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Improved production of chitin from shrimp waste by fermentation with epiphytic lactic acid bacteria

Shan Duan*, Lei Li, Zejuan Zhuang, Wenya Wu, Shuying Hong, Jiehua Zhou

College of Food Science, South China Agricultural University, No. 483, Wushan Street, Tianhe District, Guangzhou City 510642, PR China

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ABSTRACT

The epiphytic *Lactobacillus acidophilus* SW01 isolated from shrimp waste (SW) was used in SW fermentation. During the fermentation the lag phase of SW01 was hardly observed. The pH of the fermentation broth decreased to 3.86 within 12 h and reached the lowest point at 3.73 after 48 h. This indicates a quick and deep acidification process by SW01. Besides, SW01 was observed to have high protease activity. As a result, the minerals and protein in SW were quickly removed with their contents decreasing to 0.73% and 7.8% respectively after 48 h fermentation. In the pilot scale fermentation, the pH was 3.99 and 3.86 respectively after 12 and 24 h fermentation. The mineral and protein contents were 0.98% and 8.44% respectively after 48 h fermentation. The residue of the fermented SW contains less than 1% minerals and can be easily transformed into chitin by a mere bleaching treatment.

1. Introduction

Chitin, the second largest carbohydrate source in nature, is widely distributed as a structural component of exoskeletons of crustaceans, insects and other arthropods, as well as a component of the cell walls of most fungi and some algae. It is an insoluble linear homopolymer of β-(1→4)-linked-*N*-acetyl-D-glucosamine (GlcNAc). The repeating structural unit of chitin is the dimer of GlcNAc (Muzzarelli et al., 2012). Chitin and its derivatives, such as chitosan and chito-oligosaccharides, have many important applications in the fields of pharmaceutical, food, agriculture, bio-engineering and cosmetics and other related fields. Therefore chitin has attracted the attention of people for decades.

Nowadays, a widely used chitin source is shrimp waste (SW), which mainly consists of cephalothorax and exoskeleton and accounts for ca. 40% of the shrimp weight. The output of SW is very high worldwide. For example in China over 500 kilotons are produced annually. Chitin might be more profitably recovered from SW if an effective technology is developed.

In SW, chitin is linked with proteins and forms chitin-protein complex. Interspersed with the complex are minerals and a small amount of lipids and carotenoid. Traditionally, chitin is extracted from SW using strong acid and alkali to remove minerals, protein and lipids. This method has some disadvantages. For one, it causes part hydrolysis of chitin (Brine & Austin, 1981; Healy, Romo, & Bustos, 1994). Secondly, it produces a considerable amount of alkali waste water, which contains a lot of protein, lipids and carotenoid. In some cases the waste water is discharged without any treatment, which leads to serious environmental pollution. Furthermore, the protein, lipids and carotenoid cannot be recovered. Even though nowadays the waste water can be treated by couple-membrane filtration and the protein and sodium hydroxide can be recovered (Zhao, Xia, and Zhao, 2011), the capital and operating costs for such treatment are very high. A newer approach in chitin production is the use of proteolytic microorganisms (Jo et al., 2008) or protease (Ghorbel-Bellaaj et al., 2011) to remove the protein in SW. However, this approach leaves the minerals intact. As a result, strong acid has to be used to remove the minerals to produce chitin.

To solve the above problems, some researchers have tried to use lactic acid bacteria (LAB) to ferment SW to extract chitin. Under this method, lactic acid removes most minerals from SW. And most protein is hydrolyzed by protease and transferred into the fermentation broth. Chitin, protein, and carotenoid in SW can be obtained simultaneously. Moreover, the above-mentioned environmental pollution is avoided. Besides, the chitin obtained in this way has a higher molecular weight and crystallinity than the chemically extracted chitin (Pacheco et al., 2011).

This method is very promising. As a result, it has been developed, improved and optimized by different researchers. For example, Zakaria, Hall, and Shama (1998) studied lactic acid fermentation

Abbreviations: SW, shrimp waste; LAB, lactic acid bacteria; DH, degree of hydrolysis.

* Corresponding author. Tel.: +86 20 85292803; fax: +86 20 85280270.
E-mail address: duanshan@scau.edu.cn (S. Duan).

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of scampi waste in a rotating horizontal bioreactor for chitin recovery. Shiraia et al. (2001) studied the effect of glucose concentration and inoculation level in lactic acid fermentation of SW for chitin recovery and SW preservation. Cira, Huerta, Hall, and Shirai (2002) studied the lactic acid fermentation of SW in pilot scale for chitin recovery. Bhaskar, Suresh, Sakhare, and Sachindra (2007), Sachindra, Bhaskar, Siddegowda, Sathisha, and Suresh (2007) and Choorit, Patthanamanee, and Manurakchinakorn (2008) separately optimized the lactic acid fermentation process for chitin and carotenoid recovery from SW. Pacheco et al. (2009) studied the effect of temperature in lactic acid fermentation of SW on chitin and carotenoid recoveries. Duan, Zhang, Lu, Cao, and Chen (2011) studied the fermentation of SW with 3 strains of symbiotic LABs to recover chitin and protein.

However, a problem common to all the above-mentioned researches is that the removal of minerals is insufficient. As a result, the fermented SW has to be treated with hydrochloride acid for further mineral removal. An important reason for this is the insufficient acidification in fermentation.

To improve the acidification in SW fermentation and promote the removal of minerals, our group has attempted different approaches. One way was to isolate epiphytic LABs from SW and use it in SW fermentation. This method was based on the assumption that epiphytic LABs were likely to grow better than others in SW and produce more acid. As expected, 3 strains of epiphytic LABs were isolated form SW. When growing in SW, one of them was found capable of producing much more acid than the other two. It was identified as *L. acidophilus* and named as SW01. The factors influencing its acid production and protein hydrolysis in SW fermentation was explored (Li, Zhuang, Duan, Liu, & Lin, 2011).

In this research, SW01 was further compared with other LABs in terms of acid production and protein hydrolysis in SW fermentation. The ingredient changes in SW fermentation with SW01 were investigated. Finally pilot scale fermentation of SW with SW01 was conducted.

2. Materials and methods

2.1. Materials

Shrimp (*Penaeus vannamei*) waste was provided by the Kangwei Seafood Co., Ltd. in Yangjiang City, Guangdong Province, China.

Lactobacillus acidophilus SW01 was isolated from shrimp waste. *L. acidophilus* CICC6074, *L. plantarum* CICC6234, *L. plantarum* CICC6009, *S. thermophilus* CICC6038, *S. thermophilus* CICC6063 were purchased from China center of industrial culture collection. *L. plantarum* GIM1.191, *L. acidophilus* GIM1.208, *L. delbrueckii subsp. bulgaricus* GIM1.204, *L. bulgavicus* GIM1.189, *L. helveticus* GIM1.157 were purchased from Guangdong culture collection center.

2.2. Methods

2.2.1. Acid tolerance test of LABs

The MRS medium was adjusted with lactic acid to pH 4.0 and pH 3.5 respectively. The LABs to be tested were inoculated in the pH 4.0 and pH 3.5 MRS broths respectively and then cultivated at 37 °C for 24, 48 and 72 h. The survived LABs was counted and expressed as colony forming units (cfu). Its survival rate was defined as the ratio of survived LAB population to initial LAB population.

2.2.2. Fermentation of SW in laboratory

The starter culture for SW fermentation was prepared by cultivating *L. acidophilus* SW01 in MRS broth (de Man, Rogosa and Sharpe broth) at 37 °C for 18 h. 100 g minced SW (not sterilized), 15 g glucose and 10 ml water were put into a 250 ml conical flask and mixed together. Then 5 ml starter culture was inoculated into the mixture with the initial inoculum density ca. 1.1×10^6 cfu/ml. Then the flask was covered with an air permeable silica gel stopper and put at 37 °C for 168 h. The fermentation temperature, the inoculum size, the amount of glucose and the volume of added water were according to the results of our previous research (Li et al., 2011).

2.2.3. Analysis of samples during SW fermentation

Various parameters of the fermentation broth of SW were measured as follows.

The pH was measured using a pH meter (Sartorius PB-10). The acidity was determined by titrating samples with 0.1 mol/l NaOH solution to pH 8.0 and expressed as lactic acid (AOAC, 1984). The amino nitrogen content was determined by formaldehyde titration method (Adler-Nissen, 1986), and further the degree of hydrolysis (DH) of protein was calculated according to Adler-Nissen's method (1986). The glucose content was determined by Fehling reagent (AOAC, 1984). The TVBN content was measured by the method of Conway's dish (Cobb, Aoaniz, & Thompson, 1973), and was expressed in mg/100 g SW. The LAB population was determined by plate count method on MRS agar plates.

The protein and mineral content of the fermented SW was respectively determined by micro kjeldahl method (AOAC, 1984) and by burning samples at 550 °C in a muffle furnace (AOAC, 1984).

3. Results and discussion

3.1. Selection of starter culture and laboratory scale fermentation of SW

L. acidophilus SW01 was compared with other LABs in terms of acid production level and DH of protein in SW fermentation. As shown in Table 1, *S. thermophilus* CICC6038 and CICC6063 didn't grow in SW. While, *L. acidophilus* SW01, *L. acidophilus* GIM1.208, *L. acidophilus* CICC6074 and *L. plantarum* CICC6234, especially *L. acidophilus* SW01, grew well in SW and produced much more acid than other LABs. As for the DH of protein, the sample fermented by *L. acidophilus* SW01 achieved a much higher level than all other samples. This illustrates that *L. acidophilus* SW01 has much higher protease activity than other LABs. Since most LABs lack protease activity, the high protease activity of *L. acidophilus* SW01 in this research is unusual.

L. acidophilus SW01, *L. acidophilus* GIM1.208, *L. acidophilus* CICC6074 and *L. plantarum* CICC6234, which produced more acid in SW fermentation, were further compared in terms of their acid tolerance ability. In pH 4.0 and pH 3.5 MRS medium, the survival rates of *L. acidophilus* SW01 and GIM1.208 were approximately equal, but higher than that of CICC6074 and CICC6234 (Fig. 1). This means that *L. acidophilus* SW01 and GIM1.208 are more endurable in high acidic environment, and therefore more suitable for SW fermentation.

Compared with GIM1.208, *L. acidophilus* SW01 has much higher protease activity. The higher protease activity will promote the removal of protein. Therefore, *L. acidophilus* SW01 is finally selected as the starter culture for SW fermentation.

L. Acidophilus are facultative anaerobic bacteria. Though *L. Acidophilus* may live with or without oxygen, whether oxygen influences SW fermentation with SW01 needs to be investigated. The investigation steps went as follows.

Two 100 ml conical flasks were used and each of them was filled with 20 g SW and 40 ml water. Then both were sterilized to eliminate the influence of other bacteria. Glucose was autoclaved separately. After cooling, each flask was added with 3.0 g sterilized glucose and 2.0 ml starter culture. One conical flask was covered with an air permeable silica gel stopper to let air exchange in and out of the flask freely. The other was covered with an air proof

Table 1
Results of shrimp waste fermentation by different strains of LAB.

	24 h later			48 h later			72 h later		
	pH	Acidity (mmol/100 ml)	DH of protein (%)	pH	Acidity (mmol/100 ml)	DH of protein (%)	pH	Acidity (mmol/100 ml)	DH of protein (%)
SW01	3.72	14.36	18.8	3.47	21.75	19.3	3.40	25.91	20.7
L. helveticus GIM1.157	5.91	2.32	6.1	5.17	3.36	8.7	5.04	3.61	6.9
L. bulgavicus GIM1.189	5.88	2.37	7.1	5.20	3.17	5.3	4.07	12.50	1.8
L. plantarum GIM1.191	5.92	2.01	7.9	5.24	2.96	9.1	5.12	3.41	5.3
L. delbrueckii subsp. bulgaricus GIM1.204	6.42	1.48	3.9	6.10	1.94	6.1	5.93	2.52	6.3
L. acidophilus GIM1.208	3.95	13.83	9.3	3.73	20.59	10.3	3.63	23.74	8.9
L. plantarum CICC6009	6.76	1.21	7.3	6.21	1.72	9.7	6.07	2.13	8.7
L. acidophilus CICC6074	3.97	13.59	8.5	3.73	20.59	9.5	3.64	23.50	8.5
L. plantarum CICC6234	3.98	13.19	6.1	3.87	19.22	8.9	3.66	22.89	6.9
S. thermophilus CICC6038	–[a]	–	–	–	–	–	–	–	–
S. thermophilus CICC6063	–[a]	–	–	–	–	–	–	–	–

DH: the degree of hydrolysis. Fermentation conditions: Shrimp waste was sterilized and mixed with 15% glucose and 150% water. Each of the LAB slant cultures was separately inoculated into MRS broth and cultivated at 37 °C for 18 h. Then the shrimp waste was separately inoculated with 3% of each of the MRS broths and fermented at 37 °C.
[a] The LAB didn't grow.

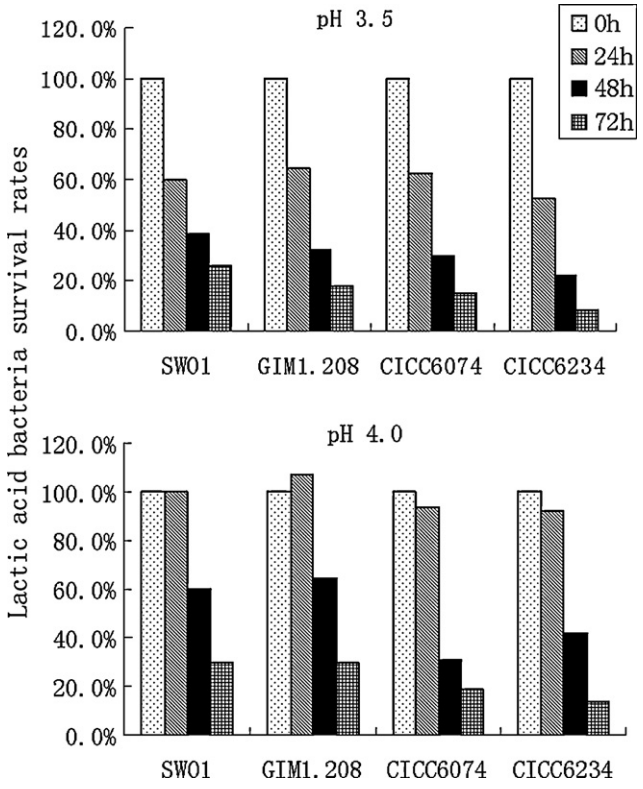

Fig. 1. LAB survival rates in acidified MRS medium.

rubber stopper. The air proof stopper was fitted with a length of glass tube to release the large amount of carbon dioxide produced in fermentation (because the carbonate in shrimp waste was dissolved by lactic acid in fermentation); the outer end of the glass tube was submerged in water to prevent ambient air from leaking into the flask. Finally the two conical flasks were incubated at 37 °C for 96 h. During the incubation period, samples were taken periodically for analysis.

The result of this investigation is shown in Fig. 2. It can be observed from Fig. 2 that the changes of pH, acidity and DH values in sample 1 and 2 were quite similar, though the two samples were under different aeration conditions. Variance analysis showed no significant difference in pH, acidity and DH values between the two samples ($p > 0.05$). This result illustrates that oxygen concentration has no significant influence on SW fermentation with *L. acidophilus* SW01. Therefore, it is unnecessary to consider the ventilating or air proof problem in SW fermentation with SW01.

Laboratory scale fermentation of SW with *L. acidophilus* SW01 was further conducted and the ingredient changes in fermentation were investigated. As shown in Fig. 3, In SW fermentation with *L. acidophilus* SW01, the pH of the fermentation broth decreased drastically in the initial 12 h and then decreased slowly. After fermentation for 8, 12 and 24 h, the pH reached 4.37, 3.86 and 3.73 respectively. 24 h later, the pH almost kept stable. In contrast to pH, the acidity of the fermentation broth increased drastically in the initial 24 h, and kept increasing within 48 h with its highest level reaching 46.5 mmol/100 ml. Compared with other related researches (Bhaskar et al., 2007; Choorit et al., 2008; Cira et al., 2002; Evers & Carroll, 1996, 1998; Fagbenro, 1996; Pacheco et al., 2009; Sachindra et al., 2007), the acid production in this fermentation was quicker and deeper.

The protein in SW was quickly removed in the initial 12 h with its content decreasing from 49.7% to 11.2% (dry weight) and then being removed at a much slower rate. After 48 h fermentation, the protein content decreased to 7.8%. At the end of this fermentation the protein content in SW decreased to 5.4% (Fig. 3). Ca. 97.4% protein was removed. This removal rate of protein was much higher than that reported by Cira et al. (2002), Pacheco et al. (2009) and Duan et al. (2011) and was close to that reported by Bhaskar et al. (2007).

During the fermentation, the mineral content of SW quickly decreased from 22.0% to 1.68% in the initial 12 h, and then slowly decreased to 1.09% and 0.73% (dry weight) respectively 24 h and 48 h later. At the end of the fermentation (168 h) the mineral content was 0.50% (Fig. 3). Cira et al. (2002) reported that the SW sediment contained 6.31% calcium after 6 d fermentation with *lactobacilli* spp. Bhaskar et al. (2007) and Choorit et al. (2008) reported that 72.5% and 83.47% minerals were removed respectively in lactic acid fermentation of SW. In our previous research (Duan et al., 2011) SW was fermented with 3 strains of symbiotic LABs, 91.3% calcium was removed after 168 h fermentation. Compared with the above-mentioned researches, the removal of minerals in this research was remarkably improved.

A noteworthy point is that in this research the mineral content of SW has decreased to 0.73% (dry weight) after 48 h fermentation. This level of mineral content has reached China's standard of chitin (according to China's national standard, the mineral content of chitin should be below 1.0%). Thus, the fermented SW obtained in this research can be easily transformed into chitin by a simple bleaching procedure. However, the fermented SW obtained from the above-mentioned researches had to be treated with hydrochloric acid for further mineral removal to produce chitin.

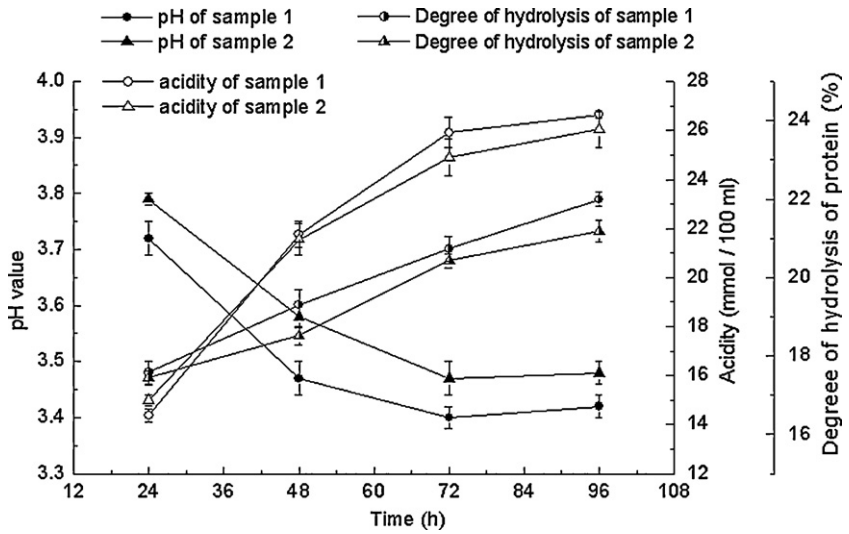

Fig. 2. The influence of oxygen on shrimp waste fermentation with SW01. Sample 1 is the shrimp waste fermented in air permeable conical flask; sample 2 in air proof conical flask.

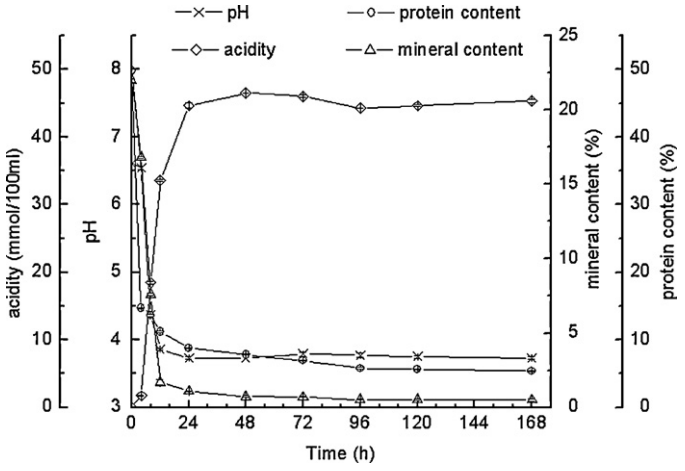

Fig. 3. Changes of pH, acidity, minerals and protein content in shrimp waste fermentation with SW01. Minerals and protein content was calculated on dry base.

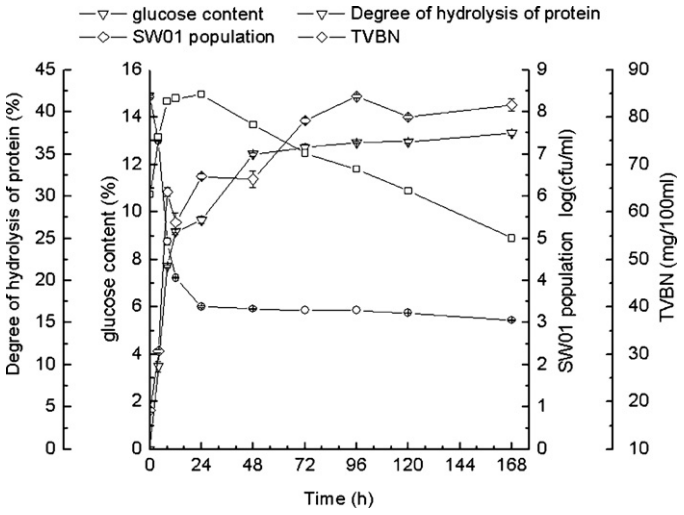

Fig. 4. Changes of SW01 population, glucose and TVBN content in shrimp waste fermentation.

It is certain that the stronger acidity formed by *L. acidophilus* SW01 in this fermentation promotes the removal of minerals, as has been reported by Cira et al. (2002) that lactic acid reacts with the minerals carbonate in SW and leads to the removal of minerals. However, a comparison of this research with our previous ones (Duan et al., 2011; Lin, Zhuang, Liu, Li, and Duan, 2010) showed that at the same acidity level, the removal of minerals varied and in this research more minerals were removed. This phenomenon implies that besides acidity, some other factor(s) also influence the removal of minerals. According to Muzzarelli (2011), the carapace of crustaceans is a multiphase nano-composite consisting of an organic matrix (crystalline chitin and non-crystalline proteins) and minerals, and the minerals are embedded in the chitin-protein matrix. Therefore a reasonable explanation for the improvement in the removal of minerals in this research is that *L. acidophilus* SW01 has much stronger protease activity than other LABs (Table 1), the removal of protein is improved in this research. With more protein removed in shrimp carapace, more minerals are exposed to acidic environment, which has led to the improvement of the removal of minerals.

The level of TVBN was low throughout the whole fermentation period, though some increase was observed (Fig. 4). This indicates that putrefaction is effectively inhibited. Lactic acid fermentation of fish waste also resulted in much lower formation of TVBN (Dapkevicius, Batista, Nout, Rombouts, and Houben 1998).

The DH of protein quickly increased in the initial 48 h and then increased at a much slower rate (Fig. 4). However the DH of protein was much higher than that of our previous researches (Duan et al., 2011). The higher DH of protein is caused by the high protease activity of *L. acidophilus* SW01. Pacheco et al. (2009) and Duan et al. (2011) indicated that the protein is removed by the protease, including the endogenous protease of SW and the protease produced by LAB. The higher DH of protein explains the higher removal rate of protein in this fermentation (Fig. 3).

The lag phase of *L. acidophilus* SW01 was hardly observed (Fig. 4). This illustrates that SW01 is well adapted to growing in SW. The quick increase of SW01 population in the initial 24 h indicated that it was in logarithm phase. 24 h later, SW01 population came to decrease (Fig. 4) and the acidity almost reached its highest level (Fig. 3). This result indicated that *L. acidophilus* SW01 was inhibited by the strong acidic environment and gradually entered autolysis phase 24 h later.

The glucose concentration quickly decreased in the initial 24 h with its concentration decreasing from 14.87% to 6.01% (Fig. 4),

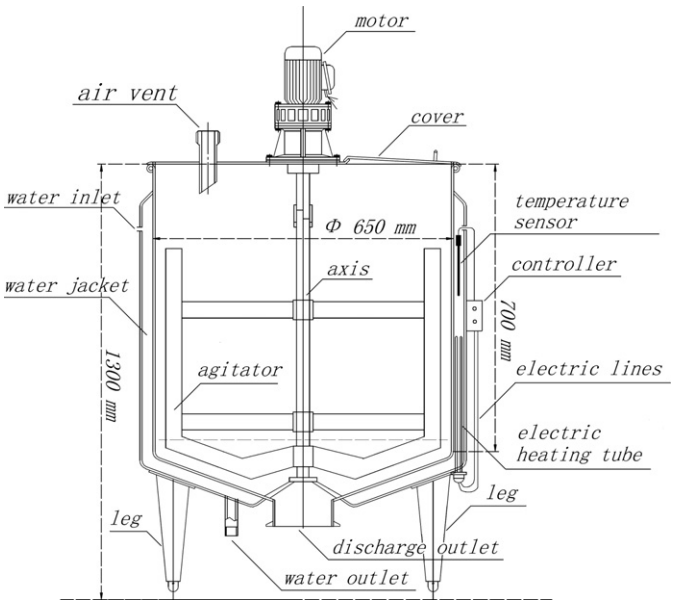

Fig. 5. The schematic diagram of the 200 l pilot scale fermenter.

which meant that 59.6% of total glucose was consumed during the period. 24 h later, hardly any glucose was consumed and its concentration just decreased to 5.44% till the end of fermentation.

In the initial 24 h, *L. acidophilus* SW01 was in logarithm phase and needed much glucose to maintain the multiplication and growth of the cells. Besides, much glucose was converted into lactic acid, which could be reflected from the quick removal of minerals and the quick increasing of acidity in the initial 24 h (Fig. 3). 24 h later much less glucose was consumed, because: first, *L. acidophilus* SW01 gradually entered autolysis phase; second, the acidity of fermentation broth almost reached the highest level; third, little minerals were removed from SW during the period. Evidently the change of glucose concentration is well in accordance with the changes of *L. acidophilus* SW01 population, the removal of minerals and the increasing of acidity. The changing patterns of glucose and lactic acid in this research are very similar to the result of Shiraia et al.'s report (2001).

3.2. Pilot scale fermentation and recovery of protein and chitin

Pilot scale fermentation was carried out with *L. acidophilus* SW01 in a 200 l fermenter. The schematic diagram of the fermenter is shown in Fig. 5. The inner wall of the fermenter and the frame type agitator are made of acid-resistant stainless steel. The fermentation was repeated for five cycles. In each cycle 50 kg minced SW (not sterilized), 7.5 kg glucose and 5 l water were mixed together, and then 2.5 l starter culture was inoculated. The fermentation was carried out at 40 °C rather than 37 °C, the temperature of the laboratory fermentation. For the work was done in summer, the workshop, where the work was conducted, was not air-conditioned and the ambient temperature reached 35 °C in the daytime. Because the cooling of the fermenter depended on air, it was impossible to cool the fermenter to 37 °C in the daytime. Moreover, according to our previous research (Li et al., 2011), the growth and acid production of SW01 almost remains constant within the range of 37–40 °C. Therefore, 40 °C was adopted in the pilot scale fermentation.

Around 4th to 12th hour of the fermentation, much foam was produced in the fermenter because the carbonate in SW was dissolved by lactic acid and much carbon dioxide was released. To prevent overflow, the agitator was turned on and rotated at 15 rpm

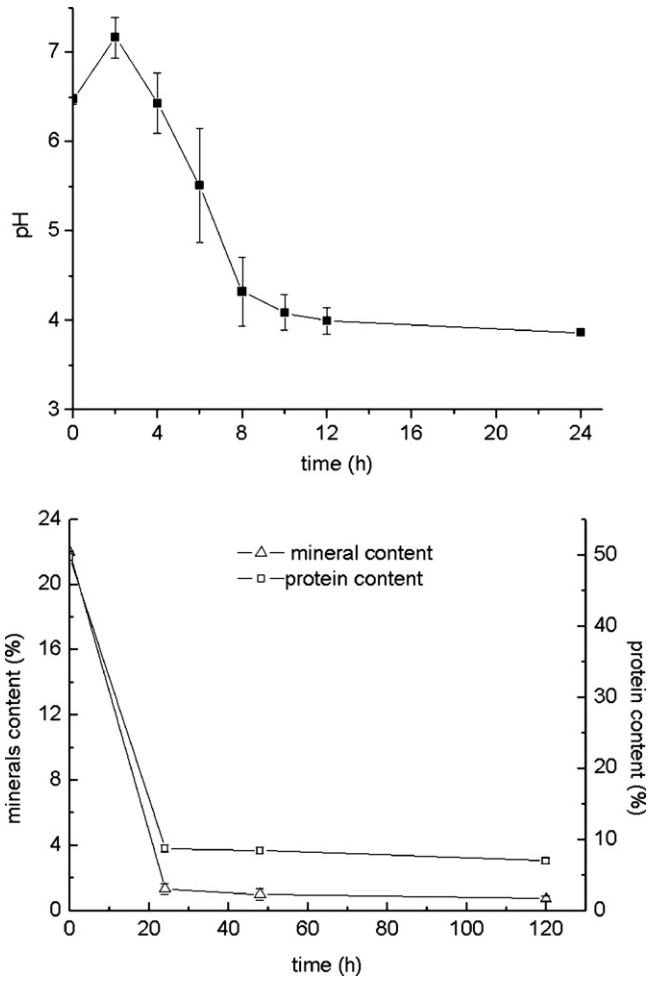

Fig. 6. Change of pH, protein and minerals content in pilot scale repeated-batch fermentation of shrimp waste with SW01. The value of each point is the mean of five cycles.

to eliminate the foam during the period. After 24 h fermentation, the fermentation broth and sediment were withdrawn from the fermenter and put at ambient temperature for 96 h (the ambient temperatures were between 28 and 35 °C).

During the pilot scale fermentation the pH of the broth quickly decreased to 4.32 and 3.99 respectively after 8 and 12 h fermentation. 24 h later it further decreased to 3.86 (Fig. 6). An interesting phenomenon was that the pH of the broth increased from 6.48 (the natural pH of SW is 8.0, which, after inoculation, became 6.40 and 6.60 with the mean at 6.48 because of the acidity of starter culture) to 7.17 2 h later after inoculation (Fig. 6). Two reasons accounted for the increasing of pH. One was that much amino acids and peptide, which had strong buffering capacity, were produced by the hydrolysis of protease from SW and *L. acidophilus* SW01. The other was that the minerals carbonate in SW consumed some lactic acid.

The mineral content in SW quickly decreased from 22.0% to 1.34% within 24 h, and further decreased respectively to 0.98% and 0.72% 48 h and 120 h later (Fig. 6). Protein in SW was removed in a trend similar to that of minerals. Its content decreased from 49.7% to 8.74% within 24 h and further decreased respectively to 8.44% and 7.01% 48 h and 120 h later (Fig. 6). Though some differences existed in the fermentation conditions, the results of pilot scale fermentation were very close to that of laboratory experiments (Fig. 3). This fact indicates that fermentation is stable and *L. acidophilus* SW01 is suitable for industrial application.

After fermentation the SW turned into two parts – the fermentation broth and the sediment (the crude chitin). The two parts were separated by filtration. Analyses of the two parts showed that 96.5% protein was removed from SW and 90.3% protein transferred into the liquid part. The 6.2% loss of protein was due to the formation of volatile nitrogen compounds in fermentation and the adsorption by the residue.

To transform the after-fermentation residue of SW into chitin, the residue was soaked in 1.0% potassium permanganate solution for 1.5 h and then in 1.0% oxalic acid solutions for 0.5 h for bleaching purpose. After that, the bleached SW was washed with water till neutral and put in the air to dry. The result of this work is white-flaked chitin.

4. Conclusions

Fermenting SW with epiphytic *L. acidophilus* SW01 remarkably improves the removal of minerals and protein. The fermented SW can be easily transformed into chitin by a mere bleaching treatment. SW01 is well adapted to growing in SW, and is hardly influenced by oxygen and requires no other ingredients except glucose in SW fermentation. The result of pilot scale fermentation is very close to that of laboratory experiments. These facts illustrates that the epiphytic *L. acidophilus* SW01 is very suitable for SW fermentation and very promising for industrial application.

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