Optimization of Keratinase Production by *Aspergillus terreus* Thom by Statistical and Immobilization Methods

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Abstract: The production of extracellular keratinase from *Aspergillus terreus* Thom, locally isolated, was studied. Medium optimization was carried out using Plackett-Burman design. The optimized media that were achieved by statistical design raise the keratinase activity as it reached about 1.63 times higher than that obtained from the control medium. The best solid substrate for keratinase activity was coconut powder. Alginate with 2.5 ml volume was the best immobilization gel materials for keratinase activity. Adsorption of fungal mycelia on clay particles showed high keratinase activity (0.314 U/ml) and protein content, however lower than that of free cells. Reusing entrapped and adsorbed cultures of A. terreus led to a gradual decrease of keratinase activity in the cultures of the 2nd run and continued to decrease up to the 5th run.

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1. Introduction

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Microbial keratinases (EC 3.4.21/24/99.11) are amongst the industrially important enzymes. They are proteolytic enzymes capable of hydrolyzing highly rigid keratin which is recalcitrant to commonly known proteases such as trypsin, pepsin and papain (Syed et al., 2009). New avenues for commercial exploitation of keratinase have yielded better animal feed additives from feather hydrolysates of keratin degrading bacteria (Williams et al., 1990). Also, they are finding applications in leather and detergent industries, textile, waste bioconversion, medicine and cosmetics for drug delivery through nails and degradation of keratinized skin (Ali et al., 2011). Keratinolytic enzymes are widespread in nature and are elaborated by a compendium of microorganisms as *Bacillus*, *Lysobacter*, *Nesternokia*, *Kocurica*, *Microbacterium*, *Vibrio*, *Xanthomonas*, and *Chryseobacterium* (Lucas et al., 2003). The most keratinolytic group among fungi are *Aspergillus, Alternaria, Fusarium, Geomyces, Paecilomyces and Penicillium* (Gupta and Ramnani, 2006). For a broad application, the cost of bio-products is one of the main factors determining the economics of a process. Reducing the costs of enzyme production by optimizing the fermentation medium is the basic research for industrial application. The use of different statistical designs for medium optimization has been recently employed for many enzymes, antibiotics, and metabolites (Francis et al., 2003). Solid-state fermentation (SSF) is the fermentation involving solids, substrate must possess enough moisture to support growth and metabolism of micro-organism (Mitchell and Lonsane, 1990; Pandey et al., 2001). Filamentous fungi are the best studied for SSF due to their hyphal growth, which have the capability to not only grow on the surface of the substrate particles but also penetrate through them (Pandey et al., 2000).

For industrial applications, the immobilization of protease on a solid support can offer several advantages including advantages of porosity, repeated use of enzyme, ease of product separation, improvement of enzyme stability and continuous operations in packed bed reactor. Immobilization of microbial cells for higher alkaline protease production using different methods namely adsorption, covalent bonding, cross-linking, entrapment, and encapsulation was studied. Several gel materials of synthetic or natural origin were used for microbial cell entrapment mainly in the form of beads (Abdel Naby et al., 1998).

2. Materials and Methods

Microorganism and Maintenance of Culture

In the present work, many fungal species were isolated from different sources (sewage, seawater and soil samples from different places in Alexandria, Egypt). Only one of these species showed keratinolytic activity. This species was isolated from a soil sample from El-Tabia in Alexandria city, Egypt and it was identified as *Aspergillus terreus* Thom by the Mycological Center, Faculty of Science, Assiut University, Egypt according to the regulations and keys described by Domsch et al. (2007). The strain used throughout this study was isolated on Czapek-

Dox agar plates containing (g/l): sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; agar, 15; the pH was adjusted at 8 and autoclaved at 121ºC for 15 min. The plates were incubated at 25°C for 5 days. Isolated culture was streaked on medium agar slants and was sub-cultured monthly. The entrapped and adsorbed fungal mycelia were scanned using electron microscope. This was done in the Electron Microscope Unit in Faculty of Science, Alexandria University.

Fermentation Medium Composition for Keratinase Production

Cultivation of the strain for the production of keratinase, was carried out in 250 ml Erlenmeyer flasks, containing 50 ml of basal broth medium of the same composition as that used for isolation, inoculated with one 0.5 cm agar cube of 48 h old cultured Czapek-Dox agar plate. The flasks were incubated at 25°C in static incubator.

Estimation of Total Protein and Keratinase Standarad Assay

Total protein was determined by the Lowry's method using bovine serum albumin as a standard (Lowry et al., 1951). Keratinase activity was performed by determining the rate of hydrolysis of 3% keratin in distilled water containing the enzyme as described by Greenberg and Rothstein (Greenberg and Rothstein, 1957). One unit of keratinase activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine under the specified conditions.

Determination of Dry Weight

The fungal mats were separated by filtration, washed with distilled water then dried at 70°C in oven until constant weights were obtained.

Selection of the Influential Medium Components for Process Modeling

Seven factors of medium components were screened in eight combinations organized according to the Plackett-Burman design matrix. For each variable, high (+1) and low (-1) levels were tested (Plackett and Burman, 1946; Rajendran et al., 2007). The factors were $NaNO_3$, K_2HPO_4, sucrose, $MgSO_4$, KCl, inoculum size and inoculum volume. All trails were performed in duplicates and the average results were treated as the responses. The main effect of each variable was determined with the following equation: $E_{xi} = (\sum M_{i+} - \sum M_{i-}) / N$ where E_{xi} is the variable main effect, M_{i+} and M_{i-} is the activity in trails where the independent variable (xi) was present in high and low concentrations, respectively, and n is the number of trials divided by 2. A main effect with a positive sign indicates that the high concentration of this variable is near to the optimum and a negative sign indicates that the low concentration of this variable is near to the optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

Production of Keratinase by *A. terreus* by Solid State Fermentation

Five grams of solid substrates were transferred to 250 ml Erlenmeyer flasks and moistened with 5 ml of Czapek-Dox medium without sucrose and $NaNO_3$. After sterilization, each flask was inoculated with 0.5 cm agar cube of the cultured Czapek-Dox agar plates. At the end of the incubation period the fermentation mass was extracted by the simple method of extraction using distilled water as extracting agent. Fifty ml distilled water was added for one hour on a rotary shaker (200 rpm) followed by centrifugation (5000 rpm) for 10 min (Balch, et al., 1979). The supernatant was used for estimation of protein content and keratinase activity.

Immobilization of *A. terreus* Cells by Entrapment in Ca-alginate

Sodium alginate 2% solution was prepared by dissolving 2 g in 90 ml distilled water and then autoclaving at 108°C for 10 min. Ten ml of fungal spore suspension obtained from 2 days old slant culture, were added to the sterile alginate solution to obtain 2 % final concentration. Different volumes of the alginate-fungal mycelia mixture were drawn with the aid of a sterile syringe and allowed to drop through the needle into a cross linking solution (100 ml of 2% $CaCl_2$ solution) in 250 ml Erlenmeyer flask to obtain spherical beads (3-4 mm diameter) of calcium alginate gel entrapping the fungal mycelia. The beads were left in the calcium chloride solution for one hour and then washed several times with sterilized distilled water. The resulted beads were added to 25 ml sterile Czapek-Dox medium in 250 ml Erlenmeyer flask which were incubated at 25°C for 5 days (Eikmeier et al., 1984).

Entrapment in Agar

The gel was prepared by dissolving 2 g agar in 90 ml distilled water by heating at 100°C then sterilized by autoclaving at 121°C for 20 min. After cooling to 50°C, 10 ml spore suspension was added and mixed well (Chapatwala et al., 1993). Fifteen ml of this mixture were aseptically poured into a petri-dish. After solidification, the gel was cut with a sterile cutter into small cubes of about 0.5 cm in length. The small gel cubes were transferred to 25 ml sterile Czapek-Dox medium in 250 ml Erlenmeyer flask. The flasks were incubated at 25°C for 5 days.

Immobilization of *A. terreus* Cells by Adsorption

A half cm agar cube of the 2 days cultured Czapek-Dox agar plates was added to the Erlenmeyer flasks containing 50 ml sterilized culture medium and solid supports. The flasks were incubated at 25°C for 5 days.

3. Results

Factors Affecting Keratinase Production of by *A. terreus*

Plackett-Burman design was applied to reflect the relative importance of various fermentation factors involved in the production of keratinase in the fungal cultures. Examined levels of seven culture variables were presented in Table 1.

Table 1. Different levels of the seven independent variables used in the Plackett-Burman design

Different factors (g/l)	Symbol	Level		
		-1	0	+1
$NaNO_3$	Na	1	3	5
K_2HPO_4	K_2	0.5	1	1.5
Sucrose	Su	20	30	40
$MgSO_4$	Mg	0.1	0.5	1
KCl	K	0.1	0.5	1
*Inoculum size (cube)	IS	0.5	1	1.5
Culture volume (ml)	CV	25	50	75

* Inoculum size was added (cube) of 48 hours culture

The design was applied with nine different fermentation conditions as shown in Table 2. The culture filtrate of each culture was taken where keratinase activity of each trial was determined. Table 2 shows that, trial 4 followed by trial 6 yielded the highest keratinase activity (0.352, 0.212 U/ml) respectively. The degree of significance of $NaNO_3$ concentration was the highest (95%). The increase in $NaNO_3$ concentration resulted in an increase in the production of keratinase activity. Also, these results recorded that the degree of significance of inoculum size concentration was (90%). The decrease in its concentration resulted in an increase in the enzyme activity. It was deduced from Table 3 that $NaNO_3$ was the most significant variable for keratinase activity. Maximum keratinase activity was obtained at 5 g/l $NaNO_3$ concentration. By decreasing the concentration of inoculum size to 0.5 cm agar cube, the keratinase activity reached its maximum value. According to these suggestion dedicated by the applied Plackett-Burman experiment it can be predicted that the optimum medium for producing an extracellular keratinase from the culture of *A. terreus* with a relatively high activity was (g/l): sucrose, 30; $NaNO_3$, 5; K_2HPO_4 , 1; $MgSO_4$, 0.5; KCl , 0.5; inoculum size, 0.5 cm agar cube/50 ml.

Table 2. The Plackett –Burman experimental design for seven factors

Trials (n)	Independent variables							Keratinase activity (U/ml)
	Na	K_2	Su	Mg	K	IS	CV	
1	-1	-1	-1	1	1	1	-1	0.0036
2	1	-1	-1	-1	-1	1	1	0.205
3	-1	1	-1	-1	1	-1	1	0.098
4	1	1	-1	1	-1	-1	-1	0.352
5	-1	-1	1	1	-1	-1	1	0.106
6	1	-1	1	-1	1	-1	-1	0.212
7	-1	1	1	-1	-1	1	-1	0.033
8	1	1	1	1	1	1	1	0.092
Control medium	0	0	0	0	0	0	0	0.216

Table 3. Degree of positive and negative effects of independent variables on the production of keratinase enzyme activity by *Aspergillus terreus* Thom according to levels in the Plackett–Burman experiments

Variable	$NaNO_3$		K_2HPO_4		Sucrose		$MgSO_4$		KCl		IS		CV	
	+	-	+	-	+	-	+	-	+	-	+	-	+	
	0.205	0.0036	0.098	0.0036	0.106	0.0036	0.0036	0.205	0.0036	0.205	0.0036	0.098	0.205	
	0.352	0.098	0.352	0.205	0.212	0.205	0.352	0.098	0.098	0.352	0.205	0.352	0.098	
	0.212	0.106	0.033	0.106	0.033	0.098	0.106	0.212	0.212	0.106	0.033	0.106	0.106	
	0.092	0.033	0.092	0.212	0.092	0.352	0.092	0.033	0.092	0.033	0.092	0.212	0.092	
Mean	0.215	0.06	0.144	0.132	0.111	0.165	0.138	0.137	0.101	0.174	0.083	0.192	0.125	
Main effect	0.155		0.012		-0.054		0.0014		-0.073		-0.109		-0.025	
T-value	2.712		0.14		-0.645		0.016		-0.895		-1.464		-0.29	
Deg.of sign.	95%		90%		90%		90%		90%		90%		90%	

` $t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439. Deg. of sign. : Degree of significance

In order to evaluate the accuracy of the applied Plackett-Burman screening test, a verification experiment was carried out in triplicate and represented in Table 4 where the keratinase activity reached about 0.352 U/ml, which was approximately higher than that obtained from the previously used control medium.

Production of keratinase by *A. terreus* in Different Solid State Cultures

In this experiment, several substrates in the powder form as coconut, wheat bran, oat, sesame, soybean and corn flour, were used as solid substrates. The highest keratinase activity (0.546 U/ml) was obtained when coconut powder was used as a solid substrate (Table 4).

Table 4. Production of keratinase from *Aspergillus terreus* Thom by optimized, antioptimized, and control media, and by solid state fermentation using different solid substrates

		Keratinase activity (U/ml)	Protein content (mg/ml)
Type of media	Optimized media	0.352	4.559
	Control media	0.216	2.096
	Antioptimized media	0.098	2.072
Solid substrate	Coconut	0.546	4.283
	Wheat bran	0.245	5.194
	Oat	0.132	6.428
	Sesame	0.194	7.986
	Soybean	0.077	10.879
	Corn flour	0.201	2.347
	Control	0.352	4.559

Effect of Entrapping Mycelia with Different Gel Materials on Keratinase Production

Different gel materials namely; Na-alginate and agar were used for entrapment of *A. terreus* mycelia. The gel-mycelia beads with different volumes (0.25, 0.5, 0.75, 1, 2.5, 5, 7.5 ml) were used to inoculate 25 ml production medium. The results illustrated in Table 5 shows that keratinase activity with free mycelia was higher than that of entrapped mycelia in general. The results also showed that the culture filtrate of the medium inoculated with 2.5 ml alginate mycelia mixture had the highest keratinase activity (0.249 U/ml) while the filtrate of the medium inoculated with 7.5 ml alginate mycelia mixture had the lowest activity (0.015 U/ml).

Effect of Adsorption of *A. terreus* on Keratinase Production

Different support materials namely; sponge cubes, luffa pulp, pumice and clay particles were used for adsorption of *A. terreus* mycelia. Mycelia adsorbed on supports were used to inoculate 50 ml of production medium. Table 5 shows that the optimal keratinase activity (0.314 U/ml) and protein content (1.622 mg/ml) were obtained by the clay particles as a support; however this activity was lower than that obtained from free mycelia. The lowest activity (0.081 U/ml) was obtained from mycelia adsorbed on sponge cubes.

Scanning electron microscopy (SEM) of immobilized *A. terreus* shows an irregular pattern of the microorganism immobilized in alginate beads and adsorbed on the particles of clay as shown in Figure 1.

Semi-continuous Production of Extracellular Keratinase by Entrapped and Adsorbed *A. terreus*

Keratinase production by *A. terreus* entrapped in 2.5 ml alginate, 15 ml agar and adsorbed on clay particles were studied in repeated batch cultures. For this purpose, 25 ml fractions of the production media were used. The entrapped and adsorbed mycelia were reused for 5 successive cycles covering a period of 25 days. At the end of each reuse, the culture media were decanted and fresh media were added under aseptic conditions to the alginate beads, and clay particles. The time interval for the reuse was 5 days. Table 5 shows that by reusing mycelia entrapped in alginate or agar, and adsorbed on clay particles, the enzyme activity gradually decreased in the cultures of the second run and continued to decrease up to the 5th run.

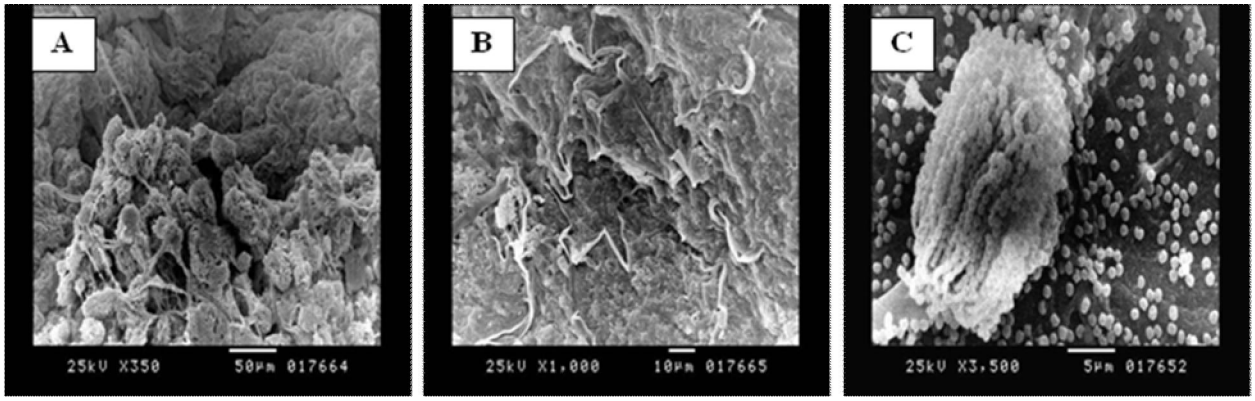

Figure 1. Scanning electron micrograph showing: (A 350× and B, 1000×) conidial heads and interwoven hyphae of *Aspergillus terreus* Thom entrapped in alginate gel materials; (C, 3500×) conidial chains of the fungus adsorbed on the surface of clay particles.

Table 5. Production of keratinase by entrapped and adsorbed cells of *Aspergillus terreus* Thom, and immobilized cells of *Aspergillus terreus* Thom in different materials reused several times

		Keratinase activity (U/ml)	Protein content (mg/ml)
Gel materials Volume of gel mixture (ml/25ml media)	**Agar**		
	3.75	0.0402	1.805
	7.5	0.048	1.894
	11.25	0.073	1.904
	15	0.095	2.198
	Alginate		
	2.5	0.249	2.404
	5	0.183	2.869
	7.5	0.015	2.306
	Free cells	0.352	4.559
Support materials	Sponge	0.081	1.507
	Pumice rock	0.259	0.999
	Clay	0.314	1.622
	Luffa	0.129	1.289
	Free cells	0.352	4.559
Reused Materials (Number of reuse)	**Agar**		
	Initial	0.095	1.894
	First	0.088	2.368
	Second	0.062	2.834
	Third	0.043	3.013
	Forth	0.028	3.111
	Fifth	0.022	2.994
	Alginate		
	Initial	0.249	2.404
	First	0.216	1.894
	Second	0.176	2.27
	Third	0.066	2.332
	Forth	0.015	3.951
	Fifth	0	3.244
	Clay		
	Initial	0.314	1.622
	First	0.099	1.768
	Second	0.099	1.659
	Third	0.006	2.594
	Forth	0	2.434
	Fifth	0	2.796

4. Discussions

In this study, the statistical optimization and immobilization of mycelia of *A. terreus* for keratinase production was closely investigated. The medium nutrient components were screened by applying the Plackett-Burman matrix. It was deduced that sodium nitrite and size of inoculum were the most significant variables for keratinase production. A previous study revealed that significant variables affecting keratinase production by *Bacillus subtilis* NCIM 2724 using Response Surface Methodology (RSM) were feather, ammonium chloride, magnesium sulphate, dipotassium hydrogen phosphate (Harde et al., 2011). Also, previous studies showed that keratinolytic proteases may be inducible enzymes, and their maximum activities were recorded in keratin-containing substrates (Gupta and Ramnani, 2006; Brandelli et al., 2010), while other studies demonstrated that keratinases can be excreted constitutively when keratin is absent in the substrate (Apodaca and Mc-Kerrow, 1989). Therefore, it could be stated that keratinase enzyme produced by *A. terreus* in the present study is a constitutive enzyme which can be produced in a keratin-deficient medium.

Production of keratinase by *A. terreus* cultures using solid state fermentation was the highest

when coconut powder was used as a solid substrate. These results are different from that obtained by Sandhya et al. (2005) who carried out solid state fermentation using various agro-industrial residues such as wheat bran, rice bran, coconut oil cake, sesame oil cake and olive oil cake for neutral protease production by *Aspergillus oryzae* 1808. It was shown that wheat bran was the most effective substrate for protease production. While other results showed a significant increase in keratinase production by *Bacillus subtilis* RM-01 using chicken feather supplemented with maltose and sodium nitrite as a substrate (Rai et al., 2009).

In practical utilization of fungal mycelia entrapped in gel matrix, the results showed that keratinase activity with free mycelia was higher than that of entrapped mycelia in general. This is because they represent a heterogeneous catalysis fermentation, in which the activity or rather synthesis of primary or secondary metabolites is dependent upon the mass transported and adequate oxygen supply. Also, the nutrients are lowering accessible to the immobilized cells. On the other hand, immobilization process leads to changes in the micro environmental conditions, and some metabolic and morphological alteration in the cells may occur (Ahmed and Abdel-Fattah, 2010). Interestingly, microbial proteases were successfully produced by entrapped microbial cells. And the results were agreed with many other searchers who utilized calcium alginate as an effective matrix for higher alkaline protease productivity from *Bacillus subtilis* PE-11 and alkaline keratinase from thermophilic *Bacillus halodurans* JB 99 respectively, compared to the other matrices as K-carrageenan, agar, polyacrylamide and gelatin (Adinarayana et al., 2005; Shrinivas et al., 2012). Adsorption was the most suitable immobilization technique for production of the keratinase by *A. terreus*, more than that produced by entrapped cells and the clay particles was the preferred matrix. The fluctuation in the amount of keratinase produced by cells entrapped in various gel matrices than adsorbed one was attributed to variation in aeration and diffusion of nutrient between adsorbed and entrapped cell system (Riley et al., 1999). Some studied the immobilization of *Bacillus circulans* MTCC 7906 in activated charcoal and kieselguhr (Mishra et al., 2012).

Semi-continuous production of mycelia entrapped in alginate or agar, and adsorbed on clay particles led to decrease in enzyme activity gradually in the cultures of the second run and continued to decrease up to the 5th run. These results are in agreement with those obtained by many investigators (Ahmed and Abdel-Fattah, 2010). Moreover, Shrinivas et al. (2012) found that in the semi-continuous mode of cultivation, immobilized cells of the thermo-alkalophilic *Bacillus halodurans* JB 99 under optimized conditions produced an appreciable level of protease in up to nine cycles and reached a maximal value after the 7th cycle.

The results of the present study indicate the possibility of using *A. terreus* Thom for the production of high active keratinase enzyme which can be applied in many fields including leather, pharmaceutical, and detergent industries, production of fertilizers and amino acids.

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