EXPRESSION OF CHITINASE GENE FROM *Bacillus Licheniformis* DSM13 IN *E.Coli* T7 AND BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT ENZYME

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ABSTRACT

In this study, the mature gene encoding for the enzyme endochitinase from the Gram positive bacterium *Bacillus licheniformis* DSM13 (ATCC 14580) was cloned into the expression vector pET-21d, and then overexpressed in *E. coli* T7. A high expression level was indicated by SDS-PAGE and an enzyme activity assay using pNP-$(GlcNAc)_2$ 0.18 mM as the substrate. The activity of the enzyme expressed in *E. coli* T7 was approximately 8 times higher than that previously expressed in *Lactobacillus plantarum*. Recombinant chitinase was purified from cell extract and characterized using coloidal chitin 2% as the substrate. The enzyme showed good thermostability (half-lives of 15 days and 8 days at 37 and 50°C, respectively), and good stability in the pH range of 5 - 9. The main product of colloidal chitin hydrolysis as indicated by thin layer chromatography was diacetyl glucosamine. The results demonstrated that this enzyme was promising for chitin waste bioconversion into different chitin-oligosaccharides, such as functional diacetyl glucosamine.

Keywords: *Bacillus licheniformis* DSM13, chitinase, *E. coli* T7.

Biểu hiện gene mã hóa chitinase từ *Bacillus licheniformis* DSM13 trong *E.coli* T7 và xác định đặc tính của enzyme tái tổ hợp

TÓM TẮT

Trong nghiên cứu này, gene mã hóa cho enzyme endochitinase của vi khuẩn gram dương *Bacillus licheniformis* DSM13 (ATCC 14580) được chuyển vào vectơ biểu hiện và biểu hiện trong tế bào *E. coli* T7. Mức độ biểu hiện cao của enzyme được thể hiện qua SDS-PAGE và hoạt tính của enzyme khi sử dụng pNP-$(GlcNAc)_2$ 0,18 mM là cơ chất. Hoạt tính của enzyme được biểu hiện trong *E.coli* T7 là cao khoảng gấp 8 lần so với enzyme biểu hiện ở *Lactobacillus plantarum* (nghiên cứu trước). Chitinase tái tổ hợp được tinh sạch và xác định đặc tính bằng cách sử dụng chitin 2% là cơ chất. Kết quả chỉ ra rằng, enzyme tái tổ hợp bền nhiệt (enzyme còn lại một nửa hoạt tính sau 15 và 8 ngày ủ ở 37 và 50°C tương ứng), bền ở giải pH từ 5 - 9. Sản phẩm chính thủy phân chitin huyền phù được xác định bằng phương pháp sắc ký bản mỏng là diacetyl glucucosamine. Các kết quả nghiên cứu này chỉ ra rằng enzyme tái tổ hợp này có tiềm năng trong việc ứng dụng sản xuất chitin-oligosaccharide, như là diacetyl glucucosamine chức năng từ việc chuyển hóa phế phụ phẩm chitin

Từ khóa: *Bacillus licheniformis* DSM13, chitinase, *E.coli* T7.

1. INTRODUCTION

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyze the hydrolytic degradation of chitin, which is an insoluble linear *β*-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc), and is the second most abundant polysaccharide in nature after cellulose (Kurita, 2001). Chitin is widely distributed in nature and forms a major constituent of the shells of crustaceans such as crabs, shrimps, lobster, and squid, which are readily available in huge

amounts in countries with a strong aquaculture industry such as Vietnam (Nguyen *et al.*, 2011). This waste material causes environmental pollution if not processed and disposed of properly, but could serve as a valuable chitin source for the production of chitin-oligosaccharide functional food ingredients.

Chitinases have been purified from many sources and their enzymatic activities have been investigated. This includes plants, fungi, yeasts, bacteria, insects, and even vertebrates (Clarke and Tracey, 1956; Hsu and Lockwood, 1975; Htakara *et al.*, 1979; Kramer and Koga, 1986; Hearn *et al.*, 1996; Kasprzewska, 2003; Ajit *et al.*, 2006; Akagi *et al.*, 2006). Chitinase from *Bacillus* has received considerable attention as it often has high activity and thermostable characteristics, especially *Bacillus licheniformis* (Barboza-Corona *et al.*, 1999; Toharisman *et al.*, 2005; Yamabhai *et al.*, 2008; Songsiriritthigul *et al.*, 2010;). The complete genome of *Bacillus licheniformis* DSM 13 has been elucidated and was found to contain a number of genes coding for polysaccharide-degrading enzymes including a gene for chitinase (glycoside hydrolase family GH18) with the GenBank accession number AAU21943 (Veith *et al.*, 2004). This gene has been cloned and expressed in *Lactobacillus plantarum* using the pSIP vector, and the recombinant chitinase was purified and characterized. Results indicated that this chitinase was a promising enzyme for the production of chitin-oligosaccharides (Nguyen *et al.*, 2011). However, the main drawback was the fact that the expression level of the enzyme was quite low (*ca.* 5 mg of recombinant protein per liter of cultivation medium), and thus, it is impossible to apply in industrial scale.

The aim of this study was to enhance the expression of the chitinase gene (AAU21943) from *B. licheniformis* DSM13 by using the pET-21d expression vector in *E. coli* T7. The recombinant enzyme was also purified and characterized in order to find the effect of the new expression system on enzyme characteristics.

2. MATERIALS AND METHODS

2.1. Enzymes, substrates and chemicals

Restriction enzymes and T4 DNA ligase were supplied by Fermentas (St. Leon-Rot, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was from Roth (Karlsruhe, Germany). Chromogenic substrates, *p*NP-$(GlcNAc)_2$, and chitin were purchased from Sigma Aldrich (St. Louis, MO). Diacetyl chitobiose was purchased from Megazyme (Bray, Ireland). All other chemicals were of reagent grade and obtained from commercial sources.

2.2. Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Roberts and Selitrennikoff (1988) with slight modifications. Briefly, 5 g of chitin from crab shells (C7170, Sigma Aldrich) was gradually added into 100 ml of cold concentrated HCl with gentle agitation on a magnetic stirrer for 18 h at 4°C. The mixture was then added to 500 ml of ice-cold 96% ethanol and left for 24 h with rapid stirring at 4°C. The precipitate was harvested by centrifugation at 8000 g for 20 min at 4°C and washed repeatedly with sterile distilled water until the pH reached 6. The colloidal chitin was kept at 4°C until further use. Approximately 95 ± 4 g of colloidal chitin was obtained by this procedure from 5 g of chitin powder.

2.3. Bacterial strains, plasmids, and media

Bacillus licheniformis DSM13 (=ATCC 14580, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown in M1 medium at 37°C with shaking at 125 rpm. Genomic DNA was extracted using the DNA Isolation Kit (Norgen, Thorold, Canada). Plasmid pET-21d (Novagen, Merck, Darmstadt, Germany) was used as the expression vector. *E. coli* T7 One Shot Chemically Competent (Invitrogen, Carlsbad, CA) was used as the expression host. *E. coli* T7 carrying pET-21d with the gene of interest was grown in LB broth with ampicillin (100 µg/ml) for expression of the recombinant protein.

2.4. Cloning and expression of the chitinase encoding gene

A forward primer (GCGGCCATGGATTCCGGAAAAAACTAT) and a reverse primer (TAATCTCGAGTTCGCAGCCTCCGATCAGCC) containing *Nco*I and *Xho*I recognition sites (underlined), respectively, were designed based on the sequence of a gene encoding a chitinase from *Bacillus licheniformis* with the GenBank accession No. AAU21943 (Veith *et al.*, 2004). Amplification conditions for a 25 µl standard PCR reaction were as follows: 1 cycle at 98°C for 3 min; 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min; and an extra extension at 72°C for 5 min for the final cycle. The amplification product was purified from an agarose gel using the Wizard SV Gel PCR Cleanup system (Promega, Madison, WI) and digested with *Nco*I and *Xho*I before directly being cloned into the *Nco*I and *Xho*I sites of pET-21d. Insertion of the amplicon resulted in translational fusion of the chitinase open reading frame with the vector-encoded 6x His-tag (N-terminal tag). Plasmids were transformed into *E. coli* T7. Positive colonies were confirmed by colony PCR and DNA sequencing. After culturing, cells were stored in 30% glycerol at -80°C.

Overnight cultures of *E. coli* T7 harboring pET-21d with the chitinase gene were used to inoculate 250 mL of fresh LB medium supplemented with 100 µg/mL of ampicillin. The cultures were incubated at 37°C and 200 rpm until the OD_{600} reached 0.5. IPTG was added to the cultures to a final concentration of 0.4 mM. Post-induction cultivation was done at 18°C and 25°C, 200 rpm for 16 hours. The cells were harvested by centrifugation (6000 rpm, 20 min, 4°C) and washed two times with 50 mM sodium phosphate buffer pH 6. The biomass was stored at -20°C for protein purification

2.5. Protein purification

The cells were disrupted 3 times in a French press (Amicon, Jessup, MD) in 50 mM sodium phosphate buffer, pH 6. A cell free lysate was obtained by ultracentrifugation at 30,000 rpm for 30 min at 4°C. Protein purification was done by immobilized metal affinity chromatography (IMAC), as follows: 10 mL of crude protein extract was loaded onto a 15-mL column of Profinity IMAC Ni-Charged Resin (BioRad, Hercules, CA) pre-equilibrated with buffer A (Na_2HPO_4 20 mM, NaCl 0.5 M, imidazol 20 mM, pH 6.5). After washing the column with two column volumes of buffer A, the enzyme was eluted with a flow rate of 0.5 mL/min using a gradient from 0% to 100% of buffer B (Na_2HPO_4 20 mM, NaCl 0.5 M, imidazol 0.5 M, pH 6.5). Fractions containing the highest enzyme activity, detected using pNP-$(GlcNAc)_2$ as the substrate, were pooled, and then the buffer was exchanged to 50 mM sodium phosphate buffer pH 6.0. The samples were concentrated by 10 kDa cutoff Amicon Ultra Centrifugal filter tubes (Millipore, Billerica, MA). The purified enzyme was stored in 50 mM sodium phosphate buffer pH 6.0 at 4°C and used for characterization

2.6. Protein electrophoresis and molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (Laemmli *et al.*, 1970) was performed to verify the purity of the enzyme preparations and to determine the molecular mass of the enzyme using 10% gels and a PerfectBlue™ vertical electrophoresis system (Peqlab, Erlangen, Germany).

2.7. Enzyme assays and protein determination

Standard enzyme assay using pNP-$(GlcNAc)_2$ as substrate

Enzyme activity was determined using p-NP-chitobiose (pNP-$(GlcNAc)_2$) as substrate. The assay was performed in principle as described by Yamabhai *et al.* (2008) with some modifications. The reaction was initiated by adding 20 µl of enzyme solution to 100 µl of 0.18 mM pNP-$(Glc\text{-}NAc)_2$ in 50 mM sodium phosphate buffer pH 6, and then the mixture

was incubated for 30 min at 37°C and 600 rpm using an Eppendorf thermomixer. After incubation, the reaction was stopped by adding 480 µl of 0.5 M Na_2CO_3. p-Nitrophenol liberated during the reaction was determined spectrophotometrically at 405 nm. One unit of enzyme is defined as the amount of the enzyme releasing 1 µmol of p-nitrophenol per minute under the given conditions.

Enzyme assay using colloidal chitin as substrate

The reaction mixture consisted of 250 µl of a 2%-solution of colloidal chitin in 50 mM sodium phosphate buffer pH 6 and 250 µl of appropriately diluted enzyme solution. After incubation for 30 min at 37°C and 600 rpm on a Thermomixer Compact (Eppendorf; Hamburg, Germany), the reaction was stopped by heating it at 100°C for 10 min and then centrifuged at 10,000 rpm for 5 min. The concentration of reducing sugars in the supernatant was determined based on the dinitrosalicylic acid (DNS) method using Glc-NAc as the standard as described previously (Miler *et al.*, 1959). One unit of chitinase activity was defined as the amount of enzyme releasing one 1 µmol of reducing sugar per minute under the specified assay conditions. This assay was used as the standard enzyme assay for the characterization of recombinant chitinase.

2.7. Protein determination

Protein concentration was determined according to Bradford (1976) using the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard.

2.8. Effect of temperature and pH on enzyme activity

The temperature optimum of the recombinant enzyme was determined using standard assay conditions with 2% colloidal chitin as the substrate in the temperature range from 20°C to 90°C. The thermal stability of the enzyme was studied by incubating the purified enzyme in 50 mM sodium phosphate buffer pH 6.0 at 37°C and 50°C. At certain time intervals, samples were taken and the residual activity was measured with colloidal chitin as the substrate under standard assay conditions.

The pH optimum was determined by the standard assay with 2% colloidal chitin in the pH range from 4 to 10 using Britton-Robinson buffers (20 mM sodium citrate, 20 mM sodium phosphate, and 20 mM borate, adjusted to the required pH with NaOH).

To determine the stability of chitinase at different pH, the purified enzyme was incubated at 37°C in Britton-Robinson buffers with various pH values. The remaining enzyme activity was measured at different time intervals using 2% colloidal chitin as the substrate under standard assay conditions.

2.9. Analysis of hydrolysis products

Chitinase-catalyzed hydrolysis of di-N-acetyl chitobiose $(GlcNAc)_2$ was followed by incubating 100 µl of a 10 mM-solution of the respective substrate in 50 mM sodium phosphate buffer pH 6 with 4 mU of purified enzyme, using an Eppendorf thermomixer set at 37°C and 600 rpm. Samples (10 µl) were taken at various time points and chitinase was inactivated by incubating samples at 100°C for 5 min.

Hydrolysis of colloidal chitin was studied using the reaction mixtures (960 µl) containing 2 or 20% of colloidal chitin together with 80 mU of purified chitinase. These mixtures were incubated at 37°C, 600 rpm using an Eppendorf thermomixer. Samples (20 µl) were taken regularly and the enzyme was inactivated as described above.

The products released by the chitinase from these carbohydrate substrates were analyzed using thin layer chromatography (TLC) based on methods of Rauvolfová *et al.* (2004). Aliquots (1 µl) of the reaction mixtures were loaded onto high-performance TLC silica plates (Kieselgel 60 F245, Merck) and run against a mobile phase of isopropanol/water/28% ammonia (7:2:1, v:v:v). Plates were dried and sprayed with 5% H_2SO_4 in ethanol, followed by baking at 220°C in an oven for 10 min to develop the spots on the TLC plates.

3. RESULTS

3.1. Overexpression of a chitinase-encoding gene from *Bacillus licheniformis* DSM13 in *E. coli* T7

The chitinase gene of *Bacillus licheniformis* DSM13 was successfully cloned into pET-21d and the protein was expressed in *E. coli* T7 driven by the T7 RNA polymerase promoter. Cells from a 250-mL culture induced with 0.4 mM IPTG at both 18°C and 25°C for 16 hours were disrupted and checked for gene expression level by using SDS-PAGE (Figure 1), enzyme activity using pNP-$(GlcNAc)_2$ 0.18 mM as a substrate, and specific activity (Table 1).

The presence of a large protein band with a molecular weight of approximately 65 kDa (Figure 1) indicated that the gene encoding for chitinase from *Bacillus licheniformis* DSM13 was successfully overexpressed in *E. coli* T7 using pET-21d. Volumetric activity and specific activity of the enzyme expressed at 18°C were 12.90 U/L and 0.97 U/mg, respectively, and the numbers were approximately two times higher than those obtained at 25°C (Table 1). These enzyme activity data agree well with the protein band intensities obtained by SDS-PAGE (Figure 1). These results also indicated that protein inclusion bodies might have formed during protein expressing at 25°. The results presented in Figure 1 and Table 1 also demonstrated that chitinase expressed in *E. coli* at 18°C (12.9 U/l) was approximately 8 times higher than that reported previously by Nguyen *et al.* (2011) in *L. plantarum* (1.56 U/l). Chitinase obtained at 18°C was used for further purification and characterization.

3.2. Purification of recombinant chitinase

Cells from a 250-mL culture induced with 0.4 mM IPTG and grown for 16 hours at 18°C after induction were disrupted, and chitinase was purified from the cell free extract by one-step IMAC. The procedure yielded 68.2% chitinase recovery (Table 2). Typically, close to 20 mg of pure recombinant enzyme was obtained from 1 L of culture with a total activity of around 8.8 U and a specific activity of about 0.44 U/mg of protein.

3.3. Characterization of purified recombinant chitinase

Effect of temperature and pH on the activity and stability of purified enzyme.

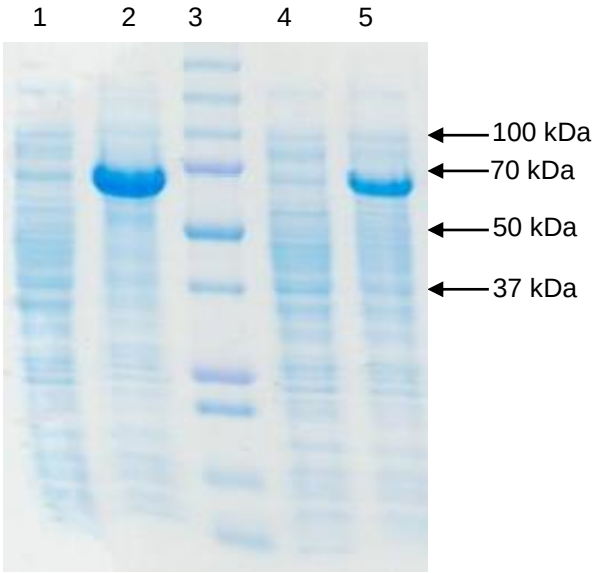

Figure 1. SDS-PAGE of chitinase expressed in *E. coli* T7 at 18°C and 25°C

Note: 1. Non-induced 18°C; 2. Induced 18°C by 0.4mM IPTG at OD 0.5; 3. Ladder; 4. Non-induced 25°C; 5. Induced 25°C by 0.4mM IPTG at OD 0.5

Table 1. Chitinase activity in *E. coli* T7 induced by the addition of 0.4 mM IPTG at OD 0.4 - 0.5. Chitinase activity was measured using pNP-$(GlcNAc)_2$ as substrate

Temperature of expression	Non-induced/induced by IPTG 0.4mM	Specific activity (U/mg)	Volumetric activity (U/L)
18°C	Non-induced	0.020	0.97
	Induced	0.240	12.90
25°C	Non-induced	0.002	0.19
	Induced	0.120	7.20

Table 2. Purification of recombinant chitinase from *B. licheniformis* DSM13 in *E. coli* T7 at 18°C (from 1 L of LB culture broth)

Purification steps	Total Activity[(a)] (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	12.9	54	0.24	
Purified enzyme	8.8	20	0.44	68.2

Note: [a]pNP-$(GlcNAc)_2$ was used to determine enzyme activity

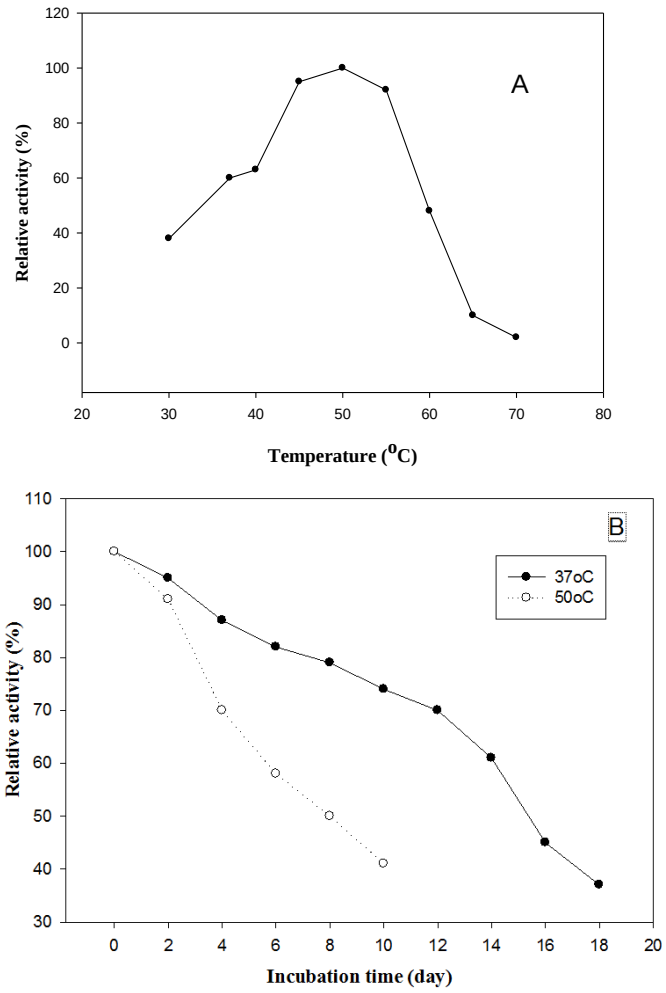

Figure 2. Effect of temperature on activity (A) and stability (B) of recombinant chitinase from *B. licheniformis* DSM13

The optimal temperature of the purified recombinant chitinase was 50°C when using 2% colloidal chitin as a substrate for a 30-min assay (Figure 2A). After 8 days of incubation at 37°C and 50°C, the enzyme still remained 80 and 50% of initial activities, respectively (Figure 2B). The results showed that the enzyme has good thermostability (half-lives of 15 days and 8 days at 37°C and 50°C, respectively), and good stability in the pH range from pH 5 to pH 9.

Figure 3 indicates that the pH optimum of recombinant chitinase is in the range of 7 - 8 and highest at pH 7.5 (Figure 3A). The enzyme was very stable at pH 6, 7, and 8 (remained over 60% of activity after 15 days of incubation).

These results were comparable with that described by Nguyen *et al.* (2012), in which the chitinase gene was cloned and expressed in the expression system of *Lactobacillus plantarum*. The characteristics of the recombinant chitinase were not significantly different when expressed in *E. coli* or *L. plantarum*.

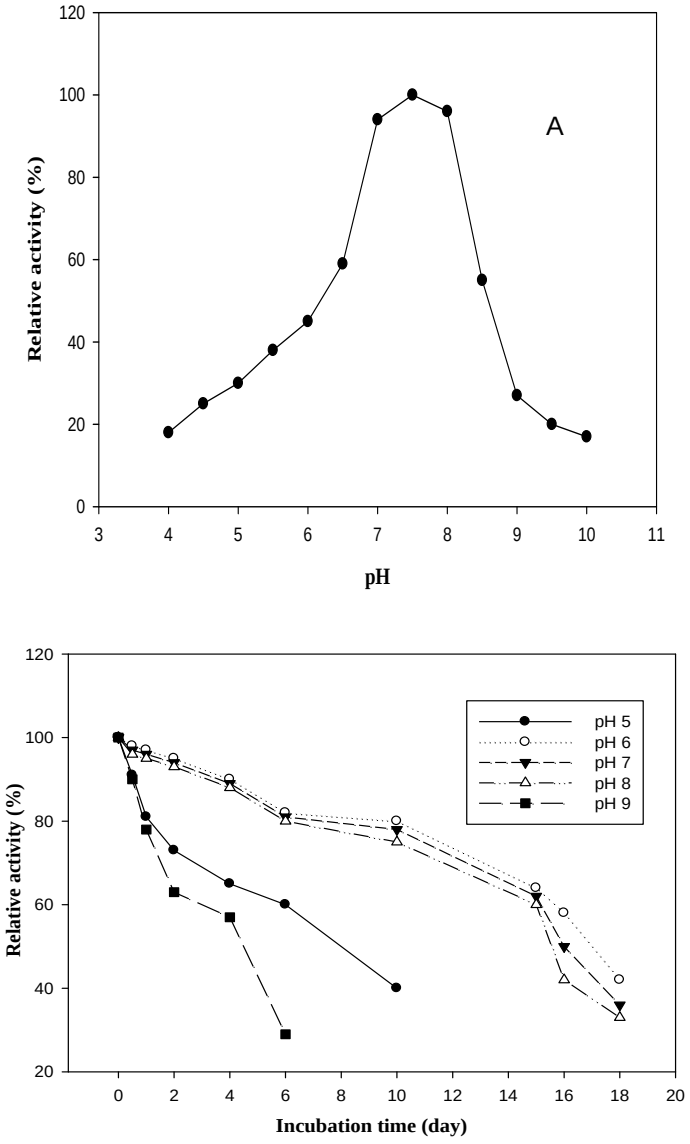

Figure 3. Effect of pH on activity (A) and stability (B) of recombinant chitinase from *B. licheniformis* DSM13

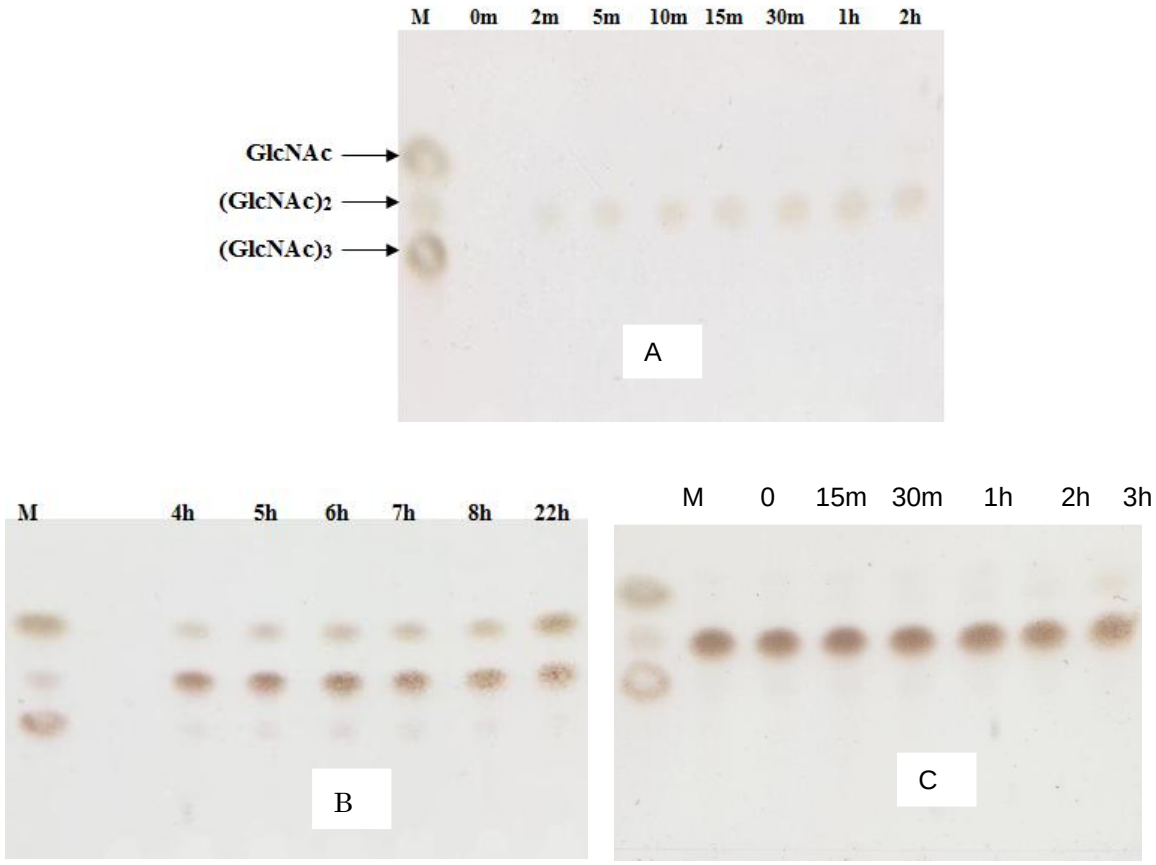

Figure 4. Thin layer chromatography showing colloidal chitin hydrolysis products of purified recombinant chitinase using 2% colloidal (A), 20% coloidal chitin (B), and 10 mM diacetyl chitobiose (C) as substrates, M: a standard mixture of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$

Analysis of hydrolysis products using TLC

The products of the chitinase-catalyzed hydrolysis of colloidal chitin (2% and 20%) and di-acetyl chitobiose were studied by thin layer chromatography. The main hydrolysis product of colloidal chitin was diacetyl chitobiose (Figures 4A and 4B), while pure di-acetyl chitobiose apparently was not hydrolyzed within 8 h under the conditions selected (Fig. 4C). This result was quite similar to that studied by Nguyen *et al.* (2012).

4. CONCLUSIONS

Recombinant chitinase activity expressed in *E. coli* T7 using pET-21d was approximately 8 times higher than previously reported in *L. plantarum*. The characteristics of recombinant enzymes expressed by these two systems were almost identical. The recombinant enzyme was stable at both 37°C and 50°C, and in the pH range of 5 - 9. The main hydrolysis product was di-acetyl chitobiose. The recombinant chitin from *B. licheniformis* DSM 13 expressed in *E. coli* T7 is promising for the production of functional diacetyl-chitooligosaccharides from chitin-rich aquaculture byproducts.

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REFERENCES

Ajit, N. S., Verma, R., Shanmugam, V. (2006). Extracellular chitinases of fluorescent pseudomonads antifungal to *Fusarium oxysporum* f. sp. *dianthi* causing carnation wilt. Curr. Microbiol., 52: 310 - 316.

Akagi, K., Watanabe, J., Hara, M., Kezuka, Y., Chikaishi, E., Yamaguchi, T., Akutsu, H., Nonaka, T.,Watanabe, T.,Ikegami, T. (2006). Identification of the substrate interaction region of the chitinbinding domain of *Streptomyces griseus* Chitinase C. J. Biochem., 139: 483 - 493.

Barboza-Corona, J. E., Contreras, J. C., Velazquez-Robledo, R., Bautista-Justo, M., Gomez-Ramirez, M., Cruz-Camarillo, R., Ibarra, J. E. (1999). Selection of chitinolytic strains of *Bacillus thuringiensis*. Biotechnol. Lett., 21: 1125 - 1129.

Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem., 72: 248 - 254.

Clarke, P. H., and Tracey, M. V. (1956). The occurrence of chitinase in some bacteria. J. Gen. Microbiol., 14: 188-196.

Hearn, V. M., Escott, G. M., Evans, E. G. V., Adams, D. J. (1996). Chitinases of the cell surface and wall of *Aspergillus fumigatus*. Chitin Enzymology, 2: 261-271.

Hsu, S. C., and Lockwood, J. L. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl. Microbiol., 29: 422-426.

Kasprzewska, A. (2003). Plant chitinases- regulation and function- a review. Cell. & Mol. Bio. Lett., 8: 809-824.

Kramer, K. J., and Koga, D. (1986). Insect chitin: physical state, synthesis, degradation, and metabolic regulation. Insect Biochem.,16: 851 - 877.

Kurita, K. (2001). Controlled functionalization of the polysaccharide chitin. Prog. Polym. Sci., 26: 1921-1971.

Laemmli, U. K. (1970). Cleavage of structural proteins during assemblyof head of bacteriophage T4. Nature., 227: 680-685.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Nguyen HA, Nguyen TH, Nguyen TT, Peterbauer CK, Mathiesen G, Haltrich D: Chitinase from *Bacillus licheniformis* DSM13 (2011). Expression in *Lactobacillus plantarum* WCFS1 and biochemical characterisation. J. Protein expression and Purification, 59(10): 5617-5624.

Ohtakara, A., Mitsutomi, M., Uchida, Y. (1979). Purification and some properties of chitinase from Vibrio sp. J. Fermet. Technol., 57: 169-177.

Rauvolfová, J., Kuzma, M., Weignerová, L., Fialová, P., Prikrylová, V., Pisvejcová, A., Macková, M., Kren, M. (2004). β-N-acetylhexosaminidase-catalysed synthesis of nonreducing oligosaccharides. J. Mol. Catal. B. Enzyme, 29: 233-239.

Roberts, W.K., Selitrennikoff, C. P. (1998). Plant and bacterial chitinases differ inantifungal activity. J. Gen. Microbiol., 134: 169-176.

Songsiriritthigul, C., Lapboonrueng, S., Pechsrichuang, P., Pesatcha, P., Yamabhai, M., (2010). Expression and characterization of *Bacillus licheniformis chitinase* (ChiA), suitable for bioconversion of chitin waste. Bioresour. Technol., 101: 4096-4103.

Toharisman, A., Suhartono, M.T., Spindler-Barth, M., Hwang, J.K., Pyun, Y.R. (2005). Purification and characterization of a thermostable chitinase from *Bacillus licheniformis* Mb-2, World J. Microb. Biotechnol., 21: 733-738.

Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K.H., Ehrenreich, P., Baumer, S., Henne, A., Liesegang, H., Merkl, R., Ehrenreich, A., Gottschalk, G. (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential, J. Mol. Microbiol. Biotechnol., 7: 204-211.

Yamabhai, M., Emrat, S., Sukasem, S., Pesatcha, P., Jaruseranee, N., Buranabanyat, B. (2008). Secretion of recombinant Bacillus hydrolytic enzymes using *Escherichia coli* expression systems. J. Biotechnol., 133: 50-57.