African Journal of Biotechnology Vol. 11(89), pp. 15523-15532, 6 November, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1596
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Chitinolytic activity of highly halotolerant *Streptomyces tendae* against *Fusarium oxysporum* PTK2

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Accepted 16 October, 2012

Fifteen (15) highly halotolerant *Streptomyces* isolates were isolated from saline soil of Teachers College garden in Riyadh city. Chitin nitrate agar medium containing 20% (w/v) NaCl was used for the isolation purpose. These chitinolytic *Streptomyces* isolates were purified and sub-cultured on chitin nitrate broth medium containing 20% NaCl (w/v). The dry weight and absorbance (585 nm) were measured for each isolate to determine the best chitinolytic isolate. The best chitinolytic isolate was symbolized HS-5 which in turn was identified as *S. tendae* using morphological, physiological and biochemical characteristics as well as 16S rRNA gene partial sequence. The later revealed that there was 98% similarity between isolate HS-5 and *S. tendae* M23 which has accession number HM594286.1 as in Gene Bank. Optimization was studied to produce the maximum yield of chitinase enzyme. High chitinase productivity was obtained at 1.6 × 10^7 CFU ml^{-1} inoculum size, 3rd day incubation period, 35°C incubation temperature, 8.5 pH value and eventually with casein; was the best nitrogen source. Chitinase enzyme was precipitated from the filtrate of *S. tendae* at 50% saturated ammonium sulphate and purified using sephadex G200 column chromatography. Chitinase enzyme was separated at 20 KDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by protein gel electrophoresis, and stained by coomassie blue dye. Amino acids of chitinase enzyme and their concentrations were determined using High-performance liquid chromatography (HPLC) device.

Key words: Chitinolytic activity, enzyme purification, halophytic actinomycetes, soil salinity.

INTRODUCTION

Lysis of the host structure by secretion of extracellular lytic enzymes is one of the important mechanisms that are involved in the antagonistic activity of biocontrol agents (Saksirirat and Hoppe, 1991; Mathivanan et al., 1997; Kim et al., 2001). Among these, chitinase (EC 3.2.1.14) plays a vital role in the biological control of many plant diseases by degrading the chitin polymer in the cell walls of fungal pathogens (Haran et al., 1993). It affects fungal growth through the lysis of cell walls (Kunz et al., 1992), hypal tips, and germ tubes (Gunarantna and Balasubramanian, 1994).

Chitinolytic *Streptomyces* sp. PTK19 produced 16.53 U ml^{-1} extracellular chitinase using submerged fermentation and broth medium contained chitin (0.4% w/v) as sole carbon source, at 30°C under shaking condition at 120 rpm with sucrose 80 mM as carbon source and (0.1% w/v) peptone as nitrogen source and initial pH at 7 (Thiagarajan V et al., 2011). After cellulose, chitin is the most abundant biopolymer found in nature (Tracey, 1957). Chitin, a β-(1,4) polymer of N-acetylglucosamine, is a structural component of the arthropod exoskeleton and is a common constituent of fungal cell walls (Peberdy, 1990). Chitin in soil can be degraded by a wide variety of microorganisms including fungal and bacterial species. Chitinolysis, namely hydrolysis of the glycosidic bonds of chitin by chitinases, is probably the most important pathway of degradation of chitin in soil (Gooday, 1990).

Streptomycetes are soil-dwelling mycelial bacteria that produce a large number of secreted proteins and many secondary metabolites, including important antibiotics. Chitin is a major nutrient source for many Streptomycetes and these microorganisms have developed complex extracellular systems for chitin utilization (Chater et al.,

2009). Chitinases play an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. Bacteria produce chitinases to digest chitin primarily to utilize it as a carbon and energy source. *Streptomyces* strains are regarded as the major producers of chitinases in soils. Much research has been carried out to date on the purification and characterization of family 18 chitinases from *Streptomyces*. Because of their inhibitory abilities, *Streptomyces* sp. have been actively studied and utilized as biocontrol agents against various plant pathogens (Merriman et al., 1974; El-Abyad et al., 1993; Hiltunen et al., 1995; Jones and Samac, 1996). Chitin and its derivatives are of commercial and biotechnological interest because they have various biological activities and wide range of applications in areas ranging from waste water treatment to agrochemical and biomedical uses (Pascual and Julia, 2001; Hirano, 1996; Nagahama et al., 2008; Kim et al., 2008).

The goal of this research was to optimize the chitinase production from *S. tendae*. The cultural characters were optimized by amending with different incubation period, various carbon and nitrogen sources, optimum pH, temperature and inoculum size were studied then scale up to the fermentation in shaked flask (2 l). It contained 1 L medium under optimum conditions. The crude chitinasewas tested against *Fusarium oxysporum* PTK2 for cell wall degradation.

MATERIALS AND METHODS

Microorganisms and maintenance

The *S. tendae* was isolated from saline soil of Teachers College garden, King Saud University in Riyadh city and was maintained on chitin nitrate agar slants which contained 20% NaCl (w/v). The plant fungal pathogens *F. oxysporum, Alternaria alternata* and *Rhizoctonia solani* were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C.

Inoculum preparation

50 ml of sterile glass distilled water was added to 250 ml conical flask containing the culture and shaken well to harvest the spores. Then, 2 ml was inoculated into 50 ml of production medium. The cell concentration was maintained (1.6×10^7 CFU ml^{-1}) and was adjusted using sterile glass distilled water.

Chitinase screening

The chitinolytic activity of *S. tendae* was tested through the chitin nitrate broth medium which was incubated at 35°C for three days. Dry weight of mycelial growth was measured as well as absorbance at 585 nm using spectrophotometer (UNICO SQ2800).

Cup-plate assays

Chitinase activity was assessed in cup-plate assays as described by Bertheau et al. (1984). 0.1% (w/v) of colloidal chitin dissolved in sodium acetate buffer (0.05 M, pH 5.2) was used as a substrate in 1% of agar medium.

Preparation of colloidal chitin

5 g of chitin powder taken from crab shells sigma - USA was added slowly to 60 ml of concentrated HCl and left at room temperature overnight with vigorous stirring. The mixture was added to 200 ml ice cold 95% ethanol and incubated overnight at room temperature with vigorous stirring. The precipitate was collected by centrifugation at 5000 *g* for 20 min at 4°C and transferred to a glass funnel with filter paper (80 g). The colloidal chitin was washed with the sterile distilled water until colloidal chitin became neutral (pH 7.0); the colloidal chitin retained on a filter paper was removed, weighed and stored in a dark place at 4°C.

Chitinase assay

Colloidal chitin was used as a substrate to assay chitinase activity; 0.2 g in a 2 ml acetate buffer (50 mM, pH 5.0) was incubated with 1 ml of enzyme at 30°C for 1 h. The product was measured in 1 ml of filtrate by the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of N-acetylglucosamine per ml in 60 min. Exochitinase (N-acetyl-β-glucosaminidase) activity was assayed by mixing 0.1 ml of the aliquot of the appropriately-diluted enzyme with 0.5 ml of 1.75 mM *p*-nitropheny-β-N-acetylglucosaminide in a 50 mM acetate buffer (pH 6.0). After incubation at 30°C for 30 min, the reaction was terminated by adding 0.9 ml of 0.2 M Na_2CO_3 and *p*-nitrophenol was measured at 405 nm.

Identification of *Streptomyces tendae*

The morphological and cultural characteristics of *S. tendae* were examined according to Shirling and Gottlieb (1966). For electron micrograph, ISP4 agar medium was inoculated and incubated for seven days at 35°C. A plug of the culture was removed and fixed in glutaraldehyde (2.5% v/v), washed with water and post-fixed in osmium tetraoxide (1% w/v) for 1 h. The sample was washed twice with water and dehydrated in ascending ethanol before drying in a critical point drying apparatus (Polaron E3000) and finally coated in gold and examined in a JEOLISM 541OLV scanning electron microscope at 15 kv.

16S rRNA identification by PCR

Identification was carried out by 16S rRNA sequencing. 16S rRNA was amplified in a thermocycler (Perkin Elmer Cetus Model 480) by using universal primers of 27f (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1525r (5'-AAG GAG GTG ATC CAG CC-3') under the following condition: 94°C for 5 min, 35 cycles of 94°C for 60 s, 55 for 60 s, 72°C for 90 s and final extension at 72°C for 5 min. The product was directly sequenced by a BigDye terminator cycle sequencing kit (PE Applied Biosystems USA) on an ABI 310 automated DNA sequencer (Applied Biosystems, USA). Homology of the 16S rRNA sequence of isolate was analyzed by using BLAST program from Genbank database (Prapagdee et al., 2008).

Determination of optimum factors

Effect of inoculum size

A wide range of inoculum size was used (20 to 220 µl). Calculation

of the viable count in each inoculum was performed according to Isenberg (1992). The phytopathogenic fungi were inoculated in Erlenmeyer flasks (100 ml) that contained PDA as test organisms. The seeded media were poured and left for solidification. Agar wells technique was used and these wells were filled by different inoculum sizes as mentioned above from the filtrate of *S. tendae* using micropipette. Diameter of inhibition zones was measured to determine the best chitinolytic activity correlated to the best inoculum size. Microbial count of the best inoculum was calculated using haemocytometer slide method to determine the inoculum size by Cell Forming Unit (CFU).

Effect of incubation period

Erlenmeyer flasks (250 ml) containing 100 ml chitin nitrate broth medium were inoculated and incubated on shaking incubator at 120 rpm and 35°C for various incubation periods (1 to 7 days). At each incubation period, the chitinolytic activity was tested against all phytopathogenic fungi which were mentioned (Isenberg, 1992).

Effect of incubation temperature

Erlenmeyer flasks (250 ml) containing 100 ml chitin nitrate broth medium were inoculated and incubated on shaking incubator at 120 rpm for three days at different temperatures (25 to 45°C). At each incubation temperature, the chitinolytic activity was tested against all phytopathogenic fungi mentioned (Isenberg, 1992).

Effect of pH

Erlenmeyer flasks (250 ml) containing 100 ml chitin nitrate broth medium were inoculated and incubated on shaking incubator at 120 rpm for three days at 35°C and various values of pH (6 to 10) using a phosphate buffer. At each initial pH value, the chitinolytic activity was tested against all phytopathogenic fungi mentioned (Isenberg, 1992).

Effect of nitrogen source

In this experiment, ammonium nitrate, peptone, urea, casein, and beef extract were tested as substitutive nitrogenous sources. Potassium nitrate as a sole nitrogenous source of chitin nitrate medium was substituted with the different nitrogenous sources mentioned. Erlenmeyer flasks containing chitin nitrate broth medium were inoculated and incubated at 35°C for three days and adjusted at 8.5 initial pH value. With each nitrogenous substance, the chitinolytic activity was tested against all phytopathogenic fungi mentioned (Vanderzant and Splittstoesser, 1992).

Preparation of cell free extract

The modified chitin nitrate broth medium was inoculated by *S. tendae* and then incubated at 35°C on shaking incubator at 120 rpm for 3 days at pH 8.5. After incubation period, the filtrate was centrifuged at 10,000 *g* for 10 min. The supernatant (cell free extract) that contained chitinase was taken and subjected to precipitation by ammonium sulphate.

Precipitation by ammonium sulphate

Chitinase enzyme was precipitated by saturated ammonium sulphate at wide range of concentrations (10 to 90%). Each fraction was left for 2 h at 4°C and followed by centrifugation at 8000 *g* for 20 min at 4°C. The precipitate was dissolved in 10 ml phosphate buffer (pH 8.5), and centrifuged at 8000 *g* for 20 min at 4°C. The resulting supernatant was brought to definite concentration saturation with ammonium sulphate, further centrifugation, and the precipitate dissolved in 10 ml phosphate buffer (pH 8.5). The fraction contained chitinolytic activity was dialyzed using plastic dialysis bag against distilled water overnight.

Quantitative estimation of total protein content

1 ml of chitinase containing fraction was pipetted out, then 5 ml of working alkaline copper reagent was added, and standing was allowed at room temperature for 15 min (Lowry et al., 1951). Immediately, 0.5 ml of diluted folin reagent was mixed and allowed to stand at room temperature for 30 min. The colour intensity of the sample was measured by spectrophotometer at 280 nm. Blank was 5 ml of copper reagent with 0.5 ml of diluted folin reagent.

Gel filtration

10 g of sephadex G-200 was dissolved in 400 ml phosphate buffer at pH 7.5, boiled in water bath for 6 h, then cold to 50°C and packed in column (2.5 × 50 cm). Gel filtration was done basically according to Andrews (1969). Active fractions were pooled and dialyzed in distilled water and then poured in sephadex G-200 column chromatography which was pre-equilibrated with phosphate buffer at pH 7.5, at a flow rate of 5 ml/25 min.

Electrophoresis

Chitinase enzyme was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, which was carried out with 10% polyacrylamide gel as described by Blackshear (1984), in the presence of 0.1% sodium dodecyl sulphate at pH 8.8 and 1 mM dithiothreitol. Gel and gel buffers were prepared as described by Laemmli (1970) and Jackowski (1990). Gels showing the formed bands clearly were stained by Coomassie Blue dye as described by Fairbanks et al. (1971).

Amino acids analysis by HPLC

The apparatus used was Spectra-Physics Analytical, Inc. A0099-600 with spectra focus optical scanning detector and spectra system UV 2000 detector and ultra sphere C_{18} Beckman column. The analysis was carried out using a gradient of Pico-Tag solvent A and B at 40°C and flow rate of 1 ml/min. Detection of the separated Pico-Tag amino acids was at 254 nm wavelength. Before injecting of the sample, the illustrated was calibrated by two injections of the standards (Steven et al., 1989).

RESULTS AND DISCUSSION

Saline soil sample was collected from the garden of Teachers College in Riyadh city. The soil sample was dried in the air for three days and analyzed to determine its characteristics, especially sodium ratio (Table 1). Chitin nitrate agar medium was prepared (colloidal chitin 0.1 (w/v), KNO_3 2 g, $CaCO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 200 g, Agar-Agar 25 g and

Table 1. Soil sample characteristics.

pH	Electrical conductivity (ds/m)	Na^+ (%)	Organic C (%)	Number of organisms/g of dry soil	
				Heterotrophic bacteria	Actinomycetes
8.0	17.5	25.3	0.35	2.9×10^6	2.5×10^3

Table 2. Color series of halophytic-chitinolytic *Streptomyces* isolates.

Series	Total	%	Chitinolytic isolates	Mycelium dry weight mg/ml	Absorbance
Gray	7	46.7	HS-1	0.541	0.547
			HS-2	0.658	0.614
			HS-3	0.895	0.774
			HS-4	0.941	0.791
			HS-5	1.942	0.847
			HS-6	1.754	0.721
			HS-7	1.025	0.597
White	4	26.7	HS-8	1.254	0.641
			HS-9	0.987	0.741
			HS-10	0.874	0.701
Red	3	20	HS-11	1.547	0.792
			HS-12	1.775	0.797
			HS-13	1.125	0.761
			HS-14	0.985	0.624
Green	1	6.6	HS-15	1.547	0.801
Total	15	100	----------	----------	----------

Table 3. Cultural characteristics of *Streptomyces tendae.*

Medium	Growth	Aerial mycelia	Substrate mycelia	Diffusible pigment
Tryptone yeast extract broth	Poor	264 L.Gray	79 l.gy.YBr	No
Yeast-Malt extract agar	Moderate	10 Pk Gray	70 l.oy	No
Oat-meal extract agar	Good	263 L.Gray	76 l.yBr	79 l.gy.YBr
Inorganic salts starch agar	Moderate	10 Pk Gray	79 l.gy.YBr	No
Glycerol asparagine agar	Good	264 L.Gray	79 l.gy.YBr	No
Peptone yeast extract iron agar	Poor	10 Pk Gray	79 l.gy.YBr	No
Tyrosine agar	Poor	10 Pk Gray	79 l.gy.YBr	No

L. Gray, light gray; l.gy.YBr, light gray yellowish brown; PK. Gray, pinkish gray; l.oy, light orange yellow; l.yBr, light yellowish brown.

deionized water 1000 ml at pH 7.8 ± 2) for isolation of chitinolytic halophytic *Streptomyces* bacteria. 15 chitinolytic-halotolerant *Streptomyces* bacteria were isolated, purified and slanted on the same medium and then maintained at 4°C. Chitinolytic-halophytic *Streptomyces* isolates were classified into four series according to the color of spore mass (Table 2).

These isolates were inoculated in chitin broth medium and incubated at 35°C for three days to determine the best chitinolytic isolate by measuring the absorbance at 858 nm and also mycelium dry weight was measured. HS-5 is the code of the best chitinolytic *Streptomyces* isolate which in turn was tested against plant pathogen *F. oxysporum* PTK2 which gave severe inhibition. HS-5 halophytic isolate was identified as *S. tendae* using morphological characteristics, illustrated spiral spore chain and warty surface of spores (Figure 1); cultural characteristics illustrated the rate of growth, color of aerial and substrate mycelia and color of diffusible pigments on different seven recommended media as mentioned in ISP (Table 3). Biochemical and physiological characteristics were studied according to Shirling and Gottlieb (1966) (Table 4). Identification was also carried out using PCR technique to determine 16S

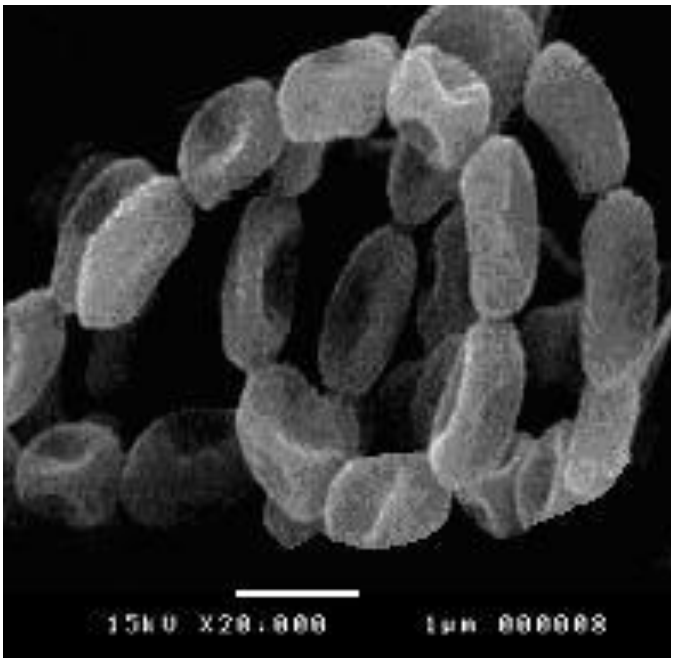

Figure 1. Scanning electron micrograph of *Streptomyces tendae* showing spiral spore chain and warty spore surface.

rRNA gene sequence which revealed 98% similarity between HS-5 isolate and *S. tendae* M23 with accession number HM594286.1 in Gene Bank.

Optimum environmental and nutritional factors of *S. tendae* were studied to increase the chitinase activity. The best inoculum size was determined at 1.6×10^7 CFU. The best incubation period was at the 3rd day (Figure 2). Chitinase production was initially found after 24 h of incubation and reached maximum levels after 60 h of cultivation. Chitinase synthesis was found to decline as the incubation period further extended. Nawani et al. (2002) reported the maximum production of extracellular chitinase by *Microbispora* sp. V2 after 48 h of incubation, which declined subsequently. The highest chitinolytic activity was determined at 35°C incubation temperature (Figure 3). The best pH value was 8.5 (Figure 4). In another study, high chitinolytic activity was determined at optimum temperature of 35°C (Nawani et al., 2002). The pH stability of the chitinolytic enzymes (FI and FII) before and after purification was determined. The inhibitory effects of the crude and purified enzymes on *F. oxysporum* were studied. The pH stability of the crude enzyme was at pH 6 to 8; FI was at pH 6 to 9 and FII was at pH 5 to 9 (San et al., 2002).

Casein is the best nitrogen source for production of chitinase (Figure 5). However, there was a significant increase in chitinase production in CYS medium amended with organic nitrogen sources as yeast extract (0.4%) and soybean meal (0.6%). Vaidya et al. (2001) reported that organic nitrogen sources as yeast extract and peptone significantly increased the chitinase production by *Alcaligenes xylosoxydans.* In the present study, CYS medium amended with starch (chitin 1%, starch 0.2%, yeast extract 0.4%, K_2HPO_4 0.2%, $MgSO_4.7H_2O$ 0.1% and $FeSO_4.7H_2O$ 0.01%), and with pH 6, was found to be suitable for maximum production of chitinase by the strain cultured at 35°C for 60 h.

S. tendae was inoculated in 1 L batch of amended chitin nitrate broth medium, taking into account the optimum factors which were mentioned. Cell free extract was obtained by high centrifugation and supplemented by saturated ammonium sulfate, with wide range from 10 to 90% (along 9 days) to precipitate chitinase enzyme. Chitinolytic activity had been detected in the fraction at 50% saturated ammonium sulfate. This active fraction which had brown color was exposed to dialysis against distilled water overnight to eliminate the impurities and toxic ions. Fraction containing chitinase enzyme was poured gently in packed sephadex G-200 column chromatography and then eluted by phosphate buffer at pH 7.5. 50 fractions were taken (5 ml/25 min) and the chitinolytic activity was tested in each one. Chitinolytic activity was constricted in only two fractions (24 and 25) which led to sharp peak (Figure 6). The two fractions containing chitinolytic activity had been pooled and exposed to dialysis against distilled water overnight. Purified single band of chitinase enzyme was separated at 20 KDa using electrophoresis.

Ueno et al. (1990) reported that two distinct chitinases were produced from the same *Streptomyces* strain while 47 kDa chitinase was present even in the inductor-free inoculums; the other four enzymes appeared in

Table 4. Morphological and biochemical characteristics of *Streptomyces tendae.*

Parameter	Characteristic	Result
Morphological characteristics	Spore chains	Spiral
	Spore mass	Gray
	Spore surface	Smooth
	Substrate mycelium	Yellowish brown
	Diffusible pigments	Yellowish brown
	Motility	Non motile
Cell wall hydrolysis	Diaminopimelic acid (DAP)	LL-DAP
	Sugar pattern	Not detected
Physiological characteristics	Amylase, protease, pectinase, catalase and cellulase	+
	Lipase and lecithinase	-
	Melanoid pigment production	+
	Degradation of xanthine and esculine	+
	H_2S production	-
	Nitrate reduction	+
	Streptomycin resistance	-
Utilization of different carbon sources	D-glucose	+
	D-galactose	+
	Sucrose	+
	Mannitol	+
	L-arabinose	-
	Raffinose	+
	meso-inositol	+
	D-fructose	+
	Xylose	-
	Rhamnose	+
Utilization of different nitrogen source	L-cystiene	+
	L-valine	+
	L-histidin	+
	L-alanine	+
	L-lysine	+
	L-leucine	+
	L-tyrosine	+
	L-phenylalanine	+
	L-proline	+
NaCl tolerance	1 to 7%	+
	10%	+

+, growth; -, no growth.

fermentations, with chitin as a substrate initially at 92 and 70 kDa and then consecutively at 30 and 20.5 kDa. The purified chitinase from *Streptomyces* sp. M-20 revealed homogeneity of a single protein band on 12% native PAGE. Its molecular weight was estimated as 20 kDa by SDS-PAGE (Kyoung et al., 2003). Different molecular masses that ranged from 35 to 45 kDa have been reported for other fungal chitinases (Ulhoa and Peberby, 1992; Gunaratna and Balasubramanian, 1994; Sakurada et al., 1996).

Amino acids content of chitinase enzyme and their concentrations were determined using HPLC. The amino

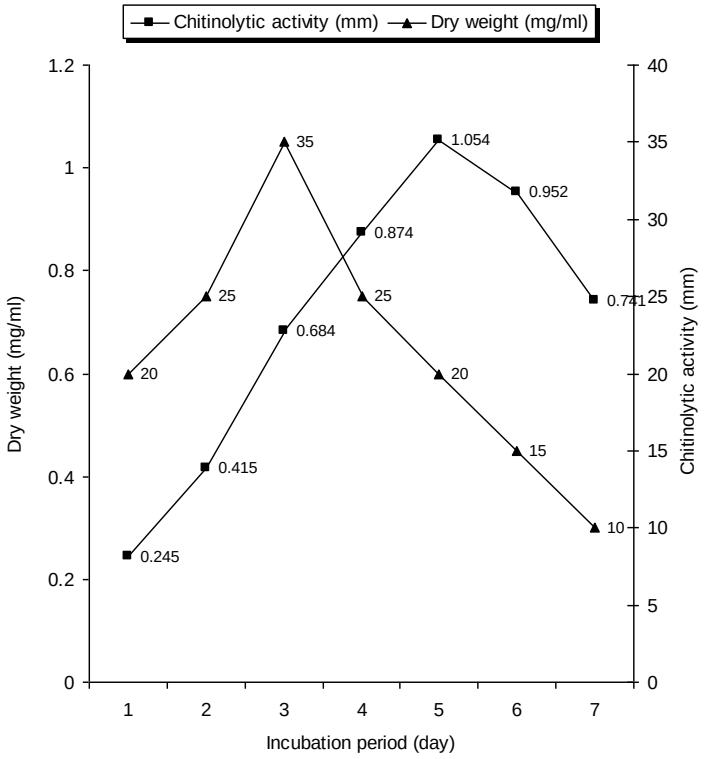

Figure 2. Effect of different incubation periods on chitinolytic activity of *Streptomyces tendae* against *Fusarium oxysporum* PTK2.

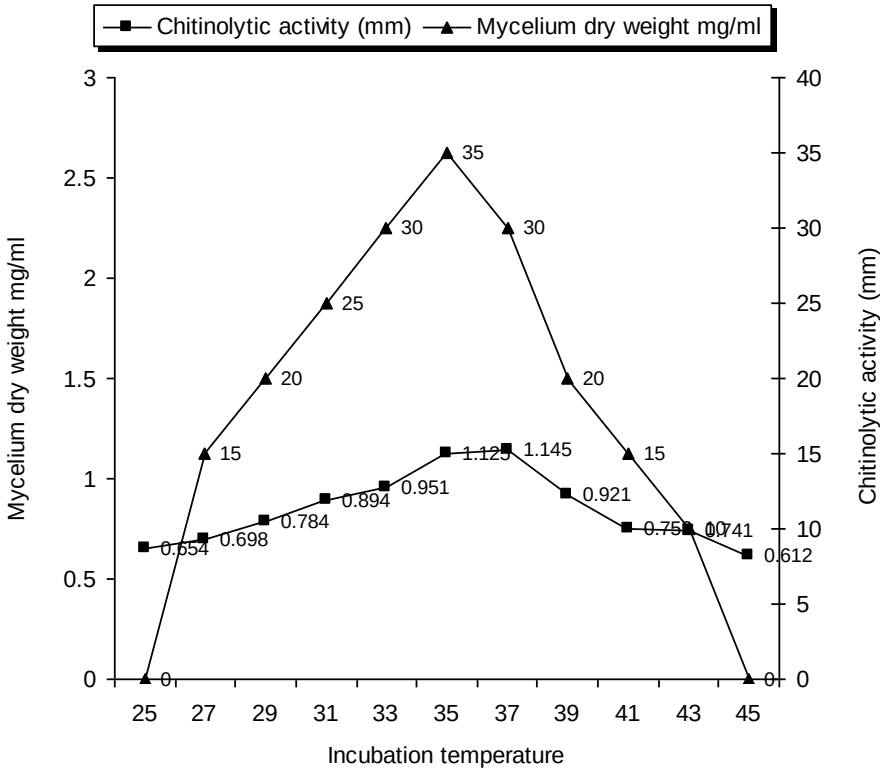

Figure 3. Effect of different incubation temperatures on chitinolytic activity of *Streptomyces tendae* against *Fusarium oxysporum* PTK2.

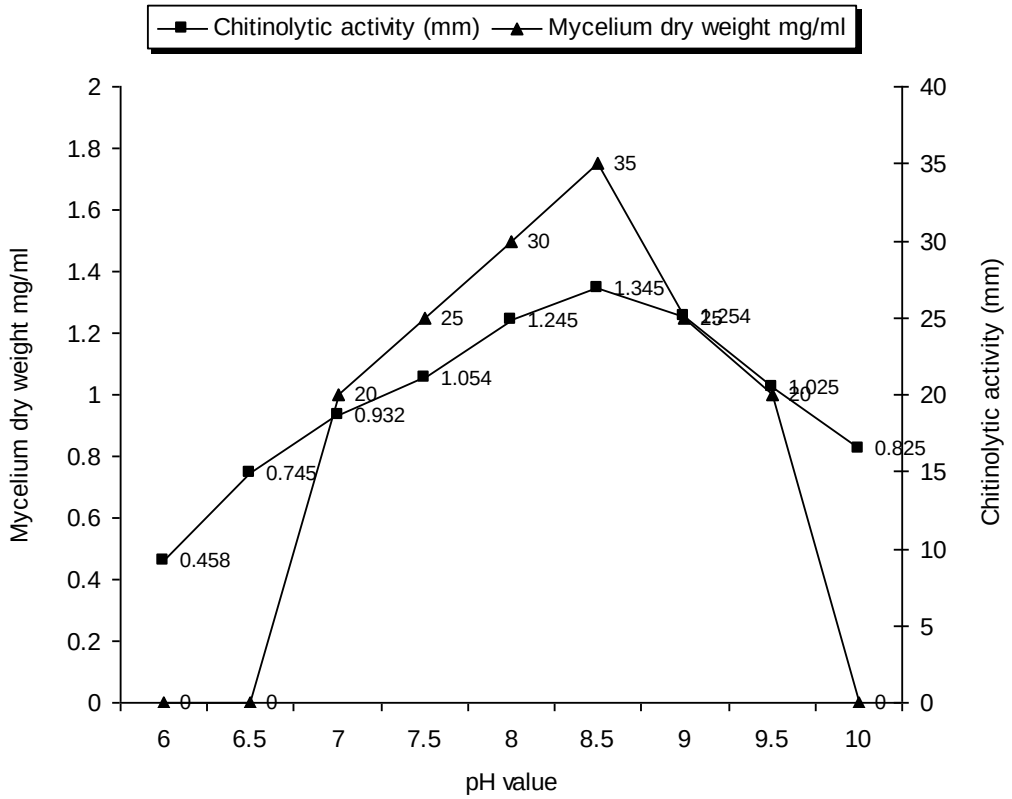

Figure 4. Effect of different pH on chitinolytic activity of *Streptomyces tendae* against *Fusarium oxysporum* PTK2.

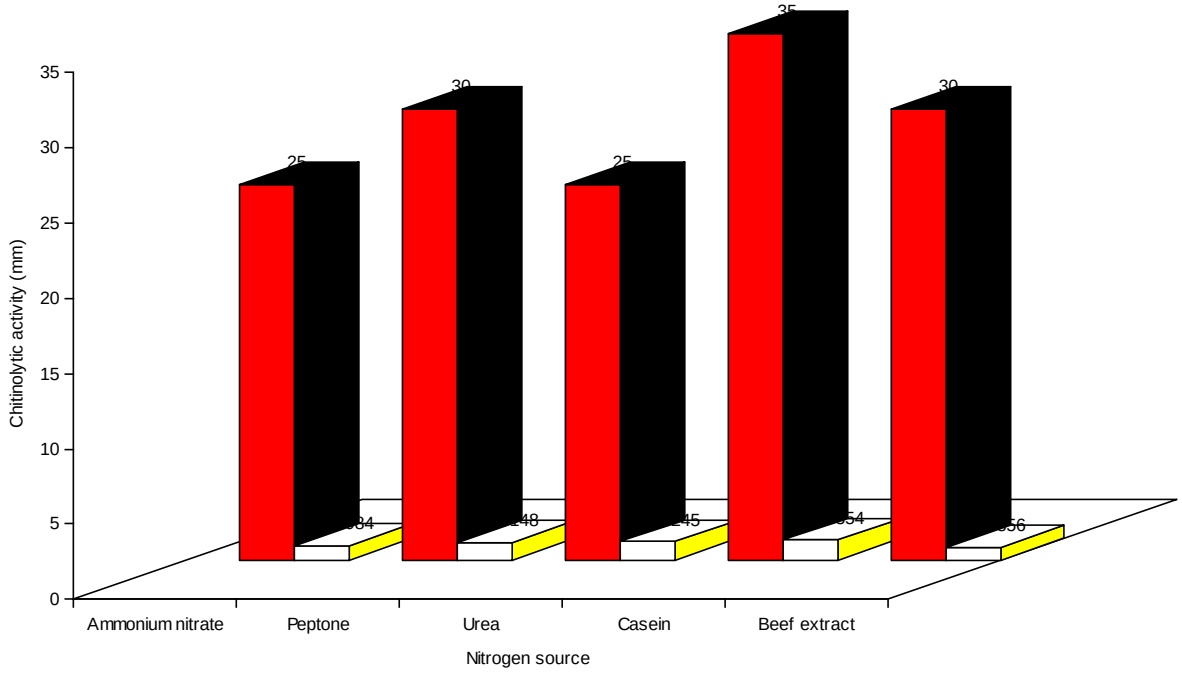

Figure 5. Effect of different nitrogen sources on chitinolytic activity of *Streptomyces tendae* against *Fusarium oxysporum* PTK2.

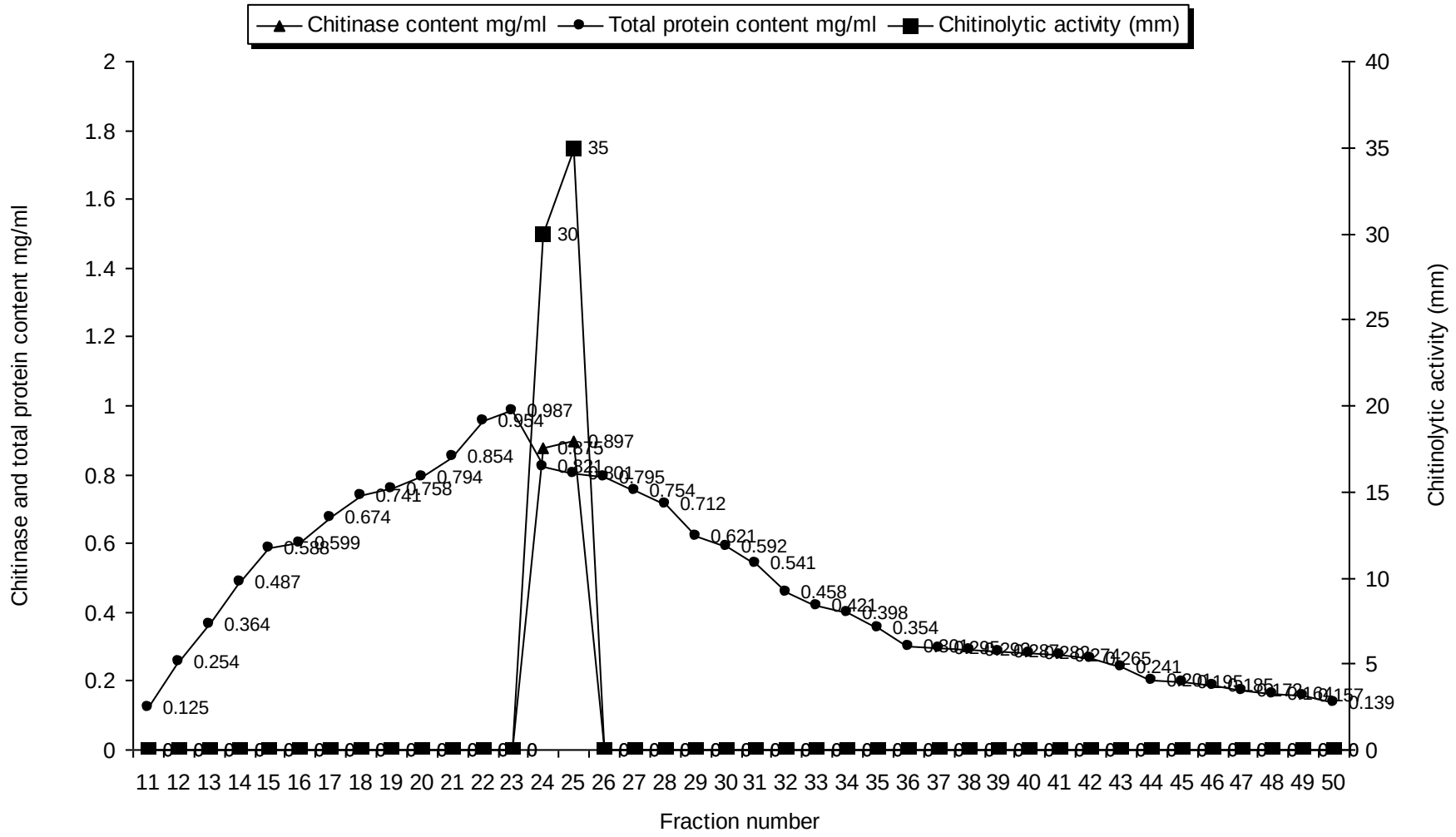

Figure 6. Purified chitinase enzyme using gel filtration with assayment of chitinase and total protein content.

acid sequences at the amino-terminal end showed no homology. We determined the carboxyterminal amino acids by cleavage by the method of Klemm (1984), with the modification that SDS was diluted to 0.01%. Threonine was the C-terminal amino acid in the two inducible chitinases tested, but no sequence could be determined because the activity of carboxypeptidase Y against the chitinases was very low. On proteolytic digestion of the 70, 47, 30, and 20.5 kDa chitinases with Serum amyloid P component (SAP), which had earlier been shown to cleave proteins reproducibly

(Cleveland et al., 1979), fragments of the same molecular masses (17.8, 14.5, and 13.7 kDa) appeared. Because of the very low concentration of 92 kDa chitinase in the extracellular protein, no proteolytic experiments have yet been performed.

ACKNOWLEDGEMENT

The author extends his appreciation to the Research Center, Teachers College, King Saud University which provided the required fund to this work.

REFERENCES

Andrews P (1969). Estimation of the molecular weight of proteins by sephadex gel filtration. Biochem. J. 91:222-223.

Bertheau Y, Madgidi-Hervan E, Kotoujansky A, Nguyen-The C, Andro T, Coleno A (1984). Detection of depolymerase isoenzymes after electrophoresis or electrofocusing, or in titration curves. Ann. Biochem. J. 139:383-389.

Blackshear PJ (1984). Systems for polyacrylamide gel electrophoresis. Methods enzymol. 104:237-255.

Chater KF, Biro S, Lee KJ, Palmer T, Schrempf H (2009). The complex extracellular biology of *Streptomyces*. FEMS Microbiol. J. Rev. 34:171-198.

Cleveland DW, SG Fischer, MW Kirschner, UK Laemmli (1979). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. Biol. Chem. J. 252:1102-1106.

El-Abyad MS, El-Sayed MA, El-Shanshoury AR, El- Sabbagh SM (1993). Towards the biological control of fungal and bacterial diseases of tomato using antagonistic *Streptomyces* spp. Plant Soil J. 149:185-195.

Fairbanks G, Steck TL, Wallach DFH (1971). Electrophoetic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.

Gooday G (1990). The Ecology of Chitin Degradation. Adv. Microb. Ecol. 11:387-430.

Gunarantna KR, Balasubramanian R (1994). Partial purification and properties of extracellular chitinase produced by *Acremonium obclavatum*, an antagonist to groundnut rust, *Puccinia arachidis.* World J. Microbiol. Biotechnol. 10:342-345.

Haran S, Schickler H, Chet I (1993). Increased constitutive chitinase activity in transformed *Trichoderma harzianum*. Biol. Control J. Theory Appl. Pest Manag. 3:101-108.

Hiltunen LH, Linfield CA, White JG (1995). The potential for the biological control of basal rot of *Narcissus* by *Streptomyces* sp. Crop Prot. J. 14:539-542.

Hirano S (1996). Chitin biotechnology applications, Biotechnol. J. Ann. Rev. 2:237-258.

Isenberg H D (1992). Clinical Microbiology Procedures Handbook, Washington, D.C.: American Society for Microbiology.

Jones CR, Samac DA (1996). Biological control of fungi causing alfalfa seedling damping-off with a disease-suppressive strain of *Streptomyces.* Biol. Control J. 7:196-204.

Kim IY, Sio SY, Moon HS, Yoo MK, Park IY, Kim BC, Cho CS (2008). Chitosan and its derivatives for tissue engineering applications, Biotechnol. Adv. J. 26:11-21.

Kim SK, Kim YT, Byun HG, Park PJ, Ito H (2001). Purification and Characterization of antioxidative peptide from Bovine skin. Biochem. Mol. Biol. J. 34:219-224.

Klemm P (1984). Carboxy-terminal sequence determination of proteins and peptides with carboxypeptidase Y. Methods Mol. Biol. J. 1:255-260.

Kunz C, Ludwig A, Boller T (1992). Evaluation of the antifungal activity of the purified chitinase I from the filamentous fungus *Aphanocladium album*. FEMS Microbiol. Lett. J. 90:105-109.

Kyoung-Ja K, Yong-Joon Y, Jong-Gi K (2003). Purification and Characterization of Chitinase from *Streptomyces* sp. M-20. Biochem. Mol. Biol. J. 36(2):185-189.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat. J. 227:680-685.

Lowry OH, Rosebrough J, Farr AL, Randall RJ (1951). Protein measurements with the folin phenol reagent. Biol. Chem. J. 193:265-275.

Mathivana N, Kabilan V, Murugesan K (1997). Production of chitinase by *Fusarium clamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*, *Indian.* Exp. Biol. J. 35:890-893.

Merriman PR, Price RD, Kollmorgen JF (1974). Effect of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. Aust. J. Agric. Res. 252:19-226.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31:426-428.

Nagahama H, New R, Jayakumar S, Koiwa T, Furuike H, Tamur J (2008). Novel biodegradable chitin membranes for tissue engineering applications. Carbohydr. Polym. J. 73:295-302.

Nawani NN, Kapadnis BP, Das AD, Rao AS, Mahajan SK (2002). Purification and characterization of a thermophilic and acidophilic chitinase from *Microbispora* sp. V2. Appl. Microbiol. J. 93:965-975.

Pascual E, Julia MR (2001). The role of chitosan in wool finishing, Biotechnol. J. 89:289-296.

Peberdy JF (1990). Fungal cell walls. A review,. In PJ Kuhn, APJ Trinci, MJ Jung, MW Goosey, and LG Copping (ed.), Biochemistry of cell walls and membranes in fungi. Springer, London pp. 5-30.

Prapagdee B, Kuekulvong C, Mongkolsuk S (2008). Antifungal Potential of Extracellular Metabolites Produced by *Streptomyces hygroscopicus* against Phytopathogenic Fungi. Int. Boil. Sci. J. 4(5):330-337.

Saksirirat W, Hoppe HH (1991). Secretion of extracellular enzymes by *Verticillium psalliotae* and *Verticillium lecanii* during growth on uredospores of the soybean rust fungus in liquid cultures. Phytopathol. J. 131:161-173.

Sakurada M, Morgavi DP, Komatani K, Tomita T, Onodera R (1996). Purification and characterization of cytosolic chitinase from *Piromyces* communities OTS1. FEMS Microbiol. Lett. J. 137:75-78.

San-Lang W, Ing-Lung S, Tze-Wun L, Chi-Hau W (2002). Purification and Characterization of Two Antifungal Chitinases Extracellularly Produced by *Bacillus amyloliquefaciens* V656 in a Shrimp and Crab Shell Powder Medium. J. Agric. Food Chem. 50:2241-2248.

Shirling EB, Gottlieb D (1966). Methods for characterization of *Streptomyces* species. Int. Syst. Bact. J. 16:313-340.

Steven AC, Michael M, Thomas LT (1989). Manual of advanced techniques for amino acid analysis the Pico-Tago Method, Millipore co-operation. Printed in U.S.A, 4189 WMO_2, Rev. I, and USA.

Thiagarajan V, Revathia R, Aparanjinib K, Sivamanic P, Girilala M, Priyad CS, Kalaichelvan PT (2011). Extracellular chitinase production by *Streptomyces* sp. PTK19 in submerged fermentation and its lytic activity on *Fusarium oxysporum* PTK2 cell wall. Int. J. Curr. Sci. 1:30-44.

Tracey MV (1957). Chitin. Rev. Pure Appl. Chem. J. 7:1-14.

Ueno H, K Miyashita, Y Sawada, Y Oba (1990). Purification and some properties of extracellular chitinases from *Streptomyces* sp. S-84. Gen. Appl. Microbiol. J. 36:377-392.

Ulhoa CJ, Peberdy JF (1992). Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. Enzyme Microb. Technol. J. 14:236-240.

Vaidya RJ, Shah IM, Vyas PR, Chhatpar HS (2001). Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential in antifungal biocontrol. World J. Microbiol. Biotechnol. 17:691-696.

Vanderzant C, Splittstoesser D (1992). Compendium of methods for the Microbiological Examination of Foods, 3rd ed. Washington, D.C.: American Public Health Association.