International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(2): 2610-2619

Received: 14-01-2020
Accepted: 16-02-2020

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Optimization of cultural conditions for enhanced keratinase production by *Bacillus cereus* N14 obtained from the poultry farm of Himachal Pradesh (India)

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DOI: https://doi.org/10.22271/chemi.2020.v8.i2an.9145

Abstract
To enhance the keratinolytic potential of bacterial strain *Bacillus cereus* N14, isolated from poultry farm of Sundernagar, District Mandi, Himachal Pradesh, different process parameters like temperature, pH, incubation time, inoculum size, inoculum age etc. were optimized using One Variable at a Time (OVAT) approach and Response Surface Methodology (RSM). *Bacillus cereus* N14 showed maximum enzyme activity (19.5 U/mL) after 3rd day of incubation at 35°C, pH 9.0, 12.5 (%) of inoculum size using 3 days old culture and 2.5 and 2.0 per cent of maltose and yeast extract as best carbon and nitrogen source, respectively in the presence of Mn^{2+} as divalent metal ion and EDTA as best media additive. Central composite design (CCD) of RSM was successfully applied to investigate the effect of 5 independent variables *viz*., incubation time (days), inoculums size (%), inoculum age (days), concentration of carbon and nitrogen source (%) on keratinase production. The maximum enzyme activity (74.86 U/mL) was attained by the *B. cereus* N14 at 22nd run. The main highlight of the present study is that a significant increase of 27.5 per cent in keratinase production after optimizing different process parameters, by *Bacillus cereus* N14 was observed.

Keywords: Cultural conditions, poultry farm, bacterial strain

Introduction
Globally, immense quantities of feathers are generated annually as a by-product of poultry-processing industries and poultry farms in association with poultry meat and eggs, creating a serious solid waste problem (Godheja *et al*., 2014) [15]. Their proper and ecofriendly disposal is a major constraint for these processing plants and poultry farms. Additionally, the potential risks due to disposal of these wastes are aggravated as a consequence of the decreasing amount of land available for their disposal as well as environmental pollution of both air and underground water resources along with protein wastage. Therefore, the need of the hour is to utilize/recycle this waste in a technological way to avoid environmental pollution and health hazards. Microbial keratinases offers an economic and eco-friendly alternative for degrading and recycling keratinous waste into valuable by-products (Sharma and Devi, 2018) [41].
Keratinases [E.C.3.4.21/24/99.11] are a specific type of proteolytic enzymes that have the tendency to hydrolyze highly stable, fibrous and insoluble protein which is the major constituent of skin and its appendages *viz.*, feathers, nails, hairs, wools, hooves, scales, and stratum corneum, known as keratin. Keratin protein can be divided into soft keratin (<10% cysteine) and hard keratin (~10-14% cysteine) based on their sulfur content (Henry *et al*., 2012) [23]. On the basis of secondary structural conformation, keratins have been classified into α (α-helix of hair and wool) and β (β-sheets of feather) keratins (Akhtar and Edwards 1997) [2]. These are highly resistant to hydrolysis by weak acids, alkalies, ethanol or salt solution and also to enzymatic digestion.
The keratin-degrading microorganisms are ubiquitous in nature that thrives either obligatorily or facultatively on keratin rich substrates. Keratinolytic enzymes produced by keratinolytic microorganisms are widely distributed in nature and have been frequently isolated from different environmental niches including soil (Lateef *et al.*, 2010; Han *et al.*, 2012) [27, 20], agro industrial residues (Mazotto *et al.*, 2011) [30], alkaline mud (Gessesse *et al.*, 2003) [14],

limestone habitat (Ningthoujam *et al.*, 2016) [32] and extreme environments (Rissen and Antranikian, 2001; Nam *et al*., 2002) [36, 31] like hot springs and soda soils etc. A vast variety of bacteria especially of the genus *Bacillus* like *B. cereus, B. halotolerans, B. subtilis, B. licheniformis* etc. (Cedrola *et al.*, 2012) [8], actinomycetes like *Streptomyces fradiae, Sacchrothrix xinjangensi, Nacardiopsis halotolarns, Amycolatopsis keratiniphila* etc. (Jaouadi *et al.*, 2010) [24] and some species of saprophytic and parasitic fungi like *Chrysosporium indicum, Aspergillus flavus, Fusarium* sp., *Trichophyton* sp., *Purpureocillium lilacinum* etc (Gradisar *et al*., 2005) [17] are known to be potential keratinase producers that have the special ability to degrade recalcitrant keratin.

Cultivation conditions are essential in the successful production of an enzyme (Allure *et al*., 2015) [3]. Optimization of various process parameters like pH, carbon sources, incubation time, nitrogen sources, media additives, inoculum age, inoculum size, temperature, media composition etc. is a crucial step that has a great significance in developing the optimum conditions for obtaining higher yield of enzymes.

'One variable at a time' (OVAT) is a conventional approach of optimization that does not establish any combined interactions among various variables. Optimization of the process parameters through statistical approach, such as response surface methodology (RSM), the concomitant effect of a specific set of values for the input variables on enzyme production levels can be easily tested and graphically visualized hence, representing an effective way to optimize cultural conditions using a reduced number of experiments (Cai and Zheng, 2009; Fakhfakh-Zouari *et al*., 2010; Tiwary and Gupta, 2010; Govarthanan *et al*., 2015) [7, 10, 45, 16] and to improve the yield significantly and lowering the production cost (Puri *et al*., 2002) [33]. Keeping in view, the above facts, the present study was mainly focused on the optimization of cultural conditions for enhanced keratinase production using OVAT and RSM approaches.

Material and methods

Keratinolytic bacterium namely *Bacillus cereus* N14, isolated from Breeding Farm and Chicken Hatchery Sundernagar, Distt. Mandi was used in the present study. The bacterial strain was available in the germpool of Microbiology section of the department of Basic Sciences, UHF-Nauni, Solan, Himachal Pradesh. Chicken feathers procured from the respective Chicken Hatchery were used as substrate for keratinase production.

Keratinolytic activity exhibited by bacterial isolate was estimated using the method of Gradisar *et al*., (2005). One unit (U/mL) of keratinolytic activity was defined as an increase of corrected absorbance at 280 nm (A_{280}) with the control for 0.01 per minute under standard assay conditions. The keratinase activity was estimated using the following formula:

$$U = [4 \times N \times A_{280}] / 0.01 \times 10$$

Where 4 = Total final volume; N = Dilution factor; 10 = Incubation time

Before applying Response Surface Methodology for the optimization of process parameters, preliminary studies with selected variables *viz.*, inoculum age, incubation time, inoculum size, carbon sources, best carbon sources, nitrogen sources, best nitrogen source, pH, temperature, divalent ions and media additives was carried out using One variable at a Time (OVAT) approach. Optimized concentrations of medium components and incubation parameters for keratinolytic bacterial strain *Bacillus cereus* N14 were added to the production medium for its mass cultivation and subsequently subjected for optimization using central composite design (CCD) of RSM. The experiment was conducted in quarduplicates and the results were analyzed statistically (OPSTAT software) using one-way analysis of variance (ANOVA). The means were compared for significance at $p \leq 0.05$.

Results and discussion

The activities of microorganisms are affected by accurate optimization process which is desirable for minimizing the processing cost. Therefore, it is pertinent to improve the performance of the system and subsequently increase the yield without increasing the production cost in order to meet the growing demands of enzymes (Gangadharan *et al*., 2008) [13].

Since, the growth of the organisms and eventually the enzyme production by them are strongly influenced by different process parameters *viz.*, inoculum age, temperature, pH, incubation time, inoculum size, carbon source, concentrations of best carbon source, nitrogen source, concentrations of best nitrogen source, divalent ions and organic solvents and surfactants, eleven different process parameters were optimized through OVAT approach for enhanced keratinases production by selected *Bacillus* spp. In this approach, only single variable effect rather than the interaction of different parameters was studied.

To examine the effect of different incubation times on keratinase production by *B. cereus* N14, keratinase activity was assayed at different incubation times varied from 3 to 13 days (Figure 1). The results reveal that different incubation times have significantly affected the keratinases production. *Bacillus cereus* N14 showed maximum keratinase production of 19.56 U/mL at 3rd day of incubation. Further incubation showed a gradual decline in the enzyme yield upto 9th day of incubation. Thereafter, a quick decline was observed in the enzymatic activity and minimum enzymatic activities of 3.26 U/mL were observed for *B. cereus* N14 at 13th day of incubation. The continuous depletion of nutrients from the production medium and build up of toxic metabolic wastes that results in the death of microbial cells at rapid and uniform rate could be the probable reason behind this trend (Benavente Valdes *et al*., 2016) [6]. The decrease in enzyme activity upon prolonged incubation may be due to irreversible adsorption of enzyme to substrate or due to feedback inhibition/denaturation of enzymes, resulted from the lesser cellular metabolism with time during fermentation (Liu and Yang, 2007; Xin and Geng, 2010) [28, 46].

To examine the effect of inoculum ages on keratinase production by *B. cereus* N14 (Figure 2), keratinase activities were assayed using different inoculum ages that varied from 1 to 6 days. The results reveal that different inoculum ages have significantly affected the keratinases production. *Bacillus cereus* N14, an increase in the enzymatic activity was noticed as the age of inoculum was increased from 1st to 3rd day. However, use of more older cultures (4th – 6th days old cultures) declined the enzymatic activity. *B. cereus* N14 showed maximum keratinase production of 20.01 U/mL with three days old culture. Whereas, minimum enzymatic activitiy of 2.16 U/mL was noticed for *B. cereus* N14 with 6th days old inoculums. Various researchers have reported that higher keratinase production is obtained at higher percentage of inoculum ages. Our observations are in agreement with the

findings of Kahrdenavis *et al*., (2009) [25] who reported 72 h old culture to be the optimum inoculum for keratinase production by *Serratia* HPC1383.

Inoculum size is the required concentration of expected microorganism for a standard test. The effect of inoculum amount on the production of keratinases by *B. cereus* N14 was studied using inoculum sizes varied from 2.5 to 13.0 per cent (v/v) (Figure 3). A statistical significant difference in the keratinases production by *B. cereus* N14. As the inoculum sizes increased, an increase in enzymatic activity was observed upto 12.5 per cent. Further increase in inoculum size showed a sharp decline in the keratinase activities exhibited by both the isolates. *B. cereus* N14, while used 12.5 percent of inoculum size, maximum keratinase production (6.73 U/mL) was obtained which was found to be statistically at par with 10 per cent of inoculum size (5.52 U/mL).

Low density of the inoculum cells decreases the number of the grown cells in the enzyme production phase. On the other hand the high density of cells in the culture increases the viscosity and the competition for the nutrients which also decrease the possibility of reaching the cells to the production phase and hence the enzyme production (Fattah *et al*., 2018).

Incubation temperature is a critical factor in enzymatic productivity (Seyis and Aksoz, 2003) [39]. Hence, the effect of incubation temperatures on the keratinase production by *B. cereus* N14 was determined by carrying out the enzyme assays at different temperatures ranged from 25-75°C (Figure 4). A statistical significant difference in the keratinase production by *B. cereus* N14. *B. cereus* N14, maximum keratinase production (17.96 U/mL) was observed at 35°C which was found to be statistically at par with the keratinase production at 25 and 45°C indicating the mesophilic and thermotolerant nature of *B. cereus* for the growth and eventually keratinases production.

Maximum enzyme production is produced at optimum temperature and the decrease in enzyme production at lower or higher temperatures is due to the fact that at these temperatures, growth of the organisms was inhibited, causing a decrease in the synthesis of the enzymes (Simoes *et al*., 2009) [42]. Production of more activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. Our observations are in agreement with the findings of Fattah *et al*. (2018) [1] who studied the effect of incubation temperature on keratinase production by *B. licheniformis* ALW1 and reported that highest level of the produced enzyme (33.2U/ml) was obtained at an incubation temperature of 42°C.

The effect of different pH on keratinases production by *B. cereus* N14 was examined by carrying out the enzymatic assays at different pH that ranged from 4 to pH 12 (Figure 5) .The data presented in Figure 5 reveals that pH has significantly affected keratinases production by *B. cereus* N14. *B. cereus* N14 showed maximum keratinase production (16.84 U/mL) at pH 9.0 while, minimum (6.43 U/mL) was observed at pH 12.0.

The pH factor is fundamental for influencing the physiology of microorganisms by affecting nutrient and adsorption solubility, enzyme activity, morphological cell membrane, by-product formation and oxidative-reductive reactions. Lower keratinase production below and above optimum pH may be due to the growth inhibition of strains at such high acidic and alkaline pH (Hamiche *et al*., 2018) [19]. The findings of present investigation are in agreement with the findings of other researchers who reported that alkaline pH possibly favors keratin degradation as higher pH modifies cystine residues to lathionine (Frankena *et al*., 1986; Rissen and Antranikian 2001; Selvam *et al*., 2013) [11, 36, 38] in order to make keratinase action easy. The resulting lanthionine residue makes keratin structure vulnerable to keratinase hydrolytic action (Friedrich and Antranikian, 1996) [12]. Gessesse *et al*. (2003) [14] reported that *Bacillus pseudofirmus* AL-20 was active in a broad pH range displaying over 90 per cent of its maximum activity between pH 7.5 and 11.5 with a peak at pH 10.

Carbon sources used in the medium are also essential elements for the growth and metabolism of microorganisms thus ultimately affect enzyme production (Satyanarayanan, 2007; Nager *et al*., 2010) [37, 31]. Many different carbon sources like sucrose, fructose, maltose, glycerol, starch etc. have been used by various researchers to examine their effect on keratinase production (Harde *et al*. 2011; Lo *et al*. 2012; Tiwary and Gupta, 2012) [21, 29, 45].

To examine the effect of different carbon sources on keratinases production by *B. cereus* N14, production medium was supplemented with six different carbon sources *viz*., lactose, maltose, fructose, sucrose, starch and xylose and subsequently keratinolytic activities were determined under standard assay conditions (Figure 6). A statistical significant difference in keratinase production by *B. cereus* N14 using different carbon sources. *B. cereus* N14, showed maximum keratinase production (27.88 U/mL) with maltose as carbon source while minimum (5.78 U/mL) was recorded with xylose as carbon source. This may be attributed to the fact that the choice of carbon source for growth and enzyme production by bacteria vary from species to species (Rai *et al*., 2009) [34].

Our observations are in agreement with the findings of Parameswari *et al*., (2015) [33] who studied the effect of different carbon sources *viz*., Glucose, Fructose, Galactose, Maltose, Mannitol and Glycerol on keratinase production by *Staphylococcus aureus* and reported that among all the carbon sources, maltose with 0.045 (µmol/min) activity showed the maximum yield of keratinase. Similarly, maltose was found to be best carbon source for the production of keratinases by both *Bacillus* spp. and *Aspergillus* spp. according to Balakumar *et al.*, (2013) [5].

Carbon source concentration as a substrate is an influencing factor that affects the yield and initial rate of enzymatic hydrolysis. Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. In the present study, the effect of best carbon source (maltose) concentrations that ranged from 0.5 to 3 per cent (Figure 4.7), on the production of keratinases by *B. cereus* N14 was determined by assaying out enzymatic activities under standard assay conditions. Maximum keratinase production (24.64 U/mL) was attained by *B. cereus* N14 at 2.5 per cent maltose concentration. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis (Da Silva *et al*., 2005; Regina *et al*., 2008) [9, 34]. Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production (Liu and Yang, 2007 and Singhania *et al*., 2007) [28, 43].

To examine the effect of diverse nitrogen sources on keratinolytic enzyme production by *B. cereus* N14,

production medium was supplemented with six nitrogen sources (1 per cent w/v each) *viz.*, yeast extract, peptone, beef extract, sodium chloride, urea, and sodium nitrate and subsequently keratinolytic activities were determined under standard assay conditions (Figure 8). Different nitrogen sources have significantly affected the keratinase production by *B. cereus* N14. Among all nitrogen sources, yeast extract was found to be best nitrogen source for *B. cereus* N14 with maximum enzyme yields of 41.30 U/mL. This could be ascribed to the fact that in the presence of two substrates, one which is structurally more resistant and compact (feather) and the other which is more accessible and small protein supplement (yeast extract), the bacteria may preferentially use the latter. Our observations are in conformity with the findings of Kainoor and Naik (2010) [25]who reported that feather medium supplemented with 0.1 per cent (w/v) yeast extract as an external organic nitrogen source showed maximum production of keratinase by *Bacillus* sp. JB 99.

The effect of best nitrogen source (yeast extract) concentrations on keratinases production by *Bacillus cereus* N14 was investigated by supplementing the production medium with different concentrations of yeast extract (0.5-3.0%) (Figure 9) and eventually keratinolytic activities were determined under standard assay conditions.

Keratinases production is significantly affected by changing the concentration of yeast extract. Maximum enzyme yields of 38.77 U/mL was shown by *B. cereus* N14 with 2.0 per cent concentration of yeast extract. Further increase in the concentration of yeast extract in the medium, drastically reduced the keratinolytic activity and minimum keratinase production of 3.12 U/mL was recorded at 3.0 per cent yeast extract. The decline in the enzyme production in the presence of higher concentration of yeast extract could be ascribed to phenomenon of catabolic repression (Saibabu *et al*., 2013) [37]. The findings of present study are in conformity with the findings of Kainoor and Naik (2010) [25] who reported that 0.1 per cent yeast extract enhanced keratinase production by *Bacillus* sp. JB99. Lakshmi *et al*., (2013) [26] reported that yeast extract was the other nitrogen source that supported the keratinase production.

The effect of various metal ions on keratinase production by *B.* cereus N14 is shown in Figure 4.10. Addition of Mn^{2+}, Mg^{2+} and Zn^{2+} in the medium showed increased keratinase production of 15.41 U/mL, 15.29 U/mL and 14.26 U/mL, respectively and were found to be statistically at par. Whereas, lowest levels of enzyme production i.e 6.19 U/mL and 3.77 U/mL were noticed with Ca^{2+} and Fe^{2+} ions, respectively. It is therefore evident from these results that keratinases from both the isolates are metallo-type or metal ions are required for enzyme activity or stability (Bhange *et al*., 2016) [7].

Stimulation of keratinase activity in the presence of metal ions like Mn^{2+} could be ascribed due to salt formation or an ion bridge which maintain the conformation of enzyme as substrate complex (Balaji *et al*., 2008) [4]. Inhibition of keratinase activity by metal ions may be attributed to the formation of bridges between metal monohydroxide and catalytic ions at the active site (Sivakumar *et al*., 2012) [44]. Our observations are in agreement with the findings of other researchers who accounted that addition of Ca^{2+}, Mg^{2+} and Mn^{2+} ions stimulated the production of some keratinases (Nam *et al*., 2002; Riffel *et al*., 2003) [31,35] .

The effect of different organic solvents and surfactants *viz*., H_2O_2, SDS, EDTA, β-mercaptoethanol, glycerol, DMSO and Triton X-100 (Figure 11) on keratinase production by *B. cereus* N14 was studied by measuring the enzyme activity in the presence of respective chemical (1mM concentration) under standard assay conditions. A statistical significant difference in the keratinase production by *B. cereus* N14 in the presence of different organic solvents and surfactants. *B. cereus* N14, among all organic solvents and surfactants, EDTA significantly produced appreciable level of keratinase with maximum yield of 11.70 U/mL followed by β-mercaptoethanol (9.25 U/mL), Triton X-100 (8.95 U/mL), SDS (8.66 U/mL), DMSO (8.00 U/mL), Glycerol (7.30 U/mL) and H_2O_2 (4.65 U/mL). The main role of organic solvent and surfactants in the fermentation medium is to increase the secretion of proteins by increasing cell membrane permeability (Hashemi *et al*., 2010) [22].

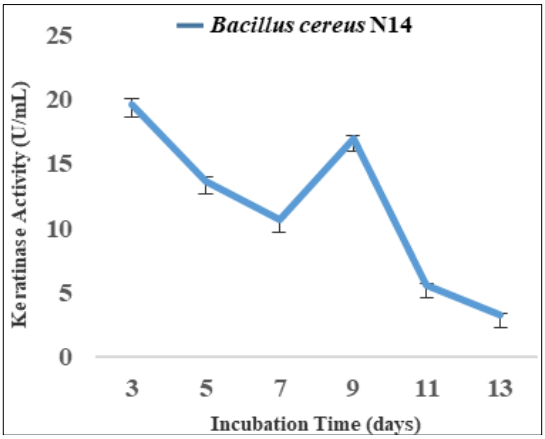


Fig 1: Effect of different incubation time (days) on keratinase production by *Bacillus cereus* N14

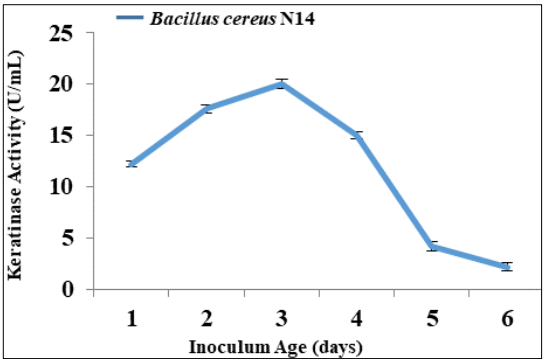


Fig 2: Effect of different inoculum age (days) on keratinase production by *Bacillus cereus* N14

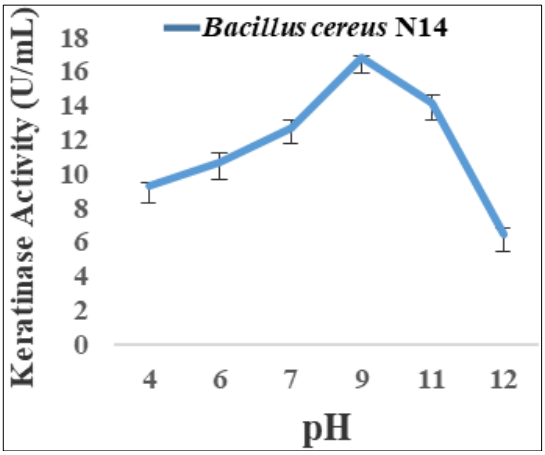


Fig 3: Effect of different pH on keratinase production by *Bacillus cereus* N14

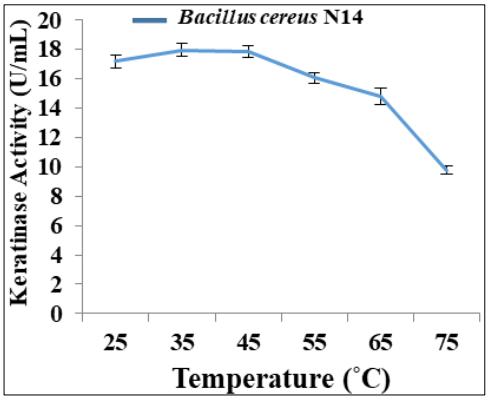


Fig 4: Effect of different temperature (°C) on keratinase production by *Bacillus cereus* N14

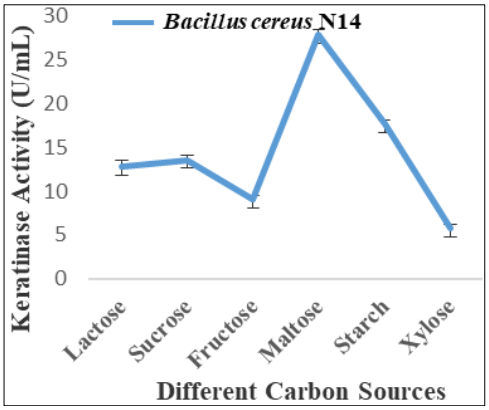


Fig 5: Effect of different carbon sources on keratinase production by *Bacillus cereus* N14

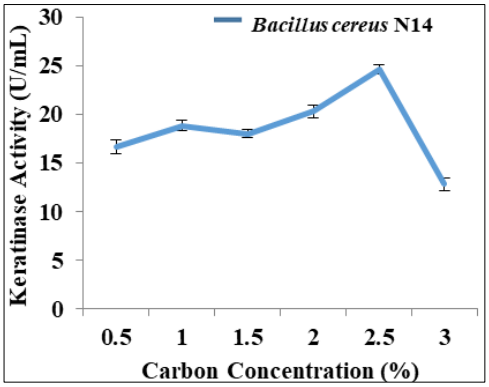


Fig 6: Effect of best carbon source concentrations on keratinase production by *Bacillus cereus* N14

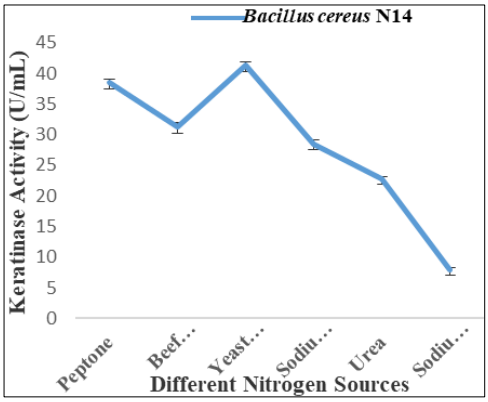


Fig 7: Effect of best carbon sources on keratinase production by *Bacillus cereus* N14

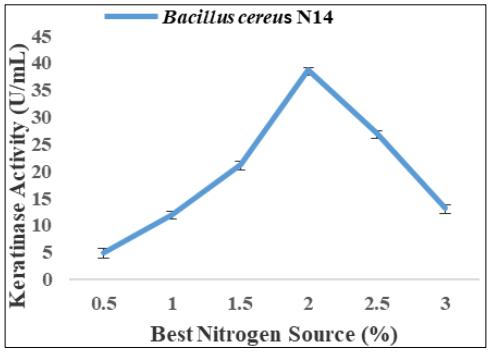


Fig 8: Effect of best nitrogen source concentrations on keratinase production by *Bacillus cereus* N14

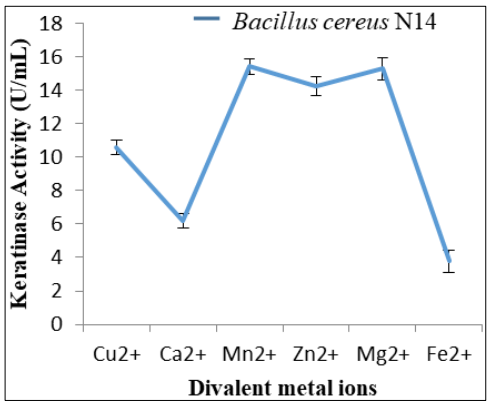


Fig 9: Effect of different divalent metal ions on keratinase production by *Bacillus cereus* N14

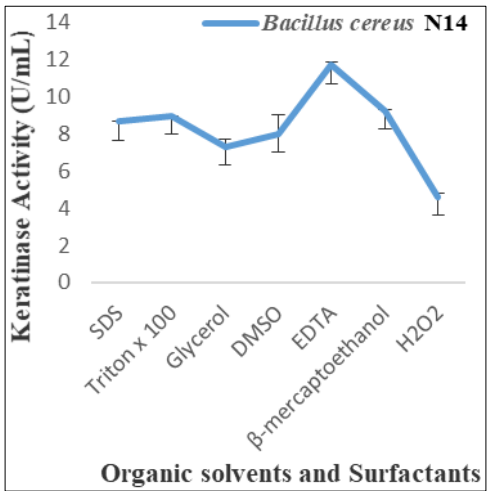


Fig 10: Effect of different organic solvents and surfactants on keratinase production by *Bacillus cereus* N14

Response surface methodology (RSM) for enhanced keratinases production by *Bacillus cereus* N14 and *Bacillus halotolerans* L2EN1

In the present study, Central Composite Design (CCD) of RSM was successfully applied for the optimization of cultural conditions using five independent variables *viz.*, incubation age (days), inoculums size (%), inoculum age (days), carbon source concentration (%) and nitrogen source concentration (%) for enhanced keratinase production by *Bacillus cereus* N14. In total, 27 sets of experiments were performed.

Experimental design: A second-order experimental design i.e. Central Composite Design (CCD) with five factors at five levels was employed to investigate the first and higher order effects of each factor and interactions among them. The full experiment plan as per the experimental design and the

minimum and the maximum range of the variables along with the low values and high values for the isolates i.e *Bacillus cereus* N14 Table 1. The statistical software package STATISTICA (StatSoft, OK, USA) was used to generate polynomials and the response surface plots (three dimensional plots and interaction graphs). All experiments were carried out in triplicates. For a five factor system, the following model equation was generated:

$$Y=\beta0+\beta1A+\beta2B+\beta3C+\beta4D+\beta5E+\beta11A^2+\beta22B^2+\beta33C^2+\beta44D^2+\beta55E^2+\beta12AB+\beta13AC+\beta14AD+\beta15AE+\beta23BC+\beta24BD+\beta25BE+\beta34CD+\beta35CE+\beta45DE$$

Where Y was response variable, β0 was intercept, β1, β2, β3, β4 and β5 were linear coefficients, β11, β22, β33, β44 and β55 were quadratic coefficients, β12, β13, β14, β15, β23, β24, β25, β34, β35 and β45 were the second-order interaction coefficients and A, B, C, D, A^2, B^2, C^2, D^2, E^2 AB, AC, AD, AE, BC, BD, BE,CD, CE and DE were the levels of independent variables.

Table 1: Variables and their levels for Response Surface Methodology (RSM) for *Bacillus cereus* N14

Independent Variables	*Bacillus cereus* N14	
	Low Value	High Value
Carbon (%)	2.5	3.0
Nitrogen (%)	1.5	2.5
Inoculum age (days)	2.0	4.0
Inoculum size (%)	5.0	10.0
Incubation time (days)	1.0	5.0

Results for *Bacillus cereus* N14 from CCD of RSM

For five factor system, the following model equation was generated:

$$\text{Response (U/mL) } Y= 67.40-5.37X_1-2.07X_2+2.39X_3-4.57X_4-7.23X_5-1.92X_1X_2-1.57X_1X_3-1.46X_1X_5-3.42X_2X_3 - 1.85X_2X_5+3.32X_3X_4+1.22X_3X_5-1.92X_4X_5$$

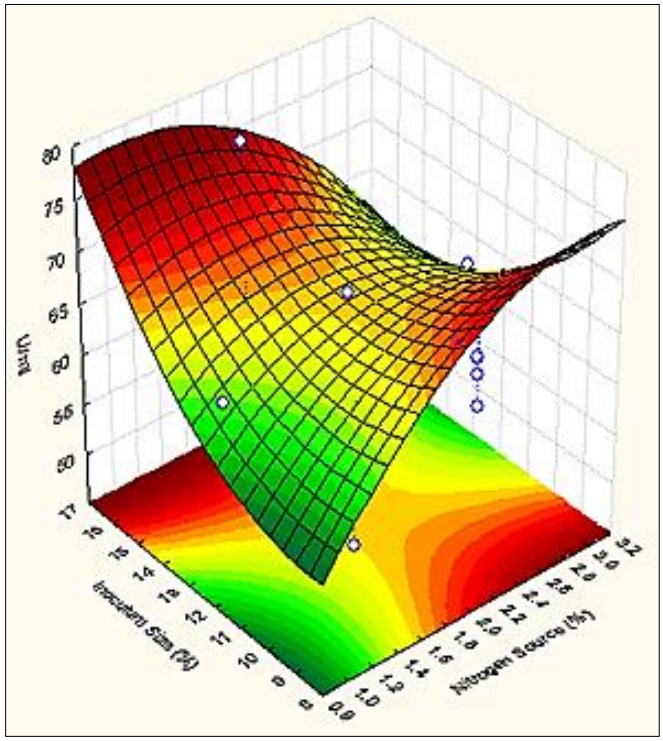
Where, X_1= Carbon source; X_2= Nitrogen source; X_3= Inoculum size; X_4= Inoculum age and X_5= Incubation time

The equation clearly explains that the presence of variables X_1, X_2, X_4 and X_5i.e carbon source nitrogen source, inoculum age and incubation time negatively influenced the production of keratinases. Whereas, inoculum size (X_3) positively affected the keratinase production. The interacting effect of X_3X_4 and X_3X_5 showed the positive effect on keratinase production whereas, X_1X_2, X_1X_3, X_1X_5, X_2X_3, X_2X_5 and X_4X_5 shown to have negative affect on keratinase production. Central values of independent variables for *B. cereus* N14 were obtained at 2.5 percent (w/v) carbon source, 2.0 percent (w/v) nitrogen source, 16.5 per cent inoculum size, 2 days inoculum age and 3 days of incubation where maximum response (Y) i.e 74.86 U/mL was obtained (Table 2).

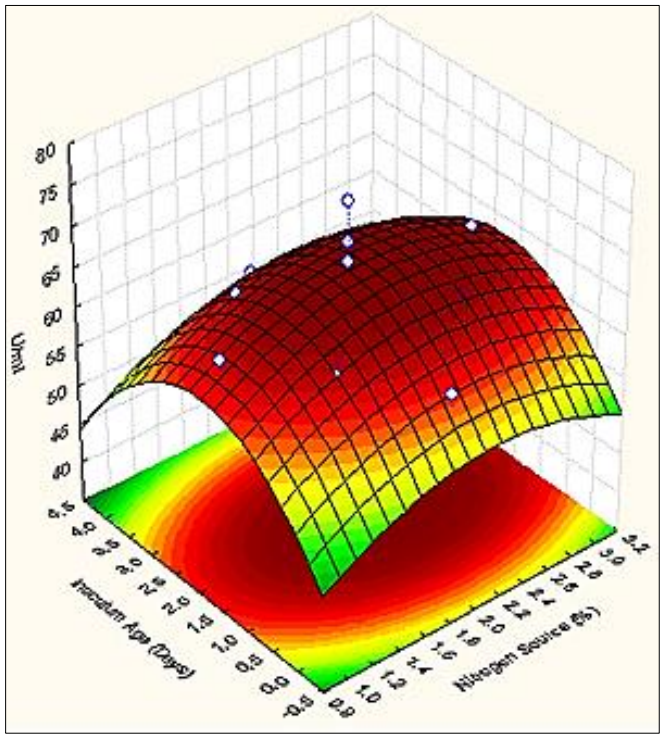
Table 2: Central Composite design (CCD) for *Bacillus cereus* N14 using five variables, showing observed and predicted value

Runs	Carbon Source (%)	Nitrogen Source (%)	Inoculum Size (%)	Inoculum Age (Days)	Time (hrs)	Observed Value (U/mL)	Predicted Values (U/mL)
1	2	1.5	10	1	5	60.55	60.51
2	2	1.5	10	3	1	50.78	50.53
3	2	1.5	14	1	1	54.46	54.96
4	2	1.5	14	3	5	62.81	63.01
5	2	2.5	10	1	1	62.80	62.82
6	2	2.5	10	3	5	58.18	58.03
7	2	2.5	14	1	5	61.30	61.98
8	2	2.5	14	3	1	59.52	59.84
9	3	1.5	10	1	1	58.44	58.36
10	3	1.5	10	3	5	56.14	55.87
11	3	1.5	14	1	5	61.50	61.98
12	3	1.5	14	3	1	60.47	60.60
13	3	2.5	10	1	5	62.98	63.00
14	3	2.5	10	3	1	61.25	61.06
15	3	2.5	14	1	1	53.49	54.09
16	3	2.5	14	3	5	56.62	56.92
17	1.5	2	12.5	2	3	57.50	57.14
18	3.5	2	12.5	2	3	56.62	56.41
19	2.5	1	12.5	2	3	62.81	62.76
20	2.5	3	12.5	2	3	64.54	64.03
21	2.5	2	8.5	2	3	69.86	70.72
22	2.5	2	16.5	2	3	74.86	73.92
23	2.5	2	12.5	0	3	59.82	59.01
24	2.5	2	12.5	4	3	57.48	57.71
25	2.5	2	12.5	2	-1	50.62	50.38
26	2.5	2	12.5	2	7	56.08	55.75
27	2.5	2	12.5	2	3	67.44	67.52

i) AB interaction (Inoculum size (%) and Nitrogen source (%))



ii) AC interaction (Inoculum age (days) and Nitrogen source (%))



iii) AB interaction (incubation time (days) and Nitrogen source (%))

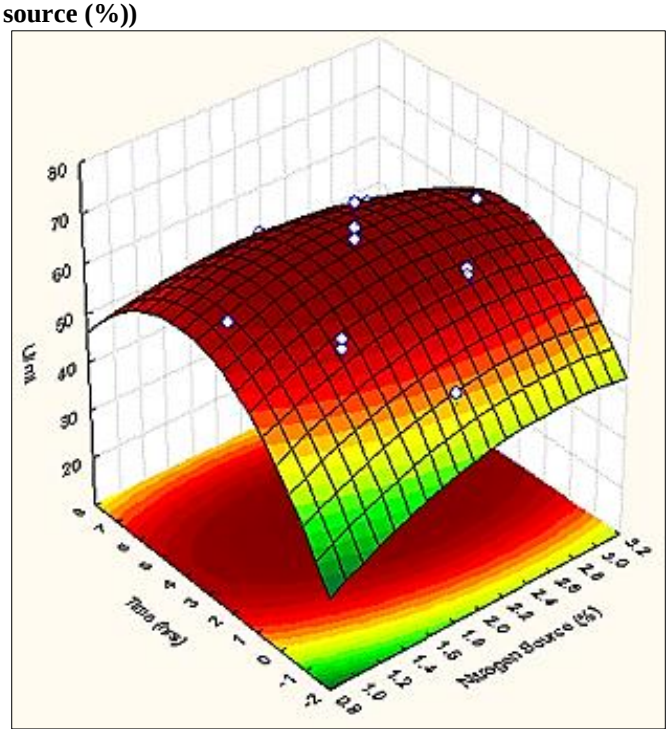


Fig 4.12: Effect of interaction of various factors on the production of keratinase by *Bacillus cereus* N14

Figures 12, i), ii) & iii) are three dimensional counter plots with the combinations of inoculum size and nitrogen source; inoculum age and nitrogen source; time and nitrogen source which suggested that these combinations exert a better effect on the keratinase production by *B. cereus* N14. Significant influence of different independent variables (nitrogen source, carbon source and inoculum size) on keratinase production have been highlighted by many researchers (Shankar *et al*., 2013; Lakshmi *et al*., 2013) [40,26]. Interactions of other factors were also found equally important for keratinase production. These experimental findings are in close agreement with the model predictions.

Analysis of variance (ANOVA) for Response surface of *Bacillus cereus* N14

Analysis of variance provided response (U/mL) as a function of initial values ofincubation time, inoculums size, inoculum age, concentration of carbon and nitrogen sources as depicted in Table 3. The coefficient of determination (R^2) was calculated as 0.9937 for the *B. cereus* N14 indicating that the statistical model can explain 99.37 per cent of variability in predicting the response. The closer the R^2 to 1.0, the stronger the model and better it is predicted (Haaland, 1989) [18].The value of multiple regression coefficient (R^2= 0.99) indicates agreeable correlation between predicted and observed value hence, representing good fit. The purpose of statistical analysis is to determine the experimental factors which generate signals that are large in comparison to noise. The adjusted R^2 of 0.97292 was in reasonable agreement with R^2 (coefficient of determination) i.e 0.9937.

Table 3: Analysis of variance (ANOVA) for *Bacillus cereus* N14

Factors	SS	DF	MS	F	P
(X_1) Carbon Source (%)(L)	0.0038	1	0.0038	0.0049	0.946498
Carbon Source (%)(Q)	116.5012	1	116.5012	149.0731	0.000018
(X_2) Nitrogen Source (%)(L)	13.1873	1	13.1873	16.8743	0.006302
Nitrogen Source (%)(Q)	17.2088	1	17.2088	22.0202	0.003352
(X_3) Inoculum Size (Days)(L)	0.9653	1	0.9653	1.2352	0.308944
Inoculum Size (Days)(Q)	24.5202	1	24.5202	31.3757	0.001379
(X_4) Inoculum Age (%)(L)	13.0055	1	13.0055	16.6417	0.006506
Inoculum Age(%)(Q)	84.6135	1	84.6135	108.2701	0.000046
(X_5) Time (Days)(L)	33.6976	1	33.6976	43.1190	0.000598
Time(Days)(Q)	210.8291	1	210.8291	269.7737	0.000003
X_1X_2	14.8148	1	14.8148	18.9568	0.004802
X_1X_3	10.0975	1	10.0975	12.9206	0.011442
X_1X_4	2.1638	1	2.1638	2.7688	0.147174
X_1X_5	8.5673	1	8.5673	10.9626	0.016185
X_2X_3	47.7842	1	47.7842	61.1440	0.000231
X_2X_4	0.0037	1	0.0037	0.0048	0.947230
X_2X_5	13.7418	1	13.7418	17.5839	0.005728
X_3X_4	45.0402	1	45.0402	57.6328	0.000272
X_4X_5	6.0862	1	6.0862	7.7878	0.031550
X_4X_5	14.8610	1	14.8610	19.0159	0.004767
Error	4.6890	6	0.7815		
Total SS	750.3658	26			
ANOVA; Var.:U/mL; R-sqr=.99375;Adj.=.97292 (Spreadsheet13)5 factors, 1 Blocks, 27 Runs; MS Residual=.7815036DV: U/mL					

Overall percent (%) increase in keratinases production before and after optimization

The main highlight of the present study is that a significant increase in keratinase production from 20.57 U/mL to 74.86 U/mL after optimizing different process parameters by *Bacillus cereus* N14 was observed thereby indicating 27.5 per cent increase in keratinase production (Figure 13).

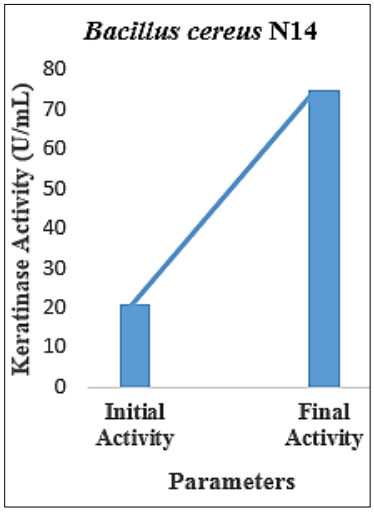


Fig 13: An overall per cent increase in keratinase activity of *Bacillus cereus* N14 after optimization of variable parameters

Conclusion

The keratinolytic potential of *Bacillus cereus* N14 has increased after optimizing its cultural conditions using OVAT and RSM approaches which is accounted for 27.5 per cent increase in keratinase production.

Acknowledgements

The authors gratefully acknowledge Integrated Eco-development Research Programme (IERP), Gobind Ballabh Pant Institute of Himalayan Environment & Development Kosi –Katarmal, Almora, Uttrakhand- 263643 (India) for providing financial help to carry out this work.

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