International Journal of Bioprocess & Biotechnological Advancements
IJBBA, 1(1):1-17
www.scitcentral.com

ISSN: 2380-0259

Original Research Article: Open Access

Influence of Autoclaving of Shrimp Shells on Proteinase Enzyme Production and Protein Hydrolysis by *Aspergillus niger*

Nesreen Mahmoud[1] and Abdelkader Ghaly[2*]

[1]*Department of Agricultural Engineering, Faculty of Agriculture, Cairo University, Giza, Egypt*
[2]*Department of Process Engineering and Applied Science, Faculty of Engineering, Dalhousie University, Halifax, Nova Scotia, Canada*

Received March 26, 2015, Accepted April 6, 2015, Published April 14, 2015

ABSTRACT

The aim of the study was to evaluate the ability of the fungus *Aspergillus niger* to carry out the deproteinization process of a carbon supplemented shrimp shells and to study the effect of autoclaving the shrimp shells on the performance characteristics of the deproteinization process. The temperatures of the shrimp shells and the exhaust gas declined during the lag period as the heat losses from the bioreactor were higher than the heat generated by metabolic activities. Once the exponential growth phase started, the temperatures of the shrimp shells and exhaust gas and CO_2 concentration increased reaching maximum values of 37.5 and 33.6ºC, 28.9 and 27.9ºC and 0.37% and 0.32% for the runs with autoclave and intact shrimp shells, respectively. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was obtained. The pH of the shrimp shells initially decreased with time reaching 7.05-7.34 and then increased reaching 7.35-8.16. The decrease in the pH was due to acid protease production while the increase in the pH was due to the buffering capacity of the calcium carbonate released from the shrimp shells and the production of ammonium nitrogen. The galactose concentration and moisture content decreased gradually over time reaching about 1.48% and 2.67% and 25.54% and 26.72% at the end of the experiment for the autoclaved and unautoclaved shells, respectively. The protease activity of the autoclave shells was significantly higher (1.7 fold) than that of the unautoclaved shells. The deproteinization efficiencies of autoclaved and unautoclaved shells were 33.23% and 25.62% for, respectively. The reduction in the protein concentration at the end of the deproteinization process did not correspond to the increase in the protease activity. The low deproteinization efficiency observed in this study could be due to the high initial pH of the shrimp shells and the high temperature and the low moisture content observed during the deproteinization process that might have interfered with protease synthesis and/or activity. The low deproteinization attained with unautoclaved shells might be due to the growth of the indigenous microorganisms in the shrimp shells which might affect the growth and metabolic activity of *A. niger*. The chitin concentration increased from 16.56 to 22.68% and 20.93% over the course of the deproteinization process for the autoclaved and unautoclaved shrimp shells, respectively. The spent shrimp shells had a pale pink-orange color with some tan patches. The pink-orange color was an indication of the presence of pigments. The extent of shrimp shell agglomeration and presence of white precipitant were much higher in case of unautoclaved shrimp shells. Unidentified microorganisms were found in both the autoclaved and unautoclaved shells with larger population in the case of unautoclaved shells.

Keywords: Shrimp shells, Protein, Chitin, Minerals, Deproteinization, *Aspergillus. niger*, Protease activity, Galactose, Temperature, pH, Moisture content, Autoclaving.

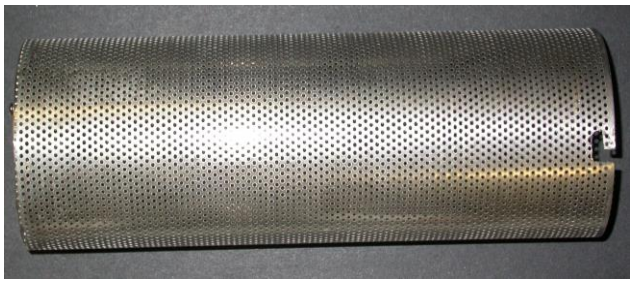
INTRODUCTION

The shell fish processing industry generates large amounts of solid wastes which have become a major environmental concern to producing countries worldwide due to the high perishability and the bulky nature of the waste material [1, 2]. Northern Pink Shrimp (*Pandalus borealis)* is commonly fished in the North Atlantic both on the East Coast of Canada and the West Coast of Norway. These shrimp are caught as part of the offshore Northern Shrimp Fishery by a vessel owned by Clearwater Fine Foods Inc. and then individually quick-frozen on board the vessel. Upon arrival at the cooking/peeling plant, they are cooked in boiling salt water for 10 minutes and then sent to automated peeling machines where the shell and meat portions were separated. This species has a mean length of 22-25 mm at maturity [3] and the processing discards of these shrimp account for up to 80% of its original weight [4]. The total

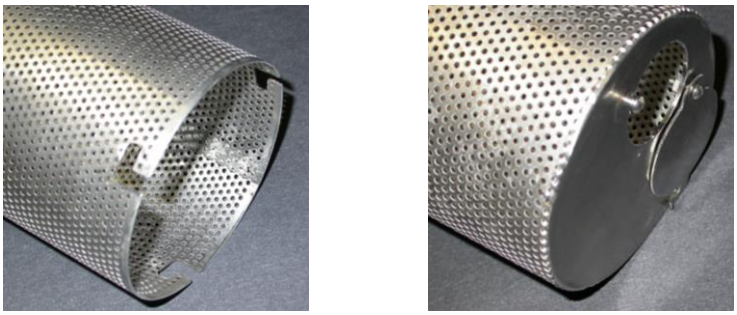
Corresponding author: Professor A. E. Ghaly, Department of Process Engineering and Applied Science, Faculty of Engineering, Dalhousie University, Halifax, Nova Scotia, Canada; Tel::902-494-6014; Email: abdel.ghaly@dal.ca

Citation: Mahmoud N & Ghaly A(2015) Influence of Autoclaving of Shrimp Shells on Proteinase Enzyme Production and Protein Hydrolysis by *Aspergillus niger*. International Journal of Bioprocess and Biotechnological Advancements, 1(1):1-21

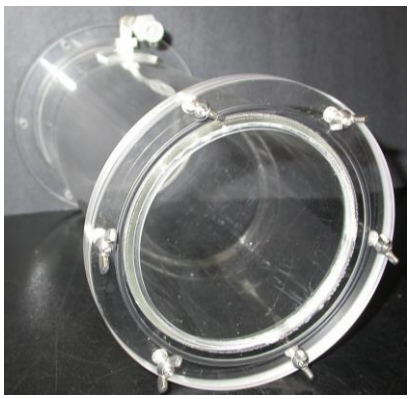
(a) Stainless steel mesh



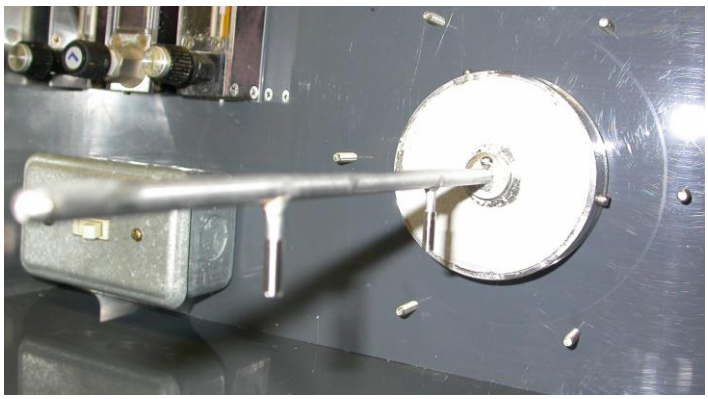
(b) Stainless steel rotating basket



(c) Both ends of the rotating basket



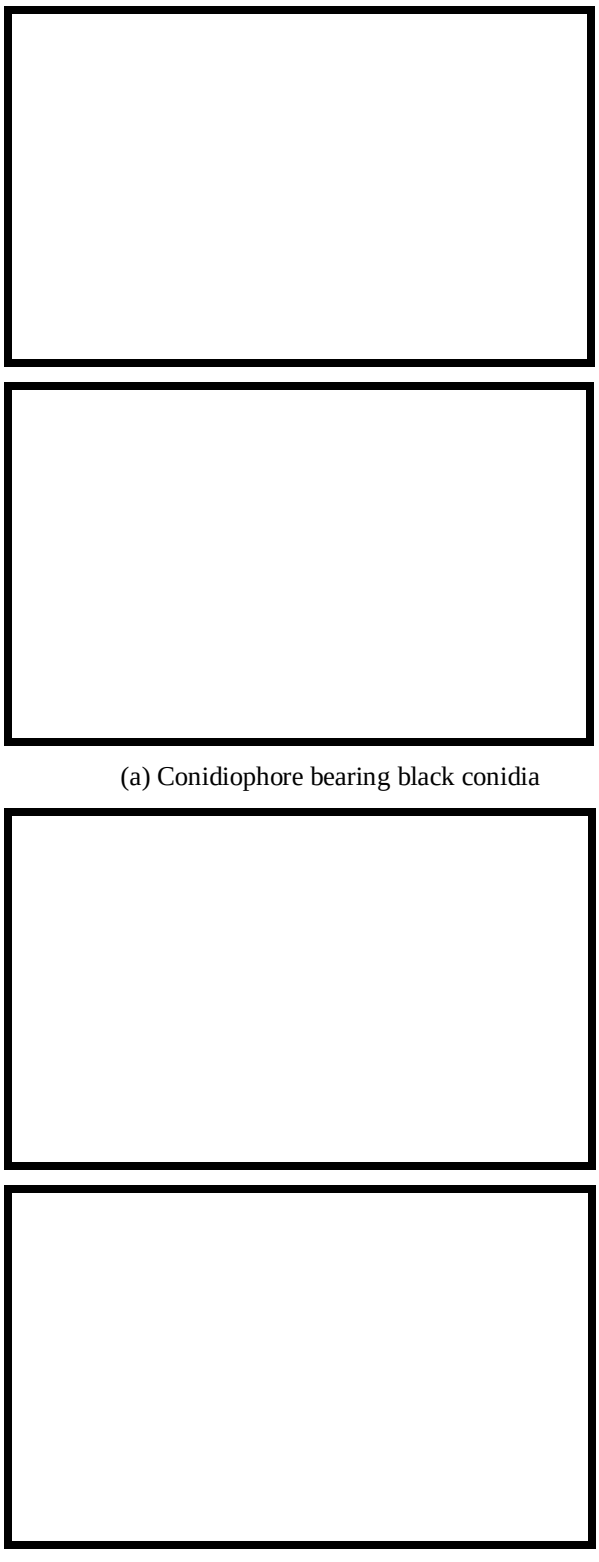
(d) Plexiglas cylinder



(e) Rotating disc, aeration tube and thermocouples

Figure 2. The drum bioreactor.

was prepared by dissolving 0.1 mL Tween 80 in 1 L distilled deionized water and then autoclaving (Model No. STM-E, Market Forge Sterilmatic, New York, USA) at 121˚ C and 103.4 kPa for 30 minutes. The spore concentration was determined using direct standard plate count method according to the procedures described in the Standard Method for the Examination of Dairy Products [26]. The prepared suspension was stored in the refrigerator at about 4 ºC until needed.



(a) Conidiophore bearing black conidia

(b) Culture and colonial morphology

Figure 3. *Aspergillus niger* colonies.

Shrimp Shells

The shells of the Northen Pink Shrimp (*Pandalus borealis)* were obtained from a shell processing plant in Mulgrave, owned by Ocean Nutrition Ltd. of Bedford, Nova Scotia. The shrimp shells were stored at about -25 ºC in the Biotechnology Laboratory till needed. The shrimp shells were dried and then ground using a conventional food processor (General Electric Company, Wal-Mart Canada Corporation, Mississauga, Ontario, Canada). The ground shrimp shells were autoclaved (Model No. STM-E, Market Forge Sterilmatic, New York, USA) at 121˚C and 103.4 kPa for 45 min before use. Table 1 shows some of the characteristics of the shrimp. Table 2 shows the particle size distribution of the ground and intact shrimp shells.

Experimental Protocol

The effect of autoclaving on the deprotenization process of shrimp shells was studied. The experimental conditions of the deproteinization process are shown in Table 3. The sugar solution was prepared by dissolving 20g galactose in I L deionized distilled water and then autoclaving (Model No. STM-E, Market Forge Sterilmatic, New York, USA) at 121˚C and 103.4 kPa for 30 min. Each reactor was loaded up to 75% of its capacity (200 g shells based on dry weight). An inoculum concentration of 1×10^7 spores per 1g shrimp shell waste was used. The initial moisture content of the shrimp shells was adjusted to 60% with the addition of sugar and spores solutions and the material was mixed thoroughly. Air was introduced inside each reactor at a flow rate of 5 VWM (I m air per g shells per minute). The experiment ran for 6 days. At the start of the experiment, the reactors were rotated (1 rpm) continuously for 30 min and then intermittently for 15 min every hour.

Experimental Analyses

The particle size distribution, moisture content, pH, galactose concentration, ammonium nitrogen, total Kjeldahl nitrogen, protein and chitin measurements were performed on the shrimp shells. During the course of the deproteinization process, shrimp shell samples of 10 g each were collected every 12 h and analyzed for moisture content, pH, galactose concentration, protease activity, ammonium nitrogen content, total Kjeldahl nitrogen content and protein content. Exhaust gas samples were also taken every 12 h and analyzed for carbon dioxide concentration. The bulk temperature was monitored and recorded every 10 minutes. The deproteinized shells were analyzed for chitin and appearance.

Particle Size Distribution

A known weight of shrimp shells were sieved for 30 min using a sieve shaker (Model RX-86, Fisher Scientific, Montreal, Quebec, Canada) with 7 different sieve sizes (6.300, 4.000, 2.000, 0.850, 0.300, 0.180, 0.075 mm aperture size). Each particle size fraction obtained was weighed and

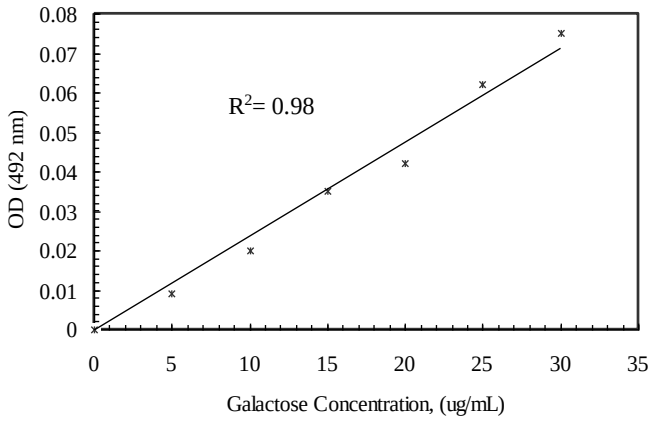


Figure 4. Galactose concentration standard curve.

using a microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

Protease Activity

Protease produced by *A. niger* was first extracted from the shrimp samples (1 g each) using 20 mL deionized distilled water and kept at room temperature for 30 min with continuous stirring using a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada). The extract was then filtered under suction using coarse porosity filter paper (Reeve Angel Grade 202, Whitman Inc., Clifton, New Jersey, USA) and the supernatant was used for the assay of enzyme. Protease activity was measured using Protease Colorimetric Detection Kit (Product Code PC0100, Sigma, Saint Louis, Missouri, USA). The assay was based on using a casein substrate, which is cleaved by the protease to trichloroacetic acid soluble peptides. The formed peptides contain tyrosine and tryptophan residues, which react with the Folin and Ciocalteu's reagent causing color change, which can be estimated calorimetrically at 660 nm using a microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

Chitin

The chitin content was determined based on the fact that chitin contains about 6.89% organic nitrogen [28]. In order to determine the chitin nitrogen, samples were first deproteinized and demineralized.

The deproteinization process was performed using 5% (w/v) NaOH solution. One gram of ground shrimp shell sample (dry weight) along with 100 mL of NaOH solution were placed in a 250 mL wide-mouth flask and the flask was covered with a piece of tin foil and sealed with a rubber band to ensure the retention of all reacted materials. The flask was then placed in a boiling water bath (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) for 1 h. The sample was filtered under suction through a Buchner funnel with coarse porosity filter paper (Reeve Angel Grade 202, Whitman Inc., Clifton, New Jersey, USA) and washed thoroughly with deionized distilled water. The deproteinized sample was dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 ºC till constant weight. The weight of the recovered dry deproteinized sample was determined.

The deproteinized sample along with 50 mL of 1.0 M HCl were placed in a 250 mL wide-mouth flask and the flask was covered with a piece of tin foil and sealed with a rubber band to ensure the retention of all reacted materials. The flask was then placed in a boiling water bath for 1 h. The demineralized sample was then filtered under suction through a Buchner funnel with coarse porosity filter paper (Reeve Angel Grade 202, Whitman Inc., Clifton, New Jersey, USA) and washed thoroughly with deionized distilled water. The deproteinized-demineralized sample was then dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 ºC till constant weight. The weight of the recovered dry deproteinized-demineralized sample was determined. The ammonium and total kejldhal nitrogen analyses were performed on the dry deproteinized-demineralized sample and the chitin content was then calculated as follows:

$$(\text{Org.-N})_c = [(\text{TKN}_c - (\text{NH}_4\text{-N})_c] \times W_c/W_s \quad (5)$$

$$\text{CH}_c = (\text{Org.-N})_c \times 14.51 \quad (6)$$

Where:

CH_c is chitin content (mg/kg)
$(\text{Org.-N})_c$ is organic nitrogen of the recovered chitin
TKN_c is total Kjeldahl nitrogen of the recovered chitin (mg/kg)
$(\text{NH}_4\text{-N})_c$ is ammonium nitrogen of the recovered chitin (mg/kg)
W_c is weight of recovered chitin (g)
W_s is weight of sample (g)

Ash Content

The dried shrimp shells were analyzed for their ash content. A known weight of the material was placed in a preweighed aluminum dish. The dish and content were weighed and then placed in a muffle furnace (Isotemp® Muffle Furnace model 186A, Fisher Scientific, Montreal, Quebec, Canada) at 700 ºC for 2 hours. The dish with the content was taken from the muffle furnace, placed in a desiccator to cool down and then weighed. The ash content was determined as follows:

$$AC = \frac{W_{ds} - W_a}{W_{ds}} \times 100 \quad (7)$$

Where:

AC is the ash content (%)
W_a is the weight of the ash (g)

Minerals

The dried shrimp shells were analyzed for their minerals content. Quantitative trace element analyses (magnesium,

calcium, manganese, potassium, sodium, iron, silicon, aluminum, titanium and copper) were performed on the ash using an Atomic Absorption Spectrophotometer (SpectrAA 55B, Varion, Mulgrave, Victoria, Australia) in the Minerals Engineering Center, Dalhousie University, Halifax, Nova Scotia. For magnesium, calcium, manganese, potassium, sodium, iron and copper analyses, the samples were first digested with hydrochloric, nitric, hydrofluoric and perchloric acids (30, 10, 10 and 5 mL/g sample, respectively) in a closed vessel at a temperature of 100 ºC and then the elements were determined by flame atomic absorption with detection limit of 1 ppm. For silicon, aluminum and titanium analyses, 1 g of the sample was fused with a flux of lithium metaborate and lithium tetraborate and leached with 1:9 nitric acid. Sulfur was determined with Leco Sulfur analyzer along with Leco Induction Furnace (Leco Corporation, St. Joseph, Michigan, USA). Phosphorus was determined as P_2O_5 by a colorimetric method using spectrophotometer with micro flow-thru system (Spectronic 100, Bausch & Lomb Incorporation, Rochester, New York, USA) at 430 nm.

Visualization of Shrimp Shells

The shrimp shells were visually inspected at the end of the deproteinization with the naked eye as well as under the incident light stereomicroscope (Carl Zeiss Stemi SV8, Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) at a magnification of 60X. The stereoscope was equipped with a cold light source (SCOHTT KL 1500, SCHOTT North America Inc., New York, USA) and a single chip CCD color video camera (Sony DXC-101, Sony of Canada Ltd., Toronto, Ontario, Canada).

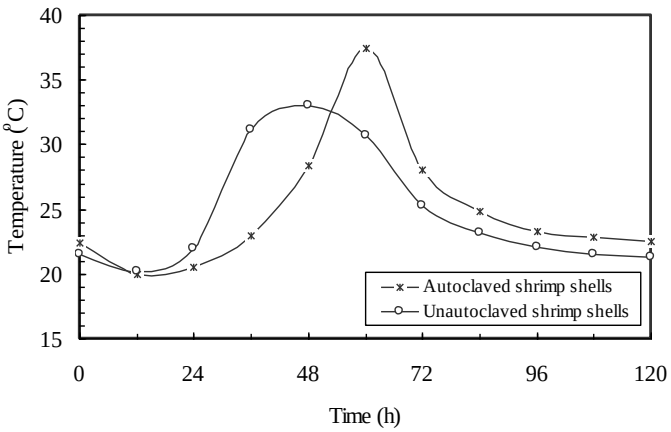
RESULTS AND DISCUSSION

The effect of autoclaving on various deproteinization parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration) of ground shrimp shells supplemented with galactose as a carbon source was investigated.

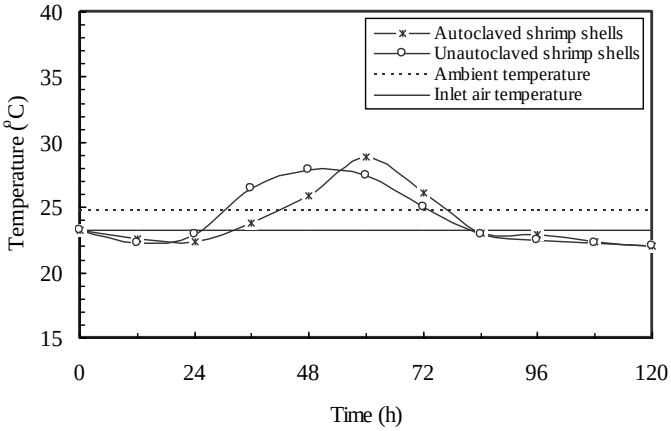
Temperature

Figure 5 shows the changes in the temperature of the shrimp shells and the exhaust gas during the course of deproteinization as affected by autoclaving. The values are the average of three replicates. The coefficient of variation ranged from 0.40 to 4.32.

The solid state fermentation process is an exothermic reaction in nature generating heat that gives rise to the temperature of the medium [29]. The microorganisms utilize organic carbon and micronutrients for synthesis of new microbial cells, product formation and energy generation [20,30]. The heat stored in the bioreactor is the net of metabolic heat production minus the heat losses (by conduction through the shrimp shells and the bioreactor



(a) Shrimp shells



(b) Exhaust gas

Figure 5. Effect of the initial autoclaving of shrimp shells on the temperatures of the shrimp shells in the bioreactor and the exhaust gas.

walls, by convection with the exhaust gas and latent heat through water evaporation from the shrimp shells).

In this study, the average inlet air temperature and initial temperature of the shrimp shells were 23.3 ± 0.54 °C and 21.8 ± 0.34 °C, respectively. The temperatures of the shrimp shells and the exhaust gas declined at the beginning of the fermentation process (lag period) as the heat losses from the bioreactor were higher than the heat generated in the bioreactor by microorganisms. After 12h, the temperature of the shrimp shells started to rise as the heat generation by metabolic activity exceeded the heat losses. The rise in the exhaust gas temperature was due to heat losses from the shrimp shells by convection. The temperatures of the shrimp shells and exhaust gas reached maximum values of 37.5 and 33.6 ºC and 28.9 and 27.9 ºC after 60 and 72 h for the autoclaved and unautoclaved shrimp shells, respectively.

Ghildyal et al. [31] and Pandey [32] reported that temperatures in the middle of the bed can reach about 20 ˚C higher than the temperature of the inlet air. Saucedo-

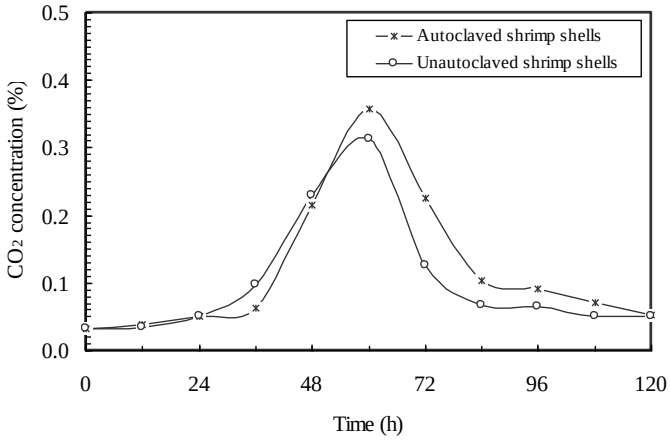


Figure 9. Effect of the initial autoclaving of shrimp shells on carbon dioxide concentration in the exhaust gas.

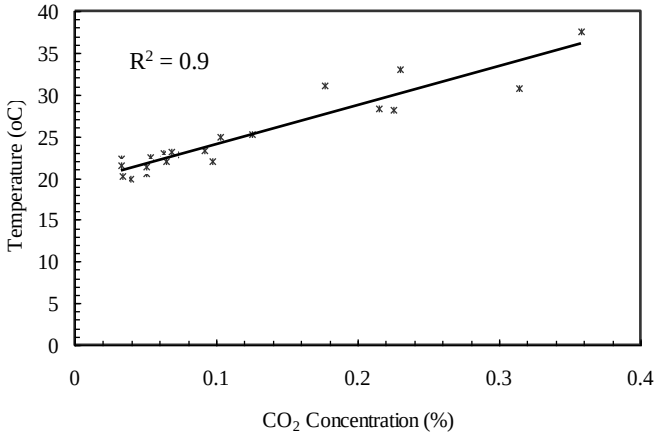


Figure 10. Correlation between the carbon dioxide concentration in the exhaust gas and the temperature of the shrimp shells.

dioxide measurements for the determination of microbial growth of *A. niger* culture on cassava flour. Rathbun and Shuler [45] stated that the relationship of CO_2 evolution and microbial growth is accurate and used the rate of CO_2evolution (gas flow time mole fraction CO_2) as a measure of the rate of microbial growth. In this study, The CO_2 concentration also followed the same trend as the temperature of the shrimp shells. A strong correlation (R^2 = 0.9) between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was obtained (Figure 10).

Protease Activity

Figure 11 shows the effect of autoclaving of shrimp shells on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shells and the values are the average of three replicates. The coefficient of variation ranged from 2.20 to 6.98%.

The results obtained from this study revealed the ability of *A. niger* to produce extracellular proteases that resulted in deprorinization of the protein in the shrimp shells. Fish proteins are complex molecules consisting of chains of amino acids linked together by peptide bonds. Proteases are proteins structured in such a way that allow them to act as catalysts in the breakage of peptide bonds through a process called hydrolysis according to the following equation:

$$\text{Fish protein} \xrightarrow[\text{Hydrolysis}]{\text{Protease}} \text{Polypeptides + Peptides + Amino acids} \quad (8)$$

Bustos and Healy [8] stated that the degree to which a microorganism will hydrolyze a protein substrate depends on its capacity to produce the required protease and the stability of a such protease under the reaction conditions. The results obtained from this study showed that the protease activity was higher in case of autoclaved shrimp shells. The protease activity increased with time from an initial value of 0.67

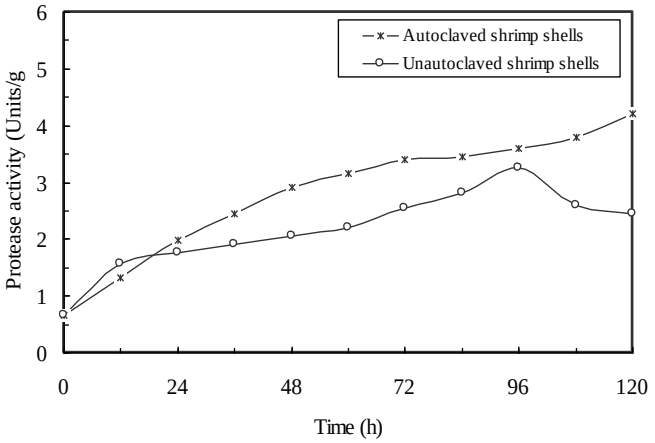


Figure 11. Effect of the initial autoclaving of shrimp shells on protease activity.

unit/g dry shrimp shell to final values of 4.21 and 2.44 unit/g dry shrimp shell after 5 d for the autoclaved and unautoclaved shrimp shells, respectively. Ashour et al. [46] reported that protease yield from the fungus *A. niger* during cheese whey fermentation increased with incubation period and reached a maximum value after 6 days. Teng et al. [19] reported protease activities in the range of 0.8-6.8 units (one unit activity was defined as 1 µM of tyrosine produced in 1 min) for 17 *A. niger* strains after 5 days of incubation of the spores.

Protein Concentration

Figure 12 shows the effect of autoclaving of shrimp shells on chitin content of the shrimp shells during the course of deproteinization. The values are presented based on the dry weight of the samples and are the average of three replicates. The coefficient of variation ranged from 1.22 to 6.18%.

The results showed that protein concentration of the shrimp shells decreased with the deproteinization time as a

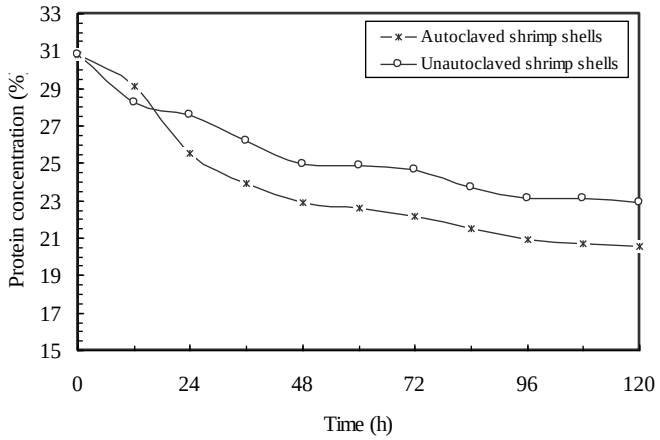


Figure 12. Effect of the initial autoclaving of shrimp shells on residual protein in the bioreactor.

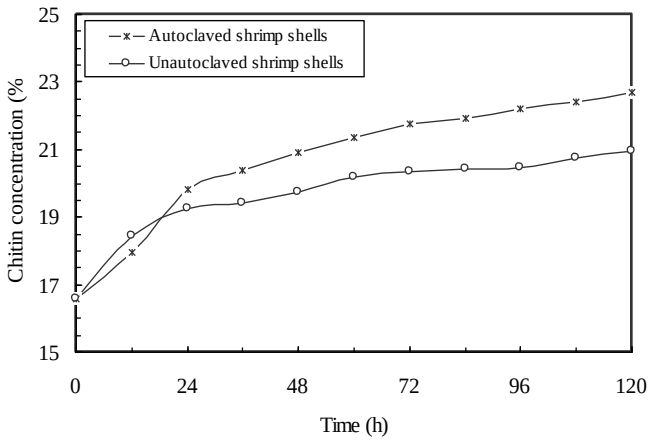


Figure 13. Effect of the initial autoclaving of shrimp shells on chitin concentration.

result of protein break down by the proteolytic enzymes produced by *A. niger*. However, the protein concentration in the shrimp shells was lower in case of autoclaved shrimp shells. The protein concentration decreased from the initial value of 30.84 to final values of 20.59% and 22.94% by the end of the deproteinization process for the autoclaved and unautoclaved shells, respectively. Zakaria et al. [2] used lactic acid fermentation for chitin recovery from scampi waste and reported 77.5% deproteinization efficiency after 5 days. Beaney et al. [12] reported 50% decrease of the original protein concentration in prawn shell waste using lactic acid fermentation and stated that complete deproteinization through a purely biotechnological process seems hard to achieve.

The low deproteinization efficiency observed in this study could be due to the high pH of the shrimp shells that might have interfered with protease synthesis and/or activity. Diniz and Martin [47] stated that the extent of hydrolytic degradation of protein depends on pH, temperature, extend of native protein denaturation, concentration and specificity of the enzyme, composition and the molecular weight distribution of the peptides in the protein and presence of inhibitory substances. The optimum pH of acid protease production from *A. niger* lies in the range of 2.0 - 3.0 and the pH of the shrimp shells during the entire deproteinization process was far (7.05-8.64) from being optimum for enzymes production.

Chitin Concentration

Figure 13 shows the effect of autoclaving of shrimp shells on residual protein in the shrimp shells during the course of deproteinization. The values are presented based on the dry weight of the samples and are the average of three replicates. The coefficient of variation ranged from 2.32 to 4.48%.

The chitin concentration was determined in the deproteinized samples without demineralization. The shrimp shells used in this study contained31.73% minerals. The results showed that the chitin concentration in the shells increased from an initial value of 16.56 % to final values of 22.68% and 20.93% over the course of the deproteinization process for the autoclaved and unautoclaved shrimp shells, respectively. Zakaria et al. [2] reported an increase in the concentration of chitin from 12.05% to 17.48% as a result of lactic acid fermentation of scampi waste. Cira et al. [48] reported increases in chitin concentration from 11.4-13.1% to 20.3-23.2% as a result of lactic acid fermentation of shrimp waste.

Visualization of Shrimp Shells

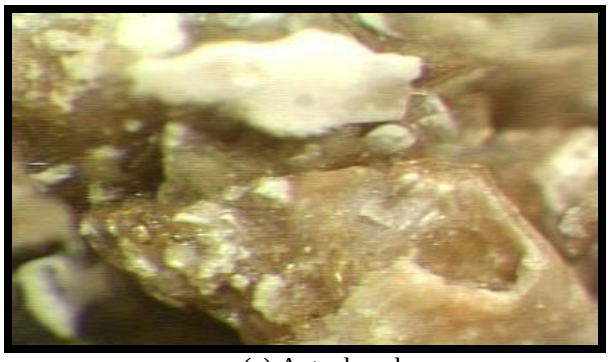
Figure 14 shows the visual appearance of autoclaved and unautoclaved shrimp shells at the end of the deproteinization process (120 h). Figure 15 shows the appearance of autoclaved and unautoclaved shrimp shells under the microscope (60X magnification) at the end of the deproteinization process. Samples of the autoclaved and unautoclaved shrimp shells were plated on potato dextrose agar after 24 and 72 h of deproteinization. Figure 16 shows the effect of autoclaving on the microbial population after 24 and 72 h fermentation time.

The spent shrimp shells obtained from both runs had a pale pink-orange color with some tan patches. More of the white precipitant accumulated on the surface of the autoclaved shrimp shells compared to the one that was accumulated on the surface of the unautoclaved shrimp shells as noticed under the microscope (60X magnification). The existence of the pink-orange color was an indication of the presence of pigments, which were not utilized during the fermentation process. The extent of shrimp shell agglomeration was much higher in case of unautoclaved shrimp shells than in the case of autoclaved shrimp shells. The plates showed the existence of unidentified microorganisms on both autoclaved and unautoclaved shells. However, the population of the unidentified microorganisms was much higher in the case of unautoclaved shells.

(a) Autoclaved shrimp shells

(b) unautoclaved shrimp shells

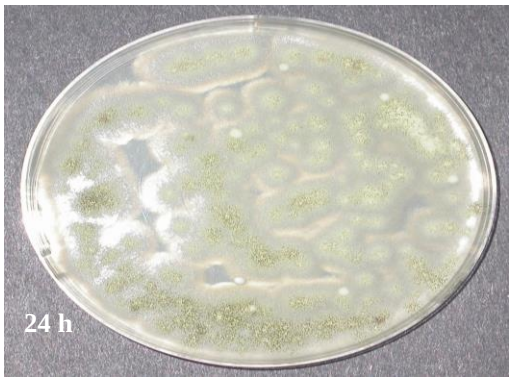
Figure 14. Appearance of shrimp shells after 120 h of deproteinization.



(a) Autoclaved

(b) Unautoclaved

Figure 15. Images of shrimp shells under the microscope (60 X) after 120 h deproteinization.



(a) Autoclaved shells

(b) Unautoclaved shells

Figure 16. Plated autoclaved and unautoclaved shrimp shell samples after 24 and 72 h of deproteinization.