Chitinase Isolated from Water and Soil Bacteria in Shrimp farming Ponds

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Abstract

Chitinases have received attention because of their wide applications in the medicine, biotechnology, agriculture, waste management and industrial applications such as food quality enhancer and biopesticide. Excessive use of insecticides has led to several problems related to pollution and environmental degradation. In this study, isolation and identification of native bacterial strains with chitin hydrolysis activity, took place from water and soil of shrimp culture ponds in Bushehr and Abadan. To investigate the capacity of our chitinase for using in insecticide, biochemical properties of selected chitinase obtained in this research were compared to that of produced by *Bacillus cereus* p-1, isolated from an insecticide tablet. In this research, three mesophilic strains containing: *Serratia marcescens* B4A, *Citrobacter freundii* B1A and *Bacillus cereus* B3R were isolated. Results showed a 1600 bp band corresponding to chitinase gene. The similarity between temperature and pH profile and stability of chitinase was extracted from native bacteria and ones was obtained from *Bacillus cereus* p-1 implied that chitinase extracted from *Serratia marcescens* B4A has potential application in industry.

Keywords: Chitinase, Chitin, Shrimp pond, Bacteria

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Introduction

Chitin is the second source of natural organic compound on the earth after cellulose. This long biopolymer contains N-acetyl D-glucosamine (GLcNAc) monomer from covalent ß-1, 4 linkages. Chitin occurs mainly as a structural component in the exoskeleton of crustaceans, mollusk shells and insects. It is also found to lesser extents in other animals, plants, fungi and bacteria (Ikeda et al., 2009; Lee et al., 2009). Shrimp culture was started in Iran in 1994 (Kakoolaki et al., 2010) and Indian white shrimp, has been considered as the main endemic culture species so shrimp culture ponds are one of the most plentiful chitin resources (Emadi et al., 2010; Zhang et al., 2010). Production of chitin from shell of shrimp, crab, lobster and *Artemia urmiana* has been reported in Iran (Asadpour et al., 2003).

Chitinases, which hydrolyze chitin, are largely found in nature, for example, in stomachs of fish (Gutowska et al., 2004; Molinaria et al., 2007) and the livers of squid (Matsumiya et al., 1998; Matsumiya et al., 2002). Recently, the different applications for chitinase have been discovered, such as: biocontrol of fungal diseases in plants (Demarco et al., 2000; Chang et al., 2003), using in biopesticides (Mendonsa et al., 1996), production of single cell protein from shellfish waste (Raveh and Carrod, 1981; Vyas and Deshpande, 1991), isolation of protoplast from fungi (Dahiya et al., 2005), production of chitooligosaccharides, glucosamine and GLcNAc by chitinase extracted from *Burkholderia cepacia* TU09 for the hydrolysis of chitin (Pichyangkura et al., 2002) and medical application (Dahiya et al., 2006). The chitinase has been extracted from number of microorganisms such as *Trichoderma harzianum* 8 (Seyedasli et al., 2004), *Bacillus subtilis* SG2 (Khorramzadeh et al., 2005) and *Trichoderma atroviride* PTCC5220 (Harighi et al., 2006) in Iran.

The ability of chitinase for digesting insect chitin raises the idea of using it for controlling insects (Mendonsa et al., 1996).

The aim of this research was a comparison between biochemical properties of native bacterial strains isolated, with commercial ones in terms of their chitinolytic activities. These species with chitinolytic activity were isolated from soil and water; the desired samples that hydrolyze chitin effectively, were belong to *Bacillus* sp., *Enterobacter* sp., *Aeromonas* sp. and *Serratia* sp. respectively. These bacteria can be used to control plant pathogenic fungi and biopesticides (Dahiya et al., 2006).

Materials and methods

Chemical compounds such as 3, 5-dinitrosalicylic acid (DNS), N-acetyl D-glucosamine and Bovine Serum Albumin (BSA) were obtained from sigma (St. Louis, Mo. USA). Taq DNA polymerase and 1 kb DNA ladder were obtained from Cinna Gene and Fermentas, respectively. All other chemicals were purchased from Merck (Darmstadt, Germany) and were reagent grade.

The modified method of Takiguchi (1991) was used for obtaining chitin powder (Fig. 1). Shrimps (*Penaeus*

indicus) were obtained from the fresh hunting and then shells were isolated, cleaned, washed and dried. For elimination of mineral, dried shells were kept in HCl 10% for 24 h. After washing, NaOH was added for 24 h. Then shells were dried again and powdered (Fig. 1).

Figure 1: Chitin powder extracted from shrimp shells.

Commonly, colloidal chitin is used as a water insoluble substrate for studying of chitinase. Colloidal chitin was prepared by the modified method of Roberts and Selitrennikoff (1998). 12 g of chitin powders that obtained from shrimp shells was poured quietly into 380 ml of HCl and kept at 4 ˚C overnight on stirrer. Then 4 L of ice-cold 95% ethanol was added to mixture and left at 4 ˚C with vigorous stirring overnight. Centrifugation at 5000 g for 20 min at 4 ˚C was done to collect the sediment. The precipitate washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0).

Microorganisms isolated from shrimp farming soils, water and wastewater at different locations in southern part of Iran. All morphological contrasting colonies were purified by streaking in Animal & Marine Biotechnology Lab in National Institute of Genetic Engineering and Biotechnology (NIGEB). Sampling was accomplished in June and October 2008. (Fig. 2a).

At the time of sampling, the important physical and chemical parameters of water such as salinity, temperature, dissolved oxygen and pH were measured (YSI MODEL 63) at three part of each pond in morning and evening. Some of the isolated microorganism from water and wastewater of shrimp culture ponds was shown in Fig. 2b.

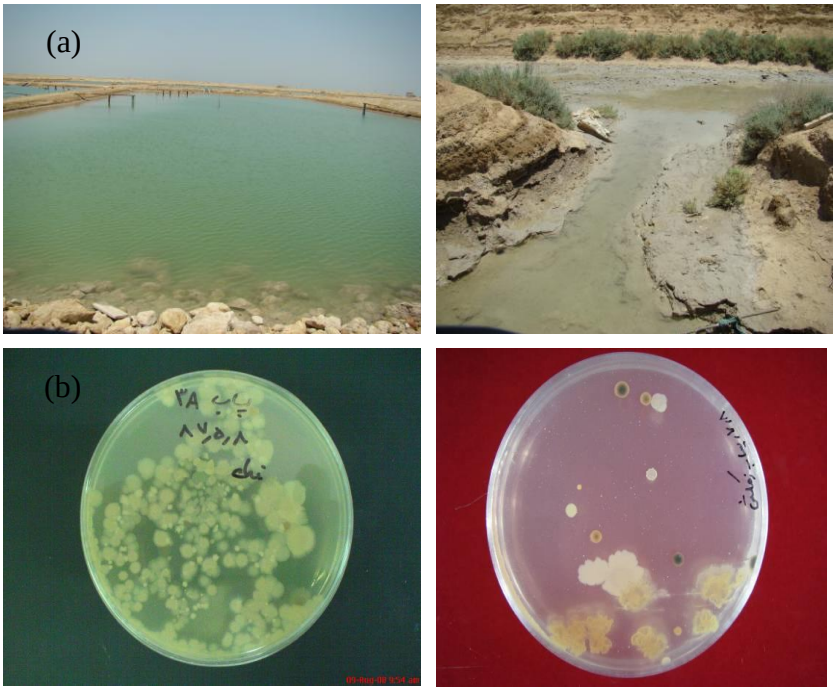

Figure 2: (a) Shrimp culture ponds and their wastewater as a sampling regions. (b) Some of microorganisms isolated from soil, water and wastewater.

Nutrient agar (N.A) for isolation and maintenance contained nutrient agar (2% w/v) supplemented with 0.1% w/v chitin (pH 7.5). For primary screening culture medium contained chitin 5 g; peptone 0.3 g; yeast extract 0.3 g; K_2HPO_4 0.7 g; KH_2PO_4 0.3 g; $MgSO_4$.$7H_2O$ 0.5 g; Agar 15 g; NH_4NO_3 2 g; NaCl 1 g in 1 lit H_2O. To determine the chitinase production on agar plates, 1% of chitin was incorporated in a buffered agar solution. Clear zone reactions produced by chitinolytic bacteria were measured after routine casting the gels onto Petri dishes (Dingle et al., 1953). For preculture, medium contained nutrient broth 8 g; malt extract 10 g; peptone 10 g; chitin 5 g; NaCl 1 g in 1 lit H_2O. Liquid culture medium for chitinase production contained chitin 5 g; peptone 0.3 g; yeast extract 0.3 g; K_2HPO_4 0.7 g; KH_2PO_4 0.3 g; $MgSO_4.7H_2O$ 0.5 g; $(NH4)_2SO_4$ 1 g; NaCl 1 g; $MnSo_4.2H_2O$ 16 mg; $ZnSo_4.7H_2O$ 14 mg; $FeSo_4.6H_2O$ 50 mg; $Cacl_2$ 20 mg in 1 lit H_2O.Solutions that used in measurement of chitinase activity were (Miller et al., 1959): (1) Enzyme solution: the selected strain was cultured in the production medium for 48 h at 30 ˚C and then solution was centrifuged and supernatant was collected. (2) 1% w/v colloidal chitin solution: for obtaining this solution 50 ml colloidal chitin was added to 50 ml phosphate buffer 20 mM (pH 7.2). (3) Phosphate buffer 20 mM (pH 7.2). (4) Color solution of 3, 5-dinitrosalicylic acid (DNS): for preparing this, 1 g of DNS was added to 50 ml distilled water on stirrer and then, 30 g potassium sodium tartrate was added slowly. 1.6 g NaOH was dissolved in 20 ml water previously. This solution was also added to the previous solution. For

enzyme assay, 0.5 ml of enzyme solution was added to 0.5 ml substrate (colloidal chitin). In blank sample 0.5 ml phosphate buffer was added instead of enzyme solution. Then samples were placed in 45 ˚C for 1 h. The reaction was stopped by addition of 3 ml DNS. The reagents were placed in boiled water for 5 min. After centrifugation, the absorbance of the supernatant was measured at 530 nm (Miller, 1959). In Fig. 3 changing color of DNS by enzymes was shown.

For determination of enzyme activity standard curve was drawn. At first, different and consecutive concentrations of N-acetyl D-glucosamine (From 0 to 50 mM) were prepared. 1 ml DNS was added to 1 ml of above solutions and placed in 45 ˚C bathwater. Then enzyme unit was calculated by using the below formula;

Unit = µ mol N- acetyl D-glucosamine released / time (min)

By definition, one unit (U) of the chitinase activity equals as an amount of enzyme required to produce one mmol of reducing sugar per minute.

The protein concentration of unpurified and partially purified chitinase was determined by Branford's method (Bradford, 1976) using 1 mg/ml bovine serum albumin (BSA) as a standard solution (table 1). In this method, 100 mg Coomassie Brilliant Blue G250 was dissolved in 50 ml 96% ethanol and then 100 ml 85% phosphoric acid was added. Final volume of the solution was received to 1 lit by distilled water. The absorbencies of solution were measured at 595 nm. The following reagents were added to the PCR tube for further reaction (Table 2).

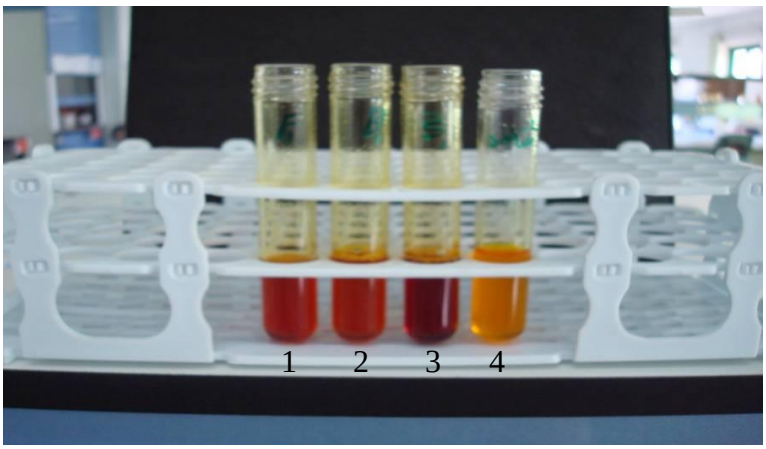

Figure 3: Enzyme assay with DNS method. Tube 4 was blank and 1 to 3 was samples. Chitinase extracted from selected strain made strong color in tube 3.

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Table1: Solution which used to protein determination

Tube / Solution (µl)	1	2	3	4	5	6	7	8	9	Unknown protein
Standard protein	10	20	30	40	50	60	70	80	90	
Distilled water	90	80	70	60	50	40	30	20	10	
Sample										100
Introducer (ml)	5	5	5	5	5	5	5	5	5	5
Protein concentration	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	?

Chitinase gene was amplified using below primers:
Forward: (5'- ATG CGC AAA TTT AAT AAA CCG CTG- 3') and Reverse (5'- TTA TTG AAC GCC GGC GCT ATT GCC- 3').

Table2: Reagents which used to PCR amplification

Reagent	Volume / Concentration
PCR buffer	2.5 µl
$MgCL_2$	1.5-2 mM
dNTP	0.5 mM
Distilled water	10 µl
DMSO	2.5 µl
Forward primer	0.5 pmol/µl
Reverse primer	0.5 pmol/µl
Template DNA	1 µg
Taq DNA polymerase	2.5 unit

PCR amplification was conducted according to the thermal cycle of; 1 cycle of 94 ˚C for 5 min: 35 cycle of 94 ˚C for 30 s, 55 ˚C for 60 s and 72 ˚C for 1.5 min: final extension for 5 min at 72 ˚C.

To determine temperature and pH profile and stability of *Bacillus cereus* isolated from an insecticide tablet, one insecticide tablet was dissolved in preculture medium for 18 h at 30 ˚C on a shaker incubator (200 rpm). Microorganisms that growth in preculture medium, were cultured in nutrient agar plates and agar plates contain 0.5% chitin, 0.03% peptone, 0.03% yeast extract, 0.07% K_2HPO_4, 1.5% agar, 0.1% NaCl and 0.1% v/v trace elements. One strain that growth on agar plate with chitin was cultured in the production medium and used for enzyme assay, temperature profile, temperature stability, pH profile and pH stability.

The results are the average of at least three repeated experiments in a typical run in order to confirm reproducibility.

Results

In this research, nearly 300 g chitin powders were extracted from 1 kg shrimp

shells. The average of important physical and chemical parameters of water such as salinity, pH, temperature and dissolved oxygen was tabulated in Table 3. ANOVA statistical test didn't show any significant differences between water physical and chemical parameters in 2 sampling areas. Biochemical and microbiological analysis was performed to characterize some screened strains (Table 4). Qualitative cup-plate assay for chitinase production indicated that three strains: *Serratia marcescens* B4A, *Citrobacter freundii* B1A and *Bacillus cereus* B3R were the most active strains (Fig. 4). In the other words the above-mentioned strains consumed chitin at a higher rate, and produced a clear zone with larger diameters (Fig. 4). Therefore some of them were selected for further studies.

Table 3: Physicochemical parameters of ponds water

Place of sampling / Physical - chemical index	Abadan (Choebdeh)	Bushehr (Helleh)
Salinity of No. 1 pool (ppt)	35.5	49.2
Salinity of No. 2 pool (ppt)	36.0	48.6
Salinity of No. 3 pool (ppt)	32.8	48.2
Temperature of No.1 pool (°C)	25.8	33.5
Temperature of No.2 pool (°C)	25.5	33
Temperature of No.3 pool (°C)	25.9	33.5
Oxygen of No.1 pool (ppm)		
Morning	3.8	3.7
Evening	5.5	5.4
Oxygen of No.2 pool (ppm)		
Morning	3.5	3.3
Evening	5.8	5.9
Oxygen of No.3 pool (ppm)		
Morning	3.6	3.4
Evening	5.8	5.6
pH of No.1 pool		
Morning	8.4	8.4
Evening	8.6	8.5
pH of No.2 pool		
Morning	8.3	8.4
Evening	8.5	8.6
pH of No.3 pool		
Morning	8.3	8.5
Evening	8.6	8.6

Table 4: Biochemical properties of some screened bacteria

Species / Biochemical properties	*Citrobacter freundii* **B1A**	*Bacillus cereus* **B3R**
Indole production	*	*
Methyl red (MR)	+	*
Voges proskauer (VP)	-	*
Citrate consumption	+	-
H_2S	-	*
Hydrolysis of urea	-	-
Motion in 36 ˚C	+	+
Hydrolysis of gelatin at 22 ˚C	-	+
Glucose (Formation of gas)	+	+
Fermentation of lactose	+	*
Fermentation of sucrose	*	*
Fermentation of mannitol	+	*
Fermentation of dulcitol	-	*
Fermentation of sorbitol	+	*
Fermentation of arabinose	+	*
Fermentation of xylose	*	-
Fermentation of galactose	*	-
Ortho-nitrophenyl-β-D-galactopyranoside (ONPG)	*	*
Lysine decarboxylase	-	*
Ornitin decarboxylase	*	*
Haemolysis	*	+
Growth at 37 ˚C	*	+
Growth at 50 ˚C	*	+
Growth in 10% w/v NaCl	*	-

–: 10% strains were positive

+: 90%-100% strains were positive

*: this test didn't do.

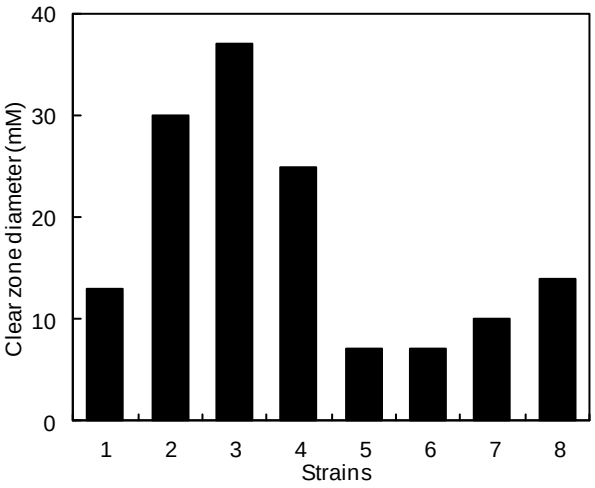


Figure 4: Clear zone diameter (mm). Qualitative cup-plate assay for chitinase production in some isolated strains was detected that 3 strains: number 2 (*Citrobacter freundii* B1A), 3(*Serratia marcescens* B4A) and 4(*Bacillus cereus* B3R) that showed much more activity from others (1, 5, 6, 7 and 8 are unknown bacteria).

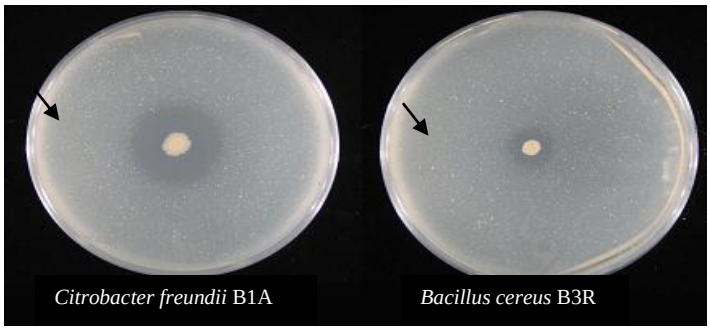


Figure 5: Comparison of diameters of clear zone produced by some isolated chitinolytic bacteria (*Citrobacter freundii* B1A and *Bacillus cereus* B3R).

Temperature, pH profile and temperature, pH stability of *Serratia marcescens* B4A chitinase (Zarei et al., 2010) were compared by chitinase produced by *Bacillus cereus* p-1 that isolated from an insecticide tablet for probable capacity of *S. marcescens* chitinase for using in insecticide. The commercialized chitinase had the optimum temperature 50 ˚C. The enzyme was completely inactivated at 75 ˚C (Fig. 6a). This chitinase was stable under 50 ˚C for 20 min (Fig. 6b). The optimum pH for activity of the enzyme was measured 5 (Fig. 6c). Chitinase was stable at pH 3 to 10 for 90 min at 25 ˚C (Fig. 6d). Analysis of amplified *Serratia marcescens* B4A chitinase DNA on 1% agarose gel was shown in Fig. 5. As we see in Fig. 7, size of chitinase gene was about 1600 bp.

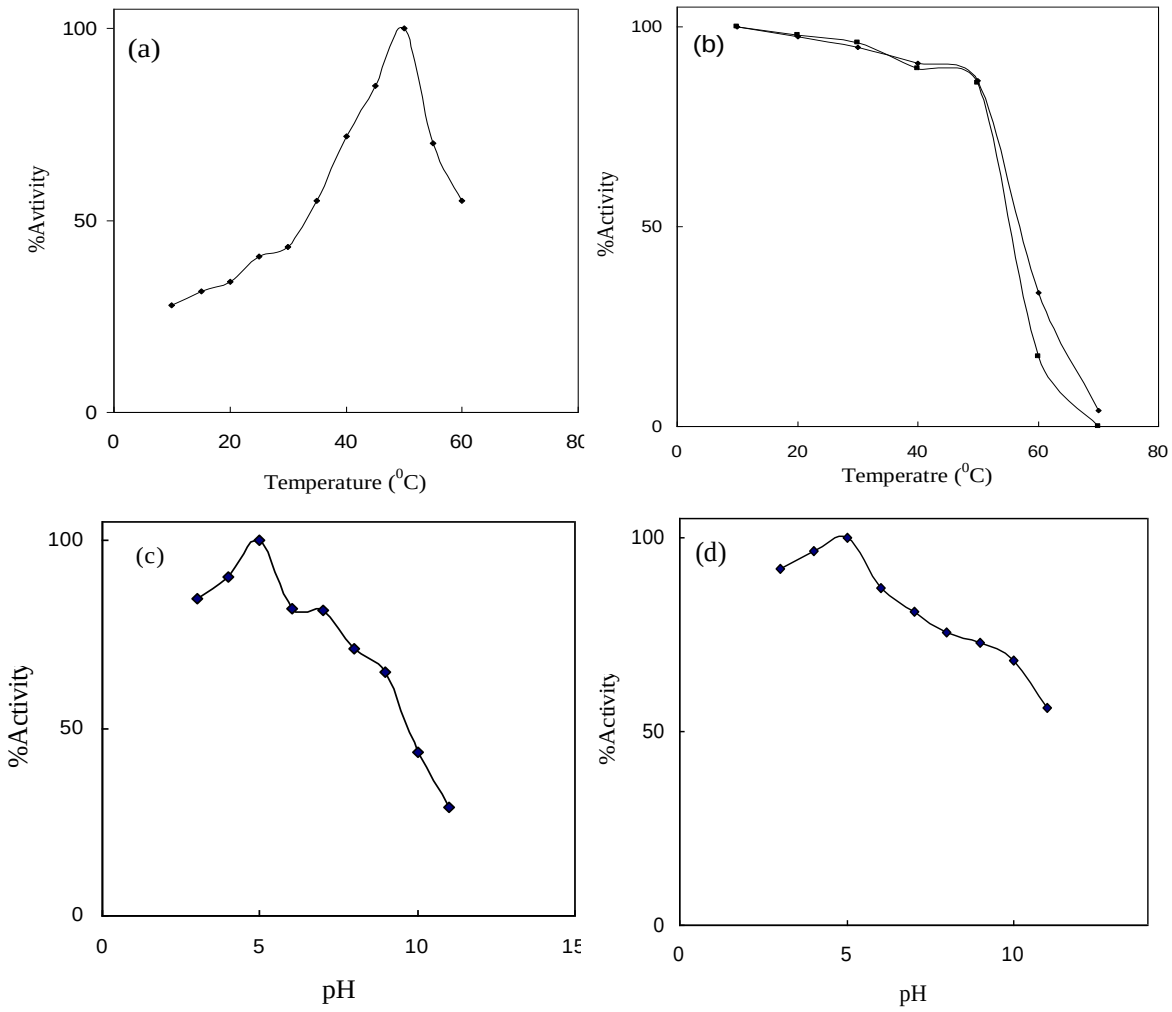
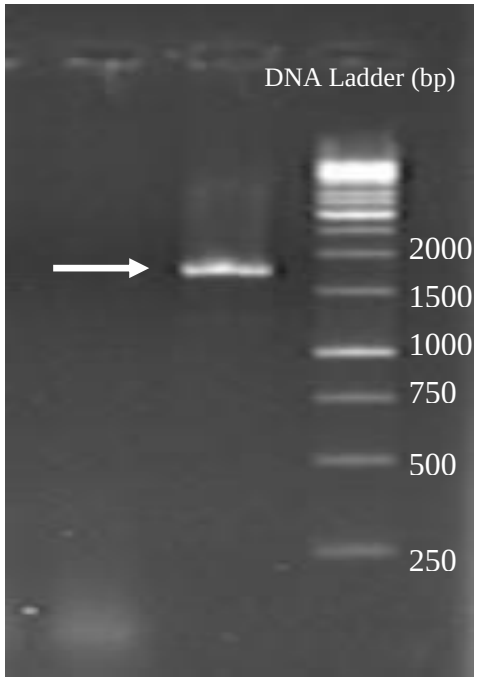


Figure 6: (a) Temperature profile of enzyme (produced by *Bacillus cereus* P-1 in insecticide tablet) in 20 mM phosphate buffer, pH 5.0. (b)Temperature Stability of enzyme (produced by *Bacillus cereus* P-1) at 10 (♦), and 20 (■) minute from 10 to 60 ºC. (c) Effect of pH on enzyme (produced by *Bacillus cereus* P-1) activity at 25 ºC. A mixture of glycine, acetate, and phosphate buffer at a concentration of 50 mM was used. (d) pH stability of enzyme (produced by *Bacillus cereus* P-1) at pH 3.0-11.0 for 90 min at 25 ºC.



Serratia marcescens B4A amplified chitinase gene

Figure 7: Analysis of amplified DNA on 1% agarose gel.

Discussion

In this research, 3 mesophilic strains, *Serratia marcescens* B4A, *Citrobacter freundii* B1A and *Bacillus cereus* B3R was isolated from Bushehr and Abadan ponds. Considering Table 3 and the fact that sampling from Bushehr ponds was done in June and temperature of water was relatively high, it seems that sampling season has not relation to finding the thermostable strains.

The number of chitin scientific reports in the 1990s were very high, for example, in 1998 more than 260 articles about chitin has been published in science direct (Khor, 2002). This can confirm the ever-increasing importance of this biopolymer. Approximately, 75% of the total weights of shellfish, such as shrimp, crab and krill is considered waste, and chitin comprises 20 to 58% of the dry weight of the said waste (Wang and Chang, 1997). There is a great supply of crustacean's shells in Iran, which often burned or reminded in nature useless (Asadpour et al., 2003). Extraction of chitin from shrimp shells has been done previously in Iran but its efficiency to change to colloidal state has not been investigated. Therefore, the performance for extraction of chitin from crustacean's shells in a semi-industrial scale, in addition to environmental advantages, the fisherman in south of Iran can improve their economical condition.

As it is shown in Fig. 7, the size of chitinase gene was almost 1600 bp.

Molecular weight of partial purified chitinase was estimated by SDS-PAGE to be almost 54 kDa (Zarei et al., 2011). This was shown the homogeneity of partial purified chitinase and synthesis of chitinase gene.

Based on Fig. 6c, the optimum pH for activity of the *Bacillus cereus* p-1 chitinase is 5 and for *Serratia marcescens* B4A is also 5 (Zarei et al., 2010). Both chitinases were stable at pH 3 to 10 for 90 min at 25 °C (Fig. 6d). The optimum temperature of *Bacillus cereus* p-1 and *Serratia marcescens* B4A for chitinase production was 50 °C (Fig. 6a) and 45 °C (Zarei et al., 2010), respectively.

The similarity between temperature and pH profile and stability of these two strains determined that chitinase extracted from *Serratia marcescens* B4A has potential use in insecticide. Further work on the application of this enzyme and its economic/commercial feasibility is currently underway.

Acknowledgements

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