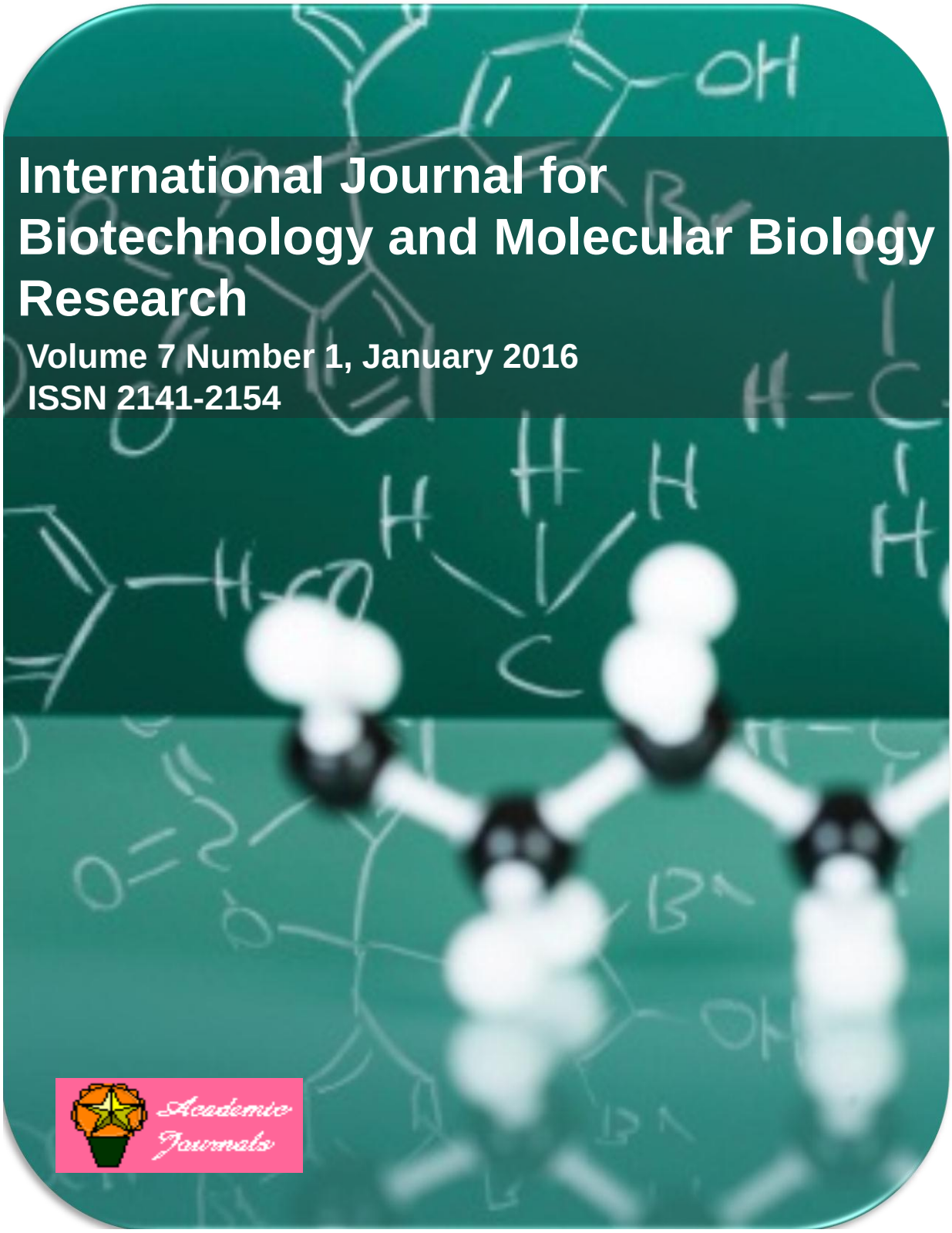

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Examples:

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Effect of the initial pH on the performance characteristics of the deproteinization process of galactose supplemented shrimp shells by *Aspergillus niger* in a solid state drum bioreactor

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The aim of the research was to study the effect of adjusting the initial pH of shrimp shell (8.64 vs. 7.00) on the ability of the fungus *Aspergillus niger* to carry out deproteinization process of galactose supplemented shrimp shells and to investigate the performance characteristics of the deproteinization process. The results showed that the initial pH of the shells has a significant effect on the temperature, carbon dioxide emission, moisture content, galactose, proteinase activity, protein, chitin and final appearance of shells. 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 microbial activities in the bioreactor. They started to rise during the exponential growth when the heat generation by metabolic activity exceeded the heat losses, reaching maximum values of 37.5 and 30.6°C after 60 h and of 29.1 and 27.2°C after 72 h for the runs with the initial pH of 8.64 and 7.0, respectively. The carbon dioxide increased with time reaching maximum values of 0.49 and 0.22% and then declined reaching 0.06 and 0.08% by the end of the experiment for the runs with initial pH of 8.64 and 7.00, respectively. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was observed. The initial moisture content of 60% fell below 25.5 and 21.5% and the galactose concentration decreased from the initial value of 20 to 1.48% and 6.17% by the end of the deproteinization process for the runs with initial pH of 8.64 and 7.00, respectively. Although the protease activity increased by 3.3-6.3 fold, the reduction in the protein concentration did not correspond to the increase in the protease activity due to the high pH of the shrimp shells. The chitin concentration increased from an initial value of 16.56 to final values of 22.68 and 21.35 for the initial pH of 8.64 and 7.00, respectively. The spent shrimp shells obtained from the run with the initial pH of 8.64 were wet and had a pale pink-orange color with some tan patches, whereas the spent shrimp shells obtained from the with the initial pH of 8.64 appeared dry and had a gray-black color due to the presence of *A. niger* spores. The use of lactic acid to lower the pH of shrimp shells inhibited the growth of *A. niger* and protease production and activity and enhanced sporulation. The existence of the pink-orange color was an indication of the presence of pigments, which were not utilized during the fermentation process.

Key words: Shrimp shells, *Aspergillus niger*, deproteinization, protein, protease, chitin, galactose, temperature, pH, moisture content.

INTRODUCTION

Chitin is a polymer of nitrogen containing polysaccharide $(C_8H_{13}O_5N)_n$] which render it a tough protective covering or structural support in certain organisms. It is found in plants and shell fish and it makes up the cell walls of fungi and exoskeleton of insects. Chitin and its derivatives are versatile environmentally friendly modern materials that have been used in virtually every segment of the economy because of their wide range of properties. The applications for chitin and its derivatives include water treatment, pulp and paper, biomedical and therapeutic devices, cosmetics, biotechnology, agriculture, food science and membrane technology (Abdullah, 1995; Kumar, 2000; Sashiwa and Aiba, 2004; Pillai et al., 2009).

Chitin present in the crustacean waste is associated with proteins, minerals (mainly calcium carbonate) and pigments. The traditional method of chitin extraction from crustacean waste involves the use of strong acid (HCl) for demineralization and strong alkali (NaOH) for deproteinization (Zakaria et al., 1998; Bustos et al., 1999; Ghanem et al., 2004). The disadvantages of this method are: (a) it yields chitin with variable physiochemical properties, (b) it results in a wastewater that creates costly disposal problems, (c) it wastes other valuable components such as protein and pigments present in the waste material and (d) it is very costly. Therefore, a less expensive environmentally friendly method for chitin extraction from crustacean waste is needed.

Several authors reported on biological deproteinization of shrimp shells using proteolytic microorganisms (Wang and Chio, 1998; Teng et al., 2001; Ghaly and Mahmoud, 2015) or purified proteolytic enzymes (Wang and Chio, 1998; Mahmoud and Ghaly, 2015) and organic acids for demineralization (Hall and Reid, 1994; Beaney et al., 2005). However, the use of purified enzymes is not as effective as living microbes. The presence of microorganisms provides a constant and gradual increase of protease throughout the fermentation (Teng et al., 2001). Thus, biological chitin extraction using microorganisms appears to be a good alternative to the harsh chemical extraction because: (a) it is considered less expensive as compared to the chemical method, (b) it results in an effluent that is less harmful to the environment and (c) it preserves the natural state of the biopolymer.

Aspergillus niger is an important microorganism that has been used in different industrial applications including production of organic acids such as citric and gluconic acids (Ruijter et al., 1999; Ali etal., 2002; Auta et al., 2014) and extracellular enzymes such as pectinase, amylase, protease, maltase, lactase, catalase, proteinase, trehalase, tannase, dipetalase, polypetalase, lipase, cellulose, amidase, glucose oxidase, glucose dihydrogenase, urease, insulase, melibase and zymase (Naidu and Panda, 2003; Mahmoud and Ghaly, 2015). *A. niger* is also considered a source of chitin as it contains up to 42.0% chitin of the dry weight of the fungal cell wall; the cell wall weight is 20-40% of the total dry cell weight (Ghaly and Mahmoud, 2015). Therefore, *A. niger* has a great potential for deproteinization of the crustacean waste in a solid state fermentation as 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 (Teng et al., 2001).

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. *Pandalus borealis* has a mean length of 22-25 mm at maturity (Hansen and Aschan, 2000). The total landing of Northern shrimp in Eastern Canada in 2013 was 185974 tonnes (DFO, 2015). Based on the processing, removal of these shrimp (80% of its original weight), the total amount of waste produced in 2013 was 148000 tonnes most of which was dumped in the ocean or landfills.

The aim of this study was to evaluate the performance of a solid state deproteinization of shrimp shells supplemented with galactose as a carbon source by the fungus *A. niger* under different pH regimes. The specific objectives of the research were: (a) to study the effect of adjusting the initial pH of shrimp shells (7.00 vs. 8.64) on the ability of the fungus *A. niger* to produce the protease enzyme and carry out the deproteinization process of shrimp shells and (b) to investigate the changes in the kinetic parameters (pH, moisture content, galactose content, protease activity, protein content and chitin content) of the deproteinization process and the final shells appearance

MATERALS AND METHODS

Experimental apparatus

The slid state deproteinization system (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

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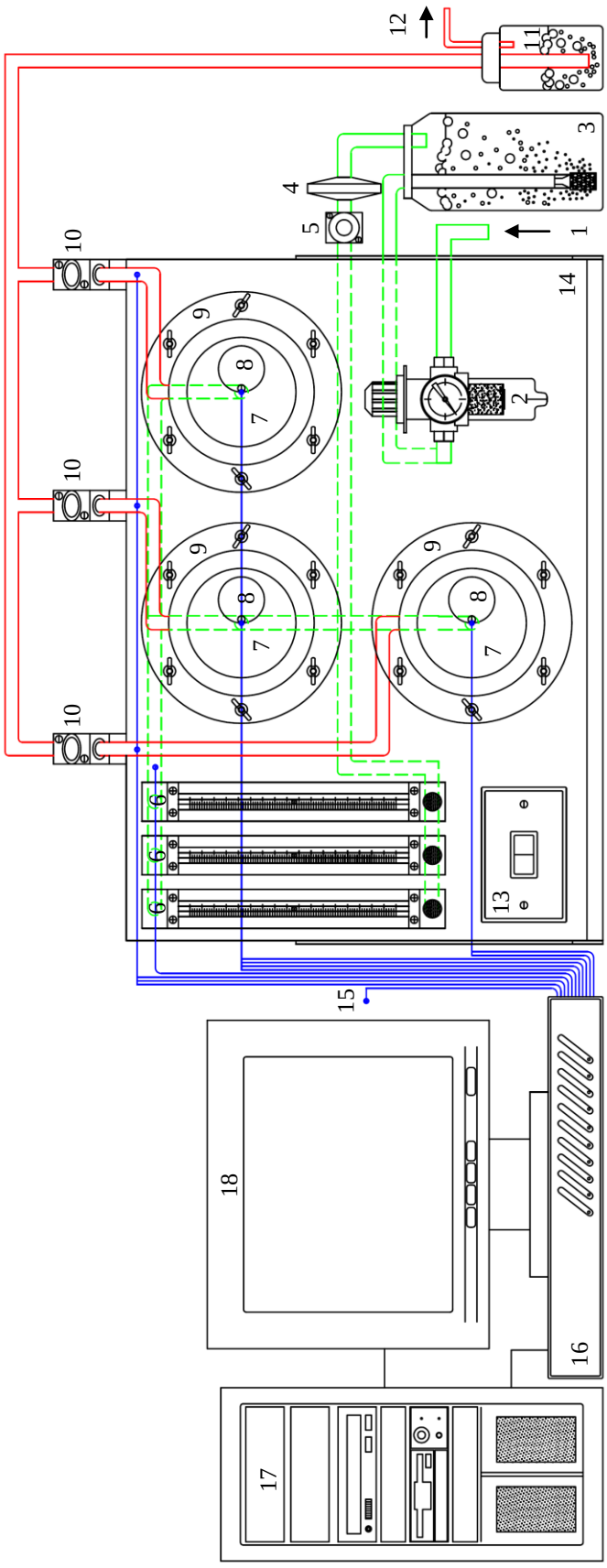
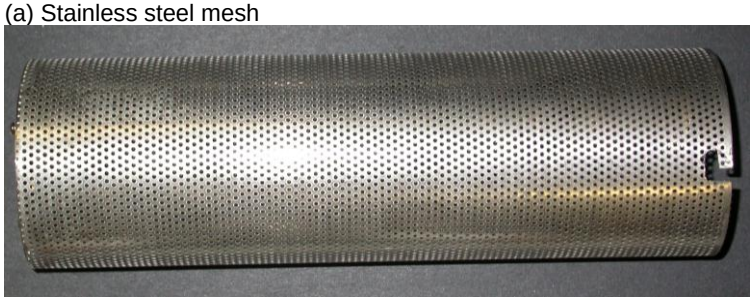
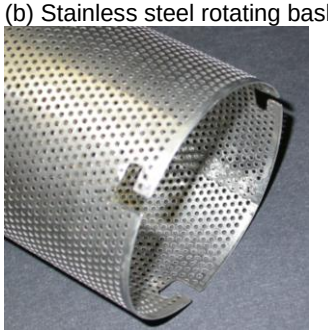


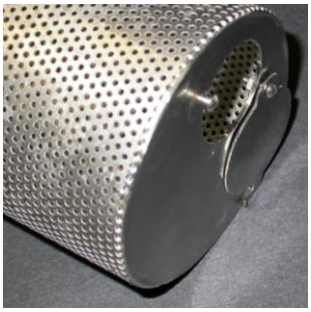
Figure 1. Experimental setup.

(a) Stainless steel mesh

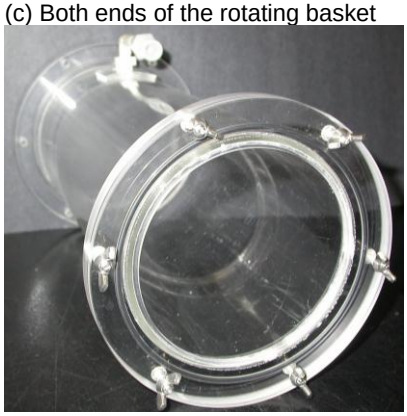


(b) Stainless steel rotating basket

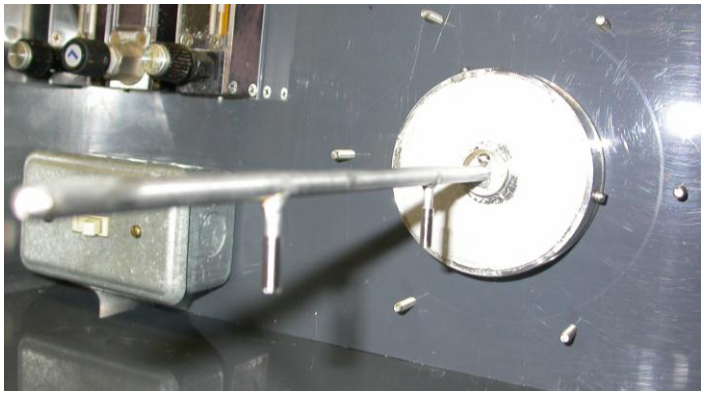




(c) Both ends of the rotating basket



(d) Plexiglas cylinder

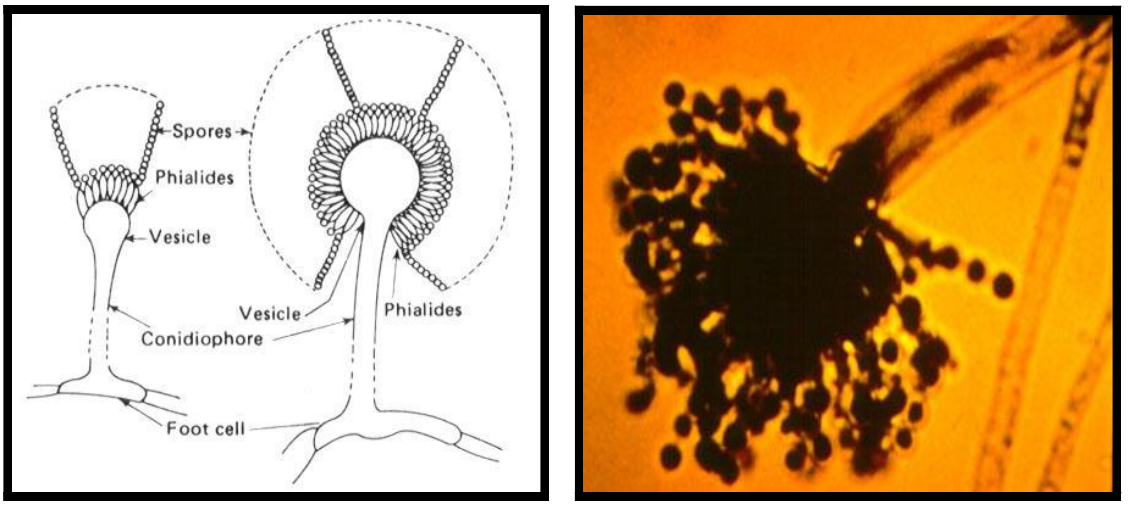


(e) Rotating disc, aeration tube and thermocouples

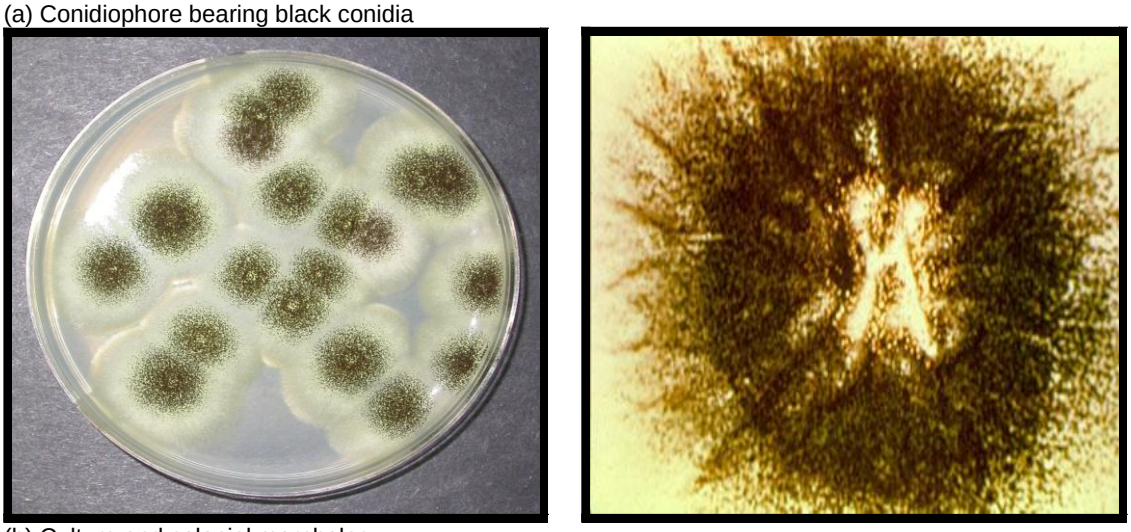
Figure 2. The drum bioreactor.

manifold, tubing and sampling ports, the thermocouple wires, the mixing motor along with the transmission system and the switch.

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 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, 20-34245G-24007, Xerox127P1292/B,



(a) Conidiophore bearing black conidia



(b) Culture and colonial morphology

Figure 3. *Aspergillus niger* colonies.

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 kept at room temperature. The humidified air was passed through a bacterial filter and then through a flow meter (No. 60648, Cole-Parmer Instrument Co., Illinois, USA) and 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. The other five thermocouples were used to measure the ambient temperature (1), inlet air temperature (1) and exhaust gas temperature of each bioreactor (3). The temperature data were monitored and stored using a data acquisition system which consisted of a master unit (Multiscan 1200, Omega, and Stamford, CT), a thermocouple/volt scanning card (MTC/24, Omega, Stamford, Connecticut, USA), a software (Tempview, Omega, Stamford, Connecticut, USA) and a personal computer.

Microorganisms

The fungus *A. niger* (Figure 3) was chosen for this study because of its ability to produce acid protease and the presence of chitin in its cell wall. The genus *Aspergillus* is characterized by a well-developed foot cell at the base of the conidiophore. The colony consists of colorless mycelium from which conidiophora arise. The spores develop the black color and the powdery appearance of the colony surface as sown in Figure 3.

A. niger (ATCC 16513) was obtained from the American Type Culture Collection (Rockville, Maryland). The freeze dried culture was revived in 6 mL of 0.1% sterilized peptone solution, which was prepared by dissolving 1 g Becto-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 min. The rehydrated culture was kept in the peptone solution in a capped test tube for 24 h at room temperature (21°C). 1 mL of the rehydrated *A. niger* was transferred to each of three test tubes containing 9 mL potato dextrose broth (PDB), which contained infusion from 200 g potatoes (4 g/L) and 20 g/L Bacto dextrose. The test tubes were kept tightly capped for 48 h at room temperature (21°C) and then stored in the fridge at 4°C and subcultured 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°C) for 4 days. The conidia were harvested from the surface by adding sterilized deionized distilled water containing 0.01% (v/v) Tween 80 (prepared by dissolving 0.1 mL Tween 80 in 1 L distilled deionized water and then autoclaved at 121°C and 103.4 kPa for 30 min) and gently scrape the surface with a sterile spatula. 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 (Wehr and Frank, 2004). The prepared suspension was stored in the refrigerator at about 4°C until needed.

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°C in the Biotechnology Laboratory till needed. The 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.

Experimental protocol

The effect of initial pH (8.64 and 7.00) on the deproteinization process of shrimp shells was studied. The experimental conditions of the deproteinization process are shown in Table 2. The sugar solution was prepared by dissolving 29 g galactose in 1 L deionized distilled water and then autoclaved (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 1 g 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. Lactic acid was used to adjust the pH to 7. Air was introduced inside each reactor at a flow rate of 5 VMM (volume air in mL per shell waste mass in grams 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 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 concentration, protease activity, ammonium nitrogen, total Kjeldahl nitrogen and protein. Exhaust gas samples were also taken every 12 h and analyzed for carbon dioxide concentration. The bulk temperature was monitored and recorded every 10 min. 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 the percentage from the total weight was calculated. Table 3 show the particle size distribution of the shrimp shells used in this study.

Moisture contents

A known weight of shrimp shells sample was placed in a preweighed aluminum dish. The dish and content were weighed and then placed in an oven (Isotemp Oven, Model 655F, Fisher Scientific, Montreal, Quebec, Canada) at 105°C for 24 h. The aluminum dish along with the dried sample 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).

pH

20 mL of deionized distilled water was added to one gram of shrimp shell sample 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).

Ammonium nitrogen

Samples 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°C till constant weight. The dried shells were ground using a small conventional grinder (Hamilton Beach, Markham, Ontario, Canada). Ammonium nitrogen (NH_4-N) of dry ground samples was determined directly using Kjeldahl system (KJELTEC AUTO 1030 Analyzer, Fisher Scientific, Montreal, Quebec, Canada).

Total Kjeldahl nitrogen

Samples 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°C till constant weight. The dried shells were

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.

ground using a small conventional grinder (Hamilton Beach, Markham, Ontario, Canada) and then digested by heating the sample 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 (420°C). 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 as determined using Kjeldahl system (KJELTEC AUTO 1030 Analyzer, Fisher Scientific, Montreal, Quebec, Canada).

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); $(Org.\text{-}N)_c$ is organic nitrogen of the recovered chitin (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).

Galactose concentration

20 mL of deionized distilled water was added to one gram of fermented shrimp shell sample 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

Table 2. Experimental conditions of the shrimp shells deproteinization process.

Parameter	Value
Studied Parameter	Initial pH
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	Intact Shrimp Shells (0.075-6.30 mm)
Autoclaving	Autoclaved Shrimp Shells
Initial Galactose Concentration	20 % w/w
Initial pH	7.00 and 8.64

Table 3. Particle size distribution of the shrimp shells.

Size (mm)	Under (%)	Above (%)
6.300	98.11	1.89
4.000	88.97	9.13
2.000	59.33	29.67
0.850	35.74	64.23
0.300	11.25	90.75
0.180	3.96	96.04
0.075	0.92	99.08
< 0.075	0.24	99.78

Jersey, 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 when reacting with phenol and concentrated sulfuric acid (Chow and Landhausser, 2004). 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 values of the absorbance were plotted against the known galactose concentrations (µg/mL) as shown in Figure 4. The following linear relationship between the galactose concentration and the absorbance was obtained (R^2= 0.98):

$$C_{ga} = 416.67\ (Ã_{492}) \quad (4)$$

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 min 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, Toronto, Ontario, Canada) at 30°C for 15 min after which the contents were mixed again using a vortex mixer. The tubes were allowed to stand for 30 min at room temperature. 200 µL of each tube were carefully loaded into duplicate wells in a microtiter plate and the absorbance was measured using a microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

Protease activity

Protease produced by *A. niger* was first extracted from the 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, Whatman 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 (Zakaria, 1997). 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 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 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°C 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 (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) 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°C till constant weight. The weight of the recovered dry deproteinized-demineralized sample was determined. The ammonium and total kejldhal nitrogen analyses were performed on

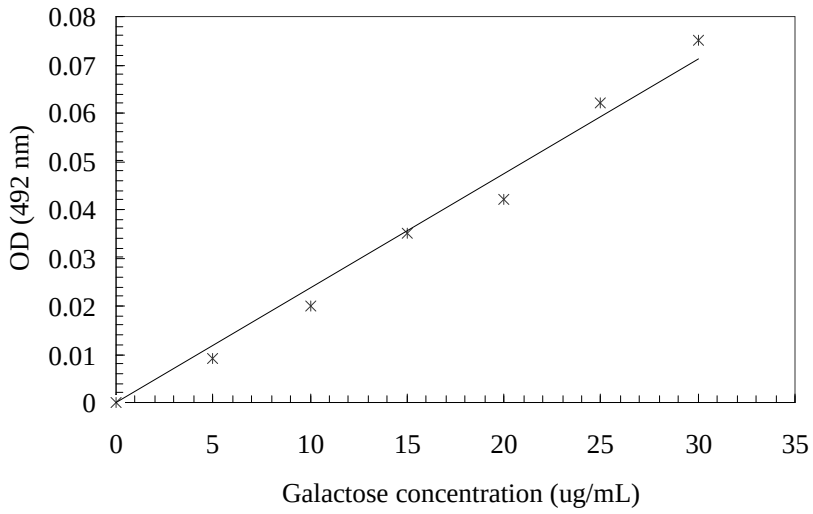


Figure 4. Galactose concentration standard curve.

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); $(Org.-N)_c$ is organic nitrogen of the recovered chitin; TKN_c is total Kjeldahl nitrogen of the recovered chitin (mg/kg); $(NH_4-N)_c$ 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°C for 2 h. The dish and content was taken from the muffle furnace, placed in a desiccator to cool down and then weighed. The ash content was determined as follows:

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

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°C 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 (Spectoronic 100, Bausch & Lomb Incorporation, Rochester, New York, USA) at 430 nm.

Visualization of shrimp shells

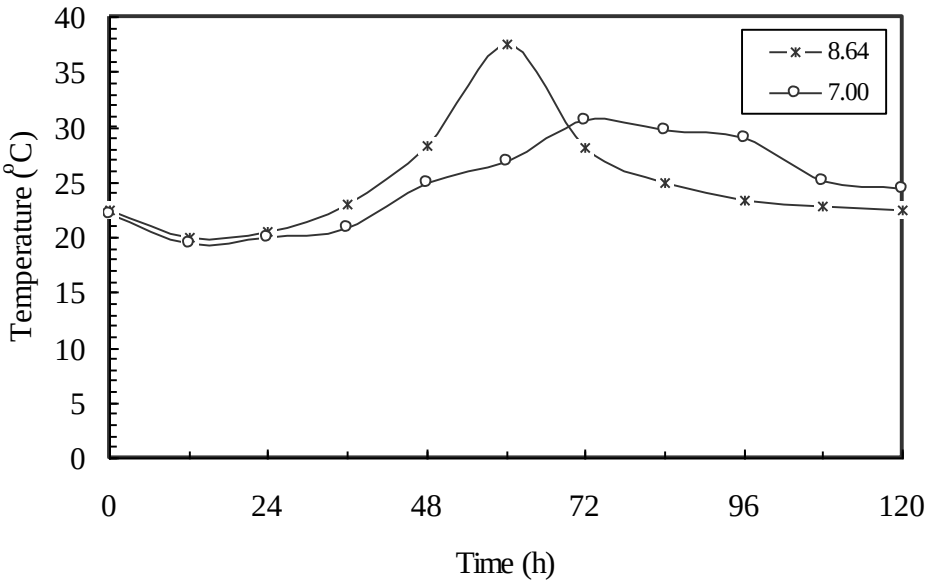
The shrimp shells were visually inspected at the end of the deproteinization with the naked eye as well as under the incident light stereomicroscope (Carl Zeiss Stemi SV8, Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) at a magnification of 60x. The stereoscope was equipped with a cold light source (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

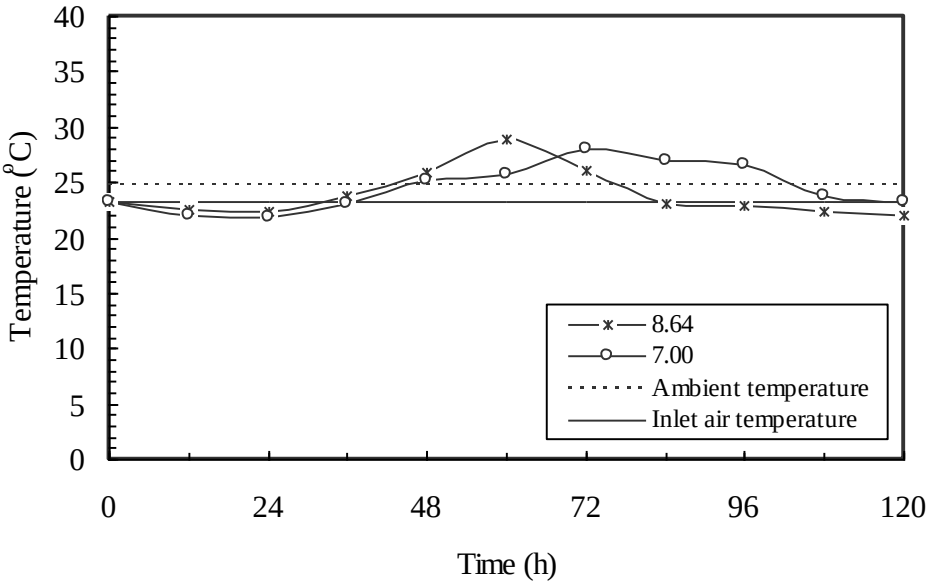
The effect of initial pH on various kinetic parameters (temperature, pH, moisture content, galactose utilization, carbon dioxide evolution, protease activity, residual protein and chitin concentration) of the deproteinization process was investigated.

Temperature

Figure 5 shows the changes in the temperatures of the shrimp shell bed and the exhaust gas during the course of deproteinization as affected by the initial pH. The values are the average of three replicates. The coefficient



(a) Shrimp shells



(b) Exhaust gas

Figure 5. Effect of initial pH on the temperatures of the shrimp shells and exhaust gas.

of variation ranged from 0.29 to 4.97. The fermentation reaction of shrimp shells is exothermic in nature in which heat is generated giving rise to the temperature of the medium. The fungus utilized organic matter for synthesis of new microbial cells, product formation and energy generation. The heat stored in the bioreactor is the net of metabolic heat produced minus the heat lost (by conduction through the shrimp shells and the bioreactor walls, by convection with the exhaust gas and latent heat through water evaporation from the shrimp shells).

In this study, 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 due to water evaporation (latent heat) and the cooling effect of the inlet air were higher than the heat generated in the bioreactor by microorganisms.

After 24 h, the temperature of the shrimp shells started to rise as the heat generation by metabolic activity

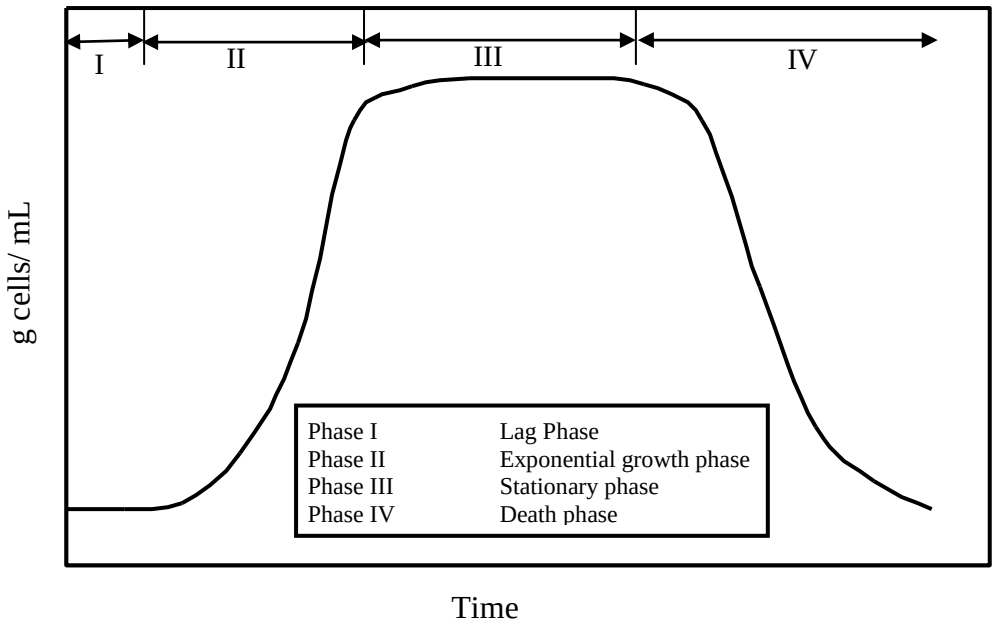


Figure 6. Typical batch growth curve.

exceeded the heat losses. However, the temperature profiles for the runs with the initial pH of 8.64 and 7:00 were different for both the shrimp shells and exhaust gas. For the shrimp shells, temperature peak of 37.5 and 30.6°C were noticed after 60 and 72 h for the runs with the initial pH of 8.64 and 7.0, respectively. Similarly, the temperatures of exhaust gas reached maximum values of 29.1 and 27.2°C after 60 and 72 h for the runs with initial pH of 8.64 and 7.0, respectively.

Ghaly and Mahmoud (2015) and Mahmoud and Ghaly (2015) reported similar temperature profiles during solid-state fermentation of shrimp shells. Pandey (2003) reported that temperatures in the middle of the bed can reach about 20°C higher than the temperature of the inlet air. Saucedo-Castañeda et al. (1990) 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 shells material was 13.5 and 6.6°C higher than that of the inlet air temperature for the runs with initial pH of 8.64 and 7.00, respectively. There were no temperature gradients in the radial or axial direction because of mixing.

A typical microbial batch growth curve (Figure 6) includes: (a) lag phase, during which microbial population remains unchanged and adaptation to the surrounding environment takes place, (b) exponential growth phase, during which the specific growth rate is constant (c) deceleration growth phase, during which growth decelerates either because of the depletion of an essential nutrient or the accumulation of an inhibitory product, (d) stationary phase, during which the net growth rate is zero as a result of the balancing of reproduction rate by an equivalent death rate and (e) death phase, during which cells die faster than new cells are produced. Zakaria (1997) stated that temperature is one of the most significant parameters affecting the growth and activities of microorganisms in solid-state fermentation. Ghaly et al. (2003) and Mahmoud and Ghaly (2015) showed that the microbial growth and the temperature curves are similar in shape and the temperature curve can be used to determine microbial kinetic parameters.

The growth kinetic parameters shown in Table 4 were determined from the temperature data. The length of the lag period was 12 and 18 h, the exponential growth phase was 48 and 60 h, the stationary phase was 4 and 24 h and the maximum temperatures of 37.5 and 30.6°C were attained after 60 and 72 h of deproteinzation for the runs with initial pH of shrimp shells of 8.64 and 7:00, respectively. Decreasing the initial pH from 8.64 to 7.00 decreased the specific growth rate of *A. niger* from 0.022 to 0.008 h^{-1}.

The significant reduction in the specific growth phase for initial pH of 7.00 could be the result of the inhibitory effect of the lactic acid used to lower the initial pH of the shrimp shells.

pH

Figure 7 shows the change in the pH of shrimp shells during the course of deproteinization as affected by the initial pH. The values are the average of three replicates. The coefficient of variation ranged from 0.49 to 3.62%.

Table 4. Growth kinetic parameters calculated from the temperate curve.

Parameter	pH	
	8.64	**7**
Lag phase (h)	12	18
Exponential phase (h)	48	60
Specific growth rate (h^{-1})	0.022	0.008
Stationary phase (h)	4	24
Initial temperature (°C)	21.8	21.8
Maximum temperature (°C)	37.5	30.6
Time of maximum temperature (h)	60	72

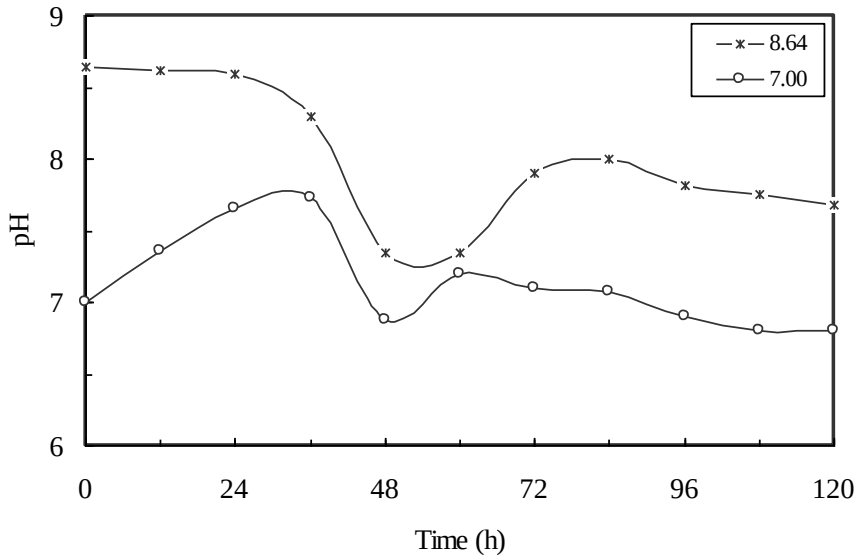


Figure 7. Effect of the initial pH on the pH of deproteinized shrimp shells.

For the run with the initial pH of 8.64, the pH of the shrimp shells decreased during the first 60 h reaching 7.34, then increased reaching 8.06 at the 83 h and finally declined to 7.86 by the end of the deproteinization process (120 h). For the run with the initial pH of 7.00, the pH of the shrimp shells increased during the first 36 h reaching 7.73, then decreased reaching 6.88 at the 48 h, then increased to 7.3 at the 60 h and finally declined to 6.75 by the end of the deproteinization process (120 h).

Zakaria et al. (1998) reported a drop in the pH to a value of 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. Beaney et al. (2005) reported rapid decrease in pH to 3.5 over 7 days during lactic acid fermentation of prawn shells as a result of metabolic lactic acid production. Andrade et al. (2002) reported that the medium pH decreased during the period of protease production using *Mucor circinelloides* as a result of metabolites accumulation resulting from D-glucose degradation. Beaney et al. (2005) reported rapid decrease in pH to 3.5 over 7 days during lactic acid fermentation of prawn shells as a result of metabolic lactic acid production.

The decrease in the pH of the shrimp shells observed in this study 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 the shrimp shells as well as the production of ammonium nitrogen as reported by Yang and Lin (1998) and Ghaly and Mahmoud (2015).

Moisture content

Figure 8 shows the change in the moisture content of the shrimp shells during the course of deproteinization as affected by the initial pH. The values are the average of

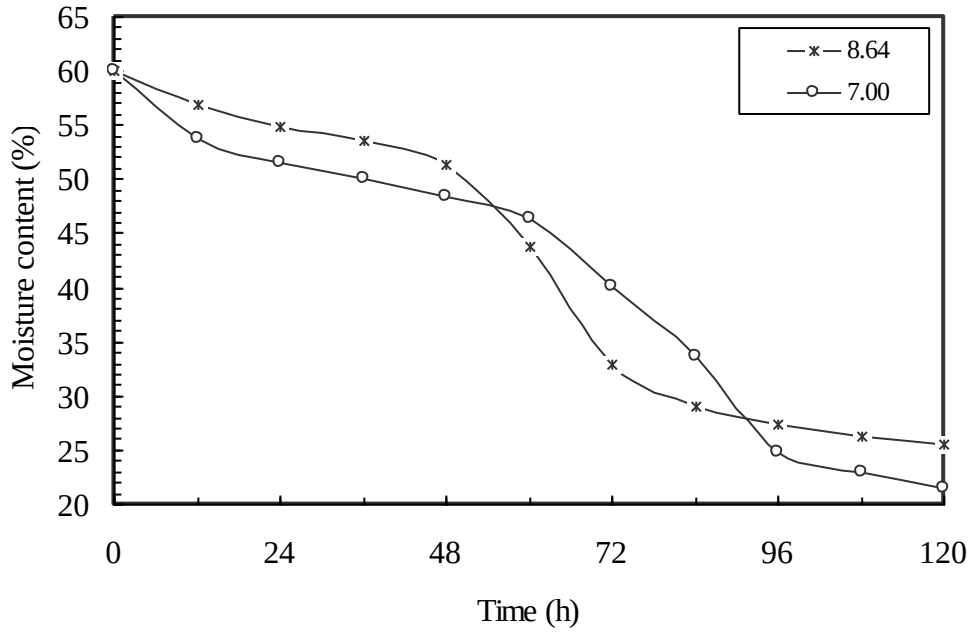


Figure 8. Effect of the initial pH on moisture content of shrimp shells.

three replicates. The coefficient of variation ranged from 0.24 to 4.53%. The net moisture content of the shrimp shell bed can be defined as follows:

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

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 moisture content of the shrimp shells declined first slowly in the first 48 h from the initial value of 60(%) and 52(%) and 47% and then sharply decreased reaching final values of 25.5(%) and 21.5% by the end of the deproteinization process for the runs with the initial pH of 8.64 and 7.00, respectively. The initial slow decrease in the moisture content was due to the initial low temperature which affected the amount of water lost by evaporation while the steep decline in the moisture content observed thereafter was due to the loss of moisture through evaporation because of the high temperature observed during the exponential growth phase. This indicated that water evaporation was higher than the metabolic water production.

The moisture content of the substrate has a significant impact on solid-state fermentation. Yang and Lin (1998) stated that low moisture content caused the substrate to be too dry for microbial growth and product formation and high moisture content caused packing of the substrate and prevention of gas exchange. Mahmoud and Ghaly (2015) reported optimum initial moisture content between 50-60% for the production of protease by solid-state fermentation of sweet potato residue supplemented with rice bran and minerals using *A. niger*. An initial moisture content of 70% can cause the substrate to stick together preventing gas exchange and causing low protease production whereas an initial moisture content of 40% can make the substrate to be too dry for mycelial growth causing inhibition of microbial activities and very poor protease production.

In this study, the initial moisture content of the shrimp shells was adjusted to 60%, which fell below 40% after 66 and 76 h and reached 25.5 and 21.5% by the end of the deproteinization process for the runs with initial pH of 8.64 and 7.00, respectively. This low moisture content affected fungus growth and protease production and in turn the hydrolysis of protein. 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.

Galactose utilization

Figure 9 shows the residual galactose concentration in the shrimp shells during the course of deproteinization as affected by the initial pH. The values are the average of three replicates. The coefficient of variation ranged from 2.91 to 3.39%.

The optimum galactose concentration of 20% w/w was used in this study as recommended by Mahmoud and

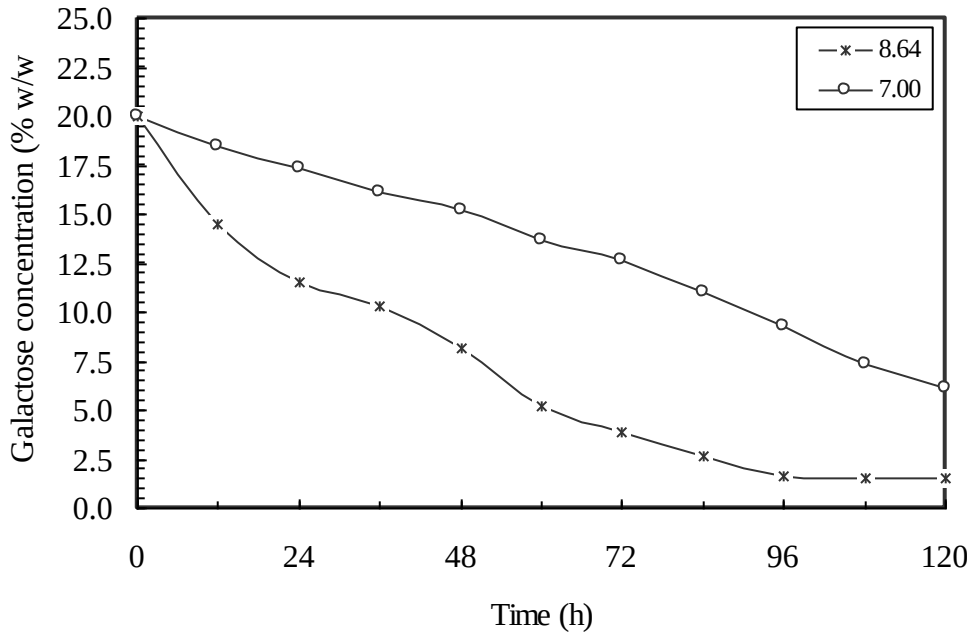


Figure 9. Effect of the initial pH on residual galactose.

Ghaly (2015). The galactose concentration decreased gradually from 20 to 1.48% and 6.17% over the course of the deproteinization process (120 h) for the runs with initial pH of 8.64 and 7.00, respectively. The high residual galactose concentration experienced by the end of the deproteinization process with initial pH of 7:00 could be the result of the inhibitory effect of lactic acid which was used to adjust the initial pH of the shrimp shells.

Carbon dioxide evolution

Figure 10 shows the effect of the initial pH on carbon dioxide concentration in the exhaust gas. The values are the average of three replicates. The coefficient of variation ranged from 1.99 to 5.34%.

Temperature and carbon dioxide evolution are considered strong indicators of microbial activity during solid-state fermentation (Bellon-Maurel et al., 2003). The CO_2 evolution is related to microbial growth and the rate of CO_2 evolution can be used as a measure of the rate of microbial growth (Mahmoud and Ghaly, 2015).

In this study, the carbon dioxide increased with time reaching 0.49 and 0.22% at 60 and 78 h and declined reaching 0.06 and 0.08% by the end of the experiment for the runs with initial pH of 8.64 and 7.00, respectively. The lactic acid used to adjust the initial pH of the shrimp shells may have inhibited the microbial activities and as a result the production of carbon dioxide. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was obtained (Figure 11).

Protease activity

Figure 12 shows the effect of the initial pH on protease activity during the course of deproteinization. The values are presented in units per gram dry shrimp shell waste and are the average of three replicates. The coefficient of variation ranged from 2.92 to 5.84%.

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:

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

The degree to which a microorganism will hydrolyze a protein substrate depends on its capacity to produce proteases and the stability of such protease under the reaction conditions.

The results obtained from this study revealed the ability of *A. niger* to produce extracellular proteases that resulted in deproteinization of shrimp shells to a certain degree. The protease activity increased from 0.67 to 4.21 units/g dry shrimp shell and from 0.71 to 2.34 units/g dry shrimp shell over the course of the deproteinization process (120 h) for the runs with initial pH of 8.64 and7.00, respectively. The protease production was greater during the exponential growth phase and was low during the stationary phase.

Ashour et al. (1996) reported that protease yield from

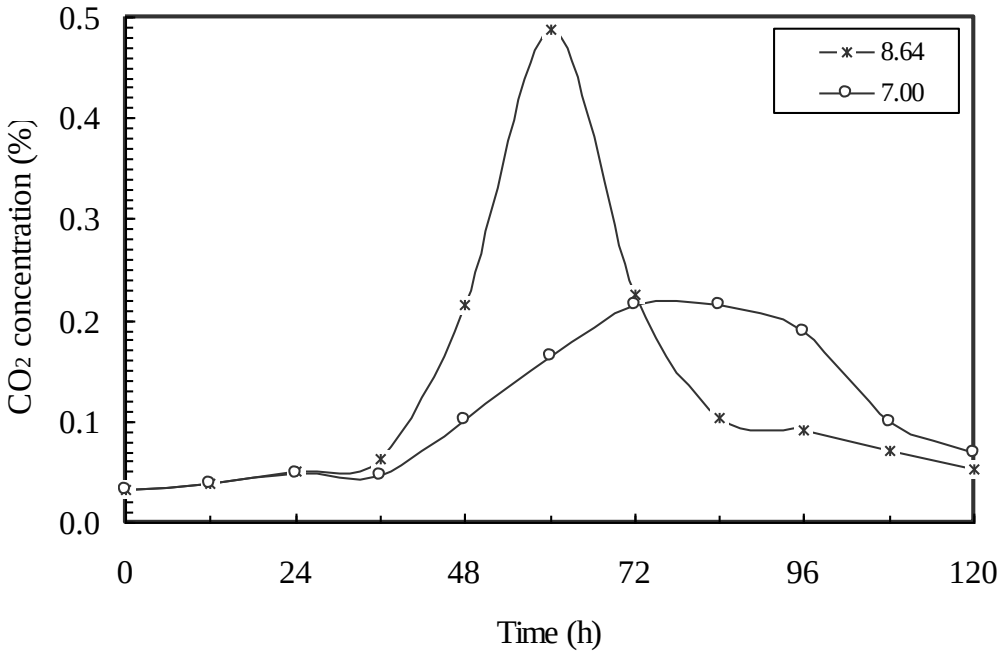


Figure 10. Effect of the initial pH on carbon dioxide concentration in the exhaust gas.

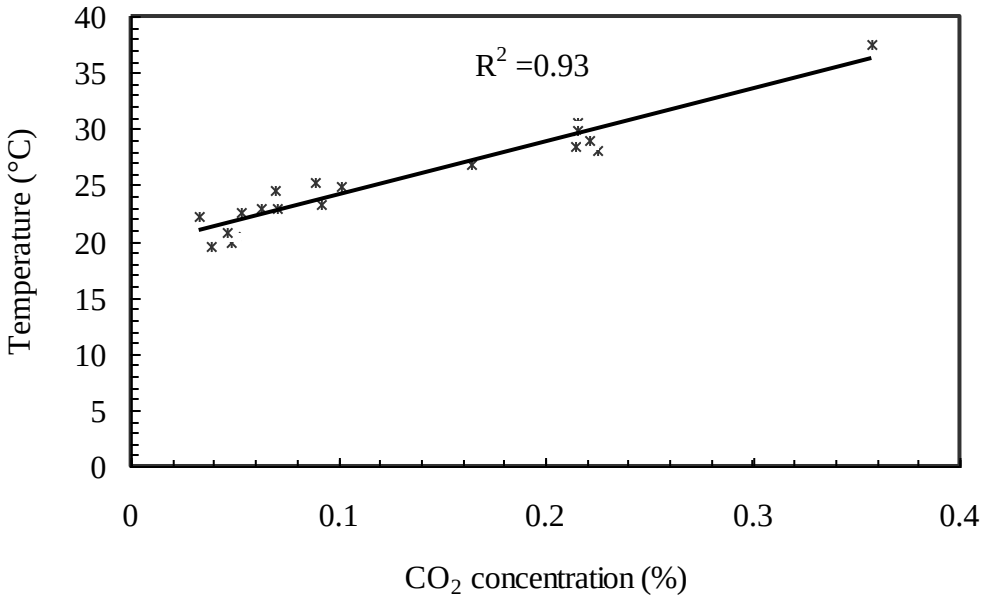


Figure 11. Correlation between the carbon dioxide concentration in the exhaust gas and temperature of the shrimp shells.

the fungus *A. niger* during cheese whey fermentation increased with incubation period and reached a maximum value after 6 days. Teng et al. (2001) reported protease activities in the range of 0.8 ± 0.3 to 6.8 ± 0.4 units (one unit activity was defined as 1 µM of tyrosineproduced in 1 min) for 17 *A. niger* strains after 5 days of incubation of the spores at a medium pH of 7.4.

The protease of *A. niger* is an acid resistant protease with optimum pH of 2.3. Thus, lowering the initial pH of the shrimp shells is expected to enhance the deproteinization process by: (a) achieving the optimum pH for fungal growth and protease production, (b) achieving the optimum pH for the proteolytic activity and (c) preventing the risk of the growth of undesirable microorganisms.

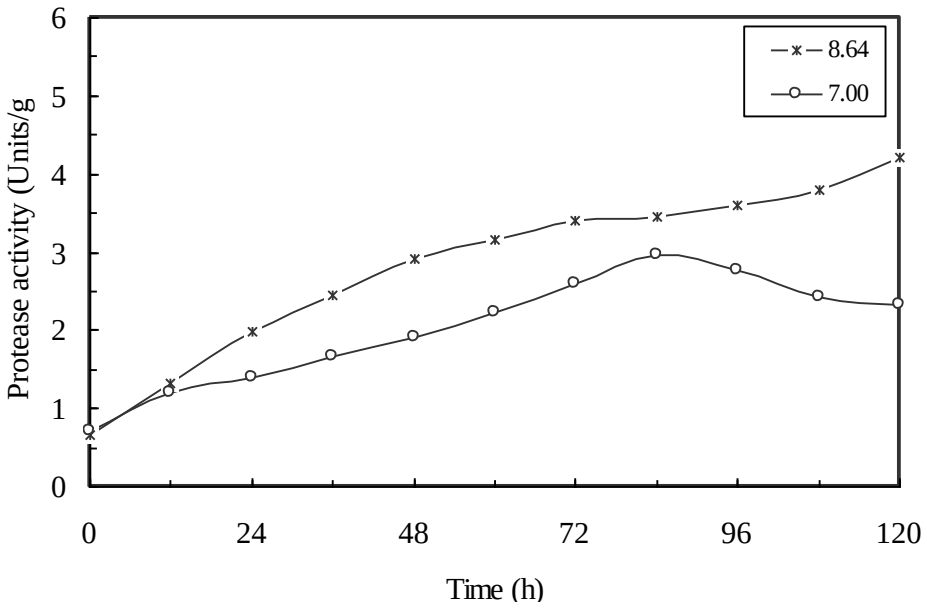


Figure 12. Effect of the initial pH on protease activity.

However, the use of lactic acid to lower the pH of shrimp shells in the current study inhibited the growth of *A. niger* and the activity of acid protease and the absence of lactic acid resulted in 1.8 fold increase in the protease activity.

Protein concentration

Figure 13 shows the effect of initial pH on 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 3.91 to 6.82%.

The results showed that protein concentration of the shrimp shells decreased with time as a result of protein break by the proteolytic enzymes produced by *A. niger*. The protein concentration decreased from an initial value of 30.84% to final values of 21.64 and 25.30% over 120 h of deproteinization for the runs with initial pH values of 8.64 and 7.00, respectively. This resulted in protein removal efficiency of 29.7 and 17.9% for the runs with initial pH values of 8.64 and 7.00, respectively. Zakaria et al. (1998) used lactic acid fermentation for chitin recovery from scampi waste and reported that about 77.5% of the original protein was solubilized during 5 days. Beaney et al. (2005) 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: (a) high pH of the shrimp shells that might have interfered with protease synthesis and/or activity, (b) protein denaturation that might have happen during shrimp shells autoclaving, (c) high temperature and/or low moisture content noticed during the deproteinization process that might have affected the production and/or activity of protease and (d) the large particles size of shrimp shells used in the study. Diniz and Martin (1997) 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.

Chitin concentration

Figure 14 shows the effect of initial pH on chitin content of 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 3.11 to 5.72%.

The shrimp shells used in this study contained 31.73% minerals. The chitin concentration was determined for the deproteinized samples without demineralization. The chitin concentration increased from an initial value of 16.56 to final values of 22.68 and 21.35 for the runs with initial pH of 8.64 and 7.00, respectively.

Zakaria et al. (1998) 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. (2002) 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. Beaney et al. (2005) reported chitin concentrations of 67.9-72.3% after lactic

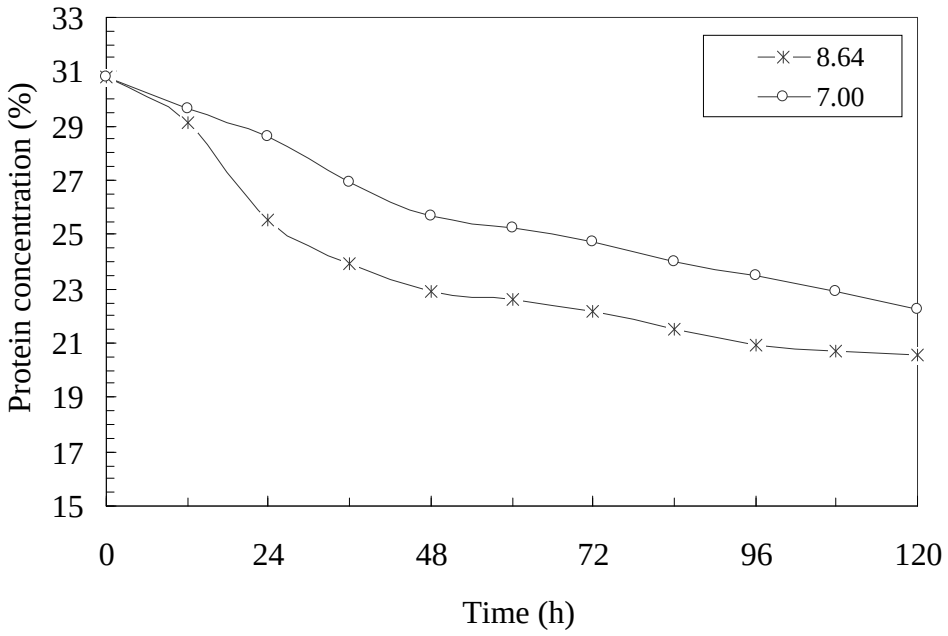


Figure 13. Effect of the initial pH on residual protein.

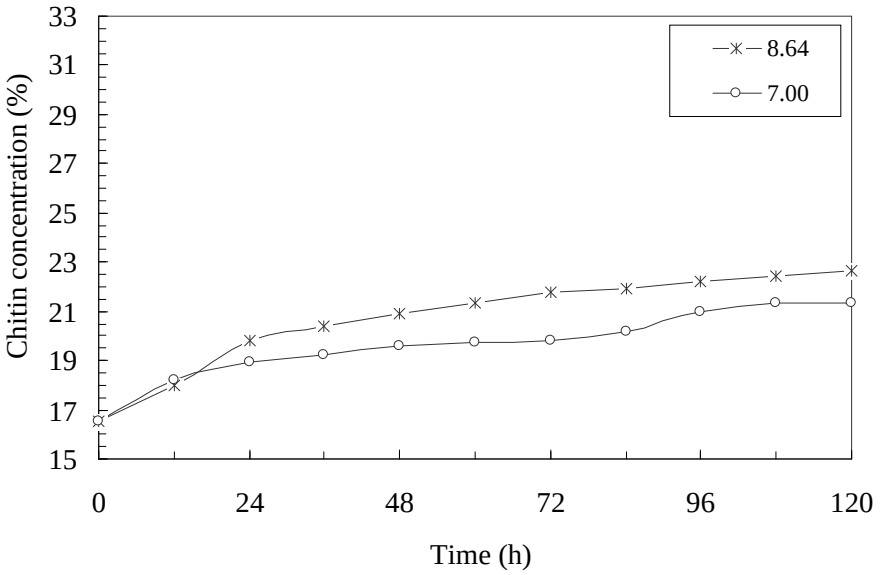
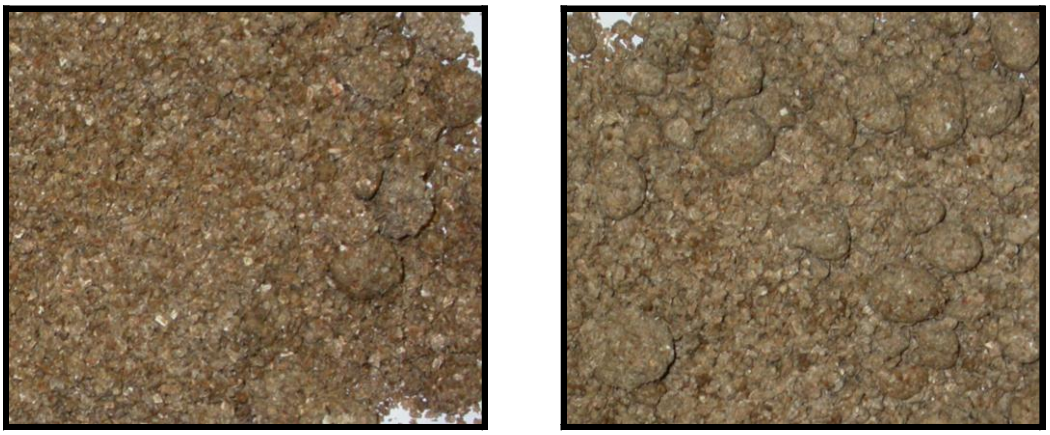


Figure 14. Effect of the initial pH on chitin concentration.

acid fermentation of prawn shells. The low increase in the chitin concentration observed in this study is due to the low deproteinization efficiency.

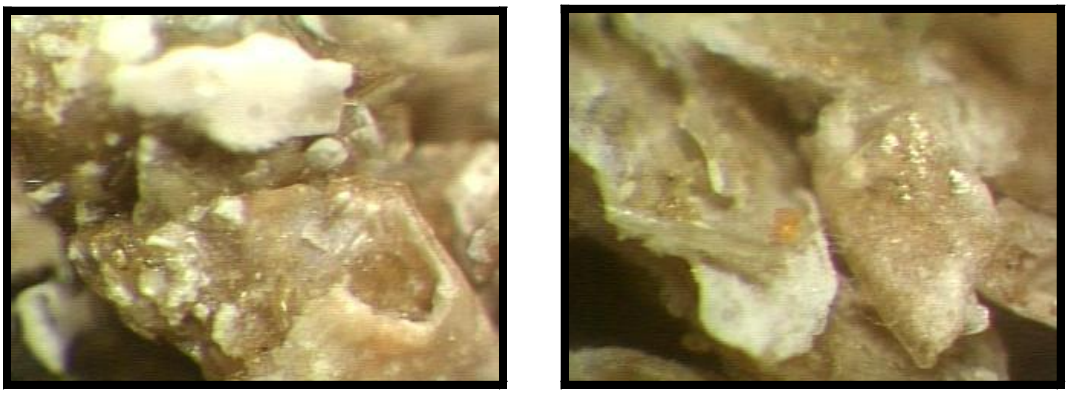
Visualization of shrimp shells

Figure 15 shows the appearance of the shell waste at the end of the deproteinization process. The spent shrimp shells obtained from the run with an initial pH of 8.64 were wet and had a pale pink-orange color with some tan patches whereas the spent shrimp shells obtained fromthe run with an initial pH of 7.00 appeared dry and had a gray-black color due to the presence of *A. niger* spores. The use of lactic acid to lower the pH inhibited the growth of *A. niger* and enhanced sporulation. The existence of the pink-orange color was an indication of the presence of pigments, which were not utilized during



(a) pH 8.64 (b) pH 7.00

Visual appearance of spent shrimp shells



(a) pH 8.64 (b) pH 7.00

Microscopic images (60x) of spent shrimp shells

Figure 15. Appearance of shrimp shells after 120 h of deproteinization.

the fermentation process.

Conclusions

The temperatures of the shrimp shells and the exhaust gas declined during the lag phase as the heat losses from the bioreactor due to water evaporation and the cooling effect of the inlet air were higher than the heat generated in the bioreactor by microbial activities. Once the microbial exponential growth phase was initiated, the temperature of the shrimp shells and exhaust gas started to rise as the heat generation by metabolic activity exceeded the heat losses. The temperature attained with high pH was much higher than that attained with the low pH. A strong correlation between the concentration of carbon dioxide in the exhaust gas and the temperature of the shrimp shells was observed. The initial moisture content of 60% fell below 40 which have negatively affected fungus growth and protease production and in turn the hydrolysis of protein. In order to maintain the desired moisture content in the bioreactor, the exhaust gas should be passed through a condensation tower and the recovered water pumped back into the bioreactor through the aeration tube. The initial decrease in the pH was due to the production of acid protease while the subsequent increase in the pH was due to the buffering capacity of the calcium carbonate released from the shrimp and/or the production of ammonium. The reduction in the protein concentration did not correspond to the increase in the protease activity and the high pH of the shrimp shells is believed to be the main reason for the low efficiency of the deproteinization process since the optimum pH of acid protease production by *A. niger* is 2-3. Low pH favors microbial growth and acid protease production, creates a however, lower pH using lactic acid which inhibit the microbial growth and protease production. The spent shrimp shells obtained from the run with high pH of 8.64 were wet and had a pale pink-orange color with some tan patches whereas the spent

shrimp shells obtained from the run with low pH appeared dry and had a gray-black color due to the presence of *A. niger* spores. The use of lactic acid to lower the pH inhibited the growth of *A. niger* and enhanced sporulation. The existence of the pink-orange color was an indication of the presence of pigments, which were not utilized during the fermentation process.

Conflict of Interests

The authors have not declared any conflict of interests.

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