Serratia marcescens B4A chitinase product optimization using Taguchi approach

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Abstract
Chitinase production by newly isolated *Serratia marcescens* B4A was optimized following Taguchi's array methods. Twenty-three bacterial isolates were screened from shrimp culture ponds in the South of Iran. A chitinase-producing bacterium was isolated based on it's ability to utilize chitin as the sole carbon source. The isolate designated as B4A, was identified as *Serratia marcescens* based on its 16S rRNA sequence and key morphological, physiological and biochemical characteristics. The cultivation of *Serratia marcescens* B4A in the appropriate liquid medium resulted in production of high levels of chitinase. The malt extract and colloidal chitin represented the best nitrogen and carbon sources, respectively. Chitinase production by *Serratia marcescens* B4A was optimized following the Taguchi orthogonal array (OA) for the design of experiments (DOE). Statistical experimental design via the Taguchi method was applied to determine the optimal levels of physical parameters and key media components in the medium, such as temperature, pH, NaCl and chitin concentrations. The results of this study showed that temperature of 30ºC, pH 7.9, NaCl 0.1% (w/v) and chitin 1% (w/v) are optimal conditions for this protocol.

Keywords**:** Chitinase; Isolation; Screening; *Serratia marcescens* B4A; Taguchi method

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INTRODUCTION

Microorganisms have always been regarded as a treasure source of useful enzymes. During the last twenty years, biochemical reactions performed by microorganisms or catalyzed by microbial enzymes have been extensively evaluated. Screening may be one of the most efficient and successful ways of searching for new or suitable microbial enzymes (Scheper, 1997).

Chitin is the most plentiful source of a natural organic compound after cellulose (Yang *et al*., 2009). This long chain biopolymer containing N-acetyl-D-glucosamine (GLcNAc) monomers form covalent β-1,4 linkages (Xayphakatsaa *et al*., 2008; Kim *et al*., 2007). Chitin is widely dispersed in the structural components of many organisms that include crustacean and mollusk shells, arthropod exoskeletons and fungal cell walls (Ikeda *et al*., 2009; Lee *et al*., 2009).

Chitinases play an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. Production of chitinase is widespread in a variety of organisms such as bacteria, fungi, actinomycetes, yeasts, plants, protozoans, coelenterates, nematodes, arthropods and humans (Wang *et al*., 2009; Molinari *et al*., 2007; Matsumiya *et al*., 2006; Gutowska *et al*., 2004; Matsumiya *et al*., 2002; Bhushan, 2000; Tan *et al*., 2000; Matsumiya *et al*., 1998; Mochizuki, 1996). Chitinases have received increasing attention because of their broad applications in the fields of medicine, agriculture, biotechnology, waste management and industrial applications,

which include antifungal, hypocholesterolemic, antihypertensive activities and food quality enhancers (Bhattacharya *et al*., 2007; Guo *et al*., 2004; Khor, 2002; Gooday, 1999). To date, study on the chitinase of shrimp farming soils, water and wastewater remains very superficial.

In order to optimize culture medium for maximum production of enzymes, usually one parameter is changed while the other ones are kept constant. Although this procedure is usually used for optimizing several parameters, but it is time-consuming and highly costly. The basic principles of statistical analysis, such as randomization, replication, duplication and prediction of the accurate results can provide data with regard to the reciprocal interactions of the parameters (Box and Hunser, 1975). The Taguchi method is a statistical approach (Taguchi, 1986), which has been applied to numerous industrial projects in America and Europe (Rao *et al*., 2004; Roy, 1990). Taguchi parameter design is an engineering methodology intended to be a cost-effective approach for improving the quality of products and processes. Taguchi design has two main advantages: orthogonal arrays (OAs) can reduce the number of experiments when compared with the factorial design, and using the signal-to noise (S/N) ratio to analyze the results will reduce the sensitivity of the system to sources of variation, thus resulting in good performance (Chen and Kitts, 2008; Salehizadeh *et al*., 2007; Yasotha *et al*., 2006; Yazdian *et al*., 2005; Yang and Tarng, 1998). This method, based on (OAs), prepares off-line quality control in three phases, which include parameter, system and tolerance design. Parameter design determines the level of the factor that produces maximum product yield and process optimization. System design is applied with finding the working levels of the design factors, and tolerance design helps in fine tuning the tolerances of the factors that have important effects on the formation of products (Joseph and Piganatiells, 1998).

This study primarily aims at the isolation and identification of native chitinolytic bacterial strains and selection of those able to produce maximum levels of chitinase. Moreover, with the objective of obtaining accurate data and economizing time and materials, it was decided to use the Taguchi method, with 4 factors (NaCl%, chitin%, pH and temperature) at 3 levels, for the optimization of culture medium instead of the traditional method.

MATERIALS AND METHODS

Chemicals and reagents: Chitin powder was obtained from the shrimp shells of *Penaeus indicus* by the modified method of Takiguchi (Roberts and Selitrennikoff, 1998). Shrimps were purchased from the local markets in Abadan (Iran), their shells were then separated, cleaned, washed and dried. For the elimination of minerals, the dried shells were kept in HCl 10 % (v/v) for 24 h. After washing with water, they were soaked in NaOH (2 N) for 24 h. The shells were dried again and then grounded to powder form. 3, 5-dinitrosalisylic acid (DNS) was obtained from Sigma (St. Louis, MO. USA). All other reagent grade chemicals were purchased from Merck (Darmstadt, Germany).

Isolation of microorganisms: Samples collected from the shrimp farming soils, water and wastewater located in different areas of southern Iran, were cultured on agar plates containing nutrient agar 2% (w/v) supplemented with 0.1% (w/v) chitin (pH 7.5) for 3 days at 30°C. Morphologically distinct colonies were purified by streaking, from which eight bacterial strains were subsequently isolated. Pure cultures were sub-cultured onto nutrient agar slants and stored at 4ºC until further use.

Primary screening: The isolates were screened on agar plates for chitinolytic activity which containing 0.5% chitin, 0.03% peptone, 0.03% yeast extract, 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4$. $7H_2O$, 1.5% Agar, 0.2% NH_4NO_3, 0.1% NaCl, (w/v) and 0.1% v/v trace elements (pH 7.8). Cultures were incubated for 2 days at 30ºC.

Culture media and growth conditions: Several different media were prepared during this study. The most appropriate medium was chosen to compare the results in terms of enzyme production. For the production of chitinase, the best strain obtained from the primary screening, was cultured at first in preculture medium consisting of 0.8% nutrient broth, 1% malt extract, 1% peptone, 0.5% chitin, and 0.1% NaCl (w/v) for 24 h at 30ºC on a shaker incubator (200 rpm). A 2 ml sample of the preculture was added to 50 ml of the production medium (primary screening medium but without agar). The resultant inoculated medium was cultured at 30ºC for 7 days on a rotary shaker (200 rpm). A 2 ml sample of the culture medium was removed every 24 h and centrifuged (10000 g for 20 min at 4ºC). The resulting supernatants were then collected and used for subsequent chitinase assays using the method described below. The strain, which showed the highest chitinase activity was isolated, maintained on nutrient agar and used throughout the study.

Selection of carbon and nitrogen sources: For selection of the best source of carbon and nitrogen for chitinase production, various simple and complex carbon sources and inorganic and complex nitrogen sources were used as substitute individually for carbon or nitrogen source in the production liquid medium. To select carbon sources in the production medium without chitin, 1% w/v glucose, galactose, mannose, fructose, arabinose, lactose, sucrose, maltose, starch, chitin + glucose, and chitin powder were added to each basic medium separately. Similarly, 1% w/v peptone, yeast extract, malt extract, $(NH_4)_2HPO_4$, NH_4HCO_3, $(NH_4)_2SO_4$, NH_4Cl, and $C_2H_7NO_2$ were added to the basic medium with optimal carbon source so that that the best nitrogen sources were determined.

Qualitative cup-plate assay for chitinase production: The activity of the chitinase enzyme produced by the isolated strains was assayed qualitatively on agar plates. In order to detect chitinase production, chitin was incorporated at 1% w/v in a buffered agar solution. Clear zone reactions produced by chitinolytic bacteria were measured to determine the better strains.

Phenotypic characterization: The morphological and biochemical characterizations of the selected strain were carried out as described in *Bergey's Manual of Systematic Bacteriology* (Holt, 1984).

Genomic DNA extraction and sequencing of the partial 16S rRNA gene: Isolation of genomic DNA was performed according to the method by Saito and Miura (1963). The partial 16S rRNA gene of the selected strain was amplified by PCR (Weisburg *et al*., 1991). The sequences of two oligonucleotides used as PCR primers were 5′-GAG TAA TGT CTG GGA AAC TGC CT-3′ (forward primer) and 5′-CCA GTT TCG AAT GCA GTT CCC AG-3′ (reverse primer). Amplification was performed in a 25 ∝l reaction mixture containing the template DNA, 0.02 µg; each primer 0.2 µM; dNTPs, 200 µM; *Taq* DNA polymerase, 1.5 U and 10 × *Taq* buffer, 2.5 µl. The mixture was subjected to the following amplification conditions: 5 min at 95ºC, 30 cycles of 40 s at 95ºC, 60 s at 53ºC, 1 min at 72ºC and one final step of 10 min at 72ºC. The PCR products were electrophoresed on 1% w/v agarose gels and the target band was excised and purified with a DNA extraction kit (Roche, Germany).

The resulting PCR fragments were ligated to pTZ75R (Fermentas, Canada), using the T/A cloning procedure. The constructed vectors were transformed into competent *Escherichia coli* DH5α cells and then spread onto LB agar plates containing 20 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 0.2 mM isopropyl-1-thio-β-D-galactoside (IPTG) and ampicillin (50 µg/ml). Consequently, a white recombinant clone was obtained. The presence of the cloned fragment was confirmed by enzyme digestion of the plasmid DNA and its subsequent sequencing by Seqlab (Germany). The obtained sequences were compiled and compared with sequences in the databases (http://www.ncbi.nlm.nih.gov) using the BLAST program.

Preparation of colloidal chitin: Colloidal chitin is commonly used as a water insoluble substrate for the study of chitinase. It was prepared by the modified method of Roberts and Selitrennikoff (Takiguchi, 1991). Twelve grams of chitin powder obtained from the shrimp shell was added slowly into 380 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 4 L of ice-cold ethanol 95% (v/v) with rapid stirring and kept at 4°C overnight. The precipitate was collected by centrifugation at 5000 g for 20 min at 4°C, and was then washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0).

Measurement of chitinase activity: Chitinase activity was measured with colloidal chitin as a substrate. The reaction mixture containing 0.5 ml of 1% (w/v) colloidal chitin and 0.5 ml of the crude enzyme was incubated at 50ºC for 1 h. The reaction was stopped by the addition of 3 ml of DNS followed by heating at 100ºC for 5 min. Following centrifugation, the concentration of the reducing sugar in the supernatant was determined using the modified DNS method (Miller, 1959). The absorption of the appropriately diluted test sample was measured at 530 nm using a UV spectrophotometer (Beckman DU530, USA) along with substrate and enzyme blanks. One unit (U) of chitinase activity was defined as the amount of enzyme required to produce 1 mmol of reducing sugar per min.

To show chitinase's ability to consume the substrate, the mixture containing 1.5 ml of 1% (v/v) colloidal chitin and 1.5 ml of phosphate buffer (tube 1) accompanied by 1.5 ml of 1% (v/v) colloidal chitin and 1.5 ml of the enzyme sample (tube 2) were incubated at 50ºC for 8 h. Another mixture (tube 3) containing the same contents as the tube 2 was not incubated, so as to observe the difference due to temperature treatment. All three tubes were immediately photographed following incubations.

Scanning electron microscopy (SEM): Bacterial cells from the late phase of exponential growth were centrifuged at 6000 g for 20 min and then washed with distilled water. The samples were fixed with 0.1 M phosphate buffer (pH 7.2) containing 2.5% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde for 2 h. After washing, the fixed cells were dehydrated by being passed for 5 min through a graded ethanol series 25, 50, 75, 95, and 100 % (v/v) and subsequently freeze dried. The dried cells were sputtered coated with gold, and finally photographed by a scanning electron microscope (SEM) (LEO, 1455 vp, Japan).

Statistical optimization of growth conditions: The optimization of medium constituents was carried out to improve chitinase activity of the selected strain. The identification of important factors was carried out in accordance with the enzymatic activity of the cells after 48 h of growth; thus 4 variables at 3 levels were investigated (Table 1). To examine the effects of these factors, Taguchi's arrays were used. An L9 array was selected to determine the effect of the four 3-level factors on chitinase production. Based on the L9 Taguchi's design, 9 experiments were carried out in triplicate (Table 1). In the full-factorial experimental design procedures, at least 64 experiments are necessary to reach the same conclusions as those of the Taguchi's array method using this variety of factors (Ross, 1998; Roy, 1990). After performing the experiments, the data were analyzed by the standard

Table 1. Four variable factors, their relative levels and assignment of experimental factors of the L9 Taguchi's array and relative enzyme activities.

Test	Temperature (^oC)	pH	Chitin % (w/v)	NaCl % (w/v)	Chitinase activity (Relative%)
1	30	6.5	0.2	0.1	64.3
2	30	7.2	0.5	0.5	46.6
3	30	7.9	1	1	100
4	37	6.5	0.5	1	20.9
5	37	7.2	1	0.1	69.3
6	37	7.9	0.2	0.5	19
7	45	6.5	1	0.5	5.7
8	45	7.2	0.2	1	5.6
9	45	7.9	0.5	0.1	11.4

Chitinase activity in production medium.

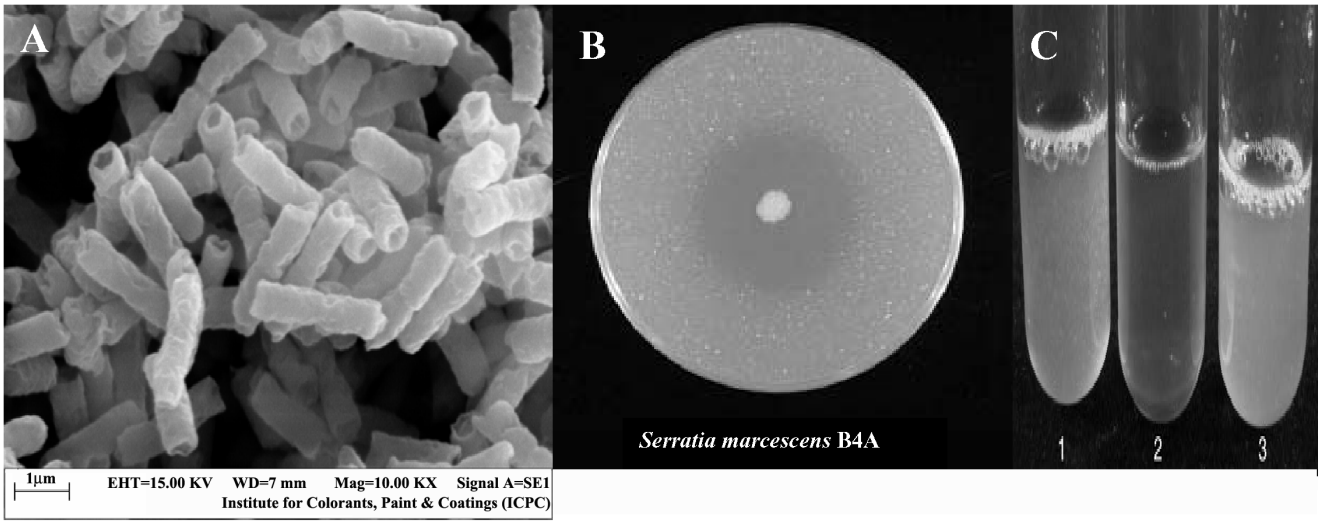

Figure 1. A: Scanning electronic microscopy (SEM) photomicrographs of *Serratia marcescens* B4A. B: Clear zone reactions produced by *Serratia marcescens* B4A. C: Substrate consumption by the enzyme. For more details, please refer to the "Materials and Methods" section.

approach, determination of main effects, and also by formation of the ANOVA (analysis of variance) Table and analysis of signal to noise ratio (S/N) using WinRobust™ and Qualitek-4™ softwares (Demo version, Nutek Inc., MI, USA).

Software: Qualitek-4 software for automatic design of experiments using the Taguchi approach was used in the present study. Qualitek-4 software uses the L-4 to L-64 arrays along with a selection of 2 to 63 factors at 2, 3 and 4 levels per factor. The automatic design option allows Qualitek-4 to select the array used and assign factors to the appropriate columns. In order to confirm reproducibility, each of the results in this study is presented as the average of at least three repeated experiments in a typical run.

RESULTS

Identification: Biochemical and microbiological analysis was performed to characterize the screened strain (Table 2). The Scanning electron microscopy (SEM) image was shown in Figure 1A revealed that the *Serratia marcescens* cells look like rod-shaped bacilli. Rods are 0.5-0.8 μm in diameter, 0.9-2.0 in length.

Phylogenetic analysis of the partial 16S rRNA sequences: According to the sequence comparisons based on the partial 16S rRNA gene of the strain in this study with sequences of the nearest type species retrieved from the ribosomal database project (RDP), this strain showed high homology with *Serratia marcescens*. Following the physiological and biochemical characteristics and comparison of its 16S rRNA gene sequence, the selected strain was identified as *Serratia marcescens*. The partial 16S rRNA gene sequence of the selected bacterial strain obtained in this study was deposited in the GenBank nucleotide sequence database under the accession number HM535665.

Selection of the strain with highest chitinase production: Qualitative cup-plate assay for chitinase production indicated that *Serratia marcescens* B4A was the most active ones. In other words the above-mentioned strains consumed chitin at a higher rate, and produced a clear zone with larger diameters (Fig. 1B). Therefore they were selected for further studies.

Table 2. Biochemical properties of the screened bacterium.

Biochemical properties	*Serratia marcescens* B4A
Indole production	-
Methyl red (MR)	(-)
Voges proskauer (VP)	+
Citrate consumption	+
H_2S	-
Hydrolysis of urea	(-)
Motility at 36ºC	+
Hydrolysis of gelatine at 22ºC	+
Glucose (Formation of gas)	d
Fermentation	
lactose	-
sucrose	+
mannitol	+
dulcitol	-
sorbitol	+
arabinose	-
xylose	-
galactose	*
Ortho-nitrophenyl-β-D-galactopyranoside (ONPG)	+
Lysine decarboxylase	+
Ornitin decarboxylase	+
Haemolysis	*
Growth at 37ºC	+
Growth at 50ºC	*
Growth in 10% (w/v) NaCl	*

–: 10% of strains were positive, (–): 11%-25% of strains were positive, d: 26%-75% of strains were positive, +: 90%-100% of strains were positive, *: this test was not carried out.

Production of chitinase from *Serratia marcescens* B4A: The Chitinase assay showed that *Serratia marcescens* B4A produced maximum levels of chitinase. Comparison of the results for this part with qualitative cup-plate assay for chitinase production guides us to conclude that one should perform chitinase assay in liquid medium for selection of the best chitinolytic microorganism (Gomez Ramirez *et al*., 2004).

Figure 1C demonstrates the consumption of substrate by chitinase incubated under different conditions. The transparency of the medium in the second tube indicates consumption of chitin by chitinase. , but in tube 3 the medium has remained offended because of no incubation time.

Effects of incubation time on chitinase production: The time course of cultivation of the above-mentioned

Table 3. The main effects of the factors at 3 levels on chitinase production.

Serial number	Factors	Level 1	Level 2	Level 3
1	Temperature (ºC)	36.6	29.6	16.9
2	pH	25.8	28.3	28.9
3	Chitin % (w/v)	25.5	26.9	30.6
4	NaCl % (w/v)	31.4	24.7	26.9

strains is illustrated Figure 2. "*Serratia marcescens* produced the highest chitinase after 2 days of incubation at 30ºC on a rotary shaker (200 rpm). Enzyme levels remaining constant during the third day of incubation. However, chitinase production started to decline, thereafter, this being perhaps due to the lack of nutrients in the medium (Aminzadeh *et al*., 2007).

Influence of carbon and nitrogen sources on chitinase production: The growth of *Serratia marcescens* and production of chitinase by this strain using different carbon sources were investigated. Biosynthesis of chitinase was induced by chitin and inhibited in the presence of easily metabolized monosaccharides (such as, glucose and fructose) and some other compounds. Glucose and fructose inhibited chitinase biosynthesis, and the enzyme was produced in relatively small amounts, demonstrating low enzymatic activities when galactose and mannose were used as carbon sources. However, Chitin led to the highest chitinase activity. Maltose, lactose and sucrose decreased chitinase production by approximately 58%, whereas starch and cellulose decreased chitinase production by approximately 43%.

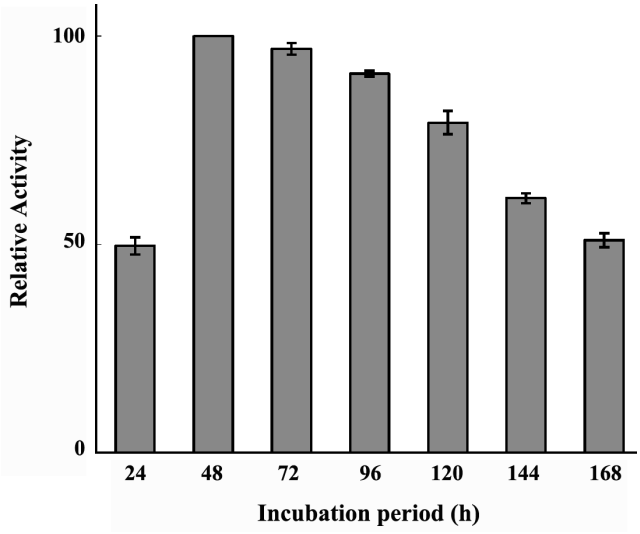

Figure 2. Effect of incubation time on chitinase production by *Serratia marcescens* B4A. For more details, refer to the "Materials and Methods" section.

In order to show the influence of nitrogen sources on chitinase biosynthesis, organic (casein, peptone) and inorganic (NH_4HCO_3, (NH_4) H_2PO_4, $(NH_4)_2SO_4$, NH_4Cl) sources of nitrogen were added to the medium containing chitin as the sole carbon source. The medium without a nitrogen source was used as a control. The medium containing malt extract and peptone led to the highest chitinase activity when compared to that of the control.

Analysis of results obtained from the Taguchi design: The main effects of the factors at three levels each, on chitinase production are shown in Table 3. According to these data, temperature and NaCl were more effective than the other factors, and pH of the medium showed the least impact factor. To understand the importance of these factors, the data obtained at different levels were compared. It was shown that temperature at level 1 had the largest value compared to the other factors at all levels (36.6).

Interaction between factors is demonstrated in Table 4. It is remarkable that the highest severity index percentage was obtained when the lowest impact factor, (pH) was resting against chitin of the medium. Whereas temperature, as the largest impact factor versus chitin, showed the least value of the severity index percentage (7). The contribution of each factor is presented in Table 5. The impact of each factor is shown in the last column of the ANOVA Table. The temperature of incubation was the most important factor for chitinase production.

Optimum conditions and the performance for maximum chitinase production are shown in Table 6. The contribution of the studied factors demonstrated that temperature at level 1, NaCl at level 1, chitin at level 3 and pH at level 3 had the strongest impacts on chitinase production, respectively. Also, data showed that the expected result under the optimum condition was 44.4, whereas the current grand average of performance was 27.7.

Table 4. Estimation of interactions among the factors.

Serial number	Interacting factor pairs	Columns[a]	SI (%)[b]	Col[c]	Opt[d]
1	pH × Chitin	3×2	56.2	1	[3,3]
2	Chitin × NaCl	4×3	44.8	7	[3,3]
3	pH × NaCl	4×2	34.8	6	[3,3]
4	Temperature × pH	2×1	26	3	[1,3]
5	Temperature × NaCl	4×1	16.6	5	[1,3]
6	Temperature × Chitin	3×1	7	2	[1,3]

[a]Columns-represent the column locations to which the interacting factors are assigned. [b]SI-interaction severity index (100% for 90° angle between the lines, 0% for parallel lines. [c]Col-shows column that should be reserved if this interaction effect was to be studied (2-evel factors only). [d]Opt-indicates the factor levels desirable for the optimum conditions (based strictly on the first two levels). If an interaction is included in the study and found to be significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum condition without considerations for any interaction effects.

Table 5. The analysis of variance (ANOVA) table (the impact of each factor was shown in the last column).

Serial number	Factors	Degree of freedom(DOF)	Sum of squares	Variance	Pure sum	Percent
1	Temperature (ºC)	2	591.9	295.9	591.9	82.2
2	pH	2	15.6	7.8	15.6	2.2
3	Chitin % (w/v)	2	42.2	21.1	42.2	5.9
4	NaCl %(w/v)	2	70	35	70.0	9.7
	Other/error	0	-	-	-	-
	Total	8	719.7	-	-	100

Pooling of factors was carried out to understand whether leaving out the factors with little impact can change chitinase production significantly or not. Thus pH and chitin were pooled, and the associated ANOVA were estimated (Table 7). Optimum conditions after pooling are shown in Table 8. Results showed that maximum production of chitinase is possible when using only significant factors, such as temperature and NaCl at their optimum levels. Thus pH can be maintained at 6.5 (level 1) without adjustment. Chitin can be kept at 0.2% w/v (level 1) for economical purposes; thus dispensing with preparation of colloidal chitin, which is a time-consuming process.

DISCUSSION

Enzymes are used extensively in modern biotechnology, with new applications of enzymes being discovered continuously. The development of enzyme products often relies on screening a large number of organisms for an enzyme activity (Aminzadeh *et al*., 2006).

Table 6. Optimum conditions and their performance for maximum chitinase production.

Serial number	Factors	Level description	Level	Contribution
1	Temperature (ºC)	30	1	8.8
2	pH	7.9	3	1.2
3	Chitin % (w/v)	1	3	2.9
4	NaCl % (w/v)	0.1	1	3.7
Total contribution from all factors	16.7	-	-	-
Current grand average performance	27.7	-	-	-
Expected result at the optimum condition	44.4	-	-	-

Table 7. Pooling of factors for understanding the omission of factors with little impact and relative ANOVA.

Serial number	Factors	Degree of freedom (DOF)	Sum of squares	Variance	define	Pure sum	Percent
1	Temperature (ºC)	2	591.9	295.9	20.5	562.9	78.2
2	pH	(2)	(15.6)	-	POOLED	(CL = NC)	-
3	Chitin %(w/v)	(2)	(42.2)	-	POOLED	(CL = 73.4%)	-
4	NaCl % (w/v)	2	70	35	2.4	41	5.7
	Other/error	4	57.8	14.5	-	-	16.1
	Total	8	719.7	-	-	-	100

Table 8. Optimum conditions and performance after pooling.

Serial number	Factors	Level description	Level	Contribution
1	Temperature (ºC)	30	1	8.8
4	NaCl % (w/v)	0.1	1	3.7
Total contribution from all factors	12.5	-	-	-
Current grand average performance	27.7	-	-	-
Expected results at the optimum condition…	40.2	-	-	-

In a world with a fast growing population and limited natural resources, enzyme technology can be helpful to a lot of industries to overcome the problems that will be encountered in the near future.

The broad variety of microbial reactions available opens the possibility of performing difficult reactions and producing the corresponding products of interest. In this study, B4A, a newly isolated chitinolytic strain, was obtained from the shrimp farming soils, water and wastewater located in different areas of southern Iran and identified as *S. marcescens* B4A based on its morphology, physiological tests, and the 16S rRNA gene sequence.

Molecular methodologies offer an alternative laboratory mechanism for the identification of such organisms, particularly employment of 16S rRNA sequencing techniques. Though the traditional characterization for bacteria depended upon phenotypic traits like biochemical testing, cellular fatty acid analysis, and numerical analysis, 16S rRNA, today is considered to be more reliable than classification based solely on phenotypes. The 16S rRNA sequencing has historically been most commonly employed and hence has the most comprehensive database for comparison during searches of unknown sequences.

In this report, the optimization of chitinase production by *Serratia marcescens* B4A is described for the first time. *Serratia marcescens* B4A was shown to produce maximum levels of chitinase during the second day of growth. A similar chitinolytic activity has been observed by a soil bacteria, *Pseudomonas* sp. TKU015 on shrimp shell based medium (Wang *et al*., 2008) and *Aeromonas schubertii* on chitin based media (Guo *et al*., 2004) after 72 h of incubation. In the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702, maximal chitinase activity has been found to occur after 40 h of incubation (Park *et al*., 2009). The main reasons for a decrease in product formation following the optimum incubation time might be due to the reduced levels of nutrients in the culture medium and/or denaturation of chitinase by proteases (Binod *et al*., 2007). In the first step of the optimization of medium components for maximum chitinase production by *Serratia marcescens* B4A, carbon and nitrogen sources were selected by the one factor-at-a-time method.

The Influence of the carbon sources on chitinase production showed that monosaccharides inhibited chitinase biosynthesis, and synthesis of the enzyme in relatively small amounts with low enzymatic activity occurred in the presence of glucose and galactose. Inhibition of chitinase production in the presence of glucose and other simple sugars might be due to catabolite repression. These results are in agreement with those of Gupta *et al*. (1995) and Ulhoa and Peberdy (1991) regarding chitinase production by *Streptomyces viridificans* and *Trichoderma harzianum,* respectively. The growth of microorganisms was found to be high in the presence of glucose and galac-

tose, but chitinase production was at its lowest. The results of this study showed that the addition of glucose to the chitin-containing medium reduced the chitinase activity of *Serratia marcescens* B4A, which was similar to the findings of Nawani and Kapadnis (2005) regarding chitinase production by *Streptomyces* sp.).

The influence of nitrogen sources on chitinase biosynthesis showed that malt extract appeared to be the most optimal nitrogen source for the production of chitinase. Comparison of the results showed that organic nitrogen sources increase chitinase activity considerably relative to inorganic ones. In confirmation of these findings, similar observations have been reported in the case of *Streptomyces* sp. Da11 (Han *et al*., 2008) and *Alcaligenes xylosoxydans* (Vaidya *et al*., 2001).

Culture conditions are critical parameters that affect cell growth and product yield (Tanfous *et al*., 2006). In this study, the effects of various variables, including incubation temperature, initial pH, and chitin and NaCl levels, on the efficiency of chitinase production were examined and were optimized by the Taguchi array method. This approach facilitates the study of the interaction of a large number of variables spanned by factors and their settings with a small number of experiments leading to considerable savings in time and costs for process optimization. Obtaining a factor/level combination that can simultaneously optimize the multiple quality characteristics is generally difficult, particularly when these multiple responses are mutually correlated (Changa *et al*., 2006). Taguchi method is not a usual way for optimizing biotechnological processes. Other methods such as response surface and Plakett-Burman may be preferred because researchers are more familiar with them. Application of the Taguchi approach appears to have potential usage in bioprocess optimization (Azin *et al*., 2007). Nevertheless, there are only a few references showing the usefulness of the Taguchi method in the optimization of biotechnological processes. This work demonstrates the effectiveness and feasibility of using the Taguchi statistical experimental design to identify better medium composition for enhanced production of thermophilic chitinase. Also, the use of this method is relatively easily, since it has the ability to include categorical factors along with the continuous ones.

The data obtained from the Taguchi experiments showed that temperature and NaCl had a more effective influence than pH and chitin percentage (w/v), on chitinase production. The temperature of incubation was the most important factor for chitinase production. The optimized culture conditions defined in this study are currently being used as starting points for the scale-up of chitinase production using a 5 lit fermentor.

CONCLUSIONS

Isolation of chitinase-producing strain from the wastewater of shrimp culture ponds indicates that this is a suitable environment for seeking chitinase-producing bacteria. This research proved the efficiency of the Taguchi statistical experimental design to determine the most suitable medium components for obtaining maximum production of chitinase by *Serratia marcescens* B4A. The Taguchi approach of OA experimental design used for the purpose of process optimization, involves a study of a given system using a set of independent variables (factors) over a specific region of interest (levels) by identifying the influence of individual factors, establish the relationship between the variables and operational conditions and finally establish the performance at the optimum levels obtained. In this methodology, the desired design is sought by selecting the best performance under conditions that produce consistent performance, thus leading to a more fully developed process. The Taguchi method facilitates the analysis of the experimental data to establish the optimum conditions for the process, understand the contribution of individual factors and to evaluate the response under optimal conditions. Application of the Taguchi approach appears to have potential usage in bioprocess optimization.

This work demonstrated that the Taguchi method is more economical as far as time and materials are concerned, when compared with the traditional methods. The obtained optimal culture conditions for chitinase production from the proposed methodology were validated by performance of the experiments with the obtained conditions. Further work on the scale-up production and application of this enzyme and its economic/commercial feasibility is currently underway.

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