Australian Journal of Basic and Applied Sciences, 4(4): 615-623, 2010
ISSN 1991-8178

A Potent Chitinolytic Activity of Marine *Actinomycete* sp. and Enzymatic Production of Chitooligosaccharides

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Abstract: locally isolated Marine Streptomyces sp. were screened for chitinolytic activity on agar plates containing colloidal chitin. The strain showed the highest activity on a colloidal chitin screening agar plate and thin- layer chromatography was identified as a strain of *Streptomyces* that may be designated *Streptomyces champavatii* AZ-1due to the production of champamycin A, B and champavatin. The strain AZ-1 produced a multi-chitinolytic enzyme complex, extracellular and cell bound activities on submerged and solid-state fermentation after 144, 168 h incubation times at 37°C. Chitinases secreted in the culture supernatant were partially purified. The optimal temperature and pH of the enzymes were 37°C and pH 5, 5.5, respectively. Production of N-acetyl-D-glucosamine (GlcNAc) and N-acetyl chitooligosaccharides is important to biotechnology sectors, facilitating its potential use in medical application as antitumor. N-acetyl chitooligosaccharides were produced from colloidal chitin by use of crude enzyme obtained from *St. champvatii* AZ-1that produced a good yield of chitopentaose $(Glc\text{-}NAc)_5$ and chitohexaose $(GlcNAc)_6$.

Key words: Chitinolytic multienzymes. Marine. N- acetyl chitooligosaccharides. Solid- state fermentation. *Streptomyces*.

INTRODUCTION

Chitin, a linear polymer of β -1,4-linked N-acetylglucosamine (GlcNAc), is synthesized in all the major groups of organisms,bacteria, fungi, plants and animals (Nopakarn *et al.* 2002). Chitinases are a group of complex chitinolytic enzyme systems capable of directly degrading chitin to its oligomers (chitooligosaccharides) and /or monomers (N-acetylglucosamine). Chitooligosaccharides and their N-acetylated analogues are useful for applications in various fields because they have specific biological activities such as antimicrobial activity, antitumor activity, immuno-enhancing effects (Gohel *et al.*2006). Some chitooligosaccharides such as $(GlcNAc)_6$ and $(GlcNAc)_7$ have been reported to possess antitumor activity (Suzuki *et al.* 1986; Liang *et al*. 2007). Chitinolytic enzymes have been widely used in various processes including the agricultural, biological and environmental fields (Chuan 2006). Several chitinolytic enzymes have been identified in various Streptomyces sp., including, *Streptomyces plicatus* (Robbins *et al.*1988), *S. lividans* (Miyashita *et al.* 1991), *S. virdificans* (Gupta *et al.*1995) and *S. halstedii* (Joo 2005).

Since high costs of commercial chitinase production restrict large-scale applications, in recent years, there has been a lot of interest in enhancing the production of chitinase using fermentation techniques. Both submerged fermentation (SmF) and solid state fermentation (SSF) were employed for microbial chitinase production. SSF offers a better alternative for cost effective production of enzymes, since it can be carried out using cheaply available agro-industrial residues such as wheat bran, rice husk, sugar cane bagasse, etc. In this study we would like to report a new type of extracellular and cell bound chitinolytic multi-enzymes from marine *St. champavatii*-AZA1 isolate which is specific for the preparation of COS.

MATERIALS AND METHODS

Chemicals:

Chitin from shrimp shells and *p*-nitro phenyl-β-D-*N*-acetylglucosaminide (PNP-β-GlcNAc) were purchased from Sigma Chemical Co. (USA). All other reagents were of the purest grade commercially available. $(Glc\text{-}NAc)_5$ and $(GlcNAc)_6$ standards were provided by Dr. Abo-Zaid M, IVF, RH, MI, USA.

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Colloidal Chitin (C.CH.) Preparation:

10 gm chitin (sigma) was treated with 100 ml phosphoric acid (H_3PO_4) according to Reid and Ogrydiazk (1981).

Preparation of Chitinous Waste:

Untreated chitinous waste was composed of (shrimp- and crab-shell wastes), collected from sea-food markets in Egypt. Partially purified chitin wastes were prepared according to the method described by Jeuniaux (1966) with some modifications. Colloidal chitin was also prepared from the chitin wastes by Reid and Ogrydiazk (1981).

Screening and Culture Condition:

Streptomyces strains locally isolated from water and sediments of the Mideterenian and Red sea coast (Ras- Ghareb) were screened for chitinolytic activity on colloidal chitin-agar plates (0.2%, w/v colloidal chitin (c.chitin) and aged sea water according to the method of Hsu and Lockwood (1975). Chitinolytic activity was expressed by a zones of clearance around individual colonies. One strain, which showed the largest zone of clearance, was identified through morphological and physiological characteristics by Micro-analytical center, Cairo University as *Streptomyces champavatii* AZ-1. The main culture medium for the production of chitinolytic enzymes was sea-water complex medium (SWC); colloidal chitin 5 g, $(NH_4)_2SO_4$ 7 g , K_2HPO_4 1 g, NaCl 1 g , $MgSO_4.7H_2O$ 0.1 g, tryptone 1 g, yeast extract 0.5 g aged sea water 1000 ml, pH 7.2. (Yuli *et al.,*2004). Inoculum was prepared by dispersing strain AZ-1 spore suspension (10^7 c.f.u./ml) of selected strain into 50 ml SWC in 250 ml Erlenmeyer flask and incubated for 3 days at 37 °C, 150 rpm. Enzymes production were achieved by transferring 5% inoculum to sterilized SWC (50 ml) and incubated for different time intervals at the same conditions. chitinolytic activity was determined in cell bound (c.b.) and culture filtrate (c.f.) after centrifugation at 10,000 × g for 20 min.

Enzyme Assay:

Endochitinase activity was determined according to the method of Jeuniaux (1966) using c.chitin as a substrate. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of *N*-acetyl-glucosamine per min under standard assay conditions. Exochitinase activity was determined according to the method of Matsumoto *et al.* (2004) using the chromogenic substrate *p*-nitro phenyl-β-D-*N*-acetylglucosaminide (PNP-β-GlcNAc) as a substrate. One unit of the enzyme activity was defined as the amount of enzyme releasing1µmol of *p*-nitrophenol per min under the specified assay conditions. The cell bound fraction (c.b.) was washed three times in 0.9 % NaCl and used for c.b. assay according to Herbert *et al*. (1971).

Protein Content:

This was estimated by the method of Bradford (1976) using Coomassie Brilliant Blue G-250 dye reagent and extrapolated from the standard curve of bovine serum albumin.

Screening of agro-industrial residues in (SSF)

A mixture of the following combinations were prepared: (a) 5g wheat bran, 0.05 g colloidal chitin and 0.05 g sugar cane bagasse; (b) 5 g chitinous waste , 0.05 g wheat bran and 0.05 g colloidal chitin; (c) 5 g chitinous waste , 0.05 g sugar cane baggase and 0.05 g colloidal chitin; (d) 5 g colloidal waste, 0.05 g Galactose and 0.05 g wheat bran; (d) 5 g sugar cane bagasse, 0.05 g wheat bran and 0.05 g colloidal chitin; (e) 5 g colloidal chitin; (f) 5 g wheat bran. Each was prepared in 250-erlenmeyer flasks and moistened with 10 ml of fermentation liquid medium at pH 7.0 then autoclaved at 121°C for 15 min (Binod *et al.,* 2007). The flasks were inoculated with freshly prepared spore suspension at (10^7 c.f.u./ml) and incubated at 37°C for 6 and 7days (the maximum periods for exo- and endochitinase respectively).

Enzyme Extraction:

The content of each Erlenmeyer flask was mixed with 100 ml of 0.1% Tween 80 solution at 30°C, 180 rpm for 1 h. Solids were separated by filtration and then centrifugation for 15 min at 4°C and the supernatants were used for enzymes and protein assays. The fermented biomass was used for c. b. assays.

Crude Enzymes Preparation:

Optimized culture supernatant (crude exochitinase) and SSF extract (crude endochitinase) were precipitated with ammonium sulfate (50, 70 %, w/v). The precipitates were obtained by centrifugation (10000 × g, 30 min,

4°C) and suspended in an appropriate volume of 0.05 M phosphate buffer (pH5.5). Cell bound extract was concentrated by ultrafiltration using an Amicon® YM10 membrane (10 kDa MW cut-off, Millipore).
Some properties of crude chitinolytic enzyme

In the preceding experiments, effect of reaction conditions (temperature and pH), and addition of metal ions on crude enzyme were investigated. The optimal temperature was determined by incubating the reaction mixture at different temperatures from 25 to 60°C.The optimal pH was determined by measuring the activities at 30 °C between pH 4 and pH 7 using sodium acetate buffer (pH 4 –5) and sodium phosphate buffer (pH 5.5 –7).

Preparation of Chitooligosaccharides (COS):

Hydrolysis of c.chitin by crude endochitinase (0.1ml, 9.67 U) was carried out in the reaction mixture containing 0.5 ml of 1% colloidal chitin in 0.05 M phosphate buffer (pH 5.5). The reaction mixture was incubated at 30 °C for 15, 20, 30, 60, 90, 120, 150 and 180 min. The reaction was terminated by immersing the tubes in boiling water for 5 min. The insoluble materials were removed by centrifugation and the water soluble supernatant was concentrated to 20% of the original volume, a yellow agglomerates were formed and the products were analyzed by TLC and HPLC for (Glc-NAc), $(Glc\text{-}NAc)_5$ and $(GlcNAc)_6$ quantification .

TLC Analysis:

Series of spots of the reaction mixture were put on a silica TLC plate (5.0 cm x 6.0 cm) against standards and then chromatographed twice in a mobile phase containing n-butanol: methanol: 30% ammonium solution : H_2O (5 : 4 : 2 : 1) (V/V). Followed by spraying with aniline- diphenylamine reagent and baking at 121 °C for 5-10 min (Tanaka *et al.* 1999).

Analysis of Hydrolysates by HLPC:

HPLC analysis of the crude enzyme hydrolysate was performed on a Hitachi L-7000 apparatus (column, Shodex Asahipak NH2P-50; mobile phase, CH3CN/H2O = 65/35; flow rate = 1.0 mL/min; detection, RI) and the oligosaccharides were compared with the standard N-acetylglucosamine, hexa- and penta-chitooligosaccharides.

RESULTS AND DISCUSSION

Screening and Isolation of Chitinase Producing Strain:

Ten marine chitinase-producing *Streptomyces* sp. were isolated on the selective medium containing colloidal chitin. The identified isolate as *Streptomyces champavatii*- AZ-1 having the largest clear zone around the developed colonies on the chitin agar plate was selected for further study (Fig. 1). The strain AZ-1 produced maximal (c.f. and c.b) exochitinase and endochitinase activities (2689, 2024 mU/ml), (271, 495 mU/ml) at 144, 168 h incubation times (Fig.2).

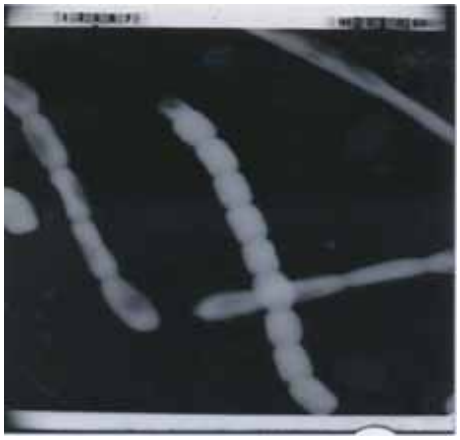


Fig. 1: Rectiflexibles and smooth surface spore chains of *S. champavatii*-AZ-1 as seen by Transmission electron microscope

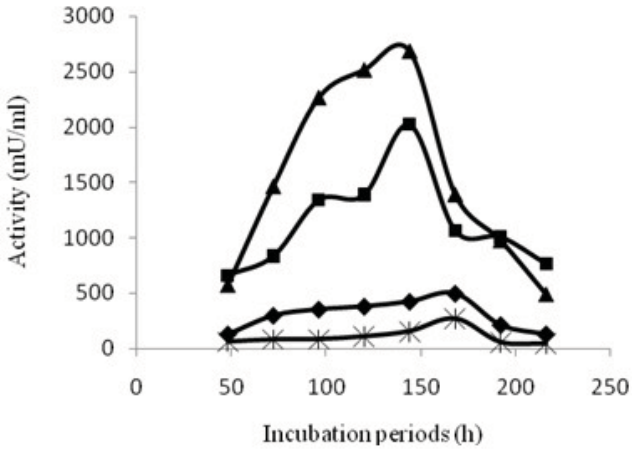


Fig. 2: Time course of c.f. and c.b. exo- and endochitinase production by *S. champavatii*- AZ-1 grown on SWC medium containing c. chitin at 37 °C, 150 rpm. Triplicate samples of culture were taken after 48 to 240 h. (▲—) c.f. exochitinase (extracellular), (■ —) c.b. exochitinase, (⁎-⁎) c.f. endochitinase, (♦—) c.b. endochitinase.

Effect of Different Nitrogen Sources:

Effect of different nitrogen sources on the enzyme production by *Streptomyces champavatii* were investigated. These include organic (yeast extract, tryptone, colloidal chitin, chitinous waste, chitin powder) and inorganic (ammonium sulfate) nitrogen sources used on equal N-basis. The secretion of endochitinase was also significantly influenced by the nitrogen source incorporated into the growth medium. Maximum c.f. and c.b. exochitinase activities were obtained in optimized medium (control), 9045, 5358 mU/ml. Endochitinases (c.f., c.b.) were highly increased using 6.9% c.chitin as a sole N and C source (2512 , 6453 mU/ml). Inorganic N source $(NH_4)_2SO_4$ was not suitable for enzymes production. Results in Fig. 3 reflect the inducible and constitutive nature of AZ-1 exo- and endochitinase enzymes with colloidal chitin.

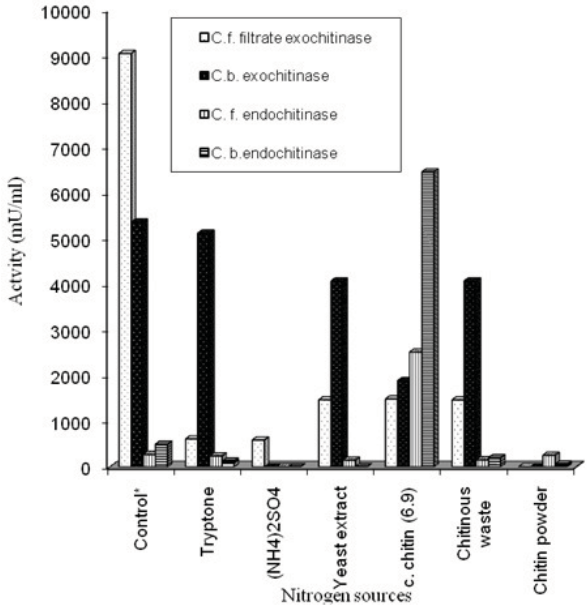


Fig. 3: The effect of different nitrogen sources on c.f. and c.b. (exo- and endochitinase) production by *S. champavatii*-AZ-1.

Solid State Fermentation (SSF):

In SSF experiments WB and SCB supplemented with1% (w/w) c. chitin induced AZ-1 endochitinase production (extracellular, c.b. (Table 1). This might be due to the fact that c.chitin was most easily accessible form of chitin source for micro-organisms. WB was a good solid substrate with respect to other substrates as it induced exo- and endochitinase activity. It was worth mentioning that (SSF) offers a better alternative for cost effective production of enzymes, since it can be carried out using cheaply available agro-industrial residues.

Table 1: Agro-industrial residues evaluated for exo-and endochitinase activity by *Str. Champavatii* AZ-1

Agricultural waste	Exochitinase activity		Endochitinase activity	
	C.f. (U/gds)	C.b.(U/db*)	C.f. (U/gds)	C.b.(U/db*)
W.B.+c.ch.+ S.C.B. [a]	12.5	1.7	74.3	6.8
C.W.+W.B+ c.ch .[b]	6.2	2.8	1.7	0.1
C.W.+ S.C.B.+ c.ch [c]	6.2	0.3	1.2	0.1
C.W.+G +W.B. [d]	9.1	0.9	9.5	0.4
S.C.B.+W.B.+c.ch.[e]	8.5	0.4	59.4	5.5
C. chitin	0.5	0.2	1.1	0.1
W.B.	26.4	2.6	53.1	7.8

* (unit/dry biomass)

Effects of pH, Temperature and Metal Ions on Crude Enzymes:

The AZ-1crude exo-and endochitinase enzymes exhibited optimal activities at pH 5 and 5.5 (16.8, 8.1U /mg protein) and optimum temperature of both enzymes was 30 °C (Fig.4). Exochitinase activity was slightly enhanced by Na molybdate and reduced by other metal ions. Mn^{+2} and Co^{+2} act as good activators to endochitinase enzyme, they increased the activity by155.2 and 63.6 % respectively. Endochitinase activity was also enhanced by Cu^{+2}, Zn^{+2} increasing the activity by 12 , 9 % respectively. The enzymes were inhibited by Hg^{+2}. TLC analysis indicated the enhanced effect of Mn^{+2} on endochitinase activity for the production of COS (Fig.5).

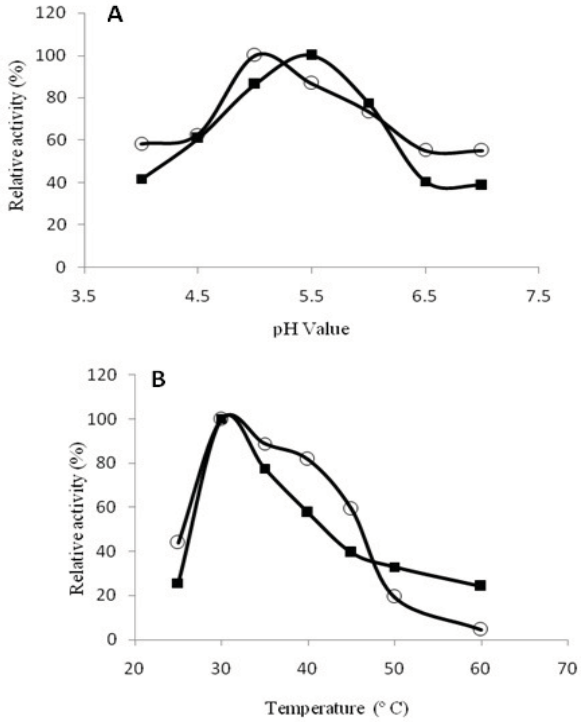


Fig. 4: Effects of pH and temperature on crude exo- and endochitinase activities from *St. champavatii*-AZA1. A). The values (Relative activity), are shown as percentages of the maximum activity, was taken as 100 % and corresponding to 16.7 U /mg protein exochitinase at pH 5 and 8.1 U/ mg protein endochitinase at pH 5.5 . B) The maximum activity of exo- and endochitinase was at 30 °C, ○—exo-chitinase activity ; ■— endochitinase activity.

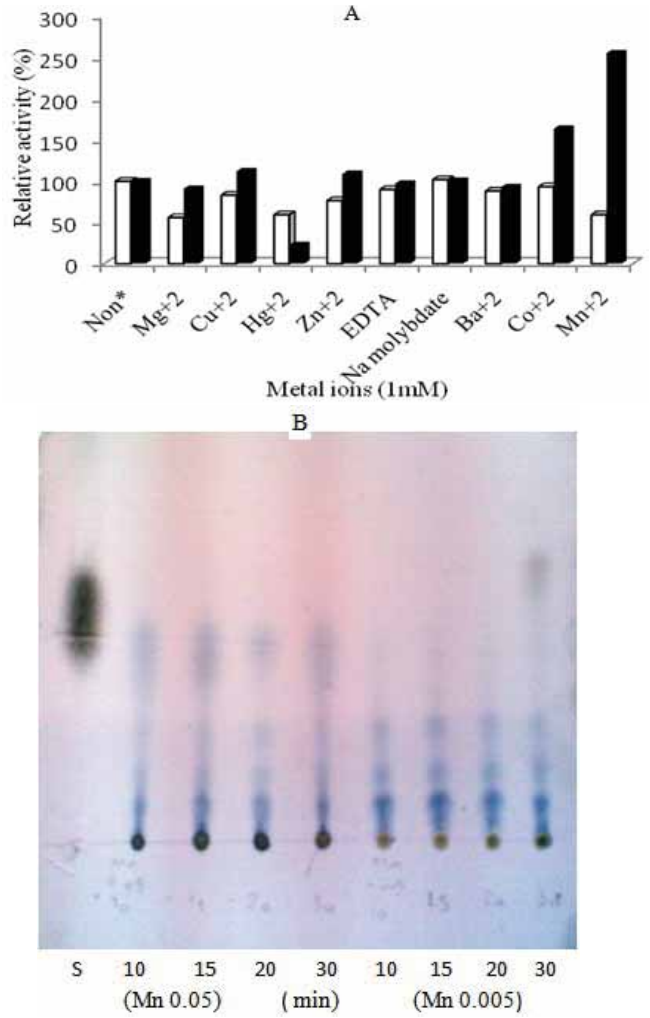


Fig. 5: Effects of metal ions on crude exo- and endochitinase activity. A) The enzyme was incubated with 0.5 ml of 1 % c. chitin in 50 mM phosphate buffer (pH 5.5) with 1mM EDTA and different metal ions at 30 ºC for 1h. Absolute activities corresponding to 100% were 16.7 and 8.1 U/ mg protein, □—exo- and ■—endochitinase relative activity. B) Time course of c.chitin hydrolysis by crude enzyme using (0.05, 0.005 mM) Mn^{+2} ion in the reaction mixture for different time intervals (10, 15, 20 and 30 min) as analyzed by TLC against (GlcNAc) standard

Preparation of Chitooligosaccharides (COS):

COS preparation was achieved by endochitinase hydrolysis of c.ch.at optimum reaction conditions. Production of $(GlcNAc)_5$, $(GlcNAc)_6$ from c.ch. Hydrolysis by crude and concentrated c.b. endo-chitinase at different time intervals was detected by TLC. The precipitates were collected and immediately vacuum-dried. (COS) were further incubated with crude enzyme to prove the active *S. champavatii*-AZ-1 endochitinase (Fig.6). The production of GlcNAc and N-acetylchitooligsaccharides except $(Glc\text{-}NAc)_5$ and $(GlcNAc)_6$ by *Paenibacillus illioisensis* KJA-424 were detected at 24h (Jung *et al.* 2005).

Analysis of Hydrolysate by HPLC:

In our work, HPLC analyses (Fig.7) detected the production and quantification of GlcNAc and N-acetyl chitooligosaccharides. The concentration of GlcNAc, $(GlcNAc)_5$ and $(GlcNAc)_6$ were 0.01, 0.41and 0.21 mg/ml respectively from the equation of Liang *et al.* (2007) who obtained $(GlcNAc)_6$ after 12h crude enzyme hydrolysis. [$(Glc\text{-}NAc)_5$ and $(GlcNAc)_6$] were not detected until 24 h during cultivation of *Aeromonase* sp.

DYU-Too7 (Lien *et al*. 2005).The amounts of (GlcNAc)n, n = 1,5,6 were estimated with the calibration curve of standard 0.3 mg/mL, (GlcNAc)n, n = 1,5,6. The yield of (GlcNAc)n , n = 1,5,6 was calculated by the following equation:

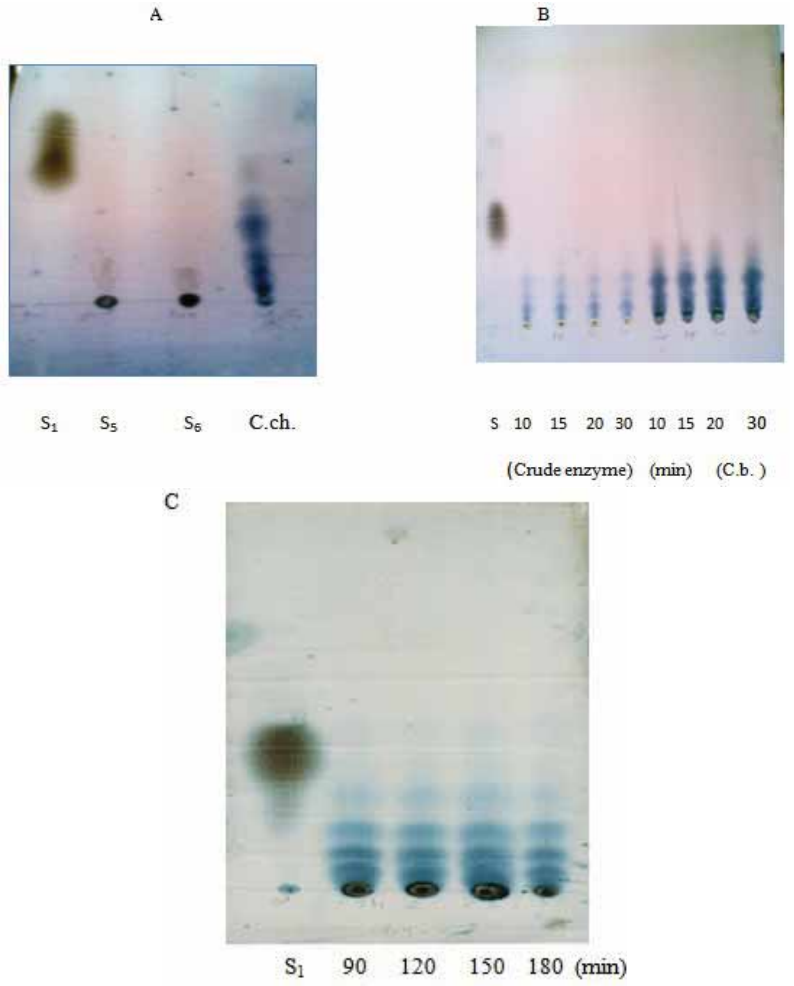


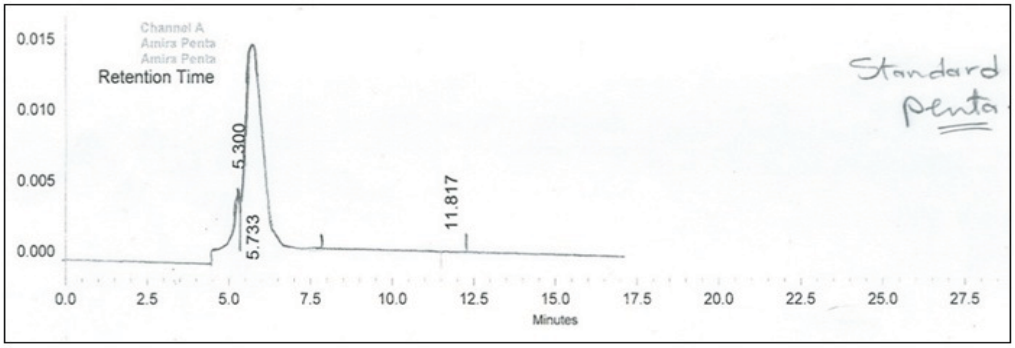
Fig. 6: A) Time course of c.ch hydrolysis was analyzed on TLC into $(GlcNAc)_n$ after 60 min against (GlcNAc) (S_1), $(GlNAc)_5$(S_5) and $(GlcNAc)_6$(S_6) standards. B) Hydrolysis of c. chitin using crude enzyme and cell bound for different intervals from 10- 30 min indicated the production of $(GlNAc)_5$, $(GlcNAc)_6$ and $(GlcNAc)_7$. C) Hydrolysis of chitooligomers (end product) by crude enzyme for different time intervals proved endochitinase action.

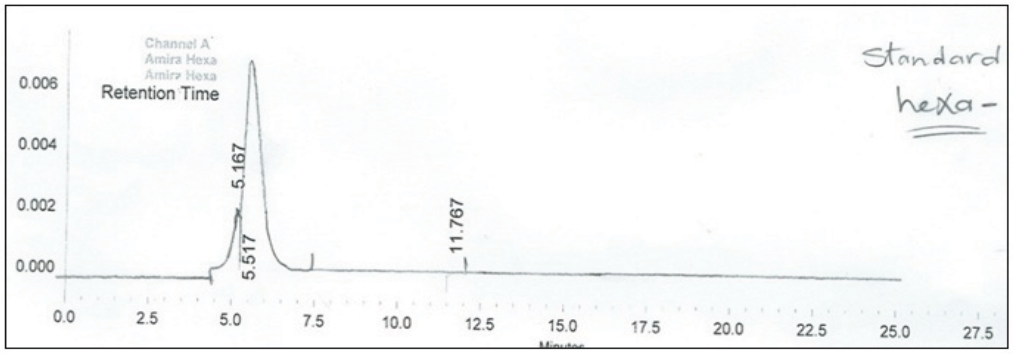
The concentration of sample mg /ml = 0.3 × the area of sample / the area of standard

In conclusion, the multi-chitinolytic enzyme complex produced by *St. champavatii*-AZ-1using low cost substrate in SSF is highly effective in the production of COS especially $(Glc\text{-}NAc)_5$, $(Glc\text{-}NAc)_6$ and $(Glc\text{-}NAc)_7$ facilitating its potential use in medical applications as antitumors.

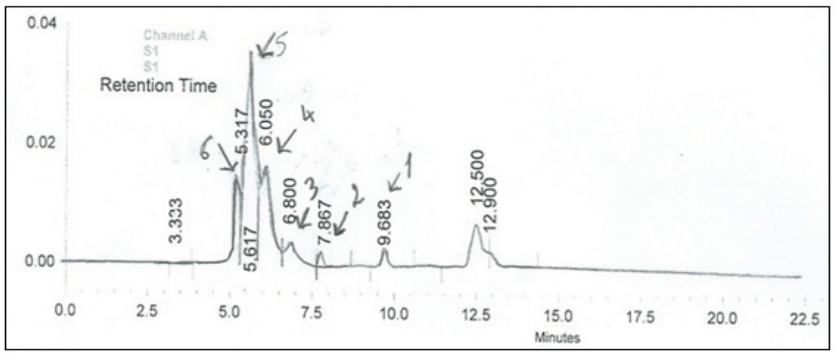
ACKNOWLEDGMENTS

The authors would like to thank Dr. Abo-Zaid M for providing the work with [$(Glc\text{-}NAc)_5$, $(Glc\text{-}NAc)_6$] standards.





Sample1



Sample 2

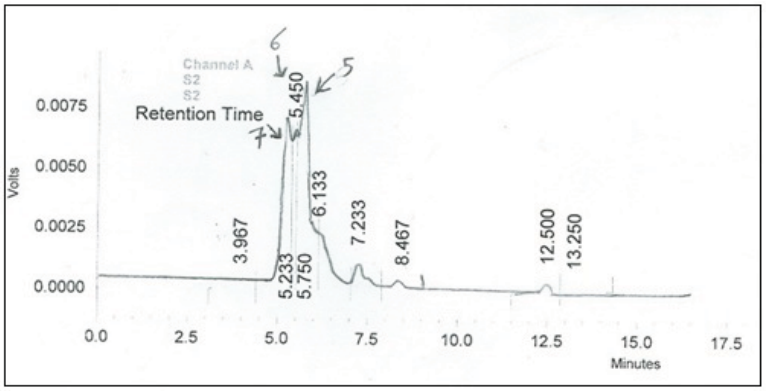


Fig. 7: HPLC analysis of the crude enzyme hydrolysates of c. chitin after 20 min . The oligosaccharides were compared with the standards (N-acetylglucosamine, hexa- and penta-chitooligosaccharides). A) HPLC analysis of another sample indicated hepta-oligomer

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