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Deproteination and demineralization of shrimp waste using lactic acid bacteria for the production of crude chitin and chitosan

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Abstract. Deproteination and demineralization efficiencies of shrimp waste using two *Lactobacillus* species treated with different carbohydrate sources for chitin production, its chemical conversion to chitosan and the quality of chitin and chitosan produced were determined. Using 5% glucose and 5% cassava starch as carbohydrate sources, pH slightly increased from the initial pH of 6.0 to 6.8 and 7.2, respectively after 24 h and maintained their pH at 6.7 to 7.3 throughout the treatment period. Demineralization (%) in 5% glucose and 5% cassava was highest during the first day of treatment which was 82% and 83%, respectively. Deproteination (%) was highest in 5% cassava starch on the 3rd day of treatment at 84.4%. The obtained chitin from 5% cassava and 5% glucose had a residual ash and protein below 1% and solubility of 59% and 44.3%, respectively. Chitosan produced from 5% cassava and 5% glucose had protein content below 0.05%; residual ash was 1.1% and 0.8%, respectively. Chitosan solubility and degree of deacetylation were 56% and 33% in 5% glucose and 48% and 29% in 5% cassava, respectively. The advantage this alternative technology offers over that of chemical extraction is large reduction in chemicals needed thus less effluent production and generation of a protein-rich liquor, although the demineralization process should be improved to achieve greater degree of deacetylation.
Key Words: waste utilization, alternative carbon source, bioprocessing, lactic acid bacteria.

Introduction. In seafood industries, shellfish waste management is a huge problem especially the crustacean sector which lacks cost-effective outlets for their waste (Raja et al 2012). About 45% of processed seafood consists of shrimp, the waste of which is composed of exoskeleton and cephalothorax (Gortari & Hours 2013). This waste represents 50-70% of the weight of the raw material, and it contains valuable components such as chitin, protein, and pigments, their amounts depending on the processing conditions, the species, the body parts, the seasonal variations, etc. (Xu et al 2008). Crustacean shells are the most important chitin source for commercial use due to their high content and ready availability (Subasinghe 1995). Chitin is a linear water-insoluble polymer consisting of β-(1→4) linked units of 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylglucosamine; GlcNAc; A-unit) (Heggset 2012). Chitosan, on the other hand, is a co-polymer of glucosamine and *N*-acetyloglucosamine which is partially deacetylated chitin (Yen et al 2009). Due to their biocompatibility, non-toxicity, biodegradability and film forming characteristics, chitin and chitosan are widely applied in agriculture (Khorrami et al 2012), biomedicine and food industries (Shirai et al 2001). The conventional methods for chitin extraction from crustaceans are chemical processes which involve the use of strong acid for demineralization and strong base for deproteination. A final bleaching step leads to a colorless chitin (Xu et al 2008). The chemical deacetylation of chitin into chitosan also requires strong chemical conditions (Stevens et al 1998). It has been reported that chemical chitin purification is extremely hazardous, energy consuming and damaging to the environment owing to the high mineral acid and base involved (Healy et al 2003).

An alternative treatment of crustacean waste with lactic acid bacteria (LAB) for the production of chitin has been studied and reported (Rao et al 2000). However, few studies have reported the use of co-culture cultivation of proteolytic LAB strains and the use of cheap carbon source such as cassava (*Manihot esculenta*) flour. Thus, this study aimed to produce crude chitin from shrimp waste through lactic acid bacteria treatment coupled with mild chemical post treatment for chitin conversion into chitosan and compare in terms of solubility and proximate composition.

Material and Method

Microorganism. Lactic acid bacteria used in this study were isolated from 'burong bangus', a traditionally low salt fermented cooked rice and milkfish (*Chanos chanos*) mixture, and raw tuna (*Katsuwonus* sp.). L137 strain was isolated from 'burong bangus' and identified in a previous study by Olympia et al (1986) as *Lactobacillus plantarum*, a starch-hydrolyzing LAB. T1 strain was isolated from raw tuna (*Katsuwonus* sp.) and identified to genus-level according to Bergey's Manual for Determinative Bacteriology (1957) as *Lactococcus* sp. These LAB strains were chosen for the study because they were found to be heterofermentative and proteolytic when tested in skim milk agar, which are important properties of LAB for deproteination and demineralization purposes. However, L137 strain, after prolonged storage, was found to be negative for amylolytic activity when tested on starch agar. These strains were stored at 4^{o}C on MRS agar (Pronadisa) as the maintenance medium at the University of the Philippines Visayas Microbiology Laboratory, Miagao, Iloilo. Tests were conducted between February and August 2014.

Preparation of inoculum. The inoculum used was a co-culture of T1 and L137 strains. Optical Density (OD) at 540 nm and corresponding CFU m L^{-1} of individual cultures of T1 and L137 in 100 mL sterile MRS broth incubated at 37°C for 48 hours were determined. To prepare the inoculum for the treatment, 200 mL sterile MRS broth in E-flasks were inoculated with 1 mL aliquot of each 48h-culture of T1 and L137 strains to serve as mother culture and incubated at 37°C for 24 hours. Prior to inoculation, OD at 540 nm and CFU m L^{-1} of the mother culture were determined.

Protease assay. Cell-free supernatants were obtained after centrifugation of the 24h co-culture of T1 and L137 strains at 1398 x g at 4°C for 40 min. The filtrate was assayed for proteolytic activity (Akinkugbe & Onilude 2013). Protease activity was conducted using the method of Sigma-Aldrich using casein as substrate. Protease activity was determined in terms of Units (U) defined as the amount (µmoles) of enzyme that catalyzes the reaction of 1 µmole of casein per minute. All assays were carried out in triplicate.

Shrimp wastes. Fifteen (15) kilograms of frozen cultured white shrimp (*Penaeus merguiensis*) (80-100 mm) were procured from a local market in Roxas City, Capiz. Frozen whole shrimp were kept in ice in a sealed polystyrene container during transport and immediately stored overnight at -20°C upon arrival at the laboratory. Whole shrimp was thawed in running tap water before use. Shrimp heads and shells (with tails) were removed and separated from the meat, washed several times to eliminate any adhering meat with running tap water and dried overnight in an oven at 105°C. The dried shrimp heads and shells were milled into flakes using a Hammer mill (Culatti).

Microbial extraction of chitin. Twenty four (24) hour co-culture of T1 and L137 strains in MRS broth were used as inoculum (10% v/w). Glacial acetic acid (approx. 5 mL) was added to bring down the pH of shrimp waste to 6.0 (Rao & Stevens 2005). Treatment of 200 g shrimp waste inoculated with 20 mL of inoculum (10%) and 5% (w/v) carbon source (Treatment A with glucose (Gibco); Treatment B with cassava flour dissolved in distilled water; and Treatment C with no added carbohydrate source as control) was conducted in duplicate 300 mL E-flasks, covered with aluminum foil, at 37°C in a controlled temperature incubator shaker for 7 days. The slurry was filtered through a

cheese cloth to separate the solid materials. This crude chitin was washed with distilled water, oven dried, weighed, analyzed and further converted into chitosan. All experiments were carried out in triplicate.

Chitosan preparation. The prepared dried crude chitin was placed into a flask with 55% NaOH solution with chitin to NaOH solution ratio of 1:25 (g mL^{-1}), in a water bath at 95°C for 4 hours (Khorrami et al 2012). The produced chitosan was washed with distilled water and dried at 105°C overnight. Fourier Transform Infrared Spectroscopy (FTIR) spectrum of chitosan samples were measured and compared among treatments. All experiments were carried out in triplicate.

Proximate analysis. Moisture content was determined using the AOAC method (1990). Ash content was determined by burning the samples in a crucible at 600°C in a furnace for 2 hours (AOAC 1990). The pH value was monitored every 24 hours using a hand held pH meter (Milwaukee pH600). Growth trends in terms of CFU mL^{-1} developed on MRS agar plates was monitored during 7-day treatment period (24 hour interval). Protein content was measured using the standard biuret protein assay in samples before and after treatment. Lowry assay was used to determine protein content in chitin and chitosan, where protein concentrations are very low. Deproteination (%DP) and demineralization (%DM) were calculated using the equations by Rao & Stevens (2005). Chitin recovery (%CR) was determined as chitin derived (g) in reference to the original amount of chitin present in shrimp heads or shells. Chitin yield (%CY) was calculated as chitin derived (dry based, g) in reference to the original wet sample quantity of heads or shells (Rao & Stevens 2005). Lipid content was measured according to the standard methods by AOAC (1990).

Characterization of crude chitin and chitosan

Solubility of crude chitin and chitosan. Solubility of chitin was determined by dissolving 1 g of dried chitin in 100 cm^3 of dimethyl acetamide/lithium chloride (DMA/LiCl) solution for 12 h and subsequently centrifuged to determine the percentage of insoluble chitin. The DMA/LiCl solution was prepared by dissolving 8 g of anhydrous lithium chloride overnight in 100 cm^3 of DMA. The solubility of chitosan was determined by dissolving 1% (w/v) chitosan in a solution of 1% glacial acetic acid for 24 h under continuous stirring (Rao & Stevens 2005). The equation was used below:

$$\% \text{ Solubility} = \frac{(\text{initial wt of tube with sample}) - (\text{final wt of tube with sample})}{(\text{initial wt of tube with sample}) - (\text{initial wt of tube})} \times 100$$

Degree of deacetylation of chitosan. The DD of chitosan was determined using a FTIR (AVATAR 330 FTIR ThermoNicolet) instrument with frequency of 4000-400 cm^{-1}. The DD of the chitosan was calculated using the baseline by Khan et al (2002).

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA). All data sets were tested for normality. If significant differences were indicated, individual groups were compared using the Tukey's Range Test.

Results and Discussion

Shrimp waste composition. Shrimp waste form the major fraction (53%) by weight of the whole shrimp as shown in Table 1. The moisture, ash, and protein content in the shrimp biowaste were 77.26, 7.49, and 7.39% respectively. Mass balance was calculated based on minerals, protein, and lipid data with an error < 10%, balance is reasonably accurate (Rao & Stevens 2005). The quality of chitin and chitosan produced from crustacean shells is partially dependent on the type of raw material used. As observed in this study, the protein and ash values of shrimp shell wastes were generally low as

compared to previous studies by Rao & Stevens (2005), Aytekin & Elibol (2010), and Jung et al (2007) possibly due to the small size of the shrimp samples used and the preparation method of the shell wastes in which all adhering meat was removed and washed. Furthermore, proximate composition of shrimps, crustaceans and other aquatic organisms has found to be varied due to the seasonal factors, climatic factors, geographic factors, habitat, developmental stage, sex, and sexual maturation (Pillay & Nair 1971).

Table 1

Composition of shrimp waste

Shrimp fraction		*Shrimp waste*			
Meat (%)	*Whole shrimp waste (%)*	*Moisture content (% wb)*	*Ash*	*Protein*	*Total lipid*
46	53	77.26±1.0	7.49±0.42	7.39±0.33	0.50±0.05

Microbial extraction of crude chitin. The pH slightly increased for the first 24 hours of treatment and decreased after 48 hours. Treatment C (control) showed pronounced increment in pH over the 7-day treatment. Treatment A (with 5% glucose) showed little change in pH reaching its lowest pH of 6.7 on days 2 and 7. Treatment B (with 5% cassava flour) also displayed minimal change in pH reaching its lowest pH of 6.6 on day 6. There was no significant difference in pH among treatments during the 7-day treatment period. Medium pH likely depends on the content of the energy source such as glucose and sucrose (Jung et al 2005). In this study, 5% carbohydrate source and 10% inoculum levels were applied, similar to the ratio applied by Rao & Stevens (2005). However, medium pH was maintained by 5% glucose and 5% cassava flour treatments possibly due to the inadequate source of carbohydrates in the substrate and the complex structure of cassava starch. Franco et al (1998) stated that starches that naturally present a porous surface, such as corn (*Zea mays*) starch, are degraded easier than those with a smooth surface such as cassava starch. Moreover, high proportions of amylose and amylopectin, 18 to 25% and 80% in cassava flour, respectively, are more resistant to enzymes (Rocha et al 2010) and generally, lactic acid bacteria are deficient in amylolytic characters especially for the highly branched starch (Boontawan 2010). In addition, the low acid production of all treatments may be caused by the heterofermentative and proteolytic properties of two LAB strains used. During heterofermentative lactic acid biosynthesis, carbon dioxide and ethanol are present, thus rendering a lactic acid yield production (Serna-Cock & Rodríguez-de Stouvenel 2005). Faithong et al (2010) reported that degradation of small fish and shrimp by proteases yields short chain peptides and free amino acids. Furthermore, lactic acid bacteria are generally recognized as non-toxicogenic, although some species isolated from fish and its products can produce biogenic amines. Biogenic amines are physiologically degraded by oxidative deamination process catalyzed by amine oxidase with the production of aldehydes, ammonia and hydrogen peroxide (Zaman et al 2009) which are basic nitrogenous compounds.

Demineralization (%) was highest during the first day of treatment with 82.4%, 82.9%, and 86.6% for treatments A, B, and C, respectively (Figure 1A) and declined thereafter. This could be due to the low carbohydrate source added (5%) which also led to low acid production during the 7-day treatment. On the other hand, Jung et al (2006) reported a high demineralization efficiency of 97.2% after 7 days of co-fermentation by *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074 and *Serratia marcescens* FS-3 of red crab (*Chionoecetes japonicus*) shell waste due to higher carbohydrate source added (10%) which yielded high lactic acid. Consequently, cell growth gradually decreased as energy sources were used up. Ghorbel-Bellaaj et al (2012) added that the addition of carbohydrate source to shrimp waste medium has no significant effect on deproteination but its effect is more important on demineralization.

Deproteination (%) was highest in treatment B (84.4%) followed by A (44.8%) and C (12.6%) during the 3rd day of treatment (Figure 1B). Owing to the efficient

deproteination, especially in treatment B with cassava as carbon source, higher value was reported in this study than the previous studies by Jung et al (2006) and Shirai et al (1998). Sumantha et al (2005) stated that the progressive decrease in proteolytic activity with increasing fermentation time could possibly be due to ending of production, as enzymes are primary metabolites and it could also be due to enzyme inactivation. Towards the end of treatment period, the control, devoid of carbon source, showed comparable values while treatment A (5% glucose) displayed lower values. According to Ghorbel-Bellaaj et al (2012), this is mainly owing to the induction of the repressive effect of glucose on protease production or catabolic repression.

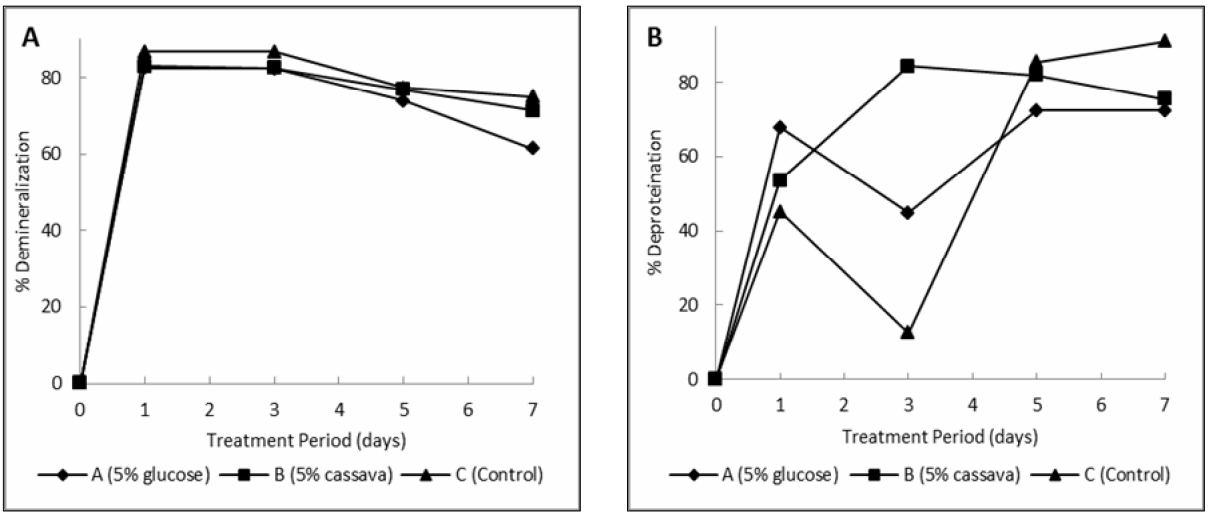

Figure 1. Changes in demineralization (A) and deproteination (B) of shrimp waste with LAB over the 7-day treatment period.

Chitin recovery (CR) was significantly highest in shrimp waste treated with 5% glucose followed by 5% cassava and control, respectively (Table 2). Chitin yield (CY) was generally low among treatments. Significantly highest CY was noted in treatment A, but showed no significant difference from treatment B. Rao & Stevens (2005) reported similar low results, wherein 50–60% of solid material, assumed to be chitin, is lost. This could be due to chitin, occurring partially in aggregates of small particle size in the shrimp heads or shells, were lost during filtration or sequential washing. In the standard procedures, chitin particles were supposed to be retained by cloth filtration, but small chitin particles might be lost during cloth filtration (Rao & Stevens 2005).

Table 2

Chitin recovery and yield (%) of shrimp waste

Sample	*Protein (g)*	*Ash (g)*	*Chitin recovery (%)*	*Chitin yield (%)*
Treatment A (5% glucose)				
Original	0.22	17.20	-	-
Residue	0.059	6.49	48.3 ± 4.51^{a}	6.5 ± 0.5^{a}
Treatment B (5% cassava)				
Original	0.24	17.73	-	-
Residue	0.059	5.1	37.3 ± 4.04^{b}	6.0 ± 1.0^{ab}
Treatment C (control)				
Original	0.29	19.78	-	-
Residue	0.026	4.95	27.0 ± 3.00^{c}	4.5 ± 0.5^{b}

Superscripts in column denote significant difference ($\alpha = 0.05$) based on one way ANOVA analysis.

Quality of crude chitin and chitosan. There were no significant ($p>0.05$) differences in protein contents among the three prepared chitins (Table 3). Protein contents of *S. marcescens* FS-3 alone and *L. paracasei* subsp. *tolerans* KCTC-3074 plus *S. marcescens* FS-3 cofermentation of red crab shell waste were 3.62% and 10.62%, respectively, after

days of fermentation (Jung et al 2006) which were higher than the protein contents in the present study. This indicated the efficiency of deproteination by co-cultures of two LAB in this study. However, the repressive effect of glucose on protease production led to high residual protein content in treatment A (0.094%). Significant difference in ash content was observed between treatments where highest residual ash content was noted in the control. The result was higher than reported by Rao & Stevens (2005). This could be due to low acid production which led to low demineralization efficiencies during treatment leaving high residual ash content. Chitin solubility (%), among the three treatments, was highest in treatment B. However, there was no significant difference in solubility between prepared chitins. Solubility was low due to the high residual ash content in the produced chitins. All treatments were brownish in color; this was probably due to the low lactic acid produced during treatment which was inefficient in removing the pigments in the shrimp waste.

The protein (%) content of produced chitosan was significantly highest ($p \leq 0.05$) in the control (Table 3). According to Rao & Stevens (2005), the use of 50% NaOH nullified possible effects that might be caused by differences in the protein content of chitin. Ash content showed significant difference between treatments; the highest residual ash was observed in the control, the reason being that; alkaline deacetylation involved only the removal of acetyl groups from the molecular chain of chitin, leaving the chitin backbone intact and producing a compound (chitosan) with a high degree chemical reactive amino group ($-NH_2$). Treatment A has significantly highest solubility (56%) among the prepared chitosans. Solubility is influenced by residual ash content; the lower the ash content, the higher the solubility, as observed in treatment A. Treatment A showed the highest degree of deacetylation (DD%) among all treatments (Table 3) however, there was no significant difference in DD% between three treatments. The study of Nessa et al (2010) on the process for the preparation of chitin and chitosan from prawn (*Penaeus indicus*) shell waste demonstrated that duration of deacetylation affected mostly the degree of deacetylation and solubility of the product. In general, prepared chitosan showed low solubility and DD (%) owing to the short duration of deacetylation process of chitin to chitosan which was four hours. Moreover, No & Meyers (1995) reported that it is estimated that deacetylation must be at least 85% complete in order to achieve the desired solubility. The color of produced chitosans was light yellow; this could be due to the quality of chitin used as raw material for chitosan conversion since characteristics of chitosan were affected by the conditions of chitin extraction.

Table 3

Quality of crude chitin and chitosan

Property	*Synthesized*		
	A	*B*	*C*
Chitin			
Protein (%)	0.094^{a}	0.085^{a}	0.081^{a}
Ash (%)	0.76^{a}	0.9^{b}	1.3^{c}
Solubility (%)	44.3^{a}	59.0^{ab}	39.0^{a}
Chitosan			
Protein (%)	0.032^{a}	0.033^{a}	0.039^{b}
Ash (%)	0.8^{a}	1.1^{b}	1.3^{c}
Solubility (%)	56.0^{a}	48.0^{b}	41.0^{c}
Degree of deacetylation (%)	33.0^{a}	29.0^{a}	26.0^{a}

Superscripts in row denote significant difference ($\alpha = 0.05$) based on one way ANOVA analysis.

Conclusions. The results of the present study showed that chitin for its conversion to chitosan can be produced through microbial treatment of lactic acid bacteria. However, the alternative process for chitin production is still less efficient than conventional chemical treatment. A ratio of 10% (v/w) inoculum and 5% (w/w) glucose and cassava starch were insufficient to produce desirable acid concentration in the demineralization

process due to inadequate energy source. However, the co-cultures of T1 and L137 showed efficient deproteination in treatment B (with added 5% cassava) despite low demineralization and that glucose (treatment A) exhibited repressive effect on protease production. Prepared chitin and chitosan showed high residual ash content; however, protein content was relatively low. Produced chitin and chitosan showed low solubility (%) due to high ash content and low degree of deacetylation (%), respectively. The produced chitin and chitosan from shrimp shell waste could be used in a variety of applications especially in food, biomedical and pharmacological industries. The advantagethis technology offers over that of chemical extraction is large reduction in chemicals needed thus less effluent production and generation of a protein-rich liquor fraction.

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