

A novel halo-alkali-tolerant and thermo-tolerant chitinase from *Pseudoalteromonas* sp. DC14 isolated from the Caspian Sea

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Abstract A novel halo-alkali and thermo-tolerant chitinase was obtained from an isolated strain found in the Caspian Sea. The effects of media composition and various fermentation conditions for the optimization of chitinase production were studied one factor at a time and by response surface methodology. The novel strain, which is designated as strain DC14 and phylogenetically related to the genus *Pseudoalteromonas*, produced chitinase after 72 h under the following optimal conditions: glucose 1 % (w/v), ammonium sulphate 0.2 % (w/v), chitin 1.07 % (w/v), pH 8, NaCl 10 % (w/v), inoculum size 2.5 % (v/v), temperature 30 °C, CaCl₂ 3 mM and MgCl₂ 4 mM. Using the statistical optimization method, chitinase production was found to increase from 2.30 to 21.90 U/dl. The enzyme showed maximum activity at 40 °C, pH 9 and 10 % NaCl. It was stable in a wide range of temperature from 15 to 65 °C, pH from pH 7 to 11 and NaCl concentration from 0 to 15 % (w/v). The molecular weight of the enzyme was estimated by SDS-PAGE to be about 65 kDa. With regard to the halo-alkali and thermo-stable properties of this enzyme, it has potential industrial activity.

Keywords Chitinase · *Pseudoalteromonas* · Extremozyme · Caspian Sea

Introduction

Chitin, a poly-β(1,4) linear-linked *N*-acetylglucosamine (GlcNAc), is the second most abundant biopolymer on earth after cellulose (Khoushab and Yamabhai 2010). Chitin polymers are mostly present in the exoskeleton components of arthropods and in the cell walls of fungi (Herring 1979; Souza et al. 2011). Chitinases (E.C. 3.2.1.14) are a group of enzymes capable of hydrolysing insoluble chitin to its oligo (chitosan and chitobiose) and monomeric (*N*-acetylglucosamine) components (Bhattacharya et al. 2007). Chitinases occur in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants and animals for offensive, defensive, morphogenetic or nutritional purposes (Park et al. 1997; Dahiya et al. 2006).

Although chitin can be obtained as a cheap renewable biopolymer, especially from marine sources, the chitinase hydrolytic products are an expensive and valuable natural material for a wide variety of biotechnological and industrial applications (Dahiya et al. 2006; Chen et al. 2010). Moreover, chitinases have attracted interest as biocontrol agents due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton (Tsujibo et al. 2003; Mostafa et al. 2009). It has been estimated that the annual production of chitin in aquatic systems is roughly equal to 10¹⁰–10¹¹ metric tons (Beier and Bertilsson 2013). In spite of the continuous production of chitin in the marine ecosystems, there is a low level of chitin accumulation in these environments (Alldredge and Gotschalk 1990). Chitinolytic bacteria, which are very abundant and widely distributed in marine environments, are responsible for

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rapid degradation of chitin in aqueous systems (Keyhani and Roseman 1996). Accordingly, there are numerous reports about the isolation of bacterial chitinase from marine environments (Tsuji et al. 1998; Ito et al. 2007; Stefanidi and Vorgias 2008). Because most industrial processes are carried out in harsh physicochemical conditions, which may not be definitively adjusted to the optimal conditions required for the activity of the available enzymes, it would be of great value to have enzymes that demonstrate optimal activities in wide ranges of salinity, temperature and pH values.

In this paper, we describe the characteristics of a haloalkali and thermo-tolerant chitinase obtained from *Pseudalteromonas* sp. DC14 isolated in the Caspian Sea and its production optimization.

Materials and methods

Microorganism isolation and identification

Sampling was carried out at 30 m depth in a southern region of the Caspian Sea (36.46°N 51.02°E). A solid media was applied for the isolation procedure, which contained (g/l): $(\text{NH}_4)_2\text{SO}_4$, 0.86; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.009; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.22; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17; KCl, 0.13; NaCl, 9.1; peptone, 10; yeast extract, 5; glucose, 2; and agar, 15. The pH of the medium was adjusted to 8.2 with 2 M NaOH. Inoculated plates were incubated at 20 °C for 2 months. After successive cultivation, a pure isolate was obtained. The bacterial isolate was maintained on the slant above-mentioned solid media at 4 °C and broth media supplemented with 30 % (v/v) glycerol at –80 °C for short and long preservation, respectively. The genomic DNA of the strain was extracted by DNA extraction kit (Thermo Scientific, Lithuania) according to the manufacturer's recommended procedure, and the 16S rRNA gene was amplified using the bacterial universal primers 27F and 1492R (Lane et al. 1985). The sequencing was conducted on an ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). Phylogenetic analysis was performed using the software package MEGA version 6 (Tamura et al. 2013).

Preparation of colloidal chitin and primary screening

Colloidal chitin was prepared from purified chitin (Merck) by the modified method of Roberts and Selitrennikoff (1988). Briefly, 5 g of chitin powder was slowly added to 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. The precipitation was collected by centrifugation and washed with 0.1 M

sodium phosphate buffer (pH 7) until the pH of the colloidal chitin became neutral. Qualitative chitinase assay was performed by spot inoculation of the bacterial strain on the solid isolation media containing 1.5 % (w/v) colloidal chitin. The chitinase activity was indicated by the clear zone around colonies after incubation for 2 weeks at 20 °C.

Measurement of chitinase production

Chitinase production was assayed with colloidal chitin as a substrate. The culture medium contained (g/l): $(\text{NH}_4)_2\text{SO}_4$, 0.86; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.009; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.22; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17; KCl, 0.13; NaCl, 9.1; yeast extract, 2; and colloidal chitin, 1.5 % (v/v). The pH was 8.2. The fermentation process was conducted in a 50-ml Erlenmeyer flask at 20 °C and 150 rpm for 72 h. The cultures were centrifuged at $10,000 \times g$ for 15 min at 4 °C, and the supernatant (crude enzyme solution) was used for chitinase assay. Enzyme solution (0.3 ml) was added to 0.3 ml of substrate solution, which contained a 0.5 % suspension of colloidal chitin in 100 mM sodium phosphate buffer (pH 8.0). The mixture was incubated at 30 °C for 45 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by Schales' method (Imoto and Yagishita 1971) and the dinitrosalicylic acid (DNS) method (Reissig et al. 1955) with *N*-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per minute.

Experimental design for enzyme optimization

To understand the effects of various conditions on the amount of chitinase production and identify the most important factors, a conventional 'one-factor-at-a-time' approach was applied. For this method, in each experiment we vary only one factor, while keeping others fixed. Various concentrations of NaCl (0–15 % w/v); different carbon sources (1 % w/v): glucose, fructose, maltose, saccharose and starch; different nitrogen sources (0.2 % w/v): peptone, yeast extract, casein, urea and ammonium sulphate; chitin substrate (0–5 % w/v); various concentrations of pH (6.0–10.0), inoculum size (0.5–4.5 % v/v), temperature (15–35 °C); and metal ions (0–4 mM) of calcium, magnesium, zinc, manganese and copper were chosen as the critical variables. To evaluate the combinatorial effects of variables and optimum condition for the chitinase production, Box–Behnken response surface methodology (RSM) was applied. Optimization studies were carried out by studying the effect of four major variables including NaCl, chitin, pH and temperature at three levels. The behaviour of the system is explained by the following empirical second-order polynomial model:

Table 1 Coded and actual values for Box–Behnken design

Independent variables	Range and level		
	–1	0	1
NaCl (% w/v, X_1)	3	6.5	10
Chitin (% w/v, X_2)	0	0.75	1.5
pH (X_3)	6	7	8
T ($^{\circ}\text{C}$, X_4)	20	25	30

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_{12}^2 + \beta_{22} X_{22}^2 + \beta_{33} X_{32}^2 + \beta_{44} X_{42}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4.$$

where Y is the predicted enzyme activity; x_i, x_j, \dots, x_k are the input variables, which affect the response Y ; $x_i^2, x_j^2, \dots, x_k^2$ are the square effects; $x_i x_j, x_i x_k$ and $x_j x_k$ are the interaction effects; β_0 is the intercept term; β_i ($i = 1, 2, \dots, k$) is the linear effect; β_{ii} ($i = 1, 2, \dots, k$) is the squared effect and β_{ij} ($i = 1, 2, \dots, k; j = 1, 2, \dots, k$) is the interaction effect. Coded levels for independent variables are presented in Table 1.

Characterization of partially purified chitinase

Optimized culture supernatants (crude enzyme) were spun in a centrifuge at $12,000 \times g$ for 20 min (4°C) and then collected for ammonium sulphate precipitation (70 %, w/v). The precipitates were formed, kept overnight and collected by centrifugation at $12,000 \times g$ for 20 min (4°C). They were then dissolved in a small amount of 50 mM Tris buffer (pH 8.0) and dialysed against the same buffer. Concentration of protein was estimated based on Bradford's method using bovine serum albumin (BSA) as standard protein.

Effects of salinity on enzyme activity and stability

The optimum NaCl concentration for chitinase activity was determined by incubation of the partially purified enzymes with the substrates at the saline buffer that ranged from 0 to 20 % NaCl, under standard assay conditions. Salt stability was measured by pre-incubation of the enzymes without substrates in sodium phosphate buffer (50 mM, pH 8.0) supplemented with 0–20 % NaCl at 4°C temperature for 30 min. Then, the remaining activity was measured under standard conditions.

Effects of temperature on enzyme activity and stability

The optimum temperature for chitinase activity was examined by incubation of the partially purified enzymes

with the substrates at temperatures that ranged from 20 to 60°C under standard assay conditions. Thermal stability was determined by pre-incubation of the enzymes without substrates in sodium phosphate buffer (pH 8.0) for 30 min at temperatures from 20 to 60°C . The remaining activity was measured under standard conditions.

Effects of pH on enzyme activity and stability

The optimum pH of chitinase was investigated by the varied pH of the reaction buffers, ranging from 6.0 to 10.0; 50 mM of each buffer was used: acetate (pH 4.0–6.0), potassium phosphate (pH 7.0–8.0) and glycine–NaOH (pH 9.0–10.0). The enzymes were pre-incubated at various pH buffers that used the above systems at 4°C for 30 min without substrates and then subjected to enzyme assays under standard conditions to determine the pH stability. All experiments were repeated at least three times.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE analysis of proteins is carried out using 12.5 % (w/v) polyacrylamide gel at 120 V for 90 min. Solutions for preparing 12.5 % (w/v) resolving gel included H_2O (6.4 ml), 30 % acrylamide mix (8.3 ml), 1.5 M Tris (pH 8.8, 5 ml), 10 % (w/v) SDS (0.2 ml), 10 % (w/v) ammonium persulphate (0.1 ml) and N,N,N,N' -tetramethylethylenediamine (0.008 ml) (Laemmli 1970).

Results and discussion

Marine environments encompass about 70 % of the earth's surface, with countless marine microorganisms containing biochemical secrets which might provide new insights into and understanding of enzymes (Das et al. 2006). Marine bacteria have been attracting more attention as a resource for new bioactive compounds recently, as the soil-derived compounds have yielded high numbers of the previously described metabolites (Toledo et al. 2006; Bhatnagar and Kim 2010; Trincone 2011). The present study was conducted to optimize a marine bacterium chitinase production and examine its properties.

Taxonomic characteristics of chitinase-producing strain

Strain DC14 was isolated from 30 m depth in the southern region of the Caspian Sea. The isolated strain was a gram-stain-negative, catalase- and oxidase-positive, non-motile rod-shaped bacterium. The clear zone was observed around the colonies on the chitin-containing solid media, which qualitatively indicated its chitinolytic activity (Fig. 1). The

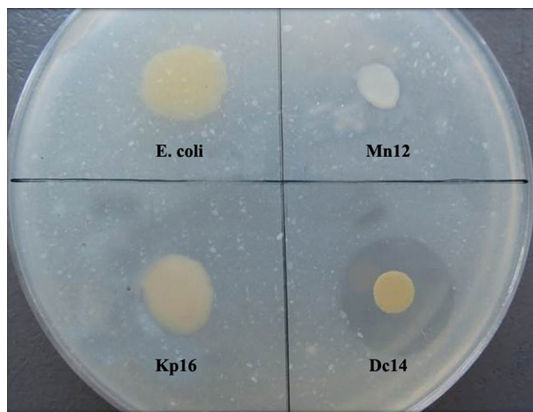


Fig. 1 Assay of chitinolytic activity on plate with solid isolation medium supplemented with colloidal chitin [1.5 % (w/v)]. Clearing zone of chitin utilization formed around the colony of strain DC12 but not around *E. coli* and two non-chitinolytic isolated strains: KP16 and MN12

16S rRNA gene sequence analysis revealed that strain DC14 is a member of the genus *Pseudoalteromonas*. The closest relatives of strain DC14 were *Pseudoalteromonas lipolytica* LMEB39^T, *Pseudoalteromonas donghaensis* HJ51^T and *Pseudoalteromonas shioyasakiensis* SE3^T, with a 16S rRNA gene sequence similarity of 99.1, 98.4 and 97.4 %, respectively. Phylogenetic analysis using the neighbour-joining algorithm supports the view that the novel strain is a member of this genus (Fig. 2). Several bacterial strains from various genera of both soil and marine ecosystems were reported to contain chitinolytic activity. Bacterial strains from *Aeromonas* (Sashiwa et al. 2002), *Alteromonas* (Tsujiibo et al. 1993; Orikoshi et al. 2005), *Arthrobacter* (Lonhienne et al. 2001; Wang et al.

2009), *Bacillus* (Alam et al. 1995; Sampson and Gooday 1998; Yuli et al. 2004), *Enterobacter* (Chernin et al. 1995), *Flavobacterium* (Campbell and Williams 1951), *Microbulbifer* (Howard et al. 2003), *Micrococcus* (Campbell and Williams 1951), *Moritella* (Stefanidi and Vorgias 2008), *Nocardia* (Bansode and Bajekal 2006), *Pseudomonas* (Wang and Chang 1997), *Salinivibrio* (Aunpad and Panbangred 2003), *Serratia* (Horn et al. 2006), *Streptomyces* (Tsujiibo et al. 1993; Tsujiibo et al. 2000; Tsujiibo et al. 2003), *Vibrio* (Fukasawa et al. 1992; Osama and Koga 1995) and *Virgibacillus* (Essghaier 2012) were noted for their chitinolytic activity. Recently, Wang and co-workers reported the isolation of chitinase-producing *Pseudoalteromonas*. That enzyme showed its maximum activity at pH 8 and 20 °C (Wang et al. 2014).

Optimization of chitinase production by the one-factor-at-a-time approach

In order to identify the most relevant factors to chitinase production with *Pseudoalteromonas* sp. DC14, the one-factor-at-a-time method was conducted as described above. The effects of various factors/levels are presented in Table 2. It showed that the key parameters were NaCl (10 % w/v), glucose (1 % w/v), (NH₄)₂SO₄ (0.2 % w/v), chitin (0.5 % v/v), pH 7, inoculums size (2.5 % v/v), temperature 30 °C, CaCl₂ 3 mM, MgCl₂ 3 mM. This resulted in the production of 8.50 U/dl chitinase after 72 h. Minimum amounts of ZnCl₂, MnCl₂ and CuCl₂ completely inhibited the enzyme production. The experimental results clearly showed that the NaCl concentration, nitrogen source, inoculums' size and temperature are the most

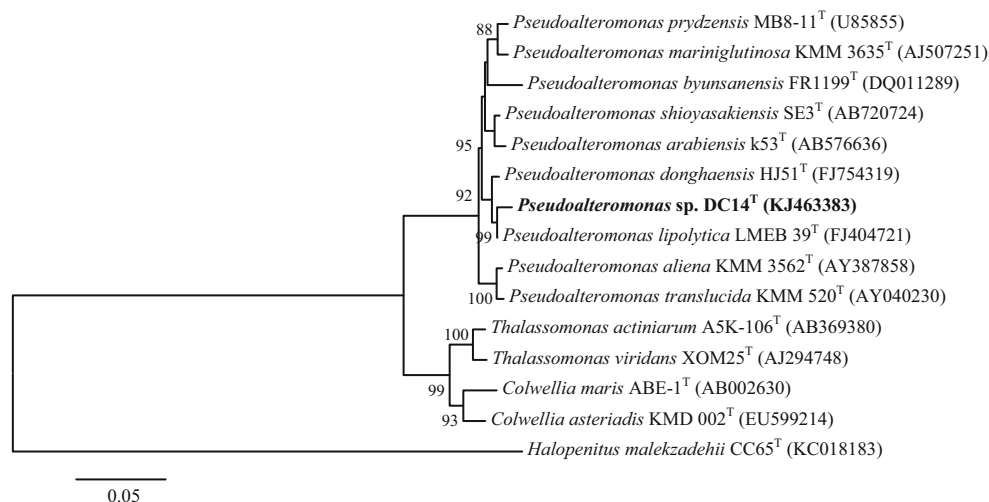


Fig. 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain DC14^T with other members of the genus *Pseudoalteromonas*. Accession numbers of the sequences are given in parentheses. The sequences of the *Halopenitus*

malekzadehii CC65^T (KC018183) was used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Bar 0.05 substitutions per nucleotide position



Table 2 The design and results of OFAT for chitinase production (optimum levels presented in bold)

Runs	Various levels/chitinase activity U/dl (in parentheses)				
NaCl	0	3	7	10	15
(% w/v)	(0)	(0.5 ± 0.01)	(0.7 ± 0.04)	(2.70 ± 0.2)	(1.10 ± 0.1)
Carbon sources (1 % w/v)	Glucose	Fructose	Maltose	Saccharose	Starch
	(3.50 ± 0.1)	(3.00 ± 0.3)	(3.30 ± 0.7)	(3.10 ± 0.3)	(3.00 ± 0.3)
Nitrogen sources (0.2 % w/v)	Peptone	Yeast extract	Casein	Urea	(NH₄)₂SO₄
	(3.20 ± 0.2)	(2.90 ± 0.4)	(2.80 ± 0.3)	(3.20 ± 0.6)	(5.50 ± 0.6)
Chitin	0	0.5	1.5	3	5
(% v/v)	(4.20 ± 0.5)	(5.80 ± 0.5)	(5.00 ± 0.3)	(3.70 ± 0.4)	(3.70 ± 0.3)
pH	6	7	8	9	10
	(4.70 ± 0.4)	(6.00 ± 0.2)	(5.50 ± 0.3)	(5.30 ± 0.4)	(4.50 ± 0.5)
Inoculum size	0.5	1.5	2.5	3.5	4.5
(% v/v)	(0.90 ± 0.02)	(4.00 ± 0.4)	(6.10 ± 0.3)	(5.00 ± 0.3)	(4.50 ± 0.4)
Temperature (°C)	15	20	25	30	37
	(1.00 ± 0.09)	(6.00 ± 0.4)	(6.60 ± 0.2)	(7.20 ± 0.3)	(2.60 ± 0.5)
Metal ions (mM)					
Ca ²⁺	0	1	2	3	4
	(0.0)	(6.90 ± 0.7)	(7.20 ± 0.6)	(8.00 ± 0.3)	(7.60 ± 0.3)
Mg ²⁺	0	1	2	3	4
	(0.30 ± 0.01)	(6.20 ± 0.6)	(7.00 ± 0.5)	(8.40 ± 0.4)	(7.30 ± 0.3)
Zn ²⁺	0	1	2	3	4
	(8.30 ± 0.4)	(4.00 ± 0.6)	(3.60 ± 0.3)	(1.50 ± 0.3)	(0.0)
Mn ²⁺	0	1	2	3	4
	(8.40 ± 0.2)	(0.0)	(0.0)	(0.0)	(0.0)
Cu ²⁺	0	1	2	3	4
	(8.50 ± 0.3)	(0.0)	(0.0)	(0.0)	(0.0)

effective parameters in chitinase production, with 27, 27, 52 and 62 % variations between their levels, respectively. This optimization enhanced the enzyme production more than 3.1 times. However, the OFAT approach has the limitation of ignoring the importance of the interaction of various physiological parameters. The statistical approach using response surface methodology for process optimization serves this purpose by finding out the optimal condition in any given system by a set of independent variables over a specific region of interest through establishing the relationship between more than one variable. It has proven to be a powerful tool in optimizing the fermentation condition (Box and Behnken 1960; Han et al. 2008).

Response surface methodology optimization

The four significant variables selected based on the results of OFAT design (NaCl, chitin, pH and temperature) were optimized using Box–Behnken response surface methodology. Experimental design and the actual yields of chitinase (response) obtained in the experiments are given in Table 3. The highest chitinase activity of 20.0 U/dl was

obtained at the following condition after 72 h (run 4): NaCl 6.5 (% w/v), chitin (0.75 % w/v), pH 8 and temperature 20 °C. Based on the analysis of the results, the predicted optimized culture condition was at NaCl (10 % w/v), chitin (1.07 % w/v), pH 8 and temperature 20 °C. It was predicted to produce chitinase at 23.0 U/dl. The model was validated for the four variables within the optimized condition. The experiment resulted in 22.1 U/dl chitinase production, which was 2.6 times better than that observed with the one-at-a-time method. The ANOVA results of RSM optimization are given in Table 4. The *P* value serves as a tool for checking the significance of each of the coefficients and is indicative of the interaction strength of each independent variable. Four interaction coefficients, including AC (NaCl/pH), AD (NaCl/Temperature), BD (chitin/temperature) and CD (pH/temperature), are significant, as evidenced by low *P* and high *F* values. The response surfaces from the interactions are illustrated in Fig. 3. *R*² value for this model is 0.9795, which indicates that the variability in the observed response can be explained by the experimental parameters and their interactions. The fermentation process at the optimal conditions predicted by this approach resulted in an 8.2 times



Table 3 Experimental design matrix and results of Box–Behnken

Run	Variables					Chitinase activity (U/dl)
	NaCl (% w/v)	Chitin (% w/v)	pH	Temperature (°C)		
1	6.5	0	8	25		4.20 ± 0.3
2	3	0	7	25		0.00 ± 0.0
3	3	0.75	6	25		2.00 ± 0.07
4	6.5	0.75	8	20		20.00 ± 0.9
5	3	1.5	7	25		0.00 ± 0.0
6	6.5	1.5	6	25		6.80 ± 0.4
7	6.5	1.5	7	30		0.00 ± 0.0
8	6.5	0	6	25		0.00 ± 0.0
9	10	0.75	7	30		6.50 ± 0.2
10	10	0.75	8	25		15.00 ± 0.9
11	6.5	0	7	30		5.80 ± 0.6
12	3	0.75	7	30		7.60 ± 0.4
13	6.5	1.5	7	20		9.10 ± 0.5
14	10	0.75	7	20		12.80 ± 0.8
15	6.5	0.75	6	30		11.20 ± 0.3
16	10	1.5	7	25		7.70 ± 0.3
17	6.5	0.75	6	20		3.60 ± 0.1
18	6.5	0	7	20		0.00 ± 0.0
19	3	0.75	7	20		0.00 ± 0.0
20	10	0.75	6	25		5.30 ± 0.2
21	10	0	7	25		2.00 ± 0.1
22	6.5	0.75	8	30		7.50 ± 0.3
23	6.5	0.75	7	25		13.60 ± 0.5
24	6.5	0.75	7	25		13.80 ± 0.3
25	6.5	0.75	7	25		13.80 ± 0.4
26	3	0.75	8	25		4.30 ± 0.1
27	6.5	1.5	6	25		0.00 ± 0.0

Bold values indicate optimum experiment

enhancement of the preliminary enzyme production. In this study, the chitinase production reached 22.1 U/dl in a 50-ml shake flask after incubating for 72 h (end of exponential growth phase) in the optimum fermentation medium (Fig. 4). The results for the reported chitinase production varied between 0.1 and more than 60 U/ml (Bhushan 2000; Vaidya et al. 2001; Singh et al. 2009; Souza et al. 2009). These variations were observed because of the strains and the level of enzyme purification. Our chitinase production is relatively low, and the application of recombinant DNA technology will be helpful to increase its production.

Effects of NaCl, pH and temperature on enzyme stability and activity

The effects of salinity, pH and temperature on the stability and activity of the chitinase from the marine-isolated strain

Table 4 Analysis of variance for response surface quadratic model

Source	Sum of square	Degree of freedom	Mean square	F	P
Model	0.081	14	0.0058	30.46	<0.05
A-NaCl	0.0104	1	0.0104	54.76	<0.05
B-chitin	0.0011	1	0.0011	5.88	0.031
C-pH	0.0106	1	0.0106	55.69	<0.05
D-temperature	0.0003	1	0.0003	2.08	0.137
AB	0.0008	1	0.0008	4.26	0.06
AC	0.0013	1	0.0013	7.18	<0.05
AD	0.0048	1	0.0048	25.33	<0.05
BC	0.0001	1	0.0001	0.89	0.362
BD	0.0055	1	0.0055	29.10	<0.05
CD	0.0101	1	0.0101	52.96	<0.05
A ²	0.0026	1	0.0102	53.94	<0.05
B ²	0.0297	1	0.0327	171.96	<0.05
C ²	0.0017	1	0.0028	14.85	<0.05
D ²	0.0016	1	0.0016	8.85	0.010
Residual	0.0022	12	0.00019		
Lack-of-fit	0.0022	10	0.00022	15.77	0.061
Pure error	0.000029	2	0.000014		
Total	0.083	26			

Pseudoalteromonas sp. DC14 are shown in Fig. 5. The optimum NaCl concentration was 10 % (w/v), while the enzyme was stable in a wide range of salt concentration from 0 to 15 % (w/v). The maximum pH activity of the obtained chitinase was at pH 9, and it could be considered an alkali-stable enzyme which was stable at an alkaline pH between pH 8 and 11. The enzyme maintained more than 60 % of its activity in a broad range of temperature from 20 to 60 °C and showed its highest activity at 40 °C. In spite of the relatively low-level production, the enzyme showed very interesting properties. It was stable in a wide range of salinity, from 0 to 15 % (w/v), pH from pH 7 to 11 and temperature from 15 to 65 °C. There are some reports about halo (Essghaier et al. 2012), alkali (Sorokin et al. 2012) and thermo-tolerant (Bhushan 2000; Christodoulou et al. 2001; Dai et al. 2011) chitinase, but the poly-extremophilic features of the novel enzyme made it a good candidate for industrial applications. The halo-tolerant nature of the enzyme is important because most industrial waste has a high level of salinity. Furthermore, halo-tolerant chitinase may be applied as biocontrol agent to control disease in salt-tolerant plants (Essghaier et al. 2012). Chitinase with alkaline pH stability has major potential in insect biological control, as their chitinous gut environments have an alkaline pH condition (Bansode and Bajekal 2006). Alkaline chitinase also has benefits in the management of alkaline chitinous waste from some



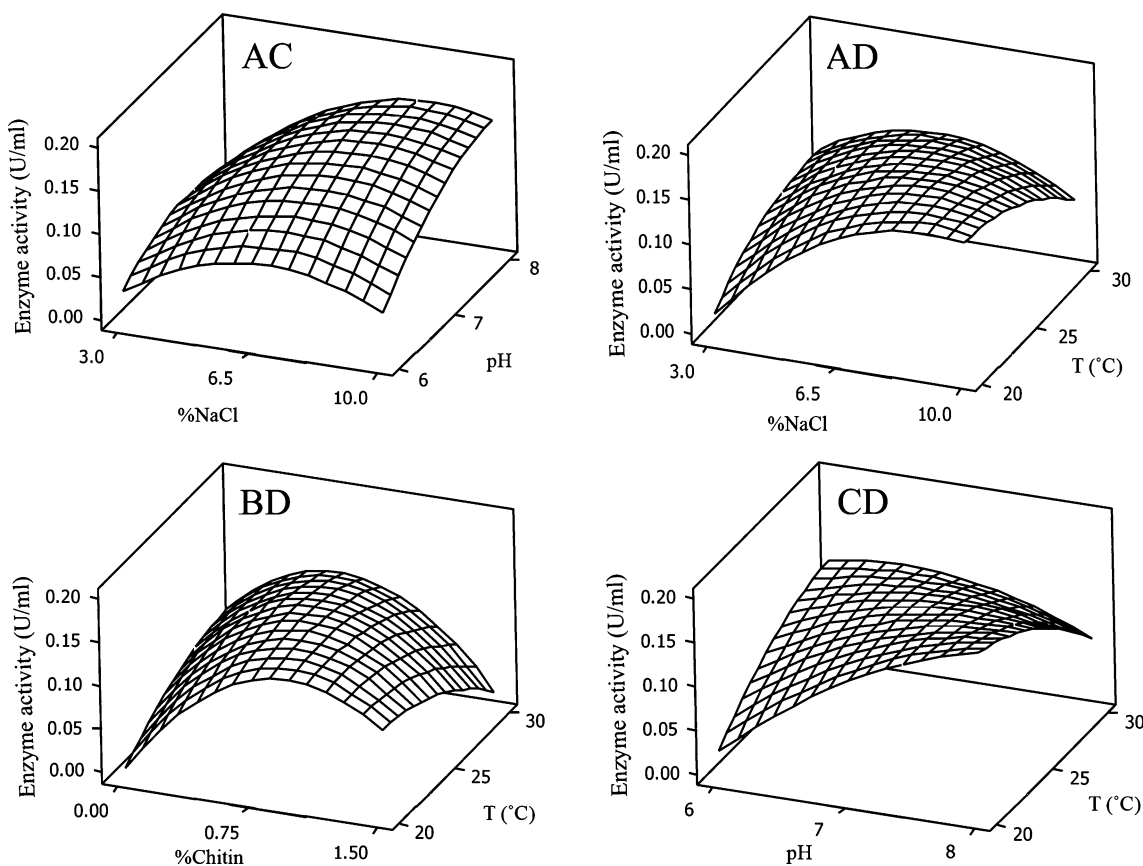
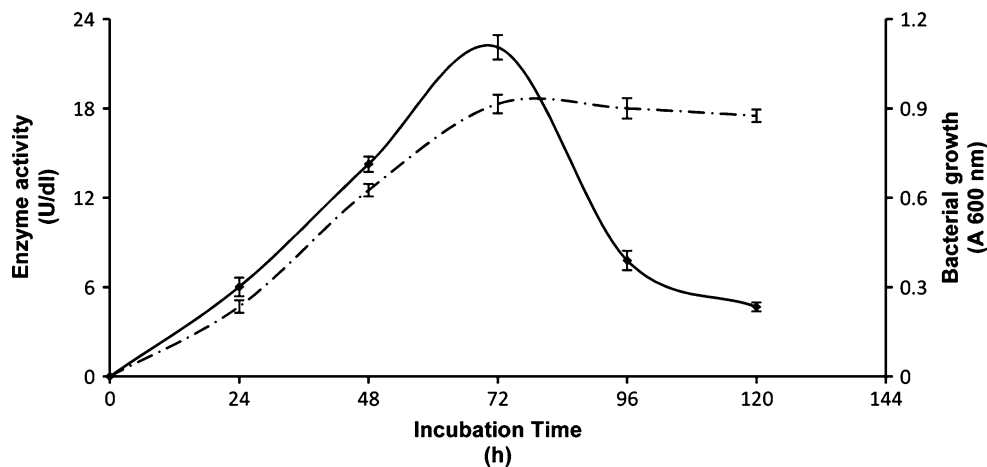


Fig. 3 Response surface plot of chitinase production by *Pseudoalteromonas* sp. DC14 showing the interactions between the NaCl and pH (AC), NaCl (% w/v) and temperature (°C) (AD), chitin (% w/v) and temperature (°C) (BD), pH and temperature (°C) (CD)

Fig. 4 Enzyme production (solid line) and bacterial growth (dashed line) in the optimal cultivation conditions



industries, like the food industry (Nawani and Kapadnis 2003). The isolated strain could produce chitinase at temperatures between 20 and 30 °C. The low producing temperature reduces the energy consumption in industry, and also the novel isolate may be applicable in field conditions against fungi and insect plant pathogens.

Molecular weight of chitinase

By the use of ammonium sulphate precipitation (70 % saturation), protein fraction was separated from theoretically optimized culture medium. Based on our process, specific activity in the culture medium was increased from

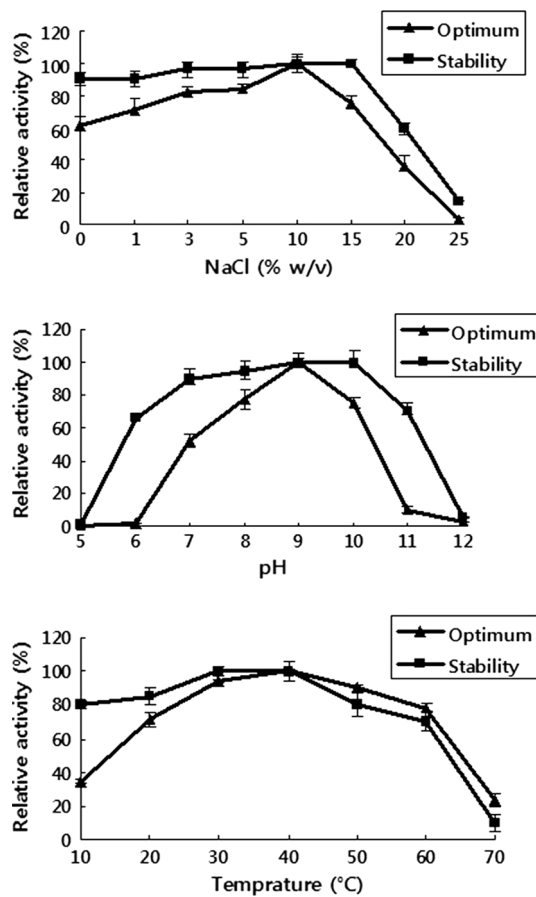


Fig. 5 Effects of NaCl, pH and temperature on stability and activity of chitinase from *Pseudoalteromonas* sp. DC14

Table 5 Partial purification of chitinase from *Pseudoalteromonas* sp. DC14

Purification step	Specific activity (U/mg)	Total protein (mg)	Total activity (U)	Purification (fold)
Culture medium	2.2	275.0	602.2	1.00
Ammonium sulphate precipitation	5.6	8.5	43.3	2.54

2.19 to 5.57 U/ml in precipitation stage. Our results also showed 2.54-fold purification (Table 5). The SDS-PAGE analysis of the purified enzyme revealed one protein band with an estimated molecular weight of 65 kDa (Fig. 6).

Conclusion

We elucidated the isolation of chitinolytic bacteria from the Caspian Sea for the first time. The results of phylogenetic analysis based on the 16S rRNA gene sequence analysis and phenotypic characterization showed that the

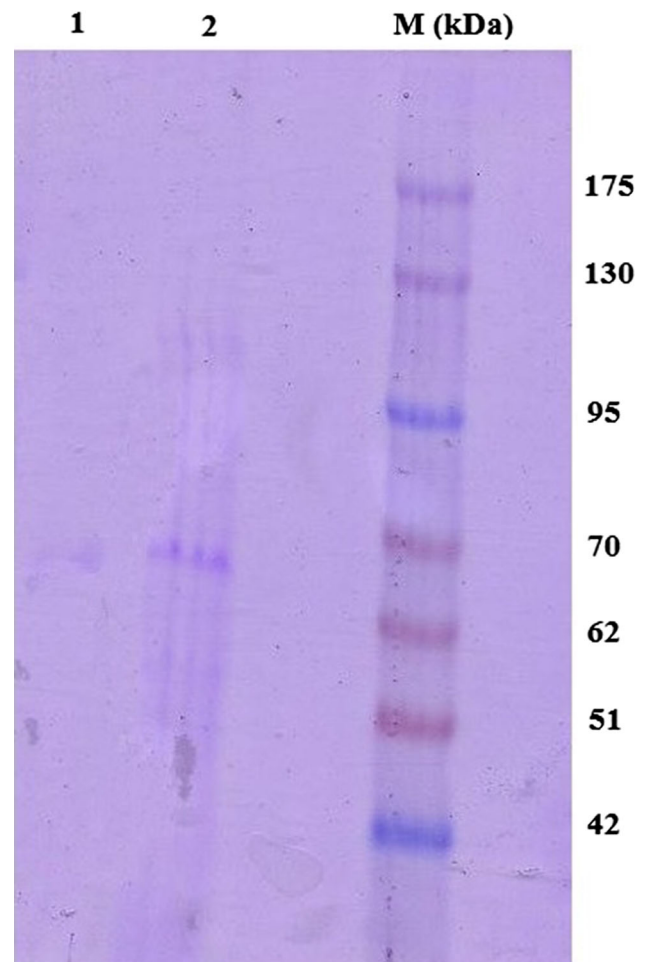


Fig. 6 SDS-PAGE of chitinase. Lane 1 supernatant of culture medium, Lane 2 ammonium sulphate precipitated protein; Lane M molecular weight markers

novel isolate belongs to the genus *Pseudoalteromonas*. The overall results show that this bacterium has great potential capability to produce *N*-acetylglucosamine in a wide range of reaction conditions. The GlcNAc and its analogues could be used to produce biofuel and also valuable pharmacological compounds for the treatment of a wide variety of ailments. The novel strain could furthermore be applied as a biocontrol agent against fungi and insect plant pathogens.

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