic) acid (15). Presently, though, lactic acid is not recovered in the current industrial process for nisin production. However, previous work by the authors has revealed that it is feasible to produce nisin and lactic acid simultaneously by fermentation, because the optimal conditions for nisin biosynthesis and lactic acid formation by *L. lactis* using cheese byproducts as feedstock are almost the same (16). Unfortunately, owing to the low concentration and high cost of separating and recovering lactic acid, the process may not be economical.

Considering that the accumulation of lactic acid in fermentation broth inhibits nisin biosynthesis (17), this study is focused on stimulation of nisin production by the removal of lactic acid via use of a mixed culture system. A mixed culture of *L. lactis* and *Saccharomyces cerevisiae* (Fig. 1) was established in order to stimulate the production of nisin via the *in situ* consumption of lactic acid by the yeast strain, which is capable of utilizing lactic acid as its carbon source. The *S. cerevisiae* in the mixed culture does not compete with the nisin producing bacteria because the yeast does not

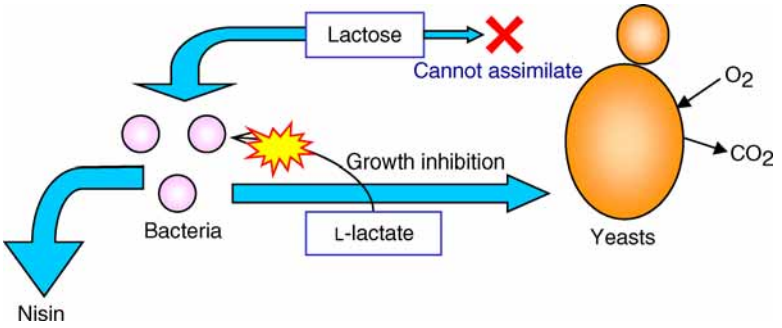

Fig. 1. The interaction between *S. cerevisiae* and *L. lactis* in a mixed culture system for nisin production.

utilize lactose, which is the major carbohydrate in whey for bacterial growth and nisin production. A pH control strategy without alkali addition was developed and nisin production in the mixed culture was compared with that in a pure culture of *L. lactis*.

Materials and Methods

Microorganisms and Media

L. lactis subsp. *lactis* (ATCC 11454) was the nisin-producing microorganism used in this work. *Micrococcus luteus* (ATCC 9341) was used as an indicating microorganism in the bioassay of nisin concentrations. The compositions of media used for the growth of these microorganisms are summarized as follows. Medium I, used for seed culture of *L. lactis* (pH 7.0), contained 5 g/L glucose, 5 g/L polypeptone, and 5 g/L yeast extract. Medium II, used for bioassay of nisin (pH 7.0), contained 10 g/L glucose, 5 g/L polypeptone, 5 g/L yeast extract, and 5 g/L NaCl. Medium III, used for the main fermentation, contained 20 g/L sweet whey powder (provided by WesternFarm Food Inc., Seattle, WA), 12 g/L yeast extract, 0.6 g/L KH_2PO_4, and 0.6 g/L $MgSO_4$.

Six *S. cerevisiae* strains, i.e., ATCC 4098, ATCC 4126, ATCC 8766, ATCC 9080, ATCC 9763, and ATCC 9841, were used in this work. The medium for yeast culture contained 1 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 1 g/L yeast extract, and 5 g/L carbon source. The pH was adjusted to 5.0 before sterilization at 121°C for 30 min. Lactose and lactic acid were applied as carbon sources, respectively, for the testing of the capabilities of the yeast strains growing on lactose and lactic acid. Glucose was used as carbon source when preparing yeast seed for further mixed culture study.

Mutagenic Treatment of S. cerevisiae

The suspension of the parental yeast strain was transferred to sterilized Petri plates. The Petri plates were then placed under a ultraviolet

(UV) lamp, (emitting the energy of 1.6 × 10 J/m/s) for 10 min. The plates were then placed in the incubator at 30°C for 3 d. The colonies showing a larger size on lactic acid containing agar as compared with the parental strain were picked up and subjected to natural selection. These mutant strains were further cultivated on lactic acid containing agar. The stable traits obtained were then compared for their capabilities for growing on lactose and lactic acid.

Analysis

Cell concentration of the pure cultures was measured as dry cell mass and optical density (OD). The viable cell concentrations of *L. lactis* and *S. cerevisiae* in mixed culture were determined as colony forming units (CFU) on selective media. Concentrations of L-lactic acid and acetic acid in the medium and fermentation broth were analyzed using a high-performance anion-exchange chromatography method *(18)*. Nisin concentration was measured by a bioassay method based on the method of Shimizu et al. *(17)*.

Cultivation Method

Before main cultivation was performed, culture size was scaled up by two steps in order to increase the amount of cells with high growth activity. Seed culture of *L. lactis* and *S. cerevisiae* was conducted in 125-mL Erlenmeyer flasks placed on an orbital shaker at 160 rpm and 30°C for 8 h. Main fermentations were performed in a 5 L Bioflo 110 fermentor (New Brunswick Scientific, Edison, NJ) equipped with temperature, pH, dissolved oxygen concentration and gas flow control systems. The working volume was 2 L. Air was supplied to the fermentor for aerobic cultivation conditions.

Results and Discussion

Nisin Production by the Pure Culture of L. lactis

The time-course of nisin production by the pure culture of *L. lactis* on a whey-based medium is shown in Fig. 2. The pH was controlled at 6.0 by the addition of NaOH. Biosynthesis of nisin increased rapidly in the first 8 h of fermentation, and reached a maximum concentration of 81.2 mg/L within the broth. Nisin concentration decreased slightly with further increase of time. Accompanied with nisin synthesis was the formation of a significant amount of lactic acid. The concentration of lactic acid increased rapidly to 10.1 g/L after 10-h fermentation, and reached 11.3 g/L in 24 h.

Lactic Acid Utilizing Yeast Strain Development

Six *S. cerevisiae* strains, ATCC 4098, ATCC 4126, ATCC 8766, ATCC 9080, ATCC 9763, and ATCC 9841, were compared for their capabilities of growth on lactic acid and lactose. The results of aerobic culture of these six yeast strains showed that none of them could use lactose as substrate. The

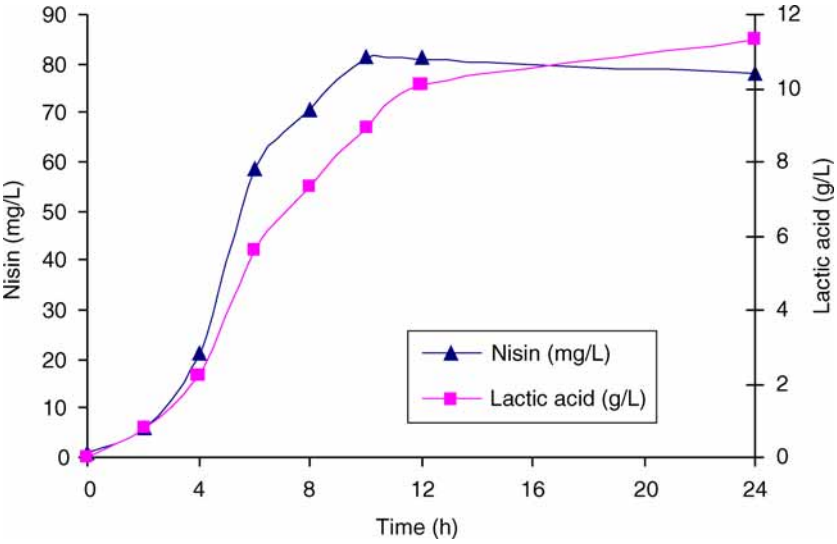

Fig. 2. Nisin production in a pure culture of *L. lactis* with pH control by alkali addition.

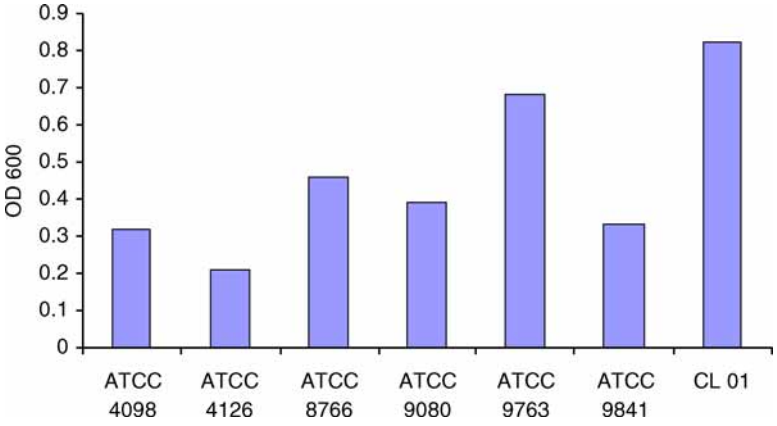

Fig. 3. The growth of different yeast strains on lactic acid-based media after 16 h aerobic culture.

growth of these six yeast strains on lactic acid was also compared. Each yeast strain was cultured on glucose-based media for 12 h, and then inoculated into lactic acid-based media. The cell growth was quantified by measuring OD_{600} after 16 h of aerobic culture. As shown in Fig. 3, the OD_{600} of ATCC 9763 was the highest among all of these six yeast strains. In order to obtain a good yeast strain for *in situ* removal of lactic acid in the mixed culture system, therefore, ATCC 9763 was mutated using UV treatment. After cultivating the mutants on lactic acid containing agar, five stable traits were obtained, and the mutant that showed the best performance for lactic acid assimilation was obtained and applied for the study of lactic acid removal in the mixed culture. The mutant was named *S. cerevisiae* CL01.

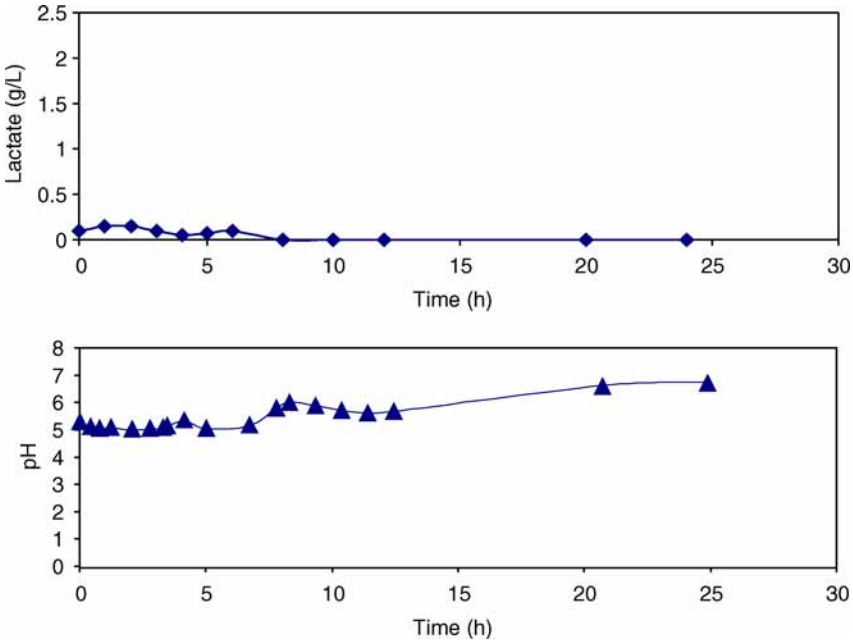

Fig. 4. Lactic acid accumulation and pH profile in a mixed culture with 3 h induction.

Effects of Induction Time on Lactic Acid Removal in the Mixed Culture

In order to overcome the inhibition of lactic acid to nisin biosynthesis, the lactic acid should be totally consumed by the yeast very quickly. In other words, there should be no accumulation of lactic acid in the fermentation broth. This objective can be achieved by activating the yeast in advance using lactic acid and providing sufficient yeast biomass in the mixed culture.

It is well known that the existence of glucose in medium will depress the capabilities of yeast to utilize other carbon source. As a result, an induction period is required to allow yeast cells to use up the glucose and shift their metabolic pathway from glycolysis to lactic acid assimilation. As shown in Fig. 4, lactic acid content in the mixed culture was well controlled at very low level (<0.2 g/L) after the yeast biomass were induced using 0.2 g/L of lactic acid for 3 h. In contrast, significant accumulation of lactic acid was observed (Fig. 5) without induction. Therefore, induction of yeast using lactic acid before mixed culture is essential for pH control. The appropriate induction time was 3 h.

Effects of Inoculum Ratio on Mixed Culture

Considering that the imbalance of the cell concentrations of the yeast and the bacteria in a mixed culture may cause the accumulation of lactic acid and thus inhibit the growth of *L. lactis* and the biosynthesis of nisin, the inoculum ratio of yeast biomass to bacteria population should be an

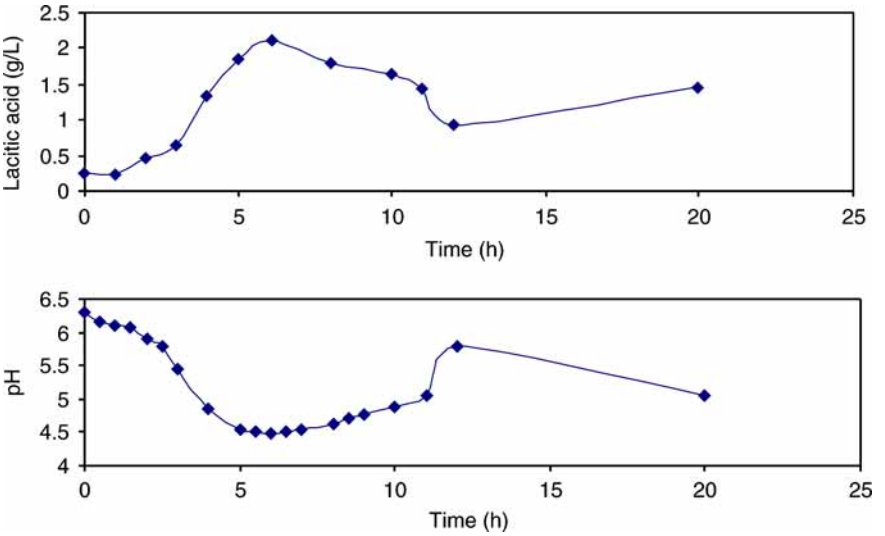

Fig. 5. Lactic acid accumulation and pH profile in a mixed culture without induction.

important parameter to achieve a stable pH control without alkali addition. Therefore, the effects of inoculum ratio on lactic acid removal were investigated. The time-course of three batches of mixed culture with different inoculum ratio of yeast biomass to bacteria population are shown in Figs. 6–8. The inoculum size of *L. lactis* in these three experiments was the same (2.4×10^6 CFU/mL), whereas the inoculum size of yeast in Figs. 6–8 was 0.8×10^6 CFU/mL, 2.2×10^6 CFU/mL, and 4.8×10^6 CFU/mL, respectively. The initial ratio of yeast biomass to bacteria population was proved to be very important for a stable pH of the mixed culture. The pH shown in Fig. 6 was not stable in the initial 8 h of fermentation, and thus caused the accumulation of lactic acid to the level of 0.1 g/L with nisin concentration reaching 135.2 mg/L. As shown in Figs. 7 and 8, with the increase of yeast inoculum, the pH of the mixed culture was well controlled around 6.0 in the first 12 h of fermentation, and nisin concentration was further increased to 150.3 mg/L. It was also noticed that the pH of the mixed culture increased gradually to 7.0. This fact could be owing to the fact that yeast assimilated not only lactic acid but also acetic acid. Analysis of the fermentation broth indicated that both lactic acid and acetic acid were removed.

The comparison of nisin production with different inoculum ratio (Figs. 6–8) indicates that sufficient inoculation of yeast is the key to a successful pH control in the mixed culture system. The inoculum ratio of the yeast to the bacteria should be 1:1. When inoculum size of *L. lactis* is greater than the expected value, or inoculum size of *S. cerevisiae* is less than the expected value, lactic acid will not be completely assimilated by *S. cerevisiae* and the pH will decrease. In such a case, growth of *L. lactis* will be inhibited. If *S. cerevisiae* grow and lactic acid concentration is decreased, both

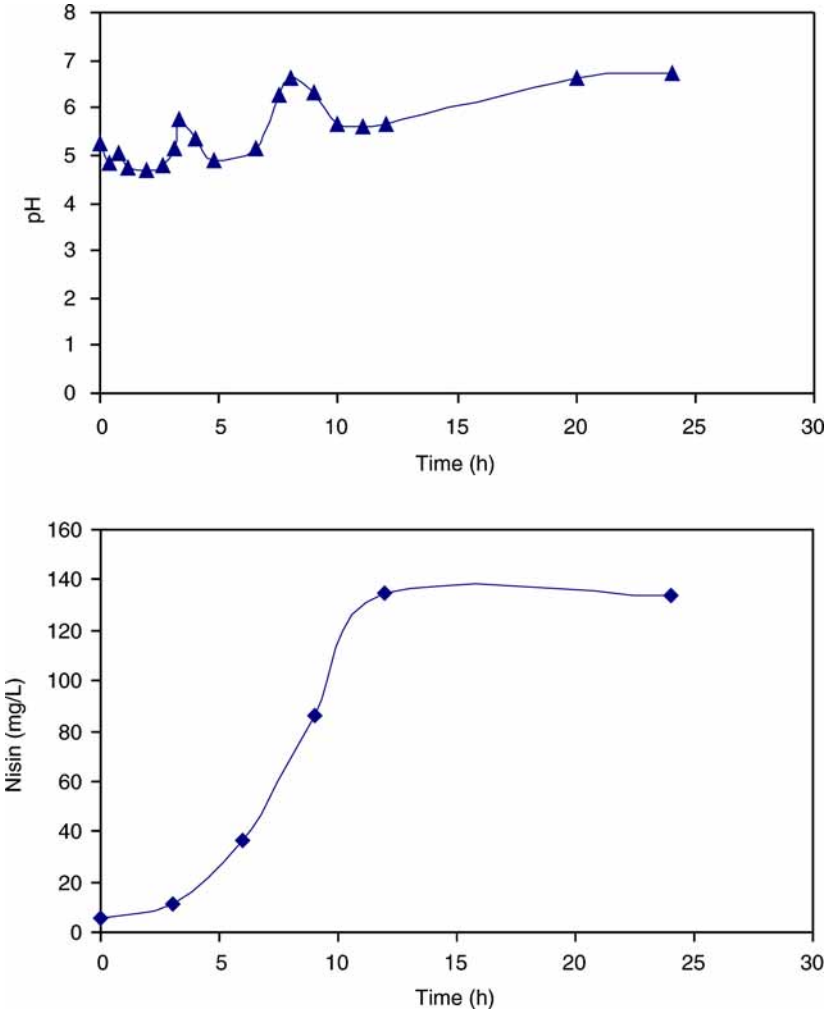

Fig. 6. The pH profile and nisin production in a mixed culture. The inoculum of yeast and bacteria was 0.8×10^6 and 2.4×10^6 CFU/mL respectively.

microorganisms are able to grow again. However, if the growth activity of *L. lactis* is lost under low pH for this period, growth of both microorganisms should be stopped *(19–22)*.

High Production of Nisin in the Mixed Culture System

By comparing the time-course of nisin production in the mixed culture shown in Figs. 7 and 8 to that in the pure culture shown in Fig. 2, nisin production was significantly stimulated in the mixed culture. Nisin concentration in the end reached 150.3 mg/L and it was 0.85 times greater than the nisin production shown in Fig. 2, which represents the production without mixed culture. Kim et al. *(23)* tried to increase nisin production

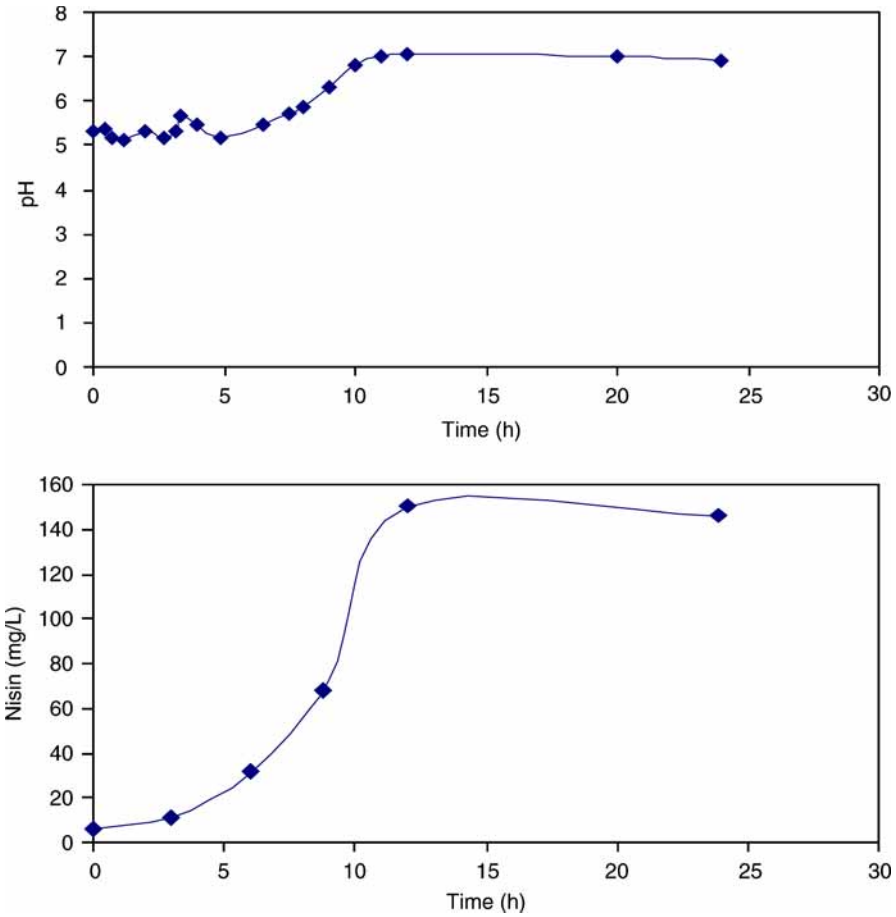

Fig. 7. The pH profile and nisin production in a mixed culture. The inoculum of yeast and bacteria was 2.2×10^6 and 2.4×10^6 CFU/mL respectively.

through genetic engineering, and they achieved 10% improvement by introducing foreign nisin immunity/resistance plasmid pND300 to *L. lactis*. The results of this study show that mixed culture improves nisin production when compared with the pure culture of *L. lactis* and is mainly limited by lactic acid inhibition rather than by the immunity/resistance of the bacteria to nisin.

Conclusions

A mixed culture of *L. lactis* and *S. cerevisiae* was established for the purpose of stimulating nisin production. The lactic acid produced by the bacteria was *in situ* utilized by the yeast and the pH of the mixed culture could be maintained at around 6.0 without alkali addition. Nisin concentration reached 150.3 mg/L and this value was 85% greater than that in a pure culture of the bacteria.

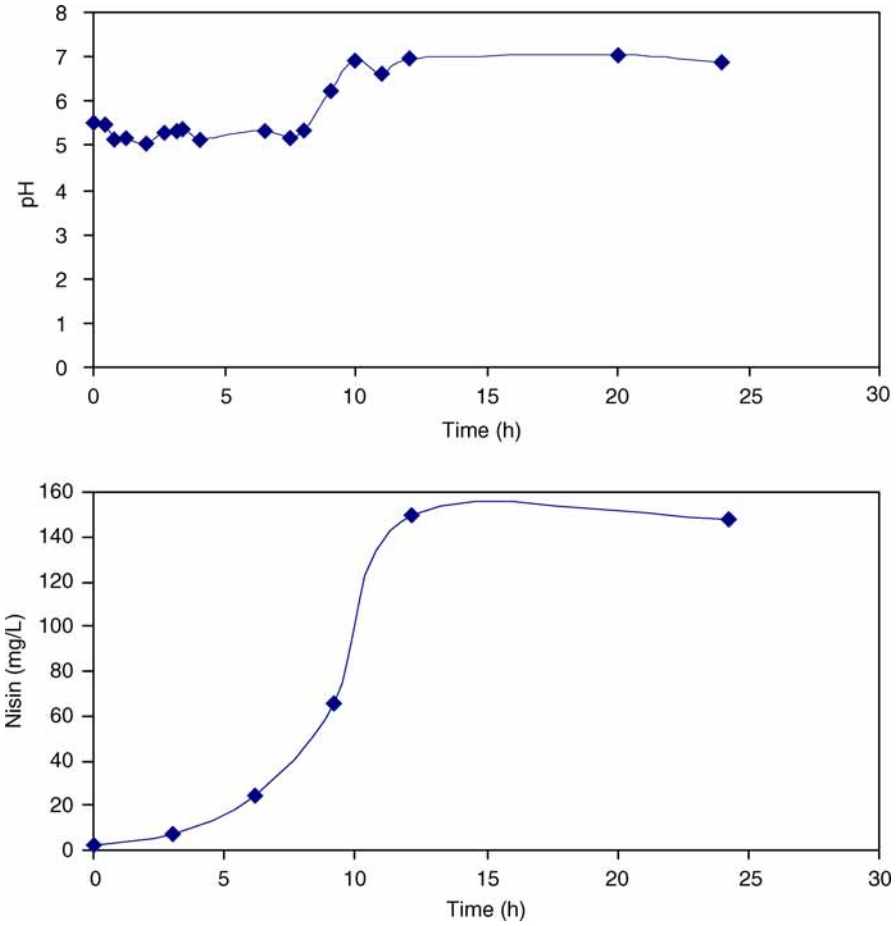

Fig. 8. The pH profile and nisin production in a mixed culture. The inoculum of yeast and bacteria was 4.8×10^6 and 2.4×10^6 CFU/mL, respectively.

Acknowledgments

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