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Full Length Article

The Availability of Astaxanthin from Shrimp Shell Wastes through Microbial Fermentations, *Aeromonas hydrophila* and Cell Disruptions

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Abstract

The removal of chitin and protein is crucial for the extraction of astaxanthin from shrimp shells. While the traditional method uses chemical extraction, the current study emphasises on the use of microbial enzyme, which is safer for the environment throughout the extraction process. *Aeromonas hydrophila* was isolated from shrimp shells and four types of sources were chosen; shrimp crab shell powder (SCSP), nitrogen sources, inorganic salts and carbon sources for the optimization of *A. hydrophila* culture media. Four types of cell disruption were introduced; autoclaving with distilled water, autolysis, heating and grinding with liquid nitrogen to further expose astaxanthin. The optimized culture media was found to be 7% SCSP, 9% bacto-peptone, 1% sodium chloride and 1% lactose at pH 7.0. This optimized media gave a high increment to both chitinase and protease activity from 0.092 and 60.816 U/mL to 1.164 and 272.565 U/mL, respectively. Mechanical cell disruptions using liquid nitrogen on pre-treated shrimp shells gave an astaxanthin recovery of 66% compared to the control. The availability of astaxanthin was made possible by the effects of optimization of the culture media in addition of mechanical cell disruptions.

Keywords: Dechitinization; Deproteinization; Enzyme; Optimization; Waste managment

Introduction

In the year 2010, total global production of fisheries by capture and aquaculture according to the world FAO statistics has accounted for up to 148 million tonnes where 59.7% (approximately 88 million tonnes) consisting of fisheries, crustacean and molluscs were captured. Shrimp wastes comprises of head, and body carapace which makes up to 48-56% of the raw shrimp weight depending on species (Pu *et al*., 2010). No doubt the shrimp wastes have been proven to be an excellent source of carotenoids such as astaxanthin, chitin, proteins and endozymes (Cao *et al*., 2008) the pigments such as astaxanthin was widely used in aquaculture feeds (Chien and Shiau, 2005), food industries, pharmaceutical, cosmetics (Seki *et al*., 2001) and medical studies (Bhuvaneswari *et al.*, 2010).

In the past two decades, scientists have been researching on the recovery of these valuable products from shrimp wastes where in early researches chemicals were used as treatment to these wastes i.e., acid and alkali for demineralization and deproteinization which offers a fast and assure result (Roberts, 2008). However, the usage of chemicals creates pollution and the fact that chemical utilization destroys most of the properties of the substances is undeniable (Giyose *et al*., 2009). In addition, the recovery of carotenoids was not made possible (Healy *et al.*, 1994). Thus, scientists are looking at a much greener technology, to treat shrimp waste through microbial fermentation and commercial enzymes. Different types of microbes (Wang and Yeh, 2006; Nadeem *et al.*, 2008) and commercial enzymes (Sumantha *et al*., 2006) were investigated and have proven to be successful with the demineralization and deproteinization rates comparable to chemical treatment.

Astaxanthin (3,3'-dihydroxy-ß-carotene-4,4'-dione) is a red coloured pigment which is responsible for the changing colour of cooked prawns, lobsters, crabs, krills and other crustaceans (Wade *et al.*, 2005; Tume *et al.*, 2009). Being well known for its antioxidant activity, astaxanthin was found abundant in the algae *Haematococcus* (Li *et al.*, 2011), *Dunaliella salina* (Abd El-Baky *et al*., 2004), crustaceans, and some microbes such as yeast (*Phaffia rhodozyma*) (Xiao *et al.*, 2009). Natural astaxanthin fetches high market value. Hence this brings for a higher demand on natural astaxanthin. Astaxanthin was known exist in three forms: sterified, free molecule or forming chemical complex molecule with protein, the carotenoprotein. This protein usually exists in crustaceans (Higuera-Ciapara *et al*., 2006) and is widely used in aquaculture feeds (to provide coloration), cosmetics, medical and as supplement for general health being.

In crustacean shells, the protein layer interlays with the chitin layer where they sometimes coexist (Wang *et al*., 2007). Due to the coexistence of both structures, the presence of both chitinase and protease is crucial to remove

both chitin and protein making astaxanthin available. Meanwhile, enzymes such as protease accounts for approximately 59% in the enzyme market (Shumi *et al.*, 2004). High protease yielding microbes includes species of *Bacillus* sp., *Alcaligenes faecalis, Pseudomonas fluorescence,* and *Aeromonas hydrophila* (Boominadhan *et al*., 2009). Alkaline and neutral proteases are usually bacterial produced mostly by the genus *Bacillus* meanwhile fungus such as *Aspergillus oryzae* produces acidic, alkaline and neutral proteases (Rao *et al*., 1998). Meanwhile chitinases are enzymes that randomly cleaves the glycosidic bond of the chitin molecule to produce oligosaccharides which are mainly chitiobiose $(GlcNAc)_2$ (Orikoshi *et al.*, 2004). Chitinases are classified under two glycoside hydrolases family, namely GH 18 and GH 19 with GH 18 being most common among bacteria, fungi, animals and plants (Killiny *et al.*, 2010).

The fact remains that both chitin and protein have the possibility to coexists chitinase plays an important role in breaking down chitin into its monomer, N-acetylglucosamine so that the interlay protein can be hydrolysed by the proteases. Moreover, deproteinization and dechitinization of crustacean wastes in the context of optimizing the activity of both enzymes through microbial fermentation has been rarely reported.

From the above background, we hypothesize that fermented shrimp shells may give a higher astaxanthin yield if protein and chitin are removed. The current study focuses on microbial fermentation by optimizing the culture media and conditions for the process of dechitinization and deproteinization to increase the availability of astaxanthin. By using the fermented shrimp shells, various mechanical cell disruptions are implied to further enhance the availability of astaxanthin.

Materials and Methods

Materials

Shrimp Crab Shell Flake (Sigma Co.) was blended into fine powder; Shrimp Crab Shell Powder (SCSP) while raw shrimp shells were collected from the wet market was washed and cleaned before oven dried at 30-35°C until a constant weight was obtained. The shells were then grinded into powder like form where this shrimp shell powder (SSP) was used throughout the experiment.

Shrimp shell powder was mixed with 2N hydrochloric acid at a ratio of 1:10, ensuring full reaction between the acid and shells. The mixture was stirred continuously for 2 h and washed with tap water until the run-off was clear of acid before final rinse with distilled water before oven dried at 30-35°C. This preparation was labelled acid treated shrimp shells (ATSS).

Isolation of Microorganism

Several field works have been carried out at Pantai Kelanang in Selangor. Samples of sand, water and crustacean shell wastes were collected where isolation was done on minimal synthetic agar and nutrient agar where they were incubated for one week at room temperature (29±1°C). Successfully growing bacteria were isolated and maintained.

Determination of Chitinase and Protease Activities of Isolates

Bacteria isolated from the samples were tested for chitinase and protease activities. Minimal synthetic media (MSM); (0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$) was prepared and mixed with 3% (w/v) of SCSP, sterilised (Yang *et al*., 2000) before added with 1×10^7 cells/mL of bacteria. All flasks were incubated at 30±0.5°C for 2 days at 150 rpm prior to chitinase and protease assay. A total of 5 replicates were prepared throughout the whole experiment unless stated.

Enzymes assay

The activity of chitinase was determined based on slight modifications of the method by Reissig *et al*. (1955), which involved the estimation of N-acetylglucosamine (GlcNAc) released from chito-oligosaccharides through β-N-acetylhexosaminidase. Slight modification was made by substituting swollen chitin with colloidal chitin. The preparation of colloidal chitin was done based on the method by Gomez Ramirez *et al.* (2004) by mixing finely grinded 10 g of chitin (Sigma) with 100 mL of 85% (v/v) phosphoric acid. The mixture was read immediately in a spectrophotometer with the wavelength of 585 nm. Enzyme activity was expressed in units where 1 unit is equivalent to the amount of enzyme required to produce 1.0 mg of N-acetylglucosamine per hour at 37°C.

The activity of protease was determined by the method of Ferrero (2001) with tyrosine as a standard. A slight modification was made by increasing the amount of substrate used where 750 µL of substrate (containing 1.0mg/mL of casein in 200 mM of sodium phosphate buffer, pH 7.4 and 5 mM phenylmethylsulfonyl flouride) was added with 150 µL of crude enzyme. The reaction was stopped with the addition of 150 µL of 10% trichloroacetic acid in an ice bath for 15 minutes before centrifuging at 8000x*g*. Then, to 3.75 µL of 0.5M sodium carbonate, Na_2CO_3 was added 750 µL of the supernatant and 750 µL of Folin-Ciocalteau reagent (threefold diluted). The mixture incubated in dark at 28±2°C for 45 min to 1 h for colour development prior to spectrophotometer reading at 660 nm. Protease activity was expressed in units where 1 unit (U) is equivalent to the amount of enzyme required to produce 1.0 mg of tyrosine at 30 min and 37°C.

Optimization of Culture Media

The highest chitinase and protease producing strain was chosen based their enzymatic activity. The chosen strain then undergoes optimization based on Cheong *et al*. (2013).

MSM was prepared in 4 sections where each section consists of SCSP, nitrogen sources (ammonium nitrate, bacto-peptone, sodium nitrate and yeast extract), inorganic salts (calcium chloride, copper sulphate, manganese (II) sulphate, and sodium chloride) and carbon sources (arabinose, glucose, and lactose) individually with the concentrations of 1, 3, 5, 7 and 9% (w/v) to be investigated for all the sources. Meanwhile a range of pH was selected to test for the optimum pH required for bacterial growth and increasing enzyme production. The pH of 5.5, 6.0, 6.5, 7.0 and 8.0 were selected and prepared. Optimization of each source was done based on the evaluation of chitinase and protease activity produced in a one type at a time manner. All the cultures were fermented at 30±0.5°C for 48 h at 150 rpm. Evaluation of the enzymatic activity of the selected bacteria was done by centrifuging the culture media at 8000x*g* for 20 min before filtering the supernatant through a 0.2 µm cellulose acetate filter.

Dechitinization and Deproteinization of Shrimp Shells

Shrimp shell powder was used together with the optimized media by substituting SCSP in the optimized amount. Fermentation was carried out for 12 days to investigate the time taken for deproteinization to complete by using optimized media, where comparison was also done in MSM media with SSP and optimized media with ATSS (demineralised). Enzymatic analysis was conducted daily.

Cell Disruption Methods

Shrimp shells that were treated with bacteria in its optimum culture media was retained and labelled as pre-treated shrimp shells (p-TSW) before subjecting it to a series of cell disruption techniques; (i) autoclaving with distilled water for 30 min, (ii) autolysis at pH 7.0 for 26 h, (iii) heating at 75°C for 30 min, (iv) grinding with liquid nitrogen, (v) negative control and vi) treating with acetic acid 4 mol/L as positive control. All the treatments were done with a 1 g/30 mL basis (Xiao *et al*., 2009). After undergoing cell disruption, the samples were centrifuged at 4000 rpm for 20 min and washed with 10 mL of distilled water (except for liquid nitrogen treatment and negative control) followed by another centrifuging at 4000 rpm, 20 min. The supernatant was discarded and the shrimp shells were extracted with absolute ethanol at room temperature for 5 min. A quick centrifugation was applied and the alcoholic supernatant was read in a UV-Vis spectrophotometer at 478 nm using a quartz cuvette, 10 mm light path way according to Schiedt and Liaaen-Jensen (1995). The carotenoid recovered was presented as astaxanthin and was calculated according to the formula below:

$$X = \frac{(A) \times (y) \times 1000}{A\,\underset{1\text{ cm}}{1\%} \times 100}$$

Where,

X = amount of astaxanthin present
Y = sample solution (mL)
A = absorption reading (O.D)
$A\,\underset{1\text{ cm}}{1\%}$ = absorption coefficient (2500) (based on Rao *et al.*, 2007)

Statistical Analysis

Data were collected and analysed by SPSS software version 16.0. Analysis of variance (ANOVA) was used to detect significant difference that occurred between groups of optimization treatment and cell disruptions.

Results

Isolation of Bacteria

A total of 25 bacterial strains were successfully isolated and tested for the presence of chitinase and protease. However, only 19 strains showed positive activity for both enzymes. Chitinase and protease producing strain was chosen based on their enzymatic activity and the strain with the highest chitinase and protease will have a better potential of degrading chitins and proteins present in shrimp shells which later on increases the availability of astaxanthin. The chosen strain was P 010 and was later being identified as *A. hydrophila* through 16S rRNA molecular analysis. Though there are few other strains having both enzymes relatively high, *A. hydrophila* was found to have an optimum enzymatic activity of 0.082 and 138.46 U/mL for both chitinase and protease, respectively compared to the rest where some were high in protease but low in chitinase and vice versa.

Optimization of Culture Media

Aeromonas hydrophila was incubated in different concentrations of SCSP ranging from 1%-9% (w/v). An increase in SCSP concentrations has increase chitinase activity in the rate of 0.023 U/mL, while protease activity (Table 1) on the other hand, remained high and significantly indifferent regardless of any concentration supplied. However, there were no significant differences ($p < 0.05$) between 3% and 5% (w/v) supply and between 7% and 9% in chitinase activity. An increase of 40% of enzymatic activity was observed with the supply of 7% SCSP from 5% with significant difference and thus 7% (w/w) SCSP was chosen to be included in the optimization media.

The pre-optimized media containing MSM and 7% (w/w) SCSP was gradually added with different nitrogen sources. Among the nitrogen sources added, it was clearly shown that 7% bacto-peptone accounts for the highest chitinase activity, 1.19 ± 0.1124 U/mL (Fig. 1a). However, at 9% concentration chitinase activity dropped slightly without any significant difference with 7% but enhances protease activity two fold (122.72 U/mL) with

Table 1: Chitinase and protease activity of *Aeromonas hydrophila* in different concentration of SCSP

Concentration (%)	Chitinase activity (U/mL)	Protease activity (U/mL)
1	0.007 ± 0.042^{a}	57.439 ± 1.9880^{d}
3	0.053 ± 0.0145^{b}	62.064 ± 2.4040^{d}
5	0.055 ± 0.0238^{b}	61.639 ± 1.3748^{d}
7	0.092 ± 0.0129^{c}	60.814 ± 3.7914^{d}
9	0.107 ± 0.0125^{c}	62.614 ± 4.1994^{d}

*Note: Different subscript denotes significance difference ($p<0.05$)

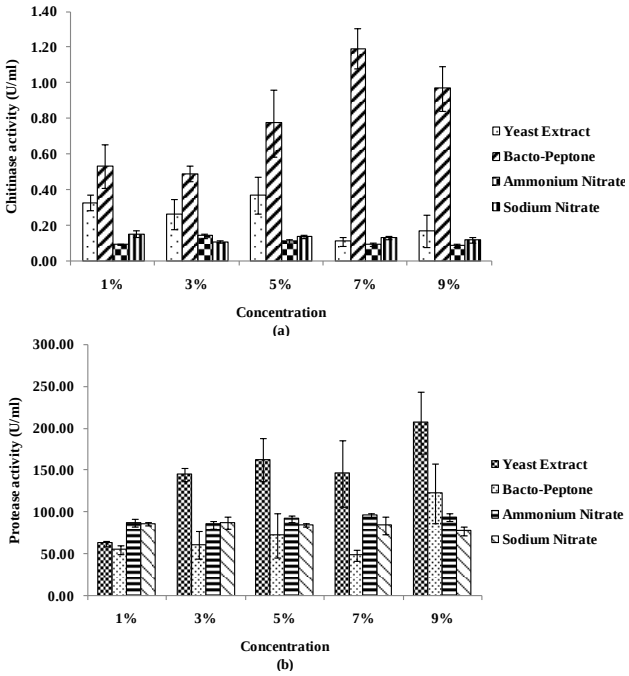

Fig. 1: The effect of different nitrogen sources in various concentrations on chitinase activity (a) and protease activity (b) by *A. hydrophila*

significantly difference ($p < 0.05$) with other concentrations. Even though protease activity was also increased drastically with the addition of yeast extract at high concentrations (7 - 9%) where protease activity reached 207.11 U/mL at 9% (w/v), chitinase activities were relatively low compare to bacto peptone. Though at high concentrations of yeast extract protease was enhanced but not for chitinase where similar reading with the control was observed, 0.092 U/mL. Thus, in order to enhance the production of both enzymes in an optimum condition, bacto-peptone at the concentration of 9% was chosen where chitinase and protease activities increased 90% and 50% respectively compared to the control media.

The addition of sodium chloride (NaCl) and calcium chloride ($CaCl_2$) in low concentrations (1% and 3%) gave a fair increase for both enzymes. NaCl was more favourable in enhancing protease activity (Fig. 2b), while $CaCl_2$ favours chitinase (Fig. 2a). In the presence of 1% (w/v) NaCl, chitinase and protease activity was recorded as 1.15 U/mL and 157.09 U/mL respectively. As the addition of

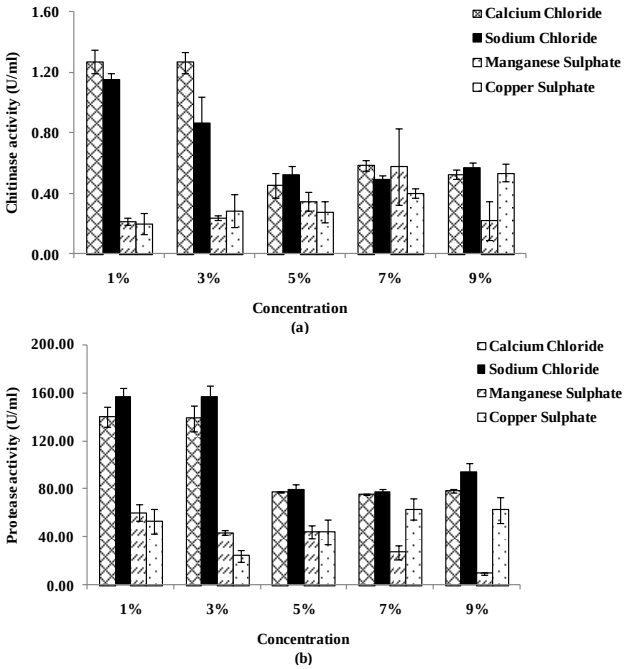

Fig. 2: The effect of various inorganic salts and concentration on chitinase activity (a) and protease activity (b) by *A. hydrophila*

$CaCl_2$ favours chitinase activity, the addition of 1% and 3% into the culture media recorded a reading of 1.26 U/mL compared to the control 0.97 U/mL but protease activity was recorded as 139.69 U/mL compared to control 122.71 U/mL. Since the addition of both sources at 1% concentration increases both enzymes, NaCl was favoured as it gives a higher increase in both enzyme activities overall.

Lactose is a disaccharide sugar comprise of glucose and galactose. The addition of lactose to the culture media increases both protease and chitinase activity, though only a fairly increase was noted. At 1% (w/v) lactose concentration, chitinase activity gave an increase of approximately 2% to 1.16 U/mL (Fig. 3a), while protease activity increased 40% to 263.21 U/mL. Although at 1% glucose (272.56 U/mL) protease activity was higher than that of lactose (Fig. 3b); however, no significance difference ($p < 0.05$) was observed between these two sources.

Optimization of pH for *A. hydrophila* to secrete extracellular chitinase and protease was being investigated after the optimization of media was done. Table 2 shows enzymatic activity for both chitinase and protease. Chitinase activity was found to be stable within the range of pH tested where at pH 7.0; enzyme activity was recorded as 1.108 U/mL. Though chitinase activity was recorded slightly lower than the optimized media, no significant difference was observed between it and the pHs tested. Protease activity showed stable activity at pH 6.0 to 8.0. Under slightly acidic condition, pH 5.5 protease activity was found to be 225.615 U/mL. Nonetheless, at pH 6.0 to 8.0 enzyme

Table 3: The optimum culture media content and culture conditions determined for inducing chitinase and protease activity in *Aeromonas hydrophila*

Optimized Culture Media		Culture Conditions	
K_2HPO_4	0.1%	pH	7.0
$MgSO4.7H_2O$	0.05%	Rotation	150 rpm
SCSP	7%	Temperature	30˚C
Bacto-peptone	9%	Time	2 days
Sodium Chloride	1%		
Lactose	1%		

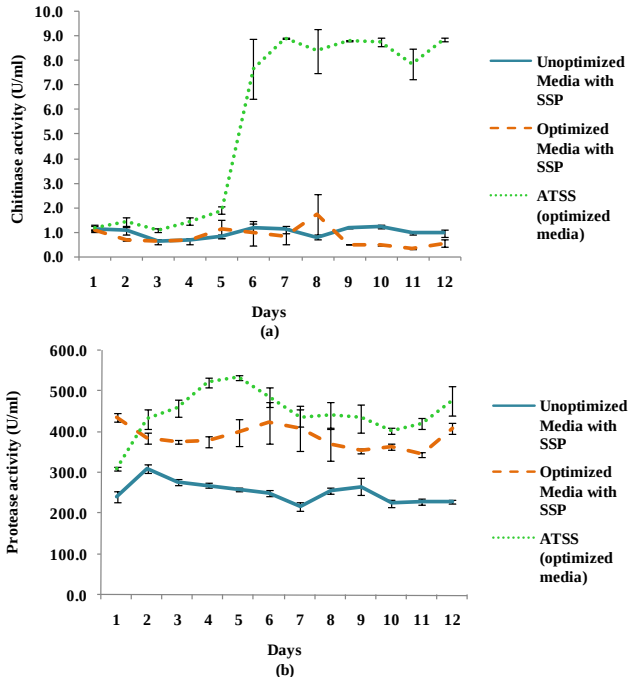

Fig. 4: Evaluation on chitinase activity (a) and protease activity (b) by *A. hydrophila* over a period of 12 days fermentation

Table 2: Chitinase and protease activity in the selected pH range

pH	Chitinase activity (U/mL)	Protease activity (U/mL)
5.5	1.080 ± 0.0828^{a}	225.615 ± 15.2158^{b}
6.0	1.051 ± 0.0507^{a}	270.438 ± 23.9219^{c}
6.5	1.110 ± 0.0867^{a}	276.215 ± 16.2126^{c}
7.0	1.108 ± 0.0990^{a}	286.315 ± 19.7753^{c}
8.0	1.049 ± 0.0631^{a}	295.690 ± 16.8059^{c}

*Note: Different superscript shows significant difference ($p<0.05$)

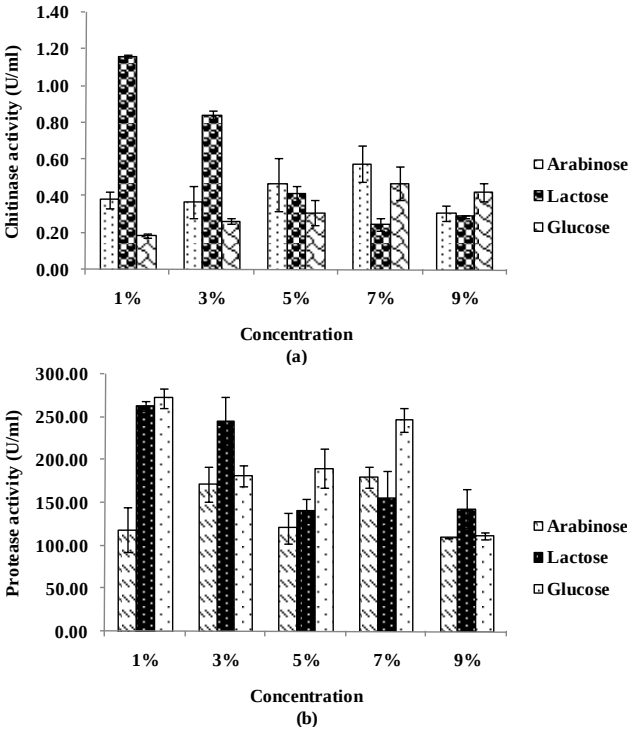

Fig. 3: The effect of different carbon sources in various concentrations on chitinase activity (a) and protease activity (b) by *A. hydrophila*

activity was almost similar with no significant difference detected.

Deproteinization and Dechitinization of Shrimp Shells

The culture media and conditions were set according to Table 3 with SSP replacing SCSP in equivalent amount and fermentation was carried out for a period of 12 days to investigate the optimum time taken and the effectiveness of dechitinization and deproteinization. Three types of different shrimp shells conditions were tested; (i) SSP (7%) in un-optimized media which contains only MSM, (ii) SSP (7%) in optimized media and, (iii) ATSS (7%) in optimized media (Fig. 4). Chitinase activity was found to be higher in ATSS whereby the highest amount of chitinase activity was denoted at day 6 with an enzymatic activity of 7.672 U/mL (Fig. 4a). Similar pattern was also observed for ATSS (day 5) where protease activity was higher than the rest (Fig. 4b).

In a period of 12 days, chitinase activity of SSP in optimized media showed only little increment compared to the optimized media with SCSP (1.108 U/mL). The highest chitinase activity for optimized media with SSP was recorded at day 8 with an activity of 1.747 U/mL before a drop was observed from day 9 onwards meanwhile for un-optimized media with SSP, chitinase activity was rather high after day 9. Though day 8 showed the highest chitinase activity in the optimized media with SSP, however chitinase activity was found to be more stable at days 5-7 with an average activity of 0.965 U/mL, slightly lesser than the optimized media with SCSP.

Protease activity on the other hand was higher in the optimized media containing SSP than the un-optimized media with SSP in all the 12 days recorded (Fig. 4b). An average of 387.40 U/mL and 252.80 U/mL was recorded for the optimized an un-optimized media respectively. However, when comparing the optimized media with SSP and optimized media with ATSS, undoubtedly protease

activity was higher in ATSS with an average protease activity of 446.34 U/mL. Highest protease activity was recorded to be 534.53 U/mL at day 5 for ATSS, which is only a 25% difference in enzyme activity compared to SSP in optimized media on the same day. These two conditions do not differ much and during prolonged fermentation, SSP in optimized media produced highest amount of protease (423.75 U/mL) recorded at day 6 which is competent to that of ATSS.

Generally, fermentation in a period of 12 days did give a rise and fall in enzymatic activities. In SSP optimized media, although at day 12 (409.47 U/mL) protease activity seem to have increased to a level almost the same as day 6 (377.16 U/mL), it was thought to be too long and might destroy the natural astaxanthin present despite in a waste of resources. In addition to the decrease in chitinase activity, day 6 was chosen to be an optimum time for fermentation.

Availability of Astaxanthin through Mechanical Cell Disruptions

The importance of dechitinization and deproteinization on carotenoproteins is essential to expose the availability of astaxanthin. Fermentation with *A. hydrophila* has turned out to be successful in dechitinazation and deproteinization. Nevertheless, the exposure of astaxanthin was thought to be more by subjecting the pre-treated shells with mechanical cell disruption in various ways.

Liquid nitrogen treatment promised a higher recovery compared to the other treatments meanwhile exceeding the negative control (pre-treated shrimp shells without mechanical cell disruption) by 29% which bring to the total availability to 3.8 µg (Table 4). As compared to acetic acid treatment, availability of astaxanthin from liquid nitrogen grinding was denoted to be 39% lower. Even though liquid nitrogen treatment recorded a lower recovery than the positive control however; it is more environmentally friendly than acid treatments. Meanwhile, mechanical cell disruptions using autoclave, autolysis and heating method yielded approximately 2 µg of astaxanthin.

Table 4: Recovery of astaxanthin from pre-treated shrimp shells undergoing various mechanical cell disruptions

Treatment	Astaxanthin recovery (µg)
Negative control	2.784 ± 0.2758^{a}
Autoclave with distilled water	2.279 ± 0.0118^{b}
Autolysis	2.589 ± 0.1784^{c}
Heating	2.338 ± 0.0642^{d}
Liquid nitrogen treatment	3.823 ± 0.5045^{e}
Acetic acid treatment	5.293 ± 0.3018^{f}

*Note: Different superscript shows significant difference ($p<0.05$)

Discussion

The selection of 7% SCSP in this study was in accordance with Yang *et al*. (2000) where *Bacillus subtilis* Y-108 was found to give optimum protease activity in this concentration. Meanwhile, Jami Al Ahmadi *et al.* (2008) showed that highest chitinase activity on *Aeromonas* sp. PTCC 1691 was observed at a supply of 7.5 g/L of chitin which was low compared to the current study (70 g/L). This was perhaps due to different species and strains having different ability to utilize SCSP. Shrimp Crab Shell Powder was chosen to induce chitinase and protease production as it was previously being reported to be the best inducer (Wang and Yeh, 2006).

Razak *et al.* (1995) working on *B. stearothermophilus* reported that protease production was the best in the presence of organic nitrogen sources. In accordance with that, in the current study bacto-peptone was found to have increased both enzymatic activities. However, Jami Al-Ahmadi *et al*. (2008) proved that ammonium sulphate with the concentration of 0.15% (w/v) was found to be the optimum source of nitrogen for *Aeromonas* sp PTCC 1691. Nevertheless, the process of optimization and the requirement for maximum enzyme production is unique to every organism.

The NaCl was known to increased metabolism. According to Watson (1970), the addition of NaCl to the culture with *Saccharomyces cerevisiae* was found to give an increase in cell metabolism up to 10 folds. In our study, as the levels of NaCl in the culture media increases, enzymatic activities began to show depletion; perhaps under high concentrations of NaCl, the cells are stressed and these *Aeromonas* cells may enter a non-culturable state (Pianetti *et al*., 2008). Thus, the quantification of enzymatic activities results in depletion.

Lactose serves as a better inducer for both enzymes compared to glucose. The reason could have been that enzyme production was controlled by the inducer catabolite repression system during enzyme production whereby non-glucose carbon sources such as maltose were seen to have augmented protease activity (O'reilly and Day, 1983). The current study has found that lactose has boosted both enzymatic activities of *A. hydrophila.* However, exception applies according to strain types (Hunt *et al*., 1981) and from the results obtained here; this strain was found to be capable in utilizing lactose. Again according to Hunt *et al.* (1981), the selection of carbon sources to be utilised depends mainly on the combination of sources during the optimization process. Here, it was found that lactose was the most suitable non-glucose carbon source.

The optimal pH for chitinase production in *A. hydrophila* was found to be at pH 7 (Wang and Hwang, 2001). In accordance with the results of this study, pH 7 was chosen since both chitinase and protease were able to tolerate a medium range of pH. Many reports have also found that pH 7 was suitable for the production of protease (Yang *et al*., 2000) and chitinase (Singh, 2010).

Previous studies done by Oh *et al*. (2000) have proven that deproteinization with *P. aeruginosa* K-187 was completed at day 7 with protein removal of 78% using natural shrimp shells, 72% in SCSP and 45% of protein in acid treated SCSP in optimized condition compare to 48, 55,

and 40% in un-optimized media respectively. Though there is a slight difference from the results of Oh *et al*. (2000) and the present study however proved that optimized media was much more efficient in protein removal. Enzyme activities were higher when optimized media was fermented with SSP compared to that of SCSP. A possible reason could be that SCSP has undergone chemical treatment and Maillard reaction (Wang and Chio, 1998) where the shells were cooked and dried under high temperature and this process has changed and destroyed most of the primary protein structure. Meanwhile SSP was processed naturally without any chemical or physical treatment thus preserving the natural properties of the shells. Treating the shrimp shells with hydrochloric acid has resulted in the removal of minerals from the shells where almost 99% of the calcium present in the shells was removed during the process of demineralization (Giyose *et al*., 2009) and the destruction of the protein primary structures (Wang and Chio, 1998). This has eventually eased the activities of enzymes in hydrolyzing chitins and proteins present in ATSS. Untreated SSP preserves the natural properties of proteins, calcium and chitins, unlike ATSS whereby demineralization occurred. Eventually this caused the production of enzymes to be lower in SSP than in ATSS. In the optimized and un-optimized media containing SSP, chitinase activity was not much induced as in ATSS probably due to this. However, the production of protease in SSP was comparable with ATSS, which is an advantage as protease is a crucial enzyme for the extraction of astaxanthin while chitinase on the other hand acts as an assistant to enhance this system.

Although in the ATSS fermentation system higher enzyme activities were denoted, nonetheless the usage of acid reduces the recovery of astaxanthin (Healy *et al*., 1994) and creates pollution, which is less favourable. Shells that are treated with acid do not maintain its natural properties anymore in addition to the decrease in astaxanthin. Evaluation of the availability of astaxanthin records acetic acid as the highest yielder due to its acidic properties that further destroy the structures of protein (as aforementioned) in p-TSW thus, eases the extraction of astaxanthin. However, due to its acidic properties, the environment is greatly harmed though it is a mild acid. Meanwhile, mechanical cell disruptions using autoclave, autolysis and heating method yielded lesser amount of astaxanthin. A possible thought was that astaxanthin was lost during the process of quantification. Nevertheless, further experimental quantification is needed to quantify the lost which is not done in this study.

In conclusion, optimization of both enzymes from *A. hydrophila* was found to be 7% SCSP, 9% bacto-peptone, 1% NaCl and 1% lactose at pH 7.0. Both chitinase and protease was augmented from 0.09 and 60.81 U/mL to 1.16 and 263.22 U/mL respectively under optimized media conditions. Dechitinization and deprotenization was optimum at day 6 when fermented with SSP. Protease activity was more favoured as the astaxanthin attaches mostly to proteins forming carotenoproteins. However, mechanical cell disruption was then applied to further enhance astaxanthin availability and liquid nitrogen was found to have 29% availability as compared to the negative control.

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