Effect of Particle Size on the Kinetic Parameters of the Deproteinization Process of Galactose Supplemented Shrimp Shells by *Aspergillus niger*

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Abstract: The aim of the research was to evaluate the ability of the fungus *Aspergillus niger* to carry out the deproteinization of shrimp shells supplemented with galactose as a carbon source and study the effect of the particle size of the shrimp shells on the kinetic parameters of the deproteinization process. Grounding of the shells resulted in a higher specific growth rate of *A. niger* and enhanced protease production by 2.6 fold. The temperatures of the shrimp shells and exhaust gas declined at the beginning as the heat losses from the bioreactor (due to evaporation) were higher than the heat generated by the metabolic activity. After 24h, the temperature of the shrimp shells and exhaust gas started to rise as a result of increased metabolic activity. Temperature peaks of 38.3 °C and 37.8 °C for the shrimp shells and 29.1 ºC and 29.0 ºC for the exhaust gas were noticed after 60 and 72 h of deproteinization for the ground and intact shrimp shells, respectively. There were no temperature gradients in the radial or axial direction because of mixing. The pH first decreased with time due to production of acid protease and then increased due to the buffering capacity of the calcium carbonate released from shrimp shells and the production of ammonium nitrogen. A significant reduction in the moisture content was noticed during the deproteinization process. In order to maintain the moisture in the bioreactor at the desired level, the exhaust gas should be passed through a condensation tower and the recovered water be pumped back into the bioreactor through the aeration tube. The galactose concentration decreased with time and the rate of galactose utilization was significantly higher in case of ground shrimp shells. Size reduction results in higher surface area and shorter pathways for nutrients diffusion. The protein removal efficiency (30.45% - 33.23%) did not correspond to the protease production. This could be a result of unsuitable pH and temperature conditions for the hydrolysis of shells proteins. The chitin concentration increased over time from the initial value of 16.59 % to final values of 21.99 %and 22.68% for the intact and ground shells, respectively. The color of the intact and ground shells was pale pink-orange with some tan patches. Some of the ground shrimp shells agglomerated and formed small balls. This problem was not observed with the intact shells. The ground shells had more white precipitants which were believed to be a result of break down of substances from the shell matrix.

Keywords: Shrimp shells, protein, chitin, minerals, deproteinization, *Aspergillus. niger*, proteases, galactose, temperature, pH, moisture content.

1. INTRODUCTION

Chitin and its derivatives are versatile environmentally friendly modern materials that have widw applications in water treatment, pulp and paper, biomedical and therapies devices, cosmetics, biotechnology, agriculture, food science and membrane technology [1, 2]. Shellfish waste is a rich source of chitin as well as proteins, inorganic material (mainly calcium carbonate), lipids and pigments [3]. Current chitin extraction from crustacean waste involves the use of acid (HCl) for demineralization and alkali (NaOH) for deproteinization [3-5]. This method: (a) yields a citin with variable and inconsistent physiochemical properties, (b) results in wastewater that creates environmental problems, (c) wastes the other valuable components (proteins, minerals, lipids and pigments) present in the waste material and (d) is costly. Therefore, a less costly environmentally friendly biological method for chitin extraction from crustacean waste is needed.

Biological extraction of chitin include the use of organic acid fermentation for demineralization [6-9] and proteolytic microorganisms [4-5, 10-14] or proteolytic enzymes [13, 15] for deproteinizationt. However, the use of microorganisms for deproteinization is more effective than purified enzymes because the presence of microorganisms provides a gradual increase of protease production throughout the fermentation period [5, 14]. Therefore, biological extraction of chitin using microorganisms is a good alternative to the harsh chemical method because it preserves the natural state of the biopolymer, is less expensive and is environmentally friendly.

Aspergillus niger has been used in different industrial applications including production of several organic acids and more than 19 extracellular enzymes [16-19]. The fungus contains up to 42.0% chitin of the dry weight of the cell wall which weigh 20-40% of the

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E-ISSN: 2311-1755/15

total dry cell weight [20]. Thus, the proteolytic enzymes released from the fungi can deproteinize the shells and the chitin in the cell wall of the fungi can be considered an additional source of chitin [13]. *A. niger* species are most suited to solid-state fermentation because their growth conditions are similar to those of the natural habitats [21]. Particle size is one of the most important parameters affecting solid-state fermentation.

2. OBJECTIVES

The aim of this study was to use a fungus known to produce proteolytic enzymes (*Aspergillus niger*) to deproteinize shrimp shells supplemented with galactose as a readily bioavailable carbon source a solid-state fermentation process and to study the effect of shrimp shell particle size (intact shrimp shells versus ground shrimp shells) on the performance characteristics (temperature, pH, moisture content, galactose uptake, carbon dioxide evolution, protease activity, protein degradation, chitin production and final color of the shrimp shells) of the deproteinization process.

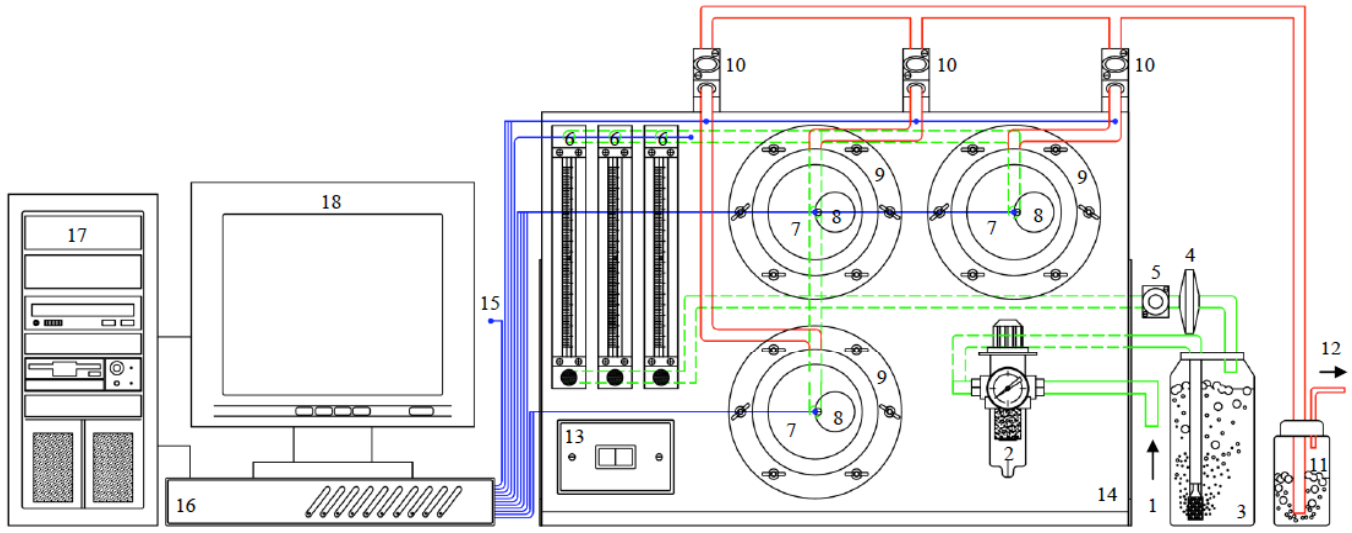
3. MATERALS AND METHODS

3.1. Experimental Apparatus

The experimental setup (Figure **1**) consisted of a main frame, three drum bioreactors, an aeration system and a data acquisition system.

The main frame was made of two polyvinyl chloride (PVC) rectangular sheets (13 mm thick) and two hexagon stainless steel sheets (3 mm thick). One of the PVC sheets (560 × 460 mm) was used as a base and the other one (560 × 380 mm) was fixed vertically on the base. The two hexagon stainless steel sheets were fixed to the two PVC sheets by means of stainless steel screws. The main frame held the drum bioreactors, the pressure regulator, the flow meters, the inlet air and exhaust gas manifolds, the thermocouple wires, the mixing motor along with the transmission system and switch, the tubing and the sampling ports,

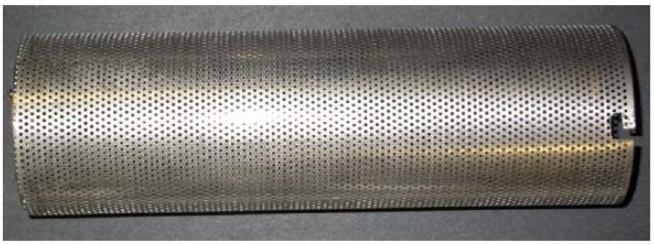
Three 1.8 L drum bioreactors with mixing motors and transmission system were used. Each drum bioreactor (Figure **2**) consisted of a removable inner stainless steel mesh (aperture of 1.5 mm) which was used as a lining for an outer stainless steel horizontally rotating basket of 88 mm diameter and 292 mm length. One stainless steel plate, with a drilled hole for sampling, was used to close one end of the rotating basket. The other end was left opened for charging and cleaning purposes and was designed so that it can be recessed and secured into a rotating disc after charging the reactor. An outer casing made from a Plexiglas cylinder of 12.5 mm diameter was installed for each bioreactor. The Plexiglas cylinder was recessed and secured into the main frame from one end by six stainless steel screws. The other end of the Plexiglas cylinder was covered by a removable circular



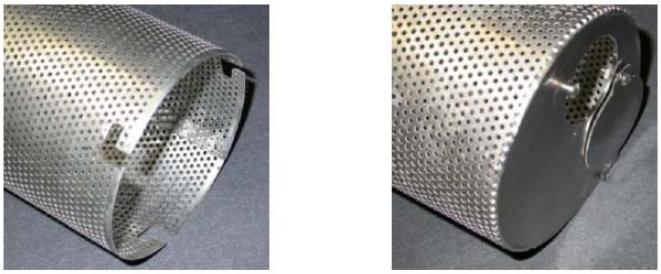
1. Laboratory air supply
2. Pressure regulator
3. Humidifier
4. Air filter
5. inlet air sampling port
6. Flow meters
7. Drum bioreactors
8. Sampling ports
9. Outer casing
10. Exhaust gas sampling ports
11. Small water container
12. exhaust gas
13. Agitation switch
14. Main PVC frame
15. Thermocouple wires
16. Data Logger
17. Computer
18. Monitor

Figure 1: Experimental setup.

(a) Stainless steel mesh

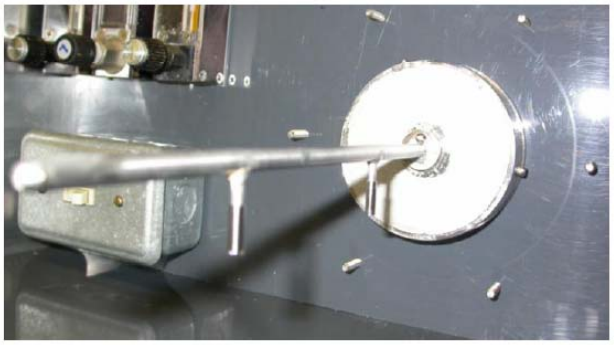


(b) Stainless steel rotating basket



(c) Both ends of the rotating basket

(d) Plexiglas cylinder



(e) Rotating disc, aeration tube and thermocouples

Figure 2: The drum bioreactor.

Plexiglas plate and was recessed and secured by six stainless steel screws and wing nuts. A rubber gasket lining (O-ring, 2.5 mm thick) was used at both ends of the Plexiglas cylinder to provide an air tight seal. A hole was drilled through the cylinder wall for the release of the exhaust gas. The rotating discs were connected to a motor (Synchronous Motor Model No. 20-34245G-24007, Sigma Instruments Inc., Braintree, Massachusetts, USA) through a transmission system.

Air was supplied continuously at the required flow rate inside each drum bioreactor from the laboratory air supply. The air passed first through a pressure regulator (ARO, Model No. 129125-510, Bryan, Ohio, USA) in order to regulate the air pressure around 5 kPa and then through a 1 L humidifier which contained 0.75 L sterilized distilled water. The humidified air was passed through a bacterial filter and then through a flow meter (No. 60648, Cole-Parmer Instrument Co., Montreal, Quebec, Canada) and was finally introduced into the bioreactor through a small perforated stainless steel tube that ran along the center of the basket. The aeration tube was fixed through the center of the rotating disc and remains stationary while the basket is

rotating. The air inlet sampling port was placed right after the bacterial filter whereas the three exhaust gas sampling ports were located on the exhaust tubes, each was made of a rubber septum. The three exhaust gas tubes were connected to a manifold and the exhaust gas was bubbled through a small container of water in order to create a slight gas pressure in the bioreactors.

Eleven T-type thermocouples (Thermo-Electric Ltd., Brampton, Ontario, Canada) were used to measure the temperature during the course of the fermentation process. Two thermocouples were threaded through the aeration tube of each bioreactor and used to measure the temperature of the material inside the bioreactor. One thermocouple was used to measure the ambient temperature, one thermocouple was used to measure the inlet air temperature and three thermocouples were used to measure the exhaust gas temperature of each bioreactor. The temperature data were monitored and stored using a data acquisition system which consisted of a master unit (Multi-scan 1200, Omega, Stamford, Connecticut, USA), a thermocouple/volt scanning card (MTC/24, Omega, Stamford, Connecticut, USA), a software (Temp-view, Omega, Stamford, Connecticut, USA) and a personal computer.

3.2. Microorganisms

The fungus *Aspergillus niger* was chosen for this study for two reasons: (a) its ability to produce acid protease and (b) the presence of chitin in its cell wall. A freeze dried culture of *Aspergillus niger* (ATCC 16513) was obtained from the American Type Culture Collection (Rockville, Maryland). It was revived in 6 mL of 0.1% sterilized peptone solution, which was prepared by dissolving 1 g Bacto-Peptone (Difco, Detroit, Michigan, USA) in 1 L deionized-distilled water and then sterilized in an autoclave (Model No. STM-E, Market Forge Sterilmatic, New York, USA) at 121 ˚C and 103.4 kPa for 30 minutes. The rehydrated culture was then kept in a capped test tube for 24 hours at room temperature (24 ºC). One mL of the rehydrated *A. niger* was transferred to three test tubes, each containing 9 mL potato dextrose broth (PDB). The PDB contained infusion from 200 g potatoes (4 g/L) and 20 g/L Bacto-Dextrose. The test tubes were tightly capped and kept for 48 h at room temperature (24 ºC). They were then stored in the fridge at 4 °C and cultured when needed.

A spore stock suspension was obtained by growing the fungus on Czapek's agar (which contained 30.00 g/L saccharose, 2.00 g/L sodium nitrate, 1.00 g/L dipotassium phosphate, 0.50 g/L magnesium sulfate, 0.50 g/L potassium chloride, 0.01 g/L ferrous sulfate and 15.00 g/L agar) at room temperature (25 ºC) for 4 days. The conidia were harvested from the surface by adding sterilized deionized distilled water containing 0.01% (v/v) Tween 80 (which was prepared by dissolving 0.1 mL Tween 80 in 1 L distilled deionized water and then autoclaving at 121˚ C and 103.4 kPa for 30 minutes) and gently scraping the surface with a sterile spatula. The spore concentration was determined using the direct standard plate count method as described in the Standard Method for the Examination of Dairy Products [22]. The prepared suspension was stored in the refrigerator at about 4 ºC until needed.

3.3. Shrimp Shells

The Northern pink shrimp (*Pandalus borealis)* is commonly fished in the North Atlantic East Coast of Canada and has a mean length of 22-25 mm at maturity [23]. The shells of the Northern pink shrimp were obtained from a shell processing plant in Mulgrave (owned by Ocean Nutrition Ltd. of Bedford, Nova Scotia, Canada), transported to the Biotechnology Laboratory of Dalhousie University in Halifax and kept at -25 ºC till needed. For the experiments in which ground shells were used, the shrimp shells were ground using a conventional food processor (General Electric Company, Wal-Mart Canada., Mississauga, Ontario, Canada). The ground and unground 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 shells. Table **2** shows the particle size distribution of the intact and ground shrimp shells.

3.4. Experimental Protocol

The effect of shrimp shells particle size (ground and intact shells) on the deprotenization process of shrimp shells was studied (Table **3**). The shrimp shells were supplemented with galactose (20 % w/w) as a carbon source. The sugar solution was prepared by dissolving the required galactose weight in the appropriate volume of deionized distilled water and then autoclaving at 121˚C and 103.4 kPa for 30 min. The initial moisture content of the shrimp shells was adjusted to 60% with the addition of sugar solution and spores solutions and the material was mixed thoroughly. An inoculum concentration of 1×10^7

Table 1: Some Characteristics[a] of Shrimp Shells

Characteristics	Value	Units
Moisture content	4.79 ± 0.03	%
Ash content	31.73 ± 0.02	%
Total Kjeldahl nitrogen	64265	mg/kg
Ammonium nitrogen	3492	mg/kg
Percent ammonium nitrogen	5.4	%
Organic nitrogen	60773	mg/kg
Percent organic nitrogen	94.6	%
Protein nitrogen	49339	mg/kg
Percent protein nitrogen	76.8	%
Chitin nitrogen	11434	mg/kg
Percent chitin nitrogen	17.8	%
Chitin[b]	165907	mg/kg
Protein[c]	308369	mg/kg
Fat	22000	mg/kg
Total carbon	305000	mg/kg
Total organic carbon	176000	mg/kg
Elements		
Magnesium	6150	mg/kg
Calcium	142000	mg/kg
Manganese	116	mg/kg
Potassium	871	mg/kg
Sodium	3480	mg/kg
Iron	308	mg/kg
Phosphorus	22400	mg/kg
Sulfur	4700	mg/kg
Silicon	794	mg/kg
Aluminum	250	mg/kg
Copper	24	mg/kg
Others	136207	mg/kg
pH	8.64	

[a]Characteristics are presented on dry basis.
[b]calculated as chitin nitrogen × 14.51.
[c]calculated as protein nitrogen × 6.25.

spores per 1g shrimp shell was used. Each reactor was loaded with shrimp shells up to 75% of its capacity (200 g shells based on dry weight). Air was introduced inside each reactor at a flow rate of 5 VMM (volume air in mL per gram shell 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.

Table 2: Particle Size Distribution

Size (mm)	Ground (% Under)	Intact (% Under)
6.300	100.00	98.11
4.000	100.00	81.97
2.000	98.20	59.33
0.850	70.25	35.74
0.300	34.84	11.25
0.180	25.14	3.96
0.075	11.97	0.92
< 0.075	3.11	

Table 3: Experimental Conditions

Parameters	Value
Studied Parameter	Particle Size
Load	75% of the Bioreactor Volume
Initial Moisture Content	60%
Inoculum Size	1×10^7 Spores/g Shell
Aeration	5 VMM
Agitation	Intermittent (15 min/h)
Particle Size	Ground (≤0.075-≤4.00mm), Intact (≥0.075-≥6.30 mm)
Autoclaving	Autoclaved Shells
Initial Galactose Concentration	20 % w/w
Initial pH	8.64

3.5. Experimental Analyses

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

3.5.1. 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 the percentage from the total weight was calculated.

3.5.2. Moisture Contents

A known weight of shrimp shells was placed in a pre-weighed aluminum dish. The aluminum dish and content were weighed and then placed in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 105 ºC for 24 hours. The aluminum dish along with the dried shrimp shells were first placed in a desiccator to cool down and then weighed. The moisture content was determined as follows:

$$MC = \frac{W_{ws} - W_{ds}}{W_{ws}} \times 100 \quad (1)$$

Where:

MC is the moisture content (%)

W_{ws} is the weight of the wet sample (g)

W_{ds} is the weight of the dry sample (g)

3.5.3. pH

20 mL of deionized distilled water was added to 1 g of shrimp shells and kept at room temperature (24 °C) for 30 min with frequent stirring using a stir plate (Thermix® Stirrer Model 120MR, Fisher Scientific, Montreal, Quebec, Canada). The sample was let stand for two minutes and the pH was then measured using a pH meter (Model 805MP, Fisher Scientific, Montreal, Quebec, Canada).

3.5.4. Ammonium Nitrogen

Samples of shrimp shells were washed thoroughly several times with deionized distilled water until the wash water was clear and then dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 ºC till constant weights. The dried shrimp shells were ground using a small conventional grinder. Ammonium nitrogen (NH_4-N) of dry ground samples was determined directly using Kjeldahl analyzer (KJELTEC AUTO 1030, Fisher Scientific, Montreal, Quebec, Canada).

3.5.5. Total Kjeldahl Nitrogen

Samples of shrimp shells were washed thoroughly several times with deionized distilled water until the wash water was clear and then dried in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 60 ºC till constant weights. The dried shrimp shells were ground using a small conventional grinder and then digested by heating the samples with concentrated sulfuric acid and kjeltabs (which contained 3.5 g K_2SO_4 and 0.0035 Se) for 45 min. The K_2SO_4 promotes the oxidation of organic matter and conversion of organic nitrogen to ammonium nitrogen by increasing the temperature of the digest to 420 ºC. Se is a catalyst which increases the rate of oxidation of organic matter by sulfuric acid. 5 mL sulfuric acid with 1 kjeltab per 0.2 g dry weight sample was used in this study. The total Kjeldahl nitrogen (TKN) of the digested samples was determined using Kjeldahl analyzer (KJELTEC AUTO 1030, Fisher Scientific, Montreal, Quebec, Canada).

3.5.6. Protein

The protein content of the samples was determined using the following equations which are based on the fact that the protein contains about 16% nitrogen:

$$(Org.\text{-}N)_s = TKN_s - (NH_4\text{-}N)_s \quad (2)$$

$$PR_c = [(Org.\text{-}N)_s - (Org.\text{-}N)_c] \times 6.25 \quad (3)$$

Where:

PR_c is protein content (mg/kg)

$(Org.\text{-}N)_s$ is organic nitrogen of the sample (mg/kg)

TKN_s is total Kjeldahl nitrogen of the sample (mg/kg)

$(NH_4\text{-}N)_s$ is ammonium nitrogen of the sample (mg/kg)

3.5.7. Galactose Concentration

20 mL of deionized distilled water was added to one gram of shrimp shells and kept at room temperature (24 °C) for 30 min with frequent 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, Whatman Inc., Clifton, New Jerse, USA) and the supernatant was used for galactose concentration measurements using the phenol-sulfuric acid method which is based on the fact that simple sugars give a stable orange-yellow color upon reacting with phenol and concentrated sulfuric acid [24]. The intensity of the color is proportional to the amount of galactose present in the sample and can be measured at 492 nm.

A standard curve was developed from solutions of galactose and deionized distilled water with different concentrations. First, a standard solution of 100 µg/mL galactose was prepared by dissolving 10 mg galactose in 100 mL deionized distilled water. Then, a set of 6 solutions with galactose concentration of 5, 10, 15, 20, 25, and 30 µg/mL) were prepared. Finally, the absorbance was measured using a microplate reader ((µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA) at 492 nm. A blank sample of pure deionized distilled water was used. The measured absorbance was plotted against the known galactose concentrations (µg/mL) as shown in Figure **3**. The following linear relationship between the galactose concentration and the absorbance was obtained ((R^2= 0.98):

$$C_{ga} = 416.67 (\bar{A}_{492}) \quad (4)$$

Where:

C_{ga} is the galactose concentration (µg/mL)

2 mL of each sample were transferred to a test tube and 1 mL of phenol solution and 5 mL of concentrated sulfuric acid (95-98%) were added to the tube. The tubes were then tight capped and the contents were mixed using a vortex mixer (Sybron Maxi Mix Model M-16715, Thermolyne Corporation, Dubuque, Iowa, USA). The tubes were allowed to stand for 10 minutes at room temperature and then the contents were mixed again using a vortex mixer. The tubes were placed in a water bath (2850 Series, Fisher Scientific, Montreal, Quebec, Canada) at 30 ºC for 15 minutes after which the contents were mixed again using a vortex mixer. The tubes were allowed to stand for 30 minutes at room temperature. 200µL of each tube were carefully loaded into duplicate wells in a microliter plate and the absorbance was measured using a microplate reader ((µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

3.5.8. Protease Activity

The protease produced by *A. niger* was extracted from the shrimp shells samples (1 g each) using 20 mL deionized distilled water at room temperature with continuous stirring for 30 min 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, Whatman Inc., Clifton, New Jersey, USA). The supernatant was used for the assay of the enzyme protease. The protease activity was measured using a Protease Colorimetric Detection Kit (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 measured calorimetrically at 660 nm using a microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

3.5.9. Chitin

The chitin content was determined based on the fact that chitin contains about 6.89% organic nitrogen [25]. 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 shells (dry weight) along with 100 mL of NaOH solution

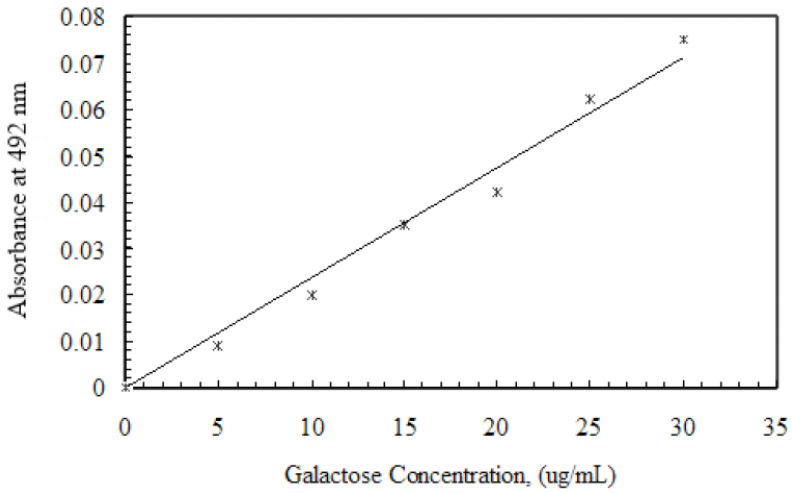


Figure 3: Galactose concentration standard curve.

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, Montreal, Quebec, Canada) for 1 h. The sample was filtered under suction through a Buchner funnel with coarse porosity filter paper (Reeve Angel Grade 202, Whatman Inc., Clifton, New Jersy, 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, Whatman 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 Kejldahl nitrogen analyses were performed on the dry deproteinized-demineralized sample and the chitin content was then calculated as follows:

$$(\text{Org.-N})_c = [(TKN_c - (NH_4\text{-N})_c] \times W_c/W_s \quad (5)$$

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

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)

$(NH_4\text{-N})_c$ is ammonium nitrogen of the recovered chitin (mg/kg)

W_c is weight of recovered chitin (g)

W_s is weight of sample (g)

3.5.10. 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).

4. RESULTS AND DISCUSSION

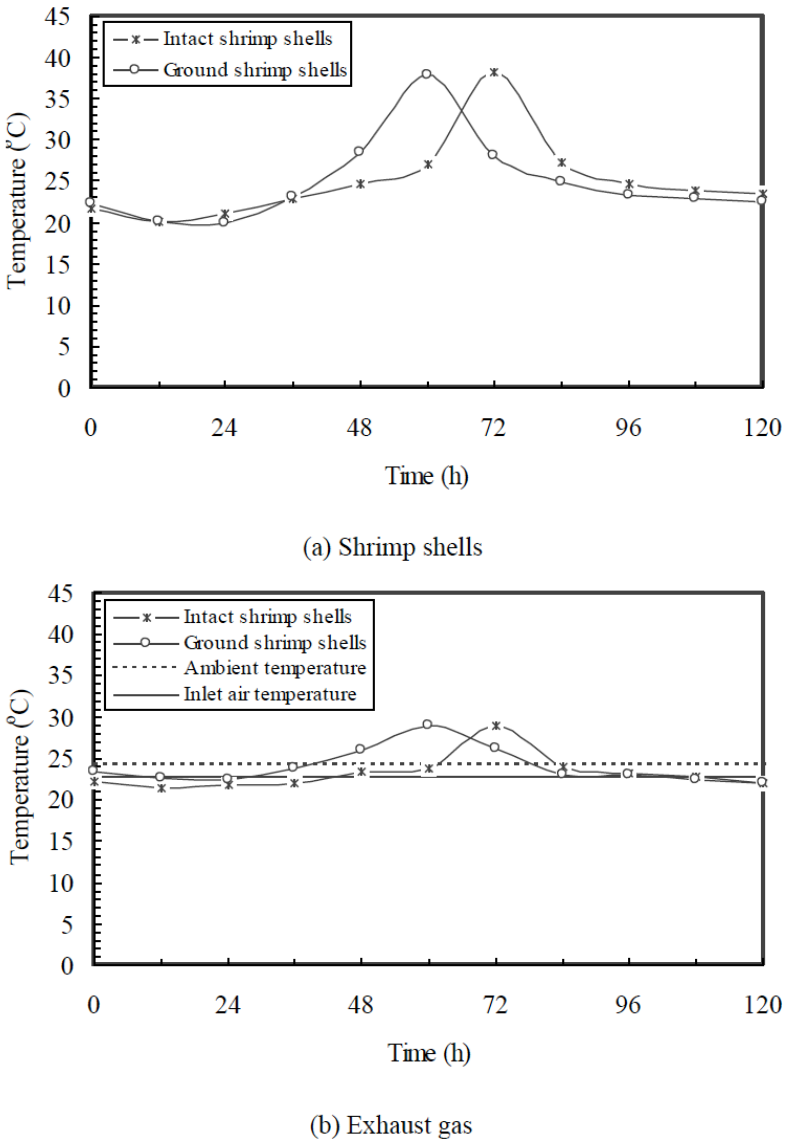
The effect of shrimp shells particle size (intact versus ground shells) on various parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein concentration and chitin concentration) during the deproteinization process was investigated.

4.1. Temperature

Figure **4** shows the changes in the temperatures of the shrimp shells and the exhaust gas during the course of deproteinization as affected by the shrimp shell particle size. The values are the average of three replicates. The coefficient of variation ranged from 0.59 to 5.29%.

Generally, microorganisms utilize organic matter for synthesis of new microbial cells, product formation and energy generation [26-28]. The temperature rises as the heat produced by metabolic activities exceeds heat losses by conduction through the shrimp shells and the bioreactor walls, convection with the exhaust gas and latent heat through water evaporation from the shrimp shells. In this study, the average inlet air temperature was 22.8 ± 0.59 °C and the initial temperature of the shrimp shells was 23.8 ± 0.50 °C. The temperatures of the shrimp shells and 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 microbial metabolism. After 24h, the temperature of the shrimp shells started to rise as the heat generation by metabolic activity exceeded the heat losses. Temperature peaks of 38.3 °C and 37,8 °C were noticed after 60 and 72 h of deproteinization for the ground and intact shrimp shells, respectively. The temperature of the exhaust gas also increased as a result of heat losses from the shrimp shells by convection. It reached maximum values of 29.1 ºC and 29.0 ºC after 60 and 72 h for ground and intact shells, respectively.

Pandey [29] reported a maximum substrate temperature of 41 ºC during solid-state fermentation of



(a) Shrimp shells

(b) Exhaust gas

Figure 4: Effect particle size on the temperatures of shrimp shells and exhaust gas.

wheat bran using *A. niger* for the production of glucoamylase. Mudgett (1986) reported that smaller particle size results in smaller packing densities of the substrate and less void space between the substrate particles which reduced heat transfer and gas exchange with the surroundings. However, this was not an issue in this study since agitation was provided.

Ghildyal *et al*. [30] and Pandey [26] reported that the temperature in the middle of the bed can reach about 20 ˚C higher than the temperature of the inlet air. Saucedo-Castañeda *et al*. [31] reported an axial temperature gradient of 0.17 ˚C/cm and a radial temperature gradient of 5 ˚C/cm in the bioreactor during the fermentation of cassava using *A. niger*. In the current study, the peak temperature of the shrimp shells was 7.7 ˚C higher than that of the exhaust gas and 17.9 ˚C higher than that of the inlet air. There were no temperature gradients in the radial and axial direction because of mixing.

Ben-Hassan *et al*. [32] and Ghaly *et al*. [33] showed that the microbial growth curve and the temperature curve were similar in shape and the temperature curve can be used to determine microbial kinetics. Table **4** shows some of the growth kinetic parameters for intact and ground shrimp shells calculated from the temperature curve. The specific growth and lag were determined graphically from the temperature data according to the procedure described by Ghaly *et al*. [34]. Lag periods of 24 and 12 h were observed for the intact and the ground shells, respectively. The exponential growth phase was 60 h for intact shells and 36 h for ground shells. The specific growth rate for *A.*

Table 4: Growth Kinetic Parameters

Parameter	Particle size	
	Intact	Ground
Lag phase (h)	24	12
Exponential phase (h)	60	36
Specific growth rate (h^{-1})	0.009	0.022
Stationary phase (h)	5	4
Initial temperature (°C)	21.8	21.8
Maximum temperature (°C)	37.8	38.3
Time to reach maximum temperature (h)	72	60

Values were calculated from the temperature curve.

niger was 0.009 and 0.022 h^{-1} for intact and ground shells, respectively.

4.2. pH

Figure **5** shows the change in pH during the course of deproteinization as affected by the shrimp shells particle size. The values are the average of three replicates. The coefficient of variation ranged from 0.81 to 4.21%.

For the run with ground shells, the pH decreased from the initial value of 8.64 to 7.34 during the first 50 h, then increased reaching 7.96 at the 82 h and finally declined to 7.68 by the end of the deproteinization process. For the run with the intact shells, the pH decreased from the initial value of 8.64 to 6.65 during the first 72 h, then decreased reaching 7.77 by the end of the deproteinization process. The decrease in the pH of the shrimp shells was due to the production of acid protease while the increase in the pH of the shrimp shells was due to the buffering capacity of the calcium carbonate released from shrimp shells (Yang and Lin, 1998) as well as the production of ammonium nitrogen [35].

Beaney *et al*. [9] reported a rapid decrease in pH (to 3.5) over 7 days during lactic acid fermentation of prawn shells as a result of production of metabolic lactic acid. Zakaria *et al*. [3] reported a drop in the pH (to 5) over the first 48 h of lactic acid fermentation of scampi waste after which the pH increased reaching a final value of 6.6 as a result of the buffering capacity of the solubilized calcium. Andrade *et al*. [36] reported a decrease in the medium pH during protease production by *Mucor circinelloides* as a result of the accumulation of metabolites produced from D-glucose degradation.

4.3. Moisture Content

Figure **6** shows the change in moisture content of the shrimp shell during the course of deproteinization

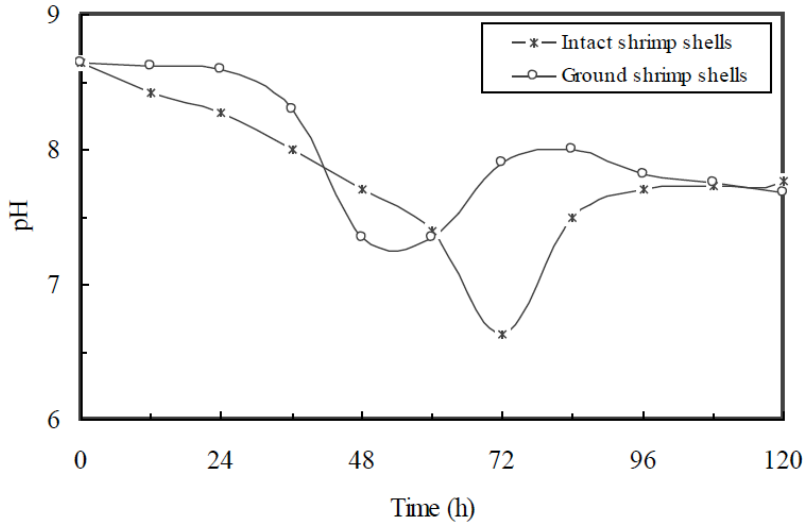


Figure 5: Effect of particle size on the pH.

as affected by the shrimp shells particle size. The values are the average of three replicates. The coefficient of variation ranged from 0.07 to 6.21%.

The net moisture content of the bed of shrimp shells can be defined by the following equation:

$$MC_{net} = MC_i + MC_m - MC_e \quad (7)$$

Where:

MC_{net} Is the net moisture content (%)

MC_i Is the initial moisture content (%)

MC_m Is the metabolic moisture content (%)

MC_e Is the moisture lost through evaporation with the exhaust gas (%)

The initial moisture content of the shrimp shells was adjusted to 60%. It declined slowly during the deproteinization process reaching 51.44% and 52.20% after 48 and 60 h and then started to decline at a faster rate reaching 25.54% and 36.63 by the end of the deproteinization process for the ground and intact shrimp shells, respectively. The results showed that the shrimp shells particle size affected the moisture content of the shells in the bioreactor during the deproteinization process. The surface area: volume ration of the ground shells was higher allowing more moisture to be evaporated from the shells. Mudgett [37] reported that smaller particle sizes cause the moisture to be distributed in thinner films on the external surfaces of the substrate that are exposed to the passing gas.

Diaz *et al*. [38] noticed that the microbial activity was inhibited when the moisture content decreased below 40% and it completely ceased when the moisture content reached 8-12 %. In this study, the moisture content of the shrimp shells fell below 40% after 64 and 84 h of the deproteinization process for the runs with ground and intact shrimp shells, respectively.

4.4. Galactose Utilization

Figure **7** shows the residual galactose concentration in the shrimp shells during the course of deproteinization as affected by the shrimp shells particle size. The values are the average of three replicates. The coefficient of variation ranged from 1.47 to 12.92%.

The galactose concentration in the bioreactor decreased with time and the rate of galactose utilization by the fungus was significantly higher in case of ground shrimp shells. Residual galactose of 1.48% and 2.33% remained unutilized by the end of the deproteinization process for the runs with ground and intact shrimp shells, respectively. Mudgett [37] reported that size reduction resulted in higher surface nutrient concentrations and shorter pathways for nutrients diffusion either on the surface or in the pores of the substrate. Molony *et al*. [39] reported that substrates with finer particles showed improved degradation as a result of the increase in the surface area. Pandey *et al*. [40] reported that substrates with finer particles resulted in high degree of nutrient solubilizeation.

4.5. Carbon Dioxide Evolution

Figure **8** shows the effect of shrimp shells particle size on the carbon dioxide concentration in the exhaust gas. The values are the average of three replicates.

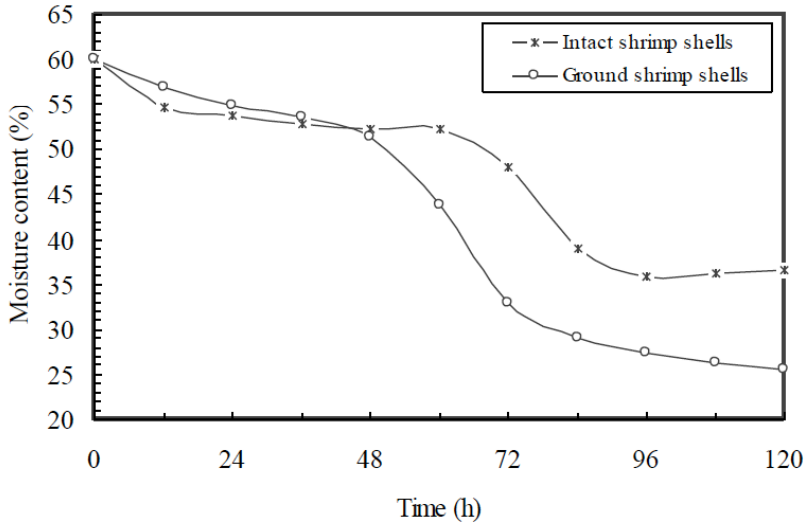


Figure 6: Effect of particle size on moisture content.

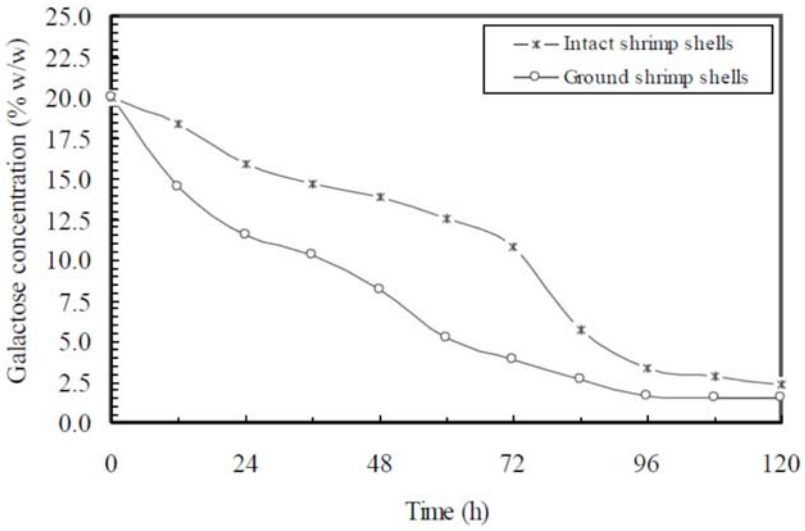


Figure 7: Effect of particle size on residual galactose concentration.

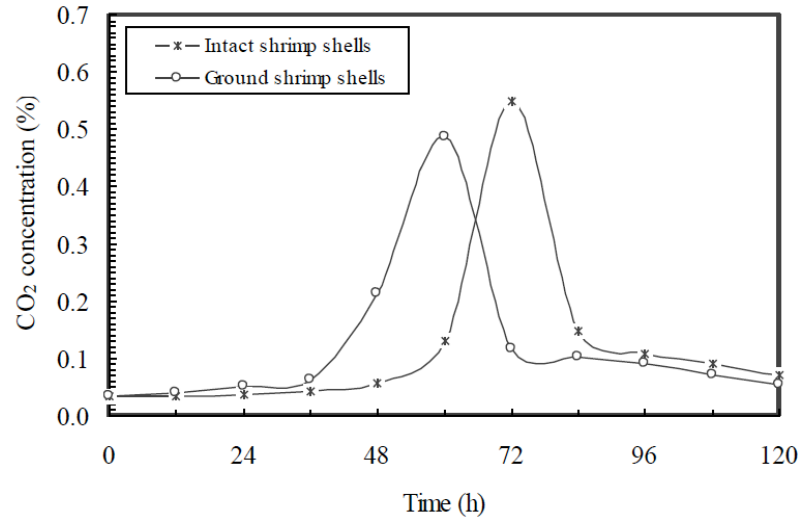


Figure 8: Effect of particle size on carbon dioxide concentration in the exhaust gas.

The coefficient of variation ranged from 1.41 to 12.58%.

The carbon dioxide increased from the initial value of 0.02% to maximum concentrations of 0.55 and 0.49% after 72 and 60 h and then declined reaching 0.07% and 0.05% by the end of the deproteinization process for the intact and ground shrimp shells, respectively. No reports were found in the literature on the direct effect of particle size on carbon dioxide evolution.

The results showed that the profile of the carbon dioxide concentration in the exhaust gas was similar to that of the temperature of the shrimp shells. A strong correlation between the carbon dioxide concentration in the exhaust gas and the temperature of the shrimp shells was obtained (Figure **9**). The rise in temperature and evolution of carbon dioxide are considered strong indicators of microbial activity during solid-state fermentation [41-42]. Carrizalez *et al*. [43] used carbon dioxide measurements for the determination of microbial growth of *A. niger* culture on cassava flour.

4.6. Protease Activity

Figure **10** shows the effect of shrimp shells particle size on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shells and are the average of three replicates. The coefficient of variation ranged from 1.41 to 2.58%.

The protease activity increased from 0.71 to 1.62 units/g shrimp shells (dry weight) and from 0.67 to 4.21 units/g shrimp shells (dry weight) over the course of the

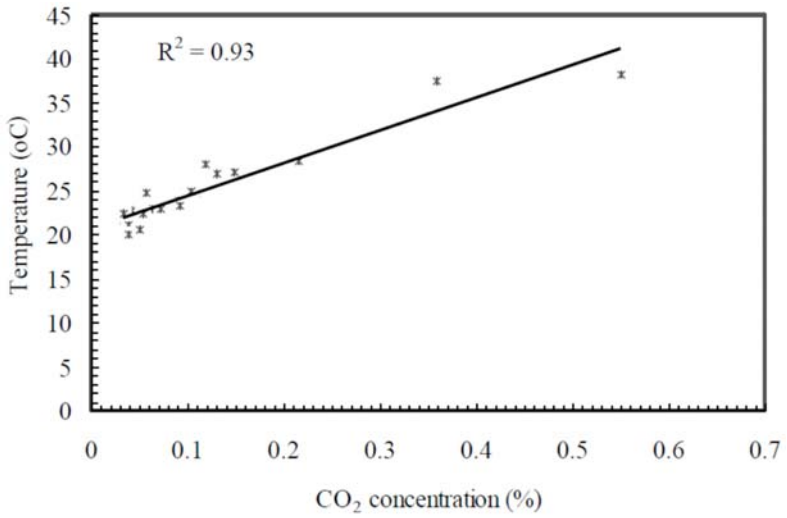


Figure 9: Correlation between carbon dioxide concentration in the exhaust gas and temperature of the shrimp shells.

deproteinization process for the intact and ground shrimp shells, respectively. Grinding the shells increased the production of proteases by 2.6 fold. Size reduction of the shrimp shells increased the surface area and made the shrimp shell protein more accessible to the microorganisms.

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:

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

Wang and Chio [13] and Andrade *et al*. [36] stated that the protein must be easily accesable for the stimulation of proteases production. Bustos and Healy [5] stated that the degree to which a microorganism will hydrolyze a protein substrate depends on its capacity to produce proteases and the stability of such protease to carry out the hydrolysis under the reaction conditions.

Zakaria [25] studied the effect of particle size on lactic acid solid-state fermentation for chitin purification from shellfish waste and fund that mincing shellfish waste (average of 600 µm) promoted good fermentation and resulted in higher level of chitin compared to large particle sizes. Pandey [44] studied the effect of wheat bran particle size on the production of the enzyme glucoamylase by *A. niger* and found that substrates of particle size in the range of 425-600 µm gave the highest productivity. Krishna and Chandrasekaran [45] reported an optimum particle size

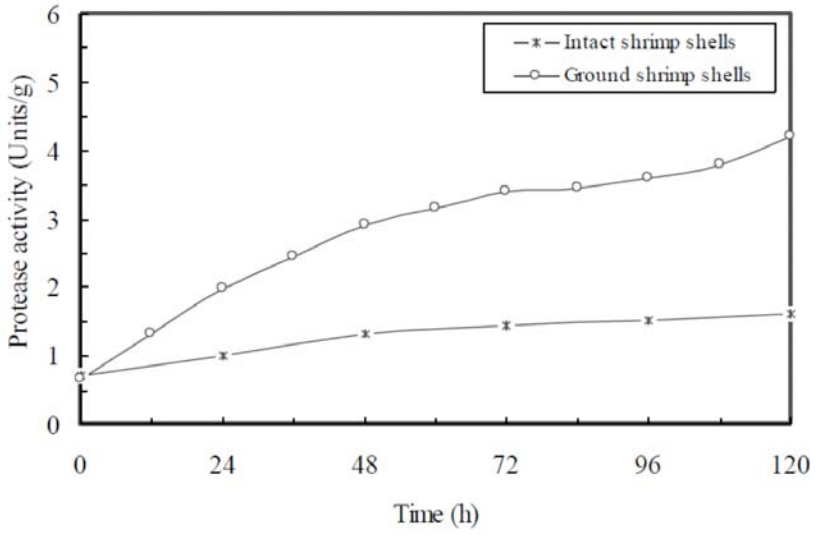


Figure 10: Effect of particle size on protease activity.

of 400 μm for the production of α-amylase from banana waste using *Bacillus subtilis* in solid-state fermentation. However, Chakraborty *et al*. [46] reported no difference in acid protease production using wheat bran of different particle sizes.

4.7. Residual Protein Concentration

Figure **11** shows the effect of shrimp shells particle size on the residual protein in the shrimp shells during the deproteinization process. 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 1.34 to 3.28%.

The results showed that the decrease in the protein concentration was faster in case of ground shrimp shells. The protein concentration decreased from the initial value of 30.84 to final values of 21.45 and 20.59% by the end of the deproteinization process for the intact and ground shells, respectively. However, the protein removal efficiency did not correspond to the protease production. This might be explained as a result of unsuitable conditions (pH and Temperature) for the produced proteases to hydrolyze the shells proteins.

Diniz and Martin [47] stated that the extent of hydrolytic degradation of protein is highly dependent on both pH and temperature. The optimum pH for cell growth and proteases production is different from the optimum pH for maximum enzymatic activity and hydrolysis of proteins. The protease of *A. niger* is acid resistant with an optimum pH of 2.3 [48]. Tthus, the high initial pH of the shrimp shells (8.64) might have interfered with protease activity Mudgett [37] and Wang and Chio [13] reported that high temperatures during deproteinization of shrimp and crab shells with the protease of *Pseudomonas aeruginosa* K-187 caused a low protein removal efficiency as a result of the Maillard reaction associated with high temperatures that renders the proteins resistant to deproteinization. In this study, autoclaving of shrimp shells may have affected the protein making it more resistant to deproteinization. Also the high temperature observed during the deproteinization process may have affected the activity of proteinase.

4.8. Chitin Concentration

Figure **12** shows the effect of shrimp shells particle size on the chitin content in the shrimp shells during the deproteinization process. 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 1.64 to 3.98%.

The results showed small differences between the chitin concentrations in the intact and ground shrimp shells over the course of the deproteinization process. The chitin concentration increased over time from the initial value of 16.59 to final values of 21.99 and 22.68% at the end of the deproteinization process for the intact and ground shells, respectively. Zakaria *et al*. [3] 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*. [49] 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. In this study. the particle size reduction of the shrimp shells caused only 3.14% increase in chitin concentration over that of the intact shells.

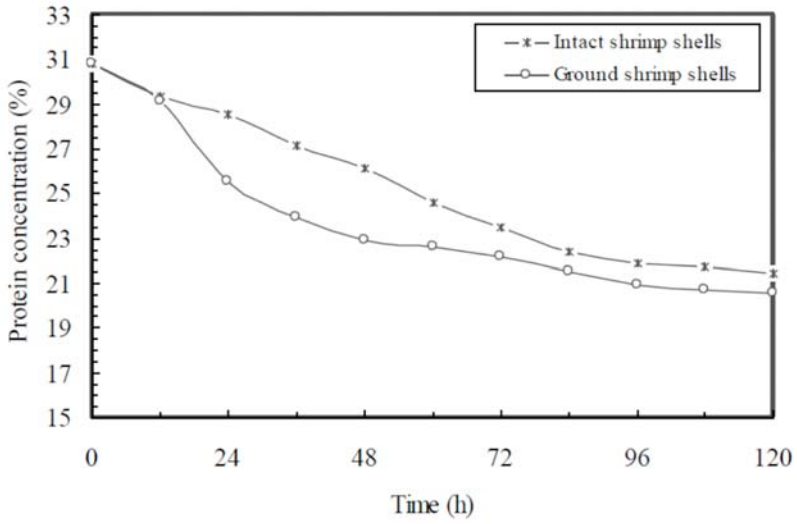


Figure 11: Effect of particle size on residual protein concentration.

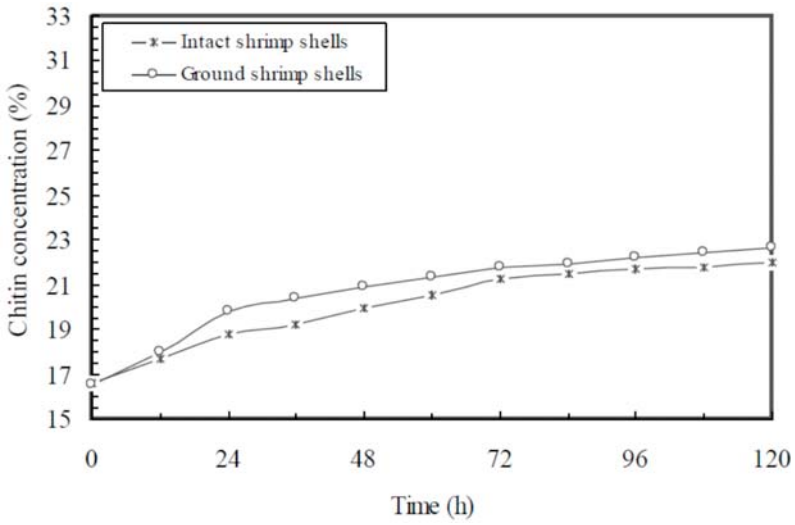


Figure 12: Effect of particle size on chitin concentration.

4.9. Visualization of Shrimp Shells

Figure **13** shows the visual appearance of the intact and ground shrimp shells at the end of the deproteinization process. Figure **14** shows the appearance of the intact and ground shrimp shells under the microscope (60X magnification) at the end of the deproteinization process. The color of the intact and ground shells was pale pink-orange with some tan patches. When the shells were inspected under the microscope at 60X magnification, it appeared that the ground shells had more white precipitants which were believed to be a result of break down of substances from the shell matrix. Some of the ground shrimp shells agglomerated and formed small balls. This problem was not observed with the intact shells. Lonsane *et al*. [50] reported that agglomeration of substrate particles into balls is a common problem when using drum type bioreactors as a result of the tumbling effect.

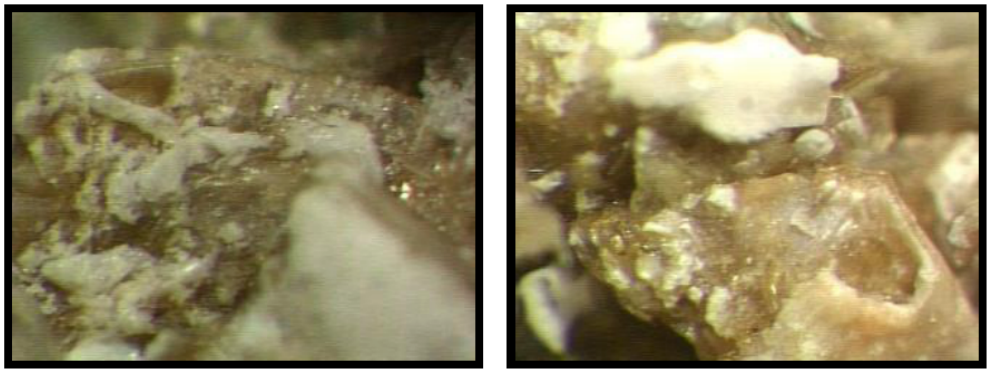
5.CONCULOSIONS

The results obtained from this study revealed the ability of *A. niger* to produce extracellular proteases in the presence of shrimp shells and supplemented galactose. The particle size of the shrimp shells affected the kinetic parameters of the deproteinization process. Size reduction (grounding of the shells) increased the surface area of the shrimp shells which resulted in a higher specific growth rate of *A. niger* and enhanced protease production (2.6 fold increase in the case of the ground shells).

The temperatures of the shrimp shells and exhaust gas declined at the beginning of the deproteinization process (lag period) as the heat losses from the bioreactor exceeded the heat generated by metabolic activity. After 24 h, the temperature of the shrimp shells started to rise as the heat generated by metabolic activity exceeded the heat losses. The rise in the

(a) Intact shrimp shells (b) Ground shrimp shells

Figure 13: Appearance of shrimp shells after 120 h of deproteinization.



(a) Intact shrimp shells (b) Ground shrimp shells

Figure 14: Microscopic images (60 X) of the shrimp shells after 120 h of deproteinization.

exhaust gas temperature was due to heat losses (from shrimp shells) by convection. Temperature peaks of 38.3 °C and 37.8 °C for the shrimp shells and 29.1 ºC and 29.0 ºC for the exhaust gas were noticed after 60 and 72 h of deproteinization for the ground and intact shrimp shells, respectively. There were no temperature gradients in the radial and axial directions because of mixing. However, the temperature observed in this study was higher than the optimum temperature range of 25-30 ^{0}C for the growth of *A. niger* and protease production.

The pH first decreased with time due to the production of acid protease and then increased due to the buffering capacity of the calcium carbonate released from shrimp shells and the production of ammonium nitrogen. A significant reduction in the moisture content was noticed during the deproteinization process because the water lost by evaporation was higher than the water released by microbial activity. To maintain the moisture of the substrate inside the bioreactor at the desired level, the exhaust gas should be passed through a condensation tower and the recovered water pumped back into the bioreactor through the aeration tube.

The galactose concentration decreased with time and the rate of galactose utilization was significantly higher in case of ground shrimp shells. Size reduction results in higher surface area and shorter pathways for nutrients diffusion. Galactose utilization of 92.6% and 88.35% was observed for the ground and intact shrimp shells, respectively. A protein removal efficiency of 30.45% and 33.23% were obtained for the intact and ground shells, respectively. The protein removal efficiency did not correspond to the protease production. This could be a result of unsuitable conditions (pH and temperature) for the produced proteases. The optimum pH for cell growth and proteases production is different from the optimum pH for the hydrolysis of proteins. The optimum pH for *A. niger* protease is 2.3 and the high initial pH of the shrimp shells (8.64) might have interfered with protease activity. Also, autoclaving of shrimp shells may have affected the protein and the high temperature observed during the deproteinization process may have affected the proteinase activity. Therefore, removal of metabolic heat during the deproteinization process might have a positive effect on the growth of *A. niger* and the production of protease.

The chitin concentration increased over time from the initial value of 16.59 to final values of 21.99 and 22.68% for intact and ground shells, respectively. The color of the intact and ground shells was pale pink-orange with some tan patches. Some of the ground shrimp shells agglomerated and formed small balls. This problem was not observed with the intact shells. The ground shells had more white precipitants which were believed to be a result of break down of substances from the shell matrix.

ACKNOWLEDGEMENT

The research was funded by the National science and Engineering Council (NSERC) of Canada. The support of Dalhousie University and Cairo University is highly appreciated

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Received on 16-03-2015 Accepted on 31-03-2015 Published on 15-04-2015

DOI: http://dx.doi.org/10.12970/2311-1755.2015.03.01.3