Simultaneous Hydrolysis and Fermentation of Defatted Rice Bran and Defatted Soybean Meal for Nisin Production with Engineered *Lactococcus lactis*

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This work aimed to study the potential of defatted rice bran (DRB) and defatted soybean meal (DSM) as carbon and nitrogen sources for *Lactococcus lactis* growth and nisin production. First, a maximum nisin yield of 3630 IU/mL was achieved using 40% DRB hydrolysates and 30% DSM hydrolysates, which was 1.13 times greater than that found in commercial media. Second, to simplify the operation and shorten the length of the entire process, the processes of combined hydrolysis of DRB-DSM followed by fermentation, and simultaneous hydrolysis and fermentation of DRB-DSM were developed. Neutral proteinase enhanced the saccharification of DRB by cellulase and α-amylase. Furthermore, the strategy of NADH oxidase expression and hemin addition was innovatively proposed to overcome the oxygen stress in a simultaneous hydrolysis and fermentation process, which could alleviate the lag period following inoculation of *L. lactis* and result in a 77.3% increase in nisin titer.

Keywords: Defatted rice bran; Defatted soybean meal; Nisin; Lactococcus lactis; Simultaneous hydrolysis and fermentation

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INTRODUCTION

Nisin, a safe and natural antimicrobial peptide, exhibits broad-spectrum antimicrobial activity against a majority of Gram-positive foodborne bacteria and some Gram-negative pathogens. It has a long and impressive history as a food preservative (Cotter *et al.* 2005; Barbosa *et al.* 2017). Additionally, nisin is also considered a novel candidate to alleviate the increasingly serious threat of antibiotic resistance. It has shown efficacy in various clinical applications, such as treatment of skin and soft tissue infections (Mygind *et al.* 2005; Fauci and Morens 2012), *Clostridium difficile*-associated diarrhea (Ali *et al.* 2013), and even cancer therapy (Joo *et al.* 2012).

Rice bran, an agricultural by-product of rice processing, is generally used for animal feed, and the potential for its global production exceeds 29.3 million tons annually (Sharif *et al.* 2014). Rice bran is widely used for the extraction of rice bran oil, a premium edible oil due to its health benefits (Sohail *et al.* 2017). However, defatted rice bran (DRB),

the residue left after extraction of rice bran oil, contains a large amount of unexploited carbohydrates, polysaccharides, and proteins (Lee *et al.* 2009). Due to the advantage of its low lignin content and the less recalcitrant structure of DRB, it is possible to use DRB as a fermentable carbon source and partial nitrogen source (Gao *et al.* 2008). Defatted soybean meal (DSM), as a major by-product of the soybean oil, is commonly applied as a substitute for fish meal or poultry feed (Kikuchi 1999; Kim *et al.* 2003). The DSM is a nutrient-rich and low-priced agricultural material containing approximately 40% proteins. Due to its low cost, nutrient availability, and abundant amino acid species, DSM has the potential to be used as a fermentable nitrogen source (Dale *et al.* 2009; Capriotti *et al.* 2014; Hur and Park 2015).

Nisin is produced by certain *Lactococcus lactis* strains. Due to the strong adaptation to nutrient-rich environments, *L. lactis* loses the synthetic ability of many metabolites. Thus, it requires considerable amounts of refined sugars and high-quality nitrogen sources, which are quite an important part of the cost source for nisin fermentation (Sauer *et al.* 2017). The high cost of industrial medium has prompted investigations into exploring cheaper growth substrates, such as agricultural wastes (Krzywonos and Eberhard 2011; Shi *et al.* 2015; Hu *et al.* 2016; Grewal and Khare 2018) and fishery residues (Vazquez *et al.* 2008; Deraz *et al.* 2011). The authors previously demonstrated that defatted rice bran, the byproduct of the rice bran oil industry, could substitute for carbon source to enable *L. lactis* growth and nisin production (Liu *et al.* 2017a). To reduce the cost of industrial nitrogen source, defatted soybean meal, the byproduct after extraction of soy oil, was hydrolyzed to better support *L. lactis* growth and increase nisin production (Liu *et al.* 2017b). However, few studies report complete replacement of both the industrial carbon source and nitrogen source by cheaper growth substrates.

Additionally, the process of nisin production from DRB or DSM in the authors' previous research contained three stages including pretreatment, enzymatic hydrolysis, and fermentation, which is referred to as separate hydrolysis and fermentation. In recent years, there has been greater interest to simultaneously conduct the enzymatic hydrolysis and fermentation in the same reactor (Guo *et al.* 2018). This strategy would simplify the operation and shorten the length of the entire process. The combined process would also ameliorate the risk of bacterial contamination (Hazeena *et al.* 2019). Therefore, the objectives of this study were to evaluate the feasibility of DRB-DSM co-utilization for nisin production by *L. lactis* and develop the process of simultaneous hydrolysis and fermentation of DRB and DSM.

EXPERIMENTAL

Materials

The DRB and DSM in this study were purchased from Huayu Co., Ltd. (Qufu, China). The materials were milled using a high-speed blender and dried at 50 °C in a drying oven until the weight remained unchanged.

The nisin Z-producing strain *L. lactis* F44 was constructed in the authors' previous study (Zhang *et al.* 2014). The seed medium was used to inoculate *L. lactis* F44 and other recombinant *L. lactis* strains containing the following (g/100 mL): yeast extract 1.5, NaCl 0.15, sucrose 1.5, $MgSO_4 \cdot 7H_2O$ 0.015, KH_2PO_4 2.0, peptone 1.5, and pH 7.2. The overnight grown culture of *L. lactis* was inoculated into a commercial fermentative medium constituted of the following (g/100 mL): sucrose 1.5, KH_2PO_4 2.0, $MgSO_4 \bullet 7H_2O$ 0.015,

NaCl 0.15, yeast extract 1.5, corn steep liquor 0.3, cysteine 0.026, peptone 1.5, and pH 7.2. *Micrococcus flavus* ATCC 10240, an indicator strain for the bioassay of nisin, was maintained at 37 °C for 24 h in the indicator medium containing (g/100 mL): tryptone 0.8, NaCl 0.5, yeast extract 0.25, Na_2HPO_4 0.2, glucose 0.5, and agar powder 1.5. *Escherichia coli* TG1 was cultured in a Luria-Bertani (LB) medium at 37 °C for plasmids preparation and enrichment.

The enzymes for saccharification of DRB included cellulase, hemicellulose, and α-amylase. Cellulase was kindly supplied by Novozymes A/S (Beijing, China). Hemicellulase was purchased from Yuanye Bio-Technology (Shanghai, China), and α-amylase was purchased from Aobox Bio-Technology (Beijing, China). Their enzyme activities as described by the supplier are 70 FPU (Filter Paper Unit)/g, 20,000 and 3,700 U/g, respectively. One FPU of cellulase activity is defined as the amount of enzyme liberating 1 μmol glucose from filter paper per minute. One unit of hemicellulase activity is defined as the amount of enzyme liberating 1 μmol of reducing sugar from hemicellulose per minute. One unit of α-amylase activity is defined as the amount of enzyme liberating 1 μmol of reducing sugar from starch per hour.

The proteases used in this study included neutral protease, Proteinase K, trypsin, pepsin, and papain. Neutral protease was purchased from Doing-higher Bio-Technology (Nanning, China). Proteinase K, trypsin and pepsin were purchased from Solarbio Bio-Technology (Beijing, China). Papain was purchased from Yuanye Bio-Technology (Shanghai, China). Their enzyme activities, as described by supplier, were 200,000, 40,000, 250,000, 3,000, and 800,000 U/g, respectively. One unit of neutral protease activity is defined as the amount of enzyme that hydrolyzes casein to produce color equivalent to 1.0 μmol of tyrosine per minute (color by Folin & Ciocalteu's Phenol Reagent). One unit of Proteinase K activity is defined as the amount of enzyme that hydrolyzes urea-denatured hemoglobin to produce color equivalent to 1.0 μmol of Folin-positive amino acids and peptides per minute. One unit of trypsin activity is defined as the amount of enzyme that produces an increase in absorbance at 253 nm of 0.001 per minute using N-benzoyl-l-arginine ethyl ester as substrate. One unit of pepsin activity is defined as the amount of enzyme that hydrolyzes hemoglobin to trichloroacetic acid-soluble components giving a change of 0.001 in absorbance at 280 nm per minute. One unit of papain activity is defined as the amount of enzyme that hydrolyzes casein to trichloroacetic acid-soluble components giving an increase in absorbance at 275 nm equivalent to 1 μg of tyrosine at 275 nm.

Methods

Strains construction

The primers used for gene cloning are shown in Table S1. Extraction of genomic DNA from *L. lactis* F44 was performed using a Bacteria Genomic DNA Extraction Kit (Tiangen Biotechnology Co., Ltd., Beijing, China). The *xylR* and *noxE* genes were obtained by polymerase chain reaction (PCR) amplification from genomic DNA with the corresponding primers. The *nprB* gene was obtained by PCR directly from *Bacillus subtilis* 168 genomic DNA. The *xylR*, *noxE*, and *nprB* genes were assembled by overlap extension PCR using PrimeSTAR Max DNA Polymerase (Takara Biomedical Technology Co., Ltd., Beijing, China) with oligonucleotides listed in Table S1 and then ligated into the digested plasmid pLEB124 using an EasyGeno Assembly Cloning Kit (Tiangen Biotechnology Co., Ltd., Beijing, China). The ligated product pLEB124-REB was heat transformed into *E. coli* TG1. The plasmid pLEB124-REB was electro-transformed into *L. lactis* F44 after antibiotics selection. The restriction enzyme digestion and DNA sequencing were

completed to verify the sequence of the plasmid. The strains and plasmids in this study are shown in Table S2.

Pretreatment of DRB and DSM

According to the authors' previous research, thermal pretreatments of DRB and DSM at a solid loading of 10% and 5% (w/v), respectively, were performed at temperature of 121 °C in autoclave sterilizers with pretreatment time of 20 min (Liu *et al.* 2017a,b). Then, the pH of DRB and DSM were adjusted to 4.8 and 7.2, respectively, after the samples were cooled to room temperature. The pretreatment experiments were performed in triplicate.

Enzymatic hydrolysis

In the process of separated hydrolysis of DRB and DSM for fermentation (SHRSF), enzymatic hydrolysis of DRB was carried out after thermal pretreatment. Then, the pH was adjusted to 4.8, and 30 FPU/g of cellulase and 150 U/g of hemicellulase were added. The enzymatic hydrolysis was processed for 45 h at 40 °C and 100 rpm. Next, 150 U/g of α-amylase was added. The enzymatic hydrolysis was processed at 50 °C and pH 5.6, 100 rpm for 3 h. Then, both the DRB and DSM supernatants were separated by centrifugation at 6,580 × g for 15 min. Enzymatic hydrolysis of DSM was performed at 40 °C, 100 rpm for 10 h after 10000 U/g of neutral protease was added. Then, the supernatants DSM were separated by centrifugation at 6,580 × g for 15 min. The supernatants were stored at 4 °C as fermentable constituents. All enzyme solutions were filter sterilized through a 0.22-mm syringe filter before use.

In the combined hydrolysis of DRB and DSM for fermentation (CHRSF), the pretreated samples, DRB and DSM, were added together into 250-mL Erlenmeyer flasks. The solid loading of DRB and DSM were 10% (w/v) and 5% (w/v), respectively. An amount of 30 FPU/g cellulase and 150 U/g hemicellulase were added. The enzymatic hydrolysis was processed at 40 °C and pH 4.8, 100 rpm for 45 h. Then, 150 U/g of α-amylase was added for enzymatic hydrolysis with pH 5.6 at 50 °C, 100 rpm for 3 h. After the pH was adjusted to 7.2, 10000 U/g of neutral protease was added. The enzymatic hydrolysis was processed at 40 °C, 100 rpm for 10 h. The samples were centrifuged to obtain the supernatant of the DRB and DSM mixture.

Simultaneous hydrolysis and fermentation of DRB and DSM (SHFRS)

In SHFRS process, the DRB and DSM mixture medium was used for simultaneous hydrolysis and fermentation including the following (g/100 mL): DRB 10, DSM 5, NaCl 0.15, KH_2PO_4 2, cysteine 0.026, $MgSO_4 \cdot 7H_2O$ 0.015, and pH 7.2. The DRB and DSM mixture medium was treated in an autoclave at 121 °C for 20 min. After the medium was adjusted to room temperature, 1800 U/g of trypsin, 100 U/g of Proteinase K, 30 FPU/g of cellulase, 150 U/g of hemicellulase, and 150 U/g of α-amylase were added. Simultaneously, 1 μg/mL of hemin (Sigma-Aldrich, St. Louis, MO, USA) was added to the DRB and DSM mixture media. Then, the samples were incubated at 30 °C with constant stirring at 100 rpm.

Degradation of nisin by protease

The nisin solution (4000 IU/mL) was added into 100-mL conical flasks, and the pH of the solution was adjusted to the optimum pH of each enzyme. Then, neutral protease, trypsin, pepsin, and papain were added to the corresponding 100-mL conical flask. At the

optimum temperature of each enzyme, the enzymatic hydrolysis was processed for 3 h with shaking at 100 rpm. After the pH was adjusted to 2.0, the nisin solution was sterilized at 121 °C for 20 min. Then, the nisin titer was detected.

Nisin titer assay

The plate diffusion method was applied to determine nisin activity. The nisin standard used for nisin titer assay was purchased from Sigma Chemical Company (Shanghai, China). The stock solution of nisin (10^4 IU/mL) was prepared by mixing 0.1 g of nisin standard powder with 10 mL of 0.02 M HCl solution and boiled for 5 min. Then, the gradient nisin solutions (25, 50, 100 and 200 IU/mL) were prepared by the dilution of prepared solution of nisin with 0.02 M HCl solution. To determine the nisin production, 500 μL of fermentation broth was diluted with 500 μL of 0.02 M HCl. For desorption of cell-bound nisin, the acidified fermentation broth was boiled for 5 min and then centrifuged at 2400 × g for 5 min. Then, the supernatant was diluted appropriately with 0.02 M HCl. The indicator strain *M. flavus* was cultured in LB medium for 24 h at 37 °C and then washed with 2 mL 0.9% NaCl solution. After autoclaving, 26 mL of indicator medium was cooled to about 50 °C, and then it was inoculated with 100 μL NaCl solution of *M. flavus* (the final concentration of the strain was 10^7 CFU/mL). The indicator medium was poured into a sterile plate and placed at room temperature for solidification. Then the plates were placed at 4 °C overnight for precultivation. Eight wells of each plate were obtained by using a 7-mm-diameter hole punch to remove agar. Then, 100 μL standard nisin solutions and test samples were added into the individual well. The diameter of inhibition zone was measured by using a vernier caliper after the plates were incubated at 37 °C for 24 h. The regression equation was obtained by processing the data from standard nisin solutions. Each sample was performed in triplicate.

Analysis methods

Two methods were used to detect the growth of *L. lactis*. One was to measure the optical density (OD) by using TU-1810 spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) at 600 nm, and the other involved detecting colony-forming units (CFU) using the plate counting method. In the simultaneous hydrolysis and fermentation, the fermentation broth was appropriately diluted with 0.9% NaCl solution. Then, the 100 μL fermentation broth was spread onto the seed medium agar plates. The CFU was detected by the plate counting method after 24 h. Each sample was performed in triplicate. The 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) was applied to detect the concentration of total reducing sugars (TRS). The pH value was monitored by a pH meter. The nitrogen solubility index (NSI) was defined as the percentage of soluble nitrogen to total nitrogen. The NSI was determined based on the method of Mune Mune and Minka (2017). The molecular weight distribution of peptides in fermentation was monitored according to the method of Liu *et al.* (2017b).

RESULTS AND DISCUSSION

Optimization of DRB and DSM Hydrolysates amounts in SHRSF process

To verify whether the use of DRB as a carbon source and DSM as a nitrogen source can ensure the normal growth of *L. lactis* and nisin production, separated hydrolysis of DRB and DSM was initially adopted (Fig. 1A). Hydrolysis of DRB and DSM was

performed according to the previous reports of Liu *et al*. (2017a,b). The TRS concentration in DRB hydrolysates was 43.8 g/L, and the NSI of 78.2% in DSM hydrolysates was achieved using neutral protease. Then, DRB and DSM hydrolysates were mixed in different proportions, and inorganic salts required for *L. lactis* growth including 20 g/L of KH_2PO_4, 1.5 g/L of NaCl, and 0.15 g/L of $MgSO_4 \cdot 7H_2O$ were added. After sterilization, fermentation was carried out using *L. lactis* F44, a nisin producing strain. The amounts of DRB hydrolysates and DSM hydrolysates were optimized based on the nisin yield at 8 h. As shown in Table 1, the maximum nisin yield was up to 3315 IU/mL with 30% DSM hydrolysates and 40% DRB hydrolysates.

Table 1. Effect of DRB and DSM Hydrolysates Amounts on Nisin Production in SHRSF Process

Nisin (IU/mL)*		DSM hydrolysates				
		10%	20%	30%	40%	50%
DRB hydrolysates	10%	713 ± 24	946 ± 30	1129 ± 43	1108 ± 67	1275 ± 59
	20%	1354 ± 55	1759 ± 79	1896 ± 84	1955 ± 94	1943 ± 114
	30%	1987 ± 121	2254 ± 122	2574 ± 167	2716 ± 74	2814 ± 196
	40%	2712 ± 112	2919 ± 172	3315 ± 114	3277 ± 145	3241 ± 158
	50%	2549 ± 74	2835 ± 156	3017 ± 125	2714 ± 98	2654 ± 132
*Values are given by mean ± standard (n=3)						

The Fermentation Performance of F44 in SHRSF Process

To evaluate fermentation performance with DRB hydrolysates and DSM hydrolysates as the sole carbon source and nitrogen source for nisin production by *L. lactis* F44, shake-flask fermentation was performed and the cell density, pH value, and nisin production were monitored in the SHRSF process. At the same time, the commercial medium for nisin production was also accomplished as comparative performance data (Fig. 2). F44 showed a lower growth rate for the first 8 h in the SHRSF medium, which may be related to certain fermentation inhibitors in the DRB and DSM hydrolysates when compared to the commercial fermentation medium. The hydrolysates of cottonseed meal and wheat bran could inhibit the growth of industrial microorganisms in the early stage (Heer and Sauer 2008). The final bacterial concentration of F44 for the commercial medium and SHRSF medium were not much different. The commercial medium had a maximum amount of nisin titer of 3211 IU/mL at 8 h, and 3630 IU/mL at 10 h. This indicated that *L. lactis* F44 could produce more nisin in the SHRSF medium per mL of biomass. This may be due to the presence of a small amount of galactose in the SHRSF medium, which has been shown to promote the early biosynthesis of nisin by increasing the transcriptional level of *nisZ* (the gene encoding the precursor peptide of nisin) (Cheigh *et al*. 2005). In contrast, the accumulation of lactic acid during the growth of *L. lactis* leads to decrease the pH of the fermentation broth, which seriously affects the growth of the cells and even causes the death of the cells. Under acidic conditions, the carbon metabolism pathway of *L. lactis* is changed and the strains tend to increase the utilization of other monosaccharides, especially galactose (Upreti and Metzger 2007; Cretenet *et al*. 2011).

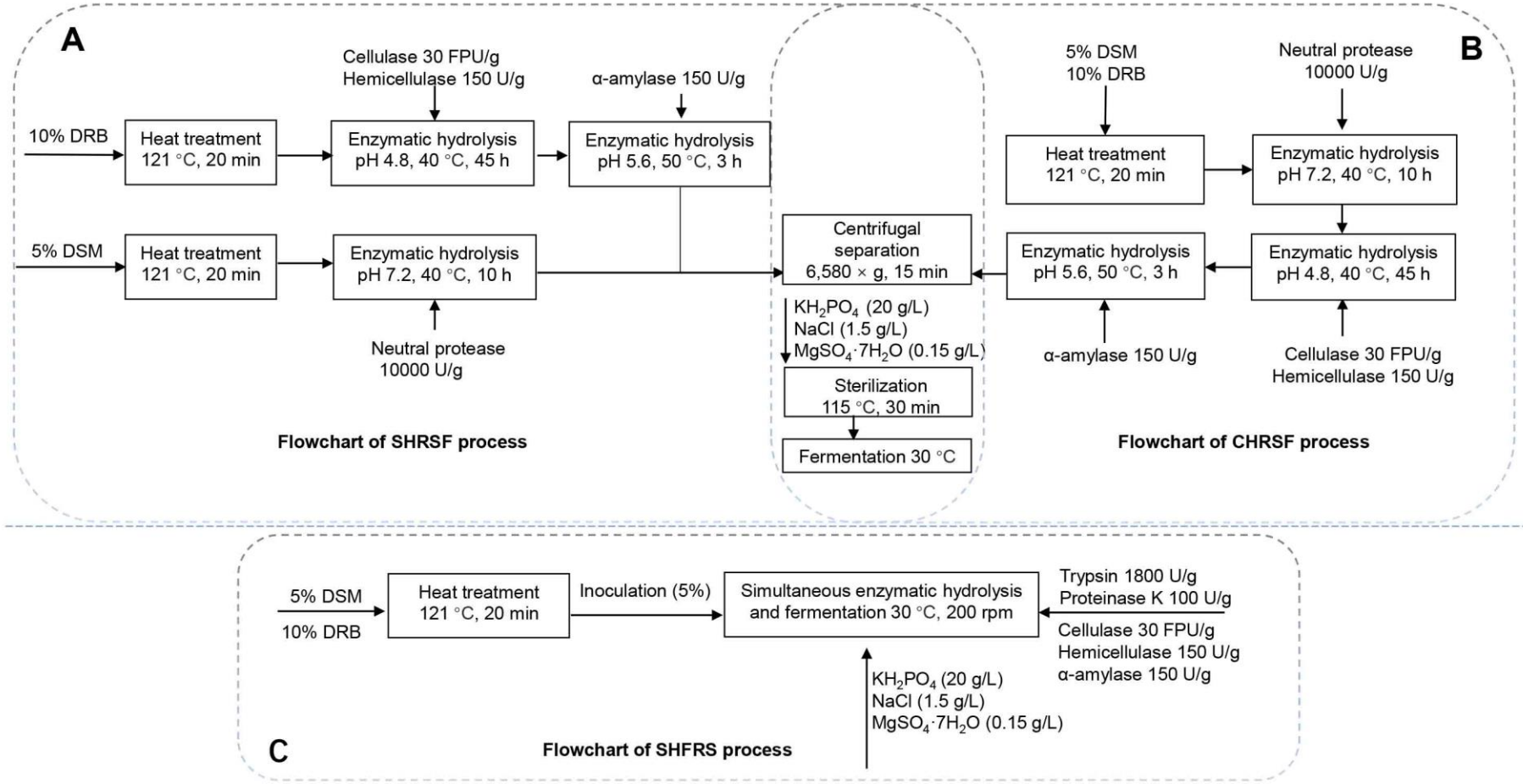

Fig. 1. Flowchart of the process for nisin production: (A) SHRSF: separated hydrolysis of DRB and DSM for fermentation; (B) CHRSF: combined hydrolysis of DRB and DSM for fermentation; (C) SHFRS: simultaneous hydrolysis and fermentation of DRB and DSM

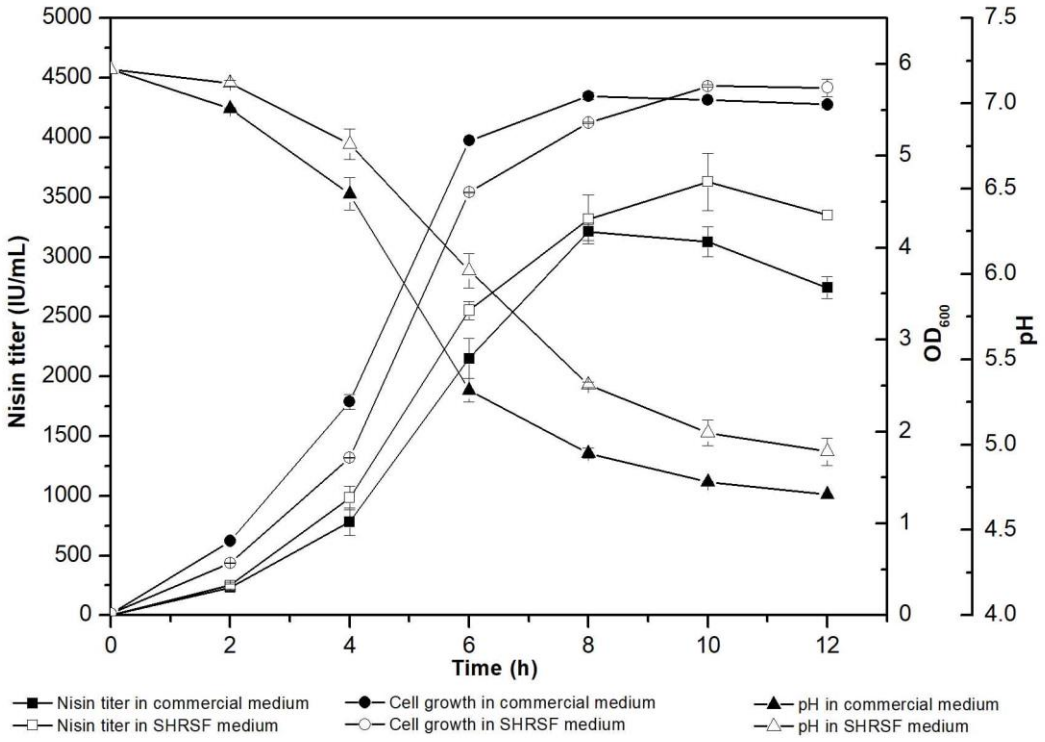

Fig. 2. Time profile of nisin titer, cell density, and pH of *L. lactis* F44 cultured in SHRSF medium and commercial fermentation medium; error bars represent standard deviations from three parallel replicates

Based on the findings, other monosaccharides in the SHRSF medium may promote the activity of the strain under acidic conditions. In addition, in the SHRSF medium, the rate of descent of pH was remarkably lower than that of the commercial fermentation medium, and the final pH of the SHRSF fermentation medium was also higher than that of the commercial medium. Some buffer substances may have been present in the SHRSF medium; further study is needed to confirm this finding.

The results of this study indicated that DRB hydrolysate and DSM hydrolysate could completely provide the carbon and nitrogen sources needed for the growth and production of *L. lactis* F44. Refined sugar and a high-quality nitrogen source did not need to be added to the prepared medium. The medium was shown to have the potential to reduce the industrial cultivation cost of *L. lactis* and the cost of nisin production.

The Distribution of Peptides Before and After Fermentation of the SHRSF Medium

For *L. lactis*, the nitrogen source that can be utilized is mainly present in the medium in the form of soluble peptides and free amino acids. The availability of soluble peptides is related to the molecular weight of peptides. Some peptides with large molecular weight can only be hydrolyzed by proteases into oligopeptides containing 4 to 18 amino acids in the oligopeptide transport system of *L. lactis* before they can be used for absorption and utilization (Sanz *et al.* 2001; Picon *et al.* 2010). The distribution of peptides of different molecular weights before and after fermentation of the SHRSF medium was observed, and the findings are shown in Table 2. In the original SHRSF medium, almost all peptides had

molecular weights above 4 kDa, indicating that the peptides in SHRSF medium were hard to be directly absorbed and utilized by *L. lactis*. After fermentation, the peptides above 4 kDa were not detected and the content of 2 to 4 kDa peptide was low, showing that *L. lactis* can effectively degrade and utilize the nitrogen source in SHRSF medium.

Table 2. Molecular Weight Distribution of Peptides of SHRSF Medium Before and After Fermentation

Weight (kDa)	0 to 0.3	0.3 to 0.6	0.6 to 1	1 to 2	2 to 4	4 to 8	8 to 12	12 to 20	> 20
Before (%)	ND[a]	ND	ND	ND	ND	29.3	21.4	28.2	21.1
After (%)	4.2	21.3	63.2	6.2	5.1	ND	ND	ND	ND
[a] ND: not detected									

Development of CHRSF Process

In SHRSF process, DRB and DSM need to be separately hydrolyzed to prepare an enzymatic hydrolysate. This causes problems including cumbersome operation and complicated process flow.

To further reduce costs, enzymatic hydrolysis of the DRB and DSM mixture was applied. The components of DRB and DSM are cross-linked. The DRB contains a small amount of protein and DSM contains a small amount of polysaccharide. Therefore, the CHRSF process was developed to not only simplify the process but also to make the full use of the nutrients of DRB and DSM. The process flow is shown in Fig. 1B.

Hydrolytic Effect of Neutral Protease on the Cellulase and α-amylase

In the CHRSF process, proteases and saccharification enzymes (cellulase, hemicellulase and α-amylase) are combined to digest DRB and DSM mixtures. Because glucoamylase is a protein, it is necessary to ensure that protease does not degrade glucoamylase. Therefore, the saccharification effect of protease on cellulase and α-amylase was verified according to the reducing sugar yield.

With the concentration gradient of different neutral proteases, 30 FPU/g of cellulase and 150 U/g of α-amylase were used to digest DRB under the optimum pH and temperature conditions, respectively. The cellulase digestion time was 40 h, and the enzymatic hydrolysis time of α-amylase was 4 h. The authors found that the addition of neutral protease not only did not reduce the yield of reducing sugar but also increased the TRS yield with the increasing of neutral protease, indicating that the neutral protease had a positive effect on the saccharification of DRB by cellulase and α-amylase. Without neutral protease, the yield of TRS obtained from enzymatic hydrolysis of DRB by cellulase and α-amylase was 8.3 g/L and 12.8 g/L, respectively.

After the addition of 10000 U/g of neutral protease, the total reducing sugar yield was increased to 10.3 g/L and 17.8 g/L, respectively (Fig. 3). In this study, the authors concluded that the protein in DRB was crosslinked with components such as cellulose and starch. The hydrolysis of protein components by neutral proteases reduced the degree of crosslinking, making cellulose and starch in DRB more susceptible to combine cellulase and α-amylase to enhance the effect of saccharification. In addition, protease can act as a surfactant, which promotes the enzymatic hydrolysis of glucoamylase (Liang *et al.* 2011).

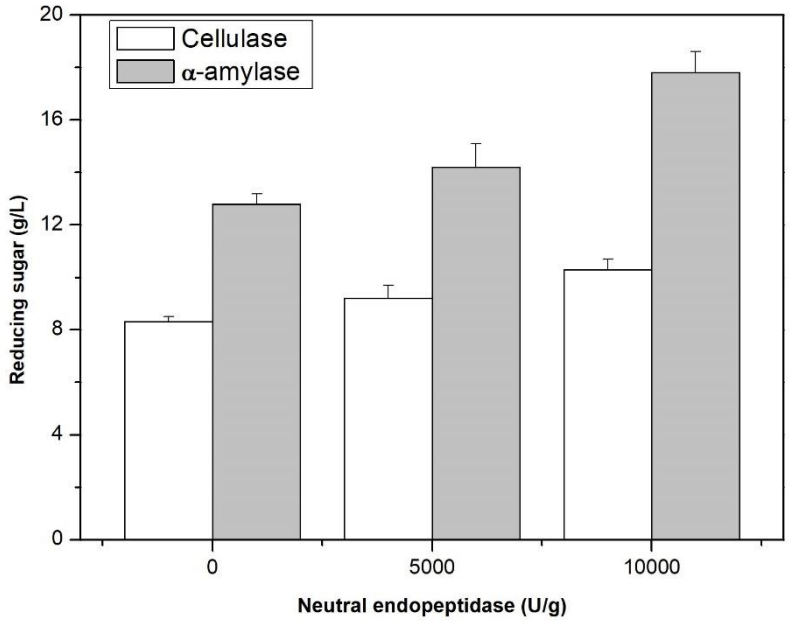

Fig. 3. Effect of neutral protease on enzymatic saccharification of cellulase and α-amylase; the enzyme dose was 30 FPU of cellulase and 150 U of α-amylase per g solids; error bars represent standard deviations from three parallel replicates.

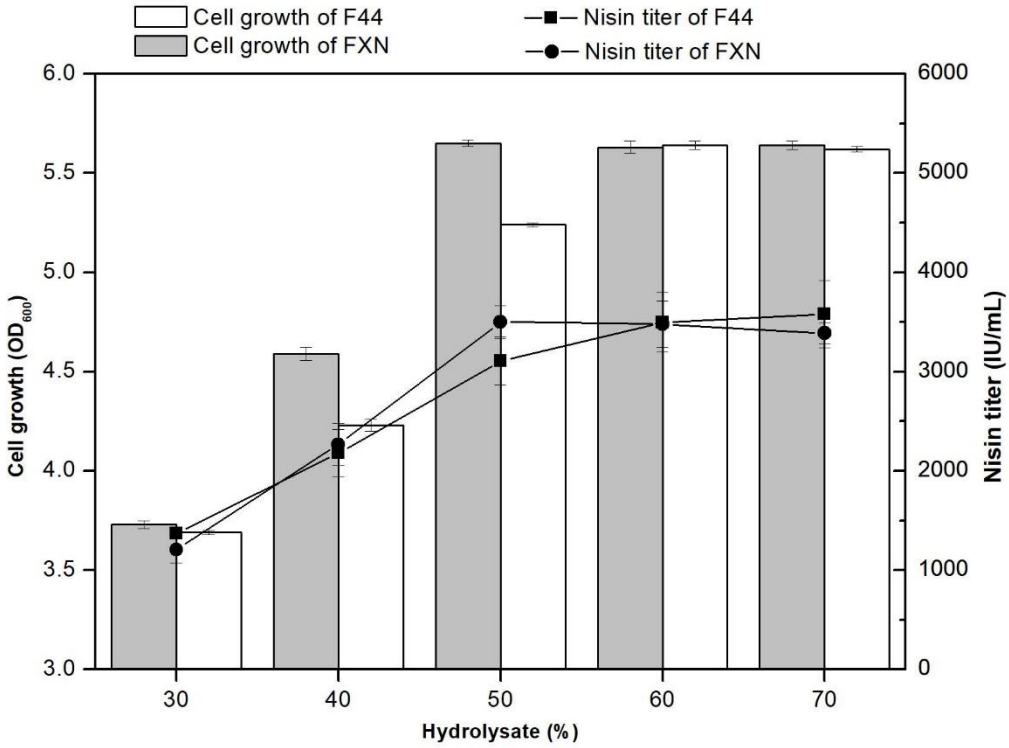

Fig. 4. Effects of hydrolysates dosage on cell density and nisin titer of *L. lactis* F44 and FXN; the average data of triplicate experiments are presented; error bars represent the standard deviation of triplicate experiments.

Construction of Engineered Strain *L. lactis* FXN and Fermentation in CHRSF Medium

The authors' previous studies showed that the ability of xylose utilization could be enhanced in DRB hydrolysate by improving the activity of the xylose transcriptional regulator xylR (Liu *et al.* 2017a). In addition, the proteolytic function of *L. lactis* F44 can increase the utilization of nitrogen sources in the DSM and reduce the amount of DSM hydrolysate (Liu *et al.* 2017b). Therefore, *xylR* gene in *L. lactis* F44 was overexpressed, and the nprB gene, encoding extracellular protease NprB derived from *B. subtilis*, was heterologously introduced into F44. As a result, the engineered strain *L. lactis* FXN was constructed. In =CHRSF process, the fermentation performances of *L. lactis* F44 and FXN were compared with different enzymatic hydrolysate contents (Fig. 4). The results showed that the engineered strain FXN could obtain the highest nisin yield and the highest amounts of bacteria with 50% enzymatic hydrolysate in CHRSF medium. The nisin yield and the cell density were equivalent to that of F44 with 60% of the enzymatic hydrolysate content. It indicated that the requirement of the FXN strain for the enzymatic hydrolysate content was lower than that of F44, which was beneficial to reducing the amount of the medium and the production cost of the nisin.

Development of SHFRS process

At present, simultaneous saccharification and fermentation (SSF) is relatively mature in the production of ethanol by *Saccharomyces cerevisiae* using lignocellulose. SSF aims to complete the saccharification and fermentation process in one step. It can simplify the production process by reducing the number of reaction tanks and operating units compared to separate hydrolysis and fermentation. The total investment cost is estimated to be reduced by more than 20% (Wingren *et al.* 2003). A further advantage is that the enzymatic hydrolysate can be rapidly utilized by the strain, reducing product inhibition by the enzymatic process. In this study, the cellulose and starch components of DRB were needed to convert into fermentable sugars; additionally, the macromolecular proteins of DSM were needed to hydrolyze into a nitrogen source that could be used for *L. lactis*. Therefore, the SHFRS process was proposed as shown in Fig. 1C. In this process, inorganic salt ions were added into enzymatic hydrolysate before the heat treatment, then enzymatic hydrolysis and fermentation were simultaneously carried out. The unfavorable factors of the process were that the optimal pH of enzyme was different from fermentation process, and the optimum pH of protease and glucoamylase were also different. However, the pH of the fermentation broth constantly decreased with the production of lactic acid during the growth of *L. lactis*, which had a beneficial effect on many enzymes used in the experiment. In addition, because the enzymatic hydrolysate can be used for growth of strains in time, the process also has the potential to increase the enzymatic efficiency.

Screening for Proteases in SHFRS Process

Because the protease and the fermentation product nisin coexist in the fermentation system in SHFRS process, it is crucial to screen for proteases that do not degrade nisin. Therefore, the degradation of nisin by neutral protease, trypsin, pepsin, Proteinase K, and papain was studied (Fig. 5), and the enzymatic hydrolysis effect on DSM was observed (Fig. S1). Neutral protease had a strong degradation effect on nisin. After treatment with neutral protease for 3 h, the nisin titer decreased to 26.7% of the amount found in the control group. Pepsin and papain treatment also reduced nisin activity. Treatment with trypsin and Proteinase K had little effect on nisin activity. Next, the enzymatic hydrolysis of DSM by

neutral protease, trypsin, and Proteinase K was studied. After digestion, the NSI of DSM by using trypsin and Proteinase K were lower than that of neutral protease. In addition, trypsin was only active in the pH range of 6 to 8 whereas the optimum pH range of Proteinase K was broadened from 4 to 12. Therefore, considering the optimum pH of the two enzymes and the enzymatic efficiency to DSM, both proteases were used in SHFRS process.

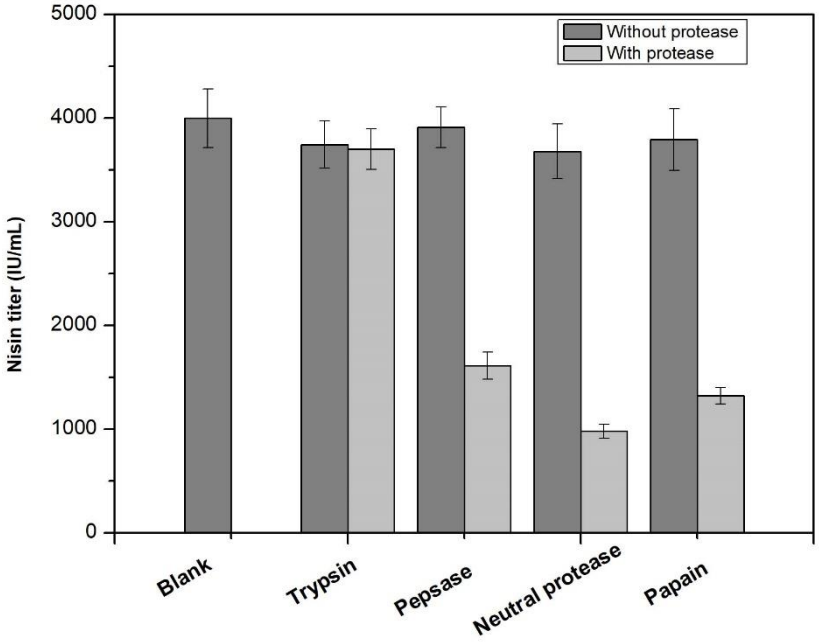

Fig. 5. Effect of protease on nisin degradation; the enzyme dose was 10 U per milliliter of nisin solution; error bars represent the standard deviation of triplicate experiments

Effect of *noxE* Overexpression Combined with Addition of Hemin in SHFRS fermentation

At present, the fermentation of *L. lactis* and the nisin production in industry are performed under anaerobic conditions. Due to the low oxygen utilization and tolerance of *L. lactis*, excessively high dissolved oxygen is unfavorable for the growth of the strain. In the SHFRS, the process must be completed under stirring conditions to ensure sufficient contact of the enzymes and feedstocks. Therefore, it is necessary to increase the dissolved oxygen of the simultaneous enzymatic hydrolysis and fermentation system. In addition, related studies have shown that the oxygen supply is helpful for the saccharification of lignocellulosic biomass (Bi *et al.*, 2016), and the respiration of the cell was stimulated with hemin to extensively enhance the nisin production (Kordikanlioglu *et al.* 2015). In SHFRS process, there was a certain delay in the growth of the strain in the early stage, probably due to the high dissolved oxygen in the simultaneous enzymatic hydrolysis system. The authors further overexpressed the *noxE*-encoded NADH oxidase into *L. lactis* FXN for constructing the recombinant strains FXNE and added 1 μg/mL of hemin to the fermentation system to activate the aerobic respiratory chain of *L. lactis*. The results showed that this strategy effectively alleviated the growth retardation of the strain in the early stage. The nisin yield of the reconstituted strain FXNE reached 1870 IU/mL at 16 h, which was 70% higher than the highest nisin yield without adding hemin (Fig. 6). In

addition, the nisin yield of the SHFRS process was remarkably lower than that of the SHFRS and CHRSF processes. This might have been due to insufficient nutrient supply during simultaneous enzymatic fermentation, but after adding 5 g/L of glucose and 5 g/L of peptone to the SHFRS medium, there was no remarkable change in nisin production. Therefore, it may be possible that the DRB and DSM solid residues in SHFRS process had an adsorption effect on nisin, resulting in a low level of free nisin detected. In the future, the process or the nisin separation method can be further improved to enhance economic benefits.

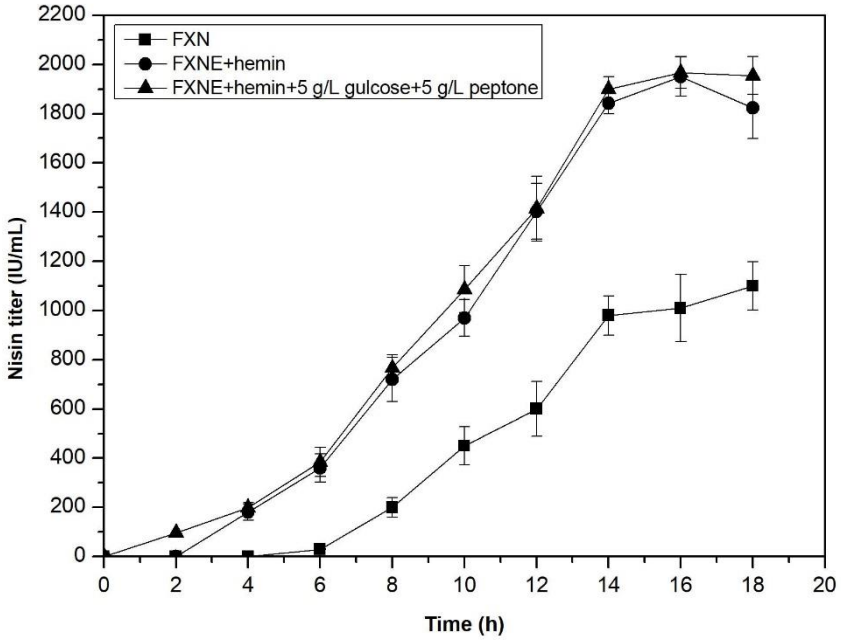

Fig. 6. Nisin production curves of *L. lactis* FXN and FXNE in SHFRS process; average data of triplicate experiments are presented; error bars represented the standard deviation of triplicate experiments

CONCLUSIONS

1. Three processes including separate hydrolysis of DRB and DSM for fermentation (SHRSF), combined hydrolysis of DRB and DSM for fermentation (CHRSF), and simultaneous hydrolysis of DRB and DSM for fermentation SHFRS were applied to realize co-utilization of defatted rice bran (DRB) and defatted soy meal (DSM) with *L. lactis* for nisin production.
2. The maximum nisin yield was up to 3630 IU/mL with 30% DSM hydrolysates and 40% DRB hydrolysates in SHRSF process, which was 1.13 times greater than that found in commercial media.
3. The neutral protease had a positive effect on the saccharification of DRB by cellulase and α-amylase.
4. *L. lactis* FXN was constructed through overexpression of XylR and heterologous expression of NrpB. The nisin yield and the cell density of FXN with 50% enzymatic

hydrolysate were equivalent to that of F44 with 60% of the enzymatic hydrolysate content in CHRSF process.

5. The strategy of NoxE overexpression and hemin addition notably increased nisin production by enhancing the ability of *L. lactis* to handle oxidative stress under aerobic condition in SHFRS process.

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APPENDIX

Supplementary Data

Table S1. Primers Used in This Study

Primers	Sequences (5 ’to 3’)
NprB-F	ACGTCATATGGATCCAAATGGTAGATTCCAAAATAGG
NprB-R	TCTCTCCTAATCACTCAGTCAGACTCTGTGTCCTGA
XylR-F	CACAGAGTCTGACTGAGTGATTAGGAGAGAATGAACATG
XylR-R	CCTATATATGGATCCTGTTAAAAATAAAGAGTCTAAAAATGA
NoxE-F	AACTTAAGTAAGCTTTGCAGAAGAGGAAGATTAGCC
NoxE-R	CGTCTGCAGAAGCTTTTAGGACATTAGTCACAAAAAA

Table S2. Strains Used in This Study

Strains	**Relevant Characteristics**	**Sources or References**
Lactococcus lactis F44	Nisin Z producer, constructed through genome shuffling of *L. lactis* YF11	Zhang *et al.* 2014
Escherichia coli TG1	Used for plasmid preparation	Laboratory stock
Micrococcus flavus	Used as an indicator strain for bioassay of nisin	ATCC 10240
Bacillus subtilis 168	Used for cloning heterologous protease	Laboratory stock
L. lactis FXN	F44 harboring pLXN	This study
L. lactis FXNE	F44 harboring pLXNE	This study
Plasmids		
pLEB124	Gram$^+$ cloning vector with an P45 promoter followed by multiple restriction sites, Em^r	Qiao *et al.* 1995
pLXN	pLEB124 carrying the *xylR* gene from F44, nprB gene from *B. subtilis* 168, Em^r	This study
pLXNE	pLEB124 carrying *xylR* and noxE gene from F44, the *nprB* from *B. subtilis* 168, Em^r	This study

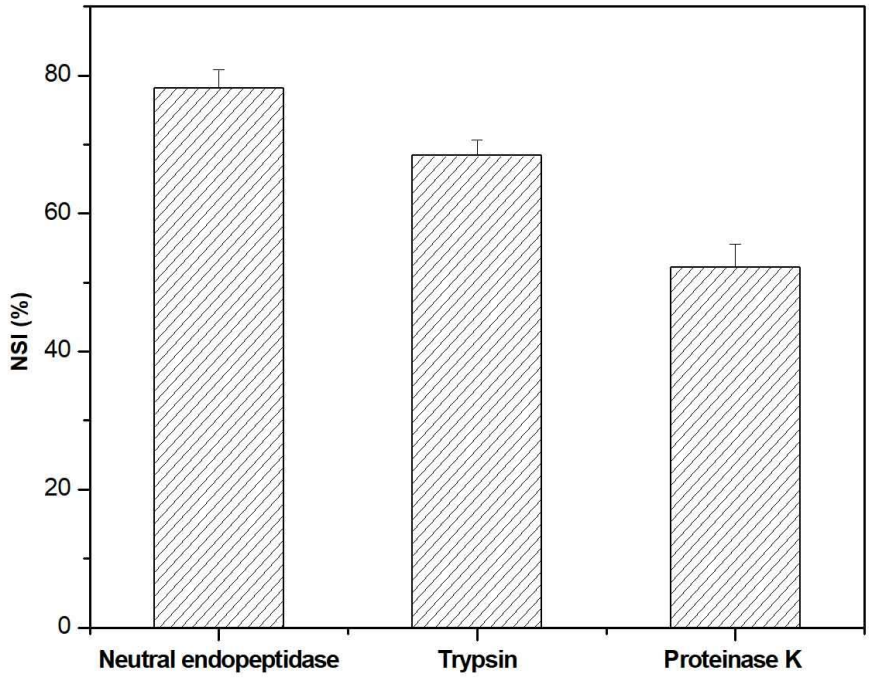

Fig. S1. Efficacy of enzymatic hydrolysis of different proteases; the enzyme dose was 10000 IU per gram DSM and the average data of triplicate experiments is presented; error bars represented the standard deviation of triplicate experiments