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Optimization of Medium and Cultivation Conditions for Chitinase Production by the Newly Isolated: *Aeromonas* sp.

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Abstract: Fifty strains of different microorganisms with ability to degrade chitin were isolated during a screening program. One of the most potent isolates (strain JK1) was identified as *Aeromonas* sp. and was deposited in Persian Type Culture Collection (PTCC 1691). Identification was carried out using morphological and biochemical properties along with 16S rRNA partial sequence analysis. This strain was able to produce high levels of extracellular chitinase in media containing chitin as sole carbon source. The effects of medium composition and physical parameters on chitinase production by this organism were studied. The optimized medium was found to contain colloidal chitin 0.75% (w/v), ammonium sulfate 0.15% (w/v), magnesium chloride 7.5 mM and Triton X-100 0.2% (v/v). The highest enzyme production by *Aeromonas* sp. JK1 was obtained at pH 8, 30°C and after 48 h growth. With respect to high amount of chitinase production by this strain in a simple medium and the relatively short time, this strain could be a suitable candidate for production of chitinase on an industrial scale and merits further investigation into its structure and characteristics.

Key words: *Aeromonas* sp., chitinase, medium optimization

INTRODUCTION

Chitin which is the second-most abundant biopolymer on the planet is an insoluble linear polymer of β-1, 4-linked N-acetyl-β-D-glucosamine (Shahidi and Abuzaytoun, 2005). It is widely distributed in nature as a structural component of crustaceans, fungi, protozoa and insects (Flach *et al*., 1992). Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyze the degradation of chitin. These enzymes have a wide range of biotechnological applications such as preparation of pharmaceutically important chitooligosaccharides and N-acetyl-D-glucosamine (Kuk *et al*., 2005; Pichyangkura *et al*., 2002; Sorbotten *et al*., 2005), isolation of protoplasts from fungi and yeast (Dahiya *et al*., 2005), preparation of single-cell protein (Vyas and Deshpande, 1991), control of pathogenic fungi (Mathivanan *et al*., 1998) and treatment of chitinous waste (Wang and Hwang, 2001). Chitinases are present in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants and animals and play important physiological and ecological roles (Cody *et al*., 1990; Duo-Chuan, 2006; Gooday, 1990). Chitinases are constituents of several bacterial species; some of the best known include the *Aeromonas*, *Serratia*, *Vibrio*, *Streptomyces* and *Bacillus* genera (Cody, 1989). Bacteria produce chitinases mainly to degrade chitin and utilize it as an energy source. In addition, some chitinases of chitinolytic bacteria are potential agents for the biological control of plant diseases caused by various phytopathogenic fungi (Chernin *et al*., 1997; Downing and Thomson, 2000).

The present study describes the results of our screening program for isolation of microorganisms producing high levels of chitinase from environmental sources. In this study one of the most potent isolates was

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identified as *Aeromonas* sp. strain JK1 and also physico-chemical parameters for the enhanced production of extracellular chitinase was optimized.

MATERIALS AND METHODS

This study was conducted in 2005-2007 by Tehran and Mashhad Universities of Medical Sciences, Ministry of Health and Medical Education, in Iran.

Screening and isolation on chitin agar plates: Various samples of chitinous waste (e.g., shrimp and crab shell waste), soil, seafood industrial waste, shrimp production pools and marine environment were aseptically collected from different locations in Iran. Screening of the chitinolytic microorganisms was performed by plating sample solutions from various samples on 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 synthetic medium and the final pH was adjusted to 6.5±0.2. The chitin/agar plates were incubated at 30°C and examined for formation of clear zones around the colonies up to 10 days. The size of the clear zones and colony size were both measured and the colonies were transferred to chitin agar slants for further studies.

Primary screening of chitinase production: Those strains which showed a higher clear zone/colony size ratio in the plate assay in a shorter time were selected and inoculated into liquid chitin medium. The fermentation media were incubated in an orbital incubator shaker at 30°C and 150 rpm. Samples of microbial cultures were taken every 24 h up to 120 h and kept at -4°C for further analysis for chitinase activity.

Taxonomic studies: Morphological, physiological and biochemical characteristics of the chitinolytic microorganism were studied according to the Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994).

To identify the bacterium at the gene level, Polymerase Chain Reaction (PCR) was performed to amplify a partial 16S rRNA gene of the bacteria. 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).

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 under vigorous stirring for 30 min and kept overnight at 25°C and then stored at -20°C until use. When in need, the precipitant was collected by centrifugation and washed with 0.1 M sodium phosphate buffer (pH 7) until the colloidal chitin became natural (pH 7) and used for further applications.

Chitinase assay: Chitinase activity was assayed with colloidal chitin as the substrate. Enzyme solution (0.3 mL) was added to 0.3 mL of substrate solution, which contained 1% colloidal chitin in a sodium phosphate buffer (100 mM, pH 8.0). The mixture was incubated at 37°C for 45 min. The reducing sugar released was measured by the DNSA method (Miller, 1959) at 540 nm using N-acetyl-D-glucosamine (GlcNAc) as standard. One unit of chitinase activity was defined as the amount of enzyme producing 1 μmol of GlcNAc per hour under the specified assay conditions.

Optimization of culture conditions: The optimum cultural conditions for the production of chitinase by *Aeromonas* sp. JK1 were carried out keeping all the factors constant except the one which was studied. The parameters studied include (a) different carbon and nitrogen sources with their various concentrations (b) effect of surfactants (c) effect of various metal ions (d) incubation temperature (e) initial pH of the medium and (f) time period of fermentation. For each parameter optimization, two sets of independent experiments were carried out and the average values are reported.

Enzyme production: Cultivation of the isolate for chitinase production was carried out in 25 mL liquid medium in 100 mL flasks for 48 h at 30°C on a rotary shaker (150 rpm). After growth, the culture broth was centrifuged at 8000 rpm for 10 min and the supernatant was used for chitinase assay. Cell growth was measured by absorbance at 600 nm.

RESULTS

Isolation of the microorganism: In a primary screening experiment, 200 colonies were isolated from 60 different samples, being capable of using colloidal chitin as a sole carbon source and forming halos on chitin-containing agar medium. Fifty strains from which the colonies formed large and clear zones in the shortest time were purified and tested for chitinase activity with DNSA method after

growth in chitin liquid medium. Most of the strains tested exhibited chitinase activity. Among them, strain JK1 selected (as one of the microorganisms with highest capability for production of chitinase) and used for further studies.

Identification of the isolate: The isolated strain JK1 was subjected to taxonomic analysis based on Bergey's Manual of Systematic Bacteriology and identified as a bacteria belonging to the genus *Aeromonas*. The organism was Gram-negative, rod-shaped, non-sporing and catalase-positive (Table 1). Further partial sequence analysis of the gene encoding 16S rRNA confirmed the isolate as being *Aeromonas* sp. related closest to the *Aeromonas* sp. U351 reported by Skrodenyte-Arbaciauskiene *et al.* (2006). According to these results, this bacterium was identified as a member of *Aeromonas* sp. Identification of the isolated microorganism was confirmed by the Persian Type Culture Collection (PTCC), Tehran, Iran, as *Aeromonas* sp. and deposited as PTCC 1691. The partial 16S rRNA sequence of the isolate was deposited in the GenBank database under accession number DQ985606.

Optimization of culture conditions: Growth was carried out in a minimal synthetic medium (M9) and gradually supplemented with the various ingredients that were investigated. The parameters optimized earlier were incorporated in subsequent experiments.

Carbon sources: The effects of various carbon sources (5 g L^{-1}) such as different monosaccharides, disaccharides and polysaccharides were tested in the absence and the presence of chitin on chitinase production.

In the control flask no carbon source was added except chitin. It was found that chitin was the best carbon source for chitinase production by *Aeromonas* sp. JK1. In a medium lacking chitin, no chitinase production was observed. However, when xylan and pectin included in the medium chitinase activity was detected to some degree. Addition of chitin increased the chitinase production after 24 h. The maximum peak of activity was observed near 72 h, after that the chitinase activity decreased slowly. These carbon sources in the presence of 5 g L^{-1} chitin caused no enhancement of chitinase production compare to the control. To optimize chitin concentration (as the main carbon source), different concentrations (1.25 to 30 g L^{-1}) were used in the production medium and maximum chitinase production by *Aeromonas* sp. JK1 was found in a media with 7.5 g L^{-1} chitin (Fig. 1).

Table 1: Morphological, physiological and biochemical characteristics of JK1

Characteristic	Result	Characteristic	Result
Gram staining	-	Indole production	+
Motility	+	Utilization of citrate	+
Spore	-	Nitrate reduction	+
Catalase	+	Gelatin liquefaction	+
Oxidase	+	Growth in NaCl	
DNAse	+	0-4%	+
Urease	-	5-7%	-
MR	+	Growth at temperature	
VP	-	10-37°C	+
Utilization of		50°C	-
Glucose, Fructose, Arabinose,		Growth at pH	
Lactose, Maltose, Manitol, Xylose	+	<5	-
Hydrolysis of Xylan, Cellulose	-	5-11	+
Hydrolysis of Starch	+		

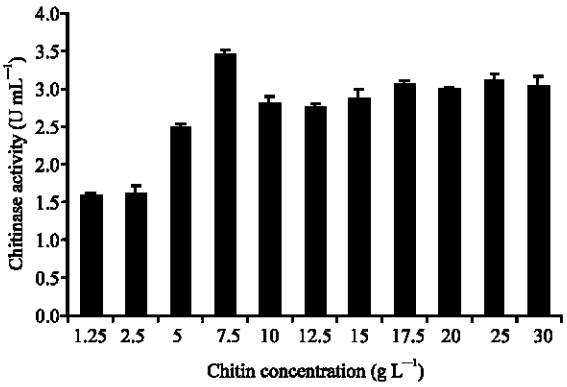

Fig. 1: Effect of different concentrations of chitin on the chitinase production by *Aeromonas* sp. JK1

Nitrogen sources: To select the best nitrogen source, the chitin medium was supplemented with different inorganic (0.5 g L^{-1}) and organic nitrogen sources (1 g L^{-1}). Among the various nitrogen sources in the basal medium, ammonium sulfate and peptone were the most effective additives resulting in the increase of the enzyme production (Fig. 2). However, in the case of meat extract, soybean powder, gelatin, ammonium nitrate, ammonium biphosphate and ammonium acetate, chitinase production was repressed from 15 to 60%. Ammonium sulfate and peptone gave nearly 15% increase in chitinase productivity. Ammonium sulfate was chosen as the simplest and cheapest nitrogen source for preparation of next media.

To evaluate the effect of ammonium sulfate, different concentrations were added to production medium (0.25 to 3 g L^{-1}). Maximum chitinase production by *Aeromonas* sp. JK1 was found in media with 1.5 (g L^{-1}) ammonium sulfate and a change from 2 to 3 (g L^{-1}) did not bring about striking variations in the amount of enzyme produced (Fig. 3).

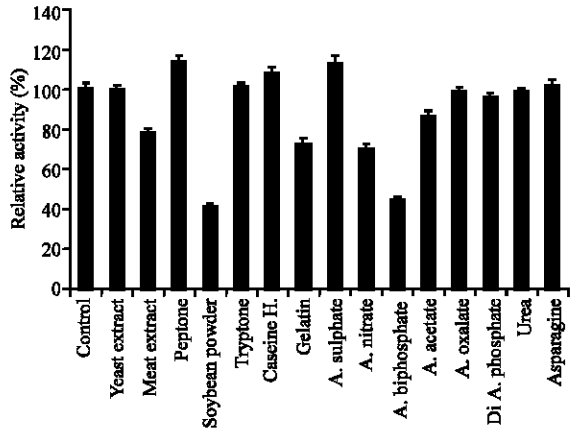

Fig. 2: Effects of inorganic and organic nitrogen sources on chitinase production by *Aeromonas* sp. JK1. Control medium contained ammonium chloride instead of nitrogen sources

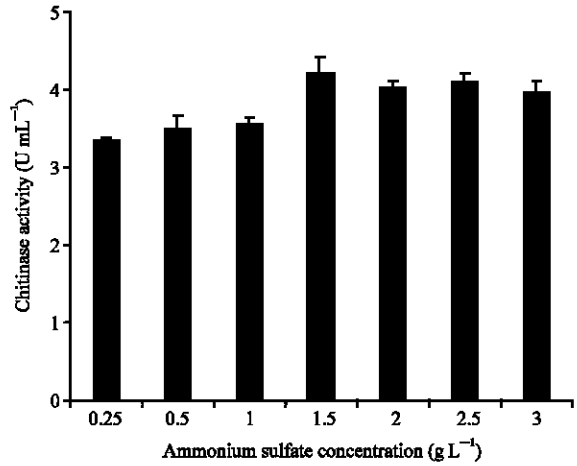

Fig. 3: Effect of different ammonium sulfate concentrations on the chitinase production by *Aeromonas* sp. JK1

Effect of surfactants: To investigate the effect of various surfactants on chitinase production, the medium was supplemented with 0.1% (v/v) Triton X-100, Tween 80, Tween 65 and Tween 20. Results showed that Triton X-100 had the best effect on chitinase production (Fig. 4). Addition of Tween 80 resulted in a slight increase in chitinase production, while other surfactants were found to inhibit chitinase production. Further studies showed that maximum chitinase production by *Aeromonas* sp. JK1 was achieved in media with 0.2% (v/v) Triton X-100.

Effect of various metal ions: The effect of various metal ions on chitinase production by *Aeromonas* sp. JK1 was investigated by supplementing the medium with 5 mM of

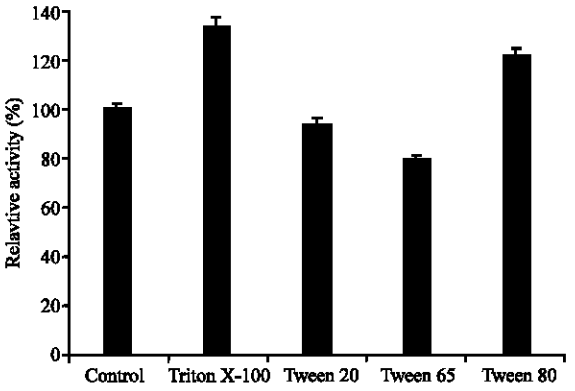

Fig. 4: Effects of non-ionic detergents on chitinase production by *Aeromonas* sp. JK1

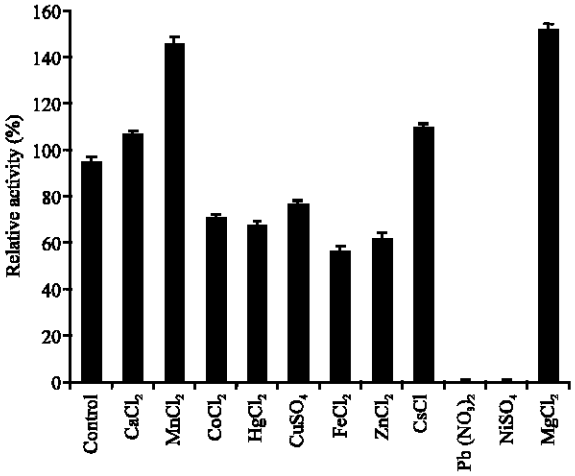

Fig. 5: Effect of different metal ions on chitinase production by *Aeromonas* sp. JK1

the respective cations. Results showed that chitinase production was most affected by the addition of Mg^{2+} and Mn^{2+}, where enzyme production was approximately 60 and 54% higher than that of control, respectively (Fig. 5). Addition of Ca^{2+} and Cs^{+} resulted in a slight increase in chitinase production, while other ions were found to inhibit enzyme production from 20 to 100%. When the concentration effect of Mg^{2+} was further studied, it was found that the optimum concentration of $MgCl_2$ for chitinase production by *Aeromonas* sp. JK1 was 7.5 mM.

pH and temperature: The optimum pH and temperature for the production of chitinase were determined in the optimal medium by varying the pH of the medium and incubation temperature. The results indicated that extracellular chitinase activity began at pH 5 and achieved its maximum level at pH 8 (Fig. 6).

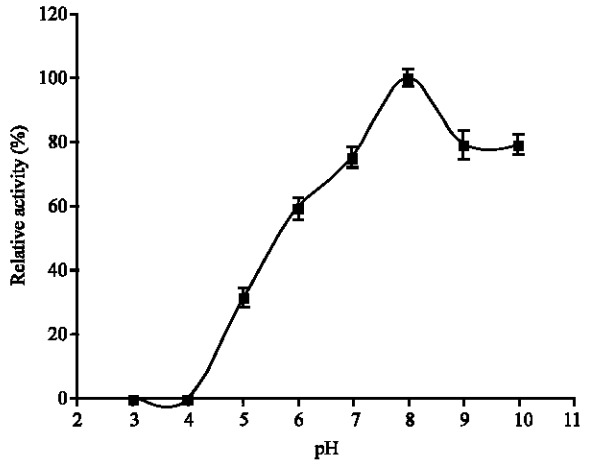

Fig. 6 Effect of pH on chitinase production by *Aeromonas* sp. JK1. The pH of production medium was adjusted before cultivation

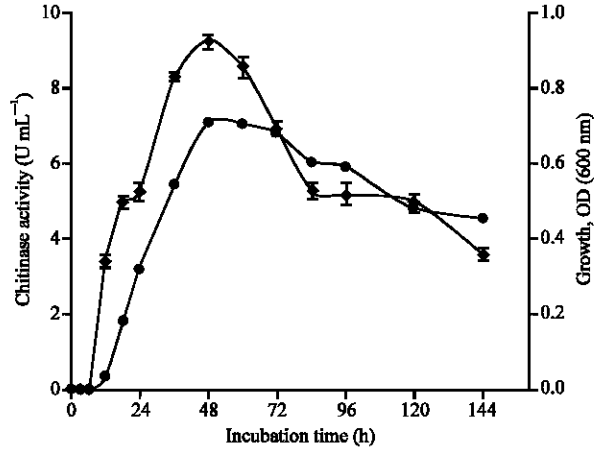

Fig. 7: Total chitinase production and cell growth by *Aeromonas* sp. JK1

Although, *Aeromonas* sp. JK1 was very sensitive to acidic pH and enzyme production at acidic pH was very low, the ability of *Aeromonas* sp. JK1 to produce chitinase in a wide range of pH, especially in alkaline pH, was relatively high. Chitinase production at pH 6 and 10 was 60 and 80% of the optimized pH. *Aeromonas* sp. JK1 gave highest yield of chitinase at 30°C. The chitinase yield of isolate at this temperature was more than two-fold higher than at 37°C and 18% more than 25°C (data not shown).

Cell growth and chitinase production: The production of extracellular chitinase was monitored during the growth of *Aeromonas* sp. JK1 (Fig. 7). The organism started enzyme production at 12 h of incubation and produced maximum enzyme at 48 h and the organism attained its stationary phase of growth after 48 h. This result indicated that the chitinase production started from the beginning of the logarithmic phase and increased linearly with the growth up to 48 h.

DISCUSSION

Microbial production of chitinase has captured the worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method. In this study a chitinase producing microorganism was isolated from the waste of shrimp shells sample collected from local area; Poonel, Gilan Province in northern Iran. Taxonomic studies showed that strain JK1 belonged to the genus *Aeromonas*. *Aeromonas* sp. is one of the most efficient bacteria for the degradation of chitin and several chitinases from *Aeromonas* sp. have been reported and studied (Chang *et al*., 2004; Guo *et al*., 2004; Huang *et al*., 1996; Kojima *et al*., 2005). In addition to other *Aeromonas* species reported in the literature, the locally isolated *Aeromonas* sp. JK1 found to have a good chitinolytic potential as evident from its growth and chitin hydrolysis on colloidal chitin agar. The ratio of the zone of chitin hydrolysis to the colony diameter was high, indicating a better diffusibility of the chitinolytic enzymes. The study of various factors influencing the chitinase production by *Aeromonas* sp. JK1 revealed that chitin was the best carbon source for the chitinase production and the best time for addition of chitin for induction the chitinase production was at the beginning time of the culture (data not shown). These results indicated that the chitinase is an inducible enzyme. The same observation was reported for *Aeromonas schubertii* (Guo *et al*., 2004), *Microbispora* sp., (Nawani *et al*., 2002), *Alcaligenes xylosoxydans* (Vaidya *et al*., 2001) and *Aspergillus fumigatus* NCPF 2140 (Escott *et al*., 1998). According to the results obtained addition of glucose to the chitin medium repressed chitinase production and the same results was reported by Miyashita *et al*. (1991). In contrast an enhancing effect of glucose on chitinase production was observed by Bhushan (1998) when glucose was used with chitin in production medium.

Regarding the effects of various nitrogen sources, the results showed that ammonium sulfate (0.15% w/v) was the most favorable nitrogen source for chitinase production, while other ammonium salts had a repressive effect. Results from the present study are supported by previous reports (Rattanakit *et al*., 2002), where among the nitrogen sources added to the basal medium, ammonium sulfate was most effective in increasing the amount of

chitinase production by *Aspergillus* sp. Sl-13 whereas peptone, yeast extract and urea had a repressive effect. With the use of the optimal culture composition, the effects of the initial pH, temperature and cultivation time on the production of chitinase were also investigated. Maximum chitinase production was achieved at pH 8 and 30°C. It is in agreement with the chitinase producer *Aeromonas* sp. GJ-18 as reported by Kuk *et al.* (2005) with maximum chitinase production at 30°C. The pH of the medium increased progressively during fermentation from the initial value to the optimum pH 8 (data not shown), probably due to the catabolism of NAG liberated by chitin hydrolysis (Forage *et al.*, 1985; Wang *et al.*, 1979). Also, the effect of cultivation time on the production of chitinase was investigated by monitoring the enzyme activity every 24 h till 5 days. The incubation time to achieve maximum chitinase production was decreased from 72 h in the initial medium to 48 h, in an improved (optimum) medium (data not shown). Wang and Hwang (2001) reported the production of chitinase by *Bacillus cereus*, *B. alvei* and *B. sphaericus* were all highest at 2 days while Sandhya *et al.* (2005) reported maximum production of chitinase by *Trichoderma harzianum* at 72 h.

With respect to present results and comparison with our best knowledge about other chitinase producers, this isolate has capability for production of chitinase on an industrial scale. This microorganism may be useful for treatment of chitinous waste and also for production of different products of hydrolyzed chitin for various applications. It would be interesting to study the molecular level and structural features of the chitinase enzyme of this isolate in future.

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