Advanced Research in Microbial Metabolites & Technology 2 (2019) 27-39

Advanced Research
in Microbial Metabolites & Technology
(ARMMT)

journal homepage: http://armmt.irost.ir

Molecular detection and optimization production of an alkaline metalloprotease from *Exiguobacterium* sp. MSB42

Matia Sadat Borhani[1], Zahra Etemadifar*1, Davood Biria[2], Eisa Jorjani[3]

[1] Biology Department, Faculty of Sciences, University of Isfahan, Isfahan, Iran

[2] Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran

[3] Biology Department, Faculty of Sciences, University of Gonbad Kavous, Golestan, Iran

Article Info

Received 29/05/2019
Received in revised form 16/09/2019
Accepted 22/09/2019

Keywords:
Alkaline protease,
Exiguobacterium,
One-factor-at-a-time strategy,
Response surface methodology

Abstract

Improvement of alkaline protease production, one of the most important types of industrial biocatalysts, is essential for commercial purposes. The present study's aim was to increase alkaline protease production of MSB42, a newly isolated strain of *Exiguobacterium* sp., through the rotatable central composite design (RCCD). In this regard, parameters affecting MSB42 enzyme production were chosen based on the results of preliminary one-factor-at-a-time (OFAT) experiments. According to the results of RCCD, the enzyme production showed its maximum level (198.48 U/ml) in the presence of 3% w/v glucose, 3% w/v peptone, at 37 °C and pH 9. Overall, the enzyme production was 2 and 3.24 times more than OFAT experiments (98.69 U/ml) and unoptimized conditions (61.29 U/ml), respectively. A polynomial model of MSB42 alkaline protease production was obtained using the experimental date and based on the significant terms. The protease gene of this strain was also amplified using the designed primers (873 bp), and based on the sequencing results, was found to be closely related to the M6 family of metalloprotease domain.

1. Introduction

Alkaline proteases (EC. 3.4.21–24, 99) have extensive industrial applications in leather, detergent, pharmaceutical, food, organic chemical synthesis, textile, wastewater treatment and other industries (Bhunia et al., 2012; Jisha et al., 2013; Naidu and Devi, 2005; Tari et al., 2006). They are usually categorized either as a serine protease or metalloprotease (Meena et al., 2013). They are ubiquitous in nature and are found in bacteria, molds, yeasts and mammalian tissues (Reddy et al., 2008). In spite of this variety, only strains that produce a high-yield of extracellular protease have been used. Currently, *Bacillus* strains are the best producer of the most commercially available proteases (Cheng et al., 2012; Sen et al., 2009; Shafee et al., 2005). However, the vast number of potential applications and high demand for alkaline proteases in various industries have caused new protease producing strains with significant enzymatic properties to be taken into consideration (Tari et al., 2006). In this regard, extremophiles can be used as valuable resources for the production of resistant enzymes in industry.

The genus *Exiguobacterium* is a bacterium with impressive extremophilic abilities such as the ability to survive in a wide range of

*Corresponding author. Tel: + 983137932367

E-mail address: z.etemadifar@sci.ui.ac.ir

DOI: 10.22104/armmt.2019.3641.1030

temperatures (-12°C to 55°C), pH (5 to 11), high salt concentration (up to 13%), and high levels of heavy metal stress. These properties recommend them as promising industrial strains, especially in the detergent industry. Although some enzyme producing *Exiguobacterium* strains have been reported, the potential of this genus has not been clearly investigated (Anbu et al., 2013; Selvakumar et al., 2009).

It is clear that enzyme production cost is an important factor limiting the wide application of alkaline proteases (Meena et al., 2013). Therefore, all parameters influencing enzyme production have to be optimized. Even a small improvement in enzyme production yield can be desirable for commercial success (Anwar and Saleemuddin, 1998). In this regard, statistical approaches are powerful tools. In the conventional one-factor-at-a-time (OFAT) approach, the nutritional or cultural variables are optimized by altering one variable while keeping others constant at the same time. The simplicity of this approach helps in the selection of significant factors influencing the enzyme yield. However, this method is time-consuming and ignores the interaction effects among variables. In contrast, statistical methods, such as response surface methodology (RSM), are also applicable for studying the effect of several factors, simultaneously. RSM also decreases the number of experiments needed for process optimization (Bhunia et al., 2012; Jisha et al., 2013). There is scarce information concerning statistical optimization of protease production using *Exiguobacterium* strains. The present study is an effort to increase the protease production of *Exiguobacterium* sp. MSB42. For this purpose, a two-step strategy was used: (1) screening of the most effective physicochemical and nutritional parameters using the OFAT method and (2) optimization of significant and effective factors through RSM. This two-step strategy was successfully adopted in earlier studies (Beg et al., 2003; Singh et al., 2011; Tari et al., 2006; Vaishnav et al., 2014). A second-order model equation was developed and then validated experimentally. In addition, we have designed and tested PCR primers as potential tools for the detection of the metalloprotease gene of the genus *Exiguobacterium*.

2.Materials and methods

2.1. Microorganism and protease production

Strain MSB42 was isolated using a sample of sausage factory wastewater (Isfahan, Iran) on a skim milk agar plate (pH 10). The method of using universal primers 27F and 1492R for molecular detection was previously described (Borhani et al., 2017). The nucleotide multiple alignments and phylogenetic analysis of this strain were done using the Muscle program and MEGA 6 software, respectively. Skimmed milk broth medium (1% w/v skim milk) was used as the initial production medium (30 °C, 180 rpm, 1% v/v inoculum). The medium was centrifuged at 10,000 rpm (10 min, 4°C) to obtain a cell-free supernatant for determining the enzyme activity after the incubation time expired for each experiment.

2.2. Enzyme activity assay

The amount of enzyme produced in each experiment was determined based on the level of enzyme activity and according to the Folin-Ciocalteau method (Cupp-Enyard, 2008). The experimental results were approved only when the difference between the mean of three independent tests was no more than 5%.

2.3. Detection of the metalloprotease gene

Research for DNA sequences of *Exiguobacterium* extracellular metalloprotease

genes in the NCBI and PIR (Protein Information Resource) databases revealed several nucleotide sequences. Genes with homologous regions were aligned using the Clustal Omega program. The conserved regions were selected as the primer targets (Table 1). The primers were analyzed using Primer Blast-NCBI. The amplification reaction contained l0 µl distilled water, l0 µl PCR Master Mix (2X) (Jena Bioscience, Germany), 5 µl DNA, 0.5 µl forward, and 0.5 µl reverse primers (10 pmol). The PCR program was 95°C (2 min), followed by 30 cycles of 95°C (30s), 61.6°C (30s), and 72°C (90s) with a final extension at 72°C (5 min). The PCR product sequencing was performed in both directions using an automated sequencer from the Bioneer Company (Korea).

2.4. Screening of effective physicochemical variables by the OFAT approach

The effects of various parameters including incubation time (6, 8, 12, 18, 24, 36, 48, 60, 72, and 96h), pH (7–12), temperatures (25, 30, and 37°C), carbon sources (glucose, soluble starch, sucrose, mannitol, lactose, and xylose), nitrogen sources (beef extract, NaNO3, NH4Cl, urea, peptone, and yeast extract), and NaCl concentrations (0-20% w/v) on MSB42 protease production were studied using an OFAT strategy.

2.5. Optimization of protease production by the RSM approach

The significant and effective independent variables identified by the OFAT results (pH, temperature, glucose and peptone concentration) were used to optimize MSB42 protease production using an RCCD. Table 2 represents the coded and real values of these variables.

Five levels of each factor (+α, -α, 0, +1, -1) were studied in a set of 60 experiments. In each experiment, protease activity was measured after a 60h incubation. Finally, the relationship between MSB42 alkaline protease production (the response) and the four studied independent variables was described as the following quadratic equation:

$$Y=b0+b1X1+b2X2+b3X3+b4X4+b11X12+b22X22+b33X32+b44X42+b12X1X2+b13X1X3+b14X1X4+b23X2X3+b24X2X4+b34X3X4$$

In the present quadratic equation, Y, X1, X2, X3, X4, and b0 are the predicted response value, pH, peptone concentration, glucose concentration, temperature (°C) and a constant, respectively. In addition, b1, b2, b3, and b4 represent the linear coefficients while b12, b13, b14, b23, b24 and b34 are the cross product coefficients Also, b11, b22, b33, and b44 are the quadratic coefficients in this equation. The validation of the obtained model was performed by using an additional six randomly selected experiments. The level of factors in these validation experiments differ from the level of factors in the 60 experiments of the design matrix.

2.6. Statistical analysis

All of the statistical analysis, including the experiments design, data analysis, regression coefficients calculation, and response surface graph drawings, were done using Design Expert 7.0 software (Stat-Ease, Minneapolis, USA). Furthermore, an Analysis of Variance (ANOVA) test was used ($\alpha = 0.05$)to check the statistical significance of the OFAT and RSM experiment results.

3. Results and discussion

3.1. Molecular detection and phylogenetic analysis of the MSB2 strain

The phylogenetic analysis of the strain

that MSB42 has a close relationship with Exiguobacterium sp. AT1b, Exiguobacterium profundum and Exiguobacterium arabatum (Figure 1). The 16S rRNA gene sequence of the MSB42 strain was submitted to the GenBank database with accession number of KT006760.

3.2. Exiguobacterium metalloprotease gene detection

The *Exiguobacterium* sp. MSB42 metalloprotease gene was amplified by PCR using the designed primers (Figure 2). Designed oligonucleotides comparison with known DNA sequences using Primer Blast-NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) revealed that the primers will amplify fragments of about 873 bp in *Exiguobacterium sibiricum* 255-15, *Exiguobacterium antarcticum* B7, *Exiguobacterium* sp. AT1b, and *Exiguobacterium* sp. MH3. Other sequences not included in the alignments, but detected by the oligonucleotides presented in this study, were *Bacillus thuringiensis* (strains: CTC and XL6), *Bacillus mycoides* strain ATCC 6462, *Bacillus weihenstephanensis* (strains: WSBC 10204 and KBAB4), and *Bacillus cereus* (strains: ATCC 4342, G9241, FM1, FT9 and 03BB87).

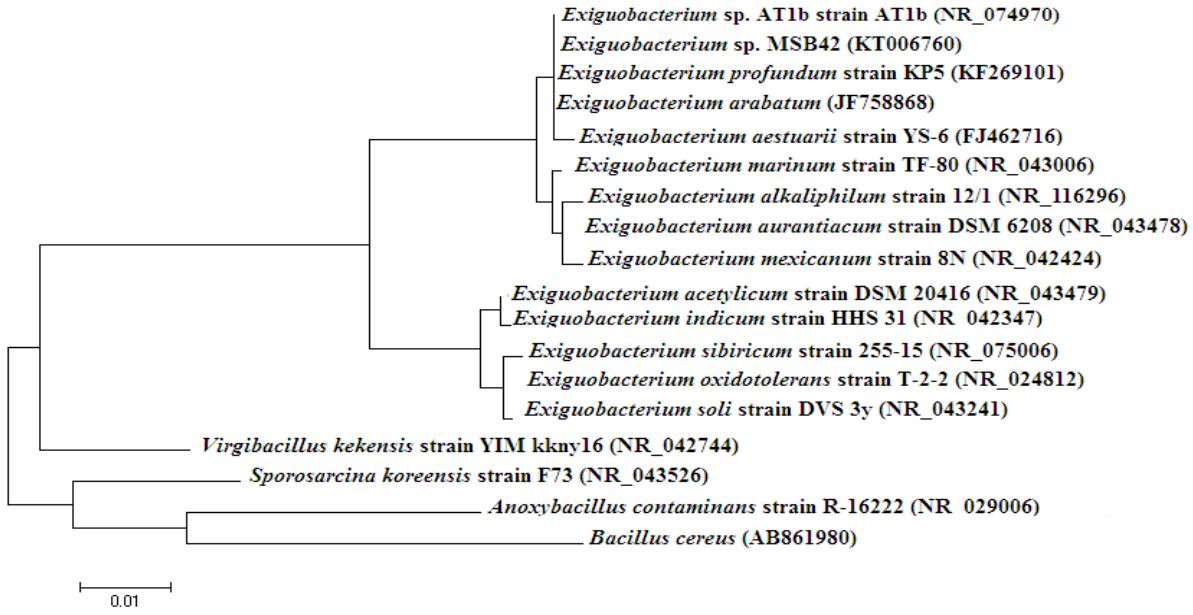

Figure 1. Phylogenetic tree showing the position of *Exiguobacterium* sp. MSB42, based on the 16S rRNA sequence comparison, obtained by the maximum likelihood method. The accession numbers of the reference strains are included in brackets. Bootstrap values are indicated on the branches.

All detected fragments had similar length and belonged to the M6 family of metalloproteases, which confirmed the accuracy of the designed primers. It is worth to note that *Exiguobacterium* is a genus derived from *Bacillus* genus and it is not surprising that similar genes were present in both genera. As defined by the MEROPS database, the M6 family of metalloprotease domain, such as an immune inhibitor A peptidase derived from *Bacillus thuringiensis,* have the HEXXHXXGXXD motif. In this motif, the catalytic residue is glutamate, and two histidines and aspartate are zinc ligands.

Unexpectedly, the PCR product had a 97% identity with a thermophilic strain of *Exiguobacterium* AT1b metalloprotease gene. Therefore, given that MSB42 is also closely related to *Exiguobacterium* sp. AT1b phylogenetically, and there is also a high percentage of similarity between the metalloprotease genes of both strains, it can be concluded that MSB42 is likely to be *Exiguobacterium* sp. AT1b.

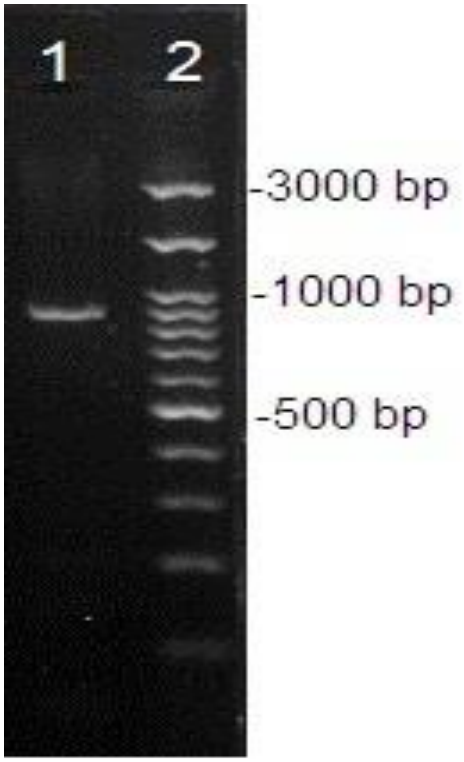

Figure 2. Gel electrophoresis of PCR product with the primers specific for the extracellular metalloproteases gene of the genus *Exiguobacterium*. Amplified product using the designed primers (lane 1), DNA size standard (lane 2).

3.3. Screening of effective physicochemical variables using the OFAT approach

Similar to previous studies, the production of the MSB42 enzyme increased during the stationary growth phase and after 60h incubation (61.29 U/ml) as shown in Figure 3 (Gupta et al., 2002; Perchat et al., 2011).

The strain showed the ability to produce protease in a wide pH range of 7-12 as illustrated in Figure 4. However, the protease production increased gradually by increasing the initial pH to the optimum value of 11 (73.95 U/ml). Among the studied temperatures, MSB42 showed higher alkaline protease production at 37ºC (Figure 4).

The strain MSB42 produced the lowest amount of enzyme with respect to the control at 1% and 5% w/v NaCl concentrations. However, the presence of more than 10% w/v NaCl in the culture medium resulted in a significant reduction or inhibition of MSB42 alkaline protease production (data not shown). These results clearly showed that the enzyme production did not depend on NaCl concentration.

Glucose (88.03 U/ml) and peptone (98.70 U/ml) significantly increased MSB42 alkaline protease with respect to other tested carbon and nitrogen sources (Figure 5).

3.4. Enzyme production optimization using the RSM approach

The design matrix, experimental, and predicted results are represented in Table 3. The model terms were considered as statistically significant when the values of "probability > *F*" were less than 0.05. ANOVA analysis indicated that the linear effects of all tested variables were statistically meaningful. The linear effect of pH was determined to be the most significant variable (*P*-value < 0.0001). In addition, all variables interactions except the interaction between glucose and peptone were also statistically significant based on the interaction terms *P* value (Table 4). The polynomial model of MSB42 alkaline protease production using the experimental date and based on the significant terms was obtained and expressed as follows:

Enzyme activity (U/ml) = $198.34 + (0.16*X_1) + (0.13*X_2) + (0.13*X_3) - (0.095*X_4) + (0.17*X_1X_2) - (0.17*X_1X_3) + (0.15*X_1X_4) - (0.11*X_2X_4) - (0.084*X_3X_4) - (0.15*X_1^2) - (0.21*X_2^2) - (0.28*X_3^2) - (0.30*X_4^2)$

in which X_1, X_2, X_3 and X_4 are pH, peptone concentration, glucose concentration and temperature (°C), respectively. The statistical significance of the model was confirmed by the results of a Fisher F-test (*F*-value 32.58) and ANOVA analysis (*p*-value < 0.0001). Based on

these results, the obtained regression model is very significant. In addition, the regression model fit, *i.e.*, a good correlation between the experimental and predicted data confirmed by the results of the non-significant lack of fit (*F*-value=2.06, *p* value= 0.2199), the pure error, the determination coefficient (R^2= 0.9682), and the adjusted determination coefficient (Adj R^2= 0.9384). Therefore, the obtained model can explain 96.82% and 93.84% of the variability in the response by considering the effect of all used independent variables in the model (R^2) and the only effective independent variables on the dependent variable (Adj R^2), respectively (Richa et al., 2013; Singh et al., 2011). Furthermore, the predicted determination (Pred R^2 = 0.8435) confirmed that the resulting model has a good predictive ability for new observations. In addition, the coefficient of variation (C.V., 0.067%) in the present study was very low. This shows the precision and reliability of the results of the experiments. The signal to noise ratio assessment, *i.e.*, an adequate precision value above 4 (18.95) also proves the established model was able to navigate the design space.

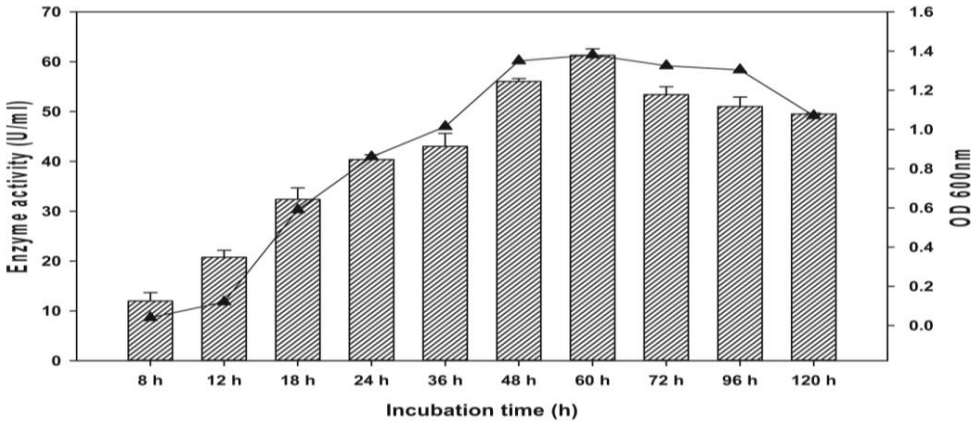

Figure 3. Kinetics of growth and protease production of *Exiguobacterium* sp. MSB42 in the basal medium. The *bars* indicate ± standard deviation.

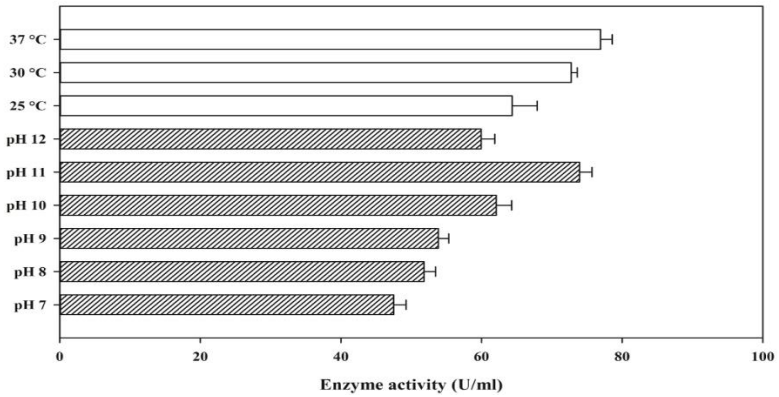

Figure 4. Effect of pH and temperature on protease production of *Exiguobacterium* sp. MSB42 in the basal medium after 60 h incubation.

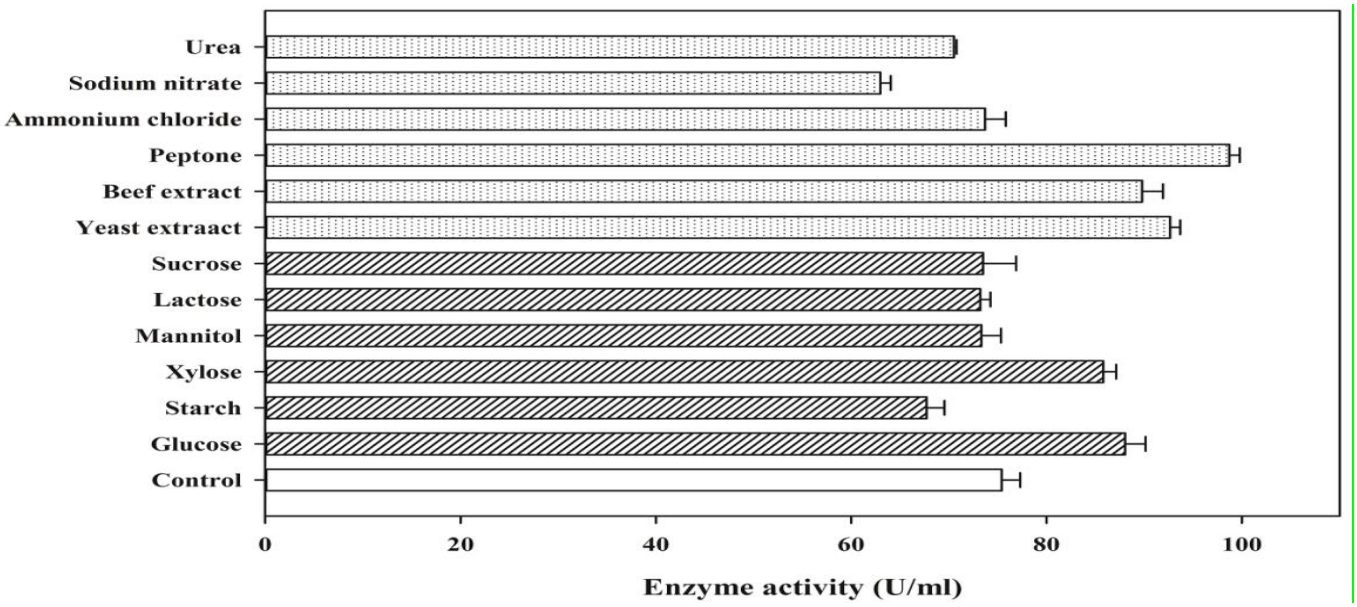

Figure 5. Effect of different carbon and nitrogen source on protease production of *Exiguobacterium* sp. MSB42 in the basal medium at a concentration of 1٪ w/v.

Table 1. Oligonucleotides used as primers for specific amplification and detection of the metalloprotease gene in the genus *Exiguobacterium.*

Oligonucleotide	Seq (5'-3')	Tm (°C)	Length of amplicon (bp)
Exig MP I	ATCATGAGTGGCGGTAGCTGG	58.5	873
Exig MP II	GACACCGAGGAATCCTTCACC	56.9	

Table 2. Experimental Range and Levels of the Independent Variables in Terms of Actual and Coded Factors

		Actual factors level at coded level				
Factor	**Name**	**-2**	**-1**	**0**	**1**	**2**
X_1	pH	7	8	9	10	11
X_2	Peptone (٪ w/v)	0	1.5	3	4.5	6
X_3	Glucose (٪ w/v)	0	1.5	3	4.5	6
X_4	Temperature (°C)	23	30	37	44	51

Table 3. Regression Analysis for Enzyme Production from *Exiguobacterium* sp. MSB42 for Quadratic Response Surface Model Fitting (ANOVA)

Run No.	X_1 pH	X_2 Peptone	X_3 Glucose	X_4 Temperature	Protease yield (U/ml)	
					Observed	Predicted
1	8.00	1.50	1.50	30.00	196.99	197.10
2	10.00	1.50	1.50	30.00	197.01	197.11
3	8.00	4.50	1.50	30.00	197.11	197.12
4	10.00	4.50	1.50	30.00	197.72	197.82
5	8.00	1.50	4.50	30.00	197.77	197.75
6	10.00	1.50	4.50	30.00	197.1	197.08
7	8.00	4.50	4.50	30.00	197.9	198.00
8	10.00	4.50	4.50	30.00	198.03	198.01
9	8.00	1.50	1.50	44.00	197.06	197.00
10	10.00	1.50	1.50	44.00	197.63	197.62
11	8.00	4.50	1.50	44.00	196.47	196.57
12	10.00	4.50	1.50	44.00	197.94	197.88
13	8.00	1.50	4.50	44.00	197.33	197.31
14	10.00	1.50	4.50	44.00	197.35	197.25
15	8.00	4.50	4.50	44.00	197.29	197.11
16	10.00	4.50	4.50	44.00	197.75	197.73
17	7.00	3.00	3.00	37.00	197.45	197.44
18	11.00	3.00	3.00	37.00	198.065	198.08
19	9.00	0.00	3.00	37.00	197.25	197.26
20	9.00	6.00	3.00	37.00	197.77	197.76
21	9.00	3.00	0.00	37.00	197.11	196.97
22	9.00	3.00	6.00	37.00	197.33	197.47
23	9.00	3.00	3.00	23.00	197.51	197.33
24	9.00	3.00	3.00	51.00	196.77	196.95
25	9.00	3.00	3.00	37.00	198.26	198.34
26	9.00	3.00	3.00	37.00	198.36	198.34
27	9.00	3.00	3.00	37.00	198.2	198.34
28	9.00	3.00	3.00	37.00	198.33	198.34
29	9.00	3.00	3.00	37.00	198.41	198.34
30	9.00	3.00	3.00	37.00	198.48	198.34

Table 4. The Regression Analysis for Enzyme Production from *Exiguobacterium* sp. MSB42 for Quadratic Response Surface Model Fitting (ANOVA)

Source	Sum of Squares	Degrees of freedom	Mean square	*F*-value	*p*-Value prob>*F*	Coefficient estimate
Model[a]	7.94	14	0.57	32.58	< 0.0001*	
Intercept						198.34
pH (X_1)	0.61	1	0.61	35.29	< 0.0001	0.16
Peptone (X_2)	0.38	1	0.38	21.68	0.0003	0.13
Glucose (X_3)	0.38	1	0.38	21.97	0.0003	0.13
Temperature (X_4) (X_4) Temperature	0.22	1	0.22	12.55	0.0030	-0.095
X_1X_2	0.47	1	0.47	26.76	0.0001	0.17
X_1X_3	0.47	1	0.47	26.76	0.0001	-0.17
X_1X_4	0.37	1	0.37	21.20	0.0003	0.15
X_2X_3	0.047	1	0.047	2.72	0.1201	0.054
X_2X_4	0.20	1	0.20	11.76	0.0037	-0.11
X_3X_4	0.11	1	0.11	6.54	0.0219	-0.084
X_1^2	0.59	1	0.59	33.70	< 0.0001	-0.15
X_2^2	1.19	1	1.19	68.24	< 0.0001	-0.21
X_3^2	2.16	1	2.16	124.07	< 0.0001	-0.28
X_4^2	2.48	1	2.48	142.38	< 0.0001	-0.30
Residual	0.26	15	0.017			
Lack of Fit	0.21	10	0.021	2.06	0.2199	
Pure error	0.051	5	0.010			
Cor total	8.20	29				

[a)] Std. Dev. (0.13); Mean (197.59); C.V.% (0.067); PRESS (1.28); R-Squared (0.9682); Adj R-Squared (0.9384); Pred R-Squared (0.8435); Adeq Precision (18.951)

*significant

The interactions between variables are also depicted (Figure 6). As shown in Figure 6a, the protease yield was enhanced by increasing the value of peptone and pH up to 4.5% and 10, respectively.

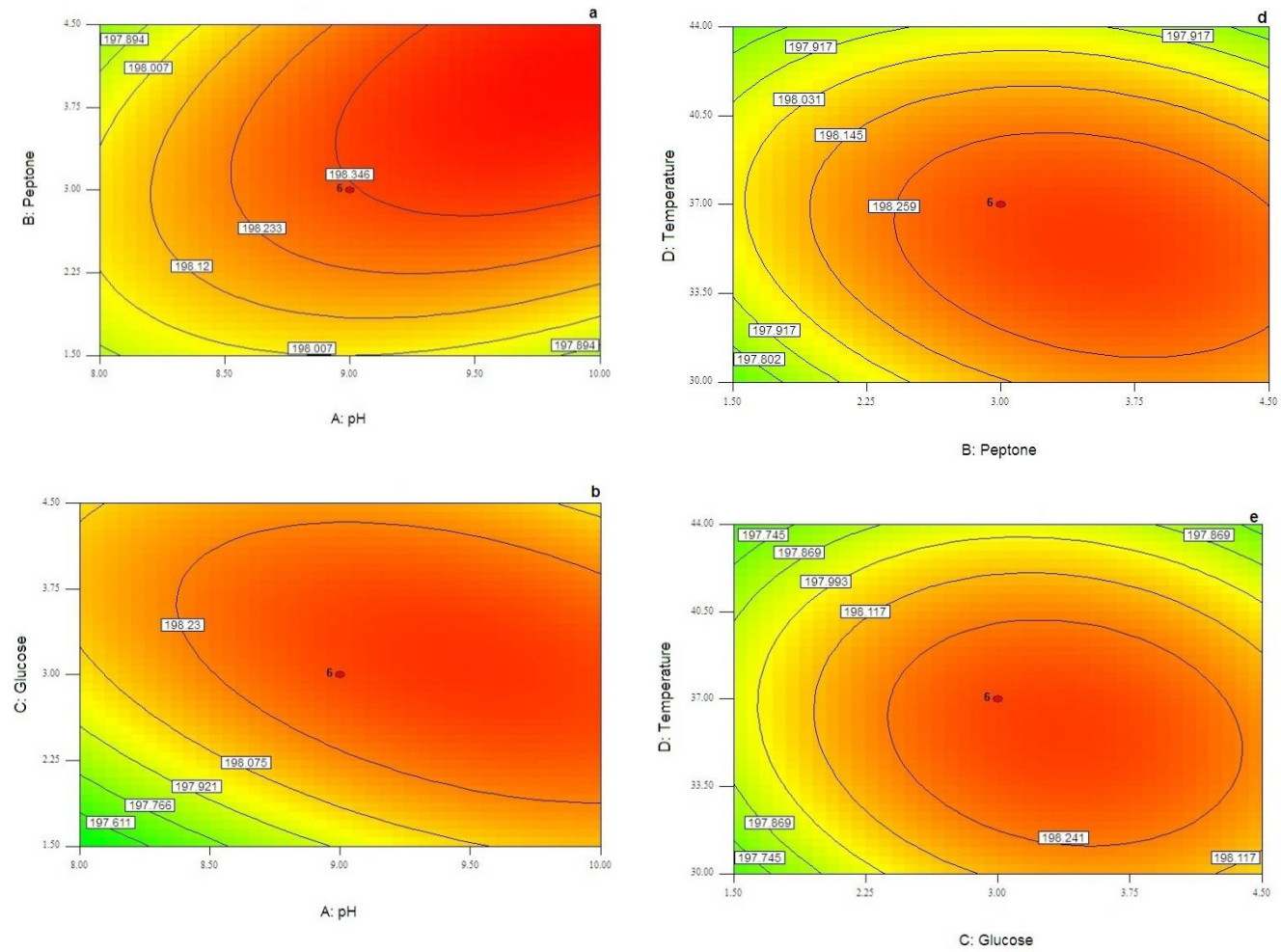

Figure 6. Contour plots of enzyme activity (U/ml) by *Exiguobacterium* sp. MSB42 as a function of pH (A), peptone (B), glucose (C), and temperature (D) in design space: a) interaction effect of pH and peptone; b) interaction effect of pH and glucose; c) interaction effect of pH and temperature; d) interaction effect of peptone and temperature; e) interaction effect of glucose and temperature.

Figure 6b shows increasing glucose concentration from 2.25 to 3.75 % w/v and pH value from 8 to10, also increased the enzyme synthesis. However, higher concentrations of glucose up to 4.5% w/v resulted in reduced protease production. Although there are contradictory reports about catabolite repression of enzyme production by glucose, this effect was

not observed in the present study (Chi et al., 2007; Patel et al., 2005). Figure 6c reveals that protease was produced more at higher levels of pH and temperature (10 and 40.5 °C) in the design range. As shown in Figure 6d, alkaline protease production was higher at higher peptone concentrations (3-4.5%, w/v) and temperatures (30-37°C). Validation of the model was performed for all four variables used in this study by performing a set of six experiments (Table 5). The experimental data of the enzyme production in these experiments were in agreement with the statistically predicted data within 5% of the predicted error. Therefore, the model's authenticity was confirmed. The steep slope or curvature for each factor in the perturbation plot represents that the response is sensitive to that factor with regard to the pH, temperature, carbon, and nitrogen source in the present study (Figure 7). In fact, the perturbation plot provides an opportunity to compare the effect of all studied factors at the center point (coded 0) in the design space.

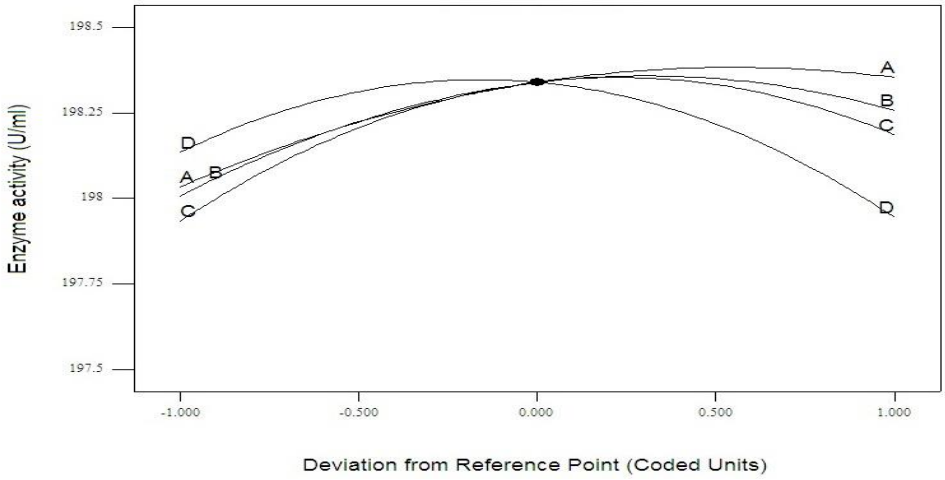

Figure 7. Perturbation plot of enzyme activity by *Exiguobacterium* sp. MSB42 as a function of pH: (A), peptone (B), glucose (C), and temperature (D).

Overall, the results of RSM confirmed the results of the OFAT strategy which stated the pH, temperature, nitrogen, and carbon sources had a considerable effect on MSB42 alkaline protease production. The enzyme production showed its maximum level at 37ºC, pH 9, 3% w/v glucose, and 3% w/v peptone (198.48 U/ml) using RSM strategy. However, the optimum levels of temperature, pH, carbon and nitrogen sources for another previously reported species of *Exiguobacterium* were 30°C, pH 9, lactose (1%) and corn steep solid (1%) (Anbu et al., 2013). This emphasizes that each organism and even each strain has its own nutritional need for optimum enzyme production (Bhunia et al., 2012).

Table 5. Validation of the Quadratic Model within the Design Space

Number	pH	Peptone (٪ w/v)	Glucose (٪ w/v)	Temperature (°C)	Actual response (U/ml)	Predicted response (U/ml)	Predicted error [a] (%)
1	9.72	4.17	2.04	35.31	195.45	198.291	1.433
2	9.80	3.35	1.50	34.47	193.33	198.073	2.395
3	8.90	4.23	4.03	31.07	201.45	198.274	1.602
4	8.66	1.91	4.02	32.00	199.98	198.011	0.994
5	8.55	2.06	1.58	37.96	192.34	197.707	2.715
6	8.32	4.41	3.12	41.86	195.47	197.651	1.103

[a] Predicted error (%) = (actual value - predicted value) × 100/predicted value.

4. Conclusions

Alkaline protease production from *Exiguobacterium* sp. MSB42 was improved by using the OFAT as well as RSM methods in this study. About a 2-fold (from 98.69 to 198.48 U/ml) increase in the protease production was observed after 60 h incubation at pH 9, 37°C and in a production medium containing 3% w/v glucose and 3% w/v peptone. Despite the differences observed between the results of the OFAT and RSM approaches, including the optimum pH of enzyme production (11 versus 9, respectively), both methods showed all tested variables had a significant influence on MSB42 enzyme production. This confirmed that the OFAT strategy can be used as a simple and practical screening method prior to RSM optimization. Furthermore, the metalloprotease gene in the genus *Exiguobacterium* was detected by using PCR. The amplified MSB42 metalloprotease gene belonged to the M6 family of metalloproteases and showed a 97% identity with a thermophilic strain of *Exiguobacterium* AT1b metalloprotease gene.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgements

This research was supported by the University of Isfahan.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. This article does not contain any studies with human participants or animals performed by any of the authors.

References

[1] Anbu P, Annadurai G, Hur BK (2013) Production of alkaline protease from a newly isolated *Exiguobacterium profundum* BK-P23 evaluated using the response surface methodology. *Biologia* 68(2): 186-193. Doi: 10.2478/s11756-013-0159-5

[2] Anwar A, Saleemuddin M (1998) Alkaline proteases: a review. Bioresour Technol 64(3): 175-183. Doi: 10.1016/S0960-8524(97)00182-X

[3] Beg QK, Sahai V, Gupta R (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochem* 39(2): 203-209. Doi: 10.1016/S0032-9592(03)00064-5

[4] Borhani MS, Etemadifar, Z., & Jorjani, E. (2017). Application of Rotatable Central Composite Design for Optimization of Extracellular Alkaline Protease Production from newly isolated *Bacillus pseudofirmus* MSB4. *Biological Journal of Microorganism*, *5*(20): 17-31

[5] Bhunia, B., Basak, B., & Dey, A. (2012). A review on production of serine alkaline protease by *Bacillus* spp. *Journal of Biochemical Technology*, *3*(4), 448-457.

[6] Cheng, S. W., Wang, Y. F., & Wang, M. L. (2012). Statistical optimization of medium compositions for alkaline protease production by newly isolated *Bacillus amyloliquefaciens*. *Chemical and biochemical engineering quarterly*, *26*(3), 225-231.

[7] Chi, Z., Ma, C., Wang, P., & Li, H. F. (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. *Bioresource Technology*, *98*(3), 534-538. Doi: 10.1016/j.biortech.2006.02.006

[8] Cupp-Enyard, C. (2008). Sigma's non-specific protease activity assay-casein as a substrate. *Journal of Visualized Experiments*, 19: 899-890. Doi:10.3791/899

[9] Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied microbiology and biotechnology*, *59*(1), 15-32. Doi:10.1007/s00253-002-0975-y

[10] Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., ... & Benjamin, S. (2013). Versatility of microbial proteases. *Advances in enzyme research*, 1(03), 39-51. Doi: 10.4236/aer.2013.13005

[11] Meena, P., Tripathi, A. D., Srivastava, S. K., & Jha, A. (2013). Utilization of agro-industrial waste (wheat bran) for alkaline protease production by *Pseudomonas aeruginosa* in SSF using Taguchi (DOE) methodology. *Biocatalysis and Agricultural Biotechnology*, *2*(3), 210-216. Doi: 10.1016/j.bcab.2013.05.003

[12] Naidu, K. S. B., & Devi, K. L. (2005). Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. *African Journal of Biotechnology*, *4*(7), 724-726. Doi: 10.5897/AJB2005.000-3132

[13] Patel R, Dodia M, Singh SP (2005) Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. *Process Biochemistry*, 40(11): 3569-3575. Doi: 10.1016/j.procbio.2005.03.049

[14] Perchat, S., Dubois, T., Zouhir, S., Gominet, M., Poncet, S., Lemy, C., ... & Lereclus, D. (2011). A cell–cell communication system regulates protease production during sporulation in bacteria of the *Bacillus* cereus group. *Molecular microbiology*, *82*(3), 619-633. Doi: 10.1111/j.1365-2958.2011.07839.x

[15] Reddy, L. V. A., Wee, Y. J., Yun, J. S., & Ryu, H. W. (2008). Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett–Burman and response surface methodological approaches. *Bioresource technology*, *99*(7), 2242-2249. Doi: 10.1016/j.biortech.2007.05.006

[16] Richa, K., Bose, H., Singh, K., Karthik, L., Kumar, G., & Bhaskara Rao, K. V. (2013). Response surface optimization for the production of marine eubacterial protease and its application. *Research Journal of BioTechnology*, *8*, 78-85.

[17] Selvakumar, G., Joshi, P., Nazim, S., Mishra, P. K., Kundu, S., & Gupta, H. S. (2009). *Exiguobacterium acetylicum* strain 1P (MTCC 8707) a novel bacterial antagonist from the North Western Indian Himalayas. *World Journal of Microbiology and Biotechnology*, *25*(1), 131-137.Doi: 10.1007/s11274-008-9874-4

[18] Sen, S., Veeranki, V. D., & Mandal, B. (2009). Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1. *Annals of Microbiology*, *59*(3), 531-538. Doi: 10.1007/BF03175142

[19] Shafee, N., Aris, S. N., Rahman, R. N. Z. A., Basri, M., & Salleh, A. B. (2005). Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. *J Applied Scientific Research*, *1*(1), 1-8.

[20] Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K., & Garg, S. K. (2011). Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic *Pseudomonas putida* SKG-1 isolate. *Microbial cell factories*, *10*(1), 114.Doi: doi: 10.1186/1475-2859-10-114.

[21] Tari, C., Genckal, H., & Tokatlı, F. (2006). Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochemistry*, *41*(3), 659-665. Doi: 10.1016/j.procbio.2005.08.012

[22] Vaishnav, D., Suthar, J., Oza, T., Dave, G., Sheth, N., & Sanghvi, G. (2014). A statistical approach for the enhanced production of thermostable alkaline protease showing detergent compatibility activity from *Bacillus circulans*. *Biocatalysis and Biotransformation*, *32*(3), 151-160. Doi: 10.3109/10242422.2014.913579.