Optimum conditions of keratinase production from *Bacillus licheniformis*

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Abstract

Forty isolates of *Bacillus* spp. were isolated from fifty samples including different source of soil to detect the ability to produce keratinase enzyme in liquid state fermentation, *Bacillus* (Bs13)was the highest keratinase producer , it was identified as a strain of *Bacillus licheniformis*. The optimum conditions for keratinase productions were in a media contains keratin 4% (hooves) as a carbon and nitrogen and energy sources, peptone 1% as a secondary nitrogen source with pH 8 , inculums size 1%, and incubated at 37C^{o} for 24 hrs.

Keywords: *Bacillus*, keratinase, keratin,liquid state fermentation, optimum condition.

الظروف المثلى لإنتاج إنزيم keratinase من بكتريا *Bacillus licheniformis*

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الخلاصة

اختبرت 40 عزلة من بكتريا *Bacillus* من خمسين عينة اخذت من مصادر مختلفة من التربة لكشف قدرتها على انتاج انزيم keratinase في الاوساط السائلة وانتخبت العزلة (Bs13) *Bacillus* لكونها الاكثر انتاجا للانزيم , وقد شخصت العزلة على انها *Bacillus licheniformis* وتم تحديد الظروف المثلى لانتاج الانزيم من العزلة المنتخبة باستخدام تخمرات الحاله السائلة ولوحظ ان اعلى انتاجية للانزيم تكون في : وسط حاوي على الكيراتين (الاضلاف) كمصدر للكاربون والنتروجين والطاقة بتركيز4% بالاضافة إلى اسخدام البيتون1% كمصدر للنتروجين برقم هيدروجيني 8 وحجم لقاح1% والحضن بدرجة حرارة 37 م^{o} لمدة 24 ساعة

Introduction

Keratinases are proteases belong to the group of serine hydrolases that are capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide, hydrogen and hydrophobic bonds [1]. Keratins which are among the hardest-to-degrade animal proteins, are the major component proteins in poultry feathers and are characterized by a tightly packed form in α-helixes and β-sheets with a high degree of disulfide bonds [2]. Microbial keratinase had become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide keratin recalcitrant to commonly known proteolytic enzymes: trypsin, pepsin, papain. These enzymes are largely produced in the presence of keratinous substrates in the form of hair, feather, wool ,hooves, nail, horn, etc. during their degradation.[3].

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Keratinase is an extracellular enzyme used for the bio degradation of keratin . It is produce only in the presence of keratin substrate. Keratinase is a potential enzyme for removing hair and feather in the poultry industry [4]. This enzyme has been produced from *Bacillus* spp. and from fungi including the species *Aspergillus, onygena, Absidia, Rhizomucor*. Some of darmatophytes including *Trichophyton mentagrophytes. T. rubrus. T. gallinae, Microsporum canis and M. gypseum*[4]. The genus *Bacillus* is an important source of industrial alkaline proteases and is probably the only genus being commercialized for alkaline protease production [5].

Materials and methods

Collection of samples:

Forty samples were collected from different sources of soil, each sample were collected in sterile containers and transported to the laboratory until usage.

Isolation of *Bacillus* spp.

One gm of each soil sample was added to 9 ml of sterile water and shacked to homogenize, and then heated to 80˚C for 15 min in water bath. Serial dilutions were made for each sample by using sterile water. 0.1ml of each dilution was spreaded on a nutrient agar plates, and incubated aerobically at 37^{o}C for 24 hrs. The growing colonies were purified by sub culturing on nutrient agar for many times until pure culture was obtained. at 37^{o}C for 24 hrs.

Microscopic and morphological characteristics

The morphology, size, shape and margin of the bacterial isolate on nutrient agar plate were studied. On the other hand, a loop full of bacterial suspension was fixed on a slide, and stained by Gram stain to examine Gram reaction, shape and spore forming.

Determination of keratinase production

Semi quantitative method [6]

The activated bacterial isolates were plated on two keratinase agar medium,the first consisted of (w/v) 10% hooves powder, 0.5% NaCl, 0.3% K_2HPO_4, 0.4% KH_2PO_4, 0.2% $MgSO_4.7H_2O$, 0.5% Yeast extract, 0.5% Peptone, 2% Agar agar, and the second consisted of (w/v) 10% wool powder, 0.5% NaCl, 0.3% K_2HPO_4, 0.4% KH_2PO_4, 0.2% $MgSO_4.7H_2O$, 0.5% Yeast extract, 0.5% Peptone, 2% Agar agar. The pH was adjusted to 7.2 and sterilized at 121^{o}C for 10 min. Production medium was inoculated with activated bacterial isolates and incubated for 24h. at 37°C. Clear zone around the spots and underneath the growth indicate keratinase production. The diameter of colonies and clear zones were measured every 24h. The ratio of clear zone diameter to colony diameter was calculated which represents a semi quantitative assay of keratinase.

Quantitative method [7]

Ten ml. of keratinase broth medium composed of (10g. hooves powder, 0.5g. NaCl, 0.3g. K_2HPO_4, 0.4g. KH_2PO_4, 0.2g. $MgSO_4.7H_2O$, 0.5g. yeast extract, 0.5 Peptone and D.W. 100ml.) Activated bacterial suspension (optical density = 0.4) at 600nm. was inoculated with 0.1 ml of 24h. Crude enzyme was extracted by cooling centrifugation for 15min. Then the enzyme activity and protein concentration was measured in the supernatant.

Assay of enzyme activity.

Keratinase Activity was determined spectrophotometrically according to Anson (8) with some modification: Enzyme extract solution (0.2ml) was incubated with 1.8ml. of reaction solution(hooves+buffer) at 40°C for 15min. The blank, consisted of 1.8ml. of reaction solution and 3.0ml. of 5% TCA (trichloroacetic acid) and 0.2 enzyme solution. The reaction was stopped by the addition of 3.0ml. of 5% trichloroacetic acid and incubated at 25°C for 10 min. The mixture was centrifuged for 10min. then supernatant was separated. Quantity 2.5ml of 0.5M Na_2CO_3 solution was added to 1ml. of the supernatant and 1ml. of Folin–Ciocalteus reagent was added Incubated at 37°C for 20 min. The absorbance (O.D.) at 600 nm. of the solution was measured. Enzyme activity was calculated according to the following.

Enzyme activity(U/ml)=O.D(600nm /slope×volume of enzyme×incubation period)

protein concentration(mg/ml)=O.D(600nm) /(slope×1000)

Calculation of specific activity

The specific activity of the enzyme was calculated as following

$$\textbf{Calculation of specific activity (U/mg protein)} = \frac{(\textbf{enzyme activity U/ml})}{\textbf{protein concentration(mg/ml)}}$$

Determination of optimium conditions for keratinase production:

Substrate specificity of keratinase

Different keratins sources were selected for the production of keratinase including (wool, hair, feathers and hoofs) they prepared according to (Vignardet 2001)(9). Keratin sources were added to production medium at concentration 1% then inoculated with 0.1ml of activated bacterial suspension ,(O.D = 0.4) and incubated at 37C^o for 48hrs,the cells were precipitated by centrifugation at 8000 rpm. The crude enzyme was assayed for enzyme activity ,protein concentration and specific activity.

Effect of nitrogen sources on keratinase production

Ten ml of production medium was inoculated with 0.1ml of activated bacterial suspension(OD=0.4 at600nm), at different nitrogen sources(peptone, tryptone, peptone+ tryptone , NH_4CL, yeast extract) at concentration 0.5% and incubated at 37C^o for 48hrs. at pH 7.2. the cells were precipitated by centrifugation at 8000 rpm. for 10 min. Supernatants were assayed for enzyme activity, protein concentration, and specific activity.

Effect of different concentration of peptone on keratinase production

Ten ml of production medium was prepared with different concentration of peptone (0.25, 0.5, 0.75, and 1%) (w/v).The medium was inoculated with 0.1ml of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at 37C^o for 48hrs . at pH7.2. with shaking conditions. the cells were precipitated by centrifugation at 8000 rpm .The activity of the enzyme, protein concentration, and the specific activity were assayed in the supernatant.

Effect of pH on keratinase production

Ten ml of production medium was prepared at different pH values (6-10) .The medium was inoculated with 0.1 ml of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at 37C^o for 48hrs. the cells were precipitated by centrifugation at 8000 rpm. Supernatants were assayed for enzyme activity, protein concentration, and specific activity.

Effect of temperature of incubation on keratinase production

Ten ml of production medium PH 8.0was inoculated with 0.1 ml of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at different temperature (37, 45 ,and 50C) for 48 hrs. at pH8.0 the cells were precipitated by centrifugation at 8000 rpm. Supernatants were assayed for enzyme activity, protein concentration and specific activity.

Effect of inoculums size on keratinase production

Ten ml of production medium containing (1% peptone) pH 8.0was inoculated with different sizes of inoculum (0.5, 1, and 2) ml. of activated bacterial suspension (OD=0.4 at600nm), and incubated at 37C for 48hrs. the cells were precipitated by centrifugation at 8000 rpm . Supernatants were assayed for enzyme activity, protein concentration and specific activity was measured.

Effect of incubation period on keratinase production

Ten ml of production medium at pH8.0 was inoculated with 0.1of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at 37˚C for different times (24, 48 and 72) hrs. the cells were precipitated by centrifugation at 8000 rpm. Supernatants were assayed for enzyme activity, protein concentration, and specific activity.

Results and discussion

Isolation and identification of *Bacillus* spp.

Forty bacterial isolates of *Bacillus* were obtained from different sources of soil in Iraq table(3-1). bacterial isolates. according to the morphological and microscopic examination [10] .

Table 1- Number of *Bacillus* isolates obtained from different soil sources.

Sources of soil	No. of samples	No. of *Bacillus* isolates
Chicken	**9**	**8**
Sheep	**19**	**15**
Cows	**15**	**10**
garden	**7**	**7**
Total	**50**	**40**

Screening for keratinase producting *Bacillus* sp.

Semi quantitative screening

Keratinase agar medium containing hooves or wool (keratin 1%) as carbon and nitrogen source, was used for semi quantitative screening of keratinase production. The result showed that thirty five of the 40 isolates were able to produce keratinase with various diameter of keratin hydrolysis zone, the ratio ranged between (1.3-3.57) as shown in (Table 2). Among the soil bacterial isolates Bs13 was the most efficient in keratinase production because it gave the highest hydrolysis ratio 3.57 compared with other isolates.

Bacterial keratinase are of particular interest because of their action on insoluble keratin substrates and generally on abroad range of protein substrates [11]. Bacteria are the most dominant group of protease producers with the genus *Bacillus* being the most prominent and serve as an ideal source of this enzyme [12].

Table 2- Ability of *Bacillus* isolates to produce keratinase and their hydrolysis ratio on keratin agar medium after incubated at 37C° for 24hrs.

No. of Isolate	Ratio of keratinase hydrolysis zone *	No. of isolate	Ratio of keratinase hydrolysis zone *	No.of Isolate	Ratio of keratinase hydrolysis zone *
Bs1	1.66	Bs15	0	Bs29	1.13
Bs2	2	Bs16	2.57	Bs30	0
Bs3	1	Bs17	1.9	Bs31	2.4
Bs4	0	Bs18	1.56	Bs32	1.5
Bs5	2.3	Bs19	2.1	Bs33	1.2
Bs6	1.7	Bs20	2.61	Bs34	0
Bs7	1.4	Bs21	1.8	Bs35	1.1
Bs8	2.2	Bs22	1.2	Bs36	1.7
Bs9	1.85	Bs23	1.9	Bs37	1.2
Bs10	2.8	Bs24	1.83	Bs38	1.33
Bs11	3	Bs25	1.87	Bs39	1.7
Bs12	1.85	Bs26	1.3	Bs40	1.7
Bs13	3.57	Bs27	0		
Bs14	1.5	Bs28	1.6		

*(diameter of zone /diameter of colony)

Quantitative screening for keratinase production

Seven isolates (Bs5,Bs10,Bs11,Bs13,Bs16,Bs20,and Bs31) which have largest keratinase hydrolysis zone were selected for quantitative screening of keratinase production. The result showed that the specific activity of keratinase produced by these isolates range between (395-521)U/mg protein (Table 3). The differences in the production of enzyme among isolates may be due to the type of isolation source or the variation in the genes coded keratinase synthesis. The stability of keratinase influenced by physical factors and chemical substances [13].

According to the previous results, *Bacillus* isolate B13 isolated from sheep soil had superior hydrolysis ratio 3.57 and higher specific activity for keratinase production (521U/mg protein). Thus isolate was selected for further study.

Table 3- Production of keratinase by *Bacillus* isolates after incubated at 37C for 48hrs.

Number of *Bacillus* isolates	Specific activity (U/mg protein)
Bs5	463.5
Bs10	449.8
Bs11	416.2
Bs13	521.5
Bs16	400.8
Bs20	395.3
Bs31	410.5

Identification of *Bacillus* Bs13 isolate

The selected isolate (Bs13) was subjected to further biochemical tests according to Bergys Manual of Systematic Bacteriology [14] and Fritze [15] as shown in Table (4).According to the results it can be concluded that the Bs13 isolate is belongs to *B. licheniformis.*

Table 4- Morphological and Biochemical characteristics of the *Bacillus* Bs13 isolate.

Characteristics	Results
Cell shape	Rod
Spore shape	Ellipsoidal
Spore site	Central
Motility	+
Gram stain	+
Catalase	+
Oxidase	+
Methly Red	-
Vogas – Proskuer	+
Starch hydrolysis	+
Gelatin liquefaction	+
Indol test	-
Simmon Citrate	+
Anaerobic growth	+
Growth at 40˚C	+
Growth at 50˚C	+
Sodium chloride tolerance	+
Carbohydrate fermentation	
glucose	+
Fructose	+
Sucrose	+
Maltose	+

+: positive result. -: negative result.

Determination of optimum condition for keratinase production from *B. licheniformis* Bs13:

Keratinous substrate specificity on keratinase production

The results showed that the hooves was the best keratin source, the specific activity reached (522)U/mg protein. While the hair gave the minimum keratinase activity, the specific activity was(410.33)U/mg protein (Figure 1).

Different keratinous wastes were used as a sole source of carbon and nitrogen in the growth medium, the complete mechanism of keratin degradation not fully understand. Basically microbial keratinolysis is a proteolytic ,protein degrading process for the simple reason that keratin is a protein [16]. The high mechanical stability of keratin and its resistance to proteolytic degradation is due to the tight packing of the protein chains through intensive interlinkage by cystine bridges [17].

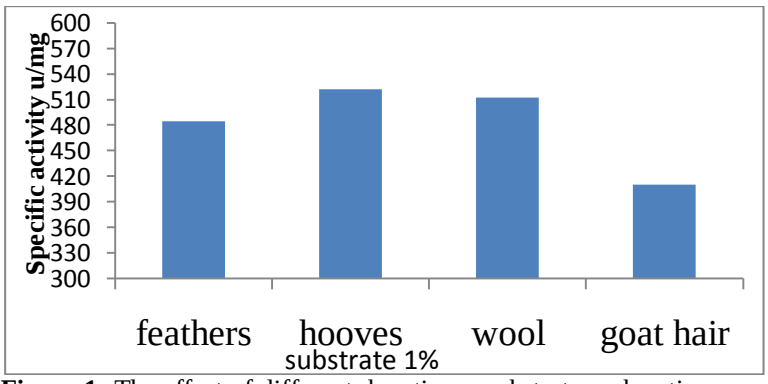

Figure 1- The effect of different keratinous substrate on keratinase production from *B. licheniformis* Bs13 after incubation at 37C˚ for 48h. at pH 7.2.

Effect of substrate concentration (hooves) on keratinase production

The results showed that the production of keratinase by *B. licheniformis* Bs13 affected by the substrate concentration of hooves (figure2). Keratinase activity was increased with the increase of the substrate concentration until 4% the specific activity reached (545.6)U/mg protein. Then slightly increased showed after 4% concentration ,the specific activity reached 583U/mg at 7%.

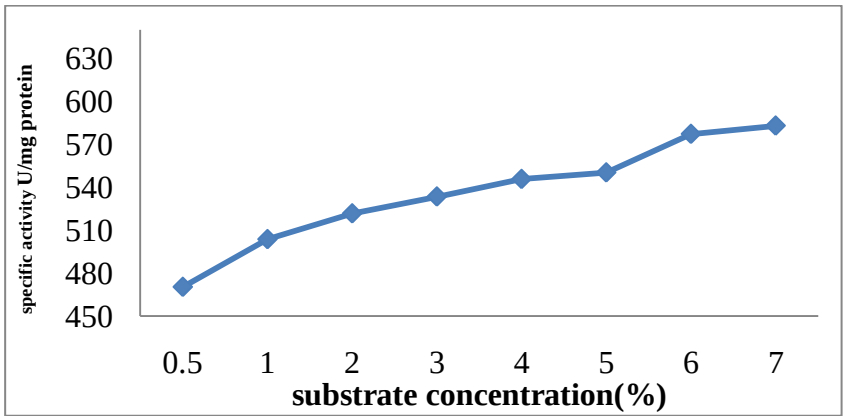

Figure 2- Keratinase production by *B. licheniformis* Bs13 at different substrate concentration after incubation at 37C° for 48h. at pH 7.2.

The amount of keratinase production depended on substrate concentration and cultivation condition. Microorganism growing on nutrient medium containing feather meal act as carbon and nitrogen source presented variable activity on keratin suggesting that this enzyme may be inductive. Substrate level in the medium may regulate enzyme secretion [18]. The increased concentration of the feather decreased the enzyme production suggesting catabolic repression on keratinase production from *B. megaterium*, the optimum concentration was 1.5% feather meal [19].

The effect of carbon sources on keratinase production

Six carbon sources were used as additional source for carbon and energy to determine the optimum production of keratinase by *B. licheniformis* Bs13 isolate, showed that the higher specific activity of enzyme(544.6) U/mg was achieved in the presence of 4% hooves only while keratinase productivity were reduced when glucose, molase, fructose, lactose, and starch were added to the production media (hooves) in both concentration (0.5 and 1) (figure3) and (figure4).

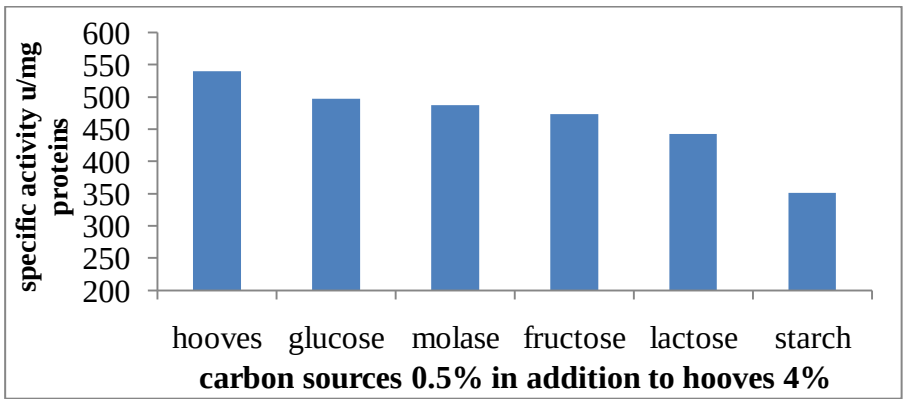

Figure 3- Keratinase production by *B.licheniformis* Bs13 cultured on keratinase production broth at different carbon sources after inoculated with 1%inoculum and incubated at 37C° for 48 hrs at pH 7.2.

Similar results were reported that the addition of glucose, fructose and sucrose inhibited growth and production of keratinolytic enzyme in *B. licheniformis* MZK-3.this might be due to the repression of expression of gene for keratinase [20].such type of inhibition by carbohydrate in the synthesis of keratinase was previously reported with *B. licheniformis* PWD-1.[21]

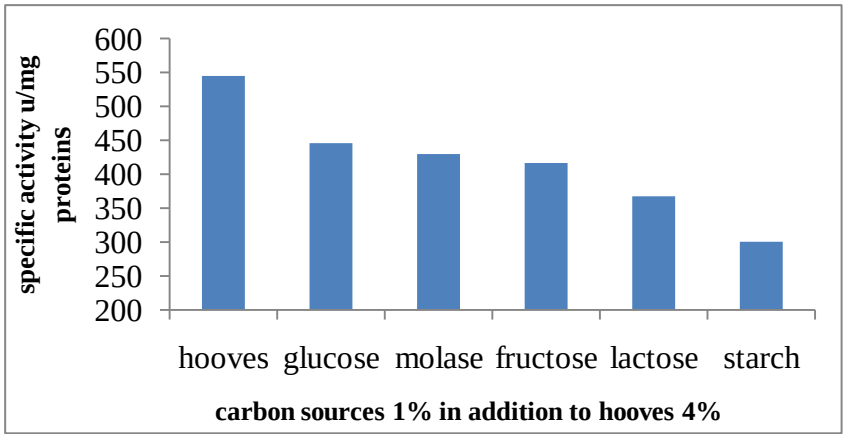

Figure 4- Keratinase production by *B. licheniformis* Bs13 cultured on keratinase production broth at different carbon sources after inoculated with 1%inoculum and incubated at 37C° for 48 hrs at pH 7.2

The effect of nitrogen sources on keratinase production

The results showed a highest specific activity (620.94)U/mg protein when peptone was used as nitrogen source while the minimum activity observed with NH_4CL the specific activity was (496.26)U/mg protein (figure 5). Result observed that 1% of peptone was the optimum concentration for keratinase production.(figure 6).

Different bacteria have different preferences for either organic or inorganic nitrogen for growth and enzyme production although complex nitrogen sources are usually used for alkaline protease production [22,23].The nitrogen sources are of a secondary energy sources for organisms which play an important role in the growth of the organism and production ,The nature of the compound and the concentration that used might stimulate or down modulate the production of enzymes [24].

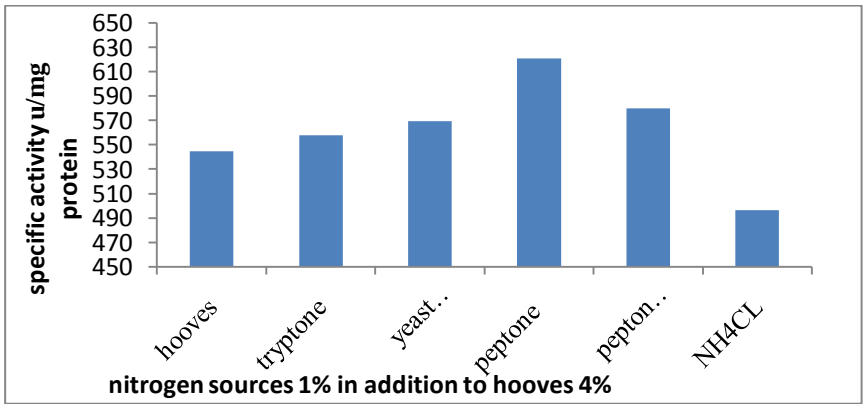

Figure 5- keratinase production by *B. licheniformis* Bs13 at different nitrogen sources after incubation at 37C° for 48 hrs at pH 7.2

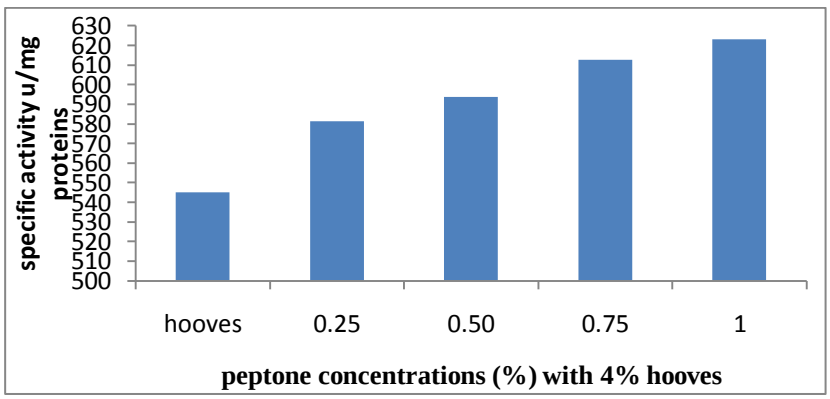

Figure 6- keratinase production by *B. licheniformis* Bs13 at different peptone concentration after incubation at 37C° for 48 hrs at pH 7.2

Effect of pH on keratinase production

A maximum keratinase production was obtained when the pH value of the production medium adjusted to 8.0 the specific activity was recorded (680.7)U/mg proteins, reduction in enzyme activity was observed at pH lower or higher than pH 8.0.(figure 7). Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion [25]. The effect of the pH value on the enzyme production resulted from its role in the solubility of nutrients, ionization of the substrates and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme [26].

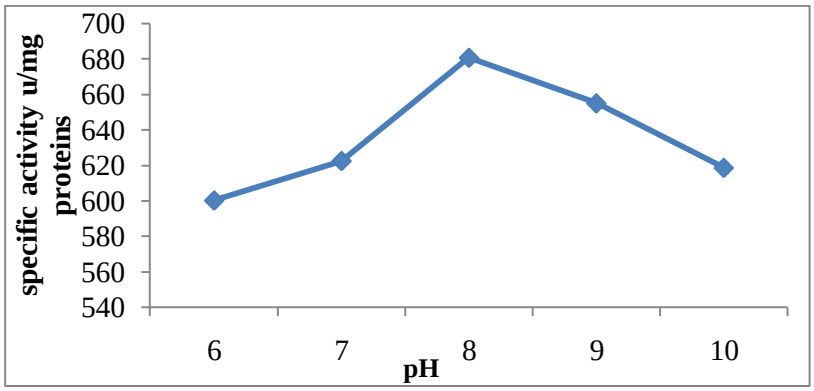

Figure 7- keratinase production by *B.licheniformis* Bs13 after incubated for 48h. at different pH values incubation at 37C°.

The effect of incubation temperature.

The results shown in figure (8) maximum production of keratinase observed at 37C° ,the specific activity for keratinase reached(681.1)U/mg. proteins, and the higher temperature were not suitable for enzyme, the specific activity decreased to (645.5)U/mg.

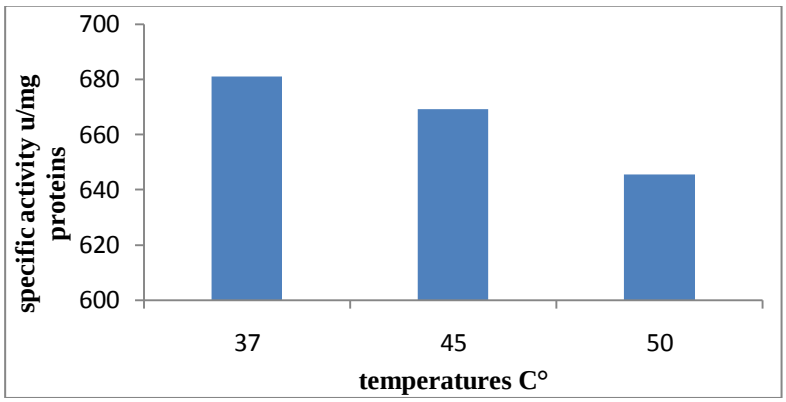

Figure 8- keratinase production by *B.licheniformis* Bs13 at different temperatures for 48 hrs after incubation at pH 8.0.

Temperature is one of the most important factors affecting the enzyme production [27]. It could regulate the synthesis and secretion of extracellular protease by microorganisms[28].A higher temperature generally result in an enzyme production , if the temperature rises beyond a certain point , however the enzyme production eventually leveled out than decline rapidly because the destroying of organism by high temperature [29]. Different conditions for growth and keratinolytic enzyme production may differ depending on the different strains of the same species [20].

Effect of inoculums size on keratinase production

The results showed in figure (9) that keratinase activity was increased with the increase of the inoculums size until 1ml. the specific activity reached (682.5)U/mg protein; then decreased to (643)U/mg protein with increase in inoculums size to 2ml. Inoculums ratio is one of important factor affecting keratinase production and feather solubilization [31].

An inoculums concentration higher than the optimum value may produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis, on the other hand, lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium, this increases the necessary time to grow to an optimum number to consume the substrate and synthesize the desired product [24].

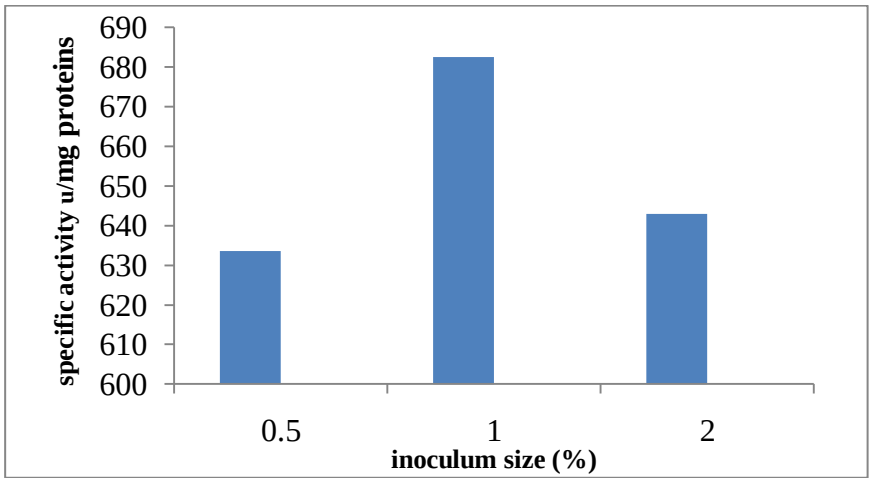

Figure 9- Keratinase production by *B. licheniformis* Bs13 at different inoculum sizes after incubation at 37C° for 48 hrs at pH 8.0.

Effect of incubation period on the enzyme production

Keratinase production from *B. licheniformis* Bs13 isolate was determined after different incubation period (24,48 and 72 hours). The production gave the maximum activity after 24 hrs. with gradual decrease in productivity with increasing the incubation period (figure 10) the specific activity reached the maximum (742 U/mg proteins) after 24hrs incubation followed by decline in specific activity reached (650.8)U/mg proteins after 72 hrs incubation at 37C°.

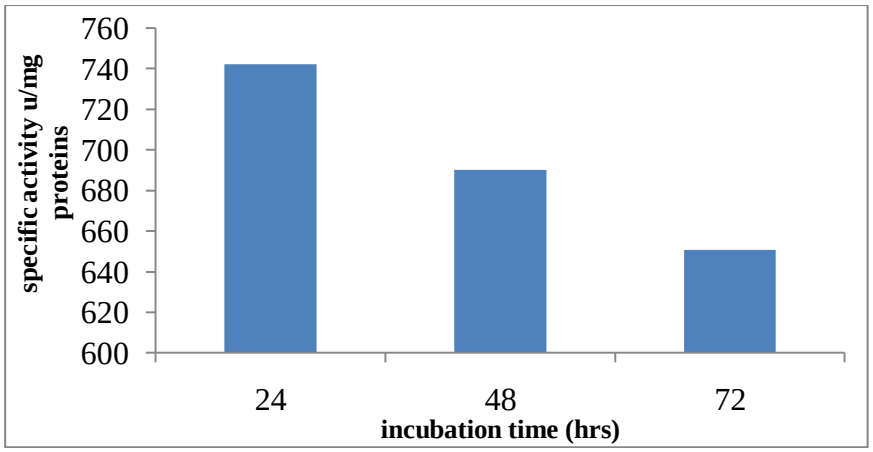

Figure 10- keratinase production by *B.licheniformis* Bs13 at different incubation periods at 37C° and pH 8.0

B. licheniformis entered the exponential phase after 6h and stationary phase after 42h[7]. While *B. licheniformis* JY4 culture entered stationary phase after 12hrs. incubation and keratinase production was greatly increased with maximum of 350 U/ml. after 72hrs, the maximum protease production was at late stationary phase[32].

Conclusion

Keratinase, produced by *B. licheniformis*.Bs13, was capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide, hydrogen and hydrophobic bonds. *B. licheniformis* Bs13 is an efficient local isolate for keratinase production, study of the optimum conditions by using liquid state fermentation medium showed the enzyme of *B. licheniformis* Bs13 was stable at different pH(7-9) and temperature (50-60)C°

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