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Optimization of Protease Production by Psychrotrophic Rheinheimera sp. with Response Surface Methodology

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Abstract

Background and Objectives: Psychrotrophic bacteria can produce enzymes at low temperatures; this provides a wide biotechnological potential, and offers numerous economical advantages over the use of mesophilic bacteria. In this study, extracellular protease production by psychrotrophic Rheinheimera sp. (KM459533) was optimized by the response surface methodology.

Materials and Methods: The culture medium was tryptic soy broth containing 1% (w v^{-1}) skim milk. First, the effects of variables were independently evaluated on the microbial growth and protease production by *one-factor-at-a-time* method within the following ranges: incubation time 24-120 h, temperature 15-37°C, pH 6-11, skim milk concentration 0-2% (w v^{-1}), and inoculum size 0.5-3% (v v^{-1}). The combinational effects of the four major variable including temperature, pH, skim milk concentration, and inoculum size were then evaluated within 96 h using response surface methodology through 27 experiments.

Results and Conclusion: In *one-factor-at-a-time* method, high cell density was detected at 72h, 20°C, pH 7, skim milk 2% (w v^{-1}), and inoculum size 3% (v v^{-1}), and maximum enzyme production (533.74 Uml^{-1}) was achieved at 96h, 20°C, pH 9, skim milk 1% (w v^{-1}), and inoculum size 3% (v v^{-1}). The response surface methodology study showed that pH is the most effective factor in enzyme production, and among the other variables, only temperature had significant interaction with pH and inoculum size. The determination coefficient (R^2=0.9544) and non-significant lack of fit demonstrated correlation between the experimental and predicted values. The optimal conditions predicted by the response surface methodology for protease production were defined as: 22°C, pH 8.5, skim milk 1.1% (w v^{-1}), and inoculum size 4% (v v^{-1}). Protease production under these conditions reached to 567.19 Uml^{-1}. The use of response surface methodology in this study increased protease production by eight times as compared to the observed before optimization.

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1. Introduction

Proteases are important in the food, detergents, and leather trimming industries and account for about 60% of the total global sales of industrial enzymes [1]. They are found in animals, plants, and microorganisms such as fungi, bacteria, and yeasts [2]. Most proteases used in various industries are de-mesophilic bacteria, whereas extremophiles microorganisms such as psychrophiles and cold-

resistant microrganisms can produce enzymes with unique features [3] (e.g. high activity at low temperatures and fast degradation). These properties made the enzymes good candidates for use in the detergent industry, leather processing, food processing, and molecular biology. Protease production in microorganisms is strongly influenced by the composition of the culture medium and such factors as inoculum size, temperature, and pH [4]. In addition, the cost of enzyme production is very important for industrial purposes [5]. So to reach the maximum production, it is necessary to optimize the enzyme production.

One-factor-at-a-time is used for initial screening of most of the significant parameters affecting protease production [6]. In this method, one factor is changed in each experiment, and other factors are kept constant [7]. To achieve more efficiency after *one-factor-at-a-time*, the response surface methodology (RSM) is used in order to evaluate the combinatorial effects of the variables [6].

Use of psychrotrophic native bacteria in different industries can reduce energy consumption and production cost. Efforts have been made on protease production with different bacteria, e.g. Pseudomonas [5], Colwellia [6], Curtobacterium [8], Acremonium [9] and Pedobacter [10] with different substrates including gelatin [5], casein [6] and skim milk [11].

Reducing the total cost of produced enzymes is still an unsolved problem. In this paper, we attempted to optimize highly significant factors affecting protease production by psychrotrophic Rheinheimera sp. isolated from Binaloud mountain first through *one-factor-at-a-time* approach, and then by Box-Behnken design [12]. This is the first RSM study on production of cold-active protease by the native bacteria in Iran.

2. Materials and Methods

2.1. Bacterial strain

Soil samples were collected from the Binaloud mountain in the northeast of Iran. After serial dilution of the soil samples, 100 µl of each dilution was cultured on tryptone soy agar and incubated at 4°C for 7 days. Primary screening for protease production was done on tryptone soy agar medium containing 2% skim milk [13]. Genomic DNA of the strain was extracted using the FastDNA® SPIN Kit (MP Biomedicals, Qbiogene) according to the manufacturer's instructions. The identification of bacterial species was performed based on the amplification and gene sequencing of 16S rRNA [14] using general primers, namely, 27F and 1492 R.

2.2. Protease production medium

Extracellular protease was produced in 20 ml TSB (tryptic soy broth) medium containing 1% (w v^{-1}) skim milk. Two hundred micro-liter of standard bacterial suspension was added to the culture medium and incubated in shaker incubator at 150 rpm and 20°C. The medium was centrifuged at 757 ×g for 10 min, and the supernatant was used as the crude enzyme [15].

2.3. Protease activity assay

Proteolytic activity was measured through Kunitz assay [16] as follows: 100 µl of cell-free enzyme solution was added to 100 µl of casein solution 0.5% (w v^{-1}) in Tris-HCl 50 mM buffer with pH 8, and incubated at 30°C for 10 min. Then 300 µl of trichloroacetic acid 10% (w v^{-1}) was added and incubated at 4°C for 15 min to stop the reaction. The reaction mixture was centrifuged at 909 ×g for 10 min, and the absorbance of supernatant was read at 280 nm. One unit of proteolytic activity is defined as the amount of enzyme that liberates 1 µg of tyrosine per minute under assay conditions. Tyrosine standard curve was used to convert the absorbance into the enzyme activity [17].

2.4. *One-factor-at-a-time*

In the *one-factor-at-a-time* method, all variables are kept constant on a contract basis at any stage of optimization, and only the effect of one variable is studied and its optimal level is determined. In the next step, the optimized variable in the previous step is used as a basis.

In order to determine the best time of incubation for protease production, the proteolytic activity was measured after 24, 48, 72, 96, 120, and 144 h. To evaluate the effect of temperature on protease production, flasks containing inoculated production medium were incubated at 15, 20, 25, 30, and 37°C. The best initial pH was determined by adjusting the pH of the production medium at 6, 7, 9, and 11. To determine the optimum level of skim milk for protease production, skim milk was used at 0, 0.5, 1, 1.5, and 2% (w v^{-1}) concentrations. The impact of different carbon sources (glucose, maltose, sucrose, and starch) was investigated by adding 1% (w v^{-1}) of each one to the production medium. Then the effect of nitrogen sources (yeast extract, ammonium sulfate, peptone, and skim milk) was evaluated by adding 1% (w v^{-1}) of each one to the production medium. To examine the effect of inoculum size on protease production, 0.5, 1, 2, and 3% (v v^{-1}) of bacterial suspension containing about 1.5×10^8 cell ml^{-1} were used [18].

2.5. Optimization with RSM statistical method

In the present study, the effect of four independent variables including temperature, pH, skim milk, and inoculum size on the production level and the interaction of these factors was evaluated based on the Box-Behnken design using Minitab program (version 16). Box-Behnken is a quadratic design at three levels, coded as -1, 0, and 1

[19]. Zero or the central level is the amount of each factor determined by *one-factor-at-a-time* as the optimal level, and levels -1 and 1 are the minimum and maximum levels, respectively. The obtained model for statistical analysis of the results is a quadratic polynomial regression equation. In this model, the dependent variable is expressed as a function of the impact of each factor and their interactions. For a system with four independent variables, this quadratic equation in Box-Behnken design is expressed as follows:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4$$

Where, Y is the predicted response (enzyme activity), β_0 is fixed model, X_1, X_2, X_3, and X_4 are independent variables, β_1, β_2, β_3, and β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, and β_{44} are quadratic coefficients, and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are interaction coefficients.

3. Results and Discussion

3.1. Microorganism isolation and identification

Rheinheimera strain (KM459533) was isolated from the Binaloud mountain in the northeast of Iran. From 145 psychrophilic and psychrotolerant isolates, 102 (70%) isolates were protease producers, among them Rheinheimera strain, which showed maximum protease production, was selected for optimization. The partial 16S rRNA gene sequencing was carried out and compared with the NCBI and Ez-taxon databases. Analysis of 16S rRNA gene sequence revealed that the isolate has 98.53% resemblance with Rheinheimera genera. The GenBank accession of the sequence is KM459533.

3.2. *One-factor-at-a-time* method

The enzyme Production and bacterial growth were studied for 144 h at 20°C and pH 7. The highest level of protease production occurred after 96 hours (70.91 Uml^{-1}) (Figure 1a). Protease production in this strain was not growth-dependent, and the majority of protease production occurred at the death phase. The effect of temperature on enzyme production and cell growth at pH 7 was investigated after 96 hours at 15-37°C. Protease production and bacterial growth reached their maximum level at 20°C, which is consistent with the definition by Morita for psychrotrophic bacteria [20]. By further increase in temperature, the enzyme production was reduced, and finally, reached to zero at 37°C (Figure 1b). This observation is reasonable for psychrotrophic bacteria as they are adapted to low temperatures. In 2008, Kuddus and Ramteke reported the best temperature for protease production of psychrotrophic Stenotrophomonas as 20°C, which was decreased at temperatures above 20°C, and was totally inhibited at 45°C [21]. This low-temperature cultivation made it avoid the risk of contamination by other microorganisms [6]. In order to evaluate the effect of initial pH on protease production and cell growth, the isolate was cultured in the production medium with pH ranging from 6 to 11 at 20°C. The highest level of protease was produced at pH 9 (418.93 U ml^{-1}). Further increase in pH of the medium greatly decreased both enzyme production and cell growth (Figure 1c).

Enzyme's substrate level, which accounts for enzyme production stimulus, is one of the most important variables in enzyme production. Therefore, skim milk was used at concentrations 0, 0.5%, 1%, 1.5%, and 2% (w v^{-1}) in the culture medium. The results showed that the studied strain can produce protease in skim milk-free medium, suggesting that the enzyme is constitutive (Figure 1d). The highest level of protease production was observed at skim milk 1% (w v^{-1}), while further increase in the skim milk decreased the enzyme production, which finally, stopped completely at skim milk 2% (w v^{-1}), despite significant cell growth in this condition.

The addition of simple sugars such as glucose, maltose, and sucrose greatly decrease protease production so that both glucose and maltose inhibit production (Figure 1e). Similar catabolite repression by simple sugars has been reported for protease production by Virgibacillus [22] and Colwellia [6]. They suggested that in the absence of the sugar, protease produces peptides and amino acids, which act not only as a nitrogen source but also as an energy source [6]. So in the presence of sugars, protease production is inhibited.

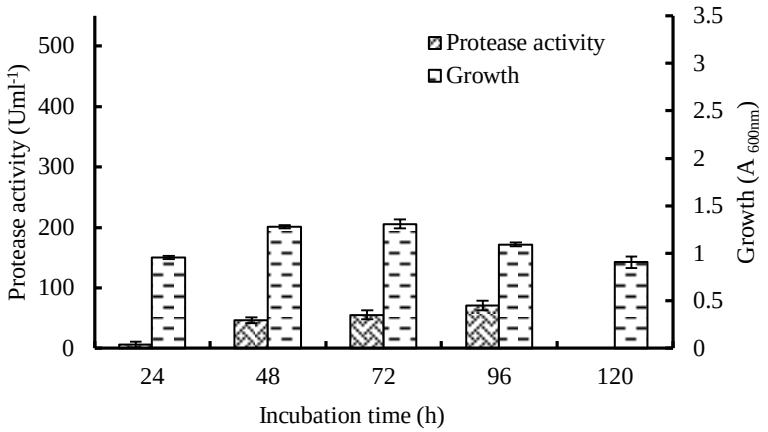
Adding 1% (w v^{-1}) nitrogen sources such as yeast extract, ammonium sulfate, peptone, and skim milk to the medium showed that the highest production of protease was achieved after addition of skim milk (433.63 Uml^{-1}) (Figure 1f). However, adding nitrogen as an inorganic source greatly decreased protease production (27.15 Uml^{-1}). Similar results have been obtained from research on cold-resistant bacteria [8,15,23].

To evaluate the effect of inoculum size on protease production, inoculum sizes of 0.5% to 3% (v v^{-1}) were used. The results showed that the inoculum size of 3% (v v^{-1}) led to the maximum enzyme production (Figure 1g).

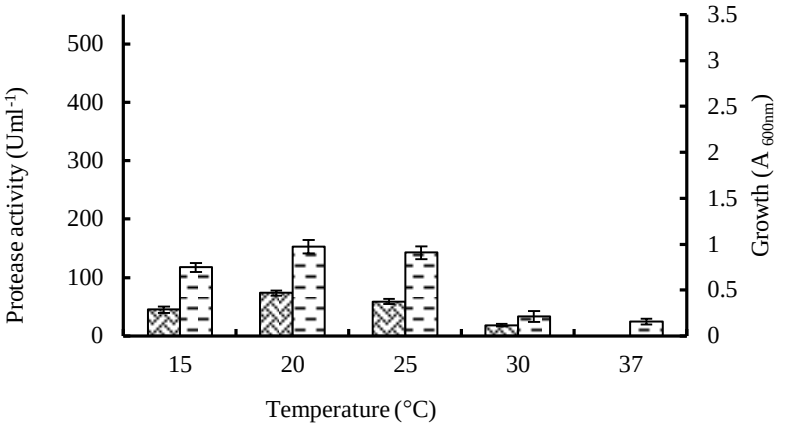
3.3. Optimization using RSM

The experiments revealed that temperature, pH, and skim milk, and inoculum size had a significant impact on the enzyme production. Accordingly, the impact of these variables on enzyme production and their interactions were studied through RSM Box-Behnken design, and 27 experiments were performed. Table 1 depicts the Box-Behnken design as well as the measured and predicted responses. The effects of different variables on the production of protease are given in Table 2.

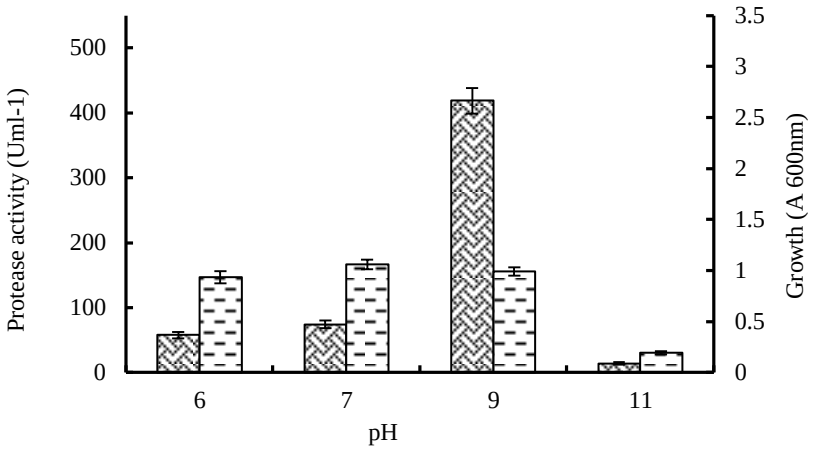
(a)


Protease activity
Growth
Protease activity (Uml-1)
Growth (A 600nm)
500
400
300
200
100
0
3.5
3
2.5
2
1.5
1
0.5
0
24
48
72
96
120
Incubation time (h)

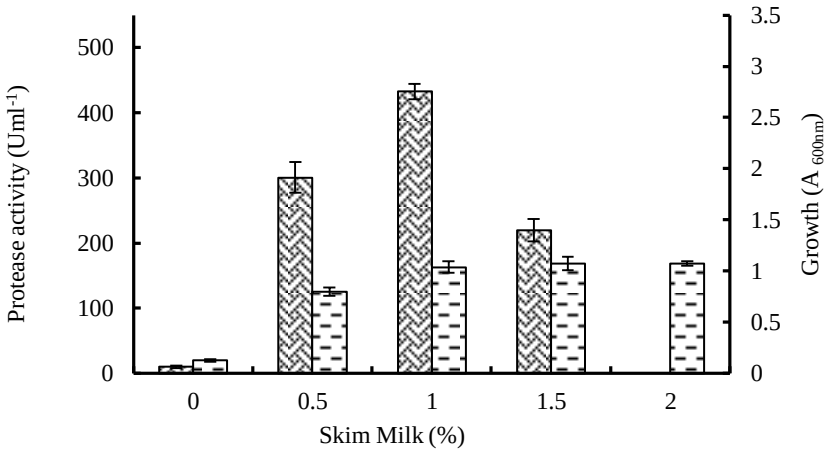
(b)


Protease activity (Uml-1)
Growth (A 600nm)
500
400
300
200
100
0
3.5
3
2.5
2
1.5
1
0.5
0
15
20
25
30
37
Temperature (°C)

(c)


Protease activity (Uml-1)
Growth (A 600nm)
500
400
300
200
100
0
3.5
3
2.5
2
1.5
1
0.5
0
6
7
9
11
pH

(d)


Protease activity (Uml-1)
Growth (A 600nm)
500
400
300
200
100
0
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2
Skim Milk (%)

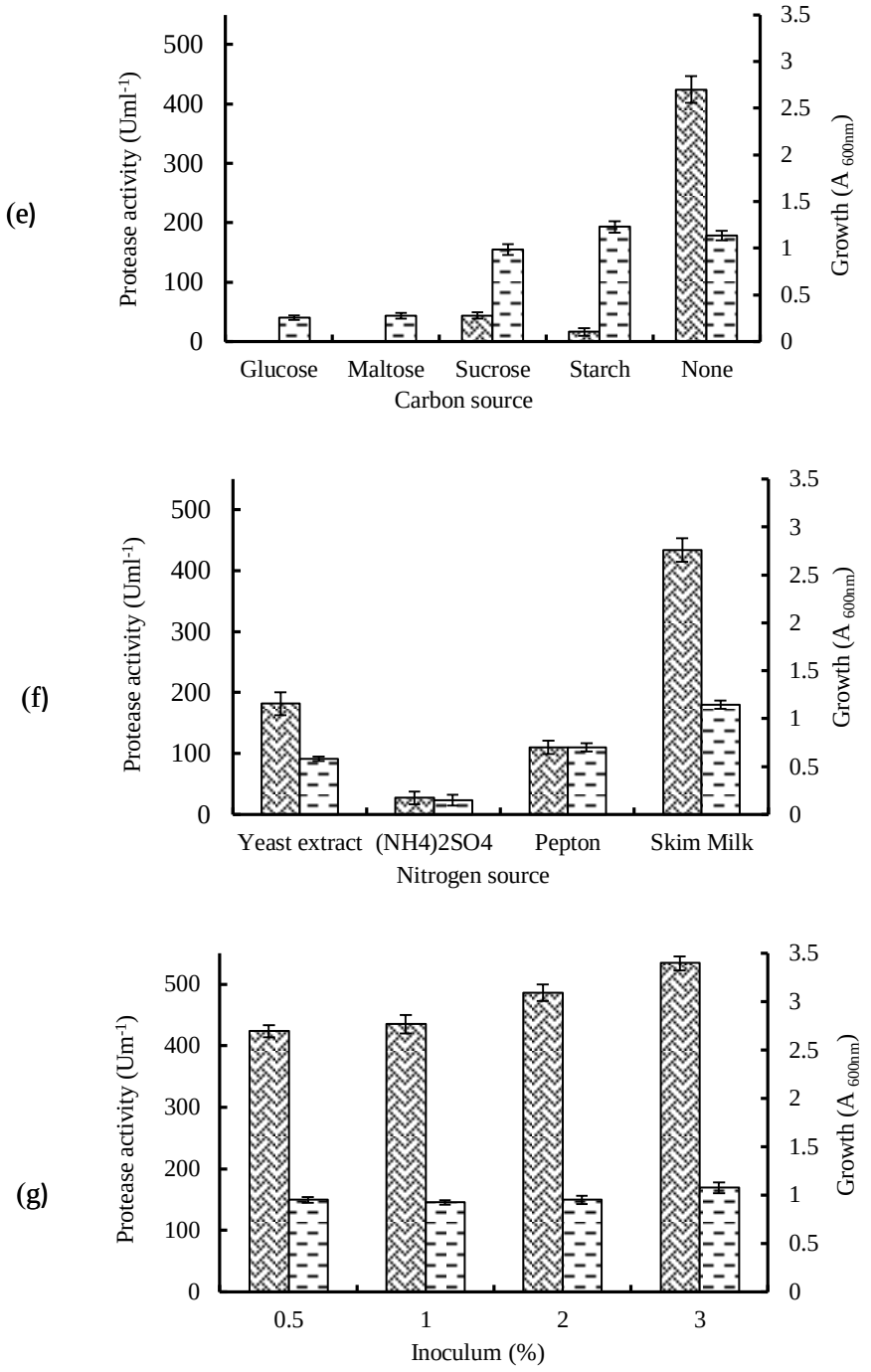
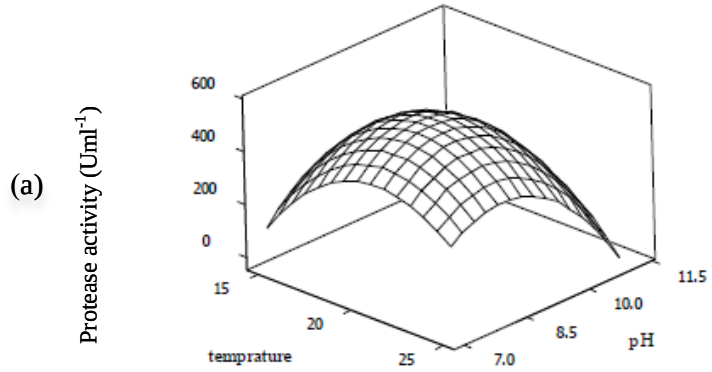
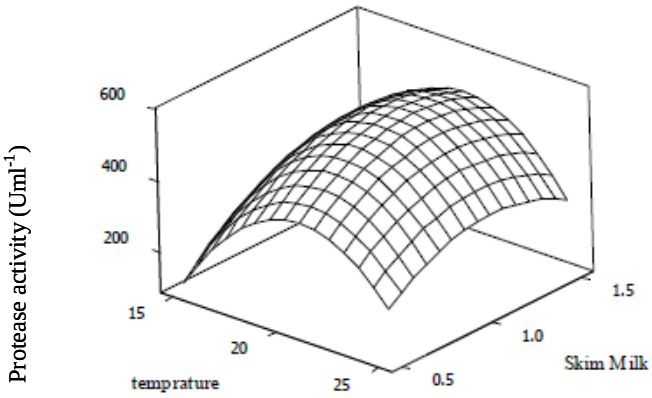


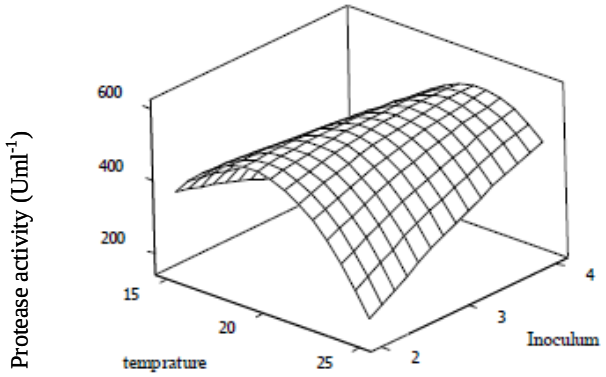
Figure 1. Effect of different variables on growth and protease production in the *one factor at a time* method



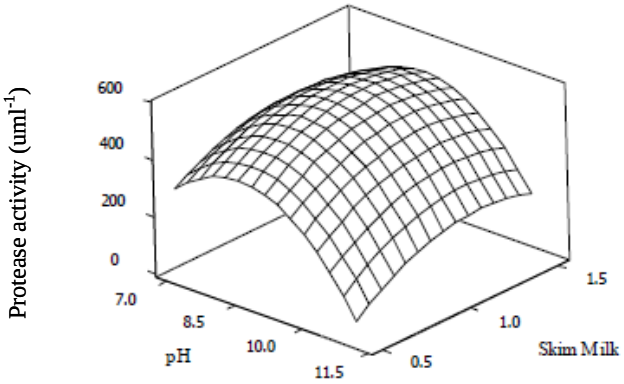
(b)


Protease activity (Uml-1)
600
400
200
15
20
temprature
25
0.5
1.0
1.5
Skim Milk

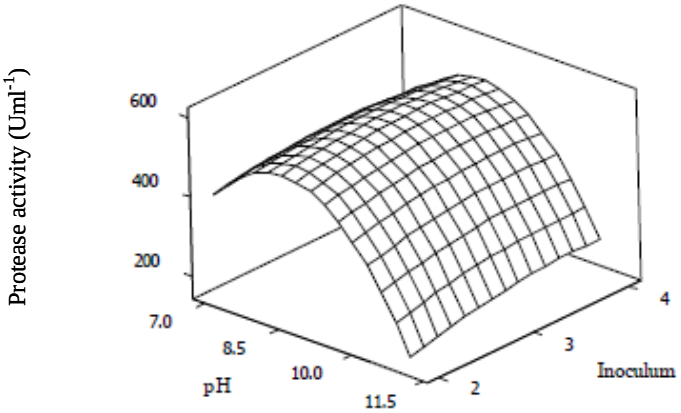
(c)


Protease activity (Uml-1)
600
400
200
15
20
temprature
25
2
3
4
Inoculum

(d)


Protease activity (uml-1)
600
400
200
0
7.0
8.5
10.0
11.5
pH
0.5
1.0
1.5
Skim Milk

(e)


Protease activity (Uml-1)
600
400
200
7.0
8.5
10.0
11.5
pH
2
3
4
Inoculum

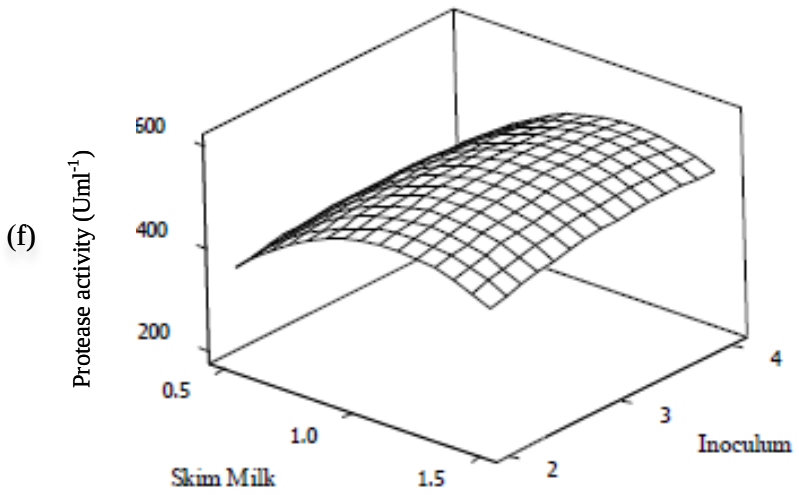


Figure 2. Three-dimensional diagrams of response surface

Table 1. RSM study to evaluate the effect of temperature, pH, skim milk and inoculum size on protease production by psychrotrophic Rheinheimera.

Run	Experimental variable				Protease yield (ml^{-1})	
	Temperature (°C)	pH	skim milk (%w v^{-1})	Inoculum (%v v^{-1})	Predicted	Observed
1	20	9	1.0	2.3	532.6986	553.08
2	20	9	1.0	3	532.6986	545.31
3	20	7	1.0	4	394.9942	423.83
4	20	9	1.5	2	451.1631	509.22
5	15	7	1.0	3	112.3378	80.9
6	15	9	1.0	4	166.6948	186.31
7	25	9	1.5	3	295.3397	296.35
8	20	7	1.0	2	402.2418	421.11
9	20	11	1.0	4	237.3589	202.52
10	20	7	0.5	3	293.5278	335.85
11	20	9	1.5	4	503.7082	489.75
12	25	11	1.0	3	0	32.83
13	15	9	1.5	3	280.8445	306.16
14	20	11	1.0	2	170.3186	125.84
15	15	9	1.0	2	366.0038	324.4
16	20	9	0.5	4	369.6276	327.41
17	15	11	1.0	3	41.6737	87.84
18	20	9	1.0	3	532.6986	500.1
19	20	7	1.5	3	364.1919	335.03
20	25	9	1.0	2	204.7447	186.86
21	25	9	0.5	3	235.547	195.57
22	20	11	0.5	3	57.9808	87.84
23	25	7	1.0	3	304.3992	274.15
24	20	11	1.5	3	210.1804	168.2
25	25	9	1.0	4	465.6583	508.95
26	15	9	0.5	3	114.1497	97.51
27	20	9	0.5	2	360.5681	388.28

Table 2. Effect of different variables on protease production

Term	Coef.	P-value
Constant	532.83	0.000
Temperature	34.30	0.037*
pH	-97.15	0.000***
Skim milk	56.02	0.002**
Inoculum	15.25	0.317
Temperature/pH	-62.07	0.030*
Temperature/Skim milk	-26.97	0.307
Temperature/Inoculum	115.05	0.001***
pH/Skim milk	20.29	0.438
pH/Inoculum	18.49	0.479
Skim milk/Inoculum	10.35	0.690

The total predictive ability of the model can be described by R^2, which is a measure of the versatility of the obtained results with the predicted results [6].

The corresponding analysis of variance (ANOVA) is presented in Table 3. The high value of the coefficient of determination (R^2 = 0.9544) indicated that only 4.56% of the total variation was not explained by the model. The high value of R^2 for enzyme production and insignificance of lack-of-fit (0.239) indicated compliance with the model. Polynomial equation was obtained from the regression analysis, in which the activity of protease (Y) is a function of the independent variables, as follows:

$$Y_{Rheinheimera}=532.83+34.30X_1-97.15X_2+56.02X_3-210.55X_1^2-210.79X_2^2-90.63X_3^2-62.07X_1X_2+115.05X_1X_4$$

Where, Y is protease activity and X_1, X_2, X_3, and X_4 are temperature, pH, and skim milk and inoculum size, respectively. The coefficient values and P-value for the impact of each factor on production are represented in Table 2, and the responses obtained based on this model are depicted in Fig. 2 (a-f). As the model showed, temperature, pH and skim milk had significant effect on the response (enzyme production), of which pH showed the greatest impact. Regarding the optimization of protease production in cold resistant bacterium (*Pseudomonas putida)*, Singh et al. [5] specified pH as the most important factor in production of protease.

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of squares	Degrees of freedom	Mean squares	F-ratio	Probability (p)
Model	643159	14	45940	17.94	0.000
Residual	30728	12	2561		
Lake of fit	29090	10	2909	3.55	0.239
Pure error	1637	2	819		
Corrected total	673887	26			
R^2 =0.9544	R^2_{Pred} = 0.7459	R^2_{adj} =0.9012			

Interaction between temperature and pH showed a negative regression (negative sign of X_1X_2). This means increasing in pH (up to 8.5) at low temperature leads to more enzyme production (Fig. 2a).The interaction between temperature and inoculum size showed a positive regression; so increasing in temperature (up to 22°C) and inoculum size (up to 4% v v^{-1}) at the same time leads to high protease production (Fig. 2c). Positive sign of the X_1X_4 coefficient in this model confirms that these two factors have a concurrent relationship. Dutta et al. [7] have recently shown a concurrent relationship between temperature and inoculum size. The results revealed that Rheinheimera sp. synthesizes more enzymes in alkaline pH and low temperatures. This emphasizes that the strain is cold and alkaline tolerant. Optimal conditions for enzyme production based on modeling and data analysis using Box-Behnken design were 22°C, pH 8.5, skim milk 1.1% (w v^{-1}), and inoculum size 4% (v v^{-1}). The production of protease at these conditions was 567.19 Uml^{-1}, which was close to the value predicted by the regression model (572.86 Uml^{-1}). These amounts were also close to the results of *one factor at a time* method, which were 20°C, pH 9, skim milk 1% (w v^{-1}), and inoculum size 3% (v v^{-1}). Optimization of protease production by psychrotrophic Rheinheimera sp. using RSM improved the enzyme production by eight times (567.19 Uml^{-1}). Wang et al. [6] were able to increase protease production by three-fold (175 Uml^{-1}) through optimization of enzyme production in psychrophilic bacterium Colwellia by RSM. They observed maximum enzyme production after 96 hours at 8°C and pH 7.5 with 5% (v v^{-1}) inoculation. Singh et al. [5] optimized the production of protease in psychrotrophic bacterium Pseudomonas through RSM. He could increase the enzyme production by nine times (617 Uml^{-1}) in 25°C, pH 8.8 and inoculum size 2% (v v^{-1}) over 72 hours. Both of the above trials are comparable with our results regarding the increasing of enzyme production for scaling up. In general, we significantly increased cold active protease production from native bacteria, which can be used for large scale fermentation and decreasing energy consumption and production cost in different industries.

4. Conclusion

Rheinheimera sp. (KM459533) is a psychrotrophic bacterium producing cold active protease in alkaline pH. Optimization of protease production by RSM significantly increases the amount of production, which would offer for large scale fermentation. The optimized conditions for protease production determined using RSM were: 96 h, 22°C, pH 8.5, skim milk 1.1% (w v^{-1}), and inoculum size 4% (v v^{-1}). Due to cold and pH tolerant activity, this enzyme can be applied in the detergent and food industries, and in polluted sites for bioremediation. This was the first report that RSM was used in the optimization for cold active protease production by a native bacterium in Iran.

5. Acknowledgement

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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