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Optimizing the fermentation conditions and enhanced production of keratinase from *Bacillus cereus* isolated from halophilic environmentS. Arokiyaraj^{a,*}, R. Varghese^b, B. Ali Ahmed^c, V. Duraipandiyar^d, N.A. Al-Dhabi^d^a Department of Food Science and Technology, Sejong University, Republic of Korea^b Department of Biotechnology, PRIST University, Thanjavur, Tamil Nadu 613401, India^c Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam^d Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia

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ABSTRACT

Keratinase degrading *Bacillus cereus* was isolated from the halophilic environment in Tamilnadu, India and keratinase production was optimized using wheat bran substrate. Of the screened bacterial isolates, four were found to have the ability to produce keratinolytic enzyme. The process parameters were optimized using one-variable-at-a-time approach and response surface methodology. Supplementation of 1% lactose supported more keratinase production (120 U/g). Among the selected nitrogen sources, addition of casein significantly enhanced maximum keratinase production (132.5 U/g). Among the ions, manganese chloride significantly enhanced keratinase production (102.6 U/g), however addition of zinc sulphate and copper sulphate decreased keratinase production. The maximum keratinase production was obtained in the wheat bran medium containing 1% lactose, 0.5% manganese with 80% moisture (292 U/g). Statistics based contour plots were generated to explore the variations in the response surface and to find the relationship between the keratinase yield and the bioprocess conditions.

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1. Introduction

Keratinolytic enzymes have attracted more attention in recent years to hydrolyze insoluble keratinolytic substrates (Brandelli et al., 2010). Halophilic organisms, including, *Bacillus* strains produce these proteases having significant activity and stability at desirable temperature and pH value (Ghasemi et al., 2011). Due to the degradation capability of microbe derived keratinases, they are considered as much more promising candidates when compared to conventional proteases. Keratinases find applications in most of the sectors in biotechnology Industry. Mainly in Animal feed, bioorganic fertilizer, functional detergent, and for various other pharmaceutical and biomedical applications (Gupta et al., 2013b). Microbial Keratinase processed feather meal is a great alternative to chemical and synthetic fertilizers as it is

economically viable and easy to produce and also due to its high nutritional value (Gurav and Jadhav (2013a, b). Further studies have indicated that it has significant positive effects on plant growth characteristics and can be a safely touted as a cheap and promising organic fertilizer (Adetunji et al. 2012). Each year there is a huge amount of keratinous waste generated primarily from Poultry related industries as well as from textile and leather industries. Approximated at a glaring 8.5 million metric tonnes and growing and this is a serious environmental issue (Gupta et al., 2013a, 2013b). As variety of other disposal and waste management techniques directly or indirectly lead to more pollution, an eco-friendly solution is to deploy keratinase producing microflora. Through this action, conversion of keratinous wastes into inorganic and sulphur will happen and these compounds can be readily be used up by the plants (Nayaka and Vidyasagar 2013). Keratinases are viewed as potential biocatalyst in pharma industry. Keratinases and Keratinolytic organisms were important in biomedical industries. This route is supposed to revolutionize the drug administering routes of the pharmaceutical industry (Gupta et al., 2013b). Keratinases has the potential to be ubiquitous with a range of applications in fields such as leather and textile industries.

Solid-state fermentation (SSF) is useful for the production of these enzymes owing to various economic advantages over submerged fermentation (Smf). The advantages of SSF include less

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pre and post-processing energy, simple management, low effluent generation and simple product recovery (Oliveira et al., 2006). The traditional one-variable-at-a-time method is generally used to screen the variables for the production of enzymes without any complicated analysis. However, this tradition method has severe flaws: interactions between selected medium components are ignored completely, the optimum level of the factors can be missed, and it is very time consuming and laborious process (Mason et al., 2003; Myers and Montgomery, 2002). The statistical experimental designs such as, fractional experimental design and RSM involve large number of factors and enhancement on enzyme production (Senthilkumar et al., 2005). RSM was previously studied using solid substrate (Vijayaraghavan et al., 2016; 2017). There is no specific culture medium that has been proposed to increase the production of keratinolytic proteases for any bacterium. Each organism required their own growth conditions for maximum production of enzymes (Kumar and Takagi, 1999). The present study aimed to use wheat bran medium for the production of halophilic keratinase for various applications.

2. Materials and methods

2.1. Screening and isolation of halophilic bacterium for keratinolytic enzyme production

The soil sample was collected from the solar salt pan, Tuticorin, Tamilnadu, India for the screening of keratinase producing organism. Soil sample was collected using spatula and was serially diluted for the screening of keratinolytic enzyme producing bacteria. The diluted sample was placed on skimmed milk agar plate and incubated for 48 h at 37 °C, zone of clearing around the colony indicated protease production. Further the positive isolates were grown on feather meal agar plates and the isolated strain, which showed maximum growth on feather meal plates, was selected for optimization studies.

2.2. Identification of protease producing organism

The keratinolytic enzyme producing bacterium was identified based on biochemical characteristics and colony morphology (Holt et al., 1994; Ilavenil et al., 2015). Based on colony morphology and biochemical characteristics the selected organism was identified as *Bacillus cereus*.

2.3. Use of agricultural byproduct in design of media

Wheat bran was used as the sole source of carbon and nitrogen. Initially the substrate (5.0 gm) was mixed with Tris – HCl buffer (3.5 ml) to attain 70% (v/w) moisture level. The medium was sterilized and inoculated with *B. cereus* at 5% inoculum level (~10⁴ CFU/ml). The fermentation process was carried out initially for 4 days and keratinase assay was carried out at every 24 h intervals.

2.4. Optimization of culture conditions for keratinase production

To study the influence of carbon source, the carbon sources such as, maltose, glucose, lactose and sucrose were supplemented at 1% (w/w) level. To evaluate the effect of nitrogen source, gelatin, skimmed milk, casein and oat meal were added with the wheat bran medium at 1% (w/w) level. The effect of additional ions on keratinase production was also evaluated. Enzyme extraction was carried out as described previously (Vijayaraghavan and Vincent, 2012),

Table 1

The factors and levels selected for CCD and response surface methodology.

Variables	Symbol	Coded values				
		−α	−1	0	1	+α
Moisture	A	66.3641	80	100	120	133.636
Lactose	B	−0.2068	0.1	0.55	1	1.30681
Manganese	C	−0.036359	0.1	0.3	0.5	0.63636

Table 2

Experimental design and results of CCD for the production of keratinase.

Std	Moisture%	Lactose%	Manganese%	Enzyme activity (U/g)
1	133.636	1	0	65
2	0	1.30681	0	120
3	0	1	0	178
4	0	1	0	156
5	0	1	0	170
6	66.3641	1	0	62
7	0	1	0	182
8	−1	1	−1	40
9	0	1	0.636359	45
10	0	1	0	170
11	1	1	−1	292
12	0	1	0	108
13	−1	1	1	249
14	0	1	−0.0363586	110
15	−1	−1	1	185
16	−1	−1	−1	88
17	1	−1	1	6
18	1	−1	−1	120
19	0	−0.206807	0	53
20	1	1	1	210

2.5. Keratinase assay

Keratinase assay was determined by following the method of Alessandro and Adriano (2006).

2.6. Optimization of keratinase production by central composite design (CCD)

RSM and the CCD were applied to optimize the significant factors influence on keratinase production. The factors such as, moisture, manganese and lactose were selected to increase keratinase production. All selected variables were analyzed at five different levels (−α, −1, 0, +1, +α) (Table 1). The keratinase assay was performed in duplicate analysis, and the average of these experimental values was taken as response Y (Table 2). The experimental results of the CCD design were fitted with a second-order polynomial equation as shown in Eq. (1).

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_3 C + \alpha_1 \alpha_2 AB + \alpha_1 \alpha_3 AC + \alpha_2 \alpha_3 BC + \alpha_1 \alpha_1 A^2 + \alpha_2 \alpha_2 B^2 + \alpha_3 \alpha_3 C^2 \quad (1)$$

where Y is the keratinase activity (U/g); A - moisture; B - lactose; C - manganese. The counter plot was analyzed to determine the optimum response on keratinase production.

3. Results and discussion

3.1. Screening of keratinase producing *B. Cereus* isolated from halophilic environment

The keratinase producing *B. cereus* was isolated from the soil sediment using skimmed milk agar plates and feather meal agar plates. Of the screened bacterial isolates, four were found to have

the ability to produce keratinase. Among the positive bacteria, *B. cereus* produced a larger halo zone in response to the colony diameter. The selected bacterium was identified as *B. cereus* on the basis of microscopic and biochemical observations. It was a Gram-positive strain, rod-shaped, spore-forming, positive to MR and VP-test, citrate-negative, positive to oxidase, catalase, casein and gelatin. It fermented the sugars such as, lactose, glucose and sucrose. This organism showed negative response towards citrate, nitrate reduction and gas production. Halotolerant proteolytic enzymes have been screened from various *Bacillus* sp. including, *Bacillus clausii* (Joo and Chang, 2005) and *Bacillus cereus* MTCC 6840 (Joshi et al., 2007).

3.2. Optimization of nutrient factors on keratinase production by one variable at a time approach

In this study, wheat bran was used for the production of keratinase. This substrate is cheap and available almost throughout the year. The important criteria for the ideal substrate should be cheap and should be available throughout the year (Vijayaraghavan and Vincent, 2012). The carbon sources such as sucrose, glucose, lactose and maltose were used to determine the production of keratinase. Supplementation of 1% lactose supported more keratinase production (120 U/g). Among the selected nitrogen sources, addition of casein significantly enhanced keratinase production (132.5 U/g) (Vidyasagar et al., 2007). However, addition of ammonium sulphate with the culture medium repressed keratinase production. Among the supplemented ions, manganese chloride significantly enhanced keratinase production (102.6 U/g), however addition of zinc sulphate and copper sulphate decreased keratinase production. Therefore, further studies were carried out using manganous as the ionic source. In *Bacillus subtilis* BS-26, manganese enhanced the production of proteolytic enzymes (Niu et al., 2008; Prakasham et al., 2006).

3.3. Central composite design and response surface methodology

The “lack-of-fit *F*-value” of the designed CCD model was 1.66 and the *p* value of the model was 0.29, which implied that the lack of fit of this designed model was non significant (Table 3). The “predicted R^2 ” of this CCD model was 0.9180 and the “adjusted R^2 ” of this model was 0.921. Figs. 1-3 illustrated counter plots on keratinase production. Keratinase production was increased at increased moisture content at certain level. The counter plots showed the increased keratinase activity at higher lactose and moisture content. RSM was widely used to optimize the process parameters to enhance the enzyme production. Tatineni and Doddapaneni (2007) optimized keratinase production from *Strepto-*

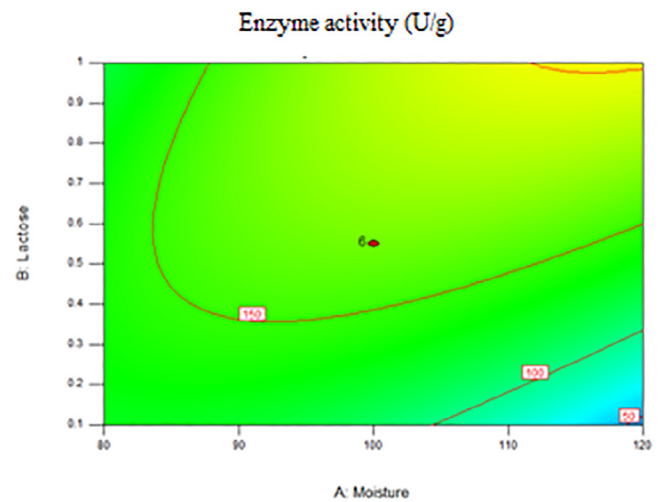


Fig. 1. 2D-contour plots of keratinase yield (U/g) between moisture content and lactose.

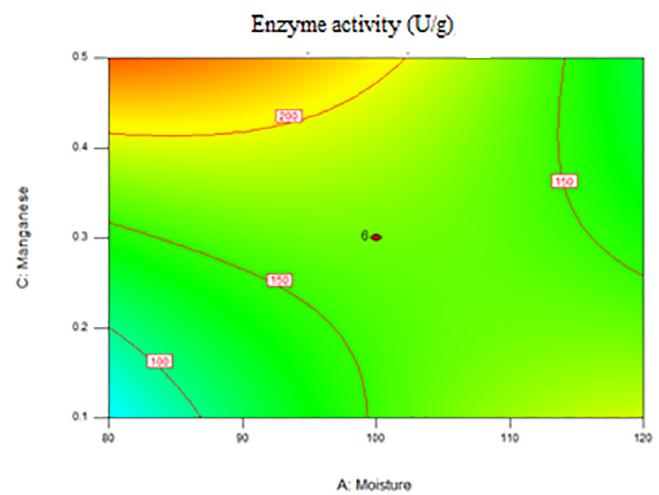


Fig. 2. 2D-contour plots of keratinase yield (U/g) between moisture content of the wheat bran medium and manganese.

myces sp7 using response surface methodology. Keratinase have significant application in poultry waste recycling to useful by-product in fertilizer industry. Hence, the present study is promising for application in keratin waste management. Keratinases has the potential to be ubiquitous with a range of applications in fields

Table 3
ANOVA for the experimental results of the CCD.

Source	Sum of Squares	df	Mean Square	F -Value	p-Value	
Model	3.28E+06	9	1.07E+04	7.32	0.0021	Significant
A	1.27E+05	1	7.30E-01	5.01E-04	0.9825	
B	2.59E+05	1	1.85E+04	11.66	0.0051	
C	1.08E+05	1	1.02E+04	7.06	0.022	
AB	1.40E+06	1	1.29E+04	9.38	0.011	
AC	3.00E+04	1	3.20E+04	22.18	0.0007	
BC	5.71E+05	1	4.64E+03	3.05	0.1061	
A ²	2.12E+05	1	8.45E+03	5.63	0.0375	
B ²	5.09E+05	1	5.17E+03	3.33	0.0933	
C ²	1.15E+04	1	2.71E+03	1.75	0.2032	
Residual	3.84E+04	10	1475.22			
Lack of Fit	29520.99	5	1843.87	1.65	0.2900	
Pure Error	8.94E+03	5	1108.27			
Cor Total	3.42E+06	19				

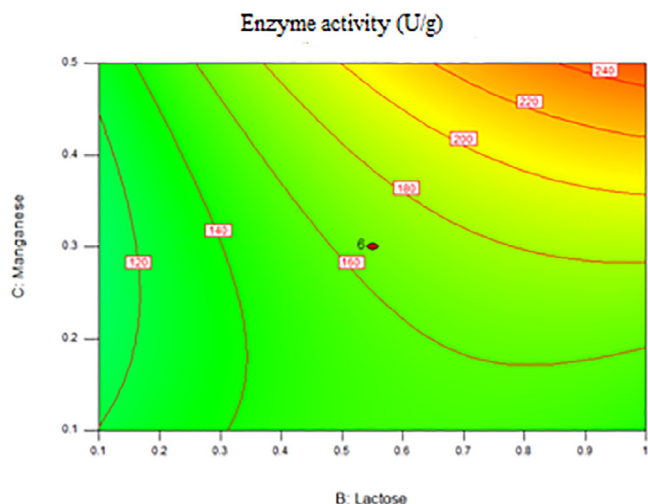


Fig. 3. 2D-contour plots of keratinase production (U/g) between lactose and manganese.

such as leather and textile industries, Hazardous waste recycling and management, bioremediation of polluted land and water, Green energy, nanobiology, food science and Animal feed technology, personal and beauty care products, Biomedical and pharma industries, agriculture (biopesticides, plant- growth promotion biofertilizers and composting,) and microbial biocatalysis.

Conflict of interest

No conflict of interest in this research work and the manuscript publications.

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