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Biotechnological process of chitin recovery from shrimp waste using Lactobacillus plantarum NCDN4

Thu hồi chitin từ phế liệu tôm bằng phương pháp sinh học sử dụng Lactobacillus plantarum NCDN4

Short communication

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Chitin in shrimp waste is tightly associated with proteins, lipids, pigments and mineral deposits. Therefore, these source materials have to be pretreated to remove these components. For a long time, chemical process has been used widely for extraction of chitin from shrimp waste. The chemical process however led to severe environmental damage and low chitin quality. The biological process has been shown promising to replace the harsh chemical process to reduce the environment impact. In our previous study chitin recovery from sterilized shrimp waste by *Lactobacillus plantarum* NCDN4 was investigated. However in large scale it is uneconomical to sterilize the shrimp waste. For that reason, in this study the microbial process using *Lactobacillus plantarum* NCDN4 for chitin recovery from unsterilezed shrimp waste has been investigated. Factors affecting the demineralization by this strain such as inoculum size, glucose concentration, initial pH, NaCl concentration and fermentation time were investigated. It was found that when unsterilized shrimp waste fermented with 20% *L. plantarum* inoculum, 12,5% glucose, and pH 6 for 4 days at 30^{o}C, 99. 28% demineralization and 48.65% deproteination could be achieved. The ash and protein content of fermented residues were 1.33% and 22.46% respectively. Compared to sterilized condition the efficiency of demineralization and deproteination was similar.

Chitin trong phế liệu tôm liên kết chặt chẽ với protein, sắc tố và khoáng. Do vậy để thu được chitin cần có các bước tiền xử lí để loại các thành phần không phải chitin ra. Phương pháp hóa học được sử dụng rộng rãi từ lâu để tiền xử lí chitin. Tuy nhiên do phương pháp hóa học gây hại cho môi trường và tạo ra chitin chất lượng thấp, các nhà khoa học nỗ lực nghiên cứu tìm ra các phương pháp thay thế. Phương pháp sinh học được xem là rất khả quan để thay thế phương pháp hóa học. Trong nghiên cứu trước của chúng tôi, quá trình lên men phế liệu tôm thanh trùng bằng Lactobacillus plantarum NCDN4 đã được nghiên cứu. Tuy nhiên việc thanh trùng phế liệu tôm không kinh tế. Trong nghiên cứu này quá trình lên men phế liệu tôm không thanh trùng bằng Lactobacillus plantarum NCDN4 đã được khảo sát. Các yếu tố như tỷ lệ giống, nồng độ đường glucose, nồng độ NaCl, pH ban đầu của môi trường lên men và thời gian lên men đã được nghiên cứu. Kết quả cho thấy ở điều kiện 20% giống theo thể tích, 12,5% dịch đường glucose, 2% muối theo khối lượng, pH ban đầu 6, sau 5 ngày lên men lượng khoáng và protein trong nguyên liệu giảm tương ứng 99.28% và 48.65%. Lượng khoáng và protein còn lại tương ứng 1.33% và 22.46% (tính theo trọng lượng khô). So với phế liệu tôm không thanh trùng, hiệu quả loại khoáng và protein là tương đương.

Keywords: chitin recovery, biological process, *Lactobacillus plantarum*, demineralisation, deproteination

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1. Introduction

Vietnam is one of the largest shrimp exporters in the world. Shrimp waste represents 50% of the weight of raw material. This waste on one hand pollutes environment, on other contains valuable components such as chitin, protein and pigments. Chitin is a non-toxic, biodegradable polymer of ß-1,4-N-acetyl-D-glucosamine, which is widely distributed in nature and found from 14-30% in shrimp waste (on dry basis). Shrimp waste was the most important source for chitin recovery. Chitin in shrimp waste is tightly associated with proteins, lipids, pigments and mineral deposits (No and Meyers, 1989). Therefore, in order to obtain purified chitin, it must be separated from them. Conventional chemical harsh treatment methods for the commercial preparation of chitin from shrimp shell involve alternate hydrochloric acid and alkali treatment stages to remove calcium carbonate and proteins. This kind of chemical method produces chitin of low quality and is extremely hazardous, energy consuming and ultimately damaging to the environment.

The alternative biological method is fundamentally based on using protease or protease producing strain for deproteination (De Holanda and Netto, 2006, Gildberg and Stenberg, 2001, Mizani *et al.*, 2005, Sini *et al.*, 2007, Synowiecki and Al-Khateeb, 2000, Waldeck *et al.*, 2006, Wang and Chio, 1998); and lactic bacteria for demineralisation (Jung *et al.*, 2005, Rao *et al.*, 2000, Xu *et al.*, 2008). This biotechnological approach leads to liquor fraction rich in proteins, minerals and astaxanthin and to a solid chitin fraction. The liquor fraction can be used as supplement to animal feed or for human consumption.

Commercial bacterial proteases derived from *Bacillus* broth such as Alcalase, chymotrypsin, papain, Neutrase has been studied (De Holanda and Netto, 2006, Gildberg and Stenberg, 2001, Mizani *et al.*, 2005). The process though effectively used in laboratory scale, causes uneconomical production costs. Therefore the use of living microbes especially *L. plantarum* facilitating efficient chitin purification is desirable. In fermentation of shrimp waste by lactic bacteria, lactic acid is produced creating low pH condition to prevent spoilage microorganisms. Furthermore lactic acid reacts with the calcium carbonate in shrimp waste to form lactate calcium which precipitates and can be removed by washing (Rao *et al.*, 2000). The efficiency of lactic fermentation depends much on fermentation conditions.

In our previous study *Lactobacillus plantarum* NDCN4 was selected due to its high protease activity and high lactic acid production. Furthermore the microbial process using *Lactobacillus plantarum* NCDN4 for chitin recovery from sterilized shrimp waste has been investigated (Le *et al.*, 2011). However in large scale it is uneconomical to sterilize the shrimp waste. For that reason, in this study the microbial process using *Lactobacillus plantarum* NCDN4 for chitin recovery from unsterilized shrimp waste has been investigated.

2. Materials and methods

2.1 Materials

Shrimp shells and heads were obtained from Quang Ninh province and kept frozen at -20^{o}C to prevent spoilage. The shrimp shell were defrozen in refrigerator at 4^{o}C overnight before use. The contents of shrimp waste were: moisture 69.75±1.34%, ash 29.32±1.9% (dry basis), 39.62±1.53% (dry basis), *Lactobacillus platarum* NCDN4 were obtained from the collections of microorganisms of Food Industry Research Institue (FIRI).

2.2 Inoculum preparation and fermentation condition

Lactobacillus plantarium NCDN4 was cultivated in MRS medium at 30^{o}C for 18 h and was ready as inoculum. 40g of shell waste was added into 250 ml flasks containing glucose solution of different concentration at ratio shirmp waste/glucose of 2/1 (v/w). The pH of media, the inoculum size and fermentation time were used as indicated in text (see below). The fermented shrimp waste was filtered to remove liquor fraction. The remaining solid chitin fraction was washed out, oven dried and used for further analysis of moisture, protein and ash content.

2.3 Analytical methods

Shrimp shells were kept in NaOH 3% solution at the ratio of 1:10 firstly overnight at room temperature and afterward at 90^{o}C for 1 h. After centrifugation at 6000 rpm for 20 min, the protein content in supernatant was measured using Biuret method. The dry weight was determined after drying for 24 h at 105^{o}C. Ash content was determined by heating at 600^{o}C for 4 h.

The deproteination was calculated using the following equation DP = [PO*O-PR*R]*100/PO*O whereas the O and R are the masses before and after fermentation respectively (g), PO and PR are the protein contents of original and residue samples respectively (g/g)

The demineralization efficiency was calculated using similar equation, instead of PO and PR, the AO and AR are the ash contents of original and residue sample respectively (g/g).

DM = [AO*O-AR*R]*100/AO*O

3. Results and discussion

3.1 Effect of inoculum concentration

Inoculum size normally influences the fermentation. Low inoculum size prolongs the fermentation whereas high inoculum size reduces the cell growth ability. The shrimp waste was mixed with 10% glucose of pH 6 and was inoculated with different inoculum size 10%, 15% and 20% (v/w). After 5 days of fermentation at 30^{o}C, the demineralisation and deproteination were determined.

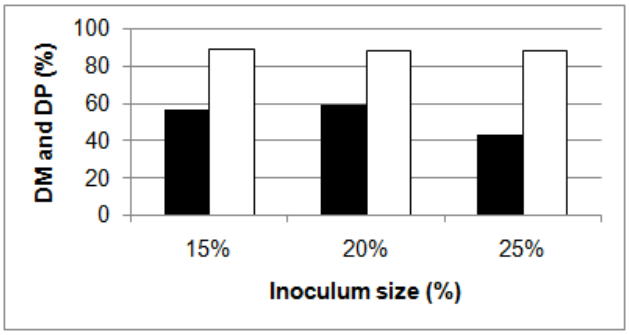

Figure 1. Effect of inoculum size on DM (white bar) and DP (black bar) efficiency

Compared to sterilized condition (Le *et al.*, 2011), whereas the inoculum size influenced the demineralization considerably, in this study the effect was insignificant (Fig 1). Increase of inoculum size from 10% to 25% resulted in only small changes of demineralization efficiency, which was around 88%.

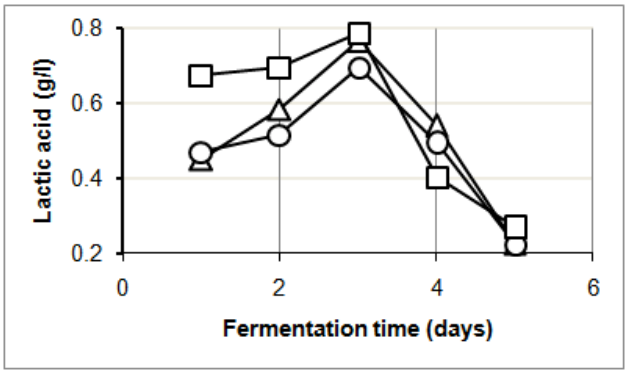

Figure 2. Lactic acid production during fermentation of shrimp waste with inoculums size of 15% (Δ), 20% (○) and 25% (□)

The results from lactic acid production (Figure 2) and pH changes during fermentation (data were not presented here) revealed that the lactic acid production at higher inoculum size was higher only during first three day and were almost similar for the last two days. Therefore the demineralisation after 5 days was similar independent of inoculums size. However the deproteination was strongly affected. Higher inoculum size of 25% reduced the deproteination from 56.06-58.52% to 42.95%. This could be explained by inhibition of deproteination process by high lactic acid production. Unlike sterile shrimp waste, where protein removal was only due to protease produced by *L. plantarum* NCDN4, the deproteination of unsterile shrimp waste was due to additional protease available in shrimp waste. The unsterile shrimp waste was rich in protease and therefore was easily autolysis (Cao *et al.*, 2009). High lactic acid concentration during first day of fermentation (Figure 1) at high inoculums size seemed to inhibit shrimp waste protease. Thus inoculum size of 20% was optimal for both demineralization and deproteination process. The optimal inoculums size for fermentation of sterile shrimp waste was 15 % (Le *et al.*, 2011), which was 1.3-fold lower. Unsterile shrimp waste required higher inoculums size to inhibit the growth of indigenous spoilage bacteria in shrimp waste.

3.2 Effect of glucose concentration

The shrimp waste was mixed with glucose at concentration of 10%, 12,5% and 15% and was inoculated with 20% inoculum of *L. plantarium* NCDN4 (v/w). After five days of fermentation at 30°C, the demineralisation and deproteination were determined.

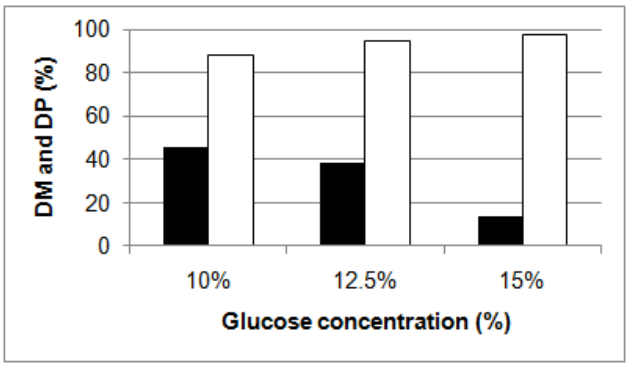

Figure 3. Effect of glucose concentration on DM (white bar) and DP (black bar) efficiency

Results from Figure 3 showed the increase of demineralization from 87.83% to 97.41% in accordance with the increase of glucose concentration from 10% to 15%. These results were in good correspondence to pH changes and lactic acid production (data were not shown). According to Rao (Rao *et al.*, 2000), pH after fermentation was lower at higher concentration of glucose since lactic acid is produced by breaking down of glucose. The deproteination was however inversely proportional to glucose concentration, which decreased almost four-fold from 45.72% to 13.81%. Thus for both good demineralization and deproteination, glucose concentration of 12.5% seemed reasonable. For similar degree of demineralization (about 95%) of sterile shrimp waste, 20% molasses was needed (Le *et al.*, 2011). Since molasses contained only 50% sugars 20% molasses corresponded to 10% glucose. This revealed that lower glucose concentration was needed for sterile shrimp waste than for unsterile one. This was explainable since spoilage bacteria in shrimp waste consumed extra glucose.

3.3 Effect of NaCl concentration

In order to prevent spoilage the NaCl normally can be added to the fermentation medium since lactic bacteria can grow in salted medium but not the spoilage one. To find out the suitable concentration of NaCl which still supports the growth of *L. plantarium* NCDN4, the NaCl was supplemented to the mixture of shrimp waste and glucose at concentrations of 2, 4, 6% (w/w). After 5 days of fermentation at 30°C, the demineralization and deproteination were determined.

From Figure 4 it can be seen that, the higher the NaCl concentration the lower demineralization and deproteination process. Thus the high NaCl concentration inhibited both *L. plantarum* and shrimp waste protease, whereas at

NaCl 6%, the protease seemed stronger affected. Thus NaCl of 2% was chosen.

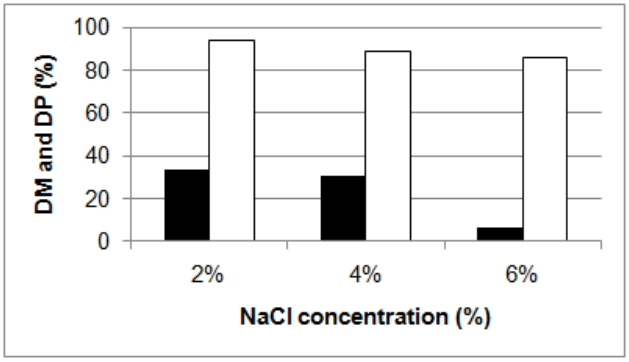

Figure 4. Effect of NaCl concentration on DM (white bar) and DP (black bar) efficiency

3.4 Effect of initial pH

Similar to other lactic bacteria, *L. plantarium* NCDN4 can grow at initial low pH. Low pH can prevent the spoilage of shrimp waste during early stage of lactic fermentation. However very low pH can affect the growth of *L. planetarium*, therefore the effect of low pH on fermentation was investigated. The shrimp waste was mixed with 12.5% glucose solution at ratio 1:2 and was inoculated with 20% inoculum (v/w). The initial pH of fermentation was adjusted to 5.0, 6.0 and 7.0.

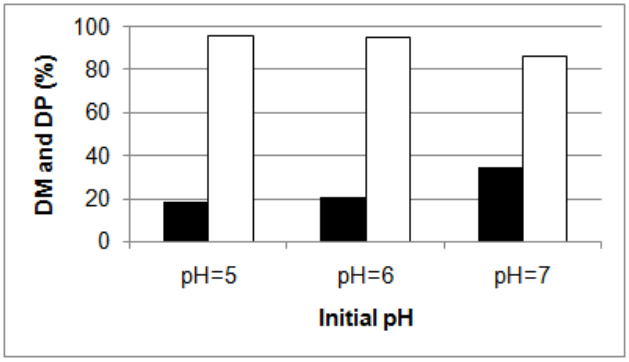

Figure 5. Effect of initial pH on DM (white bar) and DP (black bar) efficiency

The demineralization after five days was similar at pH 5 and 6, where around 95% demineralization was achieved. At initial pH 7, only 86.05% demineralization could be achieved (Figure 5). This showed that lower pH value resulted in higher demineralisation efficiency. The result was in agreement with earlier study of Rao (Rao *et al.*, 2000) and our previous study.The opposite pattern of deproteination was however observed. The deproteination was 18.46% at pH 5 and 34.68% at pH 7. This was in agreement with Cao's report(Cao *et al.*, 2009), who found the optimal pH for shrimp autolysis was 7.85. Therefore for reasonable deproteination and demineralization, initial pH 6 was chosen for the next experiment.

3.5 Effect of fermentation time

The shrimp waste was mixed with 12.5% glucose and pH was adjusted to 6. The mixture was inoculated with 20% inoculum (v/w) and NaCl was supplemented to the mixture at concentrations of 2% (w/w). The results from figure 6 suggested that prolonged fermentation has a good influence on both demineralization and deproteination. After 5 days of fermentation the demineralization and deproteination efficiencies were 99.24% and 48.60% accordingly. The ash and protein content remained about 1.19% and 24.74% after 5 days fermentation respectively.

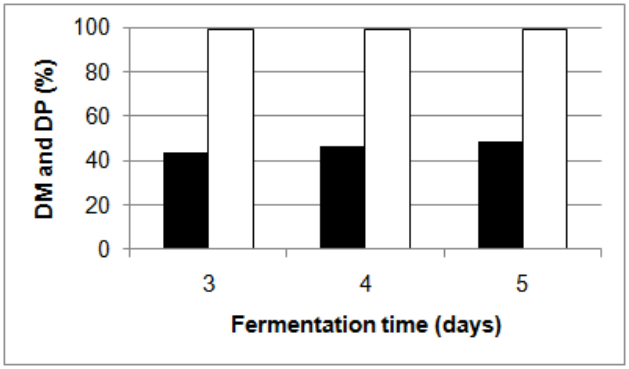

Figure 6. Effect of fermentation time on DM (white bar) and DP (black bar) efficiency

The 99.24% demineralisation and 48.60% deproteination of unsterile shrimp waste after optimization was similar to sterile conditions, where 99.51% demineralization and 47.10% deproteination were achieved. Compared to reported studies the demineralization of 99.24% was promising. The highest demineralization efficiency with lactic bacteria was only 90.23% in Rao study (Rao *et al.*, 2000), 78.8% in Aytekin study (Aytekin and Elibol, 2010) and 99.7% in Xu study (Xu *et al.*, 2008). The deproteination was however moderated in our study. Since high lactic acid concentration results on good demineralization, but inactivates shrimp waste protease, the optimal condition for high efficiency of both deproteination and demineralisation by lactic bacteria fermentation at one time seems impossible. In lactic fermentation, the good demineralization resulted in bad deproteination and vice versa was also found by Xu et al (Xu *et al.*, 2008).

4. Conclusions

Thus the optimal conditions for chitin recovery from unsterile shrimp waste by *L. plantarum* NCDN4 were 12.5% glucose, initial pH 6, 20% inoculum (v/w), 2% NaCl, 5 fermentation days at 30^{o}C. Compared to sterile shrimp waste, for which the optimal conditions for chitin recovery were 15% *L. plantarum* inoculum, 20% molasses, initial pH 6, 2% NaCl, five fermentation days at 30^{o}C, the inoculume size and sugars concentration were 1.3- fold 1.25-fold higher respectively for similar efficiency of demineralization and deproteination.

Despite indigenous protease, microbial fermentation of unsterile shrimp waste by *L. plantarum* NCDN4 could permit either good demineralization or good deproteination but not both at one time.

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