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Isolation and Characterization of a Chitionolytic Enzyme Producing Microorganism, *Paenibacillus chitinolyticus* JK2 from Iran

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Abstract: Chitinases are glycosyl hydrolases, which catalyze the degradation of chitin. These enzymes are capable of hydrolyzing chitin to its oligomers and monomer, N-acetyl-β-D-glucosamine. Fifty different chitin-degrading microorganisms were isolated in this study. One of these strains with high ability to produce chitinase was selected and identified as *Paenibacillus chitinolyticus* by morphological and biochemical properties along with 16S rDNA partial gene sequence analysis. This strain was able to produce high levels of extracellular chitinase in media containing chitin as sole carbon source. The chitinolytic activity of culture supernatant was maximal after 72 h of culture. The enzyme showed optimal activity at 37°C and a double optimum pH at pH 5 and 7. Chitooligosaccharides were the predominant products throughout the enzymatic hydrolysis of colloidal chitin, indicating that the enzyme was an endochitinase. This enzyme with these properties could be useful for waste treatment, chitooligosaccharides production and other relevant applications.

Key words: Chitooligosaccharide, Endochitinase, *Paenibacillus chitinolyticus*

INTRODUCTION

Chitin, an insoluble linear β-1, 4-linked polymer of N-acetylglucosamine (acetamido-2-deoxy-D-glucose) is the second most abundant polysaccharide in nature. It is a structural component of fungal cell wall, exoskeleton of insects and crustaceans (Flach *et al*., 1992). Derivatives of chitin, either in the form of oligomer or monomer, have many applications in a wide range of fields (Patil *et al*., 2000). Many current methods to hydrolyze chitin require harsh chemical treatments, thus an enzymatic process is preferable in order to apply a mild reaction condition, to control the extent of hydrolysis and the consistency of the product.

Chitinases (EC 3.2.1.14) are defined as enzymes that cleave the β-1, 4-glycosidic bonds of chitin. These enzymes are capable of hydrolyzing chitin to its oligomers and monomer, N-acetyl-β-D-glucosamine. Chitinases are produced by many organisms including viruses, bacteria (*Bacillus*, *Aeromonas*, *Vibrio*, *Pseudomonas*, *Serratia*, *Enterobacter*, *Actinomycete*), fungi

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(*Trichoderma* and *Aspergillus*), plants, invertebrates and vertebrates. (Flach *et al*., 1992; Gooday, 1990; Felse and Panda, 2000; Kuk *et al*., 2005; Patil *et al*., 2000; Yuli *et al*., 2004).

Chitinases are important enzymes in biotechnology and bioprocessing, because of their use as protective agent against plant-pathogenic fungi (biological control) (Mathivanan *et al*., 1998), mosquito control (Mendonsa *et al*., 1996), single cell protein production (Vyas and Deshpande, 1991) and for processing of chitin waste (Rattanakit *et al*., 2002; Wang and Hwang, 2001). Chitinases may also be used for the production of oligosaccharides and N-acetyl-glucosamine as biologically active substances (Kuk *et al*., 2005; Pichyangkura *et al*., 2002), preparation of protoplasts from fungi (Vyas and Deshpande, 1989) and as vaccine candidates to target parasitic diseases (Harrison *et al*., 1999). Screening and isolation of chitinolytic microorganisms is usually performed on chitin agar plates and then chitinase activities are assayed by determination of reducing end sugar (Cody, 1989; Wirth and Wolf, 1990).

Although chitinases have been isolated and characterized from a wide variety of sources, it is still important to screen for new sources for production of chitinases which are more economical and desirable properties to expand their usefulness. This study presents a new report on the screening program for isolation of chitinolytic microorganisms from environmental sources of Iran and characterization of a new strain, *Paenibacillus chitinolyticus* JK2 capable of producing relatively high levels of chitinase in a short time compared to other isolates.

MATERIALS AND METHODS

Materials

Chitin from crab shell and p-nitrophenyl-N-acetylglucosaminide were purchased from Sigma Chemical Co. (St. Louis. MO, USA). All other chemicals used were of analytical grade purchased from either Sigma or Merck.

Preparation of Colloidal Chitin

Colloidal chitin was prepared from purified chitin according to the method of Roberts and Selitrennikoff (1988) with a few modifications described as follows: 5 g of chitin powder was added slowly into 90 mL of concentrated HCl under vigorous stirring for 2 h. The mixture was added to 500 mL of ice-cold 95% ethanol with rapid stirring and kept overnight at 25°C and then stored at -20°C until use. When needed, the precipitant was collected by centrifugation at 6000 rpm for 10 min at 4°C and was washed with 0.1 M sodium phosphate buffer (pH 7) until the colloidal chitin became natural (pH 7) and used for further applications.

Isolation of Microorganisms

Various samples of chitinous waste (e.g., shrimp and crab shell waste), soil and seafood industrial waste from shrimp production pools and marine environment were collected. Screening of the chitinolytic microorganisms was performed by plating sample solutions from various samples on a chitin agar plates. Medium for chitin agar plate preparation was prepared by mixing 5 g of colloidal chitin as a sole carbon source and 18 g of agar in M9 medium (g L^{-1}): Na_2HPO_4, 0.65; KH_2PO_4, 1.5; NaCl, 0.25; NH_4Cl, 0.5; $MgSO_4$, 0.12 and $CaCl_2$, 0.005. The final pH was adjusted to 6.5. The plates were incubated at 30°C for up to 10 days. Microorganisms with chitinase activity were detected by the appearance of clear zones around colonies grown on chitin/agar plates.

Primary Screening of Chitinase Production

In a preliminary screening, the appearance of clear zones was considered as the criterion for chitinase production by the microorganisms. Those strains forming bigger zones in a shorter time were

selected and used to inoculate into 100 mL Erlenmeyer flasks containing 20 mL of M9 medium supplemented with 0.5% colloidal chitin as a sole carbon source. Flasks were incubated in an orbital incubator shaker at 30°C and 150 rpm. The enzyme activity was measured at different time intervals for five days.

Enzyme Assays

Chitinase activity was measured with colloidal chitin as a substrate. The amount of reducing sugar produced was measured by the dinitrosalicylic acid (DNSA) method (Miller, 1959), with N-acetylglucosamine as a reference compound. Chitinolytic activity was measured by incubating 0.3 mL of 1% colloidal chitin, pH 6.6 and 0.3 mL of supernatant samples as an enzymes source at 37°C for 45 min. The reducing sugar released was measured by the DNSA method at 540 nm using N-acetyl-D-glucosamine (GlcNAc) as standard. A unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of GlcNAc per minute under the specified assay conditions. β-N-acetylglucosaminidase was determined using the reaction mixture contained 100 μL p-nitrophenyl-N-acetylglucosaminide (5 mM), 100 μL enzyme solutions (culture supernatant) appropriately diluted in buffer and 200 μL 100 mM sodium phosphate buffer (pH 7.5). After incubation at 37°C for 4 h, 1 mL glycine-NaOH (0.2 M, pH 10.5) was added to stop the reaction. The absorbance of the p-nitrophenol released was measured at 405 nm. One unit of β-N-acetylglucosaminidase activity was defined as the amount of enzyme necessary to liberate 1 μmol p-nitrophenol per minute under assay conditions.

Xylanase and cellulase activities were determined by measuring the amount of reducing sugars released from xylan and carboxymethyl cellulose (CMC). The enzyme solution was incubated with the substrate solutions [1% (w/v) xylan, 1% (w/v) CMC, both in 100 mM sodium phosphate buffer, pH 7.5] at 37°C for 45 min. 0.6 mL dinitrosalicylic acid reagent was added and the mixture was boiled for 10 min and allowed to cool to room temperature. Reduced sugars were determined by reading the absorbance at 540 nm. One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min at 37°C, by hydrolyzing substrates.

Taxonomic Studies

Morphological, physiological and biochemical characteristics of the chitinolytic microorganism were studied according to the Bergey's Manual of Systematic Bacteriology (Seneath *et al.*, 1986).

Polymerase chain reaction (PCR) was performed to amplify a partial 16S rRNA gene of the bacteria and partial 16S rDNA sequencing was used to assist in the identification of the isolate JK2. Isolation of genomic DNA, PCR amplification and sequencing of PCR product for analysis of 16S rRNA were conducted according to Sambrook *et al.* (1986) and Marchesi *et al.* (1998). A similarity search for the nucleotide sequence of 16S rRNA of the test isolate was carried out using a Blast search at NCBI (Altschul *et al.*, 1997). The phylogenetic relationship was determined in NCBI Blast program and the option to correct for multiple substitutions.

Nucleotide Sequence Accession Number

The partial 16S rRNA sequence of the isolate JK2 was deposited in the GenBank database under accession number EF011984.

Partial Characterization of the Crude Enzyme

Effect of Temperature and pH

The effect of temperature on chitinase activity was determined by incubating the reaction mixtures at different temperature range of 20-80°C and assaying the enzyme activity under standard

assay conditions. To analyze the effect of pH, the activity was assayed at different pH, using three different buffers [sodium acetate buffer (pH 3.5-5.0); sodium phosphate buffer (pH 5.0-8.0) and Tris-HCl buffer (pH 8.0-10)] at 37°C.

Mode of Action

The mode of action of chitinase was determined by a paper chromatography method. The crude enzyme (500 µL) was added to 250 µL substrate solution (1% w/v colloidal chitin in 100 mM sodium phosphate buffer, pH 7.5). The reaction mixture was incubated at 37°C. Aliquots were taken at fixed time intervals and the reaction was terminated by immersing the sample in boiling water for 10 min. Samples were analyzed on paper chromatography, using Whatman paper (No. 7) with N-acetyl-D-glucosamine, glucose and maltose as standards. The paper was run ascendingly using the solvent system of (n-butanol, pyridine and water, 6:3:4). Spots were visualized by silver staining (Robyt and French, 1963).

RESULTS AND DISCUSSION

Isolation of the Microorganism

Selection was made through two screening experiments. The first screening was dependent on the appearance of clear zones around bacterial colonies growing on M9 minimal medium agar plates supplemented with 0.5% colloidal chitin as a sole carbon source. The second stage was performed by determining the reducing sugar released.

During the preliminary screening experiments various types of microbial colonies (more than 200 colonies from 60 different samples) were obtained. Among those stains giving diameters of clearing zones higher than 30 mm after seven days on the same condition, 50 strains were selected. Following growth in chitin liquid medium the colonies were purified and tested for chitinase activity using a dinitrosalicylic acid method (Table 1).

Most of the microorganisms screened in this study showed chitinase activity. Among the isolates two microorganisms exhibited higher enzyme activity (Table 1-group 4). Of these two strains, JK2 (isolated from Lakan forest, Gilan Province, North of Iran) exhibited higher chitinase activity, selected and maintained on storage medium (M9 medium containing 0.2% chitin) and used for further studies.

Identification of the Isolate

Taxonomic studies was carried out according to the methods described in Bergey's Manual of Systematic Bacteriology (Seneath *et al*., 1986). The physiological and biochemical characteristics of strain JK2 are shown in the Table 2. On the basis of the nucleotide sequence of the partial 16S rRNA gene, JK2 was classified as a species of the genus *Bacillus*. A Pairwise alignment (BLASTn) analysis of 16S rDNA sequence of the isolate JK2 indicated that the DNA sequence had the highest similarity with *B. chitinolyticus* (more than 99% similarity at the level of DNA). The phylogenetic tree showed that JK2 is closely related to members of the genus *Bacillus* (Fig. 1). From the above data, the isolate JK2 was identified as *Bacillus chitinolyticus*. Although in newly classification (Lee *et al*., 2004) this microorganism has been classified as *Paenibacillus chitinolyticus*.

Table 1: Relative chitinase activity of various chitinolytic isolated microorganisms

Groups	Range of relative activity (%)	No. of microorganisms
1	0-40	2
2	41-60	24
3	61-80	22
4	81-100	2

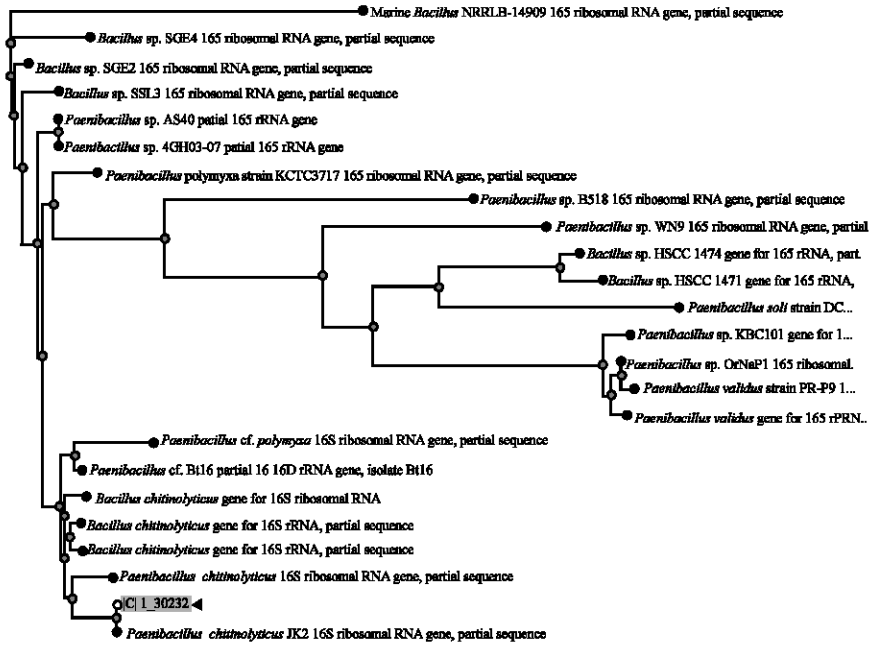

Fig. 1: Phylogenetic tree of the 16S rDNA sequences of strain JK2 and the bacteria most closely related to it

Table 2: Morphological, physiological and biochemical characteristics of the isolated strain JK2

Characters	Results
Morphology	Cells are rod shaped, gram positive and motile, spore central and oval
Motility	Positive
Catalase	Positive
Urease, oxidase, DNase	Negative
Voges-Proskauer test	Negative
Methyl red	Positive
Utilization of	
glucose, maltose	Positive
arabinose, xylose, manitol, lactose, fructose	Negative
Hydrolysis of starch	Positive
Hydrolysis of xylan, cellulose	Negative
Gelatin liquefaction	Negative
Utilization of citrate	Negative
Nitrate reduction	Negative
Formation of indole	Negative
Growth in NaCl	Up to 3%
Growth at pH	4.5-11
Growth at temperature	20°C up to 37°C

Chitinase Production by *Paenibacillus chitinolyticus* JK2

Paenibacillus chitinolyticus JK2 was grown on chitin agar plates and generated a zone of clearance due to chitinase activity (Fig. 2). The chitinase activity could be detected by reducing sugar estimation after 24 h of incubation and progressively increased to the highest value after 72 h (0.1 U mL^{-1}) and decreased thereafter (Fig. 3). Similar observations were reported in case of *Bacillus* sp. 13.26 (Yuli *et al*., 2004) and *Bacillus thuringiensis* subsp. *kurstaki* (Driss *et al*., 2005) with maximum chitinase production 28.31 mU mL^{-1} and 0.42 U mg^{-1} protein respectively, after the three days incubation. Therefore, culture supernatants of 3-day culture were used for further applications.

Fig. 2: *Paenibacillus chitinolyticus* JK2 was grown on chitin agar plate and showed clearance zone. White arrow in the picture shows size of colony and zone of clearance for the microorganism. The culture was incubated at 30°C for 48 h

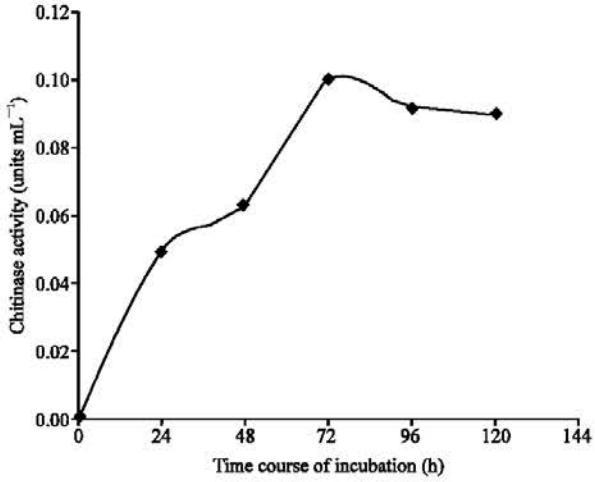

Fig. 3: Time course of chitinase production. Strian JK2 was cultivated in a M9 medium supplemented with 0.5% colloidal chitin as a sole carbon source (30°C, 150 rpm). At the indicated time, samples were taken and enzyme activity was assayed according to the procedures mentioned in text

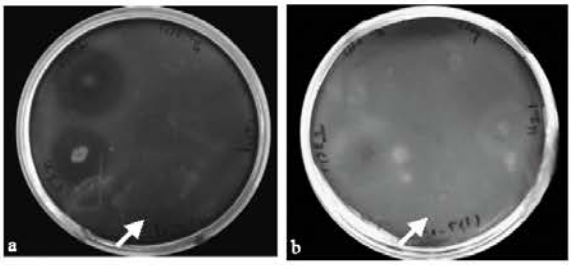

Fig. 4: *Paenibacillus chitinolyticus* JK2 was grown on: a) xylan agar plate and b) cellulose agar plate and did not show clearance zone. The cultures were incubated at 30°C for 72 h in xylan agar plate and seven days in cellulose agar plate. Finally, all plates were stained with an aqueous solution of 0.1% (w/v) Congo red, destained with 2 M NaCl and fixed with 0.5% (v/v) acetic acid to visualize zones of clearing

As shown in the Fig. 4, in minimal media with xylan or CMC as the sole carbon source, *P. chitinolyticus* JK2 did not produce other carboxylase enzymes such as xylanase or cellulase. However, the supernatant of the culture grown in medium with chitin showed xylanase or cellulase

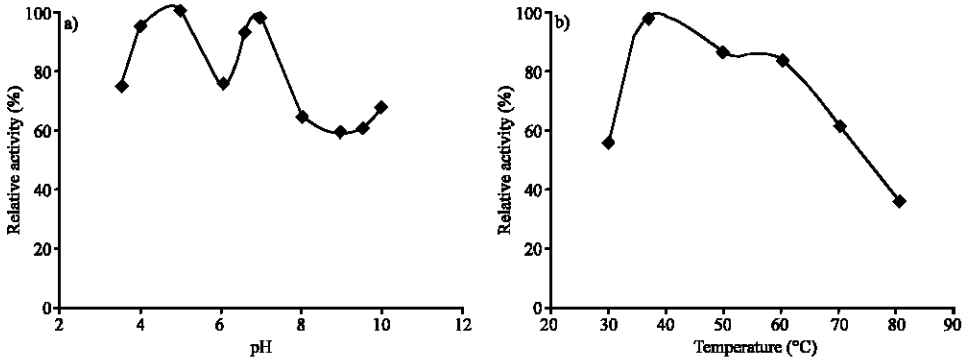

Fig. 5: (A) Optimum pH and (B) optimum temperature of the JK2 crude enzyme. Optimum pH of the enzyme was determined in various pH buffers [sodium acetate buffer (pH 3.5-5.0), sodium phosphate buffer (pH 5.0-8.0) and Tris-HCl buffer (pH 8.0-10)] at 37°C. Optimum temperature for enzyme activity was determined by incubating the reaction mixtures at different temperatures range of 20-80°C

activity, suggesting that production of xylanase or cellulase are related to chitinase production and to the presence of chitin in the medium. This suggests the possibility for arrangement of these enzymes genes as a cluster. More experiments are needed to examine other hypothesis on the multifunctionality of the chitinase.

Partial Characterization of the Enzyme

Effect of pH and Temperature

The effect of pH on enzymatic activity is shown in Fig. 5A. The crude enzyme showed a double optimum pH at pH 5 and 7. Chitinase activity showed 90 and 65% activity at pH 4 and 8, respectively indicating that this enzyme tends to be active in acidic condition than alkaline. This is similar to the pH 5 reported for *Bacillus circulans* WL-12 (Watanabe *et al*., 1992), pH 5-8 for *Aeromonas hydrophilia* H-2330 (Hiraga *et al*., 1997) and pH 4-7 for *Serratia marcescens* QMB1466 and BJL2000 (Monreal and Reese, 1969). Studies of the effect of the temperature on the chitinase activity were also performed which indicated that the activity was increased proportional to the temperature below the optimum temperature at 37°C and decreased sharply at higher temperatures. The enzyme showed 65% of maximal activity at 30°C. Near 90% of the enzyme activity was observed at 50°C (Fig. 5B).

Mode of Action

To determine the mode of cleavage by the chitinase, the reaction products were analyzed from colloidal chitin by paper chromatography. As shown in Fig 6, the final products of enzymatic hydrolysis were mainly oligomers. The paper chromatogram suggests that spots corresponding to GlcNAc and its dimer could be detected after incubation of the enzyme with substrate for 3 h. The ability of chitinase to hydrolyze *p*NP-N-acetylglucosaminide was investigated (Fig. 7) and showed that the enzyme was not able to hydrolyze *p*NP-N-acetylglucosaminide as its specific substrate for production of monomer. According to these results, the chitinase did not have β-N-acetylglucosaminidase activity but had endochitinase activity as it catalyzed the release of oligomers from colloidal chitin during different incubation times. A similar observation was reported in the case of *Bacillus* sp. MH-1, which exhibited enzyme activity with an endo type of action on the chitin polymer (Sakai *et al*., 1998).

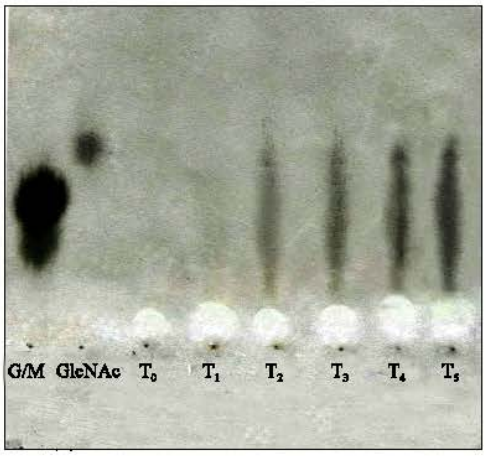

Fig. 6: Paper chromatogram of the hydrolysis products from colloidal chitin after incubation with chitinase crude enzyme. Samples (20 μL) were taken at fixed time intervals for paper chromatography [butanol/pyridine/water (6:3:4)], with detection by silver staining. Samples: G/M: glucose/maltose, GlcNAc: N-acetylglucosamine, T_0: 0, T_1:1, T_2:3, T_3:6, T_4:12, T_5:24 h incubations

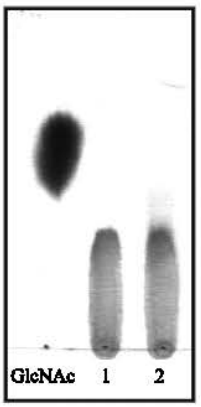

Fig. 7: Paper chromatogram of the hydrolysis product of pNP-N-acetylglucosaminide after incubation with chitinase crude enzyme. GlcNAc = N-acetylglucosamine as standard, lane 1 = the enzyme was incubated with the substrate for 2 h at 37°C. Lane 2 = the enzyme was incubated with the substrate for 5 h at 37°C

Four chitinases have been reported for *Bacillus licheniformis* exhibiting an exo-N, N'-diacetylchitobiohydrolase type of action (Takayanagi *et al*., 1991). Exochitinases, such as chitobiosidases, have been studied in *Bacillus cereus* (Wang *et al*., 2001). This strain produces a 36 kDa enzyme with optimal activity at 35°C and pH 5.8.

CONCLUSION

Although chitinases have been isolated and characterized from a wide variety of sources, it is still important to screen for new sources for production of chitinase with more economical values and enhanced properties to expand their usefulness. The present study is, to our knowledge, the first

screening program in a wide range of area for isolation of chitinolytic microorganisms from Iran. Fifty chitinolytic strains were purified and tested for chitinase activity. The strain JK2 was selected among those giving maximum production of enzyme during the shortest time. It was further identified as *Paenibacillus chitinolyticus* JK2. The strain JK2 enzymatic activity was typically endochitinolytic activity as it randomly hydrolyzed glycosidic bonds. It was apparent that this strain did not show cellulase and xylanase activities in the presence of the specific substrates (CMC and xylan). However, we detected cellulase and xylanase activities in the medium that contained chitin as sole carbon source. According the above mentioned data two possible explanations may be considered: 1) the presence of chitin in the medium culture can induce chitinase as well as cellulase and xylanase production. 2) the chitinase enzyme which was produced by this strain had other hydrolytic activities such as cellulase and xylanase activities. These hypotheses could be further examined by purification and characterization of the enzyme. Further studies on the properties of JK2 isolate and its chitinase could determine its potential for industrial application especially for waste treatment programs.

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