EXTRACTION AND CHARACTERIZATION OF CHITOSANASE ENZYME FROM *Bacillus megaterium* UNDER LIQUID STATE FERMENTATION

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Abstract

The present research work focuses on the extraction of chitosanase enzyme from soil bacteria (*Bacillus megaterium*). In this research, the soil sample was collected from Htauk-kyant Township, Yangon Region for the isolation and cultivation of bacteria. In the isolation process, bacteria were isolated from the soil sample by serial dilution plate method, followed by culture in nutrient agar medium. Ten bacterial strains (A1 to A10) were isolated from the soil sample and were characterized by microscopic examination and gram staining methods. Among these bacterial strains, A1, A2, A3, A4, A6, A8, A9 and A10 were found to be gram positive, whereas only A5 and A7 were gram negative. According to the biochemical tests, out of eight gram positive bacterial strains, only A2 was observed to give the positive results on motility tests, methyl red tests, citrate utilization tests, starch hydrolysis tests, sugar fermentation tests and negative results on indole tests, nitrate tests, (VP) Voges-Proskauer tests that similar to the characteristics of chitosanase enzyme producing bacteria (*Bacillus megaterium*). Hence, A2 might be identified as *Bacillus megaterium.* For extraction of chitosanase enzyme, the isolated bacterial strain (A2) was cultured on chitosanase producing medium of 0.6 % poly peptone, 0.1 % K_2HPO_4, 0.05 % $MgSO_4.7H_2O$, 0.6 % yeast extract, 0.1 % glucose and 0.5 % colloidal chitosan and incubated at 30 °C. The optimum incubation time (3 days) of enzyme forming bacteria, inoculum sizes of bacteria (10 %), age of culture of bacteria (3 days), temperature of enzyme-catalyzed reaction (55 °C) and pH (7.0) of chitosanase producing medium were determined based on the chitosanase activities. Turbid enzyme bacterial solution was so prepared under above conditions for preparation of enzyme bacterial solution. The enzyme bacterial solution was centrifuged with 3000 rpm at room temperature and the supernatant enzyme solution was collected. The crude chitosanase solution was obtained and then partially purified by successive precipitation method by using 30 % and 70 % saturation of ammonium sulphate. Finally the total enzyme activity, protein contents and specific activity of crude enzymes obtained from each purification step were determined.

Keywords*:* chitosanase, *Bacillus megaterium*, biochemical tests, sugar fermentation tests, chitosanase enzyme activity

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Introduction

The enzyme found in numerous bacteria, fungi, insect, plant and animal involves in natural protection mechanism. Many bacteria and fungi containing the chitinolytic enzyme convert chitin into carbon and nitrogen that can serve as energy source. Chitinolytic bacteria are typically detected and screened through the production of clearing zones on chitin containing agar as selective medium. Chitinases have received attention because of their wide applications in the medicine, biotechnology, agriculture, biocontrol of plant pathogenic fungi, waste management and industrial applications such as food quality enhancer and biopesticide (Zarei and Aminzadeh, 2012).

Chitosanase or chitosan *N*-acetylglucosaminohydrolase (EC 3.2.1.132) catalyzes the hydrolysis of glycosidic bond of chitosan. Chitosanase has been found abundant in a variety of microorganisms, bacteria, including fungi, actinomycetes and a few in plants (Zakaria and Musa, 2012). It can be extracted from various types of bacteria such as *Bacillus megaterium*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Burkholderia gladioli*, *Matsuebacter chitosanotabidus*, *Streptomyces griseus*, *Trichoderma reesei*, *Pseudomonas aeruginosa* and these bacteria can be found in soil (Johnsen *et al.,* 2010). It has been used in the preparation of bioactive chitooligosaccharide, fungal protoplasts, biocontrol agent against pathogenic fungi and insects (Thadathin and Velappan, 2014).

Chitosan is a modified natural carbohydrate polymer derived from chitin consisting of 1, 4-β-linked d-glucosamine residues, partially substituted with *N*-acetyl groups to various degrees of acetylation and the chitin has been found in a wide range of natural sources such amycelial, sporangiophore walls of many fungi, the exoskeletons of insects, crustaceans, insects and some algae. In this study, *Bacillus megaterium* could be isolated from soil to produce chitosanase enzyme used to degrade chitosan into low molecular weight chitosan.

Materials and Methods

Sample Collection

The soil sample was collected from Htauk-kyant Township, Yangon Region. Dust and dead matters on the upper layer of the sampling site was removed followed by digging down to 6 inches depth. The collected soil sample was put into the sterilized plastic zipped bag and kept in refrigerator. The isolation of chitosanase enzyme producing bacteria *Bacillus megaterium* from soil sample was conducted in the Pharmaceutical Research Department, Insein, Yangon.

Isolation and Identification of *Bacillus megaterium* from the Collected Soil Samples

A 200 mL conical flask was filled with 99 mL of distilled water and four test tubes, each filled with 9 mL of distilled water were plugged with cotton wools and labeled as 10^2, 10^3, 10^4, 10^5 and 10^6 respectively and autoclaved at 121 ˚ C for 15 min.

About 1 g of the soil sample was put into 99 mL of distilled water in the 10^2 labeled conical flask. The flask was shaken gently for a few minutes and 1 mL of the above mixture was transferred into the 10^3 test tube and then 1 mL of mixture from the 10^3 test tube was transferred into the10^4 test tubes and so on.

After that, 1 mL of each of the prepared solutions (from 10^2, 10^3, 10^4, 10^5 and 10^6 conical flask and test tubes) was added to each of five nutrient agar plates and labeled. The inoculum was spread evenly over the entire surface of nutrient agar plates until the medium no longer appeared the moist. The spreader was returned to the alcohol and reap the flaming and spreading for each of the remaining four plates. Then all the plates were incubated in 37 ˚ C for 24 h.

The separate colonies were appeared and the different types of bacteria colonies were cultured in test. The colonies were found to be white to off-white in colour with smooth edges (Rampersad and Ammons, 2005) were selected and marked on Petri dish. The selected colonies with those characteristics were categorized as possible *Bacillus* colonies. Total of 10

colonies were chosen based on colony morphology. These selected colonies were repeatedly sub-cultured to obtain pure culture (Atlas and Synder, 2006).

The isolated bacterial strains were sub-cultured on nutrient agar slant cultures to check its purity and incubated at 37 °C for 24 h. Then the purified culture was maintained at refrigerator. The isolates were subjected to various physiological and biochemical tests. The isolates were identified by using conventional biochemical tests such as motility tests, methyl red tests, urease test, citrate utilization tests, indole tests, nitrate tests, (VP) Voges-Proskauer tests starch hydrolysis tests, sugar fermentation tests (Atlas and Synder, 2006; Garcia and Isenberg, 2007).

Determination of Optimal Conditions for Chitosanase Enzyme Production from the Selected Bacterial Strain

To find out the optimal conditions for producing chitosanase enzyme from the selected bacterial strain (A2), the effect of incubation time, pH, age of bacterial culture, inoculum size and temperature of enzyme catalyzed reaction on the chitosanase enzyme activity were studied.

(a) Effect of incubation time on chitosanase enzyme activity

Chitosan was used as the substrate in the chitosanase assay. Chitosanase activity was determined by quantitative estimation of the reducing sugars produced from chitosan. To prepare the culture medium for the production of chitosanase, the peptone (0.6 g), yeast extract (0.6 g), glucose (0.1 g), dipotassium hydrogen phosphate (0.1 g), magnesium sulphate (0.05 g) and colloidal chitosan (0.2 g) were used. The initial pH was adjusted to 7 with sodium hydroxide and the medium was put into a sterilized conical flask plugged with cotton wool and then sterilized by autoclaving autoclaved at 121 ºC for 15 min. The above medium was inoculated with 2 % inoculum size from 2 days age of culture in exponential growing phase and incubated on a shaker at 30 ºC and 180 rpm. To measure the enzyme activity, the content was centrifuged at 3500 rpm for 15 min and the cell free supernatant was used for analysis. For the optimum incubation time, the reaction temperature was used at 55 ºC.

(b) Effect of pH on chitosanase enzyme activity

The effect of pH on enzyme activity was determined by incubating the culture media at different pH values from 4 to 8 at optimized incubation period and 2 % inoculum size from 2 days age of culture. To study the optimum pH, the reaction mixture consisted of enzyme solution and 1 % colloidal chitosan between pH 4 to 8 at 55 ºC for 30 min. The reducing sugar was measured immediately at 540 nm by DNS method.

(c) Effect of age of culture on chitosanase enzyme activity

The effect of age of culture on enzyme activity was determined by incubation of five cultures on chitosanase producing agar at 30 ºC. The samples were taken every one day and stored for estimation of the optimum age of culture at 55 ºC for 30 min. The 1-5 days age of cultures were grown in chitosanase producing media using 2 % inoculums size at optimized incubation period and pH.

(d) Effect of inoculum size on chitosanase enzyme activity

The above medium (45 mL) in 250 mL of conical flask was inoculated with 2 %, 4 %, 6 %, 8 %, 10 % inoculum size of pre-culture in exponential growing phase and incubated on shaker at 30 ºC and 180 rpm. The effect of inoculum size on enzyme activity was determined by incubating the culture media at optimized incubation period, pH and age of culture.

(e) Effect of temperature on chitosanase enzyme activity

To determine the optimum temperature, the supernatant and 1 % colloidal chitosan were incubated at 35 ºC, 45 ºC, 55 ºC, 65 ºC and 75 ºC for 30 min at optimized incubation period, pH, age of culture and inoculum sizes.

Extraction and Partial Purification of Chitosanase Enzyme

Chitosanase enzyme solutions were extracted from the enzyme producing bacteria solution by precipitation method using 30 % and 70 % saturation with ammonium sulphate at 4 ºC. The mixture was allowed to settle down overnight. The bacterial cell pelleted out from precipitate was separated by centrifugation (3500 rpm, 30 min). Then the precipitate was

dissolved in 50 mL of 30 mM tris HCl buffer at pH 7.0. The solution was dialyzed overnight against the same buffer at 4 ºC (Sun *et. al*., 2009).

Determination of Protein Content in Partially Purified Chitosanase Enzyme

The concentration of protein content in supernatant obtained after precipitation with ammonium sulphate was determined by using Bovine serum Albumin (BSA) as standard. The reaction mixture was prepared by mixing 1 mL of sample and 4 mL of Biuret reagent solution at room temperature and allowed to stand for 30 min. It was followed by measuring the absorbance of the solution at 560 nm (Stoscheck, 1990).

Results and Discussion

Collection of Soil Sample

The soil sample was collected from the Htauk-kyant Township, Yangon Region. The soil sample was cultured on nutrient agar medium. Ten bacterial strains were isolated from the fresh soil sample.

Isolation and Culture of Bacterial Strain in Nutrient Agar Medium

Isolation plays a vital role for the identification of bacteria and this is the separation of pure culture (i.e., one species of bacteria) from mixed culture. During the process of taking bacteria from single by using a sterilized inoculating loop, the colony transfer was carried out in duplicate near the flame of spirit burner.

In the present work, nutrient agar medium was used as culture medium. Nutrient agar medium containing agar powder, nutrient broth with the combination of distilled water was found to be suitable for the culture of isolated ten bacterial strains (A1 to A10).

Identification of the Isolated Bacterial Strains

The morphology of ten isolated bacterial strains (A1 to A10) were examined under microscope. According to the microscopic examination, all of the isolated bacteria were found to be rod shape and motile.

Gram staining or Gram stain, also called Gram's method, is a method of staining used to distinguish and classify bacterial species into two large groups (gram-positive and gram-negative). The Gram stain is almost always the first step in the preliminary identification of a bacterial organism. According to the results from the characterization of the ten isolated bacterial strains (Table 1) by using gram staining tests, the bacterial strains A5 and A7 gave red colour and the remaining strains (A1, A2, A3, A4, A6, A8, A9 and A10) showed blue colour in this test.

Table 1: Characterization of the Isolated Bacterial Strains by Gram Staining Test

Isolated bacterial strains	Observed colour	Result
A1	Blue	+
A2	Blue	+
A3	Blue	+
A4	Blue	+
A5	Red	-
A6	Blue	+
A7	Red	-
A8	Blue	+
A9	Blue	+
A10	Blue	+

(+) = gram positive (-) = gram negative

Among ten bacterial strains, only 8 strains of gram-positive bacteria (A1, A2, A3, A4, A6, A8, A9 and A10) were selected to do further identification tests. The biochemical properties of that eight selected bacteria were studied by biochemical tests. In biochemical tests, bacterial strain A2 gave negative in indole test, nitrate reduction test and Voges-Proskauer and gave positive results in catalase, gelatin, urease, citrate utilization, starch hydrolysis, sugar fermentation, methyl red tests and motility tests. The results of biochemical tests for the eight selected bacterial strains are shown in Table 2 and that from sugar fermentation tests in Table 3. According to the biochemical tests and sugar fermentation tests, the results of the isolated bacterial strain A2 were found to be identical with the reported data of *B.*

megaterium (Beeseley *et al.*, 2010) as described in Tables 4 and 5. Consequently, the isolated bacterial strain A2 was identified as *B. megaterium*.

Table 2: Results of Biochemical Tests on the Eight Selected Bacterial Strains

Biochemical tests	Observation of the selected eight bacterial strains on biochemical tests							
	A1	**A2**	**A3**	**A4**	**A6**	**A8**	**A9**	**A10**
Motility	-	+	-	+	-	-	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-
Urease	+	+	-	-	-	+	-	-
Nitrate reduction	+	-	-	+	-	-	+	+
Starch hydrolysis	+	+	+	+	-	-	-	-
Citrate utilization	+	+	-	-	-	-	-	-
Methyl red	+	+	+	+	-	-	+	+
Indole	-	-	-	-	-	-	-	-

(+) = positive result, (-) = negative result

Table 3: Results of Sugar Fermentation Tests on the Eight Selected Bacterial Strains

Fermentation tests	Observation of the eight selected bacterias strains on sugar fermentation tests							
	A1	**A2**	**A3**	**A4**	**A6**	**A8**	**A9**	**A10**
Glucose	+++	+++	+++	+++	++	+++	+++	+++
Sucrose	++	++	++	+	++	++	+	++
Mannose	++	+	++	+	++	++	++	++

(+) = acid and gas liberate (slightly)
(++) = acid and gas liberate (moderately)
(+++) = acid and gas liberate (strongly)

Table 4: Comparison of the Results from Biochemical Tests on the Isolated Bacterial Strain A2 with the Reported Data of *B. megaterium*

Biochemical Tests	Observation on A2	**B. megaterium*
Motility	+ (Growth along the stab- line)	+
Gelatin liquefaction	+ (Liquefied)	+
Voges-Proskauer	- (Not appear pink colour)	-
Urease	+ (Pinkish- red)	+
Nitrate reduction	- (Not appear red colour)	-
Starch hydrolysis	+ (clear zone)	+
Catalase	+ (Bubble appeared)	+
Citrate utilization	+ (Blue colour)	+
Methyl red	+ (Red colour)	+
Indole	- (yellow layer)	-

* Beeseley *et al.,* 2010

Table 5: Comparison of the Results from Sugar Fermentation Tests on the Isolated Bacterial Strain A2 with the Reported Data of *B. megaterium*

Sugar tests	A2 (Isolated Bacteria)	* *B. megaterium*
Glucose	+++	+++
Sucrose	++	+++
Mannose	+	+

\+ = acid and gas liberate (slightly)
++ = acid and gas liberate (moderately)
+++ = acid and gas liberate (strongly)
\- = no gas
* Beeseley *et al.,* 2010

Producation of Chitosanase Enzyme Solution from the Isolated *B. megaterium*

Chitosan was used as the substrate in the chitosanase assay. Chitosanase activity was determined by quantitative estimation of the reducing sugars produced from chitosan. The reducing sugars formed in the supernatant were estimated spectrophotometrically by using the modified dinitrosalicyclic acid (DNS) method (Miller, 1959), using glucosamine hydrochloride as standard. Firstly, a standard calibration curve was constructed as a plot of absorbance vs concentrations of glucosamine solution (Table 6 and Figure 1).

Table 6: Relationship between Absorbance and Concentration of Standard Glucosamine Solution

Standard glucosamine solution	Glucosamine concentration (mg/mL)	Absorbance at 540 nm
1	1.00	0.064
2	2.00	0.140
3	3.00	0.206
4	4.00	0.276
5	5.00	0.339

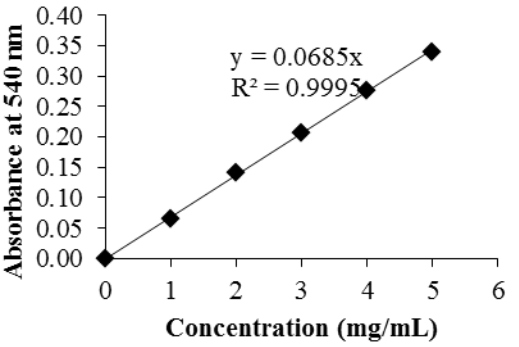

Figure 1: Standard calibration curve for glucosamine

Effect of different incubation times on enzymatic activity

The effect of incubation time on chitosanase production using pH 7, 2 % inoculum size from 2 days age of culture and reaction temperature 55 ºC is shown in Table 7. A2 bacterial strain produced the highest chitosanase activity on 3days (1.119 μmolmin^{1}mL^{1}). Enzyme production was gradually decreased after 3 days (Figure 2). One of the reasons for decreased production may be the lack of nutrients or production of toxic chemicals in the medium resulting in the inactivation of the enzyme production (Vanathia *et al*., 2016).

Table 7: Effect of Incubation Time of Chitosanase Forming Bacteria Solution on Chitosanase Activity by DNS Method

Incubation time (Day)	Absorbance at 540 nm	Chitosanase activity (μmol min^{-1} mL^{-1})
1	0.82	0.370
2	0.10	0.673
3	0.17	1.119
4	0.13	0.835
5	0.10	0.665

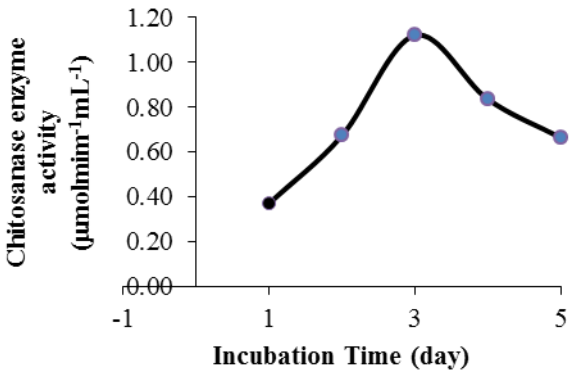

Figure 2: Plot of chitosanase enzyme activity as a function of incubation time of chitosanase enzyme forming bacteria solution

Effect of the different pH on enzymatic activity

The effect of pH of media on the chitosanase production was evaluated by bacterial cultures grown at different pH values (4 - 8) using optimum incubation time (3 days), 2 % inoculum size from 2 days age of culture and reaction temperature 55 ºC. Among the tested pH, pH 6 gave the maximum chitosanase activity (0.767 μmol mL^{-1} min^{-1}) (Table 8, Figure 3)

Table 8: Effect of pH of Chitosanase Production Medium on Chitosanase Activity by DNS Method

pH	Chitosanase activity (μmol mL^{-1} min^{-1})
4	0.709
5	0.718
6	0.767
7	0.682
8	0.639

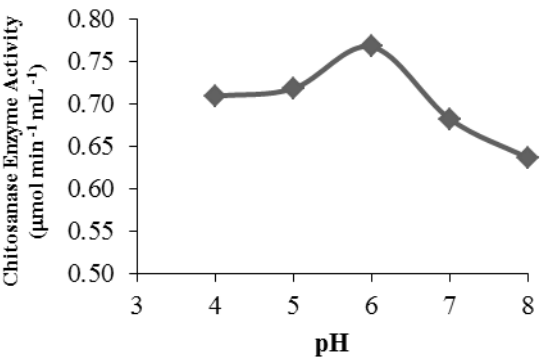

Figure 3: Plot of chitosanase enzyme activity as a function of incubation time of chitosanase enzyme forming bacteria solution

Effect of age of culture on enzymatic activity

The effect of the age of culture of media on the chitosanase production was studied up to 5 days using optimum incubation time (3 days), optimum pH 6, 2 % inoculum size and reaction temperature 55 ºC (Table 9). It indicated that chitosanase activity increased as the incubation time increased up to three days (0.840 μmol mL^{-1} min^{-1}) (Figure 4).

Table 9: Effect of Age of Culture of Bacterial Strain on Chitosanase Activity by DNS Method

Age of Culture (Days)	Chitosanase activity (μmol mL^{-1} min^{-1})
1	0.781
2	0.772
3	0.840
4	0.582
5	0.533

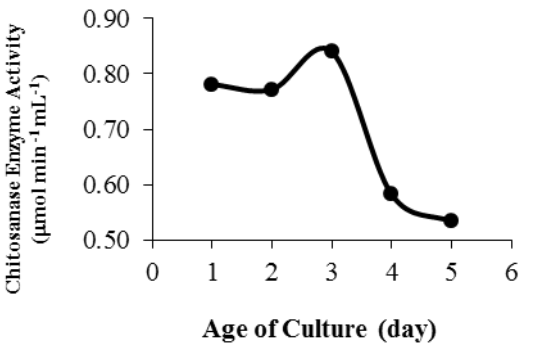

Figure 4: Plot of chitosanase enzyme activity as a function of age of culture of bacterial strain

Effect of inoculum sizes on enzymatic activity

The effect of inoculum size of media on the chitosanase production was evaluated by bacterial cultures grown at different inoculum size (2-10 %) using optimum incubation time (3 days), optimum pH 6, optimum 3 days age of culture and reaction temperature 55 ºC. Among the tested inoculum size, maximum inoculum size (10 %) gave the highest chitosanase activity (1.120 μmol mL^{-1} min^{-1}) (Table 10, Figure 5).

Table 10: Effect of Inoculum Sizes of Bacterial Solution on Chitosanase Activity by DNS Method

Inoculum Sizes (%)	Chitosanase activity (μmol mL^{-1} min^{-1})
2	0.569
4	0.605
6	0.745
8	0.840
10	1.120

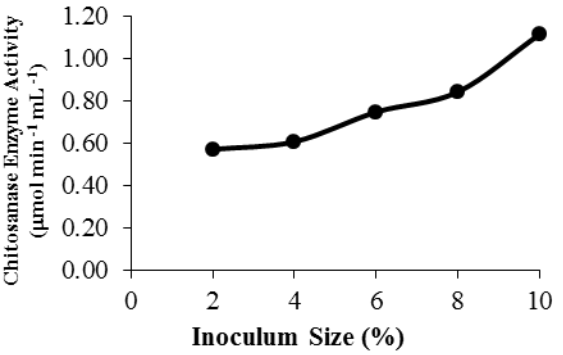

Figure 5: Plot of chitosanase enzyme activity as a function of innoculum sizes of bacterial solution

Effect of temperature on enzymatic activity

The effect of the temperature on the crude extract of chitosanase activity was studied in the range of 35 to 75 ºC using optimum incubation time (3 days), pH 6 and 10 % inoculum size from 3 days age of culture (Table 11). It was indicated that the maximum chitosanase activity (0.957 μmol $mL^{-1}min^{1}$) showed at the temperature 55 ºC (Figure 6).

Table 11: Effect of Temperature of Enzyme-catalyzed Reaction on Chitosanase Activity by DNS Method

Temperature (ºC)	Chitosanase activity (μmol mL^{-1} min^{-1})
35	0.682
45	0.827
55	0.957
65	0.890
75	0.740

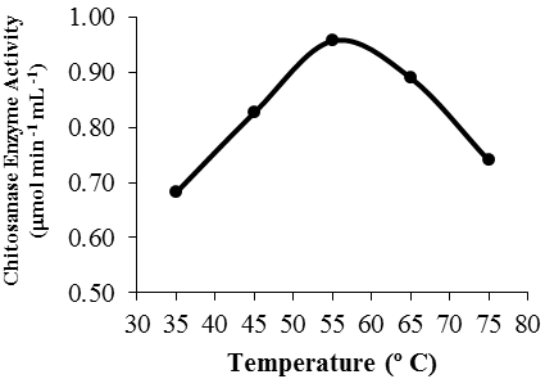

Figure 6: Plot of chitosanase enzyme activity as a function of temperature of enzyme catalysed reaction

The chitosanase activity (1.66 µ mol min^{-1}) was monitored by chitosanase assay method and the maximum activity of chitosanase was found under the condition for 3 days of incubation period of bacterial solution, pH 6 of chitosanase production medium, 10 % of inoculum size of bacteria and 3 days of age of bacterial culture and at 55 ºC of enzyme catalyzed reaction.

Partial Purification of Total Enzyme Activities of Crude Chitosanase Solution

The crude enzyme solution obtained from fermentation culture of *Bacillus megaterium* at optimum conditions was firstly purified by precipitating with 30 % saturation of ammonium sulphate solution. Then the collected supernatant enzyme solution in this step was also precipitated by using 70 % saturation of ammonium sulphate solution. The enzyme activities of the initial crude enzyme, the partially purified two enzymes by using 30 % and 70 % saturations of ammonium sulphate solutions were measured by DNS method. It was observed that the crude enzyme and two partially purified enzymes after precipitation with 30 % and 70 % saturation of ammonium sulphate solutions have 1.66 µ mol min^{-1}, 1.62 µ mol min^{-1} and 0.96 µ mol min^{-1} of enzyme activity, respectively. The degree of purity was 2.83 and 4.92 folds after 30 % and 70 % saturation of ammonium sulphate precipitation respectively (Table 12).

Total Protein Contents and Specific Enzyme Activities of the Isolated Chitosanase Enzymes

The crude chitosanase enzyme and two partially purified chitosanase enyzmes obtained by successive precipitation using 30 % and 70 % saturation of ammonium sulphate solutions were found to contain 6.43, 2.51 and 0.85 mg of protein contents, respectively. The protein contents of that enzymes were determined by Biuret assay method using bovine serum albumin (BSA) standard. It was found that the protein contents in enzymes decreased from 6.43 to 0.85 mg /mL after two steps purification.

Consequently, it leads to increase the total enzyme activity of the isolated enzymes and also to enhance the specific enzyme activity of crude enzyme from 0.26 μ mol min^{-1} mg^{-1} to 0.645 μ mol min^{-1} mg^{-1} after purified by 30 % saturation of ammonium sulphate solution and to 1.13 μ mol min^{-1} mg^{-1} after purified by 70 % saturation of ammonium sulphate solution. An increase in specific activity was observed after successively purified the crude enzyme by using 30 % and 70 % saturation of ammonium sulfate precipitation. The differences in specific activities observed at various purification steps while also comparing with that of the crude enzyme are shown in Table 12. According to the results described in the Table 12, the maximum specific activity was obtained after purified by using 70 % saturation of ammonium sulpahte precipitation.

Table 12: Total Enzyme Activity, Total Protein Content, Specific Enzyme and Degree of Purity Activity of the Isolated Chitosanase Enzymes

Purification steps	Total Enzyme activity (□mol min^{-1})	Total protein (mg)	Specific enzyme activity (□mol min^{-1} mg^{-1})	Degree of Purity (fold)
Crude enzyme solution	1.66	6.43	0.26	1.00
After 30 % $(NH_4)_2SO_4$ precipitation	1.62	2.51	0.65	2.83
After 70 % $(NH_4)_2SO_4$ precipitation	0.96	0.85	1.13	4.92

In each purification step, the total amount of chitosanase enzyme activity and protein decreased in order to salting out by high concentration of ammonium sulphate and removal of impurities. Purification of the crude chitosanase enzyme contained in the enzyme producing bacteria (*B. megaterium*) solution by 70 % ammonium sulphate precipitation resulted in increase in enzyme activity, which reflected in purification of 4.92 folds. Purification steps therefore resulted in elimination of interfering materials present in the crude cell- free extract thereby resulting in increased enzyme activity.

Conclusion

In this study, a gram-positive bacterial strain, *Bacillus megaterium* has been successfully isolated from the soil sample collected from Htauk-kyant Township, Yangon Region. The positive results were observed in biochemical tests such as catalase, methyl red, gelatin, urease, citrate utilization, starch hydrolysis, and sugar fermentation and negative results were observed in indole test, Voges-Proskauer test and nitrate reduction test.

The total enzyme activities of crude chitosanase having 1.66 μ mol min^{-1} and 6.43 mg of total protien content was produced from the isolated *B. megaterium* bacterial strain when 3 days of incubation period, pH 6 of chitosanase production medium, 10 % of inoculum size and 3 days age of bacteria culture at 55 ºC.

The crude chitosanase enzyme solution was partially purified by successive precipitation method using 30 % and 70 % saturation of ammonium sulphate solution giving 4.92 folds purity of chitosanase enzyme having 1.13 μ mol min^{-1} mg^{-1} of specific enzyme activity.

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