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Production of Keratinase Enzyme by Thermophilic *Streptomyces thermovulgaris*

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SEVENTEEN thermophilic actinomycete isolates were tested for their keratinolytic activities. Results indicate that five isolates have keratinase activity. *Streptomyces thermovulgaris* proved to be superior in keratinase formation and it was selected for the subsequent investigation.

The optimum conditions for keratinase production and feather solubilization of *S. thermovulgaris* were studied. Data revealed that *S. thermovulgaris* grew better with good yield of keratinase activity by growing it on the fermentation medium (100ml/500ml flasks), in which the nitrogen and the carbon sources were replaced with sterile chicken feather pieces (1.5g%), 1.0g% starch and 0.1g% K_2HPO_4, using tap water with a homogenized spore suspension (containing approximately $8.2x10^{-3}$ spores ml^{-1}) of 3 days old culture. Inoculated flasks were incubated at 50°C for 4 days under static conditions.

Keywords: Keratinase enzyme, Thermophibic streptomyces, *Streptomyces thermovulgaris.*

The thermoactinomycetes are found widely in nature and they can be easily isolated from different sources (Diab, 1978; Falkowski, 1978; Hamdi *et al.*, 1980; Wakisaka *et al.*, 1982; Lu & Yan 1983; Karwowski, 1986 and Abdel-Hafez *et al.*, 1995).

Thermophilic actinomycetes produce extracellular keratinases which degrade different keratinaceous wastes (Brigitte & Rudolf, 1997 and Ignatova *et al.*, 1999). Biodegradation of feather by microorganisms represents of a method for improving the utilization of feathers as a feed protein (Hussein & Swelim, 1989 and Williams & Shih, 1989), and amino acids as pure chemicals (Hussein & Elakied, 1989).

Several investigators reported that maximal production of keratinase occurs only under proper conditions of growth (Noval and Nickerson, 1959; Nickerson *et al.*, 1963; Harmon & Blank, 1968; Young & Smith, 1975; Hussein & Hamdy, 1989 and Abdel- Hafez *et al.*, 1995). The present investigation was planned to study the nutritional and environmental factors affecting keratinase production by a thermophilic *Streptomyces* strain.

Material and Methods

Microorganisms

A total of seventeen thermophilic actinomycetes previously isolated from different samples (compost, animal manure and soil) were tested for their keratinase activity. The isolates were identified according to the diagnostic key of Bergey's Mannual (1984) and the articles, of Shirling and Gottlieb (1968a, 1968b, 1969 and 1972).

Culture media

Keratinase activity of the tested isolates was determined on inorganic- salt starch medium by replacing both carbon and nitrogen sources (starch and ammonium sulphate) of the medium by 1.5% (w/v) of white chicken feather. The medium contained (g/l) the following: 1.0 g of K_2HPO_4, 1.0 g NaCl, 1.0 g of $MgSO_4$. $5H_2O$, 2.0 g of $CaCO_3$, 1 ml of trace salt solution, 15 g of white chicken feather pieces and 1000 ml distilled water. The pH of the medium was adjusted to 7.0. Feathers were washed carefully with tap water and detergents, followed by distilled water and then allowed to dry in air. The feathers were defated by washing with ethyl ether several times followed by distilled water. The medium was sterilized by autoclaving.

Fermentation procedures

Fermentation was carried out in 500 ml Erlermeyer flasks, each containing 100 ml of the above medium. The flasks were inoculated with a standard inoculum of spore suspension (2% v/v) of the tested isolates. The inoculated flasks were incubated at 50°C ± 2° under static conditions.

Samples were taken periodically every day for determination of keratinase activity and percentage of feather solubilization.

Determination of keratinase activity

Keratinase activity was determined according to the procedure of Nickrson *et al.* (1963). One unit of keratinase activity was defined as the amount of enzyme that gives 0.001 increase in absorbance at 590 nm in one hour at 55°C (Ingram *et al.*, 1983).

Determination of feather solubilization

The extent of feather digestion was measured as the percentage of its dry weight loss after incubation with the experimental strain for a specific period. Feather dry weight was determined after the end of incubation period at 105°C until reaching a constant weight.

Effect of nutritional and environmental conditions on the production of keratinase by S. thermovulgaris

Different carbon, nitrogen and phosphorous compounds were added to the fermentation medium at concentrations of 2.0, 0.2 and 0.1% (w/v), respectively

to elucidate the suitable for giving maximal production of keratinase by *S. thermovulgaris*.

The effect of addition of different microelements on keratinase activity of *S. thermovulgaris* was examined.

Different kinds of keratinaceous wastes at different concentrations (1.0, 1.5, 2.0%, w/v) were used as substitutes of chicken feather in fermentation medium. These treatments were done to select the most suitable waste material at suitable concentration for keratinase production.

The effect of initial pH value of the medium (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 or 9.5), size of spore inoculum (1.0, 2.0, 3.0, 4.0, 5.0 or 6.0%, v/v) and age of inoculum (1, 2, 3, 4, 7, 15 days) on keratinase production were examined.

Statistical analysis

LSD 0.05 and LSD 0.01 were calculated according to Snedecor (1967). If the difference between the arithmetical means of data is less than LSD 0.05, the result is non-significant. If the difference is more than LSD 0.05, the result is significant. If the difference is more than LSD 0.01, the result is highly significant.

Results

Screening of thermophilic actinomycete isolates for keratinolytic activity

Seventeen thermophilic actinomycete isolates were tested for their kernationlytic activities. Only, five isolates, namely, *Thermoactinomyces thermophilus, Streptomyces thermophilus, Streptomyces thermovulgaris, Thermomonospora alba, Microbispora thermodiastatica* were able to grow and degrade chicken feathers at 50 ± 2°. Therefore, these isolates could be considered as producers of keratinase enzyme and subjected to an evaluation of their keratinase activities under static conditions.

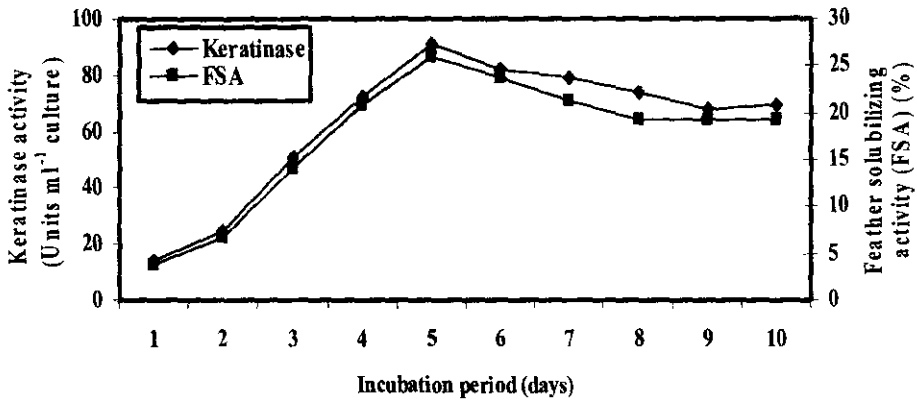
Data in Table 1 show that *Streptomyces thermovulgaris* proved to be more active in keratinase production and gave highly significant values as compared with other tested isolates and it was selected for the subsequent investigations.

TABLE 1. Screening for keratinolytic activities produced by some thermophilic actinomycetes grow in inorganics. Salt starch static cultures for 7 days at 50°C.

Actinomycete isolates	Keratinase activity (Units ml^{-1} culture)	Feather solubilizing activity (FSA) (%)
Thermoactinomyces thermophilus	59.25	16.5
Streptomyces thermophilus	77.00	19.75
Streptomyces thermovulgaris	84.00	23.75
Thermomonospora alba	57.25	17.00
Microbispora thermodiastatica	72.50	19.50
LSD 0.05	6.65	3.36
LSD 0.01	9.20	4.61

Conditions for more rapid keratinase production by Streptomyces thermovulgaris

Data in Fig.1 clearly show that the keratinase production started after the first day of incubation at 50 ± 2°C. It was found that as incubation period progressed, keratinase accumulation highly significantly increased, reaching its maximal value at the 5th day of incubation. In general keratinase productivity of *S. thermovulgaris* appeared to decrease with time after reaching the maximum.

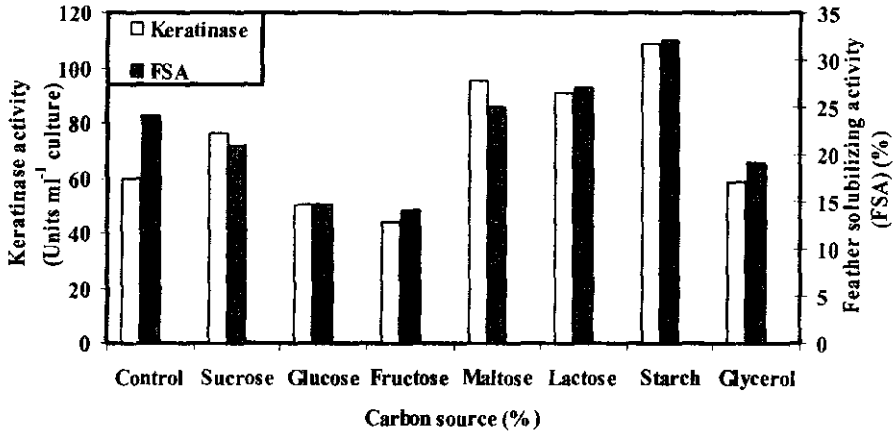


	Keratinase activity	FSA
LSD 0.05	3.71	1.24
LSD 0.01	5.00	1.67

Fig. 1. Keratinase activity of *S. thermovulgaris* at different incubation .

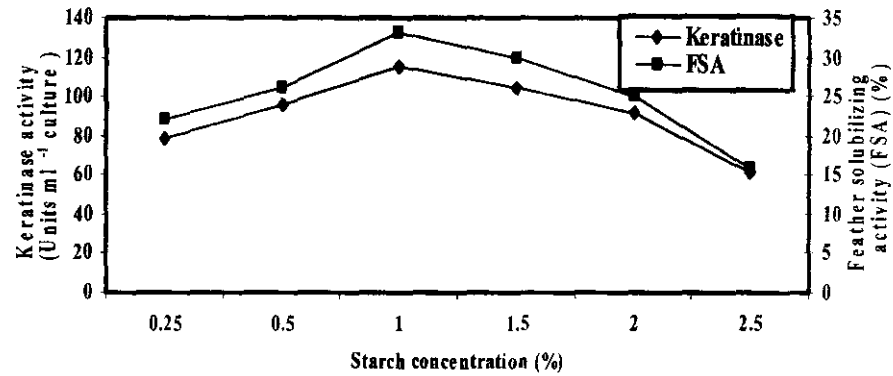
Data recorded in Fig.2 clearly indicate the addition of starch to the fermentation medium exerted highly significant stimulatory effect both in keratinase activity and the percentage of feather solubilization as compared to the control. They reached 108.38 units ml^{-1} culture and 32%, respectively, instead of 89.66 units ml^{-1} culture and 24% recorded for the control treatment. keratinase activity also showed highly significant increases when maltose and lactose were separately added to the fermentation medium. Other sugars gave the lowest values of keratinolytic activity and feather solubilizing activity (FSA).

Experiments were conducted to investigate the effect of different concentrations of starch on keratinase productivity. Results in Fig. 3 show that the maximal value of the keratinase activity was attained at starch concentration 1%, being 115.66 units ml^{-1} culture. Therefore, 1% starch was added to the fermentation medium in subsequent experiments.



	Keratinase activity	FSA
LSD 0.05	2.32	1.62
LSD 0.01	3.19	2.23

Fig. 2. Keratinase activity of *S. thermovulgaris* as affected by different carbon.

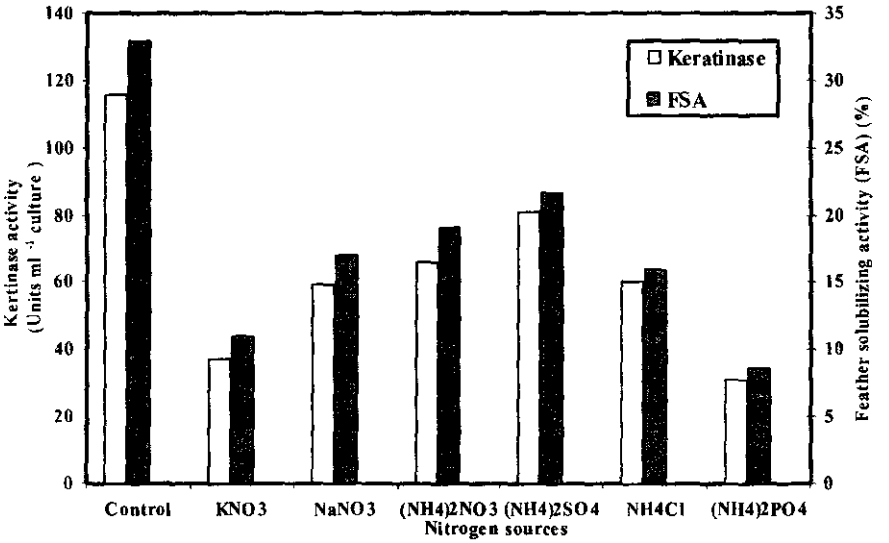


	Keratinase activity	FSA
LSD 0.05	11.15	1.45
LSD 0.01	15.63	2.04

Fig. 3. Keratinase activity of *S. thermovulgaris* as affected by different concentrations of starch.

Keratinase production showed highly significant decreases by variation of nitrogen sources as recorded in Fig. 4. The data clearly show that, when nitrate or ammonium salts were added to the medium, they gave lower keratinase activities than those recorded in control medium. The highest significant

decrease in keratinase activity was observed when ammonium phosphate was added to the fermentation medium.

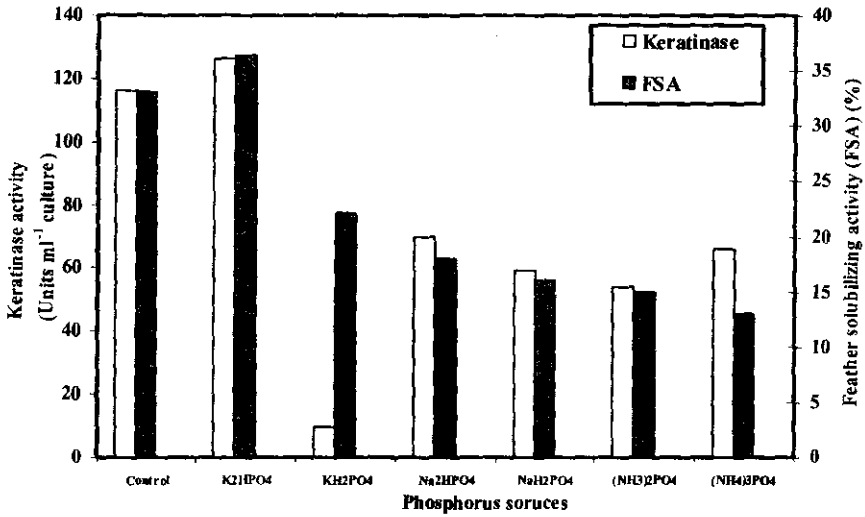


	Keratinase activity	FSA
LSD 0.05	1.67	1.58
LSD 0.01	2.31	2.19

Fig. 4. Keratinase activity of *S. thermovulgaris* as affected by different nitrogen sources.

Concerning the effect of different phosphorus compounds on keratinase productivity, dipotassium phosphate supported the highest significant level of enzyme production (Fig. 5). Other phosphorus sources have no significant effect on the enzyme activity as compared with the control treatment.

The presence of certain microelements in the fermentation medium is essential for the production of keratinase enzyme by *S. thermovulgaris*. Data recorded in Table 2 clearly show that fermentation medium prepared using tap water (control II) increased keratinase activity of the experimental strain as compared to control medium I of the same test organism. This may be attributed to the presence of all microelements in tap water needed for keratinase biosynthesis of the experimental organism. Results also revealed that omission of $ZnSO_4$, $FeSO_4$, $MnCl_2$ and NaCl from control medium I resulted in slight decrease in keratinase activity and feather solubilization, while omission of $MgSO_4$ lead to sharp decrease in keratinase activity. This result proved that $MgSO_4$ is essential for keratinase biosynthesis by *S. thermovulgaris*. An explanation of this result is that Mg^{++} may act as an activator (Co-factor) for keratinase synthesis.



	Keratinase activity	FSA
LSD 0.05	1.91	1.65
LSD 0.01	2.65	2.29

Fig. 5. Keratinase activity of *S. thermovulgaris* as affected by different phosphorus sources.

TABLE 2. Keratinase activity of *S. thermovulgaris* as affected by omission of some microelements from the fermentation medium.

Microelement	Keratinase activity (Units ml^{-1} culture)	Feather solubilizing activity (FSA) (%)
Control I*	123.25	35.25
Control II**	132.50	56.75
Element omitted from control (1):		
$MgSO_4$	53.25	15.50
$Zn\ SO_4$	119.75	32.75
$FeSO_4$	113.50	32.00
$MnCl_2$	112.75	30.75
NaCl	110.25	30.75
LSD 0.05	4.50	4.56
LSD 0.01	6.33	6.42

*Control I: Fermentation medium prepared using distilled water and with addition of trace salt solution.

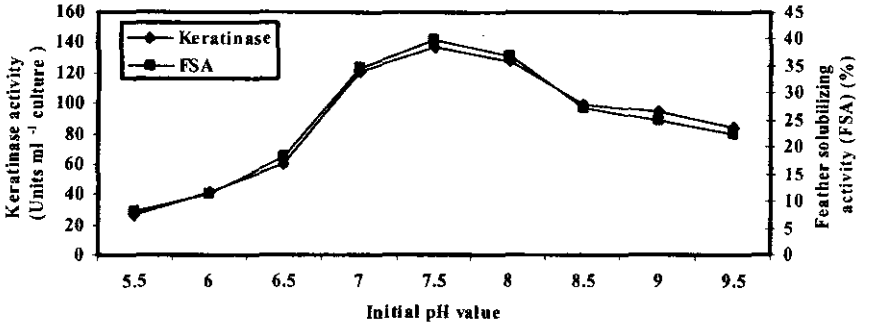
**Control II: Fermentation medium prepared using tap water and without addition of trace salt solution.

The capability of the experimental strain to degrade different available keratinaceous wastes was examined. Data of biodegradation of the keratinaceous waste materials by *S. thermovulgaris* are shown in Table 3. The data revealed that all waste materials used were actively hydrolyzed by the tested strain when added to the fermentation medium at different concentrations. The highest keratinase activity and feather solubilization of the examined strain obtained by using chicken feather in amount equal to 1.5 g/ 100 ml medium.

TABLE 3. Biodegradation of different keratinaceous wastes by *S. thermovulgaris*.

Keratinaceous waste material (%)	Concentration of keratinaceous waste materials (%)					
	1%		1.5%		2%	
	Keratinase activity (Units ml^{-1} culture)	Feather solubilizing activity (FSA) (%)	Keratinase activity (Units ml^{-1} culture)	Feather solubilizing activity (FSA) (%)	Keratinase activity (Units ml^{-1} culture)	Feather solubilizing activity (FSA) (%)
White chicken feather	114.75	31.5	130.25	27.0	123.0	34.5
Duck feather	80.50	22.75	121.25	32.25	120.5	32.25
Goose feather	42.50	11.5	52.75	14.75	50.0	14.25
Goat's hair	59.75	17.75	93.0	24.75	82.25	23.5
Buffalo's horn	74.0	21.0	115.75	31.0	103.5	27.5
Camels hair	48.5	13.25	63.75	19.0	74.75	22.0
Human's hair	38.75	12.0	61.5	17.5	62.0	17.5
LSD 0.05	1.96	1.88	2.29	2.16	2.43	2.61
LSD 0.01	2.65	2.83	3.22	3.04	3.43	3.68

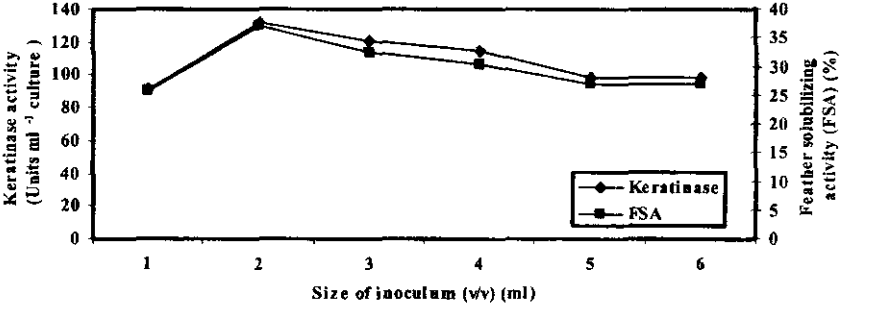
The ability of the test organism to produce keratinase enzyme is influenced by the initial pH of the medium. Results in Fig. 6 obviously show that the highest keratinase activity and feathers solubilization were obtained when the initial pH of the medium was adjusted to 7.5. Increasing or decreasing the initial pH to 9.0 or to 6.0 resulted in a considerable reduction in the feather solubilization and keratinase activity.



	Keratinase activity	FSA
LSD 0.05	1.85	1.57
LSD 0.01	3.08	2.31

Fig. 6. Keratinase activity of *S. thermovulgaris* as affected by different initial pH values.

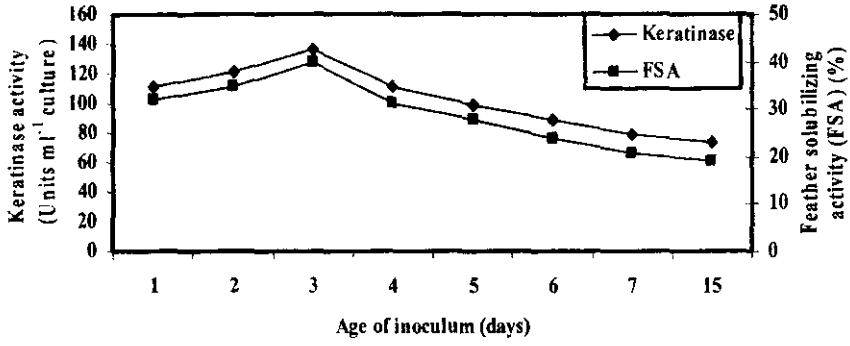
Results illustrated in Fig.7 clearly show that keratinase activities were increased by increasing the size of inoculum up to 2.0% (v/v). increasing the size of inoculum up to 6% did not cause any effect on the keratinase activity.



	Keratinase activity	FSA
LSD 0.05	9.82	1.64
LSD 0.01	12.10	2.41

Fig. 7. Keratinase activity of *S. thermovulgaris* as affected by different sizes of spore inoculum (% of the madium).

Results recorded in Fig. 8 clearly indicate that maximum keratinase production and feather solubilization by *S. thermovulgaris* was obtained when three days old culture of the spores was used. Increasing the age of inoculum up to 15 days did not cause any increase in keratinase activity.



	Keratinase activity	FSA
LSD 0.05	8.71	1.94
LSD 0.01	9.22	3.01

Fig. 8. Keratinase activity of *S. thermovulgaris* as affected by the age of inoculum.

In the light of all of aforementioned results it can be stated that *S.thermovulgaris* grew better with good yield of keratinase activity by growing it on the fermentation medium (100 ml medium/ 500 ml flasks), in which the nitrogen and the carbon sources were replaced with sterile chicken feather pieces (1.5 g%) and 1.0 g% starch and prepared in tap water with an initial pH of 7.5 and inoculated with 2% (v/v) of homogenized spore suspension (containing approximately $8.2x10^3$ spores ml^{-1}) of 3 days old culture. Inoculated flasks were incubated at 50°C for 5 days under static conditions.

Discussion

Cited literatures showed that maximal production of an enzyme occur only under proper conditions of growth (Pardee, 1962; Loginova & Usaite, 1981 and Abdel-Hafez *et al.*, 1995). In the present study, the keratinolytic activity of *Streptomyces thermovulgaris* was investigated in more details.

Culture of *Streptomyces thermovulgaris* showed highest keratinase activity after 5-days of growth. Maximum production of extracellular keratinases was reached on the sixth day for *Thermoactinomyces vulgaris* (Abdel-Hafez *et al.*, 1995) and the fourth day for *Thermoactinomyces vulgaris* (Mostafa & Hussein, 1974). These differences in maximum production in keratinolytic activity may be ascribed to either the conditions of cultivation or to species differences.

It should be stated that the production of keratinase by thermophilic actinomycetes was greatly affected by the kind and quantity of carbon source added to the basal medium. In the light of aforementioned results, starch at 1% concentration was found to be the best carbohydrate stimulator for keratinase

production and feather solubilization by *St. thermovulgaris*. Such results are in line with those obtained by Abdel-Hafez *et al.* (1995).

Our results revealed that, the supplementing basic salts feather medium with different nitrogen sources gave a negative response for lysing chicken feather by *St. thermovulgaris*. These results are in agreement with those obtained by Meevootism and Neiderpreum (1979) who reported that ammonium phosphate did not activate keratinase production when added to the basal medium. Also, the same result was obtained by Mostafa and Hussein (1974).

The omission of some minor elements from the fermentation medium of *S. thermovulgaris* greatly affected keratinase activity & feather solubilization. The results of this investigation showed that omission of $ZnSO_4$, $FeSO_4$, $MnCl_2$ and NaCl from control medium I resulted in slight decrease in keratinase activity and feather solubilization, while omission of $MgSO_4$ lead to sharp decrease in keratinase activity. This result proved that $MgSO_4$ is essential for keratinase biosynthesis by *S. thermovulgaris*. An explanation of this result is that Mg^{++} may act as an activator (Co-factor) for keratinase synthesis. Such results are in agreement with the results of Noval and Nickerson (1959) and Abdel-Hafez *et al.* (1995).

All keratinaceous waste materials were actively degraded by the tested strain when added to the fermentation medium of *S. thermovulgaris* at different concentrations . Hussein and Elakied (1989) reported that *Thermoactinomyces* keratinolyticus grows actively on chicken feather bringing its complete solubilization in 9 days of incubation at 55°C, Also, *Th. Vulgaris* and *Microbispora thermodiastatica* strains have the ability to degrade the powdered buffalo's horn when added to the medium at a concentration of 1.4% (w/v).

The size, type and age of inoculum are considered to be important factors for keratinase production by thermophilic actinomycetes (Nickerson *et al.*, 1963 and Hussein, 1989). Young and Smith (1975) used a washed spore suspension of *Streptomyces fradiae* ATCC 10745, grown on glutamate agar slants as a suitable inoculum for keratinase production. An aliquot of 3 ml spore suspension of *Micropolyspora keratinolytica* was used by Hussein (1989) for inoculation of basal chicken feather liquid medium to use for the production of keratinase enzyme. Our results indicate that maximum keratinase production by *S. thermovulgaris* was obtained when 3 days old culture of the spore was used and the size of inoculum increase up to 2.0% (v/v).

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إنتاج إنزيم الكيراتينيز بواسطة السلالة استربتوميسيس ثيرموفولجاريس المحبة للحرارة

السيد على الشربينى

قسم النبات- كلية العلوم- جامعة الزقازيق - الزقازيق - مصر .

يتضمن هذا البحث دراسة الأنشطة المحللة للكيراتين لبعض الأكتينوميسيتات المحبة للنمو فى درجات الحرارة العالية، وقد أظهرت نتائج هذه الدراسة أن ٥ معزولات لها القدرة على تحليل ريش الدواجن البيضاء عند إضافته لوسط التخمر كمصدر وحيد للكربون والنتروجين، كما أتضح من البحث أن المعزولة استربتوميسيس ثيرموفولجاريس هى اكفأ المعزولات السابقة فى إنتاج إنزيم الكيراتينيز.

كما تم دراسة العوامل الغذائية والبيئية المختلفة المؤثرة على نمو وانتاج إنزيم الكراتينيز بواسطة السلالة قيد البحث، حيث أوضحت النتائج الآتى:

١- إضافة النشا بتركيز ١% إلى وسط التخمر يعطى أعلى نمو وإنتاج للكيراتينيز حيث وصل إلى ١١٥ وحدة لكل ملليلتر من مرشح مزرعة استربتوميسيس ثيرموفولجاريس.

٢- عند إضافة إملاح الامونيوم أو النترات إلى وسط التخمر فإنها أعطت نشاطاً إنزيميا أقل بمقارنتها بالكنترول.

٣- عند إضافة فوسفات البوتاسيوم الثنائية إلى وسط التخمر فإنها تحفز إنتاج إنزيم الكيراتينيز، بينما فوسفات الامونيوم تثبط إنتاج الإنزيم.د

٤- أدت إزالة بعض العناصر النادرة من بيئة النمو له دور تثبيطى فى حين أن استبدال ماء الصنبور بدلا من الماء المقطر أدى إلى زيادة النشاط الكيراتينى حيث وصل إلى أعلى معدل له ١٣٢ وحدة لكل ملليلتر.

٥- أوضحت النتائج قدرة السلالة قيد الاختبار على تحليل مواد كيراتينية مختلفة مثل ريش الدجاج والبط والأوز ومسحوق قرون الجاموس وشعر الماعز والجمال والإنسان إذا أضيفت إلى بيئة التخمر كمصدر للكربون والنتروجين.

٦- إما من ناحية الظروف البيئية فلقد وجد أن أفضل الظروف الملائمة لإنتاج أعلى نمو ونشاط إنزيمى هى ضبط pH البيئة ما بين ٧,٥-٨ وكمية لقاح ٢% (حجم/حجم) يحتوى الملليلتر على ٨,٢ × $١٠^{٣}$ جرثومة وعمر المزرعة ثلاثة أيام فقط.