J. Agric. Sci. Mansoura Univ., 27(6): 4097 - 4113, 2002

OPTIMIZATION FOR BACTERIAL KERATINASE PRODUCTION IN A LOW COST MEDIUM

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ABSTRACT

In order to improve the production of bacterial keratinase utilizing the poultry feathers as an environmental waste, some nutritional and environmental factors were considered in a low cost medium. These factors are substrate concentration (chicken feather), pH of the cultural medium, growth temperature, inoculum size and agitation effect as well. These experiments were carried out using two bacterial strains namely *Bacillus licheniformis* CF-26 and *Bacillus licheniformis* PWD-28. Obtained results showed that both tested strains produced the maximum keratinase with 2% feather as carbon and nitrogen sources after the 9th day of fermentation. The maximum production of keratinase was found at pH value of 8.0 for *B. licheniformis* CF-26, while pH 9.0 was the best for *B. licheniformis* PWD-28. For the growth temperature at pH 7.0 of the strains No. CF-26, the keratinase activity was 3-5 fold higher at 40°C than that obtained at 55°C. At pH 9.0, opposite results were obtained by 1.9 fold. In case of the strain No. PWD-28, the production of keratinase at 40°C was lower at pH 7.5 than its value obtained at pH 9.0 by 1.2 fold after the 3rd day of fermentation. The inoculum size of 10% was the optimal for the two tested bacterial strains. In addition, the static cultivation gave higher value of keratinase activity than that of shake culture. The growth of bacterial strains on chicken feather was also followed by measuring the activity of proteinase, the total soluble proteins and the obtained free amino acids as well.

INTRODUCTION

Feedstuffs industry in Egypt is experiencing high-cost production, since raw materials for animal feeds are imported at considerably large quantities and high values of hard currency. Meanwhile, poultry processing plants generate feather (keratinaceous origin) in abundant volume as a waste every day. Keratin scleroproteins are produced by mammals and birds. This group of protein are very stable against the proteolytic enzymes action. An extreme low solubilities of native keratin makes enzymatic degradation hard to occur. The rigid structure of keratins in feather, hair, fur, and nails in attributable to their high content of cysteine (Thomas *et al.*, 1995). Because of the low degradation rate of keratin, this waste represent an environmental problem. Disposal of the waste is made complicated by the fact that spontaneous anaerobic degradation also produces obnoxious odor. Natural keratin degrading microbes have been isolated and tested for their biological activity (Scatt, 1993; El-Fadaly *et al.*, 1996; Friedrich and Antranikian, 1996; Lin *et al.*, 1996 and El-Fadaly and Zaied, 1999). The purpose of this investigation is to optimize for keratinase production by locally bacterial strains with exceptionally high keratin degrading activity converting a grim environmental burden into a marketable product containing high essential amino acids.

MATERIALS AND METHODS

I. Materials:

I.1. Chicken feather (CF):

Chicken feather (CF) samples were collected from private shops of chicken slaughtering, local market of Mansoura City, Dakahlia Governorate, Egypt. Samples were then mixed, washed twice, milled and dried at 60°C to constant weight.

I.2. Cultivation medium:

The prepared basal salts-chicken feather liquid culture medium was used for fermentation and enzyme production as recommended by El-Fadaly (1996). The ingredients of this medium were (gL^{-1}): potassium chloride, 0.2; ammonium dihydrogen phosphate, 1.0; magnesium sulphate, 0.2 and dried feather, 20. The value of pH was adjusted to 7.0.

1.3. Bacterial strains and growth conditions:

The bacterial strains used in this research work are *Bacillus licheniformis* CF-26 and *Bacillus licheniformis* PWD-28. These strains were locally isolated and identified by El-Fadaly *et al.* (2002).

II. Methods:

II.1. Bacteriological procedures:

Maintenance of isolates:

The tested isolates were maintained on NA slants at 5°C till demand. Prior to use, the microbial cultures were transferred to NA slants and reincubated again at appropriate temperature (37°C) for 24 hr. This process was repeated twice.

Preparation of standard inoculum:

Standard inoculum of each bacterial strain used was prepared by scraping the growth from the surface of nutrient agar slant in the presence of 5 ml of sterilized distilled water with the aid of platinum loop. Aliquot of desired volume of homogenized bacterial suspension was diluted up to 6 x 10^3 cfu/ml, to be used as a standard inoculum during the experimental work.

Working flasks preparation:

Six groups were prepared, each contains four flasks (three replicates and one as control) for each bacterial strain. Fifty ml of basal cultivation medium were dispensed in 250 ml Erlenmeyer flasks, then supplemented with 1% dried feather and autoclaved at 121°C for 20 min after adjusting the pH to 7.2.

Fermentation procedure:

For fermentation process, the autoclaved flasks were then inoculated with appropriate inoculum size of appropriate dilutions of 24 hr old bacterial suspension (6 x 10^3 cfu/ml). The incubation was then carried out under static conditions at 37°C. During 9 days incubation period, one group of the prepared flasks was taken every 3 days as a representative sample. For sample analysis, Keratinase activity (KA, KU/ml), proteinase activity (PA, TU/ml), total soluble peptide content (TSP, ppm), and total free amino acids

(FAA, ppm) were measured. Three replicates were analyzed and the mean value was recorded.

II.2. Biochemical analysis:

Keratinase activity measurement (KA):

The activity of Keratinase was measured after Nickerson *et al.* (1963) using pure keratin (K .0253, Sigma Co., USA). A unit of Keratinase activity was defined as that amount of enzyme in one ml of cultural filtrate that produce 1.00 μg protein in 2 hr as a product of Keratin hydrolysis. Bovine serum albumin (BSA) was used as a standard.

Protein estimation (TSP):

Total soluble protein was colorimetrically determined at 750 nm as described by Lowry *et al.* (1951) using Carl Zeiss Jena Spekol 11 colorimeter. Reference curve by using BSA (0.05 - 0.5 ml) was carried out through whole procedure.

Quantitation of proteinase activity (PA):

This assay was adopted using the modified casein digestion method described by Lupin *et al.* (1982). A unit of proteinase activity was defined as that quantity of enzyme, which produced TCA - soluble fragments giving blue color equivalent to 1.0 μg tyrosine under the assay conditions. A tyrosine calibration curve was set up.

Determination of total free amino acids (FAA):

The colorimetric method used by Lee and Takahashi (1966) was adopted. Reference curve was prepared by using glycine (10-100 μg) as a standard amino acid.

III. Statistical analysis:

All experimental data were subjected to the statistical analysis by the analysis of variance. The treatment means were compared at 0.05 and 0.01 probability levels using the Least Significant Difference (L.S.D.) method as mentioned by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Optimum percentage of feather:

In order to find out the convenient percentage of feather as keratin source to be used for keratinase production, the used basal medium was supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% of chicken feather. Obtained data are presented in Table (1). It could be seen that 2% feather was the optimum for the two examined bacterial strains, taking the 9th day of fermentation, into account. The use of more than 2% of dried feather as nitrogen and carbon source, do not increase the values of measured parameters either total soluble protein (TSP) or free amino acids (FAA). Interestingly, these values as a result of the keratinase activity start to decrease and/or increase by little differences, which can be neglected during the examined interval time. Kunert (1976) reported that the greatest percentage loss substrate was found in cultures with a small amount of hair in a large volume of medium.

Table (1): Effect of feather percent added to the cultivation medium on keratinase production.

Bacterial Strain	Feather (%) M_5	Total soluble protein (ppm)at incubation period (day) of 3	6	9	F-test	L.S.D. 0.05	0.01	Free amino acids (ppm) at incubation period (day) of 3	6	9	F-test	L.S.D. 0.05	0.01
B. licheniformis CF-26	0.5	0.123	0.129	0.205	NS			0.336	0.600	1.182	**	0.01	0.02
	1.0	0.162	0.153	0.126	**	0.002	0.003	0.673	1.697	1.932	**	0.02	0.03
	1.5	0.111	0.250	0.190	**	0.004	0.007	0.740	1.956	2.156	**	0.13	0.21
	2.0	0.120	0.249	0.273	**	0.008	0.014	1.380	2.820	2.750	**	0.03	0.04
	2.5	0.190	0.217	0.108	**	0.005	0.008	2.210	2.292	2.410	NS		
	3.0	0.165	0.246	0.256	**	0.002	0.003	2.020	2.450	3.176	**	0.36	0.59
	F-test	**	**	**				**	**	**			
	L.S.D.												
	0.05	0.005	0.003	0.004				0.025	0.31	0.07			
	0.01	0.006	0.005	0.006				0.036	0.44	0.09			
B. licheniformis PWD-28	0.5	0.12	0.11	0.13	**	0.005	0.009	0.346	0.514	1.097	**	0.005	0.008
	1.0	0.17	0.20	0.16	**	0.004	0.007	0.482	1.897	1.239	**	0.020	0.030
	1.5	0.22	0.23	0.21	**	0.007	0.011	0.645	2.560	1.030	**	0.030	0.050
	2.0	0.36	0.22	0.24	**	0.009	0.020	2.008	6.230	6.333	**	0.710	1.180
	2.5	0.25	0.29	0.24	**	0.007	0.012	1.392	2.416	1.130	**	0.040	0.060
	3.0	0.28	0.27	0.24	NS			0.114	2.910	0.374	**	0.050	0.080
	F-test	**	**	**				**	**	**			
	L.S.D.												
	0.05	0.035	0.002	0.005				0.010	0.030	0.400			
	0.01	0.050	0.003	0.007				0.020	0.040	0.600			

Williams *et al.* (1990) found that the maximum yield of FAA by *B. licheniformis* were 120, 110, 30 mM in case of feather / liquid culture ratio of 1/2, 1/4 and 1/8, respectively. Naguib *et al.* (1982) noticed that the level of keratin might indicate the existence of a certain threshold concentration of substrate for the growth and welfare of the organisms. Above which the excess keratin may be just hydrolyzed to soluble components by the produced enzymes without being used as building blocks for the organism. Hussein *et al.* (1986) stated that *Micropolyspore keratinolytica* consuming chicken feather had to hydrolyze it into proteins then to amino acids. These amino acids are used either as it is or in deaminated form to produce keto acids, which used as carbon source. The statistical analysis applied suggested the significant differences between all parameters under investigation either in feather ratio or the examined time of fermentation, except some cases as shown in the same Table (1).

Effect of initial pH value:

The initial pH of the cultivation media was adjusted before sterilization to different values by addition 0.1 N of HCl or NaOH. Recorded data in Table (2) show the experimental values of keratinolytic activity and related end-product, total soluble proteins together with the measured values of proteinase activity and closed end-product, total free amino acids produced by *B. licheniformis*, CF-26. Listed data illustrated that the optimum production of keratinase (KU/ml of cultural filtrate) was found after the 6th day of fermentation at pH value of 8.0.

Table (2): Effect of initial pH value on the keratinase production by *B. licheniformis*, CF-26.

Initial	Keratinase activity, Ku/ml at incubation periods (day) of			F-	L.S.D.		Proteinase activity, Tu/ml at incubation periods (day) of			F-	L.S.D.	
Ph	3	6	9	test	0.05	0.01	3	6	9	test	0.05	0.01
7.0	6.45	6.27	3.70	**	0.08	0.13	1.44	9.80	4.50	NS		
7.5	7.66	7.47	5.17	**	0.07	0.12	4.08	12.90	2.90	**	0.40	0.66
8.0	6.94	9.90	6.97	**	0.04	0.07	11.3	3.90	9.90	**	0.24	0.41
8.5	9.50	9.09	5.82	**	0.05	0.09	1.21	6.95	14.81	**	0.17	0.28
9.0	8.30	4.98	6.96	**	0.09	0.15	8.45	6.06	2.54	**	2.70	4.50
F-test	**	**	**				**	**	**			
L.S.D.												
0.05	0.05	0.08	0.07				0.39	1.55	0.31			
0.01	0.08	0.11	0.10				0.57	2.26	0.45			
Initial	Total soluble protein (ppm) at incubation periods (day) of			F-	L.S.D.		Free amino acids (ppm) at incubation periods (day) of			F-	L.S.D.	
pH	3	6	9	test	0.05	0.01	3	6	9	test	0.05	0.01
7.0	0.11	0.12	0.14	NS	0.001	0.002	0.625	0.770	0.579	**	0.003	0.005
7.5	0.18	0.20	0.31	**	0.002	0.004	1.108	1.070	1.008	**	0.004	0.006
8.0	0.24	0.20	0.22	**	0.004	0.010	2.017	2.070	1.650	**	0.005	0.009
8.5	0.18	0.22	0.22	**			1.164	0.822	0.858	**	0.003	0.005
9.0	0.21	0.32	0.36	NS			1.995	1.060	1.685	**	0.005	0.008
F-test	**	NS	**				**	**	**			
L.S.D.												
0.05	0.002		0.001				0.003	0.004	0.003			
0.01	0.003		0.002				0.004	0.006	0.005			

The values of keratinase decreased after the 9th day of the fermentation period. Data also exhibited that the enzyme has activity at pH range between 7.5 and 8.5. Abdel-Hafez *et al.* (1995) found that the highest keratinase activity was obtained by *Thermoactinomyces vulgaris* when the initial pH value of the medium was adjusted to 7.5.

Obtained data exhibit very interesting observation, since three different enzymes are found. The first one is at pH 7.9 being 11.3 TU/ml of cultural filttrate, then decreased to 1.2 enzyme unit and then increases again to 8.45 enzyme unit. The second one is at pH value of 7.4 to 7.5 being 12.8 TU/ml of cultural filtrate then decreases to 3.9 enzyme unit then go further in steady state. The third one is clear at pH 8.3 with value of 14.8 then the value sharply decreased to about 2.54 enzyme unit. This suggest that this bacterium *B. licheniformis*, CF-26 produces three different types of proteolytic enzymes. Meanwhile, the first enzyme is produced at the 3rd day of fermentation, the second one is after the 6th day, while the third enzyme is obtained after the 9th day (Table 2).

At all levels of examined pH values during the fermentation periods, the values of total soluble protein (TSP) gradually increase with the fermentation period increase and/or still at the same level. Even so, the values of TSP seem to be little which can be explained by that component, represents the substrate for proteinases enzymes. El-Mayergi and Smith (1971) found that the resultant curves for production of keratinase by *Streptomyces fradiae* in feather meal appeared to be diphasic suggesting that action on feather keratin involved the production of adaptive enzymes.

For the amino acids, on the other hand, the same trend was found, since the obtained values still in the same level after the third day of fermentation as can be seen in the same Table.

For keratinase activity, the second strain *B. licheniformis*, PWD-28 showed complete different behaviour compared to that of *B. licheniformis*, CF-26. (Table, 3). Listed data illustrate that good enzymatic activity was found at pH 7.0 after the three examined intervals times. Moreover, at pH 9.0, the enzymatic activity was higher than that found at pH 7.0. This mean that the activity of keratinase was higher at alkaline media than that of neutral one by 1.1, 1.3 and 1.5 fold at the 3rd, 6th and 9th day of fermentation. Regarding the proteolytic activity, again the two tested strains exhibited different behaviour in their activities. Data in Table (3) show three proteolytic enzymes in the neutral range, since the first one is obtained at pH 7.5 after the third day of fermentation. The second and the third enzymes are obtained after the 6th and the 9th day of fermentation, both of them are falled at pH 7.5.

Concerning the total soluble proteins (TSP) and total free amino acids (FAA), the values produced by *B. licheniformis*, PWD-28 showed to be in the same levels of that obtained by the other tested strain. Values of TSP show that the values decrease after the 6th day of fermentation in case of pH 7.0 and 7.5. In case of pH 8.5, the values of TSP seems to be constant after the 3rd day of fermentation. When applying the initial pH at 8.5 and 9.0, the values of TSP go further to the 9th day at increase values up to the 9th day of the fermentation process. Opposite results were obtained by Abdel-Hafez *et al.* (1995) who found that the increase or decrease the initial pH of the cultural

medium to 9.5 or to 6.0 resulted in a considerable reduction in microbial growth and keratinase activity. In contrast, the values of free amino acids exhibited the same behaviour in all examined values of pH as can be seen in Table (3). The measured values of FAA go up to the 6th day, then decreased at the 9th day of fermentation. Hussein and Elakied (1989) found that *Thermoactinomyces keratinolyticus* hydrolyses chicken feather bringing its complete solubilization in five days and releasing 52% of its amino acids content in the cultural broth.

Because of the increase both TSP and FAA within the action of keratinase during the biodegradation of chicken feather as sole source of carbon and nitrogen, evidence that these two tested bacterial strains posses a potent proteinases activities. This observation, pointed out the existence of multienzyme system. This is in agreement of that of Nickerson and Durand (1963), who demonstrated that the purified keratinase transforms keratin to peptides, but it is unable to further degradation of such compound, that what proteinase do. Furthermore, results obtained could also suggest that the two tested strains are thermoalkalophilic bacteria according to Brock (1978). The statistical analysis applied suggested the significant differences between all parameters under investigation either for pH values or the examined time of fermentation, except some cases as shown in the same Table.

Effect of growth temperature:

To determine the optimum temperature of the bacterial growth and keratinolytic enzyme production, shake flask experiments were realized on two different temperatures, 40°C and 55°C each at 7.5 and 9.0 of pH value. Data obtained are listed in Table (4). Tabulated data show that the activity of keratinase (KU) was higher at 7.5 pH than that at pH 9.0 by 3.5 times at 40°C after the third day of fermentation. Opposite result was found after the 6th day since 1.2 increase fold was found at pH 9 than that at pH 7.5 at 40°C. Regarding the total soluble protein (TSP), it could be seen that TSP value was higher by 1.2 fold at pH 7.5 than that obtained at pH 9.0. At the 6th day of fermentation, the value of TSP at pH 9.0 was higher than that at pH 7.5 by 1.14. Concerning the values of these two parameters at 55°C, the value of KU showed to be 1.9 times more at pH 9.0 than that value at 7.5, while opposite results were found to be higher at 7.5 than at 9.0 by 1.4 fold.

Table (3): Effect of initial pH values on the keratinase production by *B. licheniformis*, PWD-28.

Initial pH	Keratinase activity, Ku/ml at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01	Proteinase activity, Tu/ml at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01
7.0	11.40	8.65	7.61	**	0.11	0.18	6.36	11.06	24.41	*	12.5	20.70
7.5	8.00	7.49	7.59	**	0.15	0.25	10.73	8.27	9.34	**	0.32	0.53
8.0	6.60	10.57	7.27	**	0.27	0.46	3.03	5.44	12.04	**	0.35	0.57
8.5	6.41	8.61	9.58	**	0.15	0.25	6.70	4.10	2.31	**	0.64	1.07
9.0	12.89	10.96	11.70	**	0.10	0.17	0.90	3.75	1.98	**	0.41	0.67
F-test	**	**	**				**	**	**			
L.S.D.												
0.05	0.15	0.16	0.08				0.29	0.46	0.27			
0.01	0.22	0.23	0.12				0.43	0.67	0.39			

Initial pH	Total soluble protein (ppm) at incubation periods (day) of 3	6	9	F-Test	L.S.D. 0.05	0.01	Free amino acids (ppm) at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01
7.0	0.13	0.18	0.15	**	0.007	0.010	0.885	1.249	0.809	**	0.007	0.010
7.5	0.13	0.16	0.11	**	0.002	0.004	1.034	2.024	0.793	**	0.007	0.010
8.0	0.11	0.13	0.21	**	0.002	0.004	1.112	1.796	0.870	**	0.004	0.007
8.5	0.13	0.17	0.22	**	0.0005	0.001	1.550	2.260	1.081	**	0.010	0.020
9.0	0.24	0.21	0.20	NS			0.285	2.256	1.273	**	0.033	0.050
F-test	NS	NS	**				**	**	**			
L.S.D.												
0.05	--	--	0.004				0.018	0.013	0.001			
0.01	--	--	0.005				0.026	0.020	0.002			

Table (4): Effect of growth temperature on the keratinase production by *B. licheniformis*, CF-26.

Incub. Temp (°C)	Initial pH	Keratinase activity, Ku/ml at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01	Proteinase activity, Tu/ml at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01
40	7.5	6.00	11.98	**	1.48	3.40	0.338	0.223	**	0.020	0.05
	9.0	1.70	14.39	**	0.08	0.18	0.160	0.193	**	0.007	0.02
55	7.5	3.88	11.33	**	0.08	0.18	0.660	0.182	**	0.0001	0.0003
	9.0	7.25	8.20	**	0.06	0.13	0.588	0.217	**	0.020	0.04
	F-test	**	**				**	**			
	L.S.D.										
	0.05	0.03	0.04				0.004	0.01			
	0.01	0.05	0.05				0.006	0.02			
	Initial pH	Total soluble protein (ppm) at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01	Free amino acids (ppm) at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01
40	7.5	2.240	2.01	**	0.07	0.17	1.230	3.390	**	0.10	0.24
	9.0	1.800	2.30	NS			1.540	3.276	**	0.11	0.25
55	7.5	0.488	1.14	**	0.01	0.02	3.326	3.176	**	0.06	0.15
	9.0	0.530	2.26	**	0.40	0.80	3.255	1.504	**	0.15	0.35
	F-test	**	**				**	**			
	L.S.D.										
	0.05	0.16	0.32				0.06	0.05			
	0.01	0.25	0.48				0.10	0.07			

For the proteinase activity (TU/ml of cultural filtrate), the results show that the activity in case of pH 7.5 more than its value obtained at pH 9.0 when the microbial growth was carried out at 40°C. The total free amino acids was 1.3 higher at pH 9.0 than that of pH 7.5 after the 3rd day. After the 6th day of fermentation, opposite observation was found since the value of FAA was higher at pH 7.5 than that of pH 9.0 by 1.0 time. Meanwhile, results of the statistical analysis showed significant differences between different treatments performed and examined times as well. The least significant differences were also calculated at two different levels as shown in the same Table.

The effect of temperature was also examined for the second strain *B. licheniformis*, PWD-28 and obtained results are listed in Table (5). Tabulated data show that keratinase activity (KU/ml) was lower at pH 7.5 than its value obtained at pH 9.0 as initial pH of cultivation medium at 40°C by 1.2 time after the third day of incubation. The same enzyme shows opposite result after the 6th day of fermentation at 40°C by 1.2 time. At 55°C, the results in Table (5) are also differed according to the initial pH of the cultural filtrate for either proteolytic enzyme or free amino acids obtained. Topiwala and Sinclair (1971) noticed that increasing of cultivation temperature may appreciably change the physical properties of the culture medium and hence indirectly affect the cell metabolism. The other effect of temperature is lowering solubility of oxygen, since at 70°C is 5% of that at 20°C.

Table (5): Effect of growth temperature on the keratinase production by *B. licheniformis*, PWD-28.

Incub. temp (°C)	Initial pH	Keratinase activity, Ku/ml at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01	Proteinase activity, Tu/ml at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01
40	7.5	5.35	9.45	**	0.08	0.18	0.152	0.532	**	0.06	0.14
	9.0	6.50	8.18	**	0.32	0.75	0.358	1.01	**	0.007	0.017
55	7.5	4.57	5.43	**	0.08	0.18	0.688	0.102	NS		
	9.0	5.28	11.97	**	0.08	0.18	0.552	0.012	**	0.012	0.03
	F-test	**	**				**	**			
	L.S.D. 0.05	0.10	0.08				0.03	0.01			
	0.01	0.16	0.12				0.04	0.02			
	Initial pH	Total soluble protein (ppm) at incubation periods (day) of 3	6	F-test	L.S.D. 0.05	0.01	Free amino acids (ppm) at incubation periods (day) of 3	6	F-Test	L.S.D. 0.05	0.01
40	7.5	2.050	1.590	**	0.01	0.03	1.948	1.217	**	0.01	0.03
	9.0	1.470	1.640	**	0.03	0.07	2.470	2.915	**	0.01	0.03
55	7.5	0.156	0.187	NS			2.519	0.229	**	0.01	0.02
	9.0	0.296	1.227	**	0.02	0.05	0.698	2.690	**	0.01	0.03
	F-test	**	**				*	**			
	L.S.D. 0.05	0.016	0.018				1.6	0.01			
	0.01	0.025	0.027				2.4	0.02			

Hussein and Swelim (1989) used *Micropolyspora keratinolytica* to bring out the complete solubilization of chicken feather converting it into soluble proteins, amino acids and ammonia. They obtained 129 units of keratinase per ml of cultural broth.

For the total soluble proteins (TSP) obtained at 40°C at the two examined time intervals, it could be noticed that the produced TSP at 6th day of fermentation was lower than that of 3rd day by 1.3 time (pH 7.5). In contrast, the TSP value obtained at pH 9.0 was higher after the 6th day by 1.1 higher than that obtained after the 3rd day of fermentation at the same temperature. Abdel-Hafez *et al.* (1995) found that keratinase activity increased and reaching the maximal values at the 4th day of fermentation.

At 55°C, the activity of keratinase was higher at 9.0 pH than that at 7.5 by 1.2 and 2.2 after the 3rd day and 6th day, respectively. The total soluble protein (TSP) obtained at the 3rd day of fermentation period was higher at pH 9.0 than its value at pH 7.5 by about 1.9. At the 6th day, the produced TSP at pH 9.0 was higher than its value at pH 7.5 by 6.6 time. Recorded data in Table (5) show also that TSP obtained at pH 7.5 as initial pH of the fermentation medium was not significant at 55°C, while it was highly significant for that obtained at pH 9.0. Williams *et ai.* (1990) found that the complete degradation of the keratin to TSP and FAA after 7 to 10 days of incubation at 50°C.

The experimental values of protinase activity (PA) were found to be better at pH 9.0 than the same values measured at pH 7.5 at 40°C for the 3rd day of examined fermentation period as shown in the same Table. The same trend was found for the total free amino acids (FAA) obtained under the same cultivation conditions. Both of PA and FAA showed to be highly significant either at the level of pH tested or at examined interval times.

All parameters tested were decreased at 55°C after the 3rd day of fermentation, except the value of FAA which went further to the 6th day. Statistical analysis values (Table 5) showed high significant differences for all, except the parameters of proteinase and TSP of 55°C at pH 7.5. Hussein and Elakied (1989) applied *Thermoactinomyces keratinolyticus* to reach complete solubilization of chicken feather in 9 days of incubation at 55°C.

Obtained results suggest that the two strains used in this investigation can be considered as facultative thermophiles according to the definition of thermophily (Sonnleitner, 1984). Abdel-Hafez *et al.* (1995) found that 96.3% of the total isolates of keratinolytic actinomycetes were facultative thermophiles.

The statistical analysis applied suggested the significant differences between all parameters under investigation either in growth temperature or the examined time of fermentation, except some cases as shown in the same Table.

Effect of inoculum size:

The inoculum size was examined and obtained results are listed in Tables (6 and 7) for the two tested strains. Tabulated data (Table 6) showed that 10% of inoculum size (6 x 10^3 cfu/ml) was the optimum with the first bacterium *B. licheniformis*, CF-26. Keratinase production was better after 6 days of incubation being 3.68 KU/ml of cultural filtrate. At the same time, the

measured value of TSP was 2.52 ppm of cultural filtrate. For the proteinase activity, On the other hand, its maximum yield was found after the same incubation period to be 40.43 TU/ml of cultural filtrate as shown in the same Table. Interestingly, the produced value of free amino acids (FAA) was correlated with corresponding value of proteinase at the same time (6 days fermentation) being 3.185 ppm of cultural filtrate. Snitsar *et al.* (1975) reported that after cleavage of disulfide bridges, hydrolyzate of keratin was readily digested by gastrointestinal enzymes, hence hydrolysis turned the protein to polypeptides and free amino acids. Data presented in Table (7) showed that 10% inoculum size gave 2.61 KU/ml of cultural filtrate, which correlated with the value of TSP to be 3.41 ppm of cultural filtrate after the 9^{th} day of fermentation. Obtained results by *B. licheniformis*, PWD-28 showed also significant differences between all the treatment expressed in inoculum size used and times examined as well. On the other hand, 89.32 TU/ml was obtained after the 9^{th} day of fermentation in case of 10% inoculum size, which met 3.0 ppm of FAA cultural filtrate. Again, the statistical analysis by F-test and LSD showed significant differences at all examined levels.

Table (6): Effect of inoculum size on the keratinase production by *B. licheniformis*, CF-26.

Inoculum size (%)	Keratinase activity, Ku/ml at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01	Proteinase activity at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01
2	0.18	1.89	0.53	**	0.16	0.36	18.74	27.47	12.19	**	1.2	1.9
4	0.20	2.62	1.47	**	0.07	0.12	23.49	25.16	15.40	**	1.5	2.5
6	0.28	2.29	1.82	**	0.05	0.08	34.65	14.89	6.03	**	1.6	2.6
8	0.57	1.68	0.92	**	0.05	0.09	26.70	39.79	2.44	**	1.3	2.2
10	1.15	3.68	1.52	**	0.07	0.11	5.78	40.43	16.3	**	1.6	2.7
F-test	**	**	**				**	**	**			
L.S.D.												
0.05	0.03	0.05	0.06				0.70	0.70	1.40			
0.01	0.04	0.07	0.09				1.91	1.04	2.01			

Inoculum size (%)	Total soluble protein (ppm) at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01	Free amino acids (ppm) at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01
2	0.909	2.051	2.241	**	0.009	0.016	1.523	1.660	1.718	NS	--	--
4	0.993	1.244	2.928	**	0.003	0.005	0.857	1.284	1.257	**	0.11	0.19
6	1.013	1.330	1.723	**	0.120	0.210	2.980	1.292	1.304	**	0.02	0.03
8	1.504	1.103	2.850	**	0.250	0.410	0.975	1.086	1.173	**	0.10	0.16
10	1.908	2.516	2.663	**	0.010	0.020	2.995	3.185	3.024	**	0.08	0.13
F-test	**	**	**				**	**	**			
L.S.D.												
0.05	0.008	0.070	0.014				0.030	0.480	0.400			
0.01	0.012	0.100	0.020				0.050	0.700	0.580			

Table (7): Effect of inoculum size values on the keratinase production by *B. licheniformis*, PWD-28.

Inoculum size (%)	Keratinase activity, Ku/ml at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01	Proteinase activity, Tu/ml at incubation periods (day) of 3	6	9	F-test	L.S.D. 0.05	0.01
2	0.12	2.18	1.05	**	0.97	1.61	29.00	29.52	12.84	**	2.4	4.03
4	0.06	1.99	1.59	**	0.15	0.24	28.11	32.34	18.99	**	1.3	2.10
6	0.61	2.78	2.14	**	0.08	0.13	46.59	40.81	8.99	**	0.8	1.40
8	0.70	2.78	1.51	**	0.05	0.08	27.34	17.84	68.40	**	0.9	1.60
10	1.15	2.55	2.61	**	0.15	0.26	67.38	40.04	89.32	**	1.6	2.60
F-test	**	**	**				**	**	**			
L.S.D.												
0.05	0.07	0.08	0.60				1.60	0.70	0.50			
0.01	0.09	0.12	0.94				2.30	1.09	0.72			
Inoculum size (%)	**Total soluble protein (ppm) at incubation periods (day) of 3**	**6**	**9**	**F-test**	**L.S.D. 0.05**	**0.01**	**Free amino acids (ppm) at incubation periods (day) of 3**	**6**	**9**	**F-test**	**L.S.D. 0.05**	**0.01**
2	0.861	0.829	1.655	**	0.02	0.03	3.321	2.366	2.303	**	0.05	0.08
4	0.876	1.223	1.614	**	0.01	0.02	3.300	2.347	1.057	**	0.05	0.09
6	0.872	1.239	2.364	**	0.02	0.04	3.437	3.418	1.083	**	0.06	0.10
8	1.464	0.853	0.736	**	0.01	0.02	3.234	3.057	1.122	**	0.06	0.10
10	1.331	2.601	3.405	**	0.009	0.01	3.237	3.431	3.009	**	0.07	0.11
F-test	**	**	**				**	**	**			
L.S.D.												
0.05	0.006	0.015	0.020				0.06	0.03	0.04			
0.01	0.009	0.020	0.0[illegible]				0.09	0.05	0.05			

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إنتاج الكيراتينيز البكتيرى فى بيئة منخفضة التكاليف

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لتحسين إنتاجية إنزيم الكيراتينيز بإستخدام ريشة الدجاج كمخلف بيئى ، تم دراسة بعض العوامل الغذائية والبيئية فى بيئة غذائية منخفضة التكاليف. هذه العوامل هى تركيز مادة التفاعل (ريشة الدجاج) ، رقم الأس الهيدروجينى للبيئة الغذائية ، درجة حرارة النمو ، حجم اللقاح المستخدم .تم دراسة هذه العوامل بإستخدام سلالتين بكتيريتين *Bacillus licheniformis* CF-26 and *Bacillus licheniformis* PWD-28. بينت النتائج أن كلتا السلالتين أنتجت أقصى نشاط إنزيمى فى حالة إستخدام ٢% ريشتين دجاج كمصدر كربون ونتروجين بعد تسعة أيام من عملية التخمير. كذلك وجدت أقصى إنتاجية للإنزيم عندما كان الأس الأيدروجينى ٨ ، ٩ بالنسبة للسلالتين CF-26 ، PWD-28 على الترتيب. أما بالنسبة لدرجة الحرارة فإن CF-26 أنتجت الكيراتينيز على ٤٠ °م أعلى من المنتج على ٥٥ °م بثلاث أضعاف وذلك على رقم أس هيدروجينى ٧ . أما عندما كان الأس الهيدروجينى ٩ فقد كانت النتيجة عكسية بفارق ١,٩ مرة. أما بالنسبة للسلالة الثانية PWD-28 فإن إنتاج الإنزيم على ٤٠ °م كان عند أس هيدروجينى ٧,٥ أقل من ٩ بفارق ١,٢ مرة بعد اليوم الثالث من التخمير. بينما على ٥٥ °م كانت البيئة عكسية حيث كانت ٩ أفضل من ٧,٥ بفارق ٦ مرات بعد ستة أيام تخمير. بالنسبة لحجم اللقاح المستخدم فقد كانت نسبة ١٠% هى المثلى لكلتا السلالتين المستخدمتين.