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Optimization Conditions of Production Fibrinolytic Enzyme from *Bacillus lichniformis* B4 Local Isolate

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Abstract: The study was conducted with the aim to found local isolate belongs to *Bacillus lichniformis* to produce fibrinolytic enzyme with highest activity under optimal conditions. Forty-five local isolates belongs to the genus *Bacillus* were selected for production of fibrinolytic enzyme (E.C. 3.4.). The isolate *Bacillus lichniformis* B4 was selected due to its high productivity of fibrinolytic enzyme. The optimal conditions for fibrinolytic enzyme production were determined, using a solid Lentils medium (activity 25.25 U/mL) at pH 7.2 (65.381 U/mL), 10^5 cell/g wet weight (19.185 U/mL) 48 h as incubation time (15.766 U/mL) and shaking incubator (95.992 U/mL) were the optimal culture condition for the production of greatest amount enzyme with highest activity. The optimal carbon and nitrogen sources were mannitol and peptone or soya peptone with activity 44 and 50 unit/mL, respectively.

Keywords: *Bacillus lichniformis*, fibrinolytic enzyme, optimal conditions

INTRODUCTION

Bacillus spp produces a variety of extracellular and intracellular roteases. An alkaline protease (fibrinolytic), a neutral metalloprotease and an esterase are secreted into media, whereas at least two intracellular serine proteases are produced within *Bacillus* spp. (Kumar and Takagi, 1999). Proteases account for approximately 60% of all enzyme sales because of their varied applications in food, pharmaceutical and number of other industries (Ikasari and Mitchell, 1996) such as leather industry, manufacture of protein hydrolizates and waste processing industry (Pastor *et al*., 2001; Al-Jumaily *et al*., 2004). Alkaline protease added to laundry detergents plays a specific catalytic role in the hydrolysis of protein strains such as blood, milk, human sweat, etc. The increased usage of the protease as a detergent additive is mainly due to its environmentally acceptable cleaning capabilities. Amongst bacteria, *Bacillus* sp., is specific producers of proteases (Priest, 1977). The magnificent thing about fibrinolytic is that it appears to have many, if not most, of the benefits of pharmaceutical agents designed to regulate blood clotting (e.g., warfarin, heparin, t-PA, urokinase, etc.) without any of the side effects of these medications. It is a well known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, inoculum density, dissolved oxygen and incubation time. The effect of various carbon and nitrogen nutrient cost-effective substrates, divalent metal ions, environmental and fermentation parameters such as pH, temperature, aeration and agitation were evaluated and reported in the literature that optimum conditions of medium factors are required for maximum enzyme production. Conventional optimization procedures involve altering of one parameter at a time keeping all other parameters constant, which enables one to assess the impact of those particular parameters on the process performance. These procedures are time consuming, cumbersome, require more experimental data sets and cannot provide information about the mutual interactions of the parameters (Beg *et al*., 2003). In this study, the aim of this study was optimization conditions and production of the fibrinolytic enzyme produced from *Bacillus lichniformis* strain B4.

MATERIALS AND METHODS

Isolated *Bacillus*: Get some isolated bacteria of the same species of *Bacillus* which were taken out from different parts of the soil from Baghdad University, Baghdad city during the period from October 2011-March 2012. It was proved that these bacteria were of the same species of the *Bacillus* basing on the phenotypic characteristics of the colonies which have diameters larger than (2 mm) and of disorganized margins.

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Diagnostic tests: Initial diagnostic of *Bacillus* was done by studying the phenotypic and biochemical characteristics according to Parry *et al*. (1983) and Claus and Berkeley (1986).

The phenotypic characteristics: A microscopic examination was done for the cells taken from bacterial colonies of age 21 h stained with Gram stain and specified it s phonotypical characteristics and composition and location of spores and how its response to Gram stain.

Culture characteristics:
Nature of growth on a solid medium: The features of the growing colonies at 37°C, 24 h on the Nutrient Agar at 50°C, for 24 h in terms of shape, size, color and margin.

Nature of growth in a liquid medium: Inoculums bacteria in Nutrient broth or brain and heart infusion broth and incubated at temperature 37°C for 24 h.

Biochemical tests:
Catalase production: This test did by mixing a drop of colony of bacteria growing on brain heart infusion broth age of 24 h with a drop of Peru oxide of hydrogen 3% on a glass slide. The display of bubbles is considered a positive result for the test (Parry *et al*., 1983; Claus and Berkeley, 1986).

Lecithinase production: This test is used to detect the production of Lecithinase and lipase enzymes together. To prepare this test is to take (100) µL of nutrient agar after it is cooled down to 45°C and added 1-1.5 µL of separated egg yolk in sterilized conditions and mixes it together, then poured it in sterilized plats. The inoculated bacteria in the center of the plate and incubate it to 30°C Temp. From one day to week. The appearance of a transparent clear zone around the growth reign is a sign of producing Lecithinase anzyme (Claus and Berkeley, 1986; Collee *et al*., 1996).

Citrate utilization: Slant citrate agars were inoculated by bacteria (stabbing method) and incubated from 2 days to one week at 37°C. The growth of bacteria and the change of colors of the agar from green to blue are considered a positive result (Parry *et al*., 1983; Collee *et al*., 1996).

Anaerobic growth: Plats of nutrient agar were inoculated with bacteria (planning method) and were placed in anaerobic jar with gas generating bag. These plats incubated at 37°C for 24 h. The appearance of the growth means the test is positive.

Gelatin liquefaction test: Gelatin tubes were inoculated with bacteria (stabbing method) and incubated at 30°C and monitoring them for a whole week. Tubes were tested by removing them from the incubator and placing them in refrigerator under 4°C for an hour or more with observing the shape of the agar, it is a positive result if it stays liquid (Parry *et al*., 1983; Collee *et al*., 1996).

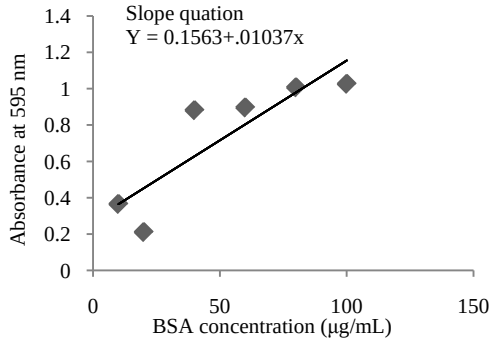

Fig. 1: Standard curve for protein determination

Mannitol fermentation medium: Prepared by adding mannitol sugar 1% percentage to the nutrient broth, phenol red added as indicator percentage of (0.04%) and adjacent the pH to 7.2, then distributed in test tubes.

Determination of protein concentration: Protein concentration was carried out using the method of Bradford (1976) as follow: A (20 µL) of (crude or purified) was mixed with (50 µL) of (1 M) NaOH with continuous shaking for (2-3 min) then (1 mL) of Coomasie Brilliant Blue G-250 was added with shaking. The absorbance was measured at (595 nm) by spectrophotometer. A standard curve bovine serum albumin was carried out using different concentrations (10, 20, 40, 60, 80 and 100 µg/mL) each was pipetted in duplicate in sterilized test tubes. The absorbancy was plotted against the corresponding concentration of bovine serum albumin (Fig. 1).

Media used in screening of Fibrinolytic enzyme: Screening of fibrinolytic enzyme was done using blood agar medium.

Fibrinolytic enzyme assay: Fibrinolytic activity was determined by conducted mixing (0.2) mL of human plasma and (0.8) mL of normal saline with (0.25) mL of (0.25%) of calcium chloride, the (0.5) mL of liquid bacterial growth in the test tube and (0.5) mL of normal saline in the control tube then mixed and incubated for one hours at 37°C. The positive result is hemolytic the clot formed. The number of units was determined according to standard curve by using bovine serum albumin (Fig. 1). The *A*595 nm for the supernatant was measured and converted to the amount of protein equivalent. One unit of Fibrinolytic Activity (FU) was defined as the amount of enzyme releasing 1 µg of

soluble protein equivalent per 30 min (Tillett and Garner, 1933).

Screening optimum enzyme producing isolates: Was chosen the isolate which gave short time to analyze the plasma clot and estimate the activity of enzyme.

Estimate the optimum conditions for enzyme production:

The optimum medium for production: Media mentioned in paragraph has been used, inoculated with 1ml of the bacterial culture with bacterial number of (3.6×10^6) cell/g and the weight of solid medium (refreshed in brain heart infusion broth in 37ºC for 24 h).

(0.2 M) Phosphate buffer pH 7 to the solid media to extract the enzyme, centrifuged the liquid and solid medium after adding of the buffer in cooling centrifuge with 3000 rpm for 15 min then accumulate the supernatant in sterile test tubes, then determinate the enzyme activity.

Screening the optimal nitrogen and carbon sources: Five kinds of nitrogen sources (casein, NH_4Cl, KNO_3, NH_3PO_4 and peptone) and six kinds of carbon sources (maltose, manitol, fructose, glucose, sucrose, lactose) were investigated. In the investigation of nitrogen sources, growth was carried out in the Minimal Synthetic Medium (MSM) containing (g/L): $K_2HPO_4.3H_2O$, 1, $MgSO_4.7H_2O$, 0.5, supplemented with 10 of glucose and the nitrogen source to be investigated. And in the process of screening carbon sources, fermentation was carried out in the same MSM (mentioned above) supplemented with the optimal nitrogen source found out above and the carbon source to be investigated.

Screening the optimal incubator for fibrinolytic production: Two kinds of incubators used (shaking incubator and constant incubator). Growth was carried out in the optimal medium and optimal other condition medium, pH, temperature, time and bacterial inoculate concentration.

The optimum inoculums percentage for enzyme production: Selected isolate has been grown in brain heart infusion broth and incubated at 37ºC for 24 h, Absorption of the bacterial culture was measured the wavelength of 600 nm and attended a series of decimal mitigations for the culture and spread every mitigation on nutrient agar plate, incubated at 37ºC for 24 h and counting the number of cells per milliliter then prepared vaccine from viable cells concentration of (10^4-10^9) cell/mL and inoculums production medium by adding (1) mL from every concentration and incubated at 37ºC for 24 h then the enzyme extracted by precipitate the cells with cooling centrifuge and estimated the enzymatic activity in the supernatant and protein concentration.

The optimal pH for enzyme production: production medium prepared in different hydrogen numbers ranged (6.2, 6.8, 7.2, 7.5, 8) by moisture the wheat bran medium with specific buffer solutions, inoculated the media by adding specific volume of the bacterial culture concentration of (6×10^7) cell/g weight of solid medium and incubated at 37ºC for 24 h then extracted the enzyme and estimated its enzymatic activity.

The optimum period of incubation for enzyme production: Inoculums the sterilized production medium with the bacterial culture of activated isolate by adding 1 mL concentration of (6×10^7) cell/g weight of solid medium and incubated the inoculated media at 37ºC for periods ranged from (1-7) days, then the enzyme extracted and estimated its enzymatic activity.

Extraction of the fibrinolytic enzyme: Sodium chloride solution (0.2) molar and phosphate buffer with pH 7 and distilled water, by adding the same volume of the solution used to moisten Wheat bran (50 mL of these solutions) to the production medium then centrifuged in cooling centrifuge with speed (5000) rpm and estimate enzyme activity of the enzyme extraction in every time, phosphate buffer used as extraction solution.

RESULTS AND DISCUSSION

Forty -five isolate from the soil belonging to the genus *bacillus* family diagnosed from other species, depending on colony, phenotypic and biochemical characters. It appeared as scattered colonies with irregular edges characterized by a cream color oblique to brown with being sticky and sometimes grows high above the surface of the agar after growth on the nutritious agar media for 24 h. The isolated swabs from these colonies were taken and staining with gram stain appeared gram positive rods often arranged in pairs or chains with rounded or square ends and usually has a single endospore either in the center of the cell or at a point close to the center (Duguid, 1996; Sneath *et al*., 1986).

Phenotypic properties: Shape of the cells long coli or moderate length, dimensions of vegetative cells Ranging from (0.5×12 µm) to (2.5×10 µm) Its response to gram stain is Positive, The form of spores elliptical Spores site in the center of the cell or to the side a little.

Colony properties: Diffuse on the solid medium and somewhat flat, growing down the middle or on the surface of the center of liquid media for being aerobic,

Table 1: The efficient *Bacillus* isolates producing fibrinolytic enzyme

Bacillus isolates	Enzyme activity (U/mL)
B1	3.182
B2	11.57
B3	3.000
B4	29.315
B5	9.566

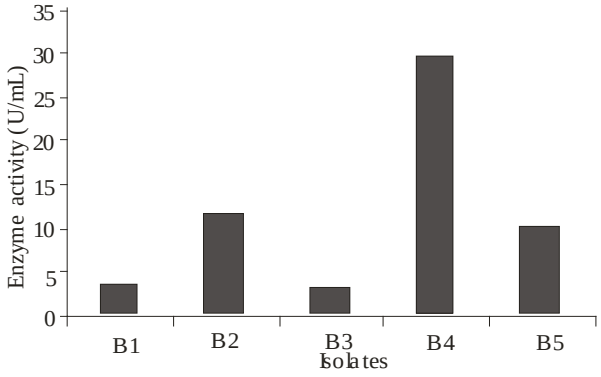

Fig. 2: The quantitative screening for efficient *Bacillus* isolates producing fibrinolytic enzyme

off white to brown colonies, Colony with circular or lobed edges, often smooth surface of the colony and sometimes sticky.

Screening the ability of isolates to produce fibrinolytic enzyme: Screenings of fibrinolytic enzyme was done using blood agar medium, some isolates which grown on nutrient broth appear inhibition zone (type ß) around the colonies on blood agar medium, five isolates mention as (B1, B2, B3, B4, B5) according to their ability to hydrolysis of blood clot, by use M-liquid medium as production medium and B4 isolate characterized with its optimize production for fibrinolytic enzyme activity was 29.315 Unit/mL. (Table 1) (Fig. 2).

Diagnosis of efficient isolate in the production of fibrinolytic enzyme: Diagnostic tests performed on the efficient isolate producing fibrinolytic enzyme as in the simplified diagnostic key proposed by Parry *et al*. (1983):

- **Phenotypic characteristics:** Bacterial cells appeared at the microscope it was gram positive bacilli, single, dual, or in series form with oval-shaped spores, central or terminal site.
- **Biochemical characteristics:** Confirmed some of the biochemical tests (Table 2), as well as phenotypic characteristics previously mentioned ownership of these isolates to the genus *Bacillus licheniformis*, As all were product of the fibrinolytic enzyme and this isolate was licithinase Productive, citrate consuming and grow an aerobically and gelatin liquefied.

Bacillus licheniformis adhered to the culture medium strongly, its spores resist difficult conditions like temperature, insufficient nutrient material and gelatin liquefied so primarily Bisset and Bartlett (1978) diagnosed it as *Clostridium licheniformis* so tests in Table 2 used to diagnosis the isolate and the result mentioned that the isolate belong to *Bacillus licheniformis* species.

Table 2 microscopic and biochemical tests of the isolates belonging to the local bacteria *Bacillus licheniformis* selected from the quality and quantity screening processes produced fibrinolytic enzyme according to the simplified key for parry *et al.* (1983).

Effect of different carbon sources on production of fibrinolytic enzyme: Six types of carbon sources were investigated: maltose, manitol, fructose, glucose, sucrose, lactose at glucose is a generally preferred carbon source for growth of bacteria and it was used as reference. The other five carbon sources had all been reported to be the best in respect of protease production by strains of *Bacillus lichniformis*. The results showed that, based on fibrinolytic production, mannitol was the optimal carbon source (46 unit/mL). Glucose, maltose and sucrose had similar positive effects, while lactose and sucrose performed poorly. So maltose was chosen as carbon source for the following investigations (Fig. 3) this accordance with the results of Wang *et al.* (2009) who showed that, based on nattokinase production, maltose was the optimal carbon source and disagreement with Bhunia *et al*. (2010) who mentioned that protease production by lactose give high activity more than maltose, mannitol, sucrose, fructose and glucose. In other study the highest nattokinase enzyme production was obtained when maltose was used.

Effects of different nitrogen sources on production of fibrinolytic enzyme: Six kinds of nitrogen sources were examined (casein, NH_4Cl, KNO_3, NH_3PO_4 and soya peptone) of all the nitrogen sources tested, soy peptone was found to be the most promising one and the corresponding fibrinolytic activity is 50 unit/mL (Fig. 4). When inorganic nitrogen sources were used,

Table 2: Microscopic and biochemical tests of the local bacteria *Bacillus licheniformis*

Isolate	Species	Gelatin liquefaction	Anaerobic growth	Citrate utilization	Licithinase production	Spore shape	Catalase	Mannitol
B4	*B. licheniformis*	+	+	+	-	Oval	+	+

+: Positive test; -: Negative test

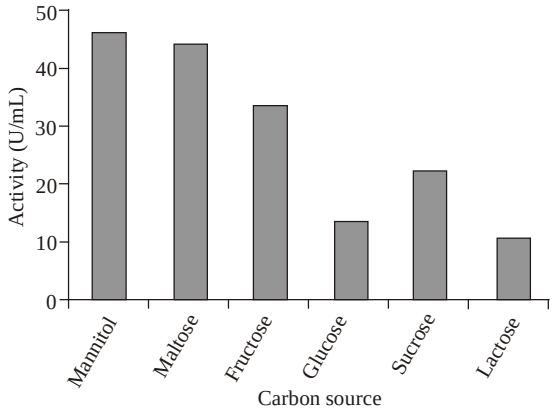

Fig. 3: Effect of different carbon sources on production of fibrinolytic enzyme

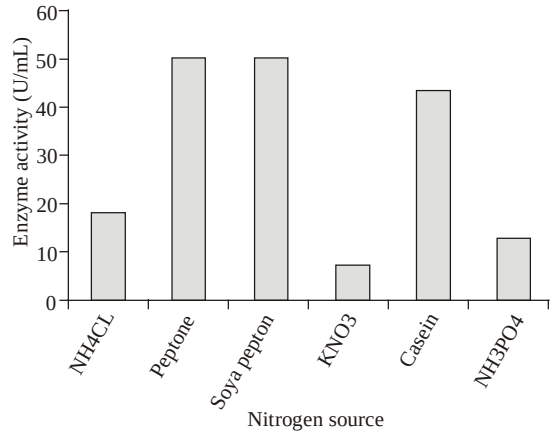

Fig. 4: Effect of different nitrogen sources on production of fibrinolytic enzyme

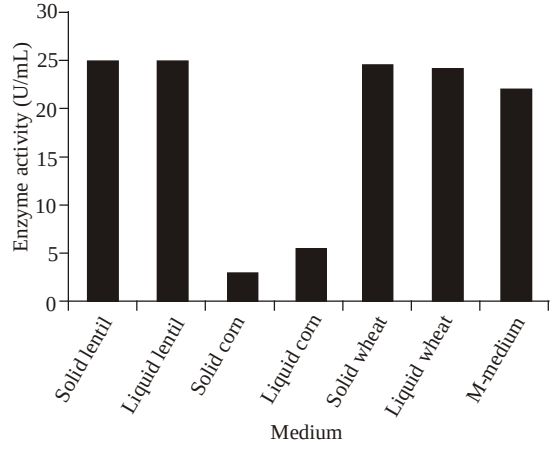

Fig. 5: Ability of the local isolates of *Bacillus licheniformis* on the growth and enzyme production in different media

very poor enzyme activities were obtained. While much higher activities were obtained by using the organic nitrogen sources. Casein gave less enzyme activity in compare with the other organic nitrogen sources. The optimal nitrogen source was soy peptone, which was used as nitrogen source in the following investigations. This result Similar to the mentioned by Liu *et al*. (2005) who show that soy peptone was found to be the best one, for fibrinolytic activity. When inorganic nitrogen sources were used, very poor enzyme activities were obtained, also agreement with Bhunia *et al*. (2010) who study the effect of different nitrogen sources on protease production, such as soya peptone, peptone, ammonium chloride, potassium nitrate and others and show that the organic nitrogen sources (soya peptone, peptone) give maximum extracellular alkaline protease production was obtained in presence of soypeptone and peptone and minimum extracellular alkaline protease production was obtained in presence of potassium nitrate and ammonium chloride. Wang *et al*. (2009) screen different nitrogen sources, soybean meals, soy peptones, sodium glutamate, ammonium phosphate and yeast extract were replaced at the same concentration and when inorganic nitrogen sources were used very low enzyme activities production were achieved much higher activities were obtained with organic nitrogen sources.

Different culture media used for growth of isolate B4 to choose the optimal medium for enzyme production. These media have varied in their content of carbonate and protein sources, which included simple carbohydrate, such as glucose and different protein sources such as yeast extract, as well as the use of plant material as culture media in their liquid and solid form to produce fibrinolytic enzyme. Observed variation in the production of enzyme for this isolate when grown in various medium and the selected isolate have given highest productivity in solid lentils medium which reached (25.25 U/mL) Followed by liquid lentils medium (25.071 U/mL), liquid wheat (24.643 U/mL) and M-liquid medium (22.469 U/mL) compared with the rest media which give the low productivity of the enzyme (Fig. 5).

The study was demonstrated that the production of enzymes in the solid-state fermentation than what it is in the fermentation liquid medium so it's best in many cases the use of solid-state fermentation production than liquid fermentation states in addition to that the ability to provide of easy handling medium and cheap cost. Wheat bran and lentils was most helpful to the enzyme yield.

Screening the optimal incubator for fibrinolytic enzyme production: Whereas the medium volume increased sequentially, the enzyme activity did not change significantly. *B. species* is an aerophilic bacteria; less medium provides a greater amount of oxygen for microorganism multiplication and metabolism. As shown in Fig. 6, the acceleration of the flask shaker led to a remarkable enhancement of enzyme production and the highest enzyme activity was 95.992 u/mL gained at 180 rpm. Higher rotating speed gave richer air for aerophilic bacteria, which is

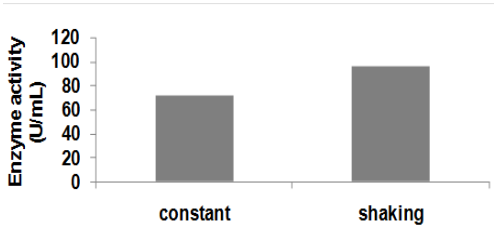

Fig. 6: The effect of shaking on production of fibrinolytic enzyme

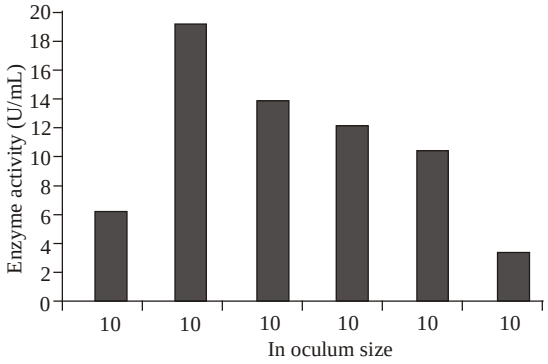

Fig. 7: The effect of inoculums volume on the enzyme production

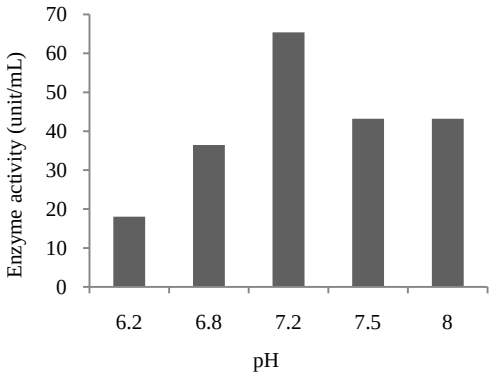

Fig. 8: Effect of pH on the fibrinolytic enzyme production

advantageous to enzyme production. Kumar and Takagi (1999) tested agitation (0, 50, 100 and 200) rpm, The optimum agitation speed was (200 rpm) which give maximum enzyme activity.

Screening the optimum inoculums percentage for enzyme production: The results showed that the production of the enzyme increases with increasing concentration of bacterial inoculums up to (10^5 cell/g wet weight) as it was effective (19.185 U/mL) and activity decreased when the amount of inoculums increased. This can be explained that when we add 10^5 (Fig. 7) the components consume the medium efficiently and at increasing the amount of the inoculums increases the number of cells that secrete enzymes analyzed the components of the medium.

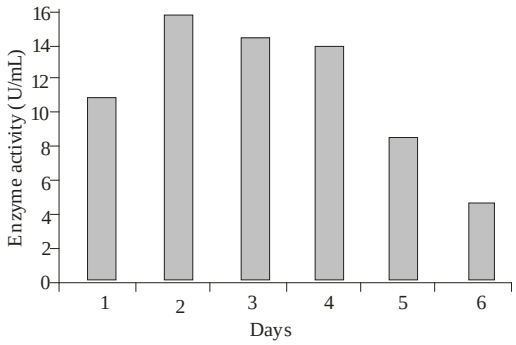

Fig. 9: Effect of incubation period on the fibrinolytic enzyme production

Affecting the contents of the medium directly in the rapid growth of microorganisms increased a certain threshold lead to congested cells and competes for nutrients and increase the amount of toxic metabolic products and rapid consumption of oxygen. The optimization of the inoculums size depends mainly on the growth period allowed (age of colony) for the applied culture, thus while the best inoculums age for production of proteases enzyme by *Bacillus subtilis* was 24 h. Inoculum size was optimum for highest protease enzyme production. The highest levels of protease production were obtained when *Bacillus species* growing in fermentor (flask) capacity 250 mL^{-1} (Ellaiah and Srinivasulu, 1996).

Screening the optimal pH for firbolytic enzyme production: The ability of the *Bacillus lichniformis* isolate to produce firbolytic enzyme using the medium of lentils in different hydrogen numbers (6.2, 6.8, 7.2, 7.5, 8) the results showed that the production of the enzyme is very low at pH (6.2) While increased productivity and reached a maximum at pH values (7.2) as it was enzymatic activity (65.381 U/mL) while there was a decrease in activity at higher pH values (7.5 and 8) as the activity at pH (8) was (43.212 U/mL) Which indicates that the optimum pH for the production of the enzyme is (7.2) (Fig. 8), This is in accordance with Siraj (2011) who mentioned that The optimum pH for cell growth and nattokinase production is (7). The concentration of hydrogen ion affect on the activities of the many micro organisms where the microstructure affects the amount and speed of growth, there are maximum and minimum concentrations for growth and differ depending on the microorganisms so we must modify the pH of the medium before culturing the microorganisms.

Screening the optimum period of incubation for fibrinolytic enzyme production: The production of fibrinolytic enzyme from the local isolated *Bacillus lichniformis* in different time periods (1-6) days, Fig. 9 showed that the production of the enzyme was low after one day of incubation, as its enzymatic activity was (10.819 U/mL) and observed an increase in fibrinolytic

enzyme production continuously for incubation date and the highest production of the enzyme after 2 days and has given the activity of (15.766 U/mL), as there was a decline in enzyme production after 3 days of incubation. Then decreased productivity until they reached their lowest levels after 6 days this means that not a little part of the enzyme product from the bacteria may be exposed to degradation either because the changes of the developments on the medium of production, especially in the pH, or because of secretions of other enzymes from bacteria itself to the medium of production and of enzymes degrading proteins (Proteases) that are likely it's part of the molecules analyzed enzyme fibrinolytic in the middle. Scientific literature varied to determine the optimal duration for the production of fibrinolytic enzyme it have been defined by each of Kwon *et al*. (2011) with 21 h, Ellaiah and Srinivasulu (1996) mention that The optimum incubation period for protease activity was 24 h (1840.77 units/mL). With 42 h while Wang *et al.* (2009) mentioned that the enzyme production peak was at 60 h.

CONCLUSION

From the results apparent that local strain *Bacillus licheniformis* B4 gave highest production fibrinolytic enzyme with high activity after optimization conditions.

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