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Original Article

Isolation and Identification of a Keratinolytic *Bacillus cereus* and Optimization of Keratinase Production

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Abstract

A keratin-degrading bacterium was selected from a bacterial collection of the Najm Biotech Company. Molecular identification indicated that the bacterium is a strain of *Bacillus cereus*, which can grow and produce keratinase in basal medium containing feather as sole source of carbon and energy. The physicochemical condition (pH, temperature, incubation time, feather concentration) of keratinase production of the isolated *B.cereus strain* was optimized using response surface methodology (RSM). A maximum keratinase production of 350 U/ml was achieved in 96 h under optimized conditions.

Keywords: Feathers, Keratinase, *Bacillus cereus*, Optimization, Response Surface Methodology

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Introduction

Feathers are mainly composed of hard keratin, an insoluble fibrous protein extensively cross-linked by disulfide bonds [1]. They are resistant to proteolysis and indigestible by common proteases. Feathers are the main by product of the poultry industry; about 8 billion tons of poultry feathers are produced annually [2] and are currently converted into feather meal through physicochemical processes [3].

However, feather meal production process requires high energy input resulted in low quality protein [3]. Keratin can also be converted into valuable biomaterial using keratinase enzymes [2, 4]. Keratinase [EC 3.4.21/24/99.11] is a serine or metalloprotease capable of degrading keratin [5]. Microorganisms, including bacteria and fungi, grow on keratin wastes by producing keratinase to use it as the source of carbon and energy. Microbial keratinolytic enzymes have applications in the detergent, feed, leather, fertilizer and pharmaceutical industries and, above all, improve the digestibility of keratinous products such as feather meal.

Many researchers have been carried out on the identification and isolation of keratinolytic microorganisms and some of the microbial keratinolytic enzymes are used in industry and have been commercialized [6-8]. Enzyme production is influenced by growth conditions and media components. Generally, each strain require specific conditions for enzyme production which should be optimized for high level enzyme production [9, 10].

In the current study a novel keratinolytic bacterium (*Bacillus cereus*) was isolated which can degrade feathers. Response surface methodology (RSM) was employed to optimize conditions for production of keratinolytic enzyme. These factors including feather concentration, incubation time, pH and temperature were optimized to improve keratinase production.

Materials and Methods

Isolation of keratinolytic bacteria

A bacterial collection, including bacteria isolated from mushroom farms [11] of Najm Biotech Company was screened for keratinolytic bacteria. In primary screening, bacteria were cultured on skim milk agar medium (2% skim milk, and 2% agar at pH 7) and incubated at room temperature for 16 h and evaluated for clear zone formation around colonies. Bacteria with clear zone around colonies were considered as proteolytic bacteria and subjected to secondary screening for keratinolytic activity. Protease positive bacteria were cultured on feather meal agar medium and incubated at room temperature for 16 h. Feather degradation was confirmed by formation of clear zone around colonies. The isolated keratinolytic bacteria were inoculated in a basal broth medium (NH_4Cl (0.5 g/L), NaCl (0.5 g/L), KH_2PO_4 (0.4 g/L), K_2HPO_4 (0.3 g/L), $MgCl_2.6H_2O$ (0.1 g/L), yeast extract (0.1 g/L) and feathers (10 g/L) at room temperature and 150 rpm. After 24 h of incubation, feather degradation was determined visually. The supernatant was used for the keratinase assay and its protein content was measured by Bradford method.

Keratinase assay

Keratinase activity was measured by the modified method of Takiuchi *et al*., [12] using 3 ml of 0.3% keratin in phosphate buffer and 2 ml of culture supernatant. After 1 h of incubation at 30°C, 1 ml of 10% TCA was added to halt the enzymatic reaction and the mixture was centrifuged for 10 min at 10000 rpm at 4°C. The optical density (A 280) of the supernatant was measured against a control. The control (medium without bacteria) was treated in the same manner, except that TCA was added before incubation. One unit (U) of keratinolytic activity was defined as a 0.01

increase in absorbance at 280 nm (A 280) under defined conditions.

Molecular identification of bacteria

Genomic DNA of the isolated keratinolytic bacterium was extracted and purified with a DNA purification kit (Najm Biotech; Iran). 1 µl of the extracted DNA and primers, fD1 (5'AGAGTTTGATCCTGGCTTAG3') (10 pmol) and rD1 (5'TAAGGAGGTGATCCAGCC3') (10 pmol) (Cinnagen; Iran), were used for amplification of the 16S rDNA. PCR was performed in a DNA thermal cycler (Techne Flexigen; USA). Amplification was carried out as 5 min of initial denaturation at 94°C, 30 cycles each at 94°C for 30 s, 50°C for 30 s and 72°C for 90 s and a final extension at 72°C for 10 min. The PCR product was cloned into the pTZ57R/T vector (fermentase) and plasmid isolation was performed using a High Pure Plasmid Isolation Kit (MBST; Iran). Cloning of 16S rDNA was confirmed by PCR using M13Fwd and M13Rev primers. Sequencing was performed in both directions to obtain a reliable sequence using M13 universal primers by the dideoxy termination method with an ABI automated sequencer (Bioneer, korea). The sequence was compared by alignment with existing 16S rDNA sequences from the GenBank database and BLASTN software at http://www.ncbi.nih.gov/blast to identify the bacteria.

Phylogenetic analysis of 16S rRNA gene

Alignment of the 16S rRNA sequence with 16S rRNA sequences available in the NCBI and EZtaxon databases was carried out using related databases. A neighbor-joining (NJ) phylogenetic tree was then created using Mega 5.0 program and the nodal support was evaluated by 500 bootstrap replicates. A phylogenetic was created with a tree view.

Experimental design

A standard RSM design named central composite design (CCD) was used for optimization of keratinase production. This method is well-suited for fitting a quadratic surface and usually works well for process optimization. The four factors evaluated were feather concentration, pH, temperature and incubation time. The design is composed of a factorial core that forms a cube for which the sides are two units coded by length (-1 to +1; Table 1).

Table 1. Factors for process optimization.

Factor	Unit	Low level (-1)	High level (+1)
Feather	g/L	5	15
pH	-	5	10
Time	h	24	72
Temperature	°C	15	30

The alpha factor of the CCD was 1.68 (α = 1.68). The two most important responses were total protein (mg) and keratinase activity (U). Design-Expert software version 7.0 was used for statistical analysis.

The experimental results were loaded into software for analysis of variance (ANOVA) and predictive mathematical model. Several models were presented as the best by the software and were run again in two repeats with a block sample under the conditions introduced.

Results

Screening of keratinase producing bacteria

The keratinolytic bacterium was selected from the mentioned bacterial collection through culture of isolates on skim milk and feather agar medium respectively to evaluate their aptitude for protein and keratin hydrolysis. Production of keratinase enzyme was evaluated semi-quantitatively as clear sector diameter/bacterial colony diameter on the feather agar plates. Three strains showed the highest ratios. The selected bacterium showed the highest feather hydrolysis (22 mm; index value of 6.0) and proteolytic activity (17 mm; index value of 4.9).

Molecular characterization

The selected keratinolytic bacterium was identified based on the 16S rDNA sequence. Sequence analysis revealed a DNA fragment about 1500 bp confirming the 16S rRNA genes. The 16S rDNA sequence was analysed with blast program in NCBI and EZtaxon web servers and was identified as *B. cereus* strain NYA101 with about 99% homology to available sequences. Also phylogenetic analysis of 16S rRNA gene confirmed it (Fig. 1).

Optimization of culture medium and growth condition

The feather concentration, temperature, incubation time and pH were optimized to increase degradation of feathers and keratinase production. Suitable condition for Keratinase production was first determined from preliminary experiments using feather as the sole carbon and nitrogen source. The optimization experiments were designed with a central composite design (CCD) and total of 21 experiments (8 as cube points, 8 as star points, and 5 as center points). The star points were located at +α and –α from the center of the experimental domain. Axial distance α was selected with a value of 1.68 to establish the rotatable and orthogonal conditions of the CCD (Table 2).

Maximum keratinase activity of about 350 U/ml was achieved in the presence of 10 g/L of feathers with a pH of 8.5 at 22°C during 96 h of incubation time. Table 3 shows the results of ANOVA and RSM. R2 and adjusted R2 were determined to find out the correlations between the experimental results and theoretical data. ANOVA of the RS reduced cubic model showed that it is highly significant (F = 245.5031). CCD optimization showed that the effects of two-factors, AB and AC, were significant (A: pH, B: feather concentration, C: temperature, D: incubation time). The correlation between variable responses was studied using the response surface plots shown in Figure 2. After keratinase production optimization, four experiments were proposed by the software to determine the optimum conditions for keratin degradation by bacterium. They were performed in two repetitions and the keratin degradation was monitored. The best keratinase activity for four experiments was 350 U and the accuracy of the prediction of model was confirmed.

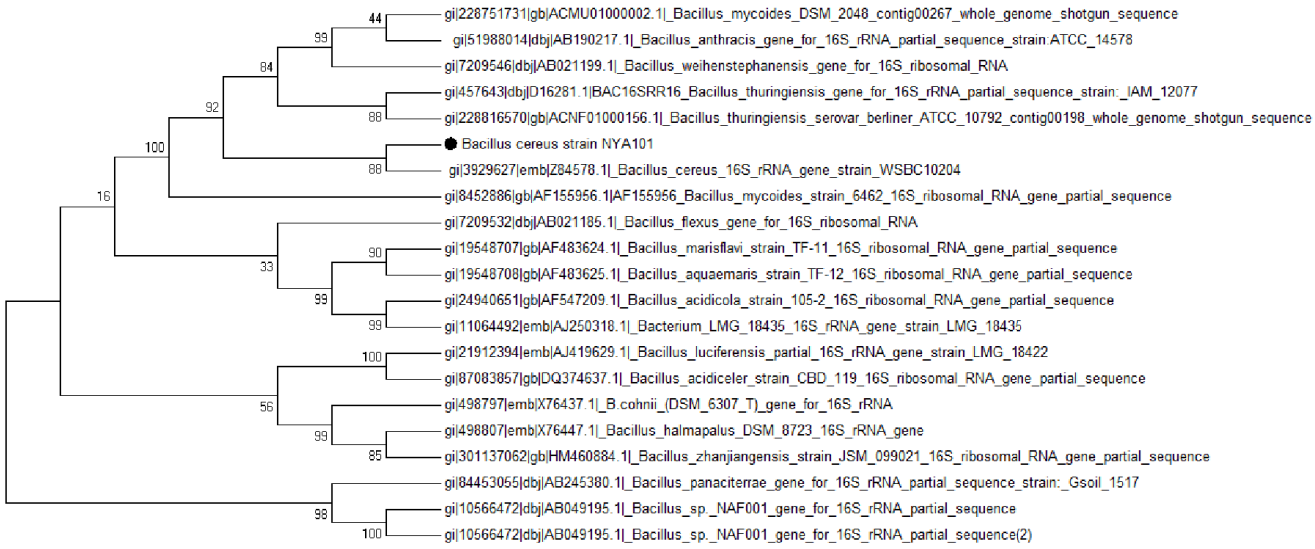


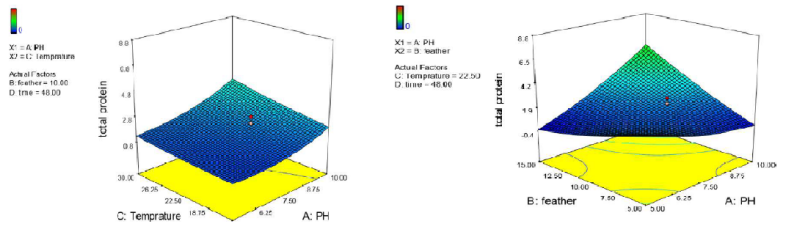
Figure 1. Phylogenetic tree of 16s rDNA nucleotide sequences. The numbers above the branches represent NJ bootstrap values (500 replicates). Accession numbers are given in brackets.

Table 2. RS reduced cubic model.

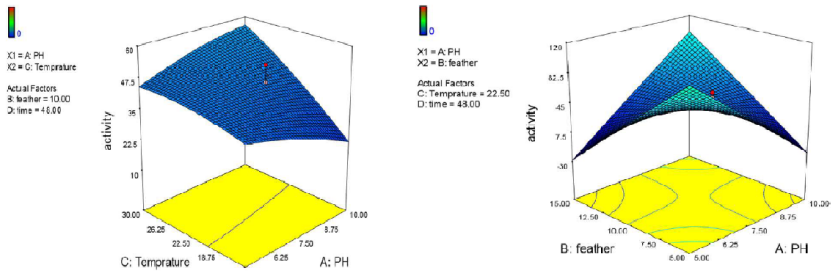
Std	Run	Block	Factor 1 A: PH	Factor 2 B: Feather	Factor 3 C: Temprature	Factor4 D: Time	Response 1 Total protein (mg)	Response 2 Activity
1	1	Block 1	12.66	15.95	31.42	19.46	9.04	220
19	2	Block 1	8.5	10	22.5	48	1.66	36
7	3	Block 1	12.66	15.95	13.58	19.46	4.95	108
21	4	Block 1	12.66	4.05	31.42	76.54	3.15	59
10	5	Block 1	1.5	10	22.5	48	2.171	38
11	6	Block 1	15.5	10	22.5	48	8.721	25
3	7	Block 1	4.34	15.95	13.58	76.54	2.015	35
8	8	Block 1	12.66	4.05	13.58	76.54	2.715	50
14	9	Block 1	8.5	0	22.5	48	0	0
12	10	Block 1	8.5	20	22.5	48	5.593	78
17	11	Block 1	8.5	10	22.5	48	2.248	43
16	12	Block 1	8.5	10	7.5	48	0.98	10
2	13	Block 1	4.34	4.05	31.42	19.46	1.846	21
13	14	Block 1	8.5	10	37.5	48	3.105	60
18	15	Block 1	8.5	10	22.5	48	1.469	38
20	16	Block 1	8.5	10	22.5	48	1.708	50
4	17	Block 1	4.34	15.95	31.42	76.54	2.94	49
9	18	Block 1	8.5	10	22.5	96	11.021	345
6	19	Block 1	4.34	4.05	13.58	19.46	2.01	30
15	20	Block 1	8.5	10	22.5	48	1.708	50
5	21	Block 1	8.5	10	22.5	0	0	0

Table 3. ANOVA for RS reduced cubic model (aliased).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	127994.5	15	8532.967	245.5031	< 0.0001	significant
A-PH	17.24872	1	17.24872	0.496265	0.5126	
B-Feather	281.3458	1	281.3458	8.094637	0.0360	significant
C-Temperature	1759.742	1	1759.742	50.62976	0.0009	significant
D-Time	57903.3	1	57903.3	1665.944	< 0.0001	significant
AB	53703.5	1	53703.5	1545.111	< 0.0001	significant
AC	1682	1	1682	48.39305	0.0009	
AD	227.8284	1	227.8284	6.554882	0.0506	
BC	1984.5	1	1984.5	57.09632	0.0006	
BD	5937.043	1	5937.043	170.8155	< 0.0001	significant
CD	800	1	800	23.01691	0.0049	
A^2	202.3	1	202.3	5.820401	0.0607	
B^2	27.65714	1	27.65714	0.795727	0.4132	
C^2	100.8	1	100.8	2.90013	0.1493	
D^2	24740.8	1	24740.8	711.8209	< 0.0001	significant
ABC	0	0				
ABD	229.5211	1	229.5211	6.603583	0.0501	
ACD	0	0				
BCD	0	0				
Residual	173.7853	5	34.75706			
Lack of Fit	2.585292	1	2.585292	0.060404	0.8180	not significant
Pure Error	171.2	4	42.8			
Cor Total	128168.3	20				



(a) (b)



(c) (d)

Figure 2. Outline plots of enzyme activity and total protein for interactions of two variables: (a) pH and temperature with total protein; (b) pH and feather concentration with total protein (c) pH and temperature with enzyme activity; (d) pH and feather concentration with enzyme activity (time: 96 h, feather concentration: 10 g/L, temperature: 22.5°C).

Discussion

A new strain of feather-degrading bacterium was selected from a bacterial collection of the Najm Biotech Co. The phenotypic characteristics and phylogenetic relationships showed that the isolated strain belongs to the *B. cereus* species. Keratinase activity of isolated bacterium was 350 U/ml after optimization of culture condition.

Feather degradation by bacteria is more common in Gram-positive bacteria (particularly in the *Bacillus* genus) [13-15] and is less common in Gram-negative bacteria [16]. The optimal temperature for enzyme production has been reported to be 20°C. In this study, *B. cereus* produced the maximum amount of keratinolytic enzymes at 22°C [17]. This optimal temperature could be the optimum growth temperature of the bacterium resulted in the increase in total amount of the enzyme activity (159 to 350 U) which showed a 2.2 fold increase in keratinase production.

Optimization of keratinase was done by RSM using a CCD model. The factors of pH, temperature, feathers, feather meal, soy peptone, sodium chloride, potassium chloride, potassium dihydrogen phosphate, glucose, soybean and incubation time have been previously used for optimization of growth condition of *B. cereus* [18]. A total of 21 experiments were used in RSM for four factors (pH, temperature, and feather concentration and incubation time) to optimize the production of keratinase in basal medium. The growth conditions play an important role in keratinase production by bacteria [18, 19]

Shankar *et al*., optimized keratinase production by *B. cereus* and the maximum activity reported by them was 63.01 U/ml [20]. Sivakumar *et al*., also reported the maximum keratinase production of about 60.67 U/ml by *B. cereus* [18]. The highest optimized keratinase activity in *B. subtilis* was 1960 U/ml which was reported by Singh *et al*., [21]. Balakumar *et al*., are showed optimized enzyme activity (156 U/ml) in Bacillus species [22]. In our study, maximum keratinase production was 350 U/ml which is one of the high production rates among the reported studies in this field.

Similar experimental setup was used for keratinase production by Tiwary and Gupta [23], Xian *et al*., [8], Zauari *et al*., [24], Sivakumar *et al*., [18] and Shankar *et al*., [20] in *Bacillus*. All above studies have mentioned the applicability and accuracy of RSM in order to optimize enzyme production. Interaction between variables on the production of keratinase was showed by Response surface plots.

Of the factors, pH and feather concentration had significant interaction affecting on keratinase production; pH and incubation time also showed interaction on response, so it seems that pH is an important factor for production of keratinases by isolated *Bacillus cereus* strain NYA101. Previous studies have shown that pH is the most important factor influencing enzymatic degradation of feather [25]. This could be due to the cooperative function of the keratinase and alkaline condition. Since keratin is a source of carbon and energy for bacteria, feather concentration could have significant effects on growth and keratinase production. At optimum values, it was shown that feather concentration has a good effect on enzyme activity.

Conclusion

Keratinase and soluble proteins from feather have important application in recycle of poultry waste to valuable byproduct in animal food, pharmaceutical, cosmetic and fertilizer industry [26]. Therefore these results and growth and enzyme production conditions of the *B. cereus* strain NYA101 isolated in this work are promising for application in industrial production of keratinase. Although more optimization in the culture condition and improvement of the strain to increase the enzyme productivity are needed that are in the way.

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