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RECOVERY OF CHITIN AND CHITOSAN FROM SHRIMP WASTE BY CHEMICAL AND MICROBIAL METHODS

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ABSTRACT

Shrimp waste is the most important chitin source for commercial use. In this study chitin and chitosan were extracted from *Penaeus semisulcatus* waste collected from a shrimp processing landing center situated at Persian Gulf in south of Iran by chemical and microbial methods. Chitin and chitosan were extracted by alkali-acid treatment and the yields were 510 and 410mg/g, respectively. Demineralization is an important step in the chitin purification process from shrimp waste. Chemical extraction method included the use of NaOH solution and acetic acid. In microbial extraction, organic acids (lactic acid) produced by probiotic bacteria was used to demineralize microbial deproteinized shrimp shells. The study showed that the effectiveness of using lactic acid bacteria especially added Fe $(NO_3)_3$ as extra nitrogen source for demineralization of shrimp shells than chemical method (1750 against 810mg/g). Chitin and chitosan extracted from shrimp waste by chemical and microbial methods was crystalline powder, non-harmful and odorless, white and off-white, respectively. The moisture content was calculated as 63.8%. The amount of Ca, Fe, Cu and Mn present in the shells was 168, 35.58, 38.28 and 6.72mg/L, obtained by atomic absorption spectroscopy, respectively. The amount of calcium in the shells was 25 times higher than manganese. The results suggested *Lactobacillus plantarum* (PTTC 1058) is an attractive source of recovery for chitin and chitosan.

Key words: Shrimp waste, chitin, chitosan, organic acids, lactic acid bacteria

INTRODUCTION

Chitin is a versatile environmentally friendly modern material (Mahmoud, 2007). It is a naturally occurring high molecular weight linear homopolysaccharide composed of N-acetyl-D glucosamine residues in α(1-4) linkage. Chitin and chitin derivatives are biodegradable and biocompatible natural polymers that have been used in virtually every significant segment of the economy (e.g. water treatment, pulp and paper industry, biomedical devices and therapies, cosmetics, biotechnology, agriculture, food science and membrane technology) (Li *et al.*, 1997).

Chitin can be found in a variety of species in both the animal and plant kingdoms. The traditional source of chitin is shellfish waste from shrimp, Antarctic Krill, crab and lobster processing (Muzzarelli, 1977; Shahidi, 1991). It is present in amounts varying from trace quantities up to about 40% of the body weight of the organism. The crustacean waste is the most important chitin source for commercial use due to its high chitin content and ready availability (Gagné and Simpson, 1993; Subasinghe, 1995). However, chitin present in the crustacean waste is associated with proteins, minerals (mainly calcium carbonate) and lipids including pigments.

Chitosan is a natural, non-toxic, co-polymer of glucosamine and *N*-acetylglucosamine obtained after partial de-*N*-acetylating of chitin, which, in turn, is a major component of the shells of crustaceans and found commercially in the offal of marine food processing industry (Tharanathan and Kitture, 2003). In spite of its abundance and various biofunctionalities, utilization of chitosan is restricted, owing to its high molecular mass, high viscosity and, thus, low absorption for *in vivo*

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applications (Shon, 2001). Recent studies on chitosan depolymerization have drawn considerable attention, since the products obtained are easily water soluble and also possess versatile biofunctional properties such as antitumour (Suzuki, 1986; Ikeda, 1995), immuno-stimulating (Shon, 2001) and antimicrobial activities (Sekiguchi, 1994; Jeon, 2001) and are being used for alleviating problems due to osteoarthritis–gastritis (Sukwattanasinitt, 2002).

In traditional chemical methods for isolating chitin from shrimp waste, 4% NaOH is used for deproteination and 4% HCl for demineralization. This process may not be considered as a good recovery option, because it is expensive and non-environmentally friendly (Rao, 2002). Partial fermentation of this biowaste using lactic acid bacteria for the production of chitin has been studied and reported (Rao, 2000).

The objective of this research was to study the effect of three strains of lactic acid bacteria on the fermentation efficiency of shrimp biowaste to recovery chitin and chitosan, its comparison with chemical extraction method and also studying the effects of intervening factors on microbial extraction.

MATERIALS AND METHODS

Shrimp waste

Shrimp waste from processing of *Penaeus semisulcatus*, comprising of head and carapace, was collected from a shrimp processing landing center situated at Persian Gulf in south of Iran. These shrimp were caught in the fall of 2007. Following cooking in boiling salt water for 10 minutes, the shrimp were sent to automated peeling machines, where the shell and meat portions were separated. The shell material was collected and dried at 50°C in oven for 24h and homogenized in a laboratory mixer before shipping for further processing. The yield of dried shell was determined by weighting after being dried. The obtained shrimp shells were stored at about-25ºC in the storage facility till needed.

Chemical extraction of chitin and chitosan

The process of extraction involved deproteinization with 2% w/v sodium hydroxide solution (30:1 v/w, 90ºC, 2h), separation of alkali-insoluble fraction (AIF) by centrifugation (4000rpm, 15min.), extraction of chitosan from AIF under reflux (10% v/v acetic acid 40:1 v/w, 60ºC, 6h), separation of crude chitin by centrifugation (4000rpm, 15min) and precipitation of chitosan from the extract at pH=9, adjusted with a 4M NaOH solution. Crude chitin and chitosan were washed on a coarse sintered-glass funnel with distilled water, ethanol and acetone and air-dried at 20ºC (Synowiecki, 1997).

Moisture content

Crude chitin sample was placed in a pre-weighted aluminum dish. The dish and contents were then placed in an oven at 105ºC for 24h. The aluminum dish along with the dried sample was first placed in a desicator to cool down and then weighted. The moisture content was determined as follows (Mahmoud, 2007):

$$MC = \frac{W_{ws} - W_{ds}}{W_{ws}} \times 100$$

Where:
MC: the moisture content (%)
Wws: the weight of the wet sample (g)
Wds: the weight of the dry sample (g)

Chitin minerals

The demineralized shell material (purified chitin) was filtered under suction condition. The recovered solids were thoroughly washed using deionized-distilled water and dried in an oven at 60ºC for 24h. The dried purified chitin samples were analyzed for their mineral content. Quantitative trace element analyses were done using an atomic absorption spectrophotometer. For Calcium, manganese, iron and copper analyses, the samples were first digested with hydrochloric and nitric acids (30, 10mL/g sample, respectively) in a closed vessel at a temperature of 100ºC and then the elements were determined by flame atomic absorption with detection limit of 1 ppm (Mahmoud, 2007).

Microorganism and culture media

Three species of *Lactobacillus* named *Lactobacillus plantarum* (PTTC 1058), *Lactobacillus acidophilus* (PTTC 1643) and *Lactobacillus rhamnosus* (PTCC 1637) were provided from Persian Type Culture Collection (PTCC) of Iranian Research Organization for

Science and Technology in Tehran, Iran. They were sub cultured on MRS broth and agar media (10g peptone from casein, 4g yeast extract, 8g meat extract, 20g D(+) glucose, 1g tween 80, 2g di-ammonium hydrogen citrate, 5g sodium acetate, 0.2g magnesium sulfate, 0.04g manganese sulfate MERCK). MRS Broth was mixed with 15g/L agar to solidify the medium and incubated at 35-37°C in the presence of 5% CO_2 for 48-72h.

Microbial extraction of chitin and chitosan

After adding 5 mL of MRS broth containing each of the three *Lactobacillus spp*. (OD_{600}=0.8-1) to the fermentative medium culture (50mL distilled water +5g of shrimp waste powder) samples were incubated for 7 days at 30°C, in the presence of 5% CO_2. The fermentative culture medium was centrifuged at 3000rpm for 5min. The recovered solids were washed thoroughly several times using deionized-distilled water. Chitin and chitosan were extracted with the same method described previously.

Optimization of conditions for microbial extraction of chitin and chitosan

The conditions for extraction were optimized with the effect of a combination of process variables (factors) such as lactose sugar, yeast extract and Fe $(NO_3)_3$ 1% was added to fermentation medium and their interactions on the response variable were determined. The extraction of chitin and chitosan and the determination of their concentration were carried out as explained earlier.

RESULTS

Result of chitin and chitosan yields in chemical extraction are present in Table 1. No significant difference was observed in chitin yields in microbial extraction by *Lactobacillus plantarum* (PTTC 1058), *Lactobacillus acidophilus* (PTTC 1643) and *Lactobacillus rhamnosus* (PTCC 1637) ($P<0.05$). But *Lactobacillus plantarum* (PTTC 1058) ability to extract chitosan was more than two other bacteria (Table 2). This was the same as chitosan yield extracted by chemical method. The microbial method could extract chitin better than chemical method (Tables 1, 2).

Table 1: Chitin and chitosan yields from chemical extraction method

Chitosan yield (mg/g)	Chitin yield (mg/g)	Biomass of shrimp waste (mg/g)
410	510	5000

Chitin and chitosan extracted from shrimp waste by chemical and microbial methods was crystalline powder, non-harmful and odorless and were white and off-white, respectively.

Table2: Chitin and chitosan yields from microbial extraction method

Microorganisms	Biomass of shrimp waste (mg)	Chitin yield (mg/g)	Chitosan yield (mg/g)
Lactobacillus plantarum (PTTC 1058)	5000	700	420
Lactobacillus acidophilus (PTTC 1643)	5000	700	310
Lactobacillus rhamnosus (PTCC 1637)	5000	700	330

The moisture content was calculated as 63.8%. The amount of Ca, Fe, Cu and Mn present in the shrimp waste was 168, 35.58, 38.28 and 6.72mg/L, respectively. The amount of calcium present in the shrimp waste was 25 times higher than the amount of manganese.

Optimization of conditions for chitin and chitosan extraction showed that, when Fe $(NO_3)_3$, as a mineral nitrogen source, was added to fermentation medium, it gave a higher chitin yield comparing with adding yeast extract (as organic nitrogen source) as at least 810 and 1750mg/g, respectively. But it did not have a significant

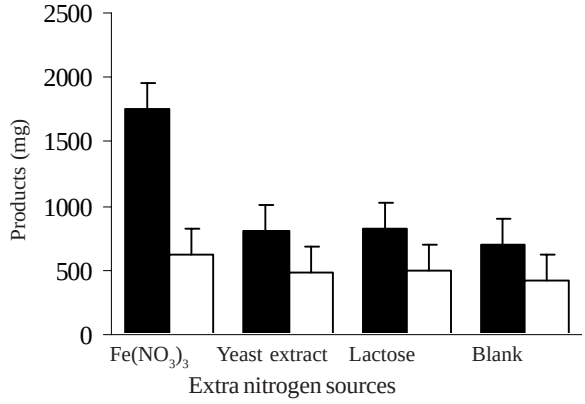

Fig. 1: Microbial chitin and chitosan extraction by *Lactobacillus plantarum* (PTTC 1058) from shrimp waste in different conditions

increasing effect on the chitosan yield (Fig.1). Results obtained from this study showed that the increasing lactose to fermentation broth, had an amplifying effect on the extraction of chitin and chitosan yield, the same as the case of yeast extract was added (Fig. 1).

DISCUSSION

Extraction of chitin and chitosan from a certain Persian Gulf shrimp species waste (*Penaeus Semisulcatus*), by chemical and microbial methods was investigated. In this study, the results showed that, microbial method is more effective especially for the recovery of chitin comparing with chemical method. Overally, extraction of chitin from biowaste by three strains was equal. The growth and lactic acid production by these bacteria increased the products. The strain 1058 showed higher fermentation efficiency, especially in chitosan yield extraction (32.25%).

According to Allan, *et al*., 1978, the traditional method involving the use of strong acids makes this process ecologically aggressive and a source of pollution. It also reduces a certain degree of depolymerization and thus chitin quality (Hansen, 1994; Simpson, 1994). The chemical treatment renders the protein component useless, which otherwise can be used as animal feed. Percot *et al*., (2003) reported that using inorganic acid such as HCl for the demineralization of chitin, results in detrimental effects on the molecular weight and the degree of acetylating that negatively affects the intrinsic properties of the purified chitin.

For microbial extraction, authors used three strains of lactic acid bacteria. Using organic acids such as lactic acid for the demineralization process is a promising idea since organic acids can be produced with low cost by bacteria, are less harmful to the environment, can preserve the characteristics of the purified chitin and the resulting organic salts from the demineralization process can be used as an environmentally friendly deicing/anti-icing agents and/or as preservatives. Fagbenro, 1996, used raw heads of Africa river prawn (*Macrobrachium vollenhovenii*) fermented with *Lactobacillus plantarum* using cane molasses. Lactic acid bacterial fermentation for demineralization has been occasionally reported for shrimp waste (Shirai, 2001), crayfish exoskeleton (Bautista, 2001), scampi waste (Zakaria, 1998), and prawn waste (Shirai, 1998). $Fe(NO_3)_3$ as a mineral nitrogen source, gave a higher chitin yield than added yeast extract (as organic nitrogen source) or lactose (as a carbon source). The most significant effect was due to $Fe(NO_3)_3$ (1750mg/g), after and then those of lactose (820mg/g), and yeast extract (810mg/g). Hall and Silva (1992) studied lactic acid bacterial fermentation of shrimp waste (*Penaeus monodon*) for chitin recovery with added carbohydrate such as lactose or cassava extract as a natural energy source. Treatment of minced scampi (*Nephrops norvegicus*) waste (supplemented with glucose) by a culture of *Lactobacillus paracasei* strain A3 was investigated by Zakaria *et al*.,(1998). The efficiency of demineralization was affected by the various inoculum amounts supplied. Also, the proportions of the additional starter and glucose were important for the lactic acid bacterial fermentation to demineralize the raw shell wastes (Meraz, 1992; Shirai, 2001; Rao, 2002).

Jung *et al*., showed that lactic acid fermentation resulted in a partial deproteination from the red crab shell waste, since the bacterium produced proteases. About 40% and 30% of proteins were removed in 5% and 10% inoculums, respectively, with 10% glucose after 5 days of fermentation. The lactic acid reacts with the calcium carbonate component in the chitin fraction, leading to the formation of calcium lactate, which precipitates and can be removed by washing. Woods, 1998 demonstrated that, deproteination of the biowaste and simultaneous liquefaction of the shrimp proteins occurs mainly by proteolytic enzymes produced by the added lactobacillus, by gut bacteria present in the intestinal system of the shrimp, or by proteases present in the biowaste.

Morita *et al*. 1997, showed that, *Lactobacillus fermentum* IFO 3956 that differed in origin converted metmyoglobin to nitrosylmyoglobin (a pentacoordinate nitric oxide (NO) complex of Fe (II) myoglobin) in MRS broth at pH=4.3. Xu and Verstraete, 2001, investigated on six strains of *Lactobacillus fermentum* and *Lactobacillus plantarum* for nitric oxide (NO) production. All

Lb. fermentum strains produced NO in MRS broth, but the NO was found to be chemically derived from nitrite, which was produced by *Lb. fermentum* from nitrate present in the medium. Indeed all *Lb. fermentum* strains express nitrate reductase under anaerobic conditions. NO and nitrite produced from nitrate by lactobacilli may constitute a potential antimicrobial mechanism. Thereby, NO_3 may play an acceleration role for Lactobacillus growth.

Results of this study showed that, increasing lactose to fermentation broth, had an amplifying effect on the extraction of chitin (820mg/g) and chitosan (500mg/g) yield.

The influence of carbon and nitrogen sources on chitin production by microorganisms was evaluated with a factorial design analysis (Andrade, 2000). It was found that chitin production was affected mostly by L-asparagine, followed by D-glucose and thiamine. The value of mineral content of the shrimp shells used in this study was within the range of 6.72-168mg/L. The most abundant minerals in the shrimp waste were calcium. Hansen and Illanes (1994) stated that the major mineral component of shellfish waste is calcium. Beaney *et al*., (2005), reported that the most abundant minerals in prawn shell were Ca, Mg, Na, Sr, K and Fe in that order and that calcium was by far the most abundant (about 17 times more than magnesium). Synowiecki and Al-Kateeb (2003) stated that the minerals fraction of shrimp shells composed mostly of phosphates and carbonates of calcium and magnesium. Mahmoud *et al*. (2007), reported, the amount of calcium present in the shells was 6 and 23 times higher than the amounts of phosphorus and magnesium, respectively. Manganese yield in shrimp waste was not determined by these scientists. Results of the manganese yield in *Penaeus semisulcatus* waste in our study was similar the magnesium yield determined by Mahmoud *et al*., (2007).

The effectiveness of lactic acid (produced by lactic acid bacteria) in removing the minerals from the shells and extracting chitin was higher than sulfuric acid in chemical method, so lactic acid fermentation could provide an alternative to chemical treatment, to extraction and recovery of chitin and its derivates.

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