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Amylase production , purification and characterization from *Escherichia coli*

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Abstract

This study was conducted in the laboratories of Biology Department, faculty of Science, which deal with isolation, purification and characterization of of amylase by *Escherichia coli* which carried out for enhanced production of amylase using starch (1%) as the substrate of enzyme , the production was carried out by submerged fermentation , the best conditions were the isolated of amylase in synthetic medium , it gave high titer of amylase activity , the ammonium sulfate as nitrogen source , incubation period 48 h , the starch as carbon source, incubation temperature 30 °C and pH = 7 , The amylase was purified using precipitation by ammonium sulphate (60%) and dialysis , the refined amylase had a maximum activity at pH = 7, the amylase was stable with pH values ranging between (7 - 8) and in temperature 30 °C also amylase was stable in (30- 40) °C analyses of the amylase for molecular weight was carried out by SDS-PAGE electrophoresis which revealed 52 KDa.

Key Words: Amylase; *Escherichia coli ;* Purification

1. INTRODUCTION

Enzymes are among the most vital produces gained for human requirements through plants , animals, and microbial source, at the present time, the enzymes use in manufacturing part is rising due to rise of industries specially in beverages , food, textile, paper and leather industries. Amylases are the enzymes that break down glycogen or starch . The Amylases can be resulting from numerous foundations such as microbes , animals and plants . The main benefit of microorganisms using for amylases production in economical bulk and microbes are also simple to manipulate to get enzyme of preferred characteristics (1) .

Amylases have been resulting from numerous yeast , bacteria , fungi , and actinomycetes but members of the genus *Escherichia* have been some of the workhorses of production of enzyme for decades, essentially for the reason that their capacity to overproduce amylase , other microorganisms producing significant amount of diverse amount of amylase enzyme are *Escherichia coli* , *Pseudomonas* spp , *Serratia* sp , *Micrococcus* sp, *Proteus* sp , and *Bacillus* sp such as *B. licheniformis* , *B. amyloliquefaciens* , *B. subtilis,* and *B. stearothermophilus,* , fungi belonging to the genus *Aspergillus* have been most generally employed for the amylase production, also *Mucor, Penicillium* ,*Candida* , *Cephalosporium* , and *Neurospora* production of enzymes by solid-state fermentation (SSF) using these moulds twisted a cost effective production technique (2) .

Spectrum of request of amylase has broadened in many sectors such as textile, baking ,food, and detergent manufacturing. as well its use in the liquefication or saccharification of starch, the enzyme is also used for the bend sizing of textile fibers, and for pretreatment of animal feed to advance digestibility (3). on the basis of the significance of amylase, the present study has been taken to production , purification and characterization of the enzyme .

2. MATERIALS AND METHODS :

2.1.Bacterial isolates :

bacterial isolates were obtained from the Al-Sader Hospital in Najaf city , and bacterial isolates identification were based on biochemical tests which described by (4) .

2.2. *E.coli* inoculum Preparation : It was ready according to (5)

(Table 1) Compositions of growth (synthetic) medium of *E. coli*.

S/N	Ingredients	Quantity(g/L
1	Starch	1
2	Peptone	6
3	$MgSO_4$ 7H2O	0. 5
4	$KCl_2.4H_20$	0. 5
5	Distilled water Up to	1000 ml mark

2.3. Amylase isolation

The selected strains of isolates were transmitted at 37°C for 24 h in 50 ml of 8% (w/v) of starch medium located in 250 ml flasks and placed in a shaker incubator operated at 120 rpm at 30°C. The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 min , the supernatant acquired was collected and used as enzyme source (5) .

2.4. Amylase assay

Amylase activity was assayed according to (6) . The enzyme activity was estimated by applying the following equation :

$$\text{Enzyme activity IU/ML} = \frac{\text{Amount of reducing sugar} \times 1000 \times \text{dilution factor}}{\text{Molecular weight of glucose} \times \text{time} \times \text{enzyme volume}}$$

One enzyme activity unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose from the substrate in 1 minute under standard assay conditions.

2-5. protein Determination : Protein content was calculated according to (7).

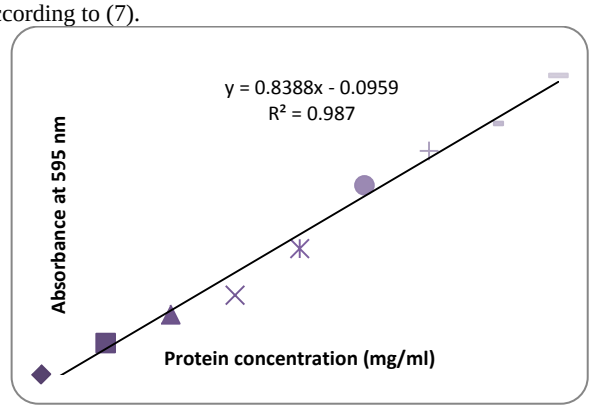

Figure (1) : Standard curve for the various concentration of bovine serum albumin (BSA) at 595 nm.

2.6. Factors affecting production of amylase: include pH regulation , medium composition, carbon and nitrogen sources, fermentation temperature and incubation period **(** 8) .

2.7.Enzyme purification:

2.7.1.Ammonium sulfate fractionation

The ammonium sulfate was added in different saturation ratio (20,40,60 and 80%) to reach the optimum ratio of ammonium

sulfate by adding gradually the amount of salt to each 20 ml of the crude amylase enzyme in ice bath and magnetic stirrer, centrifuge the solution for 25min at 6000 rpm. Dropped the supernatant and take the precipitate and dissolved it in 5 ml distilled water and both protein content and the activity of enzyme were determined for each separate fraction (9)

2.7.2. Dialysis against buffer : The gained precipitation (in solution) was introduced into dialysis bag against 1ml of 0.2 M citrate buffer (pH = 6.5) . The obtained amylase enzyme preparation was saved in the refrigerator at 4ºC for additional purification .

2.7.3. Gel filteration chromatography technique
It was measured according to (10) . Using sodium phosphate buffer (0.2M, pH = 6.5) , at flow rate 9 ml/hour (3ml for each fraction), the protein fractions was calculated at 280 nm

2.7.4.Molecular weight Determination of amylase :
The molecular weight of purified amylase was determined by SDS- PAGE according to (11).

2.8.Characterization of amylase:
2.8.1.Optimal pH for enzyme activity: The starch (substrate) was prepared with dissimilar pH ranges (4,4.5 , 5 , 5.5 ,6 ,6.5, 7 ,8 , 9).
2.8.2.Optimal temperature for enzyme activity : The starch (substrate) was prepared in tubes ,the tubes were incubated in dissimilar temperatures (10, 20, 25 ,30 ,35,40 , 50) °C to 30 min for mixing .

3. Results and Discussion:

3.1: Identification of bacterial isolates :
The Table (2) shown that the results of biochemical tests which used for the *E.coli* isolates identification , this results establish all the bacterial isolates are catalase and indole test positive , ferment glucose, , As well as all the *E.coli* isolates are oxidase and urease test negative (4)

Table (2) : Biochemical tests for *E.coli* isolates

Differential tests for *E.coli* Isolates	
Oxidase	-
Coagulase	-
Citrate	-
Urease	-
Methyl Red	+
Nitrate Reduction	+
Indole Production	+
Voges- Proskauer	-
Catalase	+

3-2 : Factors affecting amylase production :
3-2-1 : finest culture medium for amylase production :
The highest amylase production by *E.coli* was occurred using the synthetic medium , it gave elevated titer of amylase (0.812 U/ml) followed by nutrient broth (0.684 U/ml) while luria broth gave low titer of amylase activity (0.085 U/ml) , Figure (2) . The choice of the suitable fermentation medium is a critical factor for microbial development and enzyme making , the growth of an organism in culture medium is inclined by the nutrient composition of the medium and the accessibility of these nutrients (12) . This study was agreed with (13) when they found the presence of $CaCl_2$, Kcl_2 and $MgSO_4$ in culture medium are implicated to play a chief role in would boost the yield of amylase and its activity from *Bacillus subtilis* .

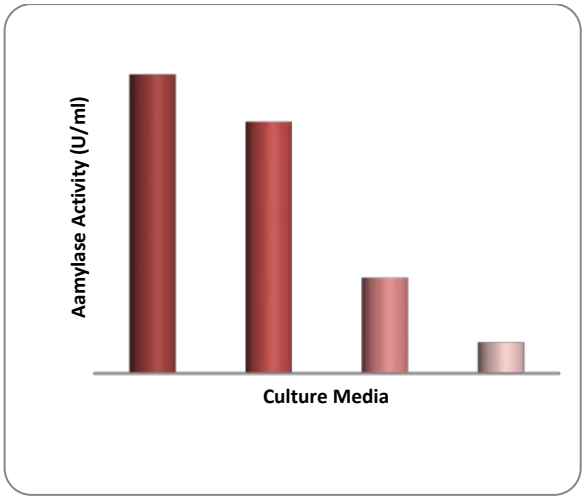

Figure (2) : Effect culture media A: synthetic medium , B: nutrient broth , C: yeast extract broth D. Luria broth on the amylase production from *E. coli*.

3-2-2 : Optimum incubation period for amylase production:
The figure (3) shown the increasing of amylase activity with increasing the incubation period until reach to greatest activity (0.746 U/ml) in second day (48 h) using the starch as a substrate of enzyme , then it began to decreased (0.497, 0.075 U/ml) in 72, 96 h respectively. This study was decided with (5) when they revealed the highest activity of amylase from *Bacillus megaterium* in 48 h (1.821 U/ml) , rise in cultivation period caused in decline in the production of amylase by *E. coli,* this may be due to the truth that after amylase maximum production , there was production of other by products and a diminishing of nutrients these by products inhibited the organisms growing and later, enzyme creation (14) .

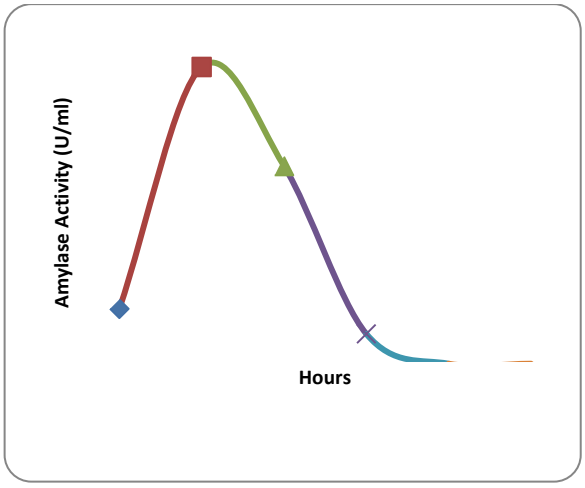

Figure (3) : Effect of incubation period on the amylase production from *E.coli.*

3-2-3 : Best temperature for enzyme production:
The effect of temperature on enzyme production exhibited that the activity of amylase increased gradually with increase in temperature from 10 °C attainment a maximum in 30°C (0.837 U/ml) above this temperature there was a lessening in the amylase activity (0.284, 0.077 U/ml) in 40 , 50 °C respectively (Figure 4) . Growth heat is a very major factor which varies from organism to organism and minor changes in growth temperature may change enzyme formation **(**15) . Other researchers (16) they showed that highest amylase production from *B.amyloliquefaciens* occurred in 42 °C .

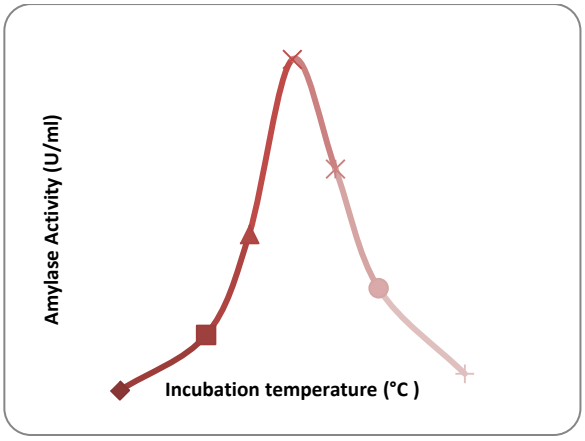

Figure (4) : Effect of incubation temperature on the production of amylase from *E. coli* .

3-2-4 : best pH for enzyme production:

The figure (5) shown the rising of amylase activity with rising the pH until reach to maximum activity (0.821 U/ml) in pH = 7 using the starch as a substrate of enzyme , then it began to decreased in higher pH values (0.483, 0.065 U/ml) in pH= 8 ,9 respectively , pH is a important feature that effects activity of enzyme, morphology of the cytoplasmic membrane, by product construction and reductive oxidative reactions, the structure of a microorganism by affecting nutrient solubility and uptake, the pH change experiential during the organism growing also affects product constancy in the pH of culture medium can have profound effects on both the synthesis of enzymes and rate of production (17) , this study was agreed with (18) they shown the chief activity of amylase from the *Bacillus* sp occurred at the same pH

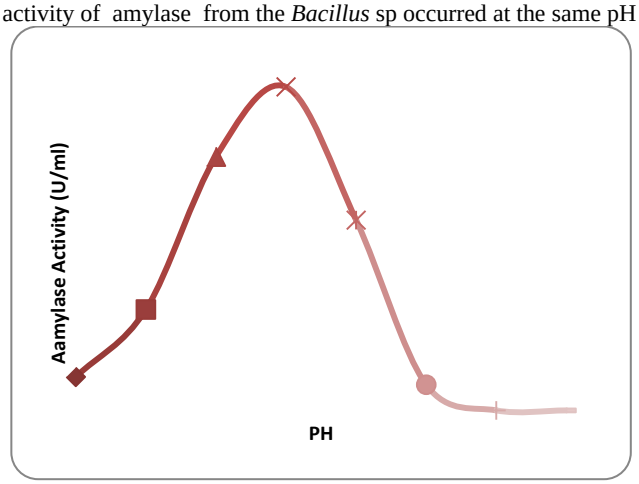

Figure (5) : Effect of pH on the amylase production from *E. coli*. 3-2-5 : finest nitrogen source for amylase production:

The highest enzyme production by *E.coli* was occurred using the ammonium sulfide as nitrogen source , it gave elevated titer of amylase activity (0.767 U/ml) followed by ammonium nitrate $(NH_4)_2NO_3$ (0.587 U/ml) , while the other sources (ammonium chloride ,pepton ,urea) gave little titer of amylase activity (0.377, 0.164, 0.089 /ml) respectively figure (6) . During fermentations of microbes, the carbon foundation not only performances as a main component for construction of cellular material , but is also used in the polysaccharide synthesis and as foundation of energy, nitrogen sources such as inorganic nitrogen is supplied as ammonium salts , ammonia gas , or nitrates , ammonium salts such as ammonium sulphate typically produces acidic situations the similar as the ammonium ion gets utilized and the free acid is activist, ammonia has been used for pH regulator , additional studies similar to (19) reported the greatest activity of amylase from *Bacillus subtilis* DM-03 using the ammonium chloride as nitrogen source .

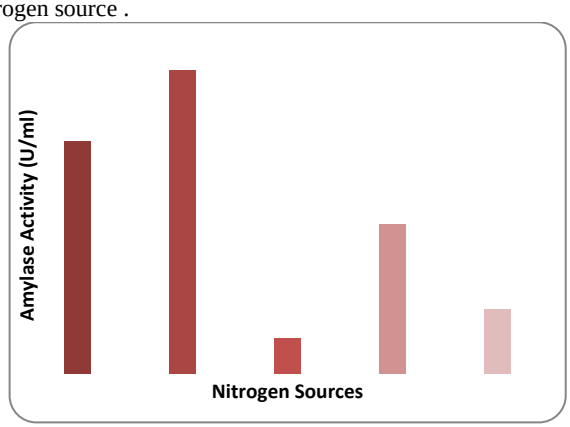

Figure (6) : Effect of nitrogen sources (A: ammonium nitrate B: ammonium sulfide, C: urea , D: ammonium chloride , E: peptone) on the production of amylase from *E.coli* .

3-2-6 : Optimum carbon source for amylase production:

The figure (7) shown the maximum amylase production by *E.coli* was occurred using the starch as the carbon source , it gave elevated titer of amylase activity (0.837U/ml) followed by sucrose (0.606 U/ml) , while the other sources (fructose ,glucose , lactose) gave small titer of amylase activity (0.441, 0.295, 0.075U/ml) respectively . further studies like (20) they shown the maximum activity of amylase from *Bacillus* sp. achieved using the maltose as the carbon source , also (21) revealed the greatest activity of amylase from *Bacillus cereus* MTCC1305 occurred using the glucose as the carbon source .

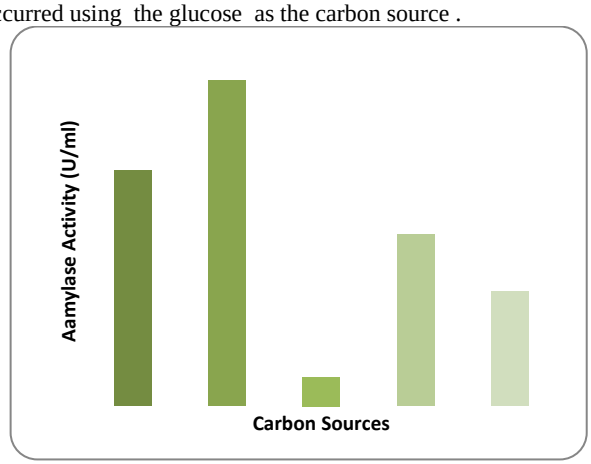

Figure (7) : Effect Carbon sources (A: Sucrose, B: starch , C: Lactose , D: fructose , E: glucose) on the amylase production from *E.coli* .

3.3.Amylase purification

3.3.1.Precipitation with ammonium sulfate :

The ammonium sulfate used in different saturation ratios (20 ,40 ,60 , and 80)% , then the 60% ratio was chosen as finest ratio for precipitate the crude extract of enzyme , when the specific activity reached to (4.119 U/mg) , with a purification fold (1.204) and yield (0.40) as shown in table (3) , while the additional saturation ratios (20 ,40 ,80) gave low down titer of specific activity (3.239, 3.689, 3.837U/mg) respectively. The ammonium sulfate was used in precipitation of the enzyme because it elevated soluble and inexpensive compared with the further salts, unchanged in pH and enzyme stability, the concentration by ammonium sulfate depending on equilibrate the charges found in protein surface and disrupt of the water layer surrounding it, that leads to precipitate it, this research was agreed with (22) when they purified amylase from *Bacillus* sp. used ammonium sulfate with 60 % saturation ratio.

Table (3): The amylase purification steps from E.coli

Purification steps	Volume (ml)	Activity (U/ml)	Total activity(U)	Protien con.(mg/ml)	Specific activity(U/mg)	Fold	Yeild %
Crude enzyme	20	2.681	53.62	0.784	3.419	1	100
Ammunium sulfate precipitation (60)%	10	2.171	21.71	0.527	4.119	1.204	0.404
Dialysis against phosphate buffer	10	1.752	17.52	0.276	6.347	1.540	0.326
Gel filtration (First step)	5	1.187	5.935	0.084	14.130	4.132	0.110
Gel filtration (Second step)	5	0.998	4.99	0.061	16.360	4.785	0.093

3.3.2.Dialysis against phosphate buffer

The acquired ammonium sulfate precipitate was introduced into dialysis bag over night against (0.2 M) phosphate buffer at (pH= 6.5) , then the specific activity reached to (6.3 47U/mg) , with a purification fold (1.540) and yield (0.32) as shown in table (3) .The additional results of (23) they purified amylase from *Bacillus subtilis* used dialysis bag against (0.1M) distilled water, the specific activity was (0.06 U/mg) with purification fold (0.54) .

3.3.3. Gel filtration chromatography

The enzyme solution produced from dialysis was passed during gel filtration using (sephadex G-200) column (2× 40 cm) that equilibrated with (0.2 M) phosphate buffer at (pH= 6.5) , the fractions were collected from column and calculated at 280 nm absorbency. In the first step of gel filtration the specific activity was the protein peak of (14.130 U/mg) with purification fold (1.436) while to the second peak of protein (21.466U/mg) with purification fold (4.132) , while in the second step of gel filtration the specific activity reached (16.360 U/mg) with purification fold (4.785) shown in table (3) . The other results recorded by (24) when they get specific activity (13,011U/mg) with purification fold (96.3) when they purified amylase from *Bacillus subtilis* KIBGE HAS by using sepharose CL 6-B column **.**

3.4. Amylase characterization:

3.4.1. The finest pH for amylase activity :

The figure (8) shown the rising the activity of amylase purified from *E. coli* with rising the pH until reach to greatest activity (0.297U/ml) in pH = 7 then it began to decreased in higher pH values (0.116 ,0.059 U/ml) in pH= 8 , 9 respectively. The change in the pH of the reaction mixture results in the change in the ionic nature of the amino and carboxylic acid components of the enzyme ,this in turn affects both the conformational status of the enzyme and catalytic site thus altering its activity (25).

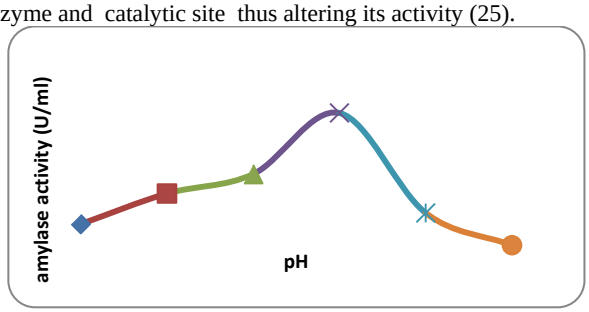

Figure (8) : Effect of diverse pH on the activity of purified amylase from *E.coli*

3.4.2. The best pH for amylase stability:

To study the effect of optimum pH for amylase stability the amylase solution was incubated with diverse buffers pH values ranging between (4-10) , for 30 minutes at room temperature , then calculated the remaining activity. The figure (9) illustrates the best pH for amylase stability ranging between (7 - 8) and the stability was decreased in great alkaline and acidic pH. The enzyme was kept 75% of activity in pH= 5 while the activity was decline in pH= 4 in pH= 9 and in pH =10 to 42%, 58 %, 35% respectively . The effect pH on the enzyme stability is belong to the effect of pH in enzyme structure lead to change the ionic state of active site or denaturation of enzyme molecule, also it effect on secondary and tertiary of enzyme structure lead to behind the activity in buffers that far away from the best pH (26) .

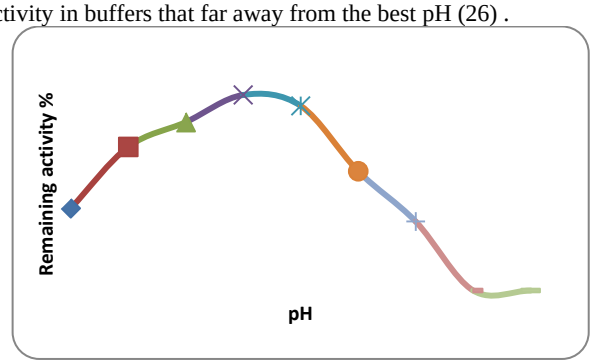

Figure (9) : Effect of pH on the stibility of purified amylase from *E.coli*.

3.4.3. finest temperature for amylase activity:

To establish the best temperature of amylase activity purified from *E.coli* ,the enzyme reaction was done in dissimilar range of temperature (10 - 60) °C , and the results shown in figure (10) rising the activity of enzyme with increasing the temperature until reached to highest activity of amylase (0.271U/ml) in 30°C then it began to decreased in elevated temperature values (0.132, 0.062 , 0.026U/ml) in 40 ,50, 60 °C respectively . Previous also amylase purified have been described for the influence of pH and temperature by [27] .

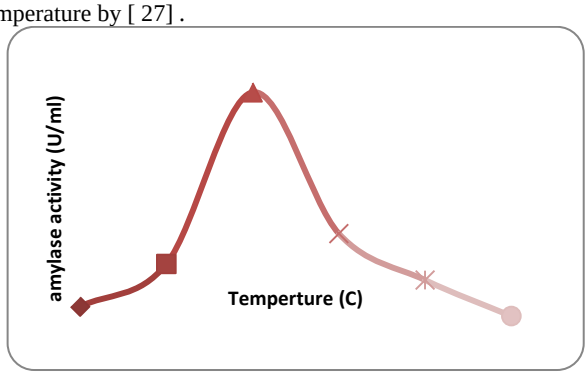

Figure (10) : Effect of diverse temperature on the activity of purified amylase from *E.coli*

3.4.4. The finest temperature of amylase stability

The figure (11) showing the results of incubation of enzyme with diverse temperature ranging between (10 -60) °C for 30 minutes , the enzyme was maintained the activity when it incubated into (30 -40) °C . while keep 71% of its activity in temperature 10 °C , while its keep only 33 % in 60 °C . Additional results were also reported by (22) when they shown the best temperature of amylase stability purified from *Bacillus* sp ranging between (10 -50) °C , the differences of thermostability values and inhibition period of enzyme depend on the molecular weight of enzyme , the enzyme source ,type of substrate,and ionic strength of buffer . (28).

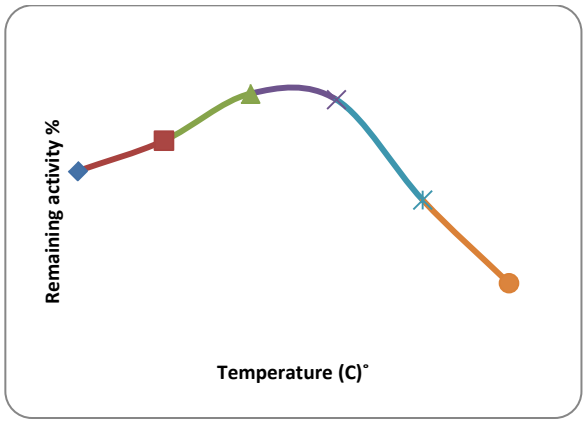

Figure (11) : Effect of different incubation temperature on the stibility of purified amylase from *E.coli*

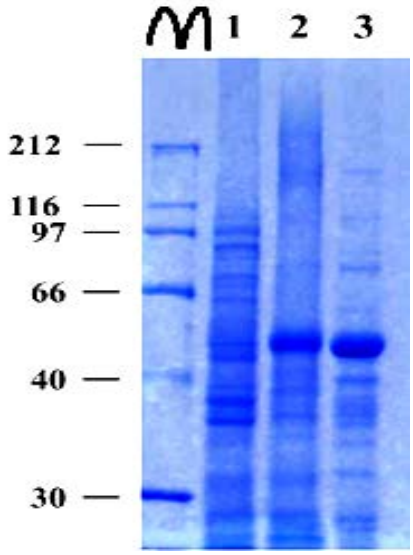

Figure (12) : The polyacrylamide gel electrophoresis of the amylase from *E.coli* under denaturing conditions . Lane (M) standard molecular weight markers, lane (1) crude extract enzyme , lane (2) purified amylase produced from the first step of gel filtration, lane(3) purified amylase produced

3.4.5. Molecular weight determination of amylase by polyacrylamide gel electrophoresis technique from the second step of gel filtration.

In order to examine the purity of the amylase , which was purified from *E.coli*, polyacrylamide gel electrophoresis under denaturing and with concentration12.5 % , the electrophoresis involve three samples , the first sample was crude enzyme extract, the second sample was the first step of gel filtration , the third sample was the second step of gel filtration, when the gel is engrossed in coomassie brilliant blue G- 250, numerous protein bands seemed with diverse molecular weight along the gel in crude extract sample, while one band appeared in the second and third sample. The appearance of many protein bands along the gel is imputed to that crude extract contains large number from different proteins with diverse molecular weights , the second and third sample was gave one band, this means that one protein with one molecular weight of approximately 52 kDa is establish, although wide variation of microbial amylases characters, their molecular weights are usually in the same range 40-70 kDa (29) , the results come closer to (30) when they found the molecular weight of the amylase isolated from *Bacillus subtilis* approximate 53 kDa when its mobility relative to those of standard proteins on SDS-PAGE.

CONCLUSION

Lastly this research it can be finished that *E.coli* isolates can be a fine foundation for the amylase manufacture being used technologically. Amylases purified here was institute to be steady in a pH extent of 7 to 8 and temperature extent of 30°C to 40°C. The efficiency of the amylase refined here is similar to the efficiency of the amylases refined previous by diverse investigators. The molecular mass was determined by SDS-PAGE and a single band was detected after staining procedures giving signal of purity of the amylase.

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