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Keratinase production by *Bacillus weihenstephanensis* PKD5 in solid-state fermentation and its milk clotting potential

Dipak K Sahoo[1], Suman K Halder[1], Arpan Das[1], Arijit Jana[1], Tanmay Paul[1], Hrudayanath Thatoi[2], Keshab C Mondal[1] and Pradeep K Das Mohapatra[1]*

[1]Department of Microbiology, Vidyasagar University, Midnapore 721 102, India

[2]Department of Biotechnology, College of Engineering and Technology
Biju Patnaik University of Technology (BPUT), Bhubaneswar 751 003, India

A low cost and energy intensive solid-state fermentation medium was formulated by employing poultry feather for the production of keratinase by *Bacillus weihenstephanensis* PKD5 following 'one variable at a time' (OVAT) and response surface methodology (RSM). After OVAT optimization, four most critical factors were identified with significant increase of enzyme production (2.95-fold). Among them, incubation period, incubation temperature, pH and nitrogen source (ammonium chloride) were further optimized statistically by Box-Behnken RSM. The results of analysis of variance and regression of the second-order model of RSM showed that among the parameters, fermentation time (2.85 d), temperature (34.12°C), pH (7.79) and ammonium chloride (0.5%) had the significant influences on keratinase production. Under the optimized conditions, a maximum enzyme production of 164.9 U/g was achieved, which was 3.17-fold higher. During further investigation on milk clotting property, the enzyme had shown the clotting activity of 43.6 SU/mL, suggesting its usefulness as new source of milk-coagulant for cheese making.

Keywords: Keratinase, milk-coagulant, OVAT, RSM, solid-state fermentation

Introduction

To meet the escalating demand of chicken, the number of poultry-processing plants and slaughter houses are increasing day by day. As an obvious consequence, feathers are accumulated in tons, which are often dumped, land filled, burned that create potential environmental pollution[1,2]. About 8.5 billion ton poultry feathers are produced each year globally, while India contributes about 350 million tons[3]. Feathers are principally composed of keratin (90%); a fibrous and insoluble structural protein. Because of a high degree of cross-linking by disulphide bond, keratin is not degraded by common proteases like pepsin, trypsin, and papain[4]. However, in nature, the native structural keratins are not accumulated because some indigenous microorganisms harbour specific proteolytic enzyme, namely keratinase [EC 3.4.21/24/99.11], which potentially degrade the rigid and tough keratin[5]. Keratinases have imperative biotechnological applications including enzymatic improvement of feather meal, production of rare amino acids (like serine, cysteine & proline) and peptides, conversion of feathers into value-added products like fertilizers, glues, films and foils, as well as used in leather, detergent, textile, medicine and cosmetic industry[5-9].

Despite such dynamic relevance, information is scarce regarding the proper utilization of poultry waste towards value addition and the large scale production of keratinase. In addition to the low activity, stability of the enzyme restricts commercial exploitation. Keratinase biosynthesis has been reported by different species of fungi[10], actinomycetes[11] and bacteria (*Bacillus*)[12], mainly through submerged fermentations (SmF).

Recently, solid-state fermentation (SSF) has been receiving more attention because of its unique advantages over SmF, like low production cost, saving of water, requirement of minimal energy and stability of the product[13,14]. In SSF, the agro-industrial wastes are employed for enzyme production, which not only lowers energy consumption but is also eco-friendly[15]. The advantages of using low cost natural material in SSF are especially interesting for countries like India where agro-industrial residues are generated in abundance[16]. There are very few reports dealing with the production of keratinase by different microorganisms using a keratinous substrate in SSF system.

*Author for correspondence:
Tel.: +91-3222-276554/555 x477; Fax: +91-3222-275329
pkdmvu@gmail.com

Milk coagulation is a basic step in cheese manufacturing by using calf rennet obtained from the fourth stomach of suckling calves after sacrification[17]. To meet the demand of the cheese and ethical restriction on animal sacrification, a sustainable alternative is essential. In this regard, various animals, plants and microbial proteases have been suggested as milk coagulant[17]. Among these, microbial sources draw more attention due to high growth rate, simple nutritional requirements as well as low cost-high productivity. Though, microbial keratinase has multi-dimensional applicability, its use in dairy industry as milk clotting agent was rarely reported.

The present study was conducted to optimize the keratinase production by *Bacillus weihenstephanensis* PKD5 through SSF, using sequential execution of one variable at a time (OVAT) and response surface methodological (RSM) approach. The application of crude keratinase as milk clotting agent was also investigated.

Materials and Methods

Microorganism and Inoculum Preparation

B. weihenstephanensis PKD5 was isolated from feather dumping soil near chicken slaughter houses at Midnapore Town, West Bengal, India and grown on keratin agar slant [composition (%, w/v): keratin, 0.5; K_2HPO_4, 0.03; KH_2PO_4, 0.04; NaCl, 0.05; $MgSO_4.7H_2O$, 0.01; agar, 1.5 (pH 7.5)] at 37°C for 4 d[18]. After growth, slants were kept at 4°C for further use.

The organism was grown in broth medium (composition stated above) in 100 mL Erlenmeyer flask at 37°C and 120 rpm for 24 h, and used as inoculum for solid-state fermentation when the cell concentration reached ~10^9 cells/mL.

Feather Substrate

Freshly plucked chicken feathers were obtained from chicken slaughter houses, washed extensively with tap water, dried at 60°C for 2 d and then kept at room temperature until further use.

Optimization of Keratinase Production in SSF

OVAT Approach

In the experimental design, varying amount of chicken feather was taken in 250 mL of Erlenmeyer flask, moistened with salt solution [containing (g%): NaCl, 0.5; $MgSO_4$, 0.1; K_2HPO_4, 0.05] and autoclaved at 121°C for 15 min. The medium was inoculated with 2 mL of the bacterial suspension and incubated in a programmable environmental chamber (REMI-CHM-6S) after thorough mixing. The parameters of SSF like incubation period (1-10 d), incubation temperature (30-45°C), pH (5.0-10.0), substrate concentration, moisture content (as solid:moisture ratio, 0.04-0.2), and supplementation with different additional nitrogen and carbon sources were investigated.

RSM Experimental Design and Statistical Analysis

Four most influencing process parameters (with respective level) were selected for further optimization through RSM. To explore the effect of these variables on the keratinase production, a Box-Behnken factorial design was employed. An experimental design (4 factors with 3 levels) comprising 29 experimental run were conducted (in triplicate). The response was keratinase activity (U/g). The minimum and maximum ranges of variables were used and a full experimental design is listed in coded form in Table 1. The relation between the coded value and actual values is described as in the following Eq. 1.

$$Xi = (xi-xo)/\Delta xi \quad \ldots (1)$$

where Xi is the independent variable coded value, xi is the independent variable actual value, xo is the independent variable actual value on the centre point and Δxi is the step change value.

A model was generated by the regression analysis of the responses, and its efficiency was tested by ANOVA and F-test. Interaction effects of the variables were represented by three dimensional (3D) response surface plots. The RSM optimization was performed by employing Design-Expert 8.0 (USA).

Extraction of Crude Enzyme

The fermentation mass was mixed with distilled water (1:20 w/v) and agitated on a rotary shaker at 120 rpm for 60 min. The slurry was then squeezed through cheese cloth followed by centrifugation at 10000× g for 10 min at 4°C. The clear supernatant was used for enzyme assay.

Enzymatic Assay

The keratinolytic activity was assayed by the modified method of Gradisar *et al*[19] using keratin powder as a substrate.

Milk Clotting Activity

Preparation of Substrate

The skimmed milk powder (10 g) was dissolved by stirring on magnetic stirrer in 100 mL of 10 mM

$CaCl_2$ and $MnSO_4$ separately. The pH of substrate was adjusted to 6.5 with 0.1 N NaOH or HCl.

Milk Clotting Assay

Milk clotting activity was determined according to the method of Arima *et al*[20] and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme that clots 1 mL of a milk substrate containing 0.1 g skim milk powder in 40 min at 35°C. In brief, 0.5 mL of crude enzyme was added to a test-tube containing 5 mL of reconstituted skim milk solution (10 g dry skim milk/100 mL, 10 mM $CaCl_2$, 10 mM $MnSO_4$, separately and in combination) pre-incubated at 35°C for 5 min. The mixture was mixed well and the clotting time T(s), *i.e.*, the time period starting from the addition of test material to the first appearance of clottings of milk solution, was recorded and the clotting activity was calculated using the following formula:

$SU = 2400 \times 5 \times D/T \times 0.5$; where T is the clotting time (s) and D is the dilution of test material.

Table 1—Experimental design used in RSM studies by using four independent variables (A, pH; B, temperature; C, NH_4Cl; D, time) each at three levels (−1, 0, +1) showing observed and predicted values of keratinase production

Run order	A	B	C	D	Keratinase (U/g) Exp	Keratinase (U/g) Pred
1	+1(9)	+1(35)	0(0.5)	−1(2)	127.5	126.83
2	0(8)	+1(35)	+1(0.75)	+1(4)	140.2	139.44
3	0(8)	+1(35)	0(0.5)	0(3)	162.5	163.22
4	−1(7)	0(35)	+1(0.75)	0(3)	135.6	136.69
5	0(8)	+1(40)	+1(0.75)	0(3)	125.9	125.95
6	0(8)	+1(40)	−1(0.25)	0(3)	128.3	127.93
7	0(8)	+1(40)	0(0.5)	+1(4)	132.4	133.29
8	+1(9)	0(35)	0(0.5)	+1(4)	115.9	115.18
9	0(8)	0(35)	−1(0.25)	+1(4)	142.7	142.62
10	+1(9)	0(35)	+1(0.75)	0(3)	120.1	120.89
11	−1(7)	0(35)	0(0.5)	−1(2)	138.3	138.18
12	0(8)	0(35)	0(0.5)	0(3)	162.5	163.22
13	0(8)	−1(30)	+1(0.75)	0(3)	140.9	140.43
14	−1(7)	−1(30)	0(0.5)	0(3)	138.2	137.67
15	−1(7)	0(35)	0(0.5)	+1(4)	144.4	144.23
16	−1(7)	0(35)	−1(0.25)	0(3)	141.2	142.02
17	0(8)	0(35)	0(0.5)	0(3)	164.4	163.22
18	0(8)	−1(30)	−1(0.25)	0(3)	141.2	140.31
19	0(8)	0(35)	−1(0.25)	−1(2)	143.2	143.17
20	0(8)	−1(30)	0(0.5)	−1(2)	148.8	149.52
21	0(8)	0(35)	0(0.5)	0(3)	163.1	163.22
22	0(8)	+1(40)	0(0.5)	−1(2)	123.5	124.29
23	+1(9)	0(35)	−1(0.25)	0(3)	116.9	117.42
24	+1(9)	−1(30)	0(0.5)	0(3)	120.1	120.42
25	+1(9)	+1(40)	0(0.5)	0(3)	104.3	104.04
26	0(8)	0(35)	+1(0.75)	−1(2)	145.2	144.49
27	−1(7)	+1(40)	0(0.5)	0(3)	128.3	127.19
28	0(8)	−1(30)	0(0.5)	+1(4)	134.1	134.92
29	0(8)	0(35)	0(0.5)	0(3)	163.6	163.22

Results and Discussion

Keratinase production under submerged fermentation by *B. weihenstephanensis* PKD5 was previously explored[18]. For economic and environmental view point, production of keratinase in SSF by supplementing chicken feathers as substrate was carried out in the present study.

Optimization by OVAT

Different incubation period (1-10 d) was employed to study their effect on keratinase production by *B. weihenstephanensis* PKD5 through SSF at a temperature of 37°C and pH 8.0. Keratinase production and keratinolytic activity increased gradually with a maximum activity of 52.0 U/g after 3rd d (Fig. 1) and decreased thereafter. Comparatively, the keratinolytic strain *B. subtilis* RM-01[21] showed keratinase production after 4th d, thus the present microbial strain taking less time in the process.

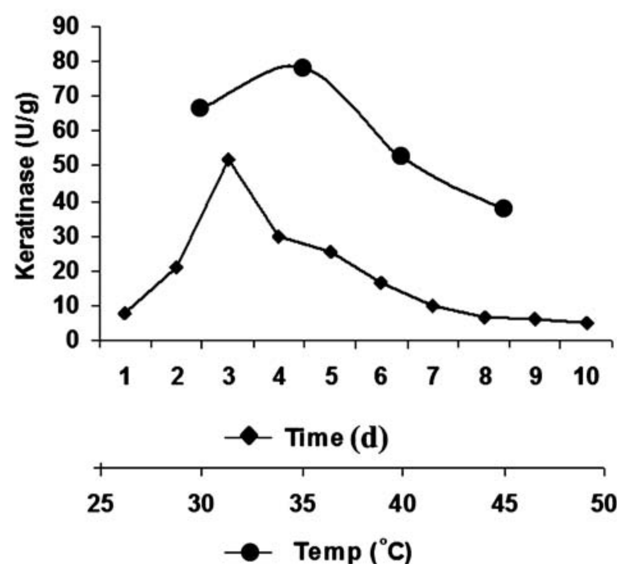

Fig. 1—Effect of fermentation time and temperature on keratinase production and biomass formation by *B. weihenstephanensis* PKD 5 under SSF.

The incubation temperature of 35°C was found the best for maximum keratinase production (78.0 U/g; Fig. 1) by *B. weihenstephanensis* PKD5. Below and above this temperature, enzyme production decreased. Many independent lines of evidence suggested that optimum temperature for keratinase production varied widely. A similar result was reported by El-Refai *et al*[22] and Kumar *et al*[23]. A lower temperature (23°C) was also reported by Cai *et al*[24] for keratinase production using *B. subtilis*. On the other hand, *B. subtilis* RM-01[21] and *Fervidobacterium islandicum* AW-1[25] showed optimum temperatures of 50° and 70°C, respectively for keratinase production.

During fermentation, highest keratinase production (81.0 U/g) by *B. weihenstephanensis* PKD5 was observed in alkaline pH range with an optimum at pH 8.0 (Fig. 2). Keratinase production by microbial strains depends strongly on the extracellular pH, which influences many enzymatic processes and transport of various components across cell membrane that in turn supports cell growth and production[26]. Possibly the alkaline pH modifies cystine residues to lathionine, making it accessible for keratinase action[21]. Generally, keratinases from different organisms have varied pH optima, acidic to alkaline range[21,27].

Different concentration of chicken feather as substrate was used for the production of keratinase by *B. wehenstephanensis* PKD5 through SSF. It was found that 1 g/flask chicken feather was the optimized amount for enzyme production (120.2 U/g). Similar result was observed for the strain *B. subtilis* RM-01 using chicken feather as substrate in SSF[21].

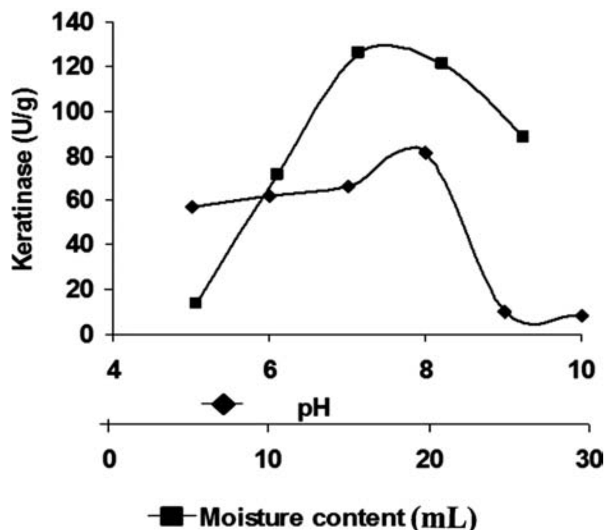

Fig. 2—Effect of fermentation pH and moisture content on keratinase production by *B. weihenstephanensis* PKD 5 under SSF.

Moisture level is considered as one of the most significant among several factors that is important for microbial growth and enzyme production under SSF using a particular substrate[28,29]. In SSF, microbial growth and product formation occur at or near the surface of the solid substrate particle[29]. Thus, it is crucial to provide an optimized water level that controls the water activity of the fermenting substrate in order to achieve the maximum amount of product. The solid substrate with moisture ratio of 1:15 (w/v) was found to be the best for enzyme (125.0 U/g) production by *B. weihenstephanensis* PKD5 (Fig. 2). At above or below moisture level, the enzyme production was found to be decreased.

Among the additional carbon sources, mannitol resulted in a significant increase in keratinase production (130.2 U/g; Fig. 3) compared to the cultivation without the supplement (98.2 U/g). In a sharp contrast, galactose, glucose inhibited the keratinase production by *B. wehenstephanensis* PKD5 strain due to catabolite repression[21].

Ammonium chloride was the most effective nitrogen source for keratinase production by *B. wehenstephanensis* PKD5 in SSF (Fig. 4). It was also revealed that 0.5% NH_4Cl was ideal for the enzyme production (153.4 U/g). Elemental nitrogen is a very essential component for growth of the microbes and production of secondary metabolites. Similarly, *B. subtilis* RM-01 has shown a preference for inorganic nitrogen ($NaNO_3$; 69.3 U/g) source compared to organic nitrogen for keratinase production[21].

Optimization through Box–Behnken Design

A Box–Behnken factorial design was performed for further enhancement of enzyme production. All the predicted and observed responses related with keratinase production are summarized in Table 1. The adequacy of the model and fitness were evaluated by

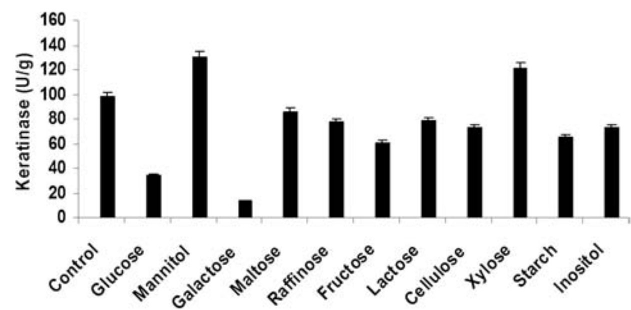

Fig. 3—Effect of different carbon sources (1%) on keratinase production by *B. weihenstephanensis* PKD 5 under SSF.

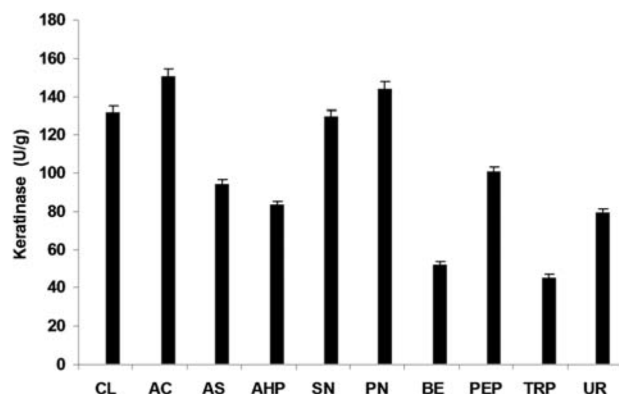

Fig. 4—Effect of different nitrogen sources (0.2%) on keratinase production by *B. weihenstephanensis* PKD 5 under SSF. [CL, Control; AC, Ammonium chloride; AS, Ammonium sulphate; AHP, Ammonium hydrogen phosphate; SN, Sodium nitrate; PN, Potassium nitrate; BE, Beef extract; PEP, Peptone; TRP, Tryptone; UR, Urea]

ANOVA (analysis of variance); while regression coefficients were used for the experimental design (Table 2). ANOVA of the quadratic regression model suggested that the model was significant with a computed F-value of 533.76 for keratinase production and P-value (Prob > F) lower than 0.0001. The fitness of the model was also examined by the coefficient of determination (R^2) and found to be 0.998, which indicates that response model can explain 99.8% of the total variations for keratinase production.

By applying multiple regression analysis on the experimental data, the following second-order polynomial equations were derived for the enzyme:

$$Y_{Keratinase}=163.22-10.1A-6.716666667B-0.466666667C-1.4D-1.475AB+2.2AC-4.425AD-0.525BC+5.9BD-1.125CD-22.64333333A^2-18.24333333B^2-11.31833333C^2-9.468333333D^2$$

Where A=pH, B=temperature, C=NH_4Cl and D=incubation time.

From the ANOVA study, it was clear that keratinase production by *B. weihenstephanensis* PKD5 in SSF was mostly influenced by pH, followed by fermentation temperature. 3D plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones. Response surface 3D plots were generated by plotting the response on the z-axis against any two independent variables, while keeping the other variables at their experimental level. The most significant (P < 0.05) mutual interaction for keratinase production was in between the fermentation temperature and the fermentation time (Fig. 5).

Table 2—ANOVA analysis of RSM model for keratinase production

Source	Sum of squares	df	Mean square	F-value	P-value
Model	6877.315	14	491.2368	533.7597	<0.0001 Significant
A: pH	1224.12	1	1224.12	1330.083	<0.0001
B: Temperature	541.3633	1	541.3633	588.2253	<0.0001
C: NH_4Cl	2.613333	1	2.613333	2.839551	0.1141
D: Time	23.52	1	23.52	25.55596	0.0002
AB	8.7025	1	8.7025	9.455813	0.0082
AC	19.36	1	19.36	21.03586	0.0004
AD	78.3225	1	78.3225	85.10232	<0.0001
BC	1.1025	1	1.1025	1.197936	0.2922
BD	139.24	1	139.24	151.293	<0.0001
CD	5.0625	1	5.0625	5.500724	0.0343
A^2	3325.755	1	3325.755	3613.642	<0.0001
B^2	2158.827	1	2158.827	2345.702	<0.0001
C^2	830.9492	1	830.9492	902.8785	<0.0001
D^2	581.5092	1	581.5092	631.8463	<0.0001
Residual	12.88467	14	0.920333		
Lack of Fit	10.29667	10	1.029667	1.591448	0.3468 Not significant
Pure Error	2.588	4	0.647		
Cor Total	6890.2	28			

Model Validation

Using Design Expert 8.0.3, numerical optimization subroutine design space was explored with a fitted quadratic model to arrive at an optimum fermentation pH, fermentation temperature, NH_4Cl and fermentation time. Solutions with higher desirability gave an optimum fermentation pH 7.8, fermentation temperature 34.1°C, NH_4Cl 0.5% and fermentation time 2.85 d. Under these conditions, confirmation experiments were conducted in three replicates. The observed mean keratinase activity was 164.68 U/g. The value was largely consistent with the predicted value of 164.92 U/g. Enhanced synthesis of keratinase after statistical optimization has been reported by several investigators. It is very difficult to compare the keratinase activity observed during fermentation using chicken feather or other types of solid substrates due to the fact that the chemical compositions of the solid substrates vary diversely, which strongly influence the rate of fermentation and subsequent enzyme production. Further, the differences in

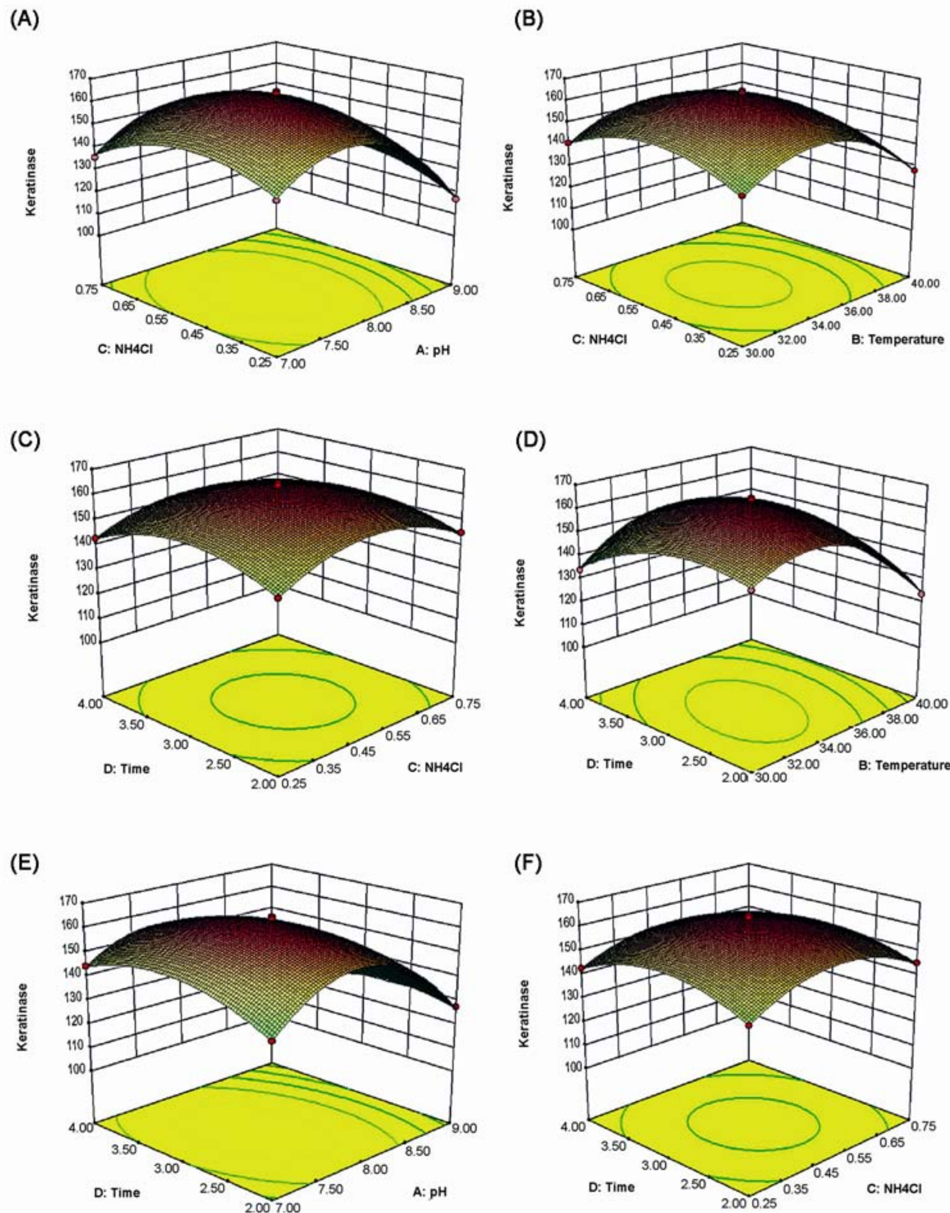

Fig. 5 (A-F)—Response surface and contour plots of the most effective interactions among the variables on keratinase production.

keratinase yields could be related to the type of organism, fermentation vessel and methods of keratinase assay.

Milk Clotting Activity

Milk clotting activity was seen in crude enzyme and also in the presence of $CaCl_2$ and $MnSO_4$, in single or in combination. In the presence of both $CaCl_2$ and $MnSO_4$, the clotting activity was the highest compared to individual $CaCl_2$ or $MnSO_4$. The milk clotting was observed in the order of keratinase+$CaCl_2$+$MnSO_4$ (43.6 U/mL) > keratinase+$CaCl_2$ (39.3 U/mL) > keratinase+$MnSO_4$ (36.3 U/mL) > keratinase (30.7 U/mL). Calcium exists in milk in soluble as well as colloidal forms. The equilibrium between both the forms is especially dependent on pH, temperature and ionic strength[30]. The addition of calcium increases the ionic concentration in milk, reduces clotting time, enhances its retention in the cheese, and improves dry wt as well retention rate of protein and fat[31]. The metal ion Mn^{++} may stimulate the milk clotting activity of

keratinases due to the maintenance of the active enzyme conformation and thus stabilize the enzyme-substrate complex[32]. Additionally, it may protect the enzyme against thermal denaturation[33].

In conclusion, the experimental organism *B. weihenstephanensis* PKD5 can utilize low cost chicken feather as substrate for keratinase production in SSF. Moreover, the enzyme can be explored as milk-coagulant, which will be immensely beneficial for dairy industry. Calf rennet is generally used as milk coagulant for cheese making. With increasing demand of cheese and its related products, it is necessary to search an alternative. In this regard, the crude keratinase of *B. weihenstephanensis* PKD5 will be very much ideal due to unlimited production through fermentation, cost effectiveness and acceptable by lacto-vegetarians.

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