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Statistical Optimization, Immobilization and Partial Purification of Keratinase Production from Newly Isolated *Bacillus subtilis*

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In the present study, keratinase production by *Bacillus subtilis* DALEX isolated from chicken feathers was evaluated. The bacterium showed a proteolytic activity when grown on skimmed milk agar plates. Best enzyme activity (1.26 U/mg protein) was obtained on using chicken feathers compared to other substrates such as wool and rabbit fur. Best activity (1.35 U/mg protein) was achieved when *B.subtilis* DALEX was grown in shaken cultures of Feather basal medium supplemented with 10g feathers meal and incubated at 37°C for 3 days. Lower activities were observed with static cultures or in solid state fermentation. Placket-Burman design was employed to reach the near optimum condition for keratinase activity. Immobilization of bacterial cells on different supports was employed to study the effect of immobilization on enzyme activity. The highest keratinase production (1.83 U/mg protein) was reached by immobilized *B. subtilis* DALEX with 4 % alginate concentration which was higher than that obtained with free cells (1.33 U/mg protein).The fraction precipitated at 85% acetone concentration was considered as the partially purified enzyme. Optimum enzyme protein and substrate concentrations were 2.5 mg and 10 mg / reaction mixture, respectively. The optimum reaction temperature for the immobilized purified keratinase was 45°C. Partial purified enzyme showed a maximum activity at a reaction pH of 9.0 and a relatively high activity in a pH range of 7.0 to 9.0. The semi-purified enzyme was strongly stable at 50°C for 15 min and showed thermal stability even after 30 min exposure to 50 °C.

Key words: Keratinase, Solid state fermentation, Placket-Burman, Immobilization, Partial purification.

Keratin is the principle structural material making up the outer layer of human skin. It is also the key structural component of hair, nails and feathers. Feathers represent over 90% protein, the major component being keratin. Owing to their insoluble nature, feathers are resistant to degradation by common microbial proteases. Thus, several million tons of feathers generated annually by the livestock industry lead to environmental pollution and wastage of protein-rich reserve (Gousterova *et. al.*, 2005). Several species of bacteria, fungi, and actinomycetes are able to produce keratinases and degrade keratin-rich materials like feathers (Mabrouk, 2008; Kumar *et.al.*, 2011; El-Borai *et.al*., 2013 Shankar *et.al*., 2013; Laba *et.al.*, 2014).

The use of microbial Keratinase for keratin degradation is the innovative solution for recycling feather wastes and reducing pollution (Kulkarni *et.al.*, 2014). After hydrolysis, the feathers can be converted into feedstuff, fertilizers, glues, films and as the source of rare amino acids, such as serine, cysteine and proline (Gupta and Ramnani *et. al.*, 2006; Cao *et. al.*, 2009,).

Statistical designs for medium

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optimization of microbial cultures, offer several advantages over conventional methods being rapid and reliable. They help understanding the interactions among the nutrients at various concentrations and reduce the total number of experiments tremendously resulting in saving time, glassware, chemicals and manpower (Sivakumar *et.al*., 2011; Gupta *et.al.*, 2013).

Solid-state fermentation (SSF) is the fermentation involving solids, substrates must possess enough moisture to support growth and metabolism of microorganism (Pandey *et.al*., 2001 and Rodrigues de Siqueira *et.al*., 2014).

Immobilized cells have many advantages, such as high biomass, high metabolic activity, strong resistance to toxic chemicals and the reuse of beads without significant loss of activity (Martims, *et. al.*, 2013).

Due to the high costs of complete purification and because preparation of industrial enzymes, neither pharmaceutical nor other medical uses of the enzyme, needs to involve minimum number of purification stages that are compatible with their usage, partial purification was performed (Suneetha *et.al*, 2010; Suneetha *et.al*, 2012; Mandal *et.al.*, 2013). Therefore, this study was concerned with studying the production of extracellular keratinase from *B. subtilis* DALEX using free and immobilized cells. The study also focused on optimizing the factors affecting enzyme production using the Plackett –Burman design. In addition, partial purification and characterization of the enzyme was performed.

MATERIALS AND METHODS

Microorganism

Bacillus subtilis used in this study was locally isolated from chicken feathers and identified phenotypically according to Bergey's Manual of Systematic Bacteriology (Vos *et.al*., 2011). This isolate was assigned as *Bacillus subtilis* DALEX.

Isolation of keratinolytic bacteria

Isolation was done from soil, chicken feathers, rabbit fur and wool from poultry farming in Alexandria, Egypt. 1 g of soil or 10 g of other substrates were suspended in 10 ml sterile distilled water. After sedimentation, 0.1 ml of the supernatant was plated on Feather Basal medium **(FBM)** adopted by **Lakshmi (2013)** after slight modification**.** The medium had the following composition (g/L) (NH_4Cl, 0.5 ; NaCl, 0.5 ; K_2HPO_4, 0.3 ; KH_2PO_4, 0.4 ; MgCl- $6H_2O$, 0.1 ; yeast extract, 0.1) supplemented with 10 g of feathers meal. The plates were then incubated at 37°C for 48 h.

Screening for proteolytic activity

For primary screening of proteolytic enzyme activity, pure isolates were streaked on milk agar plates (5 g/l peptone, 3 g/l yeast extract, 10 g/l sterile non-fat milk and 20 g/l agar) and incubated at 37 °C for 24 h. Isolates producing clear zones around colonies were selected for further studies.

Keratinase production by *B. subtilis* DALEX

For solid state fermentation (SSF), 3 g of dry milled feathers, fur of rabbit or wool, were placed in 250 ml flasks and moistened with 10 ml of FBM. The flasks were sterilized at 121 °C for 20 min and inoculated with the bacterium. The flasks were allowed to stand for 6 days at 37 °C in static conditions. The fermentation mass was extracted with 50 ml of distilled water at room temperature for 1 h on a rotator shaker (150 rpm), followed by centrifugation (5000 rpm) for 20 min (Balch, *et. al*., 1979). The supernatant was used for determination of the enzyme activity and protein content.

For submerged fermentation, 50 ml of FBM containing 10g of chicken feathers meal were dispensed in 250ml Erlenmeyer flasks and incubated at 37°C, shaken at 200 rpm for 3 days. The supernatant representing the crude enzyme was obtained by centrifugation of the whole culture at 5000 rpm. Protein content and enzyme activity were measured in the crude enzyme.

Plackett-Burman design

Application of a complete factorial design would require 2n experiments if n factors have to be investigated. In the present case, seven variables would lead to 128 trials, which is a very large number. Using a fraction of the factorial design without losing information about the main effect of variables (Ooijkaas et al., 1998), a fractional factorial design, (Plackett and Burman, 1946; Yu *et.al*., 1997) was used in this research to reflect the relative importance of various environmental factors on the activity of keratinase enzyme. In this experiment, eleven independent variables were screened in twelve combinations organized according to the Plackett-Burman design matrix described in the results section. For each variable,

a high (+) and low (-) level was tested. All trails were performed in duplicates and the averages results were treated as the responses. The main effect of each variable was determined with the following equation: Exi = (? Mi+ - ? Mi-) / N Where Exi is the variable main effect, Mi+ and Mi- are the activity percentage 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.

Estimation of protein content

The protein concentration was measured according to Lowry *et.al*., (1951).

Assay of keratinase activity

The keratinase activity was assayed by the modified method of Cheng *et.al*., (1995).

Immobilization

Immobilization of *B.subtilis* DALEX cells was performed by three approaches; entrapment in calcium alginate according to Eikmeier *et.al*. ,(1984), in agar (Chapatwala *et.al.,* 1993),or by adsorption on different solid supports such as luffa pulp, pumice, sponge and clay particles cut into small cubes (EL-Naggar *et.al*., 2006).

Scanning electron microscope

The bacterial growth within different porous supporting materials was collected, washed with phosphate buffer and fixed with 2% glutaraldehyde followed by 1% osmium tetraoxide treatment. Samples were then washed in a buffer solution dehydrated in ethanol and completely dried in a critical point dryer. Afterwards they were coated with gold in JEOL-JFG 1100 E ion-sputter-coater. Specimens were viewed in JEOL-JSM 5300 scanning electron microscope operated at 20 kV with a beam angle of 45°.

Partial purification of keratinase enzyme produced by *B. subtilis* DALEX

Partial purification was achieved by salting-out with ammonium sulphate and by fractional precipitation with acetone and ethanol. The crude culture supernatant was obtained from 3 days old immobilized culture of *B.subtilis* DALEX **(**Beltagy, 2010, Saibabu *et.al.,* 2013; Suneetha *et.al.,* 2013**).** The supernatant was precipitated at different concentrations of each precipitant in a sequential manner.

Fractionation by salting-out with ammonium sulphate

A 200 ml volume of the crude culture filtrate was fast rotated in a cooling centrifuge 6000 rpm at 4°C to remove the residual waste and bacterial cells. The protein content and the keratinase activity of the enzyme solution were determined as previously described. The whole enzyme solution was kept in an ice bath. This was followed by adding ammonium sulphate very slowly while stirring to the ice cold enzyme solution until the desired saturation of ammonium sulphate was reached. The solution was left for 2 h and then centrifuged for 15 min at 7000 rpm in a cooling centrifuge. The precipitate (Fraction1) was removed and further ammonium sulphate was added to the supernatant fluid to obtain the next fraction. The process was repeated until 100% saturation was reached. Each precipitated fraction was dissolved in about 10 ml distilled water and dialyzed against distilled water (or buffer) in a cellophane bag in a refrigerator until the water outside the bag gives no precipitate with 1% barium chloride solution, indicating that the enzyme solution inside the bag became free of sulphate. This was achieved by changing the water (or buffer) outside the bag several times. After complete dialysis, each enzyme solution was dried by evaporation under low temperature. The protein content and keratinase activity of each solution was measured.

Fractional precipitation with acetone

Acetone (Analar Reagent) was cooled at 4°C one day before starting the precipitation. The culture supernatant sample was identical to that used for salting-out and was treated by the same manner before precipitation with acetone. The whole enzyme solution was kept in an ice bath; a certain volume of acetone was added slowly while stirring until the required concentration was reached. After removing the precipitated fraction by centrifugation at 5000 rpm at 4°C for 15 min, in a cooling centrifuge, further acetone was added to the supernatant fluid and the process was repeated until the acetone reached a final concentration of 80 %. Several enzyme fractions were thus obtained (35%, 50%,65%,75% and 85%). Acetone fractions were dried over anhydrous calcium chloride under reduced pressure at room temperature and then dialyzed against distilled water.

Fractional precipitation with ethanol

Absolute ethanol (A.R.) was cooled at 4°C overnight and precipitation with ethanol was

performed using the same procedure as that of acetone precipitation. The protein contents and enzymatic activities of each targeted enzymes were measured.

RESULTS

Seven bacterial isolates were obtained through isolation process from different keratin-rich substances. They were screened on agar plates containing feathers meal as substrate. Selection was based on size of clear zone formed around colonies. Isolate showing the largest clear zone was thus selected for further experiments. Cells were Gram positive spore former straight rods, aerobic and motile.

Effect of substrate type on *B.subtilis* DALEX keratinolytic activity using solid state fermentation

Data in Figure 1 show that cells were able to grow and utilize rabbit fur, wool and chicken feathers as sole carbon source. Maximum activity (1.26 U/mg protein) was recorded after 4 days of incubation with feather carbon source.

Keratinolytic activity under static and shaken cultures

Enzyme activity in submerged cultures was investigated under static and shacked conditions. Data in Fig. 2 reveal that after 3 days of

Table 1. Screening for growth factors affecting keratinase activity by *B.subtilis* DALEX and their levels in Plackett-Burman design

Factors (g/l)	Symbol	Level		
		-1	0	1
NH4Cl	NH	0.3	0.5	0. 7
K_2HPO_4	K_2	0.1	0.3	0.5
$KH2PO_4$	K	0.2	0.4	0.6
NaCl	Na	0.4	0.5	0.6
MgCl	M	0.75	0.1	0.125
Yeast extract	YE	0.05	0.1	0.15
Feathers	F	5	10	15

Table 2. The applied Plackett- Burman experimental design for seven cultural variables and their keratinase activity results

Trials	Different factors							Keratinase activity
	NH	K_2	K	Na	M	YE	F	(U/mg protein)
1	-1	-1	-1	1	1	1	-1	1.14
2	1	-1	-1	-1	-1	1	1	1.29
3	-1	1	-1	-1	1	-1	1	1.46
4	1	1	-1	1	-1	-1	-1	1.09
5	-1	-1	1	1	-1	-1	1	1.23
6	1	-1	1	-1	1	-1	-1	1.18
7	-1	1	1	-1	-1	1	-1	1.19
8	1	1	1	1	1	1	1	1.26
9	0	0	0	0	0	0	0	1.43

Table 3. Statistical analysis of the Plackett-Burman experiment by using feathers under shaken conditions

Variable	Keratinase specific activity(U/mg protein)	
	Main effect	t-value
NH	-0.05	-0.59
K_2	+0.04	+0.47
K	-0.03	-0.35
Na	-0.1	-1.31
M	+0.06	+0.72
YE	-0.02	-0.23
F	+0.16	+2.84

The Plackett–Burman experimental design matrix for 9 trials at two levels of concentration for each variable with three center points along with the respective experimental responses are given in Table 2.

The main effect of each variable upon enzyme production was estimated and graphically presented in Fig 3. A main effect figure 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. Data indicate that the presence of high levels of K_2HPO_4,

shaken incubation ,the activity reached a value of 1.35 U/mg protein which is higher than static condition (1.28 U/mg protein) and also higher than the value obtained with SSF after 4 days (1.26 U/mg protein).Thus,optimization of culture conditions was carried in submerged fermentation in shaken flasks.

Application of Plackett-Burman for the determination of factors affecting the production of keratinase by *B.subtilis* DALEX

The examined levels of 7 culture variables are represented Table 1**.**

Table 4. Production of keratinase enzyme by entrapped (in alginate and agar) and immobilized (adsorbed) *B. subtilis* DALEX cells in different support materials

		Keratinase activity (U/mg protein)	Protein content (mg/ml)
Gel materials	Agar2.5	1.52	0.894
Volume of gelmixture	5	1.56	0.928
(ml/50mlmedia)	7.5	1.57	0.931
	10	1.58	0.982
	Alginate2.5	1.31	0.216
	5	1.81	0.612
	7.5	1.82	0.614
Support materials	Sponge	1.70	0.615
	Pumice rock	1.72	0.832
	Clay	1.50	0.206
	Luffa	1.83	0.968
	Free cells	1.33	0.322

Table 5. Fractional precipitation of keratinase from *B. subtilis* DALEX cultures with ammonium sulphate, acetone, and ethanol

Purification Agent	Concentration %	Protein content (mg/ml)	Recovered protein (%)	Keratinase activity (U/mg protein)	Recovered activity (%)
Culture filtrate		140.05	100	1.33	100
Ammonium sulfate	25	12.01	6.23	0.67	2.80
saturation	35	7.26	3.77	1.18	2.98
	50	6.93	3.60	0.75	1.81
	60	11.42	593	1.19	4.73
	75	18.35	9.52	1.22	7.80
	85	18.64	9.67	0.81	5.26
	Total	74.61	38.72	-	25.39
Acetone	25	13.93	7.23	1.14	5.53
	35	15.12	7.85	1.17	6.16
	50	18.11	9.40	1.20	7.57
	60	19.45	10.09	1.24	8.40
	75	21.67	11.25	1.25	9.44
	85	23.26	12.07	1.28	10.37
	Total	111.54	57.89	-	47.46
Ethanol concentration	25	8.15	4.23	0.89	2.53
	35	9.22	4.78	1.07	3.44
	50	11.03	5.74	1.09	4.20
	60	12.45	6.46	1.13	4.90
	75	15.60	8.10	1.14	6.19
	85	13.12	6.81	0.92	4.20
	Total	69.61	36.12	-	25.46

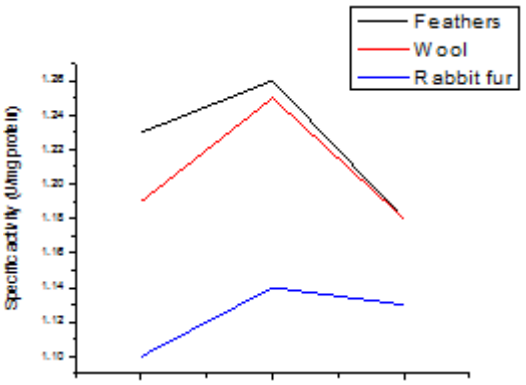


Fig. 1. *B.subtilis* **DALEX** keratinase activity using different substrates as carbon source in solid state fermentation.

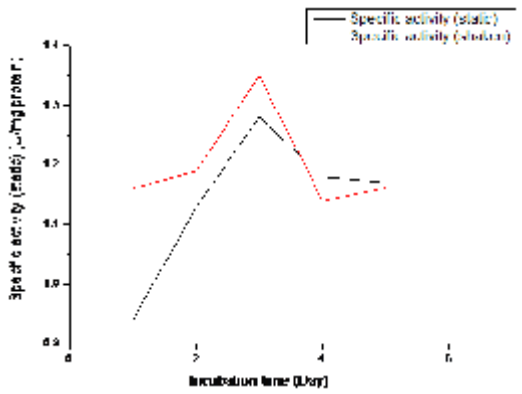


Fig. 2. *B. subtilis* **DALEX** enzyme activity in shaken and static cultures using chicken feather as a substrate. Cells were incubated in FBM aliquots and incubated at 37 °C

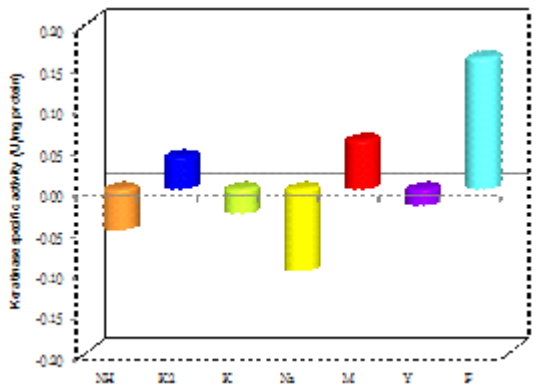
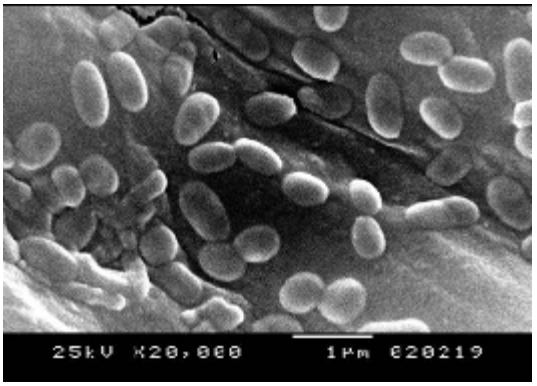
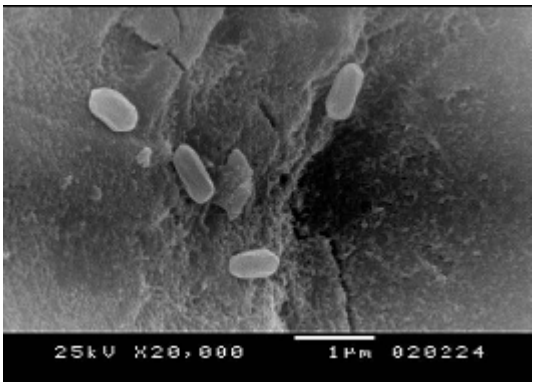


Fig. 3. Elucidation of fermentation factors affecting enzyme activity under shaken conditions when using feathers as a substrate





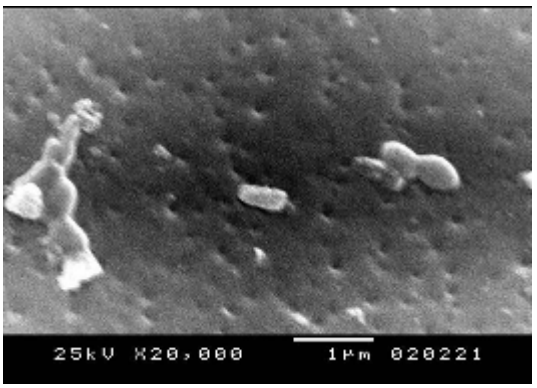


Fig. 4. Scanning electron micrographs of *B. subtilis* DALEX cells immobilized in a) luffa pulp, b) pumice and c) clay

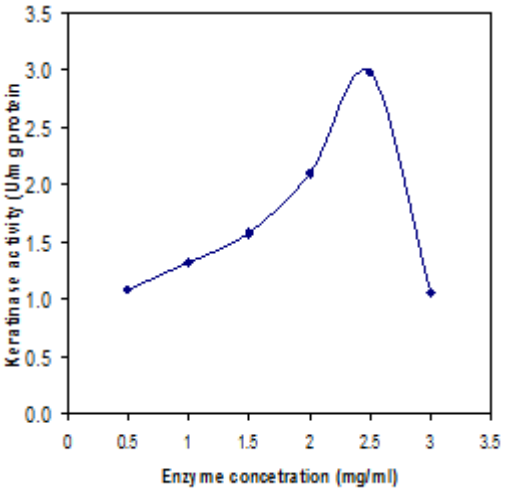


Fig. 5. Effect of enzyme concentration on the activity of the semi-purified keratinase preparation obtained from *B. subtilis* DALEX.

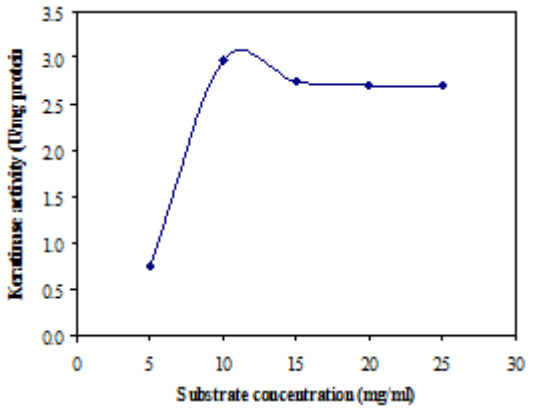


Fig. 6. Effect of substrate concentration on the activity of partial purified keratinase preparation obtained from *B. subtilis* DALEX

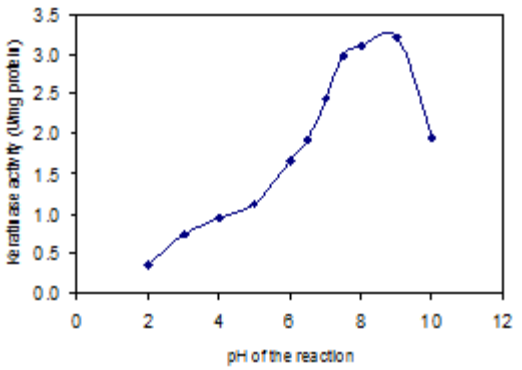


Fig. 7. Effect of pH of the reaction on the activity of the partial purified keratinase preparation obtained from *B. subtilis* DALEX.

Table 6. Thermal stability of the semi-purified keratinase obtained from *B. subtilis* DALEX

Temperature °C	Time of exposure(min)	Specific activity (U/mg protein)
Control	0	3.22
37	1530	3.223.22
50	1530	3.223.00
60	1530	2.982.84
70	1530	2.111.20
80	1530	0.290.17

$MgCl.6H_2O$, and feathers in the growth medium affect keratinase production by *B. subtilis* DALEX positively. This means that these variables should be present in their high level. On the other hand, the same figure suggested that the presence of NH_4Cl, KH_2PO_4, NaCl, and yeast extract at their lowest levels would result in high keratinase activity.

Statistical analysis shown in Table 3 was done using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

According to this suggestion dictated by the applied Plackett-Burman experiment it can be predicted that the optimum medium for producing an extracellular keratinase from the culture of *B. subtilis* DALEX with a relatively high activity should be (g/l): NH_4Cl, 0.3; NaCl, 0.4; K_2HPO_4, 0.5; KH_2PO_4, 0.2; MgCl- $6H_2O$, 0.125; yeast extract, 0.05; and inoculum size 1ml .

Production of extracellular keratinase by immobilized *B. subtilis* DALEX

B.subtilis DALEX cells were immobilized using different methods to compare enzyme activity with free cells. Fig 4 shows a scanning electron micrograph of *B.subtilis* DALEX cells immobilized in different supports.

The results represented in Table 4 show that the enzyme activity produced by immobilized cells entrapped in alginate was higher than that produced from cells entrapped in agar, also higher than free cells. The results also show that the enzyme activity of immobilized cells adsorbed on luffa pulp was higher than pumice, sponge cubes, clay particles, or free cells.

Partial Purification of extracellular keratinase produced by *B. subtilis* DALEX

The results in Table 5 show the fractional precipitation with different concentrations of

ammonium sulphate, acetone and ethanol .As shown in the table ,purification with 86% acetone recovered the best activity (10.37 %) compared to the 7.80% highest recovered activity obtained when using ammonium sulfate and 6.19% recovered activity when using ethanol by 75% solvent.

Properties of semi purified keratinase

Effect of different enzyme concentrations at constant substrate

The concerning effect of enzyme protein concentration on keratinase activity is given in Figure 5. It was observed that a parallel relationship occurred between the enzyme concentration and keratin hydrolysis. However, maximum specific activity was obtained at an enzyme protein concentration of 2.5 mg/ml reaction mixture. Lower protein concentration showed a decrease in the enzymatic activity. Therefore, this enzyme concentration was used in the next experiment.

Effect of substrate concentration

The result illustrated in Fig. 6 indicates that optimum substrate concentration for the partial pure enzyme was 10 mg/reaction mixture. At this concentration, the highest activity was recorded. Further increase of the substrate concentration yielded slightly lower enzyme activity. According to the previous results, a substrate concentration of 10mg/reaction was used in the next experiment.

Effect of pH of the reaction

The result in Fig. 7 indicates that the optimum pH value for the partial pure enzyme was 9.0 where the highest specific activity was obtained. Higher or lower pH values showed an adverse effect on the activity and the lowest activity was obtained at pH 2.0. However, the enzyme showed a relatively high activity in a pH range from 8.0 to 9.0.

Thermal stability

The results recorded in Table 6 indicate that the stability of the partial pure enzyme in the absence of keratinase depends on temperature of heating and the time of exposure. The enzyme was stable and its optimum temperature was 50°C. after 15 min. When exposed to 80°C for up to 30 min, the enzyme lost 94.7% of its original activity.

DISCUSSION

In the present study seven bacteria were isolated from keratin rich substrates. The most promising isolate capable of producing protease when grown on skimmed milk agar and keratinase using feathers as a sole carbon and nitrogen source was identified as *Bacillus subtilis*. *Bacil-lus* species are one of the best keratinase producers that have high enzyme secretion capacity (Ana Maria *et. al*.,2013, Shankar *et.al*.,2013).

B.subtilis DALEX was tested on different keratin containing wastes (feather meal, wool, fur of rabbit) under solid state fermentation. Chicken feathers proved to be the best substrate yielding a maximum enzyme activity (1.26 U/mg protein) after 4 days of incubation. In accordance with ourresults those obtained by *Bacillus subtilis* RM-01 using chicken feather supplemented with maltose and sodium nitrite as a substrate (Rai *et.al*., 2009). It was illustrated that keratinolytic potential of tested microorganisms, regardless of their significant dissimilarity in degradation mode, was dis-tinctly directed against ²-keratin of feathers rather than ±-keratin of hair, wool or bristle (Laba and Rodziewicz, 2014). Solid state fermentation was employed for keratinase production by other researchers (Ghribi *et.al*., 2012 ; de Siqueira *et. al*., 2014). As elucidated by Mazotto *et.al*. (2010), keratinic substrate pretreatment could be of significant importance in terms of improving keratinase production. Specifically, applica-tion of feather meal instead of raw feathers in cultures of two *B. subtilis* strains resulted in evident enhancement of keratinase biosynthesis, accompanied with elevated level of soluble proteins. In this study feather meal was used to improve enzyme yield.

Thus, in our study SSF was employed and compared to submerged fermentation (shaken and static).The shaking speed at 150 rpm yielded the maximum keratinase production after 3 days of shaken incubation. The activity reached a value of 1.35 U/mg protein which is higher than that at static conditions (1.28 U/mg protein) and also higher than the value obtained with SSF after 4 days (1.26 U/mg protein).

The use of statistical model to optimize culture medium components and conditions has increased in present day biotechnology due to its easy applicability, reliability and validity. To develop a process for maximum production of keratinase from poultry feather, standardization of media components is crucial. Statistical optimization of Keratinase production by *Bacillus*

cereus (Sivakumar *et.al*., 2011) and *B.subtilis* (Gupta and Singh, 2011) was investigated. In this work the significant variables necessary for the enhancement of Keratinolytic enzyme production was selected using Plackett-Burman design. The Plackett – Burman experimental design (Plackett and Burman, 1946) proved to be a valuable tool for the rapid evaluation of the effect of the various medium component. Under our experimental condition, it was deduced that K_2HPO4, MgCl and Feathers 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).

The biosynthesis of keratinase by *B. subtilis* DALEX immobilized by entrapment in different gel materials was studied. The gel materials tested, alginate gel polymer and agar were the effective ones for production of keratinase. Utilization of bacterial cells in gel matrix, the results showed that keratinase activity with immobilized cells was higher than that of free cells in general. The results 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). 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).

The enzyme was partially purified by using ammonium sulphate, acetone and ethyl alcohol precipitation. Purification with acetone was the best, as recovered activity of 10.37 % and 1.28 U/mg protein specific activity were obtained upon using 85% solvent, thus showing that acetone precipitation is the most suitable method for keratinase purification. Ali *et.al*., (2011) and Suntornsuk *et.al*.,(2004) purified keratinase from *B. licheniformis* FK 14 with 86 fold purification, specific activity of 218 U/mg and 25% recovery. Among all the obtained fractions, the 85% acetone fraction showed the highest specific activity; therefore it was selected for further experiments.

The optimum pH value for the partial pure enzyme was 9.0 where the highest specific activity was obtained. The thermal stability of the partial purified enzyme preparation was studied at pH 9.0.The enzyme was stable and its optimum temperature was 3.33°C for 30min. The maximum activity of the keratinase from *B. subtilis* **DALEX** was observed at pH 9.0 and 50ºC for 15 min. Rozs *et.al.*, 2001 and Saibabu *et.al*., 2013 reported maximum keratinase activity at pH 8.5 and 50ºC with *B. licheniformis* K-508; pH 10 and 40ºC was reported by (Bernal *et.al*., 2006) with *K. rosea*.

CONCLUSION

The results of the present investigation collectively indicate the possibility of using free or immobilized cells of *Bacillus subtilis* DALEX for the production of a relatively highly active keratinase preparation, which can be applied in many fields such as feed stuff, fertilizers detergent applications, and leather industry. Also indicate the possibility of using different keratin containing wastes like feather, wool, fur of rabbit, as a sole carbon and nitrogen source for production of keratinase by *B. subtilis* DALEX.

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