Optimization of Extraction Parameters for Keratinase Recovery from Fermented Feather under Solid State Fermentation by *Streptomyces* sp. NRC 13S

Hoda Mohamed Abdel Halim Shata · Mohamed Abdel Fattah Farid

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Abstract The effects of solvent type and concentration, solid/liquid ratio, extraction time and repeated extraction on recovery of keratinase from solid-state fermentation (SSF) of chicken feather by a local *Streptomyces* sp. NRC 13S were investigated in order to establish the experimental conditions for keratinase yield. Among solvents tested, 0.5% (v/v) glycerol was the best. Box-Behnken design was used to investigate the effect of relevant variables on keratinase recovery. The factors investigated were solid/liquid ratio (1:1.66–1:6.66 g/mL), glycerol concentration (0.5–5% v/v) and repeated extraction (1–5 cycle). The results showed that the maximum recovery of keratinase (6933.3 U/gfs) was obtained using 0.5 (v/v) glycerol as extracting solvent, in a solid /liquid ratio of 1:5 and three extraction cycles.

Keywords Box-Behnken design · extraction · keratinase · optimization · solid-state fermentation

Introduction

Keratinases (EC 3.4.21/24/99.11) have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide “keratin” recalcitrant to the commonly known proteolytic enzymes trypsin, pepsin and papain. These enzymes are largely produced by a variety of different microorganisms in the presence of keratinous substrates in the form of hair, feather, wool, nail, horn etc. during their degradation

(Gupta and Ramnani, 2006). Besides their use in traditional industrial sectors like detergent, medicine, cosmetics, leather and feed (Farag and Hassan, 2004; Cai et al., 2008), keratinases also find applications in newer fields like prion degradation for treatment of the dreaded mad cow disease, biodegradable plastic manufacture and feather meal production and thus can be aptly called “modern proteases” (Langeveld et al., 2003).

Solid state fermentation (SSF) can use wastes or agro-industrial substrates for enzyme production. It is a low energy-consuming and environmentally friendly process that greatly decreases pollution problems. Because feathers and their meal are available in large amounts, they could be an interesting low-cost substrate in the fermentation industry for the production of commercial enzymes as well as for biotechnological applications (Pandy et al., 2000; Holker et al., 2004). However, one of the important aspects of SSF is the adequate recovery of enzymes from the fermented solids (Castilho et al., 1999). According to Ikasari and Mitchell (1996) extraction efficiency is a critical factor determining the economic feasibility of SSF for enzyme production. In order to make SSF applicable for the production of high-purity enzymes, some studies are found about extraction of enzymes in SSF. Therefore, when studying the solid–liquid extraction of enzymes, it is of crucial importance to test the influence of five different parameters (type of solvent, solid/ liquid ratio, temperature, agitation, and contact time) and the interrelation among them. Few studies on enzyme extraction in SSF have been reported in literature. Some reports investigated the extraction of pectinases obtained by SSF, verifying the influence of temperature, stirring rate, and time of extraction in batch and fixed bed multiple stages (Castilho et al., 1999). The extraction of xylanases obtained by SSF was also studied using experimental design technique, where the kind of solvent, the extraction temperature, the solid/liquid ratio, the stirring rate, and the extraction time were investigated (Heck et al., 2005; Diaz et al., 2007).

The purpose of this study was to investigate the extraction conditions of keratinase obtained from SSF using chicken feather

Md A. F. Farid (✉)
Natural and Microbial Products Department, National Research Center,
Dokki, Cairo, Egypt
E-mail: nrcfarid@yahoo.com

H. Md A. H. Shata
Microbial Chemistry Department, National Research Center, Dokki, Cairo,
Egypt

as a substrate by *Streptomyces* sp. NRC 13S. The effects of the solvent type and its concentration, solid/liquid ratio, extraction time and repeated extraction on recovery of keratinase were studied. Extraction process was optimized using Box-Behnken design.

Materials and Methods

Microorganism. *Streptomyces* sp. NRC 13S was originally isolated from Egyptian soil and preliminarily identified as a producer of keratinase in our laboratory, Natural and Microbial Products Department, National Research center, Dokki, Cairo, Egypt. *Streptomyces* sp. NRC 13S was maintained on ISP2 agar slants containing (g/L): yeast extract 4, glucose 4, malt extract 10, and agar 25 (Shirling and Gottlieb, 1966).

Inoculum preparation. The spores from a fully sporulated *Streptomyces* sp. NRC13S slant grown on ISP2 agar slants at 28°C for 10 days were dispersed in three milliliters of sterile distilled water by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculums for each Petri dish (9 cm).

Solid-state fermentation. Feathers obtained at a local poultry processing were washed with warm tap water, distilled water and then dried at 45°C for 48 h in a drying oven. After drying, feathers were cut into small fragments and milled using electric mixer. SSF was carried out by taking one gram of feather substrate in each Petri dish, and the moisture content of the meal was adjusted, unless other wise, to 66% with distilled water. After sterilization (121°C, 30 min) and cooling, each Petri dish was inoculated with 1ml of spore suspension and incubated in an incubator with control of temperature at 30°C for the desired period.

Enzyme extraction. Three grams of the fermented mass in a 250 mL conical flask was extracted with the selected solvents by soaking the fermented feather and the mixture was mixed thoroughly on a rotary shaker (150 rpm) at room temperature (28±2°C) for 30 to 150 min. The crude extract was then filtered through a cotton cloth and the filtrate was centrifuged at 4500 rpm for 10 min. The clear filtrate obtained was used for the keratinase assay. The parameters selected for this study were: solvent type and its concentration, solid/liquid ratio, extraction time and number of washes. In the experiment on the parameter pertaining to the number of washings, fresh lots of the solvent were added to the fermented feather recovered at the end of the previous extraction. Leachates from these extractions were collected separately and tested for the recovery of keratinase.

Selection of extraction solvent for keratinase. Different solvents were tested for extraction of keratinase: distilled water, tap water, glycerol (1% v/v), 0.1 M glycine buffer (pH 8, 9, 10, 11), 0.1 M sodium phosphate buffer (pH 6.0, 7.0, 8.0), NaCl solution (10% w/v), and non-ionic detergents such as Tween 20, Tween 40, Tween 60, Tween 80 and Triton X-100. The kinds of solvents, the range of pH and concentration were chosen based on some previous studies of extraction of proteases (Tunga et al., 1999;

Table 1 Coded values of variables used in Box-Behnken design

Independent variables	Level		
	-1	0	1
X ₁ : Solid/liquid ratio (g/mL)	1:2.5	1:5	1:7.5
X ₂ : Glycerol concentration (% v/v)	0.25	0.5	0.75
X ₃ : Repeated extractions (number)	1	2	3

Aikat and Bhattacharyya, 2000; De Azeredo et al., 2006; Sumantha et al., 2006).

Keratinase assay. Keratinase activity was determined using method of Friedrich et al. (1999) with some modifications. 0.25 mL of crude enzyme with 0.75 mL keratin powder (1% w/v) in 0.1 M glycine buffer pH 7.8 for half an hour at 40°C in a shaking water bath. The reaction was stopped by adding 0.5 mL of 15% trichloroacetic acid (TCA). After half an hour, centrifugation was done at 4000 rpm for 10 min and the released amino acids were estimated according to Lowry et al. (1951). One unit of the enzyme activity (U) was defined as the amount of the enzyme that liberates 1 µg of amino acids equivalent to tyrosine per min under the experimental conditions. Enzyme yield was expressed as units per gram fermented substrate (U/gfs).

Optimization of extraction parameters for recovery of Keratinase. Based on the results of the one-variable-at-a-time experiment, response surface methodology (RSM) was used to optimize keratinase extraction using Box-Behnken design (Box and Behnken, 1960). The behavior of the system was explained by the following quadratic equation,

$$Y = \beta_0 + \beta_i X_i + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 \quad (1)$$

Where Y is the predicted response variable; β_0 , β_i , β_{ij} , β_{ii} are constant regression coefficients of the model, and X_i , X_j ($i=1, 3$; $j=1, 3$, $i \neq j$) represent the independent variables in the form of coded values. Statistical software package Design-Expert (Version 8.0.2, State-Ease, USA) was used to design and analyze the experiment. A 2^3 factorial design, with three replicates at the centre point with total number of 15 trials was employed. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2 . The coded values of the variables at various levels are given in Table 1.

Results and Discussion

Solvent Selection. The selection of a suitable solvent is critical to the recovery of the enzyme from the solid fermented biomass and one of important aspect of SSF. An ideal solvent would extract the enzyme selectively and completely at room temperature with minimal contact time and, preferably, at the pH of the cultivated substrate (Singh et al., 1999). As shown in Fig. 1(A), among the solvents tested glycerol 1% (v/v) gave the highest yield of keratinase (3200 U/gfs) from the fermented solid feather. The other organic solvents (Tween 60, Triton X-100 and glycine buffer) gave good results for recovery of the enzyme from the fermented mass, which indicate that organic solvents are more effective as

Fig. 1 Effect of different solutions (A), glycerol concentration (B), solid/liquid ratio (C) and extraction time (D) on keratinase recovery from fermented feather under SSF.

extracting media than water or NaCl solution. Glycerol at a concentration of 2.5% (v/v) was found to be the best solvent for extraction of α -amylase from the fermented wheat bran by *Bacillus circulans* GRs313 (Palit and Banerjee, 2001). At this concentration the hydroxyl group of the glycerol forms hydrogen bond with the protein molecules, which gives better stability to the enzyme molecule. Palit and Banerjee (2001) explained this reason by Debye-Hickel theory (Maron and Prutton, 1965):

$$F = I/D / (Q_1 Q_2) / r^2$$

Where F=force of attraction or repulsion, D=dielectric constant, Q₁, Q₂=charges of the attracting molecules, r=distance of two attracting molecules. All solvents have its own dielectric constant, organic solvents possess lower dielectric constant than inorganic solvents, so water possesses higher dielectric constant than organic solvents, therefore from the above theory it can be concluded that force of interaction between keratinase and solvent may have increased due to lowered dielectric constant of the extracting

solvent, glycerol. Though other organic solvent possesses lower dielectric constant than glycerol they may have some inhibitory effect on enzyme activity.

On the other hand, the results show that phosphate buffer at all pHs proved to be better than NaCl or water. These results are in accordance with that reported by Chen et al. (2011). They mentioned that sodium acetate buffer of pH 4.5 gave the better recovery of inulinase from fermented solid than water and NaCl solution. The use of citrate-phosphate buffer (pH 4.0) to leach other enzymes such as α -galactosidase from the fermented mass has been reported before (Annunziato et al., 1986). Soares et al. (2003) also demonstrated that water was the best solvent for transglutaminase extraction on solid-state cultivation. In many works on solid-state production of xylanases, the solvent used has been either acetate buffer, pH around 5.0 or water. Water has a higher dielectric constant compared with organic solvents. When the dielectric constant is decreased, the interaction forces between xylanase and solvent may increase; therefore, loss of enzymes

would increase (Heck et al., 2002).

Glycerol concentration. Leaching, a process for recovering the solute from the solids in the form of crude extract using the appropriate solvent is an important unit operation (Treybal, 1981). Various factors are known to influence the degree of leaching of the product from the fermented substrate produced by solid-state fermentation (Lonsane and Krishnaiah, 1992). Among using different concentrations of glycerol (0.5 to 5 % v/v), Fig. 1 (B) shows that 0.5% (v/v) glycerol was the concentration which provided maximum keratinase extraction, whereas glycerol at high concentrations gave less yield in the enzyme extraction from the fermented biomass. It was reported that at high glycerol concentrations there might be some inhibitory effect due to distortion of enzyme structure (Stryer, 1975). The poor performance of glycerol at high concentration (50% v/v) during extraction of pectinase can be explained by its higher viscosity, which has a negative effect on mass transfer. However, it was mentioned that only with glycerol no decrease in both polygalacturonase and pectinase activities was observed beyond 35°C. This was due to the protective effect of glycerol on enzyme activity (Castilho et al., 2000). Glycerol forms strong hydrogen bonds with water, reducing the water activity. Enzymes which would rapidly lose their activity may keep active even at higher temperatures in the presence of glycerol (Scopes, 1982).

Solid to solvent ratio. The ratio of solvent-to-solid plays an important role in the extraction of enzymes from the fermented biomass. In the solid-state fermentation system, free flowing solvent is very much limited. Thus, adequate amount of the solvent is required to leach out the enzyme present (Palit and Banerjee, 2001). The volume of the solvent (0.5% (v/v) glycerol) was varied from 1.66 to 6.66 mL/gfs and the extraction process was carried out on a rotary shaker (150 rpm) at room temperature (28±2°C) for 60 min. It was found that solid/ liquid ratio of 1:5 (g/mL) was optimum Fig. 1(C). The total activity decreased when lower volume of the solvent was used for extraction. Recovery of the low yield of enzyme from the fermented feather with lower volume of solvent might be due to the insufficient solvent volume to penetrate the solid fermented mass. Higher solvent to solid ratios also causes the solute to be more dilute in the final extract. The increase in leaching efficiency gained at higher ratios must, therefore, be balanced against the extra effort for concentration of the dilute extract (Lonsane and Krishnaiah, 1992). Also, it was mentioned that increasing the ratio of leaching solution (5% (w/v) NaCl) to moldy bran increases protease recovery, however, with high ratios more solvent is consumed, larger vessels are required for the leaching process, and the extract is more dilute. Lower ratios give lower recoveries because a significant fraction of the leaching solution is retained in the bran (Ikasari and Mitchell, 1996).

Effect of extraction time. The volume of 0.5% glycerol in relation to the solid matrix was retained at the optimal level (1:5 g/mL), and the incubation time for extraction was further optimized for maximum enzyme recovery from the fermented feather. The time period was varied from 5 to 180 min (Fig. 1D).

Fig. 2 Effect of extraction number on keratinase recovery from fermented feather under SSF. Note: X1: solid/liquid ratio (g/mL); X2: glycerol concentration (% v/v); X3: repeated extractions (number).

Fifteen minutes extraction time was optimum, and thereafter no beneficial effect on the keratinase extraction was observed, indicating the minimum time for total penetration of the solvent through the fermented biomass. Further increase in the contact time slightly decreased the leaching of the enzyme. Lonsane and Krishnaiah, (1992) mentioned that short leaching times maximize the volumetric productivity of the leaching equipment. Solid-solvent contact time is another factor governing the efficiency of leaching. In the present study, 15 min contact time between the solvent and the solid achieved maximum recovery of the keratinase. Drop in the recovery of the enzyme at the higher contact time may be due to the inactivation of the enzyme. Soaking of the fermented bran substrate in 0.1 M citrate buffer pH 4 at 22°C for 1 h was used for the recovery of acid protease (Ikasari and Mitchell, 1996). A contact time of 180 min with occasional agitation was employed for the leaching of milk-clotting enzyme from the fermented bran, whereas 150 min soaking for amylase extraction was reported (Palit and Banerjee, 2001; Shata, 2005). On the other hand, Chandra et al. (2008) found that 90 min contact time at 30°C was optimum for extraction of filter-paperase from fermented bran by *Aspergillus niger*.

Repeated extractions. The efficiency of extraction of the enzyme from the fermented feather with 0.5% glycerol at the optimal solid: liquid ratio (1:5 g/mL) was carried out in five cycles of 15 min each. Fresh solvent was added in each cycle to the same fermented feather. As shown in Fig. 2, the first extract had maximum activity of more than 67.7%, the second extract (13.2%) and the third extract (9.34%) represent about 89.77% of the total activity. Subsequent extractions did not have any significant enzyme activity.

Optimization of extraction parameters for keratinase recovery. After establishing the different factors (solvent type, solvent concentration, solid/solvent ratio, effect of extraction time and repeated extractions) to leach the keratinase from solid matrix and

Table 2 Box-Behnken design matrix with experimental and predicted values of keratinase recovery

Run	X1 Solid: Liquid (g/mL)	X2 Glycerol concentration (%)	X3 Repeated extractions (number)	Keratinase yield (U/gfs)	
				Experimented	Predicted
1	-1	0	-1	4467	4692.96
2	-1	0	1	5324.1	5268.41
3	1	0	-1	3492.4	3548.09
4	1	0	1	6933.3	6707.34
5	0	0	0	6893.2	6887.93
6	0	1	-1	4122.8	4082.04
7	0	-1	1	5131.6	5172.36
8	0	-1	-1	3470	3229.11
9	1	1	0	4953.9	4938.98
10	-1	-1	0	4000	4014.93
11	0	1	1	5632.6	5873.49
12	0	0	0	6851.4	6887.93
13	1	-1	0	4907.4	5092.6
14	-1	1	0	5907.8	5722.6
15	0	0	0	6919.2	6887.93

Table 3 Regression analysis for keratinase recovery for quadratic response surface model fitting (ANOVA)

Source	Sum of Squares	Degrees of freedom	Mean Squares	F Value	p-value Prop>F
Model	2.11E+07	9	2.34E+06	39.17	0.0004*
X1	43232.7	1	43232.7	0.72	0.4341
X2	1.21E+06	1	1.21E+06	20.19	0.0064*
X3	6.97E+06	1	6.97E+06	116.59	0.0001*
X1 X2	8.66E+05	1	8.66E+05	14.48	0.0126
X1 X3	1.67E+06	1	1.67E+06	27.9	0.0032*
X2 X3	5760.81	1	5760.81	0.096	0.7688
X1 ²	2.02E+06	1	2.02E+06	33.83	0.0021*
X2 ²	5.36E+06	1	5.36E+06	89.68	0.0002*
X3 ²	4.41E+06	1	4.41E+06	73.79	0.0004*
Residual	2.99E+05	5	59816.13		
Lack of Fit	2.97E+05	3	98913.54	84.54	0.0117
Pure Error	2340.03	2	1170.01		
Cor Total	2.14E+07	14			
R-Squared	0.986				
Adj. R-squared	0.9608				

Note: X1: solid/liquid ratio (g/mL); X2: glycerol concentration (% v/v); X3: repeated extractions (number), Cor: correlation, * Values of “probability >F” less than 0.05 indicate model terms are significant.

in order to approach the optimum condition for keratinase extraction, response surface methodology (RSM) was used to optimize the extraction parameters for enhancing keratinase recovery. A Box-Behnken factorial design with three factors and three levels including three replicates at the center point was used for fitting a second-order response surface (Box and Behnken, 1960). Based on the above results; glycerol concentration, solid/liquid ratio and repeated extraction were selected as effective variables for keratinase extraction. A total of 15 experiments with different combinations of solid/liquid ratio (X₁), glycerol concentration (X₂) and repeated extractions (X₃) were performed (Table 2). The results were analyzed by standard analysis of variance (ANOVA)

and the following quadratic regression equation was obtained in terms of keratinase yield.

$$Y = 6887.93333 + 73.51250 X_1 + 388.51250 X_2 + 933.67500 X_3 - 465.32500 X_1 X_2 + 645.95000 X_1 X_3 - 37.95000 X_2 X_3 - 740.35417 X_1^2 - 1205.30417 X_2^2 - 1093.37917 X_3^2 \quad (1)$$

Keratinase yield varied from 3470 to 6933 U/gfs according to different levels of selected factors. The lowest yield was observed for run No. 3 and 8 while the highest yield of U/gfs was obtained with run No. 4. These results suggest that the glycerol concentration and repeated extraction strongly affect keratinase yield. However, when the solid/liquid ratio is considered, it is interesting to note

Fig. 3 Surface and contour plots of combined effect of solid /liquid ratio and glycerol concentration (A, B), solid/liquid ratio and repeated extraction (C, D), glycerol concentration, and repeated extraction (E, f) on the recovery of keratinase from fermented feather under SSF.

that keratinase yield increase with the increase solid/liquid ratio up to 1:5 (w/v) with no significant variation for ratios above this ratio.

The significance of each regression coefficient was determined by *p*-values, which are listed in Table 3. Values of “Prob>F” less than 0.05 indicate model terms are significant. In this case, glycerol concentration (X2), repeated extraction (X3), their inter

action with with solid/liqued ratio (X1X2 and X1X3) are significant model termes. The quadratic effects of X_1^2 , X_2^2 and X_3^2 are also significant model terms. The quadratic terms for the three variables indicated that they can act as limiting factors even small increase in their values will significantly increase or decrease keratinase yield, respectively (Adinarayana and Suren, 2005). Values greater than 0.1 indicate the model terms are not

significant.

The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2=98.6$) indicated that only 1.4 % of the total variation were not explained by the model. The value of the adjusted determination coefficient (Adj $R^2=0.9608$) was also very high to advocate for a high significance of the model. The “Pred R -Squared” of 0.7778 is in reasonable agreement with the “Adj R -Squared” of 0.9608. At the same time, a relatively lower value of the coefficient of variation (CV=4.64) indicated a good precision and reliability of the experiments carried out.

Figure 3 shows the response surface plots and their contour plots of keratinase yield during the extraction process. An elliptical nature of the contour plots indicates that the interactions between the independent variables are significant. Since interactions between the three independents are observed from Table 3 for $X_1 X_2$ and $X_1 X_3$, an elliptical contour plots are found in Fig. 3B and 3C.

Evidently, keratinase yield varied significantly upon changing the solid/liquored ratio, glycerol concentration or repeated extractions. Under certain condition (glycerol con.=0.525–0.55% v/v) coded as 0.1–0.2, solid/liquid ratio (1:5.25–1:5.66 g/mL) coded as 0.15–0.4, and repeated extraction coded as 0.37–0.58, a maximal contour (Y activity=7122) could be determined (Fig. 3D) meaning that further increase of X_1 , X_2 and X_3 would not increase the keratinase yield any longer.

Comparison of observed and predicted. A regression model can be used to predict future observations on the response (keratinase yield) during the hydrolysis process corresponding to particular values of the repressor variables. Table 2 shows also the observed keratinase yield (the response) and their predicted value. The results prove that the predicted data of the response from the empirical model are in agreement with the observed ones in the range of the operating variables.

In conclusion, the effect of solid/liquid ratio, glycerol concentration and repeated extraction on recovery of keratinase from fermented chicken feather by *Streptomyces* sp. NRC 13S under solid state fermentation were investigated. The extraction parameters were optimized by Box- Behnken design resulting in keratinase activity values of 6933.3 U/gfs, when using solid/liquid ratio of 1:5 (g/mL), glycerol concentration 0.5% (v/v) and three repeated extraction cycles.

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