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ORIGINAL PAPER

Optimizing the biodegradation of two keratinous wastes through a *Bacillus subtilis* recombinant strain using a response surface methodology

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Abstract Statistical optimization of the biodegradation of two keratinous wastes directed by *Bacillus subtilis* recombinant cells was carried out by means of a response surface methodology. A Box–Behnken design was employed to predict the optimal levels of three variables namely, keratin percent, incubation time and inoculum size. Analysis of variance revealed that, only keratin percent had the highest significant effect. Canonical analysis and ridge max analysis were used to get the optimal levels of the three predictors along with the optimum levels of the responses. The optimal sets of predicted and validated levels of the three variables were [7.69% (w/v) feathers, 96.58 h and 1.28% (v/v) inoculum size] and [8% (w/v) feathers, 98.45 h, 3.9% (v/v) inoculum size] to achieve the highest levels of soluble proteins (1.25–1.7 mg/ml) and NH_2-free amino groups (245.82–270.0 μmol leucine/ml), respectively upon using three optimized feathers-based media. These values represented 83.67–100% and 100% adequacy for the models of soluble proteins and NH_2-free amino groups, respectively. While, [8.23% (w/v) sheep wool, 5.52% (v/v) inoculum size and 46.58 h] and [8.33% (w/v) sheep wool, 5.89% (v/v) inoculum size and 63.46 h] were the optimal sets of predicted and validated levels of the above variables to achieve the highest yields of soluble proteins (3.4–4.6 mg/ml) and NH_2-free amino groups (290.9–302.0 μmol leucine/ml), respectively upon using three optimized sheep wool-based media. These values represented 100% adequacy for the models of soluble proteins and NH_2-free amino groups. By the end of the optimization strategy, a fold enhancement (2.14–2.43 and 1.78–2.12) in the levels of released soluble proteins and NH_2-free amino groups, respectively was obtained upon using three optimized feathers-based media. However, a fold enhancement (4.25–5.75 and 2.42–2.5) in the levels of soluble proteins and NH_2-free amino groups, respectively was obtained upon using three optimized sheep wool-based media. Data would encourage pilot scale optimization of the biodegradation of these wastes.

Keywords *Bacillus subtilis* recombinant cells · Keratinous wastes · Response surface methodology · Box–Behnken design · Canonical analysis · Ridge analysis

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Introduction

Keratinous wastes are increasingly accumulating in the environment mainly in the form of feathers, hair and wool generated from various industries. Today, it is also becoming a part of solid waste management and wastage of a protein rich reserve because it is tough to degrade by common proteolytic enzymes. Highly rigid structure rendered by extensive disulfide bonds and cross linkages contributes to this recalcitrant nature. Hence, there is an indispensable and urgent need for developing biotechnological alternatives for recycling of such wastes (Onifade et al.1998; Gousterova et al. 2005; Gupta and Rammani 2006). Despite the recalcitrant nature, keratinous wastes can be efficiently degraded by a variety of bacteria, actinomycetes and fungi due to the elaboration of keratinolytic proteases (Onifade et al.1998). Isolation and purification of several keratinolytic enzymes from keratinolytic microorganisms including bacteria, actinomyctes and a lesser from fungi (Williams et al. 1990; Böckle et al. 1995; Santos et al. 1996; Sangali and Brandelli 2000; Kim et al. 2001; Nam et al. 2002; Bernal et al. 2006; Ionata et al. 2008; Syed et al. 2009; Khardenavis et al. 2009; Cao et al. 2009; Prakash et al. 2010; Xie et al. 2010) have been reported. Moreover, the use of keratinolytic enzymes was reported to be an excellent solution for recycling of these keratinous wastes (Onifade et al. 1998; Grazziotin et al. 2006; Grazziotin et al. 2007; Deivasigamani and Alagappan 2008; Khardenavis et al. 2009).

To maximize the amount of keratinases produced by wild type keratinolytic microorganisms, few attempts have been made to clone keratinase genes from these keratinolytic microorganisms (Lin et al. 1995; Kluskens et al. 2002; Mitsuiki et al. 2004; Radha and Gunasekaran 2008; Lin et al. 2009a; Lin et al. 2009b). In most of the expression studies on keratinases, it was observed that, plasmid-based expression in *E. coli* or *B. subtilis* is unstable. Formation of inclusion bodies and complications in in vitro folding of prokeratinase presented a challenge for expression in *E.coli* and resulted in a limited enzyme yield. Furthermore, *B. subtilis* produces segregational instability during fermentation (Wang et al. 2003; Gupta and Rammani 2006). Successful expression of keratinase encoding genes is still in the stage of infancy.

On the other hand, high expression levels of a keratinolytic serine protease gene from the recombinant *B. subtilis* DB 100 (pS1) and *B. subtilis* DB 100 (p5.2) (Zaghloul et al. 1994; Oulad Haddar et al. 2009) have been reported. A sustained and promising stability of the plasmid 5.2 was observed in cultures growing on keratin-based medium (unpublished data). Keratin biodegradation by the *B. subtilis* DB 100 (p5.2) recombinant cells accompanied with production of considerable levels of soluble proteins and NH_2-free amino groups, in feathers (Oulad Haddar et al. 2009) and sheep wool (unpublished data) hydrolysates, were reported.

Successful expression of the cloned *apr E* gene in *B. subtilis* cells, sustained plasmid stability and high degradation capability of the above recombinant bacterial cells greatly addressed the indispensable need for a step wise optimization of keratin biodegradation. Moreover, each strain has its own peculiar physicochemical and nutritional requirements and no uniform defined medium for keratin biodegradation was reported till now. As a matter of fact, process optimization is a topic of central importance in biotechnological processes, in which even small improvements can be decisive for commercial success. In general, the first step in the agenda of process optimization is carried out by the traditional method using one variable at a time approach, OVAT (Gokhade et al. 1991). This method involves varying one variable while keeping the others constant. It is not only laborious and time consuming but also often leads to an incomplete understanding of the system behavior, resulting in confusion and lack of predictive values (Kalil et al. 2000; Gheshlaghi et al. 2005; Jain and Nian-fa 2007). The second step in the optimization agenda is concerned with the determination of the exact level of each key determinant. This step is carried out by using a response surface methodology (RSM) approach. RSM is a powerful and efficient mathematical approach widely applied in the optimization of fermentation processes, e.g. medium components for enzyme production (Adinarayana and Ellaiah 2002; Bernal et al. 2006; Anbu et al. 2007; Cai and Zheng 2009; Sharma et al. 2009), production of other metabolites (Zhang et al. 1996) and biomass production (Lohomme and Roux 1991; Rucka et al. 1998). The method can give

information about the interaction between variables, provide information necessary for design and process optimization, reduce the number of experiments, and give multiple responses at the same time (Box and Behnken 1960; Chang et al. 2002; Jain and Nian-fa 2007). Three variables namely keratin percent, incubation time and inoculum size involved in keratin biodegradation by the *B. subtilis* DB 100 (p5.2) recombinant cells were highlighted through an OVAT approach as a first step in the optimization strategy (unpublished data).

Present study reports the second step involved in the optimization of keratin biodegradation by applying a response surface methodology approach. The aim is extended to validate the predicted results of the model (the last step involved in the optimization strategy). To the best of our knowledge, this work is the first attempt to apply statistical optimization of keratin biodegradation particularly sheep wool waste via a keratin-degrading *B. subtilis* recombinant strain.

Materials and methods

Chemical and reagents

Yeast extract, peptone and bacteriological agar used in this study were Oxoid type. Ninhydrin, hydrindantin, Coomassiae brilliant blue G250 (CBB G250), leucine and methylcellosolve were purchased from Sigma. Bovine serum albumin was obtained from Winlab.

Bacterial strain and plasmid

Bacillus subtilis DB100 *his*$^-$ *met*$^-$ (p5.2) was used in this study to direct the process of keratin biodegradation. The p5.2 plasmid (4.7 kbp) is a pUB118 derivative plasmid, where the expression of the alkaline protease *aprE* gene (750 bp) is controlled by an early promoter P^{43}. The plasmid (p5.2) carries a kanamycin resistance gene as well (unpublished data).

Media

The bacterial strain was activated and grown on PY medium (Bernhardt et al. 1978) (Bactopeptone, 10 g; yeast extract, 5 g; and NaCl, 5 g, per liter, pH 7.0) and PA medium (PY medium supplemented with 1.5% agar). Three production media were used, keratin-based modified basal medium II (0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4 and 0.1 g $MgCl_2$ per liter), keratin-based distilled water medium and keratin-based tap water medium.

Keratinous wastes

Two keratinous wastes namely sheep wool waste and chicken feather waste were used in this study. Both wastes were washed with tap water then with distilled water. The keratinous wastes were dried at 60°C and were cut into small particles.

Determination of NH_2-free amino groups and soluble proteins

NH_2-free amino groups resulted from keratin biodegradation were determined using ninhydrin as described earlier (Pearce et al. 1988). A standard curve for leucine was established. Moreover, soluble proteins were determined as described earlier (Bradford 1976) and a standard curve using bovine serum albumin (BSA) was established.

Activation of the *B. subtilis* recombinant cells

Single fresh colonies of the *B. subtilis* recombinant cells were used to inoculate 50 ml of PY medium. The inoculated medium was incubated at 37°C for 2.5 h with agitation at 200 rpm. Three milliliters (3 ml) of the growing culture, unless otherwise stated, was centrifuged at 7,000 rpm for 3 min using a microcentrifuge. The bacterial pellet was washed with saline [0.85% (w/v) NaCl] and was used to inoculate 100 ml of keratin-based media. Each ml of the activated culture contained 3.0 $x10^8$ colony forming units (CFU).

Monitoring of the levels of NH_2-free amino groups and soluble proteins

All optimization experiments using response surface methodology were carried out in 250 ml Erlenmeyer flasks containing 100 ml of modified basal medium II supplemented with keratin. Inoculated keratin-based media were incubated at 37°C, for a maximum incubation period of 5 days, unless otherwise stated, with agitation at 150 rpm. One ml samples were

withdrawn from the cultures at the indicated times and were centrifuged at 7,000 rpm for 3 min in a microcentrifuge. The supernatants were used to determine the levels of NH_2-free amino groups and soluble proteins as mentioned above.

Box–Behnken design

Three independent variables involved in keratin biodegradation were highlighted tentatively in a previous study (unpublished data). These three key determinants were keratin percent, incubation time and inoculum size. In this study, a Box–Behnken design which is a response surface methodology, was applied to determine the optimal level of each key determinant. This model included the variables keratin percent, incubation time and inoculum size. Each variable was studied at three different levels, designated as −1 (low), 0 (middle) and +1 (high) (Table 1). Thirteen experiments (trials) have been conducted and their responses were fitted to the following second polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{33} X_3 X_3 \quad (1)$$

where Y is the dependent variable (NH_2-free amino groups or soluble proteins here in this study), X_1, X_2 and X_3 are the independent variables (keratin percent, incubation time, and inoculum size in this study). β_o is the regression co-efficient at the center point, β_1, β_2 and β_3 are linear co-efficients, β_{12}, β_{13} and β_{23} are second interaction co-efficients and β_{11}, β_{22} and β_{33} are quadratic co-efficients. For statistical calculations, each independent variable X was coded as X_i according to the Eq. 2.

$$X_i = (x_i - x_o)/\Delta x_i \quad (2)$$

where X_i is dimensional coded value of the independent variable, x_i is the real value of this variable at this coded value, x_o is the real value of this variable at the center point (zero level) and Δx_i is the step change value. The range of the independent variables, keratin percent, incubation time and inoculum size (Table 1) was chosen from a set of experiments carried out previously using one variable at a time approach (unpublished data). An estimate of a main effect was obtained by evaluating the difference in process performance caused by a change from the low (−1) level to high (+1) level of the corresponding factor. The process performance was determined by measuring the level of released end products; NH_2-free amino groups and soluble proteins.

Statistical, canonical, ridge analyses and contour surface plots

RSM package (R Development Core Team 2009), available from the Comprehensive R Archive Network at http://CRAN.R-project.org/package=rsm,

Table 1 Real values of variables chosen for the Box–Behnken design of feathers and sheep wool biodegradation optimization experiments

Independent variable	Variable symbol	Code levels		
		−1	0	+1
Feathers biodegradation system				
Chicken Feather (g/100 ml)	X_1	2	5	8
Incubation time (h)	X_2	24	72	120
Inoculum size (ml/100 ml medium)[a]	X_3	1	5	9
Sheep wool biodegradation system				
Sheep wool (gm/100 ml)	X_1	1.7	5	8.3
Inoculum size (ml/100 ml medium)[a]	X_2	0.96	6	11.04
Incubation time (h)	X_3	24	48	72

$X_1 = (x_1 - 5)/3$, $X_2 = (x_2 - 72)/48$ and $X_3 = (x_3 - 5)/4$ for feathers biodegradation system

$X_1 = (x_1 - 5)/3.3$, $X_2 = (x_2 - 6)/5.04$ and $X_3 = (x_3 - 48)/24$ for sheep wool biodegradation system

[a] Each ml of the inoculum contained 3×10^8 CFU

Table 2 Experimental data of the Box–Behnken design for feathers and sheep wool biodegradation experiments

Trials[a]	Independent variables			Dependent variables			
	X_1	X_2	X_3	NH_2-free amino groups (μmol leucine/ml)		Soluble proteins (mg/ml)	
				Feathers Y_1 response	Sheep wool Y_3 response	Feathers Y_2 response	Sheep wool Y_4 response
1	1	1	0	240	252.36	1.25	3.24
2	1	−1	0	105	250.18	0.336	3.8
3	−1	1	0	61.09	41.51	0.208	0.61
4	−1	−1	0	30.84	42.5	0.085	0.64
5	1	0	1	201.21	308.5	1.39	2.85
6	1	0	−1	218.18	187.63	1.49	3.44
7	−1	0	1	48.48	57.94	0.233	0.69
8	−1	0	−1	83.64	43.64	0.466	0.625
9	0	1	1	113.63	181.45	0.8	1.37
10	0	1	−1	170.36	125.09	1.09	1.28
11	0	−1	1	106.36	194.55	0.56	1.625
12	0	−1	−1	80.00	142.18	1.15	1.24
13	0	0	0	141.82	169.45	1.05	1.34

[a] Each trail means a combination of sheep wool, inoculum size and incubation time. The coded levels (−1, 0 and +1) of the independent variables (X_1, X_2 and X_3) have corresponding real values illustrated in Table 1

was used in this study to carry out design generation, multiple regression, canonical, ridge analyses and graphing of three dimensional contour surface plots.

Verification of the experimental model

Predicted values of the three independent variables and the response variables derived from steepest ascent of ridge analysis and those derived from canonical path were verified in the laboratory.

Results and discussion

Model fitting and analysis of variance

Response surface methodology is an empirical model technique used to evaluate the relationship between a set of controllable experimental factors and derived results. Here, Box–Behnken design experiments were used with independent variables to obtain the combinations of values that optimize the responses with the region of three dimensional observation spaces. The input independent variables that have the maximal influence on the final responses of the system were identified from one variable at a time approach previously reported (unpublished data). The input variables were keratin percent, incubation time and inoculum size. The design was applied with 13 different combinations, shown in Table 2 together with the experimental results of the levels of NH_2-free amino groups (Y_1 & Y_3) and soluble proteins (Y_2 & Y_4) for feathers and sheep wool biodegradation systems, respectively. The experimental results of the two Box–Behnken designs were fitted to the following second polynomial equations:

Feathers biodegradation system. Equation 3 describes NH_2-free amino groups model

$$\begin{aligned} Y_1 =& 141.82 + 67.54X_1 + 32.86X_2 - 10.31X_3 \\ & - 6.15X_1X_1 - 26.44X_2X_2 - 2.21X_3X_3 \\ & + 26.19X_1X_2 + 4.55X_1X_3 - 20.78X_2X_3 \end{aligned} \quad (3)$$

Equation 4 describes soluble proteins model

$$\begin{aligned} Y_2 =& 1.05 + 0.434X_1 + 0.152X_2 - 0.152X_3 \\ & - 0.29X_1X_1 - 0.29X_2X_2 + 0.14X_3X_3 \\ & + 0.19X_1X_2 + 0.03X_1X_3 + 0.08X_2X_3 \end{aligned} \quad (4)$$

Sheep wool biodegradation system. Equation 5 describes NH_2-free amino groups model

$$Y_3 = 169.45 + 101.63X_1 - 3.631X_2 + 30.49X_3 - 17.1X_1X_1 - 5.71X_2X_2 - 2.92X_3X_3 + 0.793X_1X_2 + 26.64X_1X_3 + 0.99X_2X_3 \quad (5)$$

Equation 6 describes soluble proteins model

$$Y_4 = 1.34 + 1.35X_1 - 0.103X_2 - 0.0063X_3 + 0.628X_1X_1 + 0.105X_2X_2 - 0.066X_3X_3 - 0.133X_1X_2 - 0.164X_1X_3 - 0.074X_2X_3 \quad (6)$$

These equations included all model terms regardless of their significance.

The fit of the two models was checked by the co-efficient determination R^2, which was calculated to be 0.99 for both NH_2-free amino groups and soluble proteins models in case of sheep wool biodegradation system. These R^2 values of sheep wool biodegradation system indicated that only 1% of the variability in the response could not be explained by each model. While, R^2 co-efficient determination was calculated to be 0.984 and 0.919 for NH_2-free amino groups and soluble proteins models, respectively in case of feathers biodegradation system. These R^2 values of feather biodegradation system indicated that 1.6% and 8.1% of the variability in the response could not be explained by the models for NH_2-free amino groups and soluble proteins, respectively. Generally, the closer the value of R^2 to 1.0 means the great fitness of the model.

Another correlation measure to check the fit of the models is determination of the multiple correlation co-efficient R. Generally, the closer the value of R to 1.0 is the better the correlation between the experimental and the predicted values. In these experiments, the values of R were 0.993 and 0.995 for NH_2-free amino groups and soluble proteins models, respectively in case of sheep wool biodegradation system. These values indicated high degree of correlation between the experimental and the predicted values. Similarly, the values of R were 0.99 and 0.96 for NH_2-free amino groups and soluble proteins models, respectively in case of feathers biodegradation system. Again, these values indicated high degree of correlation between the experimental and the predicted values.

The analysis of variance was performed to check the statistical significance of the quadratic regression models. Analysis of variance demonstrated that, all the four above mentioned quadratic regression models were highly significant models, as evident from Fischer's F-tests with high values (Table 3). Values of Fischer's F-tests for all the above mentioned quadratic regression models showed values (22.59 and 110.7) for feathers and sheep wool NH_2-free amino groups models, respectively and (3.9 and 23.63) for feathers and sheep wool soluble proteins models, respectively greater than F-tabulated. Again, this demonstrated the high significance of the regression models.

For each model term, the t-statistic value and the individual significance probability (*prob*) of t-value greater than t are shown in Tables 4 and 5. Generally, significance of the coefficients has been reported to be directly proportional to t-test and inversely to P-value (Douglas 2001; Heck et al. 2005). Our data revealed that, in the four above models, keratin percent (sheep wool percent and feathers percent) had the highest significance. The P-values 0.000084 and 0.00081 of sheep wool percent had the highest significant effect on the yields of NH_2-free amino groups and soluble proteins, respectively. Moreover, sheep wool exhibited a less significant effect on the yield of soluble proteins in a quadratic form. Similarly, 0.0013 and 0.021, the P-values of feathers percent, showed the highest significant effect on the yield of NH_2-free amino groups and soluble proteins, respectively. However, the variable inoculum size did not display any significant effect on the yield of soluble proteins and NH_2-free amino groups in both biodegradation systems. It seemed that, a threshold level of inoculum size was reached by using a low level of inoculum size (i.e., $-1 \leq$ inoculum level < 0). Beyond this level, cells competition would occur resulting in lack of both oxygen supply and nutritional requirements. Thus, only a limited number of cells could grow efficiently, while others could not even there is a considerable increase in the initial level of added inoculum size.

Dissimilarly, the variable incubation time displayed a little bit significance effect (P-value = 0.003 and 0.0103) in a linear manner only on the yield of NH_2-free amino groups released from sheep wool and feathers biodegradation, respectively. Moreover, the two variables feather percent and the incubation time showed a little bit interaction significant effect (P-value = 0.047) on the yield of NH_2-free amino groups. Conversely, the variable incubation time did not display any significance

Table 3 Analysis of variance (ANOVA) of regression models for feathers and sheep wool biodegradation experiments

	Feathers biodegradation system									
	Soluble proteins[a]					NH_2-free amino groups[b]				
	Sums squares	df	Means squares	*F*	*P*-level	Sums squares	df	Means squares	*F*	*P*-level
Regression	2.6267	9	0.29186	3.9	0.148	52580.36	9	5842.262	22.59	0.013
Residual	0.23010	3	0.07670			848.08	3	282.694		
Total	2.85887					53428.44				
	Sheep wool biodegradation system									
	Soluble proteins[c]					NH_2-free amino groups[d]				
	Sums squares	df	Means squares	*F*	*P*-level	Sums squares	df	Means squares	*F*	*P*-level
Regression	15.99	9	1.777016	23.63	0.0124	93747.33	9	10416.37	110.7	0.0013
Residual	0.22561	3	0.075202			282.35	3	94.12		
Total	16.219					94029.68				

[a] R^2 equals 0.92, [b] R^2 equals 0.985, [c] R^2 equals 0.99 and [d] R^2 equals 0.99

Table 4 Regression summary for the full polynomial equation for sheep wool biodegradation experiment

Coefficient symbol	Soluble proteins				NH_2-free amino groups			
	Estimate	*t*-value	*P*-value	Confidence level%	Estimate	*t*-value	*P*-value	Confidence level%
β_o	1.34	4.89	0.0164	98.36	169.45	17.47	0.00041	99.96
β_1	1.35	13.88	0.00081	99.92	101.63	29.63	0.000084	99.99
β_2	−0.1	−1.038	0.3756		−3.63	−1.01	0.368	
β_3	−0.0063	−0.065	0.9527		30.49	8.89	0.003	99.70
β_{11}	0.628	3.46	0.041	95.90	−17.1	−2.66	0.076	
β_{22}	0.105	0.579	0.603		−5.71	−0.89	0.439	
β_{33}	−0.066	−0.37	0.739		−2.92	−0.46	0.6719	
β_{12}	−0.133	−0.966	0.405		0.79	0.163	0.881	
β_{13}	−0.164	−1.19	0.318		26.64	5.49	0.0119	98.81
β_{23}	−0.074	−0.538	0.628		0.99	0.21	0.85	

Underlined model terms are significant ones

effect on the yield of soluble proteins released from both biodegradation systems. As a matter of fact, on the first days of incubation period all keratin bulk is hydrolyzed to a great bulk of soluble proteins and a minor bulk of NH_2-free amino groups. Reaching a soluble proteins peak is followed by a decline in soluble proteins peak and ascending of the NH_2-free amino groups peak. By extending the incubation time, the already released soluble proteins will be further hydrolyzed to NH_2-free amino groups. In other words, the longer the incubation time the highest levels of NH_2-free amino groups will be obtained. The consequential manner at which keratin hydrolysis assumingly occurring could greatly explain the reason behind the slight significant effect of the incubation time on the yield of released NH_2-free amino groups.

Attaining optimized conditions

The optimum conditions for feather and sheep wool biodegradation were determined by canonical and

Table 5 Regression summary for the full polynomial equation for feathers biodegradation experiment

Coefficient symbol	Soluble proteins				NH_2-free amino groups			
	Estimate	*t*-value	*P*-value	Confidence level%	Estimate	*t*-value	*P*-value	Confidence level%
β_o	1.05	3.81	0.032	96.8	141.82	8.41	0.0031	99.69
β_1	0.434	4.46	0.021	97.9	67.54	11.87	0.0013	99.87
β_2	0.152	1.56	0.216		32.86	5.78	0.0103	98.97
β_3	−0.152	−1.56	0.217		−10.31	−1.81	0.1676	
β_{11}	−0.290	−1.61	0.217		−6.15	−0.58	0.604	
β_{22}	−0.290	−1.58	0.213		−26.44	−2.48	0.0889	
β_{33}	0.140	0.76	0.515		2.21	0.21	0.8491	
β_{12}	0.200	1.43	0.247		26.19	3.25	0.047	95.3
β_{13}	0.030	0.42	0.825		4.55	0.56	0.612	
β_{23}	0.080	0.54	0.624		−20.78	−2.58	0.082	

Underlined model terms are significant ones

ridge analyses. The canonical analysis can be used to examine the overall shape of the response surface and determine whether the estimated stationary point is a maximum, a minimum, or a saddle point. The eigenvalues and eigenvectors in the matrix of second order parameters characterize the shape of the response surface. The eigenvectors point in the directions of principle orientation for the surface, and the signs and magnitudes of the associated eigenvalues give the shape of the surface in these directions. Positive eigenvalues indicate directions of upward curvature, and negative eigenvalues indicate directions of downward curvature. The larger an eigenvalue is in absolute value, the more pronounced is the curvature of the response surface in the associated direction (Myers 1976). The stationary points of the responses Y_1 and Y_3 were [(37.569) X_1, (11.764) X_2, (18.999) X_3] and [(−2.665) X_1, (−1.181) X_2, (−7.181) X_3], respectively. These two stationary points are clearly outside the explored domain, and any prediction at these points is unreliable. The predicted yields Y_1 and Y_3 at these stationary points were 1506.5 and −75, respectively. The eigen values for Y_1 model were ($\lambda_1 = 6.0534$, $\lambda_2 = -0.749$, $\lambda_3 = -35.686$) and that of Y_3 model were ($\lambda_1 = 5.155$, $\lambda_2 = -5.595$, $\lambda_3 = -25.035$). These eigen values for both models Y_1 and Y_3 have mixed signs so the stationary points are saddle points in both of them. It makes sense to use ridge analysis in both cases since, these two stationary points were some distance away (i.e., outside the explored domain). The method of ridge analysis computes the estimated ridge of optimum response at a point among all predictor combinations at radius d with increasing radii from the center of the original design (Hoerl 1959; Draper 1963). On the other hand, the stationary points of the responses Y_2 and Y_4 were [(0.97) X_1, (0.63) X_2, (0.26) X_3] and [(−0.92) X_1, (0.24) X_2, (0.97) X_3], respectively. These two stationary points are clearly inside the explored domain. The predicted yields Y_2 and Y_4 at these stationary points were 1.29 and 0.7, respectively. Eigen values of both models Y_2 and Y_4 were ($\lambda_1 = 0.142$, $\lambda_2 = -0.19$, $\lambda_3 = -0.39$) and ($\lambda_1 = 0.65$, $\lambda_2 = 0.11$, $\lambda_3 = -0.09$), respectively. These eigen values with mixed signs showed that both stationary points were saddle ones (no maximum or minimum). Additionally, the largest eigen value in the four models Y_1, Y_2 Y_3 and Y_4 were 6.0534, 5.155, 0.142 and 0.65, respectively conferred pronounced curvature in the directions of the variable (X_1) in the four models. This greatly confirms the results of regression analysis stated above that the variable (X_1) exhibited the highest significant effect in the four models. However, it is clear that, the stationary point of the models Y_2 and Y_4 is to some extent nearby, so it makes sense to start at the saddle point (rather than the origin) and follow the most steeply rising ridge in both directions. This path is obtained using the canonical path function. In this function, distance is a signed quantity, according to the direction along the ridge. Tables 6 and 7 display the results obtained

from the canonical path of canonical analysis and the steepest ascent path of ridge analysis to locate the points that get optimum responses in the four aforementioned models. Eigen values of Y_2 and Y_4 are small and greatly near from zero, consequently there is flexibility in choosing operational conditions regarding the cost effectiveness issue. For example, for selection of the optimum response (Y_2) through the canonical path (Table 6), there were two combination sets of the predictors giving the same response at two different distances at two opposite directions from the stationary saddle point. These two combination sets were (7.69% feather, 96.58 h, 1.28% inoculums size) and (8.1% feather, 108.14 h, 10.8% inoculum size) at two distances −1.2 and +1.2, respectively. Regarding cost reduction of the process, the combination (7.69% feather, 96.58 h, 1.28% inoculums size) was selected to be verified experimentally. As a matter of fact, any residual undegraded feather or sheep wool will elevate the cost involved in downstream processes upon scaling up the process of biodegradation of these keratinous wastes. Additionally, extensive incubation times without gaining considerable amounts in the yield of the response will contribute greatly in elevation of the overall cost of the process. In the light of cost effectiveness issue, the cost involved in inoculum size preparation must be reduced as much as possible to use the lowest inoculum size that would efficiently direct the process.

Conversely, upon using the canonical path to get the maximum response Y_4, the combination set (8.5% sheep wool, 6.04% inoculum size and 65.95 h) was obtained at distance +2 from the stationary point. The level of the variable ($X_1 = 8.5\%$) was outside the experimental domain (Data not shown). Upon using the ridge analysis as shown in Table 7, the combination set (8.3% sheep wool, 5.5% inoculum size, 46.58 h) was obtained at distance +1 from the center. These two combination sets had the same predicted optimal response soluble proteins (3.3 mg/ml). Regarding cost effectiveness and time saving issues mentioned above, the combination (8.3% sheep wool, 5.5% inoculum size, 46.58 h) was selected to be verified experimentally.

Concerning the response NH_2-free amino groups in Y_1 and Y_3, data derived from the ridge analysis in Tables 6 and 7 revealed that, there is a rising ridge in both cases. In other words, by increasing the level of the highly significant variable (X_1) in both cases, a corresponding elevation in the response was achieved in both cases along the path without reaching to a

Table 6 Estimated ridge of maximum response Y_1 and Y_2

Distance	Real values of independent variables			Estimated response[a] Y_1	Distance	Real values of independent variables			Estimated response[b] Y_2
	X_1	X_2	X_3			X_1	X_2	X_3	
0.0	5.000	72.000	5.000	141.82	−1.6	7.620	94.61	−0.308	1.650
0.1	5.267	74.112	4.944	149.432	−1.4	7.660	95.57	0.484	1.570
0.2	5.531	76.224	4.880	157.019	−1.2	7.691	96.576	1.280	1.494
0.3	5.795	78.384	7.812	164.675	−1.0	7.727	97.536	2.076	1.431
0.4	6.056	80.544	4.736	172.314	−0.8	7.763	98.496	2.868	1.380
0.5	6.317	82.704	4.650	180.006	−0.6	7.802	99.456	3.664	1.340
0.6	6.575	84.912	4.564	187.709	−0.4	7.838	100.416	4.456	1.312
0.7	6.833	87.120	4.472	195.457	−0.2	7.870	101.376	5.252	1.295
0.8	7.088	89.376	4.368	203.252	0.0	7.910	102.336	6.048	1.289
0.9	7.340	91.632	4.260	211.032	0.2	7.946	103.296	6.840	1.295
1.0	7.592	93.888	4.144	218.885	0.4	7.820	104.304	7.636	1.312
1.1	7.841	96.144	4.024	226.728	0.6	8.021	105.624	8.428	1.34
1.2	8.087	98.448	3.892	234.638	0.8	8.057	106.224	9.224	1.380
1.3	8.330	100.75	3.760	242.610	1.0	8.093	107.184	10.02	1.431
1.4	8.576	103.056	3.612	250.610	1.2	8.129	108.144	10.812	1.494

[a] Estimated maximum response by using steepest ascent path of ridge analysis

[b] Estimated maximum response by using canonical path (dist = seq (1.2, −1.6 by 0.2)) of canonical analysis

Table 7 Estimated Ridge of steepest ascent path for maximum responses Y_3 and Y_3 using ridge analysis

Distance	Real values of independent variables			Estimated response Y_3	Distance	Real values of independent variables			Estimated response Y_4
	X_1	X_2	X_3			X_1	X_2	X_3	
0.0	5.000	6.000	48.000	169.312	0.0	5.000	6.000	48.00	1.339
0.1	5.314	5.985	48.744	179.848	0.1	5.330	5.959	47.97	1.481
0.2	5.620	5.969	49.632	190.244	0.2	5.656	5.919	47.88	1.634
0.3	5.920	5.955	50.616	200.48	0.3	5.987	5.870	47.78	1.801
0.4	6.214	5.939	51.740	210.673	0.4	6.313	5.828	47.66	1.980
0.5	6.501	5.929	53.940	220.744	0.5	6.643	5.778	47.49	2.174
0.6	6.785	5.919	54.240	230.857	0.6	6.970	5.733	47.35	2.379
0.7	7.059	5.909	55.608	10.818	0.7	7.296	5.682	47.16	2.596
0.8	7.326	5.904	57.070	250.788	0.8	7.626	5.632	46.99	2.829
0.9	7.587	5.899	58.580	260.700	0.9	7.953	5.576	46.80	3.073
1.0	7.845	5.890	60.168	270.708	1.0	8.28	5.526	46.58	3.329
1.1	8.095	5.894	61.800	280.69					
1.2	8.339	5.894	63.460	290.609					
1.3	8.581	5.894	65.160	300.613					
1.4	8.820	5.890	66.910	310.710					

stationary point (threshold level). At a distance 1.2 in both cases, the upper level of the highly significant variable (X_1) was located. By moving to further distances (1.3 and 1.4) along the path and outside the domain, predicted levels of the response NH_2-free amino groups still increased. To get the optimal level of the response of Y_1 and Y_3, the three combination sets of the predictors at the three distances 1.2, 1.3 and 1.4 in Tables 6 and 7 were selected to be verified experimentally in both cases.

To further explore the nature of the response surface at the stationary point and to evaluate where optimum conditions exist within the experimental area covered or in what way further experiments are necessary to achieve better results, three dimensional contour plots were created. Figures (1, 2, 3, 4, 5, 6, 7, 8) display the contour surface plots for the aforementioned responses Y_1, Y_2, Y_3 and Y_4. The contour surface plots shown in Figures (1, 2, 3, 4, 5, 6, 7, 8) were based on the model, holding one variable constant at its optimal level where varying the other two variables within their experimental range. Figures 1 and 3 illustrated the response surface NH_2-free amino groups and soluble proteins for the optimal level of inoculum size. The maximal predicted levels of NH_2 -free amino groups ($\approx$290 μ mol leucine/ml) and soluble proteins (3.3 mg/ml) were obtained at higher levels of sheep wool (8.3%) along with slightly higher and moderate levels of incubation time, respectively. However, the response surface NH_2-free amino groups and soluble proteins for the optimal level of incubation time revealed that, sheep wool had the highest significant effect on both responses and inoculum size had no significant effect (Figs. 2 and 4).

Regarding surface contour plots in Figs. 5 and 7 the highest levels of the responses NH_2-free amino groups and soluble proteins were obtained when feathers levels are at highest (8%) or near highest levels (7.69%) along with moderate levels of the incubation time. Conversely, Figs. 6 and 8 exhibited dissimilar pattern. Optimal levels of the responses NH_2-free amino groups and soluble proteins were obtained upon using highest levels of feather and the inoculum size had no significant effect. The results derived from regression analysis, contour plots, ridge analysis and canonical analysis were greatly consistent with each others.

The present data were compared with others previously reported in the literature. The present finding concerning the high significance effect of feather percent on the yield of end products is in disagreement with that of a report stated that, by increasing feather percent from (2.0–6.0% (w/v) in the growth medium of *B. pumilus* FH 9 the percent of feather hydrolysis was decreased (El-Refai et al. 2005). On the other hand, our finding was in agreement with others stated that, by increasing

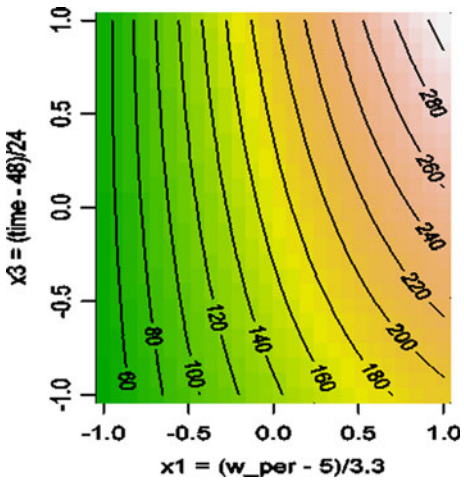

Fig. 1 Contour surface plot of the dependent variable NH_2-free amino groups versus the independent variables; sheep wool and incubation time

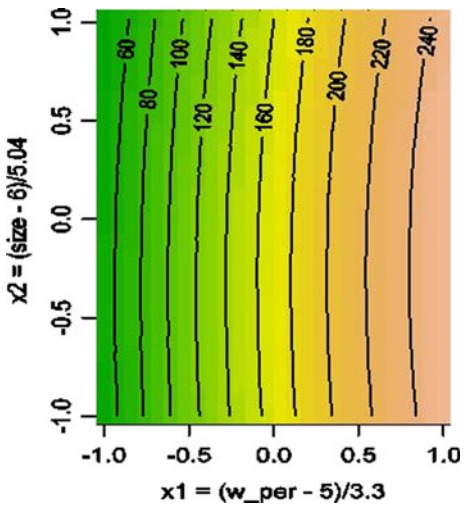

Fig. 2 Contour surface plot of the dependent variable NH_2-free amino groups versus the independent variables; sheep wool and inoculum size

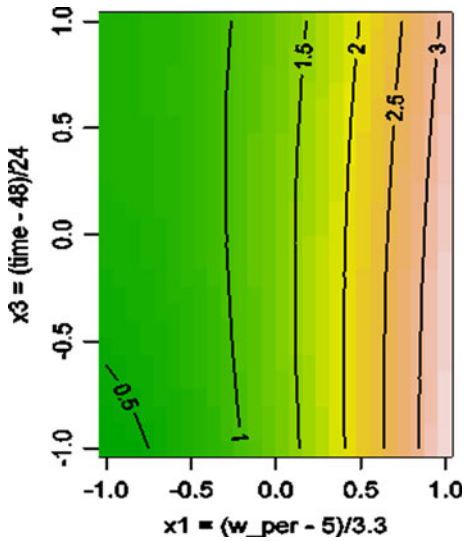

Fig. 3 Contour surface plot the dependent variable soluble proteins versus the independent variables; sheep wool and incubation time

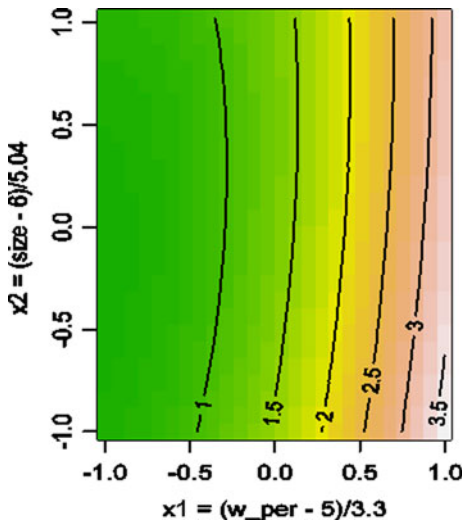

Fig. 4 Contour surface plot of the dependent variable soluble proteins versus the independent variables; sheep wool and inoculum size

feather percent from 1–10% (w/v) in the growth media of two keratinolytic bacteria *Vibrio* sp. Kr2 and mutant strain of a *B.subtilis*, the percent of feather hydrolysis was increased (Grazziotin et al. 2007; Cai et al. 2008). Concerning the insignificant effect of inoculum size, our finding is in partial agreement with that of other report stated that by increasing the inoculum ratio from 2–10% there was no increase in the percent of feather hydrolysis (Cai et al. 2008). However, this comparison may be not justified yet unless the number of colony forming units in both cases is quite similar. Conversely, our results concerning the optimal level of incubation time is in discordance with that of other report stated that, complete feather hydrolysis directed by *B. pumilus* FH 9 was achieved after 84 h of incubation (El-Refai et al. 2005).

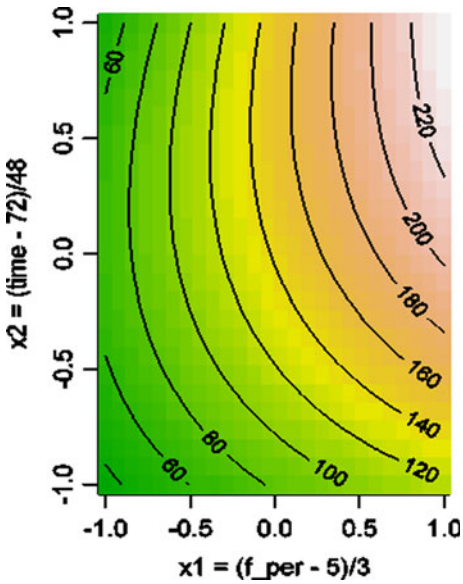

Fig. 5 Contour surface plot of the dependent variables NH_2-free amino groups versus the independent variables; feathers and incubation time

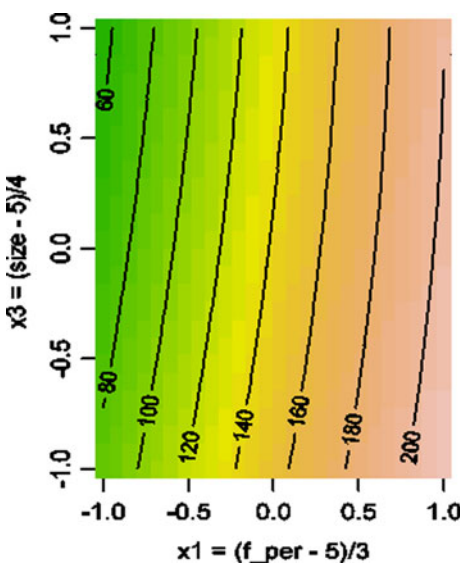

Fig. 6 Contour surface plot of the dependent variable NH_2-free amino groups versus the independent variables; feathers and inoculum size

Experiments carried out using the predicted optimal conditions

In order to determine the accuracy of the models, two experimental sets were carried out for the two biodegradation systems under predicted optimized

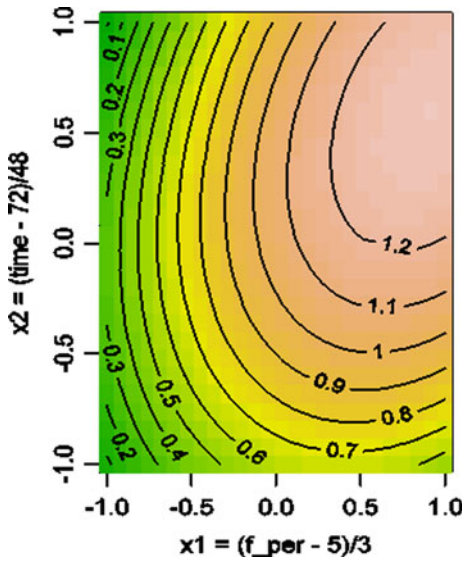

Fig. 7 Contour surface plot the dependent variable soluble proteins versus the independent variables; feathers and incubation time

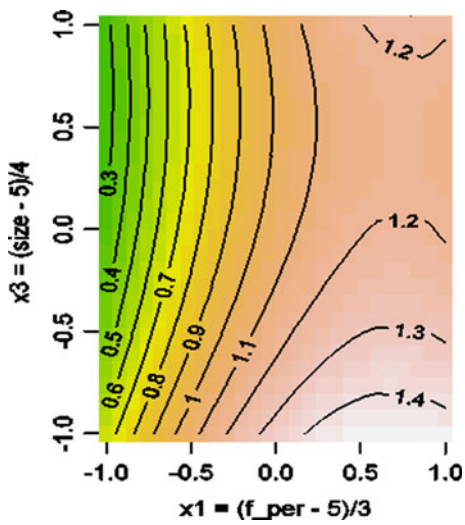

Fig. 8 Contour surface plot of the dependent variable soluble proteins versus the independent variables; feathers and inoculum size

conditions derived from contour plots, canonical and ridge analyses. The experiments were run in triplicates. For each set of experiments, three keratin-based media recommended by a previous study were used (unpublished data), keratin-based modified basal medium II, keratin-based distilled water medium and keratin-based tap water medium. The verified levels of NH_2-free amino groups and soluble proteins along

Table 8 Levels of end products resulted from sheep wool and feather biodegradation under preoptimized and non- optimized conditions

Medium	Levels of NH_2-free amino groups (μ mol leucine/ml)						Levels of soluble proteins (mg/ml)					
	Preoptimized conditions		Optimized conditions		Fold enhancement		Preoptimized conditions		Optimized conditions		Fold enhancement	
	Sheep wool	Feathers	Sheep wool	Feathers	Sheep wool	Feathers	Sheep wool	Feathers	Sheep wool	Feathers	Sheep wool	Feathers
I	≈120	141.18	302.0	270.0	2.50	1.9	≈0.8	0.54	3.4	1.25	4.25	2.30
II	≈120	126.18	290.9	268.73	2.42	2.12	≈0.8	0.70	4.4	1.50	5.50	2.14
II	≈120	138.0	290.9	245.82	2.42	1.78	≈0.8	0.70	4.6	1.70	5.75	2.43

I: keratin-based modified basal medium II

II: keratin-based distilled water medium

III: keratin-based tap water medium

Keratin here means feathers or sheep wool

with the corresponding predictors in the three optimized feathers-based media were 245.82–270 μmol leucine/ml and 1.25–1.7, respectively when these two combination sets [8% (w/v) feathers, 98.45 h and 3.9% (v/v) inoculum size] and [7.69% (w/v) feather, 96.58 h, 1.28% (v/v) inoculum size] were applied, respectively. Using higher levels of the variable feather (8.33 and 8.58%) at distances 1.3 and 1.4, respectively from the center of the design did not impose elevation in the obtained levels of the response NH_2 -free amino groups experimentally. These values represented 100% and 83.66–100% adequacy for the models of NH_2-free amino groups and soluble proteins, respectively.

On the other hand, the levels of NH_2-free amino groups and soluble proteins verified in three optimized sheep wool -based media were 290.9–302.0 μmol leucine/ml and 3.4–4.6 mg/ml, respectively when these two combination sets [8.3% (w/v) sheep wool, 5.9% (v/v) inoculum size, 63.46 h] and [8.2% (w/v) sheep wool, 5.52% (v/v) inoculum size, 46.58 h] were applied, respectively. Similarly, using higher levels of the variable sheep wool (8.58 and 8.8%) at distances 1.3 and 1.4, respectively from the center of the design did not impose elevation in the obtained levels of the response NH_2-free amino groups experimentally. These verified values represented 100% adequacy for both models. Conclusively, after verification of the optimal predicted levels derived from canonical and ridge analyses, the optimal conditins to obtain maximal levels of the four responses were located inside the experimental domain. These data indicated that the generated models were an adequate prediction for soluble proteins and NH_2-free amino groups derived from the biodegradation of feathers and sheep wool.

Enhanced levels of end products upon using optimized conditions

By the end of the optimization process, it is worthy to compare the levels of end products resulted from keratin biodegradation upon applying optimized and non- optimized conditions. Data of Table 8 display the fold enhancement in the level of released end products. Our data revealed that, 2.42–2.5 and 4.25–5.75 fold increase in the level of released NH_2-free amino groups and soluble proteins were achieved upon using three optimized sheep wool-based media. However, 1.78–2.12 and 2.14–2.43 fold enhancements in the level of released NH_2-free amino groups and soluble proteins, respectively were achieved upon using three optimized feathers-based media.

Conclusion

Optimizing the biodegradation of two keratinous wastes (feathers and sheep wool) directed by *B. subtilis* recombinant strain was conducted through a response surface methodology. Three variables namely; keratin percent, incubation time and inoculum size were studied here. The selection of these variables was

based on a previous OVAT study highlighted their positive impact on the biodegradation of keratin. A Box–Behnken design was employed to determine the optimal level of each variable. Models concerning the two keratin biodegradation systems (sheep wool and feather) have good fitness and high significance as indicated from their high R^2 values (almost equal to 1.0) and high *F*-test values. Results showed that only one variable; keratin percent displayed the highest significant effect on the yield of keratin hydrolysis end products. Contour surface plots of the responses were in a good accordance with regression analysis of the model. The optimal sets of predicted and verified levels of the three variables were [7.69% (w/v) feathers, 96.58 h and 1.28% (v/v) inoculum size] and [8% (w/v) feathers, 98.45 h, 3.9% (v/v) inoculum size] to achieve the highest levels of soluble proteins (1.25–1.7 mg/ml) and NH_2-free amino groups (245.82–270.0 μmol leucine/ml), respectively upon using three optimized feathers-based media. These values represented 83.67–100% and 100% adequacy for the models of soluble proteins and NH_2-free amino groups, respectively. While, [8.23% (w/v) sheep wool, 5.52% (v/v) inoculum size and 46.58 h] and [8.33% (w/v) sheep wool, 5.89% (v/v) inoculum size and 63.46 h] were the optimal sets of predicted and validated levels of the above variables to achieve the highest yields of soluble proteins (3.4–4.6 mg/ml) and NH_2-free amino groups (290.9–302.0 μmol leucine/ml), respectively upon using three optimized sheep wool-based media. These values represented 100% adequacy for the models of soluble proteins and NH_2-free amino groups. Data revealed that, 2.42–2.5 and 4.25–5.75 fold increase in the level of released NH_2-free amino groups and soluble proteins, respectively were achieved upon using three optimized sheep wool -based media. However, 1.78–2.12 and 2.14–2.43 fold enhancements in the level of released NH_2-free amino groups and soluble proteins, respectively were achieved upon using three optimized feathers-based media. These obtained enhanced levels of end products conferred the great importance of optimizing keratin biodegradation. Data would strongly encourage pilot scale and industrial scale optimization of these wastes. Results would greatly encourage the valorization of keratinous wastes by these *B. subtilis* recombinant cells. Moreover, reproducibility and high efficiency of keratin biodegradation by this recombinant *B. subtilis* strain are considered a great challenge to overcome low expression of the cloned keratinase genes published in the literature. End products resulted from keratin biodegradation processes could promote several new industries such as soluble proteins, amino acids, as well as enzyme industry.

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