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Value-Added Production of Nisin from Soy Whey

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Abstract The objective of this study was to evaluate the potential of low/negative value soy whey (SW) as an alternative, inexpensive fermentation substrate to culture *Lactococcus lactis* subsp. *lactis* for nisin production. Initially, a microtiter plate assay using a Bioscreen C Microbiology Plate Reader was used for rapid optimization of culture conditions. Various treatments were examined in efforts to optimize nisin production from SW, including different methods for SW sterilization, ultrasonication of soy flake slurries for possible nutrient release, comparison of diluted and undiluted SW, and supplementation of SW with nutrients. In subsequent flask-based experiments, dry bacterial mass and nisin yields obtained from SW were 2.18 g/L and 619 mg/L, respectively, as compared to 2.17 g/L and 672 mg/L from a complex medium, de Man–Rogosa–Sharpe broth. Ultrasonication of soybean flake slurries (10% solid content) in water prior to production of SW resulted in ~2% increase in biomass yields and ~1% decrease in nisin yields. Nutrient

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supplementation to SW resulted in ~3% and ~7% increase in cell and nisin yields, respectively. This proof-of-concept study demonstrates the potential for use of a low/negative value liquid waste stream from soybean processing for production of a high-value fermentation end product.

Keywords Soy whey · Nisin · Value-added production · *Lactococcus lactis* · Bioscreen · Fermentation

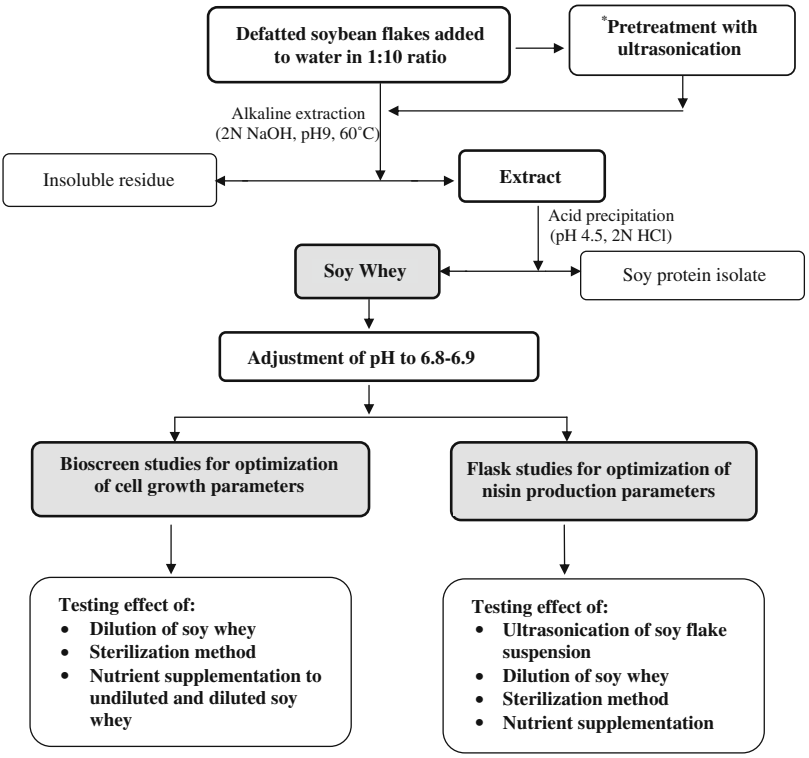
Introduction

An increasing demand for natural food preservatives has created a niche market for bacteriocins, which are natural antimicrobial peptides produced by lactic acid bacteria (LAB). Nisin is the only bacteriocin approved by the Food and Drug Administration and commercially produced by the fermentation of the lactic acid bacterium, *Lactococcus lactis* subsp. *lactis* in milk-based media under well-controlled culture conditions [1–3]. Nisin is a 34-amino acid peptide with a molecular mass of 3.5 kDa. Nisin occurs naturally in two different forms, nisin A and nisin Z. Nisin Z differs from Nisin A by one amino acid residue. It contains asparagine at position 27 instead of histidine. The two nisin variants have identical minimum inhibitory concentrations against most microbes with nisin Z showing better diffusion ability in agar. Nisin demonstrates wide-spectrum activity against almost all Gram-positive bacteria including the food-borne pathogens *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* [4].

The limitations of nisin production are low yields, low product concentrations, and high medium costs among others [5]. In view of the growing consumer demand for natural food additives, there is a great need for more cost-effective means to produce this product. Thus, there has been a rising research trend in exploring the use of food processing waste streams as low-cost, value-added growth media for nisin production. In the quest for suitable materials, dairy byproducts have been widely studied [1, 6, 7]. Recent work has also focused on the use of nondairy substrates. For example, agricultural wastes such as cull potato hydrolysate and fermented barley extracts as well as fishery byproducts, including octopus and mussel processing wastes, have been examined as possible feedstocks for nisin fermentation [8–11]. In this study, we have evaluated soy whey, a byproduct stream from the soybean processing industry for nisin production as substrate for cultivating the strain *L. lactis* subsp. *lactis* ATCC 7962. Production of nisin using a readily available and nontoxic plant product such as soy whey may help minimize processing requirements and ease barriers in regulatory approval for food use of nisin produced in this way [12]. To our knowledge, this is the first report on potential usage of soy whey to support *L. lactis* growth and nisin production.

Wet processing of soybeans is commonly used for the production of high-value products such as soy protein isolates (SPI). This process, however, leads to the generation of large volumes of concentrated liquid waste material, known as “soy whey.” As an economical recovery method is yet to be established, whey generation leads to a serious waste disposal problem [13]. Figure 1 shows a schematic of lab preparation of soy whey (*modified from* [12, 14, 15]).

Soy whey accounts for nearly one third of the defatted desolventized soybean flakes used in the production of SPI and contain about 11% (*w*/*v*) of the total nitrogen in soybean meal. In other words, every kilogram of wet-processed soybean generates almost 0.33 kg of soy whey [14, 16]. Because soy whey contains only 1–3% (*w*/*v*) solids, it is generally



* This step was performed only in some experiments (potential effects of ultrasonic pretreatment on growth and nisin production).

Fig. 1 Lab preparation of soy whey (modified from [12, 17, 23]) and experimental outline for its use as a growth medium for *L. lactis* subsp. *lactis* ATCC 7962

considered too dilute for economical recovery of the whey solids [14, 17]. Smith et al. [18] showed that soy whey solids content and composition depend on origin of the soybean meal and method of whey preparation. On a dry basis, soy whey typically contains 3.6–4.4% nitrogen (50% of which is protein nitrogen) and 25–35% soluble sugars like sucrose, raffinose, and stachyose, various glycosides, galactans, and hemicelluloses [14]. Substantial amounts of phosphorus and minerals, largely as phytate salts and numerous trace elements essential for microbial fermentation of fastidious LAB, are also present in soy whey [14, 18]. Because of its inherent nitrogen content, soluble sugar levels and high chemical oxygen demand content (25,000 mg/L), soy whey poses a burden on the environment if disposed of untreated. Although it is generally considered a waste product, we hypothesized that the high-nutrient content of soy whey could enable its value-added use as a substrate for microbial fermentation. Specifically, we sought to investigate the use of soy whey as a fermentation feedstock for the production of the high-value food preservative nisin. The goals of this study were (a) evaluation of soy whey as a substrate for LAB growth and subsequent nisin production, (b) optimization of soy whey medium and fermentation parameters, and (c) comparison of the biomass and nisin yields obtained from soy whey to de Man–Rogosa–Sharpe (MRS) broth (Difco Laboratories), a nutritionally rich complex growth medium.

Materials and Methods

Microorganisms and Media *L. lactis* subsp. *lactis* ATCC 7962 (nisin producer) and *Micrococcus luteus* ATCC 10240 (nisin indicator strain) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained as frozen stocks at −75 °C in MRS or nutrient broth (Difco Laboratories, Lawrence, KS, USA) media, respectively, containing 20% (*v*/*v*) glycerol. Working cultures of *L. lactis* and *M. luteus* were maintained as slants on MRS and nutrient agar at 4 °C, respectively.

Soy Whey Preparation and Partial Characterization Commercially available soybean flakes (Cargill, Inc., Cedar Rapids, IA) were obtained from the Center for Crops Utilization Research (CCUR) at Iowa State University, Ames, IA. Soy flakes were packed in air-tight plastic bags and stored at 4 °C until further use. According to analyses provided by the manufacturer, these flakes contained 57.3% (dry basis) crude protein and had a protein dispersibility index of 93.8. A method for lab-scale production of soy whey from hexane-defatted, desolventized dry soybean flakes was developed, yielding a final product with properties similar to soy whey generated through commercial-scale wet milling of soy beans. Briefly, ground and defatted soybean flakes (50 g) were mixed in a 1:10 ratio (*w*/*v*) with tap water [19]. The pH was raised to 8.5 with 2 N NaOH, and the resulting slurry was stirred for 30 min at 60 °C. After centrifugation at 10,000×*g* (20 min, 4 °C), the supernatant was collected. The water soluble proteins in the supernatant were precipitated by lowering the pH to 4.5 with addition of 2 N HCl. The slurry was kept at 4 °C for ~4 h and was again centrifuged at 10,000×*g* for 20 min at 4 °C. The supernatant (soy whey) was collected; the pH was adjusted to 6.8–6.9, and after sterilization, it was ready to be used as a growth medium. Use of this lab-scale method for production of soy whey yielded a reproducible substrate for our proof-of-concept studies.

The soy whey produced in this manner was characterized for its total nitrogen and protein content, as well as its sugar composition. Total nitrogen content was estimated using the Dumas nitrogen combustion method [1] with an Elementar Vario MAX CN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) and was found to be 1,210 mg/L, and after multiplying by conversion factor 6.25, the crude protein content was calculated to be 7,575 mg/L. Whey sugars were analyzed using a Varian high-performance liquid chromatograph (HPLC) with a Bio-Rad HPX-87P column for carbohydrates, a guard column, and equipped with a Varian's refractive index (RI) detector. The sample volume was 20 µl, eluant used was filtered (Millipore nylon membrane filter, pore size 0.2 µm), and degassed HPLC grade water at a flow rate of 0.6 ml/min. The column temperature was 85 °C, and run time was 22 min. Standards used were glucose, galactose, fructose, sucrose, stachyose, and raffinose prepared at a concentration of 5 mg/ml in mili-Q water. The soy whey was found to contain 7.14 mg/ml sucrose, 6.56 mg/ml stachyose, 0.84 mg/ml galactose, and 0.74 mg/ml glucose.

Inoculum Preparation In preparation for its use as a microbial growth substrate, the pH of the whey was adjusted from 4.4–4.7 to 6.8–7.0, and the whey was sterilized via heat (121 °C for 15 min) or filtration (0.2 µm nylon filter). Inocula for batch fermentation were prepared by adding 1 ml of culture stock (10^9 CFU/ml) to 10 ml of sterilized soy whey and incubating at 28 °C for 10 h under stationary conditions. Additional dilution was performed as needed to obtain a final cell concentration of 10^9 CFU/ml, equivalent to an absorbance of 0.45 at 600 nm (Abs_{600}).

Batch Fermentation Batch fermentations were carried out in 250-ml Erlenmeyer flasks containing a final volume of 100 ml of soy whey. A 10% (*v*/*v*) inoculum was added to the flasks to obtain a cell density of 10^8 CFU/ml, and flasks were incubated at 28 °C for 48 h. All soy whey batch fermentations were carried out under static conditions. At regular time intervals during the fermentation, samples were taken and pH, cell density (Abs_{600}), total sugars, total soluble proteins, lactic acid, acetic acid, and bacteriocin concentration were measured. For comparison, parallel batch fermentations were carried out in MRS broth using growth conditions identical to those used for soy whey. All batch fermentations and analytical determinations were performed in triplicate. Between 1 and 3 ml of sample was removed for analysis at each sampling time, depending on which analytical tests were performed.

Microtiter Plate Rapid Bioassay A Bioscreen C Microbiology Reader (Growth Curves, Inc., Piscataway, NJ) was used to rapidly determine the effect of various culture parameters on bacterial growth, assuming a positive correlation between cell growth and nisin production. The Bioscreen instrument is a combined incubator and microplate reader that enables collection of high-resolution optical density data for up to 200 individual wells (well capacity 400 μl). Plates can be incubated at temperatures ranging from ambient to 60 °C, and automated optical density readings of individual wells can be taken at discrete intervals to yield detailed growth curves useful in process optimization. Growth medium (200 μl) and inoculum (50 μl) were added to individual wells to yield a final cell density in each well of 10^8 CFU/ml or Abs_{600}=0.17. Inoculated plates were placed in the Bioscreen and incubated for up to 24 h at 28 °C. Abs_{600} was recorded at 30-min intervals with automatic shaking for 5 s before each reading to ensure proper mixing of well contents. All treatments were evaluated in replicates of five (n=5).

Analytical Procedures

Biomass Yields A standard curve was derived by plotting cell dry weight (CDW) measurements (grams per liter) against corresponding spectrophotometric Abs_{600} readings (Spectronic 20 Genesys, Thermo Electron, Cambridge, UK). CDW was measured by harvesting the cells of known Abs_{600} by centrifugation (12,000×g for 15 min at 4 °C), washing twice with sterile saline (0.85% (*w*/*v*) NaCl) and drying to a constant weight at 80 °C. A regression equation was obtained for CDW as a function of Abs_{600}. A correlation coefficient of 0.9955 was derived for the best-fit regression line for the CDW–Abs_{600} curve. During fermentation, absorbance readings of samples were converted into equivalent CDW values (grams per liter) using this standard curve and plotted against time (hours) to obtain growth–time curves.

Determination of Sugars, Proteins, and Fermentation End Products After removal of the bacterial cells from the culture broth by centrifugation (12,000×g, 15 min, 4 °C), the culture supernatants were tested for total sugars by the phenol–sulfuric acid method of Strickland et al.[20], with glucose as the standard. The total soluble proteins were measured by Lowry's method [21], with bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard. The sugar and protein contents were determined spectrophotometrically at 490 and 750 nm, respectively. Lactic acid and acetic acid concentrations were measured using a Waters HPLC (Millipore Corporation, Milford, MA, USA) equipped with a Waters Model 401 RI detector, column heater, auto-sampler, and computer controller. A Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad Chemical Division, Richmond, CA, USA) was

used with 0.012 N sulfuric acid as the mobile phase with a flow rate of 0.8 ml/min, injection volume of 20 µl, and a column temperature of 65 °C. All measurements were performed in triplicate (n=3).

Nisin Quantification The nisin titer in the fermentation broth samples was determined by the method of Tramer and Fowler [22], with the modifications of Wolf et al. [15]. The volume of the fermentate present in individual Bioscreen wells was too low to allow accurate manipulation of the sample needed to determine nisin titer. Therefore, nisin titer was determined only for samples prepared using Erlenmeyer flasks. Briefly, fermentation broth samples from flasks were adjusted to pH 2.0 with dropwise addition of 2 N HCl. This was done to release nisin bound to producer cell surfaces to facilitate measurement of cell-associated nisin. Samples were then heated at 90 °C for 5 min to inactivate any interfering proteases in the medium without affecting nisin, which is thermostable at acidic pH. After centrifugation at 4,000×g for 10 min at 4 °C, the clear, nisin-containing supernatant (nisin extract) was neutralized in order to eliminate erroneous inhibition zones resulting from low pH. Nisin concentration was determined by agar well diffusion bioassay [22, 23] using *M. luteus* as the indicator strain. Briefly, nutrient agar (NA) overlays containing a concentration of 10^8 CFU/ml *M. luteus* were poured over NA base plates and allowed to harden, and wells were bored in the agar aseptically using a sterile stainless steel borer (5 mm inner diameter). One hundred microliter aliquots of nisin extract were added to individual agar wells, and the plates were incubated overnight at 37 °C. The diameter of resulting zones of inhibition around each well were measured horizontally and vertically to the nearest 0.01 mm using a digital caliper (Digimatic caliper, Mitutoyo, Kanagawa, Japan) and the average value recorded. These values were used to determine the nisin concentration from the standard curve (below). Each zone of inhibition determination represented the average of three replicate wells.

Standard Curve The commercial nisin used here (Nisaplin, Sigma Chemical Co., St. Louis, MO), was reported to contain 1×10^6 IU/g. A stock solution of this nisin preparation at 1,000 IU/ml was made using sterile 0.02 N HCl. This stock solution was diluted further as needed in sterile 0.02 N HCl, neutralized, and a standard curve relating zone size to nisin concentration was prepared by plotting inhibition zones versus known units of activity (IU). The regression equation obtained for inhibition zone diameter as an exponential function of nisin concentration: Y (log nisin conc.)=0.1515 × (zone diameter)−3.9467, resulted in a correlation coefficient of 0.989. Nisin concentrations (in milligrams per liter) in test samples were estimated from inhibition zone measurements using this standard curve.

Optimization of Fermentation Parameters

Several different pre-fermentation treatments were examined for their impact on cell growth and nisin production in an effort to optimize our results. Factors examined included different methods of soy whey sterilization, physical treatment (sonication) of defatted soy flakes for possible enhanced nutrient release, the use of diluted vs. undiluted soy whey and micronutrient supplementation of diluted or full strength soy whey. These treatments are described below.

Soy Whey Sterilization Four methods for soy whey sterilization were evaluated to determine the procedure with the minimal impact on nutrient bioavailability as determined by microbial growth and nisin production. The first method tested was membrane filtration

using a Millipore nylon membrane filter of pore size 0.2 μm (Millipore Corp. Billerica, MA, USA). The second method was heat sterilization at 121 °C for 15 min. In the third method, heat sterilization at 121 °C for 15 min was followed by centrifugation at 5,000×*g* for 15 min at 20 °C to remove precipitated materials. In the fourth method, soy whey was filter-sterilized followed by heat sterilization and centrifugation as described above. Filtered and autoclaved soy whey mixed in different proportions was also examined. Heat-sterilized MRS was used as control. Cell growth and nisin production were monitored and measured at regular time intervals in the Bioscreen as well as flask level. Table 1 gives the nomenclature used in this paper to describe these different preparations of the soy whey media.

Pretreatment of Soybean Flake Suspension by Ultrasonication The impact of high-power ultrasound on sugar and protein release from soybean flakes [24, 25] and subsequent biomass and nisin yields from soy whey made with sonicated flakes was investigated. Defatted soy flakes (10 g) were dispersed into 100 ml of tap water in a glass beaker. The batch experiment was conducted in a Branson 2000 series bench-scale ultrasonic unit (Branson Ultrasonics, Danbury, CT) with a maximum output of 2.2 kW. A standard 20-kHz half wavelength titanium horn with a gain of 1:8 and a booster with a gain of 1:1.5 were used. Samples were sonicated at amplitudes 60 and 192 μm_{pp} (peak to peak amplitude in μm) for 15 and 120 s. Soy whey was prepared from the sonicated soybean flakes suspension by the aqueous alkaline method summarized in Fig. 1. Whey prepared from non-sonicated defatted soy flakes was used as a control. All whey samples were filter sterilized and characterized for protein and sugar contents and tested for their ability to support cell growth and bacteriocin production in batch fermentation studies.

Soy Whey Dilution Three different dilutions of 10%, 50%, and 80% (*v*/*v*) of Medium FW (Table 1) were prepared with sterile-distilled water in 250-ml Erlenmeyer flasks. Undiluted Medium FW was used as a positive control. All flasks were inoculated as described earlier, and samples taken aseptically were tested for cell growth and nisin concentration and the yields were compared.

Table 1 Nomenclature of soy whey media used.

Soy whey media	Composition
Medium FW	Filter sterilized (0.2 μm nylon membrane) soy whey
Medium AW	Autoclaved (121 °C for 15 min) soy whey
Medium ACW	Autoclaved and centrifuged (5,000×*g* for 15 min at 20 °C) soy whey
Medium FACW	Filtered, autoclaved, and centrifuged soy whey
Medium 1:1	1:1 Filtered/autoclaved and centrifuged soy whey
Medium 3:7	3:7 Filtered/autoclaved and centrifuged soy whey
Medium SFW	Soy whey with nutrient supplementation (FW+2% (*w*/*v*) sucrose+0.5% (*w*/*v*) KH_2PO_4+0.01% (*w*/*v*) $MgSO_4{\cdot}7H_2O$+0.1% (*w*/*v*) NaCl+0.01% (*w*/*v*) yeast extract (Difco))
Medium DFW	4-fold diluted Medium FW
Medium DFW-ss	Diluted soy whey with sucrose supplementation (4-fold diluted FW+2% (*w*/*v*) sucrose)
Medium DFW-ns	Diluted soy whey with nutrient supplementation (4-fold diluted FW+2% (*w*/*v*) sucrose+0.5% (*w*/*v*) KH_2PO_4+0.01% (*w*/*v*) $MgSO_4{\cdot}7H_2O$+0.1% (*w*/*v*) NaCl+0.01% (*w*/*v*) yeast extract)

Supplementation of Diluted Soy Whey The effect of carbon source supplementation to diluted soy whey (Medium DFW-ss) was compared to micronutrients supplementation (Medium DFW-ns), and cell yields were calculated from Bioscreen absorbance data. It was found from previous experiments that 4-fold dilution of Medium FW (Medium DFW) could support growth; hence, in this experiment, supplementation was done in Medium DFW. Unsupplemented Medium DFW and Medium FW were used as controls.

Supplementation of Soy Whey In order to evaluate the potential of enhancing nisin activity by external nutrient supplementation, different micronutrients (sucrose, NaCl, KH_2PO_4, Yeast Extract, $MgSO_4$) were added to Medium FW in defined amounts [25], and their individual as well as combined effects were studied in the Bioscreen. Unsupplemented Medium FW was used as the control.

Comparative Study with Lactobacillus casei To examine whether nisin activity was the sole cause of the inhibition zones observed for *L. lactis* grown on soy whey, a comparative study using a modified agar overlay method [26] was done using *L. casei* ATCC 7469, a homofermentative LAB that produces lactic acid but not nisin. Briefly, a colony of each organism was grown on MRS agar, a nutrient agar overlay containing 10^8 CFU/ml of the *M. luteus* indicator strain was poured over the colonies. These plates were incubated overnight at 37 °C, zones of inhibition in the overlay resulting from the production of inhibitory substances by the *L. lactis* or *L. casei* colonies were measured and additional testing was performed as needed to characterize the nature of any inhibition phenomena observed (see "Results and Discussion").

Statistical Analysis The significant difference between results obtained from different treatments was evaluated and analyzed by the Method of Derived Variables [27]. A logistic growth model was fitted to the time profile of each well, using nonlinear least squares. Using this fit, the entire time profile for each well was reduced to one number denoted by the letter, "a," which can be scientifically interpreted as the limit of bacterial growth over time. All pairwise treatment comparisons were done using the Wilcoxon Rank Sum Test. The p value was derived after comparison of the mean "a" values for each experimental sample. The p value was calculated for every experimental sample in comparison with the others. A p value <0.01 indicated statistically significant different mean values of "a" for the two samples. Samples having higher "a" values were then judged to represent the optimal condition or treatment under investigation. The performance of different media was statistically analyzed, and the ones having statistically significant differences in their performance were further selected to identify the best culture growth condition.

Results and Discussion

Sterilization Method The sterilization method of soy whey played an important role on the growth rate of *L. lactis* in soy whey. The combined treatment of filtration, followed by autoclaving and centrifugation (Medium FACW) resulted in a significantly higher ($p<0.01$) growth of *L. lactis* while the autoclaved soy whey medium AW resulted in a significantly lower growth ($p<0.01$) of *L. lactis* as compared to MRS. This is summarized in Table 2 and Figs. 2a and 3.

Table 2 Biomass and nisin yields from soy whey sterilized by different methods in static culture flask studies (n=3).

Soy whey media	Biomass yield			Nisin yield		
	Maximum absorbance at 600 nm	Maximum biomass yield (mg/L)	Maximum growth rate[b] (mg/L/h)	Maximum nisin activity (IU/mL)	Maximum nisin concentration (mg/L)	Maximum nisin production rate[c] (mg/L/h)
FW	1.03	2,151.3	218.13	23,230	580.75	53.44
AW	0.71	2,112.4	176.03	1,192	29.79	1.98
ACW	1.12	2,141.5	308.79	24,767	619.16[a]	72.58[a]
FACW	1.32	2,183.2[a]	253.86[a]	23,740	593.49	63.26
1:1-FW:ACW	1.01	2,144.0	252.24	23,988	599.7	61.24
3:7-FW:ACW	0.81	2,122.2	282.96	24,114	602.85	67.84
MRS	1.2	2,174.8	217.48	26,912	672.81[a]	57.75[a]
25%,15 s	1.23	2,175.95	217.59	23,886	597.15	54.29
25%, 120 s	1.24	2,177.37	217.74	24,279.2	606.98	55.18
100%, 15 s	1.17	2,168.10	216.81	24,248	606.20	55.11
100%, 120 s	1.22	2,174.01	217.40	24,339.6	608.49	55.32

[a] Highest yields/rates of biomass/nisin production in the group

[b] Maximum growth rate was determined by calculating the growth rates over constant time intervals during log phase of growth curve and selecting the highest

[c] Maximum nisin production rate was determined by calculating the nisin production rates over constant time intervals during entire growth curve and selecting the highest

It is possible that heat sterilization led to a loss of proteins from the whey via denaturation and precipitation. If so, then Medium ACW would likely be nitrogen deficient, which negatively impacted cell yields (Table 2). However, Medium ACW also supported the highest nisin production of all the soy media examined. Soy is known to contain protease inhibitors [28, 29]. Posttranslational modification of pre-nisin to nisin requires the action of proteases. It is, therefore, possible that heat denaturation of endogenous soy protease inhibitors could have played a role in the higher nisin activity observed in Medium ACW, as proteases responsible for activating nisin would have remained active. This observation was further supported by the fact that equal proportions of filtered soy whey and autoclaved soy whey in Medium 1:1 gave a nisin yield which was almost midway between that obtained separately from filtered soy whey and autoclaved soy whey. Medium 3:7 which had an increased percentage of autoclaved soy whey, showed an increased nisin yield but lower cell density than Medium 1:1 (Table 2).

We found that growth of *L. lactis* in soy whey differed from that previously reported for cheese whey [30]. Specifically, we found the nisin yield from soy whey was growth-independent. Among the soy whey media tested, Medium FACW led to the highest growth rate (253.86 mg/L/h) and biomass yield (2.18 g/L), while Medium ACW yielded the highest nisin activity (24,767 IU/ml, corresponding to a nisin concentration of 619.2 mg/L). Overall, MRS gave the highest nisin concentration (672.8 mg/L). However, Medium ACW resulted in a higher production rate for nisin, after an initial lag from 0–12 h (Table 2).

A similar decoupling of nisin and cell biomass production has been reported by other groups. For example, Vásquez et al. [10] found higher nisin yields under conditions

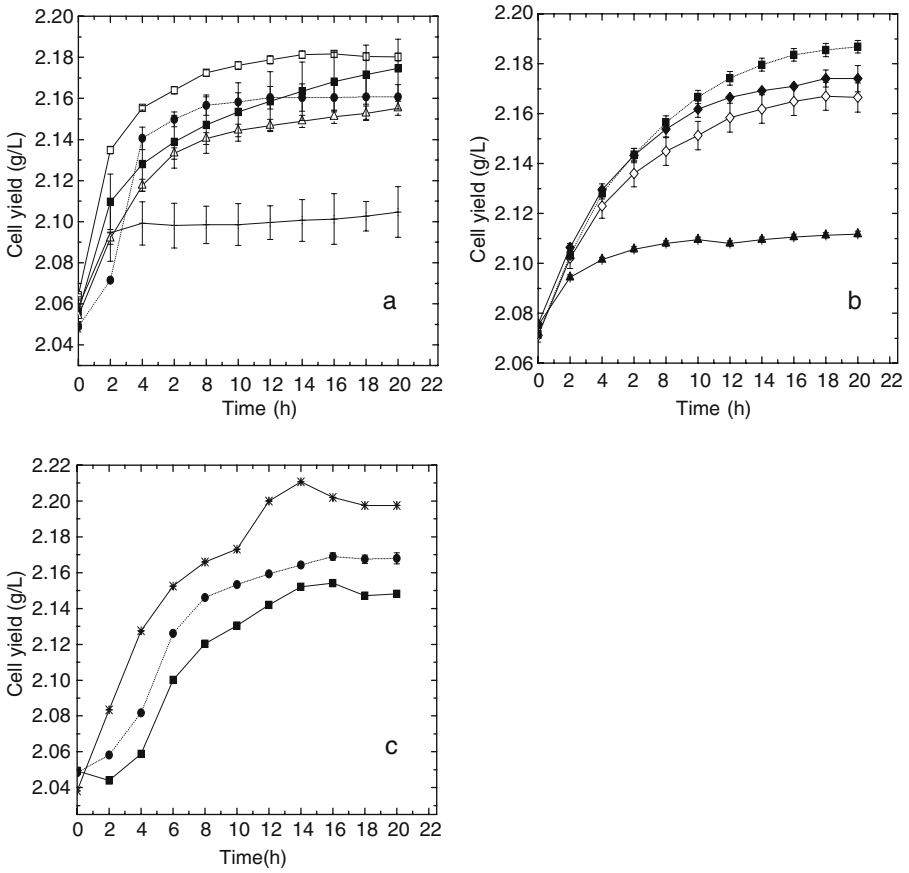


Fig. 2 Time course of growth of *L. lactis* subsp. *lactis* ATCC 7962 in soy whey media under different conditions using the Bioscreen C Microbiological Reader. **a** Soy whey sterilized by different methods as compared to standard MRS medium: *filled circles* MRS, *filled squares* Medium FW, *unfilled triangles* Medium ACW, *solid line* Medium AW, *unfilled squares* Medium FACW; (n=5). **b** Medium FW diluted to different levels as compared to original Medium FW: *filled triangles* 10% or 10-fold diluted Medium FW, *unfilled diamonds* 50% or 2-fold diluted Medium FW, *filled diamonds* 80% or 1.25-fold diluted Medium FW, *filled squares* undiluted Medium FW; (n=5). **c** Soy whey supplemented with nutrients as compared to unsupplemented soy whey and MRS: *asterisks* Medium SFW, *filled squares* Medium FW, (*filled circles*) MRS; (n=5)

conducive to lower cell growth. There is some controversy as to whether nisin should be considered a primary or a secondary metabolite [5, 30–33]. Difficulties in making direct correlations between cell growth rate and nisin production may stem from cryptic phenomena such as adsorption of the bacteriocins onto cell surfaces and/or the posttranslational processing of the prepeptides to active forms [9]. Additional media-specific variables (pH, nutrient availability, etc.) may also play a role in bacteriocin synthesis and further confound efforts at direct correlation between growth and nisin production [2, 34–36].

A clear link between the nutrient content of media in which *L. lactis* is grown and final nisin concentration has been established [37, 38]. With this in mind, it was interesting to note that the average biomass yield from the soy whey media tested (average for all

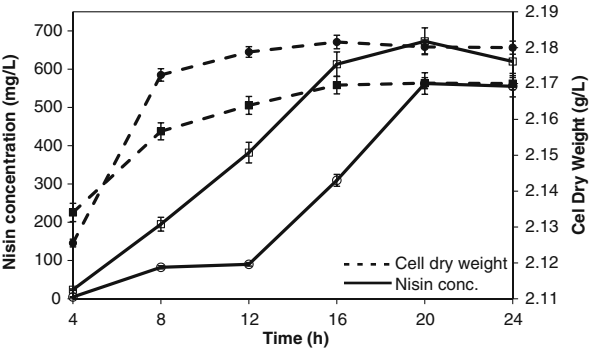


Fig. 3 Growth and nisin production rates in soy whey as compared to MRS in flask culture studies: *filled circles* Cell dry weight-MRS, *filled squares* Cell dry weight-Medium FACW, (*unfilled circles*) Nisin concentration-MRS, *unfilled squares* Nisin concentration-Medium FACW; (n=3)

treatments was 2.15 g/L) was comparable to MRS (2.17 g/L), while the growth rate in soy whey (Medium FACW, 253.86 mg/L/h) was higher than MRS (217.48 mg/L/h; Table 2). This indicates a high nutrient content for soy whey, underscoring the latent potential of this agricultural waste stream and its potential use as a value-added substrate for supporting microbial growth.

Ultrasonication of Soybean Flake Slurry Pretreatment of the soy flake slurry by ultrasonication prior to lab scale production of soy whey did not affect the protein and sugar concentration in the soy whey (data not shown). Soy whey made from sonicated soy flake slurry resulted in slight increase in cell yields and slight decrease in nisin yields (Fig. 4). These results suggest that sonication did not result in the release of nutrients essential for nisin production.

Dilution of Soy Whey Both lactic acid (R^2=0.9571) and nisin yields (R^2=0.7867) were directly related to the whey concentration (Fig. 5). However, 10-fold diluted Medium FW was still able to support *L. lactis* growth and nisin production (Fig. 2b). Hence, the nutrient level in soy whey was found to be quite high and dilution could be done without significant decrease in biomass yield.

Nutrient Supplementation to Diluted Soy Whey From the results of the dilution studies done above, it was evident that the growth of *L. lactis* in 4-fold diluted soy whey was significantly lower than undiluted whey (p<0.01). Hence, 4-fold diluted whey was used in

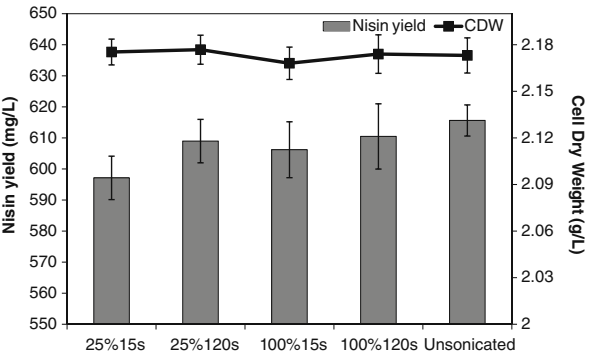


Fig. 4 Flask culture study of *L. lactis* subsp. *lactis* ATCC 7962 growth in Medium FW prepared from ultrasonicated soy flake suspension and consequent nisin yields after 48 h of incubation at 28 °C, as compared to Medium FW prepared from unsonicated soy flakes. The 25% and 100% amplitude of ultrasonic waves corresponding to 21 and 84 μm_{pp} (peak to peak amplitude in micrometers) were used for 15 and 120 s; (n=3)

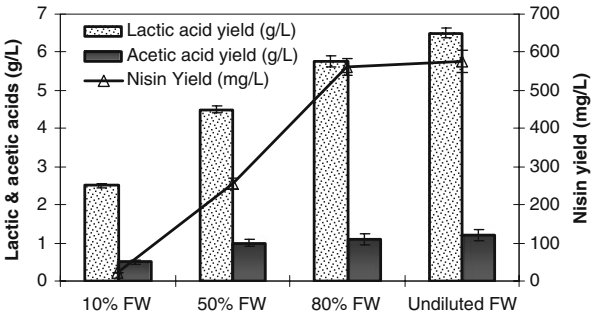


Fig. 5 Flask culture study of lactic acid, acetic acid, and nisin yields after 24 h of growth of *L. lactis* subsp. *lactis* ATCC 7962 in Medium FW at different dilution levels; (n=3)

this test to examine the effect of nutrient supplementation on *L. lactis* growth and nisin yields. Supplementation of diluted soy whey with sucrose as a carbon source (Medium DFW-ss) did not lead to a significant increase in biomass yield vis-à-vis unsupplemented, diluted soy whey (p>0.01), whereas supplementation with nitrogen and phosphorus sources (Medium DFW-ns) led to a significant increase in growth (p<0.01; data not shown). Interestingly, Medium DFW-ns was almost as efficient as undiluted soy whey in its ability to support bacterial growth. This suggests that the growth-limiting nutrient in soy whey is not the carbon source.

Nutrient Supplementation to Undiluted Soy Whey When sucrose, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, and yeast extract were individually supplemented to Medium FW, significant increase (p<0.01) in biomass yield was observed in almost every case (data not shown). Among the nutrients, KH_2PO_4 proved to be the most effective growth promoter/supplement. Our hypothesis is that KH_2PO_4 acts as a growth promoter in soy whey by supplying phosphorus, as supported by Liu et al. [30]. Soybeans do contain phosphorus, but the primary source of phosphorus in soy is phytate which is in a biologically unavailable form [39].

Biomass yield from Medium SFW was significantly higher than Medium FW and MRS both (p<0.01; Fig. 2c, Table 3). Thus, this indicated that addition of small amounts of mainly a phosphorus source could greatly increase *L. lactis* growth in soy whey.

As determined via HPLC, lactic acid production in soy whey during *L. lactis* growth was seen to rise slightly with nutrient supplementation, paralleling our observation of increased biomass yield in supplemented whey treatments. Growth of *L. lactis* was seen to reach a stationary phase before the complete depletion of sugars in the whey (Fig. 6). There could be two possible explanations to this observation; either the growth-limiting nutrient in soy whey was not the carbon source, or *L. lactis* grew and exhausted the simple sugars in the

Table 3 Cell yields of *L. lactis* subsp. *lactis* ATCC 7962 and nisin production when grown on MRS, supplemented soy whey (Medium SFW) or unsupplemented soy whey (Medium FW); (n=3).

Media	Maximum nisin activity (IU/ml)	Maximum nisin concentration (mg/L)	Maximum absorbance at 600 nm	Maximum biomass yield (mg/L)
Medium FW	23,230	580.80	1.03	2,151.3
Medium SFW	24,858	621.46	1.5	2,211.2
MRS	26,912	672.81	1.2	2,174.8

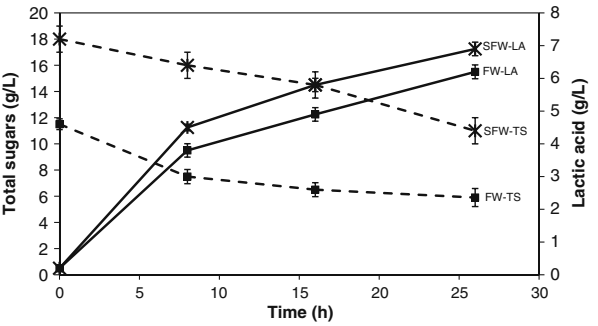


Fig. 6 Total sugar depletion and lactic acid production during flask culture study of *L. lactis* subsp. *lactis* ATCC 7962 in Medium SFW and Medium FW. *Filled squares in broken lines* Total sugars in Medium FW, *ex symbols in broken lines* Total sugars in Medium SFW, *filled squares in solid lines* Lactic acid in Medium FW, *ex symbols in broken lines* Lactic acid in edium SFW; (n=3)

whey and were not able to breakdown the remaining disaccharides and complex sugars for carbon uptake. This latter possibility is supported by the fact that *L. lactis* does not produce α-galactosidase, the enzyme needed to degrade stachyose and related sugars [40].

Antimicrobial Activity of *L. lactis* and *L. casei* Against *M. luteus*

The modified agar overlay method [26] used resulted in inhibition zones around both *L. lactis* and *L. casei* colonies (data not shown). We surmised that the unexpected zones observed for the nisin-negative *L. casei* could have arisen from the production of lactic acid, which has weak antimicrobial effects against the indicator strain. To investigate this, plates were incubated for an additional 2 to 3 days. After additional incubation, growth of *M. luteus* was observed within the previously clear inhibition zones on plates containing *L. casei* colonies. Parallel tests performed with the nisin-producing *L. lactis* resulted in persistent and clear zones of inhibition around *L. lactis* colonies. These results suggest that the antimicrobial effects initially seen with *L. casei* colonies were transitory effects due to metabolites such as lactic acid, which could be differentiated from the unambiguous inhibitory effects of nisin produced by colonies of *L. lactis*. These results, along with the fact that nisin extracts from soy whey fermentates were pH-adjusted, confirm that the clear and persistent zones of inhibition produced by these extracts were indeed due to nisin and not caused by confounding factors such as the presence of other inhibitory metabolites such as lactic acid.

Conclusions

This work provides proof-of-concept data for the use of soy whey as a suitable and inexpensive medium to grow the nisin-producing strain, *L. lactis*. Because soy whey is a byproduct of food processing operations, the nisin produced from this feedstock is not expected to face steep regulatory hurdles for its ultimate use in foods. A clear advantage of the present work is that nisin yields obtained from soy whey were substantially higher than those previously reported from other nondairy-based media [8–11, 35, 41]. In fact, soy whey, without any additional nutrient supplementation resulted in a nisin yield of 619.2 mg/L (or 24,767 IU/ml), as compared to the 92.9 mg/L reported by Liu et al. [30] from cheese whey. Other researchers have reported 88.7 mg/L of nisin from cull potatoes [8], 1,233 IU/ml of nisin from fermented barley extract [9], and 16,000 IU/ml of nisin from hydrolyzed sago starch [41], to name a few.

The ability to efficiently produce nisin using value-added agricultural waste materials such as soy whey may facilitate wider use of this once cost-restrictive antimicrobial. In the simplest example, the entire fermentate containing the soy whey, *L. lactis* and nisin could be concentrated and dried and used as a shelf life extender in foods or also as a value-added animal feed additive. Depending on the processes used, this product could potentially have both probiotic and antimicrobial properties due to the presence of viable *L. lactis* and nisin, respectively.

In summary, we have demonstrated the successful use of soy whey, a soy-processing waste material, for fermentation of *L. lactis* and subsequent high-yield production of nisin. Our ability to use this readily available and inexpensive agricultural byproduct as a base for fermentative production of nisin suggests the possibility for more cost-effective, sustainable production of nisin, which is currently produced using higher cost milk-based materials. Based on the strengths of this study, future work should be performed, focusing on the use of commercial waste soy whey and on scaled-up production of soy-fermented nisin to industrially relevant levels. Additional monitoring of *L. lactis* gene expression (*nisP*) during growth on soy whey could also enable us to more fully characterize this substrate as a medium for nisin production.

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