Genetics

Purification and characterization of chitinase secreted by *Pseudoalteromonas* sp. DXK012 isolated from deepsea sediment

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With the chitin as the sole carbon source, a chitinase-producing strain DXK012 was isolated from the deepsea sediment of 4826 m depth. The strain DXK012 belonged to *Pseudoalteromonas*, showing the closest phylogenetic affinity to *Pseudoalteromonas arabiensis* k53(T) (99.8% sequence similarity) and the stain was deposited with the number of CCTCC AB 2013143^T. Cells were Gram-negative, with the optimum growth at 37℃, pH 7.5 and with the presence of 3% (w/v) NaCl. The chitinase from *Pseudoalteromonas* sp. DXK012 was extracellular enzyme and purified to homogeneity by ultrafiltration, DEAE-cellulose and Sephadex G-100 column chromatography. Molecular weight of purified chitinase was estimated to be ~36 kDa by SDS-PAGE. The optimal temperature and pH for the chitinase activity was 40℃ and 7.5, respectively. It acted on the GlcNAc-GlcNAc bonds, probably the GlcN-GlcNAc or GlcN-GlcN bonds. Mg^{2+} had positive effect on the enzyme activity, other metals inhibited the enzyme activity. In addition, the enzyme activity was inhibited by DTT, β-Mercaptoethanol (β-Me) and EDTA. Oligosaccharides mainly between hexose and octose were produced when chitin was hydrolyzed by this chitinase. The enzyme could be potentially used in industrial applications because of its interesting characteristics. *Journal of Nature and Science, 1(2):e37, 2015.*

Chitinase | Deepsea | Enzymatic properties | Chito-oligosaccharide | *Pseudoalteromonas*

1. Introduction

Chitin, the linear homopolymer of β-(1-4) linked N-acetyl-D-glucosamine (GlcNAc) and the major structural polysaccharide in insect exoskeletons, shells of crustaceans and fungal cell walls, is the second-most abundant biopolymers on earth with an annual production of 10^{10}-10^{11} tons per annum (Wang et al. 2008), which is only next to that of cellulose(Chang et al. 2003). The application of chitin is difficulty cause it can just dissolve in concentrated HCl, phosphoric acid, or HF but not in water, dilute acid or organic solvent. The catabolism of chitin typically occurs in two steps involving the initial cleavage of the chitin polymer by endochitinase (EC 3.2.1.14) or exochitinase (EC 3.2.1.29) into chitin oligosaccharides, and then further cleavage to β-N-acetylglucosamine monomers by chitobiase (Draborg et al. 1996, Watanabe et al. 1999)，Chitin degradation products have an important role in Antitumor effect, bacteriostasis, moisture, immunoregulation and improving plant defense, which has various potential application in pharmaceutical, agricultural, biomedical and food field (Rattanakit et al. 2007, Tsai et al. 2000, Zhang et al. 2011). Compared with enzymolysis approach and chemical degradation method, enzymolysis approach has many advantages in specificity, product stability and controllability and environmentally friendly, so it will be the preferred method in the industrial application (Wang et al. 2006).

Chitinases are types of enzymes that hydrolyze chitin by cleaving its β-1,4 N-glycosidic bond (Fujita et al. 2006) generating soluble chitooligosaccharides of low mass multimers (Howard et al. 2003). Many microorganisms producing chitinase have been isolated after *Bcillus chitinovirous* identified by Benecke (W, 1995) for first time. Recently almost 100 stains in 50 genera producing chitinase distributed in actinomycetes, fungi, bacteria and other classes have been isolated and identified. Researchers have taken amount of work on characteration of chitinases and genes of chitinase (Barboza-Corona et al. 2003, Kang et al. 1999, Omumasaba et al. 2000, Songsiriritthigul et al. 2010a, Zhong et al. 2003), however, There hasn't been any report about *Pseudoalteromonas* of deepsea producing chitinase except *Pseudoalteromonas* sp. strain S9 from marine environment (Techkarnjanaruk et al. 1997) so far. In this paper *Pseudoalteromonas* sp. DXK012 from the deepsea sediment excreting extracellular chitinase was isolated and morphological observation, physiology-biochemistry tests and 16S rRNA gene sequence analysis have been conducted. The characterization of this chitinase and its degradation products had been studied, which provided theoretical basis for production and application.

2. Materials and methods

2.1. Sample, powder of shrimp and crab shell and media

The deepsea sediment was from China DaYang YiHao exploration. The sun-cured leftover shrimp and crab shell (ratio (g/g)=1:1) was crushed into 60mesh to obtain shrimp and crab shell powder(sacp) stored at 4 ℃ for use. Media: enrichment medium (5 g sacp in 100 mL seawater), screening medium (5 g colloidal chitin, 1.5 g agar for solid medium in 100 mL seawater), medium used for chitinase production (5 g sacp chitin, 0.2 g yeast extract, 1.5 g agar for solid medium in 100 mL seawater).

2.2 Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Songsiriritthigul, C(Songsiriritthigul et al. 2010b) with some modification. Twenty grams of chitin flakes (Sangon Biotech, Shanghai, China) were mixed with 200 ml concentrated HCl at RT with vigorous stirring for 4-5 h and continued incubation overnight. Afterwards, the mixture was filtered through glaswolle (New Jersey, USA) and dropped slowly into 2 L of 50% ice-cold ethanol with rapid stirring. Then, the colloidal chitin was collected by centrifugation at 8000 g, for 30 min at 4 ℃ and washed several times with double distilled water until the pH was neutral (pH 7.0).The colloidal chitin was kept at 4 ℃ for use.

2.3 Isolation and screening of chitinase-producing strains

One gram of the deepsea sediment sample was inoculated into the enrichment medium, cultured in 50/250ml shaking flasks at 37℃ and 150 rpm for 7 days. approximately 100 μL supernate was diluted by using a tenfold dilution series method with sterilized seawater. After incubation for 3-5 days at 25℃, large and transparent colonies were picked out and purified into pure culture, deposited at -80℃ for use.

2.4 Identification of the isolate and phylogenetic analysis based on 16S rRNA gene sequence

Genomic DNA was extracted using a genomic DNA extraction kit (Bioteke, Beijing, China). The 16S rRNA gene sequence was

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amplified by PCR with universal primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-AAGGAGGTGATCCAGCC-3')(Wilson *et al.*, 1990). DNA sequencing was performed by Sangon Biotech (Shanghai, China).

The 16S rRNA gene sequences of the related type strains were obtained from the EzTaxon-e(Kim et al. 2012). A neighbor-joining tree and a maximum-parsimony were constructed using MEGA 5.1(Tamura et al. 2011), Bootstrap analysis was used to evaluate the tree topology of the NJ data, performing 1,000 replicates and marked into branching points. The evolutionary distance matrix was estimated using the Kimura's 2-parameter model(Jukes 2000).

2.5 Phenotypic and biochemical characteristics of *Pseudo-alteromonas* sp. DXK012

Gram staining was conformed using the standard reaction and was performed by using the KOH test(Martinez-Checa *et al.*, 2005). Cellular morphology was surveyed using the optical microscope and transmission electron microscopy in the period of logarithmic phase. Colony morphology was observed on solid enzyme-producing medium after incubation at 37 ℃ for overnight. Growth at different temperatures (between 4 ℃ and 40 ℃), pH range (between pH 4.0 and 10.0 at intervals of 1.0 pH unit), and NaCl concentrations [0 -15% (w/v)] were determined after 1-2 days of incubation at 37 ℃.

2.6 Preparation of the crude extracellular chitinase, purification of chitinase and SDS-PAGE

To prepare the crude extracellular enzyme, the DXK012 strain was cultured at 37℃ for 48h in 50/250 mL shake flask of liquid chitinase-producing medium. Then the cultures were centrifuged at 12000g for 10 min at 4℃, the supernatant was the crude enzyme solution and stored in aliquots at -20℃ before use. 500 mL of the supernatant mentioned above was concentrated to 4mL using ultrafiltration membrane having 10 kDa molecular mass cut off value according to instruction of Amicon Ultra (USA). The concentrated crude chitinase was dialyzed against 2.5 L of 10 mM citrate phosphate buffer (pH 7.0) with 3-4 changes at the intervals of 6 h. The sample was then centrifuged for 10 min at 14000×g and the supernatant was loaded on DEAE cellulose column (6.5 ×2.0 cm) equilibrated with 10 mM citrate phosphate buffer (pH 4.0). The protein was eluted stepwise using 10 mL of NaCl (0.2–1.0 M) in the same buffer at the flow rate of 18 mL h^{-1}. Fractions of 3.0 mL were collected and analyzed for activity for the chitinase. The pooled fractions from DEAE cellulose showing maximum activity were concentrated with sucrose and dialyzed against 2.0 L of 100 mM citrate phosphate (pH 7.0) buffer, The dialysed chitinase was centrifuged at 14000 g and the supernatant was loaded on a Sephadex G-100 column (1.0 ×30.0 cm), preequilibrated with the same buffer. The flow rate was maintained at 9.0 ml/h and fractions of 1.5 mL were collected and analyzed for protein and PNL activity. the active fractions were pooled, concentrated and tested for homogeneity by electrophoresis.

2.7 Enzyme assay

Unless indicated otherwise, the colloidal chitin was used as the substrate in the purified enzyme assay. The reaction mixture contained 1 mL of 1% soluble colloidal chitin (pH 7.5 in sodium phosphate buffer) and 1 mL of purified enzyme solution. The incubation was carried out at 40 ℃ for 30 min in water bath. The amount of reducing sugar in the supernatant was measured using the modified dinitrosalicyclic acid (DNS) method(Miller 1959). One enzyme unit was defined as the amount of enzyme required to produce 1μmol of reducing sugar as glucosamine per min.

2.8 Characterization of the crude enzyme

2.8.1 Effect of pH and temperature on the crude

Temperature effects were studied at 50 mM sodium phosphate buffer (pH 7.5) in the range of 10~70℃. The pH effects were determined in the range of pH 4-10 at 40℃ using 50 mM sodium acetate (pH 4-5), sodium phosphate (pH 6-8), and glycine-NaOH buffers (pH 9-10). The relative activity was defined as the percentage of activity with respect to the maximum chitinase activity.

2.8.2 Effect of metal ions and other compounds on the crude chitinase

Metals (Na^+ , K^+ , Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Hg^{2+}), all of which were chloride salts, were added into the crude enzyme making the final concentration of metal to 0, 2, 4, 6, 8, 10 mmol/L, respectively. In order to study the effects of other chemicals on the chitinase activity, enzyme samples were incubated with the agents in 1 mmol/L β-Mercaptoethanol, EDTA, Dithiothreitol (DTT), SDS and 0.1% Triton-X100 and Tween80. After 60 min incubation at 4℃, the residual activity was measured under standard conditions. Crude chitinase activity assayed without metal ions and chemical agents was taken as 100%.

2.8.3 The substrate specificity of chitinase

The substrate specificity of chitinase was determined at 40℃ and pH 7.5 with 1% (w/v) colloidal chitin, chitin, chitosan(DDA 80%), CMC, cellulose and soluble starch, respectively.

2.9 Analysis of hydrolysis products by thin layer chromatography (TLC)

To analyze the hydrolysis products, the crude chitinase (1 mL) was incubated with 1 mL of 1% colloidal chitin at 40℃ and pH 7.5 for 30 min. The enzymatic products were subjected to TLC Silica gel 60 (Merck, Darmstadt, Germany), the plates were developed using a solvent system with a ratio of 2:1:1(v/v/v) for n-butanol: ethanol: water. The resultant Chito-oligosaccharide spots were visualized by spraying the plates with a staining solution (1% diphenylamine and aniline in acetone) and heating at 110℃ for 10 min.

3. Results

3.1 Phenotypic and biochemical characteristics of DXK012

Cells are Gram-negative, short rods, 0.8 μm-1 μm wide by 2 μm-2.4 μm long, with flagellum on the ends (one flagellum on one end and several on the other) (Fig.1A and Fig.1B). Colonies are round, faint yellow, moist, smooth surface and Edges neatly. Growth is detected at 20-45℃, with the optimum growth yield at 37℃. The pH range for growth is 6-9 with the optimum growth yield at pH 7.5. Growth occurs at 0–12 % NaCl with the optimum growth yield at 3 %.

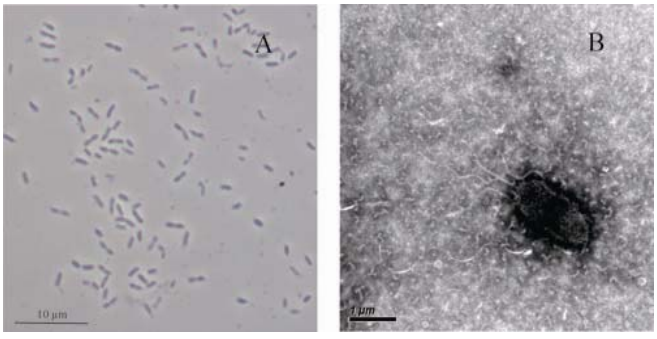

Fig.1 Optical micrograph (Fig.1A) and Electron micrograph (10000×) (Fig.1B) of strain DXK012.

3.2 Phylogenetic analysis of the 16S rRNA gene

The 6S rRNA gene sequence of strain DXK012 was determined and deposited in GenBank under the accession number JN624807. The homology search result of 16S rRNA gene sequence show a close relationship to *Pseudoalteromonas arabiensis* k53(T), *Pseudoalteromonas lipolytica* LMEB 39(T) and *Pseudoalteromonas donghaensis* HJ51(T) with 16S rRNA gene sequence similarities of 99.86%, 97.84% and 97.11%, respectively (Fig. 2). Based on morphologic, biochemical, and phylogenetic analysis of DXK012, the bacterium was identified as *Pseudoalteromonas* sp. DXK012.

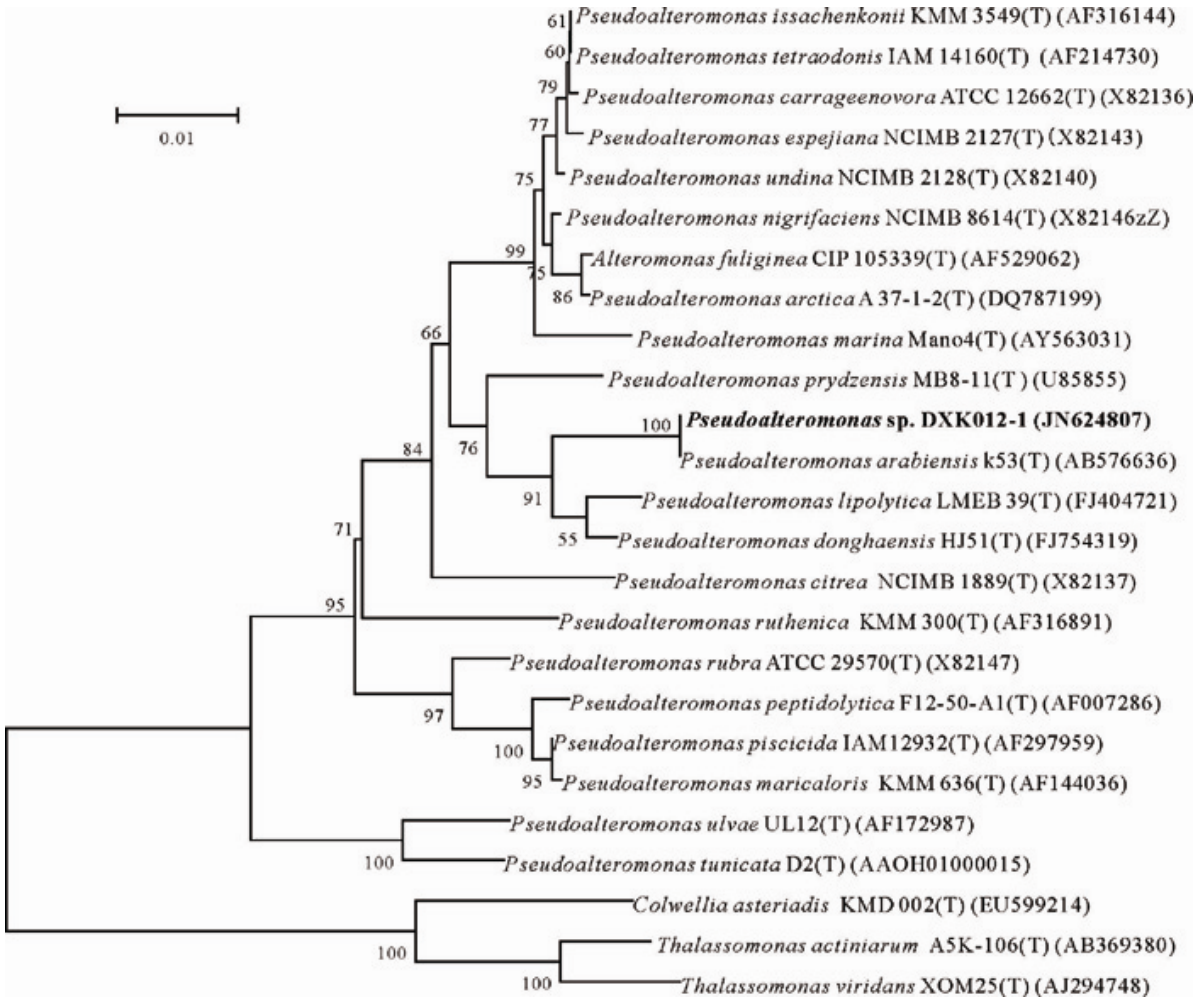

Fig.2 Neighbor-joining phylogenetic tree of strain DXK012 based on 16S rRNA gene sequences. The tree rooted was constructed by the neighbor-joining method with bootstrap values calculated from 1000 resampling. The numbers at each node indicate the percentage of bootstrap supporting.

3.3 Purification of chitinase from *Pseudoalteromonas* sp. DXK012

The crude chitinase was purified by combination of ultrafiltration, DEAE-cellulose and Sephadex G-100 column chromatography. The molecular weight of chitinase was estimated to be ~36 kDa by SDS-PAGE (Fig. 3).

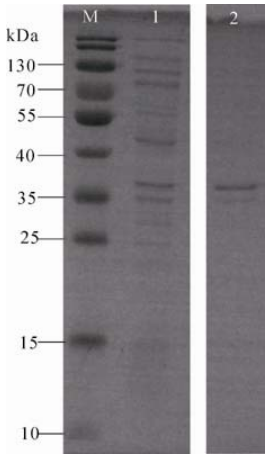

Fig. 3 SDS–PAGE of the purified chitinase from *Pseudoalteromonas sp*. DXK012. M: protein molecular markers, lane 1: crude extract, lane 2: purified chitinase.

3.4 Effect of pH and temperature on chitinase activity

The effect of pH and temperature on the catalytic activity was studied by using soluble colloidal chitin as a substrate under the standard assay conditions. The optimum temperature for the chitinase was 40℃(Fig.3A). The enzyme was stable when it was kept at ambient temperature for 5 d although enzyme activity was nearly lost 60% after 1 h at 80℃. The optimum pH for this enzyme was 7.5(Fig.3B). The activity was reduced more quickly when pH below 7.0.

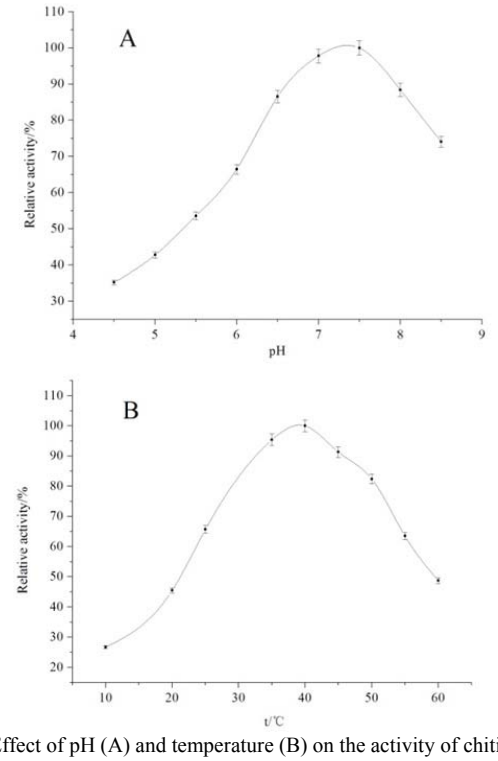

Fig.4 Effect of pH (A) and temperature (B) on the activity of chitinase

Table 1. Effect of metal ions on the activity of chitinase

metal ions		ionic concentration (mmol/L)				
		2	4	6	8	10
Cu^{2+}	Relative activity (%)	101.2	93.5	88.4	76.8	68.3
Co^{2+}		98.2	93.2	90.1	86.3	83.1
Zn^{2+}		88.5	83.2	85.6	81.2	78.6
K^{+}		96.8	98.1	93.6	92.4	90.5
Mn^{2+}		89.6	90.6	88.9	87.3	80.3
Ca^{2+}		99.3	96.8	96.5	92.6	93.6
Fe^{3+}		99.6	99.3	96.5	98.6	97.8
Mg^{2+}		111.6	125.6	129.2	135.1	130.7
Control		100				

3.5 Effect of metal ions and other compounds on chitinase activity

To further characterize chitinase of DXK012, the effect of metal ions and some on its activities was examined. As showed in Table 1. The activity for colloidal chitin hydrolysis was slightly promoted by 2mmol/L Cu^{2+}, the inhibition of Cu^{2+} was enhanced quickly the concentration increased. The enzyme activity was inhibited with most of metal ions such as K^{+} , Mn^{2+}, K^{1+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} and Ca^{2+}, with the concentration growing the inhibition became stronger. However, the enzyme activity of DXK012 was increased by Mg^{2+}(Table 1), which suggested the active center of enzyme included Mg^{2+}. The enzyme was inhibited by DTT , β-Mercaptoethanol (β-Me) and EDTA, indicating disulfide bond and metal such as Mg^{2+} was crucial in this enzyme activity(Fig. 4).

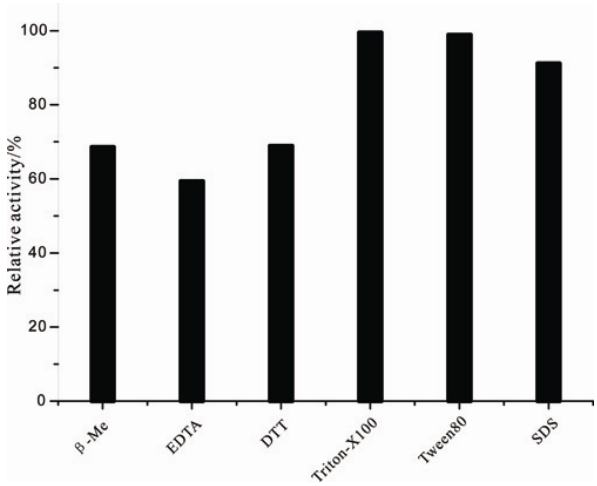

Fig.5 Effect of surfactant and enzyme inhibitor on the activity of chitinase.

3.6 Substrate specificity of chitinase

As it was showed in Table 2, chitin was clearly the preferred substrate of the chitinase, followed by chitin. It indicated this chitinase could cleave the GlcNAc-GlcNAc bond, maybe the GlcN- GlcNAc or GlcN-GlcN bond, But did not show any activity to Glc-Glc.

Table 2 The chitinase specificity of hydrolyzing substrates

substrates	chitin	colloidal chitin	Chitosan (DDA 80%）	CMC	cellulose	soluble starch
Relative activity (%)	100	87.6	8.3	0	0	0

3.7 Analysis of enzymatic hydrolysates by TLC.

The hydrolysates of chitin by chitinase of DXK012 were analyzed by thin-layer chromatography (TLC) (Fig. 5). The main products of the hydrolysis was between $(GlcN)_6$ and $(GlcN)_8$. These chitooligosaccharides were of importance in preserving moisture, reservation and other functions (Lu *et al.*, 2013, Wei *et al.*, 2013, Wu, 2012). Further study on the products should be taken.

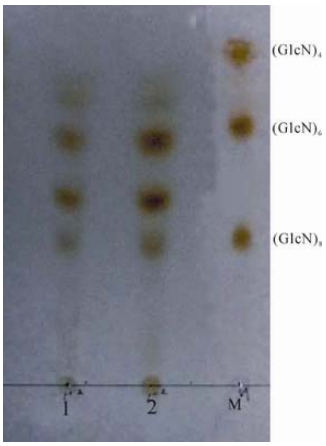

Fig. 6. Thin layer chromatography of chitinase hydrolysis products. Lane 2 is two concentration of lane 1. M denoted standard $(GlcN)_4$, $(GlcN)_6$, $(GlcN)_8$, respectively. Lanes 1-2 denoted hydrolysis products of chitin, which was hydrolyzed by the crude chitinase at 40℃, pH 7.5 for 2h.

4. Discussion

Ten of billions tons of insoluble chitins were generated in nature, but hardly accumulated in seafloor, this phenomenon was attributed to effective enzymolysis of chitinase from marine microorganisms. Marine microorganisms could push on the cycle of carbon sources and nitrogen sources through producing chitinase using the chitin as nutrient. Recently, the research on chitinase-producing microorganisms focusing on the genus *bacillus*, *Serratia* and *Streptomyces* et al.. There hasn't been any report about *Pseudoalteromonas* of deepsea producing chitinase except *Pseudoalteromonas* sp. strain S9. But the S9 produces a distinct orange pigment, and two chitinase proteins (76 and 64 kDa) were found in the culture supernatant of S9 on chitinase activity gels. So there are big differences between *Pseudoalteromonas* sp. DXK012 and *Pseudoalteromonas* sp. strain S9.

Most of chitinase/chitosanase-producing strains reported at present could only hydrolyze the deacetylation derivatives of chitin, such as colloidal chitin and chitosan. However, the preparation of colloidal chitin and chitosan involves demineralization, deacetylation and deproteinization of shell-fish waste with the use of strong acids or bases, So the superiority of the enzymatic degradation couldn't really take its advantage in actual application. The chitinase from *Pseudoalteromonas* sp. DXK012 did not only have high enzyme activity in chitin, reaching 356.2u/mL, but also process 87.63% relative enzyme activity against colloidal chitin. The enzyme was quite stable at room temperature. *Pseudoalteromonas* sp. DXK012 could grow in the medium only with the shrimp and crab shell waste as nutrients and producing the economic Chito-oligosaccharide using this chitinase.

This job laid a solid foundation for the production and application of chitinase from *Pseudoalteromonas* sp. DXK012. Optimization of chitinase-producing conditions, clone and expression of the chitinase gene from Pseudoalteromonas sp. DXK012 should be focused on the next steps, and also with purification of Chito-oligosaccharides and exploring their effects.

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