Research Journal of Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 6 (12): 839-850, 2011
ISSN 1816-4935 / DOI: 10.3923/jm.2011.839.850

Enhanced Production of Recombinant Thermostable Keratinase of *Bacillus pumilus* KS12: Degradation of Sup35 NM Aggregates

Rinky Rajput and Rani Gupta
Department of Microbiology, University of Delhi, South Campus, New Delhi 110021, India

Corresponding Author: Rani Gupta, Department of Microbiology, University of Delhi, South Campus, New Delhi 110021, India Tel: +91-11-24111933 Fax: +91-11-24115270

ABSTRACT

Production and secretion of keratinase rK_{27} from *Bacillus pumilus* was optimized by using response surface methodology. The recombinant strain *E. coli* HB101 produced the enzyme extracellularly with 1.4 KU mL^{-1} in LB-amp medium at 37°C and 300 rpm after 12 h. Its production has reached to 4.2 KU mL^{-1} when the medium was supplemented with 0.5% glucose. Further, optimization was done using CCD model comprising glucose, yeast extract and incubation time. A maximum production of 5.6 KU mL^{-1} was achieved in the extracellular broth when 0.5% glucose and 1% yeast extract were used. SDS-PAGE analysis revealed that part of the enzyme was retained in the periplasm. Hence, optimization of enzyme secretion in extracellular broth was done using CCD model which led to an overall yield of 7.3 KU mL^{-1} in the medium supplemented with 1% glycine and triton X-100. The enzyme was partially purified using thermal precipitation with a final recovery of 91% and 5.8 KU mg^{-1}. The 890 mg of enzyme was found to completely degrade 100 μg of yeast surrogate prion protein, Sup35 NM at pH 7 and 37°C.

Key words: *B. pumilus*, *E. coli*, keratinase, response surface methodology, Sup35 NM

INTRODUCTION

Keratin is hard to degrade, structural protein that comprises 90% of the dry weight of feather (Tatineni *et al.*, 2008). These proteins are resistant to degradation by a broad range of proteases like trypsin, pepsin and papain because of their tight secondary structure and presence of disulfide linkages (Pillai and Archana, 2008). Despite of their rigidity they are efficiently degraded by a large number of bacteria, actinomycetes and fungi that elaborate keratinases (Gupta and Ramnani, 2006). Keratinases have recently gained biotechnological impetus because of their ability to act on hard-to-degrade proteins such as hair, feather, nail, hoof etc and thus, becoming a part of solid waste management as recycling of these wastes is tough (Brandelli *et al.*, 2009). In nature, they have been continuously contributing to environmental cleanup of large amounts of feather by converting it into nitrogen rich feather meal (Brandelli *et al.*, 2009). Besides their use in traditional industrial sectors like detergent, medicine, cosmetics, leather and feed, they also find uses in dehairing for leather processing, as ungual enhancers to increase drug delivery (Gupta and Ramnani, 2006; Prskash *et al.*, 2010; Mohorcic *et al.*, 2007; Mitsuiki *et al.*, 2009). However, till date only few keratinases from *Bacillus* and *Streptomyces* species have been extensively studied (Brandelli *et al.*, 2009).

During the last decade, keratinases have gained significant importance due to their ability to act on proteinase-K resistant prion proteins (Brandelli *et al.*, 2009). Though prion degradation has

been studied using various enzymatic preparations, however for complete degradation harsh pretreatments of prion protein is necessary (Rao *et al.*, 1998). Hence, there is a demand of newer keratinases with better catalytic efficiency for degradation of tough proteinase-K resistant proteins.

We have earlier reported cloning, functional expression and biochemical characterization of keratinase rK_{27} from a feather degrading strain of *Bacillus pumilus* KS12 (Rajput *et al.*, 2011). The substrate specificity of rK_{27} as studied by hydrolysis of soluble and insoluble proteinaceous substrates and oxidized insulin B-chain suggested that it shared similarities with *Streptomyces* sp. protease which has already been documented for prion degradation suggesting it to be a prospective prion degrading enzyme.

Hence, the present investigation was undertaken to improve the recombinant keratinase production, secretion and expression level through optimization of medium conditions using response surface methodology. Also, enzyme was evaluated for its potential to degrade yeast surrogate prion protein Sup35 NM.

MATERIALS AND METHODS

Bacterial strain: Keratinase rK_{27} was expressed using *E. coli* HB101-pEZZ18 system (Rajput *et al.*, 2011). Stock culture of *E. coli* HB101-pEZZ18-rK_{27} was maintained at -80°C and plasmid was stored at -20°C.

Inoculum preparation: The clone was revived from glycerol stocks in 50 mL Luria-Bertani broth supplemented with ampicillin (0.27 mM) in 250 mL Erlenmeyer flask and incubated at 37°C, 300 rpm in a New Brunswick Scientific shaker (Edison, New Jersey, USA) for 18 h. A 2% (v/v) inoculum (6×10^8 cfu mL^{-1}) of this culture was used to inoculate the production medium.

Extracellular production of recombinant keratinase rK_{27}: *E. coli* HB101 with pEZZ18-rK_{27} was cultivated in Luria-Bertani broth supplemented with ampicillin (0.27 mM) at 37°C and 300 rpm. After 18 h, cells were separated by centrifugation at 7441×g for 10 min (Sigma centrifuge, Germany) and the supernatant was checked for the enzyme activity.

Keratinase production: Keratinase production was carried out in four different modified Luria-Bertani broth (10 g tryptone, 5 g yeast extract and 10 g NaCl L^{-1}, pH 7; Hi-media) supplemented with ampicillin (0.27 mM). The first medium (M_1) contained (g L^{-1}): 10 g tryptone, 5 g yeast extract and 10 g NaCl, pH 7; Hi-media. Second medium (M_2) contained (g L^{-1}) 5 g glucose in M_1 medium, pH 7 and third medium (M_3) contained following constituents (g L^{-1}): 10 g tryptone, 12 g yeast extract, 10 g NaCl and 10 g glucose, pH 7. Fourth production medium (M_4) was a modified M_3 medium supplemented with glycine (1%) and triton X-100 (1%). Production was carried out in 250 mL Erlenmeyer flask containing 50 mL medium inoculated with 2% overnight grown culture as the inoculum, at 37°C and 300 rpm in an incubator shaker.

Source of keratin substrate: Chicken feather were obtained from local poultry plants. They were washed thoroughly with triton X-100 (1% w/v) and rinsed with distilled water and autoclaved. Thereafter, feather were dried in an oven at 60°C for 1 h and passed through a sieve of mesh No.10.

Keratinase assay and protein estimation: The assay was set up with 1 mL of appropriately diluted enzyme, 4 mL glycine-NaOH buffer (50 mM, pH 9) and 20 mg feather powder and incubated at 70°C for 1 h. The reaction was terminated with 4 mL 5% (w/v) trichloroacetic acid.

Insoluble residues were removed by centrifugation at 7441×g for 10 min. Appropriate controls were also set up. Proteolytic products in the supernatant were determined at 280 nm against respective controls. An increase in absorbance of 0.01 was considered as 1 unit (U) of enzyme and expressed as KU, where 1 KU is defined as 1000 U of enzyme. The total protein was estimated by Bradford (1976) taking BSA as the standard protein.

Biomass estimation: The cell was determined by subjecting one mL culture broth to centrifugation at 7441×g for 10 min. The cell pellet was washed twice with distilled water and dried at 60°C overnight.

Experimental design for medium optimization of recombinant keratinase rK_{27}: LB-amp medium M_1 was supplemented with 0.5% (w/v) glucose to study the effect of carbon source on keratinase production from recombinant *E. coli*. From M_2 medium glucose was replaced with equivalent amounts of different carbon sources like fructose, galactose, lactose, maltose, mannitol, starch and sucrose to study their effect on enzyme production. Addition of equimolar amounts of different organic nitrogen sources like beef extract, casein, malt extract, soy flour, soybean meal and urea and inorganic nitrogen source ammonium sulfate, ammonium nitrate, on keratinase production were also studied as substitute of tryptone in M_2 medium.

Response surface methodology of recombinant keratinase rK_{27}: Medium optimization was carried out further by Response Surface Methodology (RSM) in M_2 medium where responses of three parameters, viz. glucose, yeast extract and incubation time were studied at five different levels (-α, -1, 0, +1, +α) using Central Composite Design (CCD) and are presented in Table 1. A set of 20 experimental runs including eight centre points were designed using Design expert® 6.0 (Stat Ease). Finally, cells from the experimental runs which supported maximum keratinase production were separated out by centrifugation at 7441×g for 10 min followed by keratinase activity in both cell pellet and supernatant. The model was analyzed using ANOVA, 3D response surface, contour and one-factor plots to study the interactions among various factors and also to determine the optimum concentration of each for maximum keratinase production. Accuracy of the model was determined by validating the model within and beyond the design space. Hence, M_3 medium was formulated for enzyme production. Biomass production was also considered as one of the responses to evaluate the dependency of keratinase on growth of the organism.

Optimization of secretion strategy of recombinant keratinase rK_{27} in extracellular broth: The secretion of recombinant protein in extracellular broth was further optimized using two-factorial CCD at five different levels (Table 2). A set of 13 experimental runs including

Table 1: Experimental ranges of three different parameters used in central composite design of RSM for medium optimization of keratinase rK_{27} by recombinant *E. coli* HB101

		Range coding (%)				
Run	Parameters	-α	-1	0	+1	+α
1.	Glucose (%) w/v	0.00	0.25	1.00	1.75	3.00
2.	Yeast extract (%) w/v	0.00	0.50	1.25	2.00	2.50
3.	Incubation time (h)	3.00	12.0	30.0	48.0	60.00

Production was carried out in the respective combinations at pH 7, 37°C and 300 rpm in triplicate. Keratinase assay was performed at pH 9, 70°C using 20 mg chicken feather as substrate

Table 2: Experimental ranges of two different parameters used in central composite design of RSM for secretion and expression of keratinase rK_{27} by recombinant *E. coli* HB101

		Range coding (%)				
Run	Parameters	-α	-1	0	+1	+α
1	Glycine (%) v/v	0.00	0.50	1.00	1.50	2.00
2	Triton X-100 (%) v/v	0.00	0.50	1.00	1.50	2.00

Production was carried out in the respective combinations at pH 7, 37°C and 300 rpm in triplicate. Keratinase assay was performed at pH 9, 70°C using 20 mg chicken feather as substrate

six centre points were designed using Design expert® 6.0. (Stat Ease). It was freshly cultivated in M_3 medium at 37°C, 300 rpm until the optical density of the grown culture reached to 0.6 O.D. at 600 nm. After reaching to 0.6 O.D., different concentrations of glycine (0.5, 1, 2% w/v) and triton X-100 (0.5, 1, 2% v/v) were supplemented to M_3 medium at 37°C, 300 rpm for 30 h. The cells were separated by centrifugation at 7441×g for 10 min and the activity was checked in periplasm and supernatant. The model was analyzed using ANOVA, 3D response surface, contour and one-factor plots for maximum keratinase production and also validated within and beyond the design space.

Downstream processing of recombinant keratinase rK_{27}: Two liter of culture supernatant was subjected to a 10 kDa molecular weight cutoff cassette (Millipore Inc., USA) using an ultrafiltration unit (Sartorius, Gottingen, Germany) with a transmembrane pressure of 5 psi. Thereafter, retentate and permeate were checked for enzyme activity and retentate was incubated at 80°C for 30 min. The contents were centrifuged at 7441×g and supernatant was checked for enzyme activity.

Evaluation for degradation of yeast surrogate protein (Sup35 NM) by keratinase rK_{27}

Expression and purification of Sup35 NM: The yeast surrogate prion protein (Sup35 NM) plasmid was purchased from North Carolina University, USA. It was transformed into an expression host, *E. coli* BL21 CODON PLUS. The positive transformants were stored at -80°C. It was cultivated in Luria Bertani broth supplemented with ampicillin (0.27 mM) at 37°C and 200 rpm using 1% overnight grown culture till an OD_{600} of 0.6 has reached. IPTG of 1 mM concentration was then added to the broth to induce the expression of prion protein at 37°C, 200 rpm for 6 h. Thereafter, cells were pelleted and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.2, 1 mM DTT, 1 mM PMSF, 8 M urea) followed by sonication at 3 sec pulse on and 3 sec pulse off for 10 min. The sonicated broth were centrifuged at 7441×g for 10 min and purified according to Chen *et al*. (2005) using Q-Sepharose chromatography. The purified protein was then treated with 6 M guanidine hydrochloride for 1 h and total protein was estimated at 280 nm and stored at -20°C for further use. The level of expression of Sup35 NM was checked on 15% SDS-PAGE.

Enzymatic degradation of Sup35 NM: The enzymatic hydrolysis of yeast surrogate prion (Sup35 NM) protein was achieved by treating 100 μg of Sup35 NM with 120-1200 μg of enzyme at pH 7 and 37°C for 12 h under mild shaking. The contents were centrifuged at 7441×g for 10 min and supernatant was applied to 15% SDS-PAGE along with appropriate controls followed by electroblotting.

Western blot analysis to detect Sup35 NM: Western blot analysis was performed using a modified ECL Western blotting analysis system to detect Sup35 NM (Amersham Pharmacia). Peptide, Ac-GGYQQYNPDAGYQ-amide was used to generate anti-Sup35 NM polyclonal anti-peptide against amino acids 55-68 (anti-N). This peptide was synthesized by Bioconcept (Delhi, India) and used for production of polyclonal antibodies in rabbits by Imegenex (Orissa, India). Sup35 NM proteins were analyzed by 15% SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membrane was blocked in 3% skim milk for 1 h in PBS buffer. Primary antibody (1:5000) was added and the membrane was incubated for 1 h at mild shaking and then washed three times for 10 min with PBS buffer with 0.1% Tween 20 (PBST). Secondary anti-rabbit antibody (1:5000) conjugated with alkaline phosphatase was then added and membrane was incubated for 1 h. The membrane was washed three times with PBST. The proteins were visualized with enhanced chemiluminescence reagents in alkaline phosphate buffer.

Statistical analysis of data: All the experiments were repeated twice in triplicate and the average values have been presented as Mean±Standard deviation. Linear regression analysis has been conducted and ANOVAS has also been applied to determine the model co-efficient.

RESULTS AND DISCUSSION

E. coli HB101 harboring recombinant plasmid pEZZ18-rK_{27} was developed by our laboratory and used for the present work. The clone was cultivated in Luria Bertani broth supplemented with 0.27 mM ampicillin and enzyme was produced constitutively with 1.4 KU mL^{-1} at 37°C and 300 rpm after 18 h (Rajput *et al.*, 2011).

Of all the carbon sources (0.5% w/v) tested in LB-amp medium, glucose supported the highest level of enzyme production corresponding to 4.2 KU mL^{-1}. This was enhanced upto 3-fold along with biomass of 3.9 g L^{-1} (data not shown). Other sugars though supported the growth of the organism, they repressed the enzyme production. None of the tested organic and inorganic nitrogen salts improved the production of keratinase over tryptone (data not shown). Hence, tryptone was finally selected as a nitrogen source in M_2 medium.

Response surface methodology of recombinant keratinase rK_{27}: The final optimization of culture medium by CCD revealed that both observed and predicted values obtained for each experimental set up were almost similar and are presented in Table 3. On regression analysis of the experimental data, the following quadratic equations were obtained for keratinase production (Y_1) and biomass production (Y_2): as a function of glucose (A), yeast extract (B) and incubation time (C):

$$Y_1 = +5.61-0.14*A+0.20*B+0.22*C-0.65*A^2-0.86*B^2-1.39*C^2-0.21*A*B+0.078*A*C-0.68*B*C$$
$$Y_2 = +5.24-0.24*A-0.43*B+0.66*C-0.61*A^2+0.22*B^2-1.23*C^2-0.24*A*B-0.21*A*C-0.038*B*C$$

A reduced quadratic model was selected for analysis using statistical software package Design expert® 6.0 (Stat Ease). The model indicated that model terms *viz.* A, A^2, B^2, C^2 and BC were significant for keratinase production, while B, C, A^2, AC and BC were significant for biomass production. The model coefficients determined by multiple linear regression and Analysis of variance (ANOVA) are presented in Table 4.

The coefficient of correlation (R^2) was found to be 0.9507 and 0.9552 for keratinase production and biomass, respectively. This indicates the sample variation of 95.07 and 95.52% of the total could

Table 3: Experimental design used in RSM studies carried out using three independent variables in CCD showing production and biomass of keratinase rK_{27} by recombinant *E. coli* HB101

				Keratinase production (KU mL^{-1})		Biomass (g L^{-1})	
Run	A (%)	B (%)	C (h)	Observed	Predicated	Observed	Predicated
1	0	0	0	5.92	5.96	5.12	5.83
2	-α	0	0	3.59	3.46	4.25	3.35
3	-1	-1	+1	1.77	1.83	3.36	2.62
4	-1	-1	-1	1.70	1.66	2.40	2.14
5	0	0	-α	0.09	0.08	0.12	0.08
6	0	0	0	4.65	4.30	4.25	4.32
7	0	+α	0	3.27	3.24	4.06	3.59
8	0	-α	0	3.44	3.07	3.40	3.11
9	0	0	0	5.28	5.33	3.92	4.15
10	+α	0	0	3.82	3.05	2.79	3.35
11	0	0	0	5.76	5.82	5.40	5.28
12	0	0	0	6.00	5.92	3.58	4.25
13	+1	-1	+1	0.01	0.27	0.44	0.88
14	-1	+1	+1	2.15	2.40	2.17	2.51
15	+1	+1	+1	3.43	3.24	5.61	5.27
16	+1	-1	-1	3.01	2.94	5.23	4.15
17	0	0	0	5.53	5.84	4.43	3.35
18	0	0	+α	4.74	4.44	5.37	5.28
19	-1	+1	-1	3.15	3.05	5.04	4.95
20	+1	+1	-1	2.65	2.74	5.81	5.15

Production was carried out at the respective combinations at pH 7, 37°C at 300 rpm in triplicate. The keratinase assay was performed at pH 9, 70°C using 20 mg chicken feather as substrate, where, A: Glucose (%) w/v; B: Yeast extract (%) w/v; C: Incubation time (h)

Table 4: ANOVA values of three factorial CCD used for production and biomass of keratinase rK_{27} by recombinant *E. coli* HB101

Term	Keratinase production (KU mL^{-1})	Biomass (g L^{-1})
F-value	18.55	20.16
Standard deviation	0.393	0.39
Mean	4.070	4.05
R^2	0.9507	0.9552
Adjusted R^2	0.9280	0.9345
Predicted R^2	0.9070	0.9139
Coefficient of variance	12.82	9.62
PRESS	3.806	5.97
Adequate precision	20.670	17.080

F-value of 18.55 and 20.16 implies the model is significant

be explained by the model and hence, it was selected for the present analytical work. The adjusted R^2 was also very high which was corresponding to the values of 0.9280 and 0.9345 for keratinase and biomass production, respectively. The value of Adjusted R^2 indicates that the model was significant. Furthermore, the value of predicated R^2 obtained for keratinase (0.9070) and biomass (0.9139) production, was in reasonable agreement with the Adjusted R^2 indicating a good agreement between observed and predicated values. The Coefficient of Variation (CV) measures the residual variation of the data relative to the size of the mean. Usually, the higher the value of CV, the lower is the reliability of experiment. The values of CV obtained for keratinase and biomass

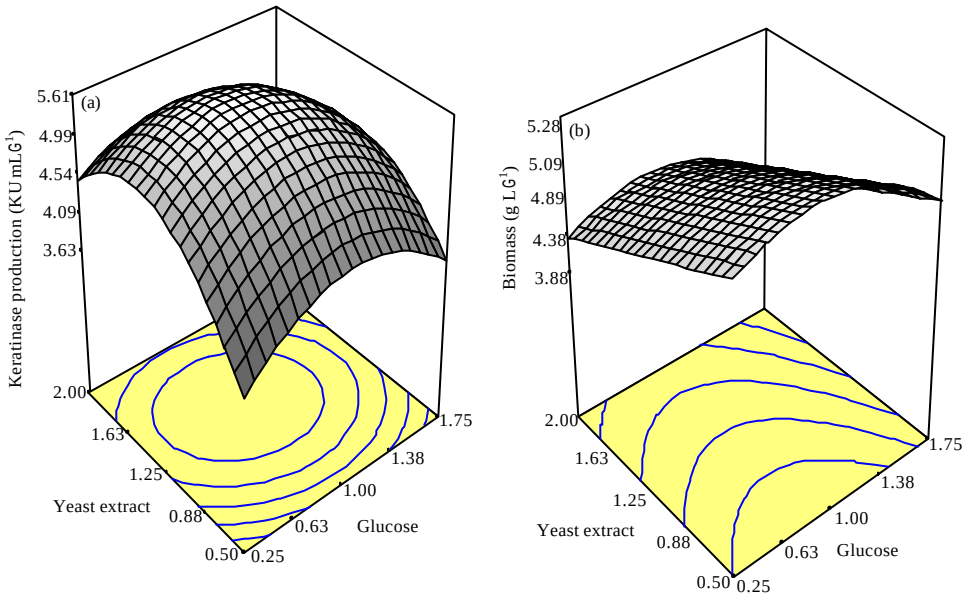

Fig. 1(a-b): 3D plot of (a) production (KU mL^{-1}) of recombinant keratinase rK_{27} by *E. coli* and (b) biomass (g L^{-1}) as a function of glucose and yeast extract after 30 h of growth at 37°C and 300 rpm

production were also desirable corresponding to 10.82 and 9.62, respectively. This indicates a greater reliability of the experiment. Also, lower values corresponding to 3.8 and 5.97 of PRESS for keratinase and biomass production implies that the model fits the data points better. The Adequate Precision obtained was also desirable corresponding to the value of 20.76 and 17.08 indicates as adequate signal and this model can be used to navigate the design space.

One-factor plots, contour and 3D response surface were analyzed to observe the interaction among glucose, yeast extract and incubation time to determine the optimum concentration of each for maximum keratinase production. On the basis of overall predictions derived from 3D, contour and one factor plots of keratinase production (Fig. 1a), glucose and yeast extract at their minimum and maximum level were found inhibitory for enzyme production while keratinase production was a linear function of incubation time upto 30 h. Maximum keratinase production corresponding to 5.6 KU mL^{-1} was produced along with biomass of 5.2 g L^{-} .[1]However, till date no medium optimization has been performed for recombinant keratinase using *E. coli* as heterologous host for proteases/keratinases as they have been reported to be largely expressed as inclusion bodies and resulted in limited enzyme production (Lin *et al*., 2009a). To overcome this problem, attempts were made to express keratinase in *Bacillus* and *Pichia* system. In earlier reports, ker A of *B. licheniformis* PWD-1 was produced in *B. subtilis* maximally with 140 U mL^{-1}, *B. licheniformis* with 35 U mL^{-1} and *P. pastoris* X33 with maximum yield of 285 U mL^{-1} after 72 h (Wang *et al*., 2003, 2004; Porres *et al*., 2002). The constitutive production of keratinases of *B. licheniformis* MKU3 was achieved in *B. megaterium* with highest production of 168.6 U mL^{-1} after 36 h (Radha and Gunasekaran, 2007). Besides, keratinases of *P. aeruginosa* were successfully expressed and produced in *B. subtilis* and *P. pastoris* with 157.5 and 1.03 KU mL^{-1}, respectively after 72 h (Lin *et al*., 2009a, b).

Table 5: Experimental design used in RSM studies carried out using two independent variables in CCD showing keratinase production and biomass of rK_{27} by recombinant *E. coli* HB101

			Keratinase production (KU mL^{-1})		Biomass (g L^{-1})	
Run	A (%)	B (%)	Observed	Predicated	Observed	Predicated
1	-1	+1	4.52	4.53	4.06	4.38
2	0	0	8.10	7.93	5.44	5.67
3	0	+α	5.60	5.56	3.92	3.96
4	0	-α	5.14	5.30	3.23	3.18
5	-α	0	4.91	4.98	5.12	4.92
6	0	0	7.70	7.79	6.63	6.64
7	0	0	7.45	7.30	5.90	5.68
8	+1	-1	5.90	5.71	3.33	3.39
9	0	0	6.95	7.18	6.06	6.07
10	+1	+1	4.45	4.28	4.96	5.02
11	+α	0	5.85	5.72	5.21	5.58
12	0	0	7.10	7.08	6.21	5.85
13	-1	-1	4.98	5.37	5.25	5.02

Production was carried out at the respective combinations at pH 7, 37°C at 300 rpm in triplicate. The keratinase assay was performed at pH 9, 70°C using 20 mg chicken feather as the substrate, where, A: Glycine (%) w/v; B: Triton X-100 (%) v/v

In order to evaluate the dependency of keratinase to the growth, predications derived from 3D, contour and one factor plots of biomass were studied (Fig. 1b) which indicated that maximum biomass corresponded to 5.2 g L^{-1} was obtained after 30 h which remained almost constant with increasing incubation time with slight decline after 48 h of incubation corresponding to 4.8 g L^{-1}. The keratinase production increased linearly with the growth and during late log phase significant enzyme production was achieved.

The model was validated with a set of twelve experiments within the design space (data not shown). The experimentally determined production values were in close agreement with the statistically predicted ones confirming the model's authenticity and are presented in Table 5. Thus, based on the response surface curves and validation experiments, the optimized medium obtained for maximum keratinase production by *E. coli* HB101 was as follows: tryptone 1% w/v, yeast extract 1.2% w/v, sodium chloride 1% w/v and glucose 1% w/v; pH 7 and incubation time of 30 h at 2% v/v inoculum at 37°C, 300 rpm where 5.6 KU mL^{-1} enzyme and 5.2 g L^{-1} biomass could be obtained in extracellular broth.

However, SDS-PAGE analysis revealed that the secretion of enzyme after 30 h of incubation was not complete in extracellular broth of *E. coli* HB101 and part of protein remained in the periplasm (Fig. 2). Therefore, osmotic environment of bacterial cells needs to be changed so as to release maximum protein of interest from periplasm. Constitutive secretion of desired protein was achieved and optimized by using two factorial CCD design for two independent variables, glycine and triton X-100. Both glycine and triton X-100 improves secretion and expression of protein of interest by altering osmotic pressure of outer membrane of bacterial cells as suggested by Jin-bao *et al.* (2007). On regression analysis of the experimental data, the following quadratic equations were obtained for keratinase production (Y_1) and biomass production (Y_2): as a function of glycine (A) and triton X-100 (B):

$$Y_1 = +7.34+1.70*A+0.43*B-1.17*A^2-1.15*B^2-0.13*A*B$$
$$Y_2 = +5.88-0.62*A-0.48*B-1.01*A^2-1.00*B^2+0.23*A*B$$

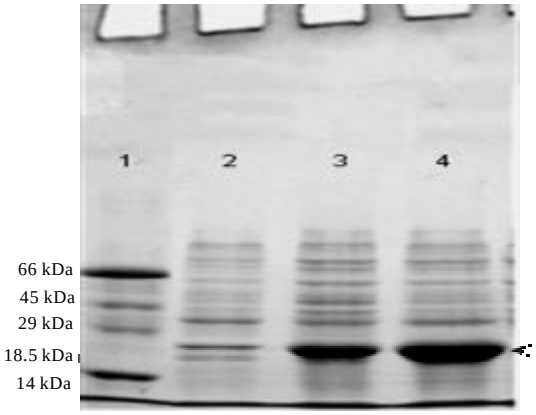

Fig. 2: Expression profile before standardizing the secretion of keratinase rK_{27} by recombinant *E. coli* HB101 in periplasm (PP) and extracellular (EC) fractions after 30 h of incubation at 37°C and 300 rpm. Lane 1: Protein marker; Lane 2: pEZZ18 only; Lane 3: PP; Lane 4: EC

Table 6: ANOVA values of two factorial CCD used for keratinase production and biomass of rK_{27} by recombinant *E. coli* HB101

Term	Keratinase production (KU mL^{-1})	Biomass (g L^{-1})
F-value	16.52	20.19
Standard deviation	0.41	0.43
Mean	4.80	7.080
R^2	0.9468	0.9290
Adjusted R^2	0.9202	0.9199
Predicated R^2	0.9039	0.9178
Coefficient of variance	9.83	6.01
PRESS	2.50	3.72
Adequate precision	13.306	10.968

F-value of 16.52 and 20.19 implies the model is significant

A reduced quadratic model was selected for analysis using statistical software package Design expert® 6.0 (Stat Ease). The model indicated that B, A^2, B^2 were significant for keratinase production, while A^2, B^2 and AB were significant model terms for biomass production. The statistical significance of the second-order polynomial equation was checked by an F-test (ANOVA) and data shown in Table 6. The overall analysis of various terms of polynomial equation indicated that the model selected was significant as described earlier for three factorial CCD.

Two factorial CCD was analyzed using one-factor plots, contour and 3D response surface which helped to determine the optimum concentration of glycine and triton X-100 for constitutive secretion of recombinant protein rK_{27}. 3D, contour and one factor plots of keratinase production predicated that 7.3 KU mL^{-1} was achieved in M_4 medium containing glycine (1% w/v) and triton X-100 (1% w/v) after 30 h at 37°C, 300 rpm (Fig. 3a). Glycine and triton X-100 at 1% concentration disrupted bacterial outer membrane without affecting the integrity of bacterial cell and effectively released the recombinant protein into the medium (Jin-bao *et al.*, 2007). However, glycine and triton X-100 at higher concentration i.e. 2% (w/v; v/v) resulted in declined in cell growth and therefore, decrease in enzyme production along with biomass as observed in Fig. 3b. Overall,

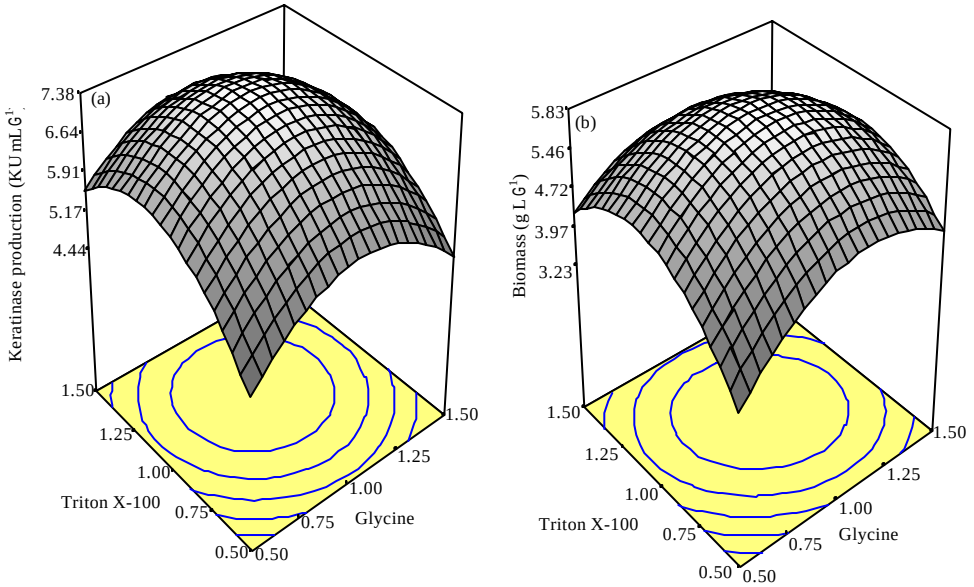

Fig. 3(a-b): 3D plot of (a) enzyme secretion (KU mL^{-1}) of recombinant keratinase rK$_{27}$ by *E. coli* HB101 in extracellular broth and (b) biomass (g L^{-1}) as a function of glycine and triton X-100 after 30 h of growth at 37°C and 300 rpm

1.3-fold enhanced secretion of enzyme in extracellular broth was achieved after 30 h which led to a total of 5.2-fold enhancement compared to the original medium.

Downstream processing of recombinant keratinase rK$_{27}$: Enzyme was concentrated by a molecular cutoff of 10 kDa followed by partial purification using thermal precipitation with an overall recovery of 91% and 5.8 KU mg^{-1} since the enzyme was highly thermostable (Rajput *et al*., 2011). Purity was confirmed by HPLC on C18 column using UV detector, where two peaks were obtained with a single major peak having retention time of 5 min (data not shown).

Enzymatic degradation of Sup35 NM: Sup35 NM is a protein from *Saccharomyces cerevisiae* which belongs to translational termination factor eRF3 family. It has the property to form prion-like proteinase-K resistant amyloid plaques which are insoluble. It is a non-pathogenic protein which has physical and chemical properties very similar to BSE prion and thus mimics prion and being used as a surrogate prion protein to study prion protein degradation (Wang *et al*., 2007). The degradation of prion replicative potential is determined by degradation of peptides N and M. There are reports where it has been demonstrated that microbial keratinases can degrade these proteinase-K resistant aggregates (Chen *et al*., 2005). However, complete degradation required pre-treatment by triton X-100 and SDS and thus till date no keratinase is known which can degrade these native aggregates. Keratinase from *Norcardiopsis* sp. TOA-1 degraded a scrapie prion protein without any pre-treatments (Mitsuiki *et al*., 2006). During present studies, it has been observed that 890 μg enzyme rK$_{27}$ completely degrade 100 μg Sup35 NM as detected by western blot analysis using N antibodies (Fig. 4).

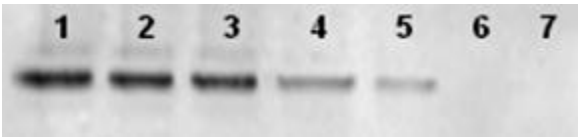

Fig. 4: Western blot analysis of degradation of prion protein Sup35 NM as a function of concentration of keratinase rK_{27} after 12 h at pH 7.0, 37°C and 100 rpm. Lane 1: Sup35 NM only (100 μg); Lane 2: Proteinase-K (500 μg) treated Sup35 NM; Lane 3: Keratinase (120 μg) treated Sup35 NM; Lane 4: Keratinase (380 μg) treated Sup35 NM; Lane 5: Keratinase treated (600 μg) Sup35 NM, Lane 6: Keratinase (890 μg) treated Sup35 NM; Lane 7: Keratinase (1200 μg) treated Sup35 NM

CONCLUSION

The 890 mg of enzyme was found to completely degrade 100 μg of yeast surrogate prion protein, Sup 35 NM at pH 7 and 37°C.

ACKNOWLEDGMENTS

Financial assistance from University of Delhi, Misc. R and D Grant and Department of Biotechnology, New Delhi through project no. BT/PR012505/PID/06/01/2009 is acknowledged.

REFERENCES

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brandelli, A., D.J. Daroit and A. Riffel, 2009. Biochemical features of microbial keratinases and their production and applications. Applied Microbiol. Biotechnol., 85: 1735-1750.

Chen, C.Y., K. Rojanatavorn, A.C. Clark and J.C.H. Shih, 2005. Characterization and enzymatic degradation of Sup35NM, a yeast prion-like protein. Protein Sci., 14: 2228-2235.

Gupta, R. and P. Ramnani, 2006. Microbial keratinases and their prospective applications: An overview. Applied Microbio. Biotechnol., 70: 21-33.

Jin-bao, T., Y. Hong-ming, S. Shu-liang, Z. Peng and J. AI-guo, 2007. Effect of glycine and triton X-100 on secretion and expression of ZZ-EGFP fusion protein. Food Chem., 108: 657-662.

Lin, H.H., L.J. Yin and S.T. Jiang, 2009a. Cloning, expression and purification of *Pseudomonas aeruginosa* keratinase in *Escherichia coli* AD494 (DE3) pLysS expression system. J. Agri. Food Chem., 57: 3506-3511.

Lin, H.H., L.J. Yin and S.T. Jiang, 2009b. Functional expression and characterization of keratinase from *Pseudomonas aeruginosa* in *Pichia pastoris*. J. Agric. Food Chem., 57: 5321-5325.

Mitsuiki, S., M. Takasugi, M. Goto, H. Kanouchi and T. Oka, 2009. Keratinolytic properties of PrPSc degrading enzyme E77. Bulletin Fac. Agri., 59: 37-42.

Mitsuiki, S., Z. Hui, D. Matsumoto, M. Sakai and Y. Moriyama *et al*., 2006. Degradation of PrPSc by keratinolytic protease from *Nocardiopsis* sp. TOA-1. Biosci. Biotechnol. Biochem., 70: 1246-1248.

Mohorcic, M., A. Torkar, J. Friedrich, J. Kristl and S. Murdan, 2007. An investigating into keratinolytic enzymes to enhance ungual drug delivery. Int. J. Pharma., 332: 196-201.

Pillai, P. and G. Archana, 2008. Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. Applied Microbiol. Biotechnol., 78: 643-650.

Porres, J.M., M.J. Bentio and X.G. Lei, 2002. Functional expression of keratinse (kerA) gene from *Bacillus licheniformis* in *Pichia pastoris*. World J. Microbiol. Biotechnol., 18: 563-568.

Prskash, P., S.K. Jayalakshmi and K. Sreeramulu, 2010. Production of keratinase by free and immobilized cells of *Bacillus halodurans* strain PPKS-2: partial characterization and its application in feather degradation and dehairing of the goat skin. Appl. Biochem. Biotechnol., 160: 1909-1920.

Radha, S. and P. Gunasekaran, 2007. Cloning and expression of keratinase gene in *Bacillus megaterium* and optimization of fermentation conditions for the production of keratinase by recombinant strain. J. Appl. Microbiol., 103: 1301-1310.

Rajput, R., R. Sharma and R. Gupta, 2011. Cloning and characterization of a thermostable detergent-compatible recombinant keratinase from *Bacillus pumilus* KS12. Biotechnol. Appl. Biochem., 58: 109-118.

Rao, M.B., A.M. Tanksale, M.S. Ghatge and V.V. Deshpande, 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev., 62: 597-635.

Tatineni, R., K.K. Doddapanem, R.C. Potumarthi, R.N. Vellanki, M.T. Kandathil, N. Kolli and L.N. Mangamoori, 2008. Purification and characterization of an alkaline keratinase from *Streptomyces* sp. Bioresour. Technol., 99: 1596-1602.

Wang, J.J., H.E. Swaisgood and J.C.H. Shih, 2003. Production and characterization of bio-immobilized keratinase in proteolysis and keratinolysis. Enzyme Microbial Technol., 32: 812-819.

Wang, J.J., R. Borwornpinyo and J.C.H. Shih, 2004. Increased production of *Bacillus* keratinase by chromosomal integration of multiple copies of the kerA gene. Biotechnol. Bioengineer., 87: 459-464.

Wang, J.J., R. Borwornpinyo and J.C.H. Shih, 2007. Sup35NM-His6 aggregates: A prion-like protein useful in prion degradation studies. Enzyme Microbial Technol., 40: 976-981.