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Ultrasonication in Soy Processing for Enhanced Protein and Sugar Yields and Subsequent Bacterial Nisin Production

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Abstract. *Soy protein recovery from hexane-defatted soybean flakes using conventional methods is generally low. Importantly, some tightly-bound sugar in the soy flakes ends up in soy protein, thereby deteriorating the usefulness and quality of soy protein as a food ingredient. This research investigated the use of high-power ultrasound prior to soy protein extraction to simultaneously enhance protein yield and facilitate more sugar release in soy whey. The nutrient-rich soy whey was then used as a cheap growth medium to produce high-value nisin* using ***Lactococcus lactis subsp. lactis****. A nisin sensitive* organism ***Micrococcus luteus*** *was used as an indicator organism for international unit determination of nisin production as compared to standard. Soy flakes and water was mixed at the ratio of 1:10 (w/w). The slurry was then sonicated for 15, 30, 60 and 120 sec at a frequency of 20 kHz. The ultrasonic amplitude was maintained at 84* μm_{pp} *(peak to peak amplitude in µm) for all sonication durations. The results showed that with ultrasound pretreatment, the protein yield*

improved as much as by 46% in soy extract and sugar release by 50% with respect to nonsonicated samples (control). To maximize nisin production from soy whey, different parameters, such as aeration/agitation and incubation period were optimized. Nisin production from standard medium, DeMan, Rogosa and Sharpe (MRS) and soy whey was tested and compared. Maximum nisin production was achieved in stationary conditions and showed a continuous increase in yield till 48h of incubation (incubation period beyond that was not tested). Maximum nisin yield of 1.78 g/L of soy whey was obtained at 30°C and pH of 4.5 as opposed to 2.96 g/L of nisin with MRS medium

Keywords. *Defatted soy flakes, Protein yield, Sugar release, Soy whey, Ultrasound pretreatment, Nisin, Antimicrobial activity*

INTRODUCTION

Soybean (*Glycine max*) is a protein-rich oil seed. Protein is extremely valuable functional component in biochemical, pharmaceutical and food applications. Soy protein is produced from defatted soybean flour, known as residual soybean meal (Lusas and Riaz, 1995; Grieshop et al., 2003). Extraction is one of the key processing steps in the recovery of tightly bound intracellular material from soybean seed. The conventional extraction procedure for protein and carbohydrates involve the hydrolysis and extraction of material from defatted soybean flake. Conditions that could be varied during extraction are solvent choice, temperature, pH, agitation, and extraction time (Mason et al., 1996; Kasai and Ikehara, 2005). Extraction with water alone without any pretreatment may not achieve a higher yield of protein and carbohydrate from defatted soy flakes since protein and carbohydrate are considered to be localized in the middle lamella and in the proteins and/or oil bodies of cell (Kasai and Ikehara, 2005).

High power ultrasound is a new method that may improve the yield during extraction of intracellular compounds from plant material. Ultrasound pretreatment produces cavitation in the slurry. This results in extreme shear force and disintegrates particles in aqueous phase. Ultrasound technology has been applied widely in various biological and chemical processes. Li et al. (2004) employed ultrasound to enhance oil extraction from soybeans. Ebringerová et al. (1998) employed ultrasound to aid the extraction of active xylan and heteroxylan from corncobs and corn hulls, respectively. However, the use of high power ultrasound in soy processing for protein recovery is a new concept. Since the release of protein from soy flakes is governed by disintegration of lamellar structures that bind the protein molecules, we hypothesize that the use of high-power ultrasound could improve the protein yield and sugar release in soy whey.

Soy whey produced in the extraction of soy protein isolate is nutritionally rich, containing over eight amino acids and numerous trace elements that could be used in microbial fermentation of fastidious lactobacilli. For this research we evaluated the ability of soy whey to support the growth of *Lactococcus lactis* for nisin production. This will not only recover a value-added product – nisin from this low value organic stream; but will also solve the problem of pollution resulting from its disposal. To our best understanding, this particular stream has never been evaluated for its potential to produce nisin.

Nisin is a FDA approved bacteriocin commercially produced by microbial fermentation using lactic acid bacteria (LAB), primarily *L. lactis*. Nisin is of considerable interest because of its increasing usage as a natural food preservative against a wide range of Gram positive bacteria, *Listeria, Clostridium* and *Bacillus,* and Gram negative *Escherichia coli* and *Salmonella typhimurium* which costs the nation $5-17 billion annually in spoiled food (Daeschel, 1993). Nisin is also used in cosmetic and health care products. There are two factors, however, which make commercial production of nisin very costly: expensive milk-based growth medium for microbial fermentation and low nisin production rates. *L. lactis* is a nutritionally fastidious microorganism requiring complex culture medium.

The goals of this study are to evaluate the effectiveness of ultrasound pretreatment on protein yield and carbohydrate release from defatted soy flakes at different sonication times, and to explore soy whey as a potential nisin producing media using LAB.

MATERIALS AND METHODS

Soybean samples

Hexane-defatted dry soybean flakes (50 kg) were obtained from Center for Crops Utilization Research (CCUR) in the Department of Food Science and Human Nutrition at Iowa State University. The soybean flakes were packed in air-tight plastic bags and stored at 4^{o}C until use.

Ultrasonic treatment

Ground defatted soybean flakes (100 g) were mixed with 500 ml tap water. Soybean-water suspension was sonicated for 15, 30, 60 and 120 sec using a Branson 2000 Series bench-scale ultrasonic unit (Branson Ultrasonics Corporation, Danbury, CT, USA). The system has a maximum power output of 2.2 kW and operates at frequency of 20 kHz. Sonication tests were carried out at amplitude of 84 μm_{pp} (peak to peak amplitude in µm) in a customized 1.2 L stainless steel sonication chamber with a cooling jacket. The horn used was standard 20-kHz half wavelength titanium with a gain of 1:2.8. After sonication, soy protein was extracted by using a conventional extraction method. Controls included the same defatted soybean flakes in water, but without ultrasound pretreatment.

Extraction procedure

Sonicated samples were mixed with 500 ml of hot water to make flakes: water ratio of 1:10 (w/w basis), and the temperature was increased upto 60^{o}C. The pH was raised to 8.5 (initial pH 6.2) by adding 2N NaOH. Extraction was carried out in water bath at constant temperature and pH of 60^{o}C and 8.5, respectively for 30 min. During extraction, samples were continuously stirred using magnetic stirrer. Following 30 min of extraction, samples were centrifuged at 10,000 *g* for 20 min at 20^{o}C and the supernatants were collected for protein and sugar determination.

Analytical Procedures

Phenol sulfuric acid assay for total sugar determination

Appropriately diluted samples (1.0 ml) were pipetted directly into a test tube (1.5 x 15 cm). Phenol reagent, 5% (v/v) (1.0 ml) was added followed by 5 ml concentrated sulfuric acid using a fast flow pipette. The solution was mixed immediately and allowed to cool down at room temperature. After 30 min, absorbance at 490 nm was determined using a spectrophotometer (ThermoSpectrogenic Genesys 2 model W1APP11, Rochester, NY, USA). Total sugar concentrations were calculated from D-glucose standard curve.

Protein determination

Protein content in supernatant and flakes was determined by Combustion Type Rapid Nitrogen analyzer. Samples were analyzed according to Dumas method (AOAC, 993.13) using Rapids N III (Elementar Americas, Inc., Mt. Laurel, NJ, USA), where liquid samples were packaged in tin capsules. Aspartic acid (A9, 310-0; Sigma-Aldrich, St Louis, MO, USA) was used as the nitrogen reference calibration. The system was calibrated each time before analysis. Oxygen dosing for optimal combustion was selected based on sample type. Dosing for blanks was 50 ml O_2/min, whereas dosing for sample was 150 ml O_2/min. After analysis of 15 samples, a run-in

was analyzed to verify satisfactory system performance. Protein content was calculated from the nitrogen content of the material using a nitrogen conversion factor of 6.25.

Microorganisms, media and cultivation

Lactococcus lactis subsp. *lactis* (ATCC No. 7962, Rockville, MD, USA) - a nisin producing microorganism was used. *Micrococcus luteus* (ATCC No. 10240, Rockville, MD, USA) was used as an indicator microorganism in the bioassay agar for nisin. *L. lactis* was grown in De Man, Rogosa and Sharpe broth (MRS) (Difco, Sparks, MD, USA) and maintained as frozen stock in MRS broth with 20% (v/v) glycerol at -70°C and as lyophilized stock cultures. The working cultures were maintained as slants on MRS agar at 4°C. *M. luteus* was maintained similarly in Nutrient Broth (NB) (Difco, Sparks, MD, USA).

Characteristics of soy whey used as the substrate for nisin production are summarized as follows. Total chemical oxygen demand (COD) - 25,000 mg/L, ammonia (as N) - 8 mg/L, organic nitrogen (as N) - 1,100 mg/L, total phosphorus (as P) - 260 mg/L, total solids - 2.74%, protein content - 7,000 mg/L, sugar content – 8,000-9,000 mg/L and pH - 4.5 to 4.8.

Fermentation

All experiments were carried out at 30°C in 250 ml Erlenmeyer flasks containing 100 ml of the medium. The medium was inoculated with 10% (v/v) of seed culture of *L. lactis*. The seed culture was grown aerobically with shaking at 150 rpm for 12 h. pH of filter sterilized soy whey medium was adjusted to 6.8 – 7.0 each time before inoculating with *L. lactis.*

Nisin activity determination

The activity of nisin is expressed in terms of international units (IU) and 1 g of Nisaplin (a commercial nisin) (Aplin and Barrett, UK), is equivalent to 10^6 IU (Davies and Delves-Broughton, 2000). Nisin activity is measured with an agar diffusion bioassay, which is based on the measurement of the zones of inhibition in indicator seeded agar plates. There is a linear relationship between the size of the zone and the $\log_{10}$ concentrations. The higher the concentration is, the greater the inhibition zone diameter. For nisin quantification of an unknown sample, the diameters of the inhibition zone are measured and correlated with a standard curve (plot of diameter of inhibition zone against $\log_{10}$ concentration of known nisin concentration in IU/ml).

A stock solution of nisin (1000 IU/ml) was prepared with commercial nisin (10^6 IU/g) (Sigma-Aldrich, St. Louis, MO) as 1 mg/ml of sterile 0.02 N HCl. Nisin activity was determined via a bioassay. A culture agar pour of the indicator organism, *M. luteus*, was prepared with NB agar plates with 1% (v/v) Tween 20 (Sigma-Aldrich, Chemical). After sterilization, the agar medium was cooled down to 40°C, inoculated with 1% (v/v), 24-h culture of *M. luteus* (10^8 CFU/ml of agar medium), and poured aseptically into sterile petri dishes. After agar gels, 3-4 holes were bored, using a 5-mm diameter stainless steel borer. The nisin samples (100µl) were added to these wells and the plates were kept at 4°C overnight for diffusion. They were then incubated at 30°C for 24 h for the indicator organism to grow, and zones of inhibition were measured by a digital caliper. The standard curve was determined by measuring the zones of inhibition for each

known concentration of nisin (100 to 1000 IU). Since 1 IU/ml of nisin corresponds to 1 mg/L of nisin, the nisin concentration for every sample could be calculated.

Optimization of fermentation conditions

Two Erlenmeyer flasks (250 ml) containing 100 ml of filter sterilized soy whey were inoculated with 10% of *L. lactis* seed culture (10^8 cfu/ml) and incubated for 48 h at 30°C. One flask was incubated at stationary condition and the other at 150 rpm. The samples were withdrawn at 12 h intervals. They were heated in a 90°C water bath for 5 min to kill the bacteria, and then centrifuged at 10,000 *g* for 10 min. The supernatant was collected and tested for antimicrobial activity. All the readings were taken in triplicates.

Comparative study

Two Erlenmeyer flasks (250 ml), one containing 100 ml of filter sterilized soy whey, and the other 100 ml of heat sterilized MRS broth were inoculated with 10% of *L. lactis* seed culture and incubated for 48 h at 30°C at stationary condition. The samples were withdrawn at 12 h intervals and processed to check their antimicrobial activity.

RESULTS AND DISCUSSION

Ultrasound Pretreatment of Soy Flakes

Effect of ultrasound on protein release

The effect of ultrasonic pretreatment on protein yield at different sonication times is shown in Figure 1. Protein yield increased in extract with increase of ultrasonic exposure time. After 120 sec of sonication at an amplitude of 84 μm_{pp}, the protein yield increased by 46% with respect to control. The protein yields improved by 20, 23, and 30%, respectively for sonication times of 15, 30 and 60 sec with respect to control.

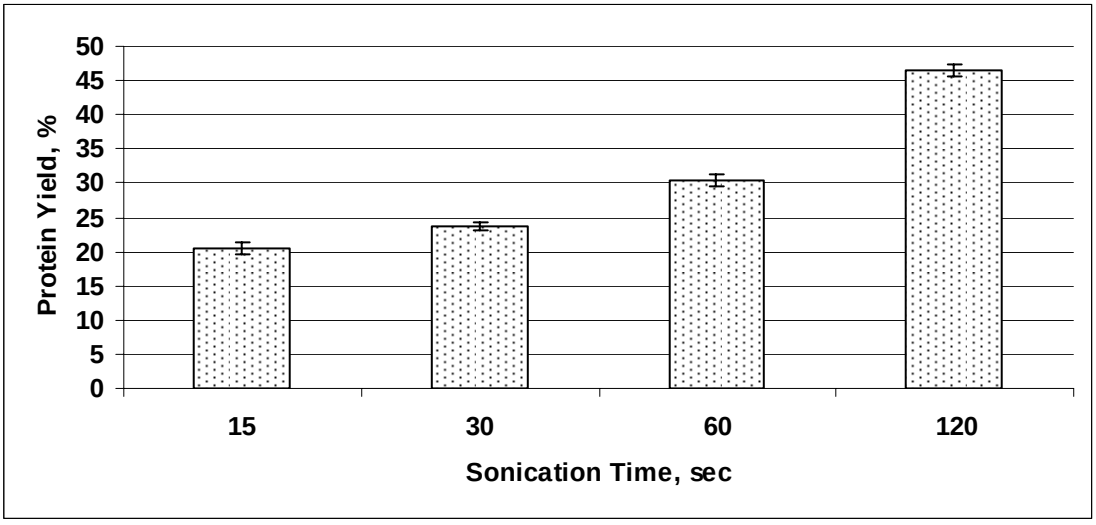

Figure 1 Protein yield at different sonication times, n= 2

Effect of ultrasound on sugar release

Total sugar released in extract at different sonication times is shown in Figure 2. As apparent from the figure, total sugar yield increased with increase in sonication time. The highest sugar concentration of 14.5 g/L was obtained during 120 sec of sonication at ultrasonic amplitude of 84 μm_{pp}. The improvements in total sugar yield were 18, 21, 32 and 50%, respectively at sonication times of 15, 30, 60 and 120 sec with respect to control.

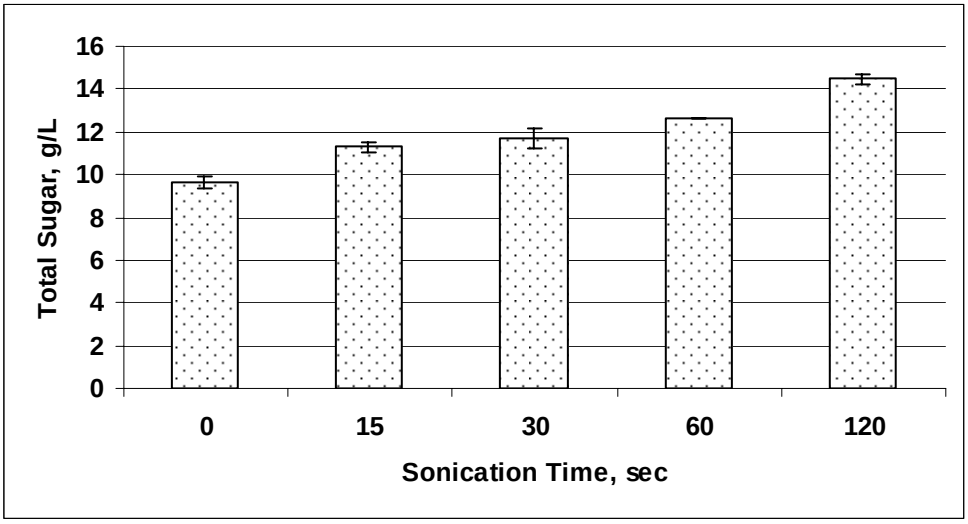

Figure 2 Total sugar in extract at different sonication times, n=2

Commercial soybean contains about 8% hull, 90% cotyledon and 2% hypocotyls. The chemical composition of soybean shows that it contains 43% protein and 29.4% carbohydrates (dry wt) in cotyledon. The interior elongated palisade-like cells of cotyledon occupies most of the protein and oil in the form of protein and oil bodies. Thus, the maximum yield of protein and carbohydrate is mostly contributed by the cotyledon of soybean seed. However, the localization of the water-extractable protein and carbohydrates in the soybean is still unknown (Kasai and Ikehara, 2005). High molecular weight protein and carbohydrates are tightly bound to the inner soybean (cotyledon) and difficult to extract by stepwise hot water extraction method alone. Study on enzymatic extraction showed that heat and humidity are important factors in the extraction of proteins from soy flakes, and the matrix formed by proteins and carbohydrates interferes with the enzymatic digestion (Fischer et al., 2001).

Thus, ultrasound provides a unique opportunity to improve the extraction of protein and sugar from defatted soy flakes. The cavitational effect is a major governing factor in improving the protein and carbohydrate yield following sonication. As the cavitation bubbles collapse in the vicinity of the plant membranes, it generates strong hydrodynamic shear forces. Such forces disrupt plant cell-walls thereby facilitating the release of extractable compounds (Vinatoru, 2001). The intense mixing during sonication also helps to better release intracellular material into aqueous phase.

Nisin Production on extracted whey

Effect of aeration and agitation

Higher yields of nisin were obtained when the soy whey was inoculated with the nisin producing strain and kept under stationary culture conditions as compared to shaking conditions (Figure 3). Maximum nisin production was 1,787 mg/L during 48 h of incubation for static culture, whereas the maximum nisin yield for shake flask cultures was 238 mg/L at 24 h. Parante and Ricciardi (1999) also reported declined nisin concentrations and yields at higher agitation speeds.

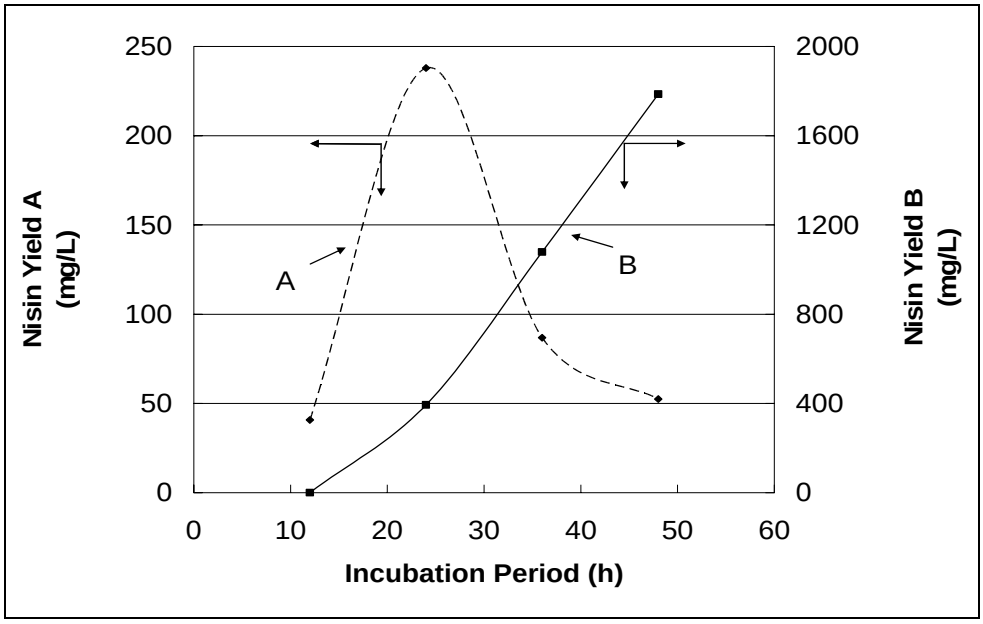

Figure 3 Nisin production (A) shake flask culture (B) stationary culture

Incubation period

Nisin production has been reported to be growth associated (a linear relationship observed between nisin production rate and growth rate). However, deviations from linearity have also been observed (Biswas et al., 1991; Kim et al., 1997). This is attributed to complex interaction of several factors, such as media composition and fermentation conditions, which affect nisin synthesis. Maximum nisin production was observed in soy whey during 48h of incubation under stationary culture condition. At this time, the bacterial culture had reached stationary growth phase (Figure 4).

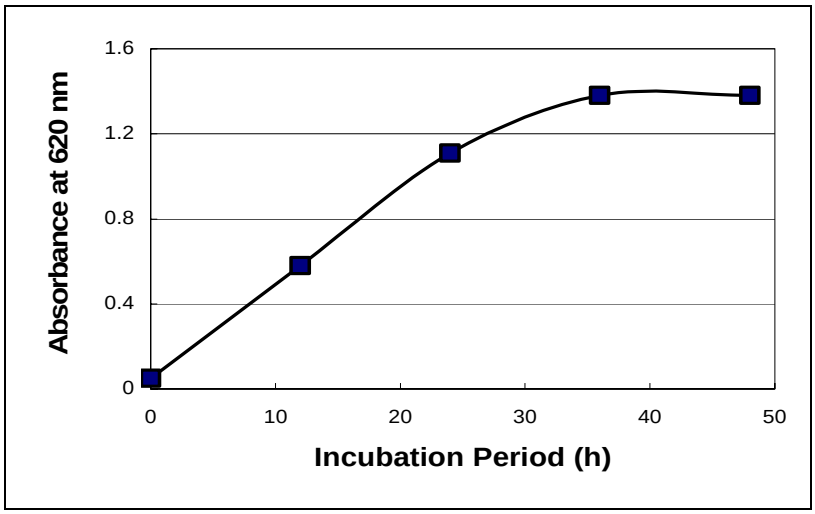

Figure 4 *L. lactis* growth curve in filter sterilized soy whey incubated as a stationary culture

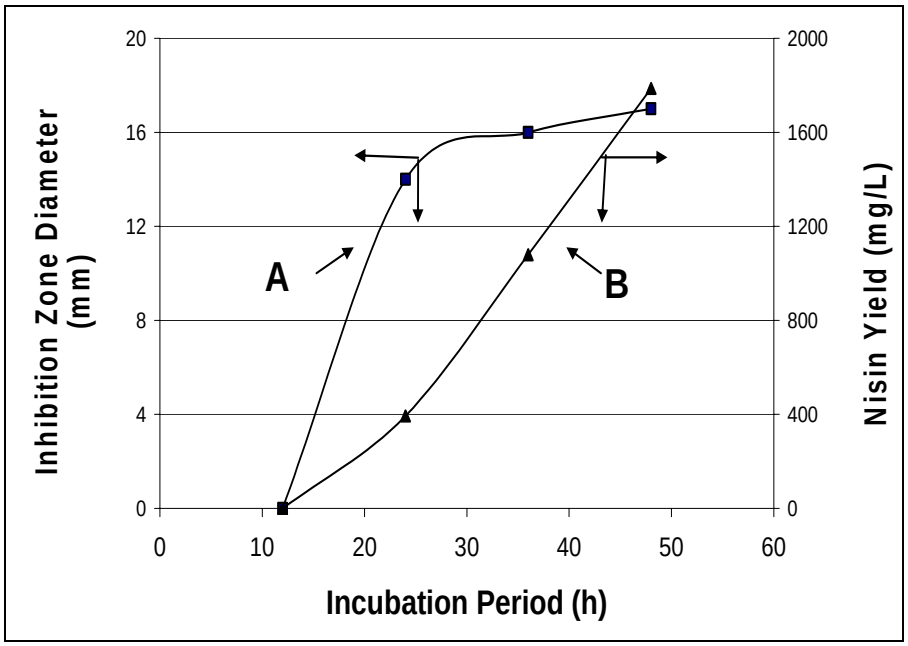

Figure 5 Antimicrobial activities at different incubation periods at stationary culture (A) Change of inhibition zone diameter (B) nisin yield at different incubation time

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Comparative study

Nisin yield of 1,780 mg/L of soy whey was obtained at 30°C, pH 4.5 stationary culture after 48 h of incubation, whereas MRS medium produced 2,960 mg/L of nisin (Figure 5). The lower yield with soy whey is mainly due to the fact that MRS is a standard medium which contains all essential micronutrients for the growth of LAB in the right proportion. This is not the same in case of soy whey, which is a waste product after separating the soy protein isolate from

soybean. But, it is also interesting to note that the yield of nisin from soy whey is quite comparable to that from MRS, and so with the proper optimization of the fermentation process much higher yield could be obtained. Though MRS gives a better yield of nisin, it's a very expensive medium as compared to a waste, like soy whey. Research is currently underway to optimize nisin production using soy whey.

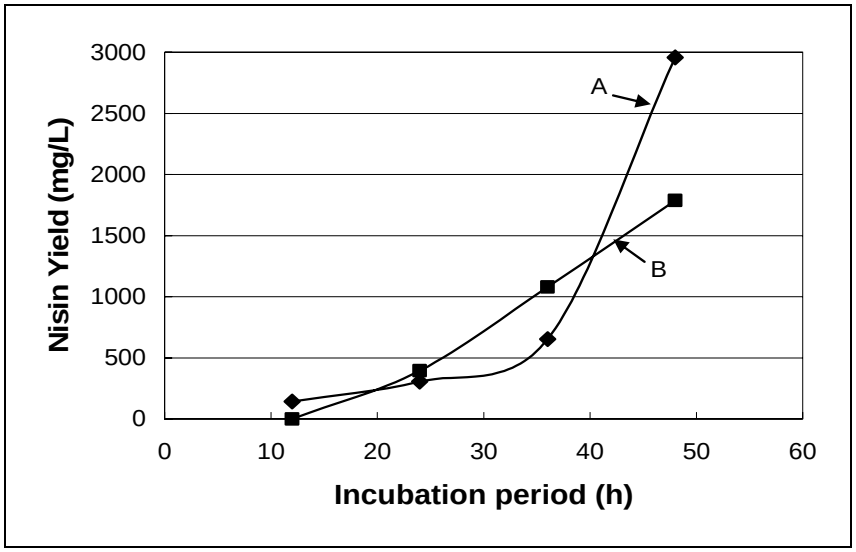

Figure 6 *L. lactis* nisin production (mg/L) in static culture using (A) MRS medium; (B) soy whey

CONCLUSIONS

The results obtained in this study have significant implications for the soy processing industry. Ultrasound has the potential to improve protein and sugar extraction. The increase in protein and sugar yields from sonicated samples was mainly due to the reduction in the particle size caused by the ultrasound, and also due to the release of tightly bound carbohydrates and protein from middle lamella and from the oil bodies. Similarly the soy whey following protein recovery could be used for producing high-value nisin. The nisin yield from soy whey was quite comparable to that of standard MRS medium. The best nisin yield was found in stationary culture conditions after 48 h of incubation. Since the use of synthetic growth media is cost prohibitive for sustainable production of nisin, an alternative source like soy whey definitely holds significant promise. Research is currently underway to optimize fermentation conditions to maximize nisin production. This research will provide an excellent opportunity to explore the potential of high value by-product recovery from low value organic streams.

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