STUDY OF OPTIMUM CONDITION FOR ALKALINE PROTEASE PRODUCTION FROM LOCAL ISOLATE OF *Aspergillus niger*

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Abstract

Extracellular alkaline protease production by *Aspergillus niger* was studied with the presence of different carbon and nitrogen sources availability in batch culture condition .*A. niger* showed better protease production in the presence of organic nitrogen sources compare to inorganic nitrogen sources in the medium, which consist of 1% glucose as carbon source, 1.5% casein hydrolysate as nitrogen source and 1% Nacl at initial pH=10 on rotary shaker incubator 140 rpm at 40 ºC for 5 days.

دراسة الظروف المثلى لانتاج انزيم البروتيز القاعدي من العزلة المحلية *Aspergillus niger* للعفن

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الخلاصة

تم إنتاج إنزيم البروتيز القاعدي من العفنِ *Aspergillus niger* باستعمال مصادر كربونية ونتروجينية مختلفة في مزارع الدفعة الواحدة ,كانت أفضل إنتاجية للأنزيم بوجود مصادر النتروجين العضوية مقارنة بمصادر النتروجين اللاعضوية في الوسط الإنتاجي الأمثل المتكون من 1% كلوكوز كمصدر للكربون و1.5% متحلل الكازائين كمصدر للنتروجين و1% ملح الطعام Nacl وبرقم هيدروجيني ابتدائي pH =10 باستعمال حاضنة هزازة وبسرعة تدوير 140 دورة/دقيقة بدرجة حرارة 40م° لمدة 5 ايام.

Introduction

Industrial enzymes have seen a spectacular rise in their production in the last three decades. The growth of industrial enzyme market has expanded to nearly 85 enzymes, which are currently in commercial production. With the discovery of a variety of new and more active enzymes, the enzyme market has been forecasted to go up to US $ 1.7-2.0 billion by 2006[1]. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes[2].

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins; proteolytic enzymes can be classified as acidic, neutral and alkaline proteases, with regard to their pH working range. Acidic proteases have application in meat tenderization, in the production of fermented foods and also in acidic cleaning compositions[3].

Neutral and alkaline proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment friendly technologies[4].

Alkaline proteases have numerous applications in food industries[5,6,7], silver recovery from X–ray film[8] and several bioremediation processes.

Plants,animals and microbial sources are employed for protease production. Microbes serve as the preferred source of proteases because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties[9]. Microbial extra cellular alkaline proteases are important enzymes and are mainly used in detergents to facilitate the release of proteinaceous stains such as blood, milk, egg and meat, food and leather industries and is an important tool in studying the structure of protein and peptides.[10,11] . They account for approximately 40 % of the total worldwide enzyme sale. Though a good number of bacterial alkaline proteases such as subtilisin and Savinase are commercially available, having their major application as detergents enzymes but alkaline proteases of fungal origin offer an advantage over bacterial proteases because (i) the mycelium can be easily removed from the final product by simple filtration, (ii) ability of the fungus to grow on cheaper substrate, (iii) easy immobilization of mycelium for repeated use, (iv) broad range of pH (4-11) and substrate specificity and hence low cost of production[10,12]. For industrial use enzyme must be produced at low cost and should be reused reproduce result with consistent efficiency, proteases are generally produced by submerged fermentation. In this paper *Aspergillus niger* was investigated for screening of medium components and culture conditions for protease production.

Experimental

Microorganism and culture conditions

A.niger was obtained from Biotechnology Department, Collage of Science Baghdad University, Iraq. The organism was maintained on potato-dextrose agar slants at 4 ºC. Spores were inoculated into the PDA plate by streaking method from the slant preserved in 4ºC refrigerator. Spores were scraped from the plate surface by adding distilled water with 0.85% NaCl and 0.01% Tween 80 to the growth agar plate which have been cultivated for 3 days. The concentration of the spore was maintained at level of 10^8 cells per mL. This solution was kept in 4 ºC refrigerator and used as the inoculums into 50 mL medium. The Czapek Dox medium has been used through out the study for protease production by the isolate *A.niger* .The composition of (g/l): K2HPO4, 1; MgSO4 ·7H2O, 0.5; KCl, 0.5; FeSO4 · 7H2O, 0.01; sucrose, 30; casein, 1% (w/v). The culture was grown for 72 h in a shaking incubator (140rpm) at 30 ºC. The pH of the medium was adjusted to 8 at the end of fermentation period, the broth was centrifuged at 10,000 rpm, 4 ºC for 20 min and the clear supernatant was used as the crude enzyme fermentation experiments were run with 50ml each of the above-described medium (was used to initiate the growth) in 250 ml Erlenmeyer flasks. 5% level of 24 h inoculums raised in the basal medium was used to initiate the growth. The inoculated flasks kept on rotary shaker incubator (140 rpm) at 30 ºC for 3 days.

Protease assay

Protease activity in the culture supernatant was determined by using the method originally described by Murachi[13] and modified by Senior[14] as following**:**

Casein (0.8ml, 0.5%, pH8) was preincubated in a water bath at 37 ºC for 10min. Unit of enzyme activity was defined as the amount of the enzyme, which gives 0.001 increases at Ab. 280nm /min. under the assay condition .The protease activity was determined by using this equation:

Protease activity U/mL = Ab. at 280nm / 0.001×20min×0.2ml.

Results and Discussion

Carbon sources

Effect of carbon sources (0.5%) was studied in protease production medium described above where Sucrose was replaced with sugars or sugar alcohols glucose, maltose, starch, fructose, lactose and glycerol. Glucose was found to be the best source which gave activity 320 U/mL compared to other carbon sources gave only (260-312 U/mL) activity compared to glucose (Fig1.) that was quite similar to that obtained by Srinubabu et al.11, Phadatare et al.16 reported highest activity with arabinose followed by sucrose while *Sutar et al.*[17] found sucrose, glucose and fructose to be equally good to be the best carbon source for protease production by Bacillus brevis14 and A.

flvaus18. Johnvesly and Naik reported19 that starch, raffinose, arabinose and fructose to be good carbon sources while glucose totally represented protease production. For commercial production, sugars like raffinose, arabinose or maltose will be prohibitive due to their cost and hence starch and glucose will be more suitable as carbon sources.

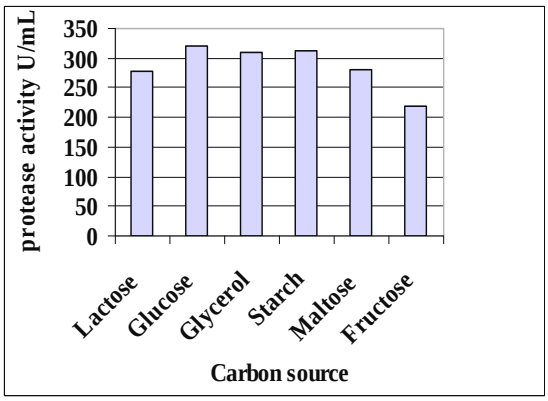

Figure 1: Effect of Carbon source on Enzyme activity.

Nitrogen sources

Protease production was studied in 0.5% glucose as a carbon source and casein was replaced with various inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, ammonium chloride and potassium nitrate) or organic nitrogen sources (peptone, gelatin, malt extract, casein hydrolysate) at equivalent nitrogen concentration. It was observed that potassium nitrate was found to be the best nitrogen source which gave activity 210U/mL compared to other inorganic sources (Fig.2). Johnvesly and Naik[18] found that nitrate nitrogen NaNO3 and KNO3 supported protease production while ammonium nitrogen completely inhibited production. Among the organic N-sources, casein hydrolysate gave highest activity 338U/mL while activities were slightly lower with, gelatin and malt extract (Fig.2) Phadatare *et al.*[16] reported that tryptone followed by peptone. and yeast extract to be good organic nitrogen sources while beef extract was poor for protease production by *C. cornatus*. Yeast extract was best followed by casein while beef extract is poor organic nitrogen source for protease production[18] in thermophilic *Bacillus* sp. JB-99. Similarly, Soya peptone was the best organic nitrogen sources for protease production [15] in *B. brevis*.

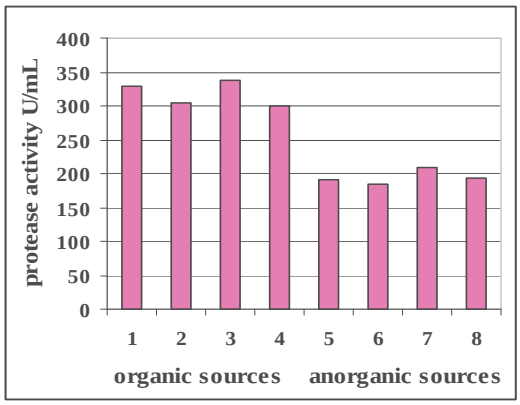

Figure 2: Effect Nitrogen source on Enzyme activity.

1=Pepton,2=Gelatin,3=casein hydrolysate,4=Malt extract,5=Ammonium nitrate,6=Ammonium sulphate,7=Potassium nitrate,8=Ammonium chloride

Effect of carbon and nitrogen sources percent on protease activity

After determination of suitable carbon and nitrogen source for optimum production of protease ,it is necessary to find out the concentration of optimum carbon and nitrogen source in the medium .For this reason the study was carried out with 0.5to2.5% glucose as carbon and casein hydrolysate as nitrogen sources keeping other experimental conditions at optimum level ,the results in (Fig.3) showed that the maximum enzyme activity 327U/mL was at 1% of glucose concentration but it decrease to170 U/mL at 3% this results agreed with Srinubabu *et al.*[11],kiramay and Lakshmi [19] and the results in (Fig.4) showed that the best activity of enzyme about 345U/mL when we used 1.5% of casein hydroltysate as nitrogen source. kiramay and Lakshmi [19] reported that the highest activity was obtained with 0.5% casein as nitrogen source while Md. Tohid *et al.* [20] found highest enzyme activity when he used gelatin as nitrogen source in 1.5% concentration.

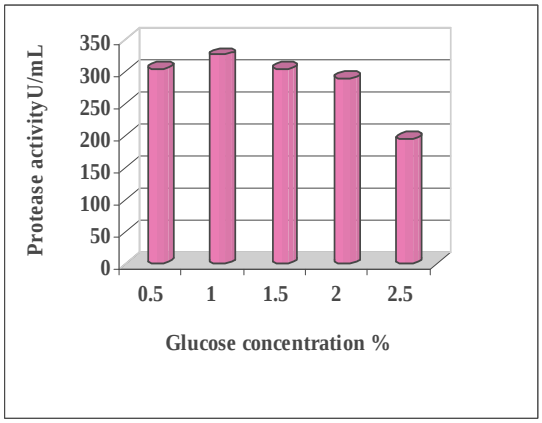

Figure 3: Effect of Glucose concentration on Enzyme activity.

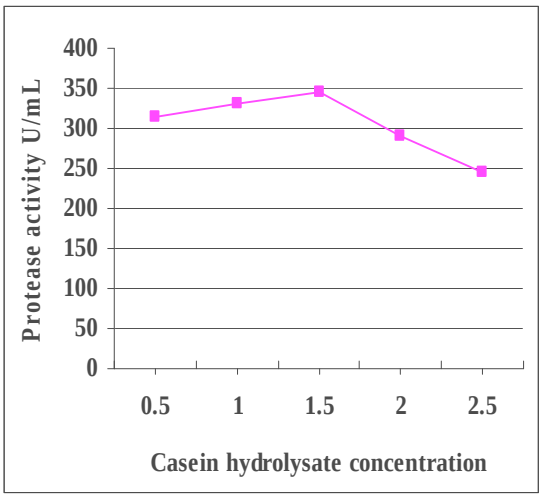

Figure 4: Effect of casein concentration on Enzyme activity.

Effect of initial medium pH on protease production.

Different initial pH values (4-12) were used to study their effect on protease production. The fermentation and assays were carried out in triplicate as the general procedure. It is clear from (Fig.5) that the organism grew well at a wide range of pH 4 – 12. The maximum cell mass and protease yield were obtained at an initial pH 10. So the optimum pH for protease production was found to be 10 (365U/mL) it was observed that in alkaline conditions, the protease yield was high when compared to acidic conditions. This result was similar to alkaline protease produced by A.oryzae in similar study reported by Srinubabu et al.[11], Kiranmayee et al.[19] reported that pH9 was the optimum for protease produce by Bacillus firmus 7728, Coral et al[21] found that the optimum pH to produced protease from *A.niger* was 9.

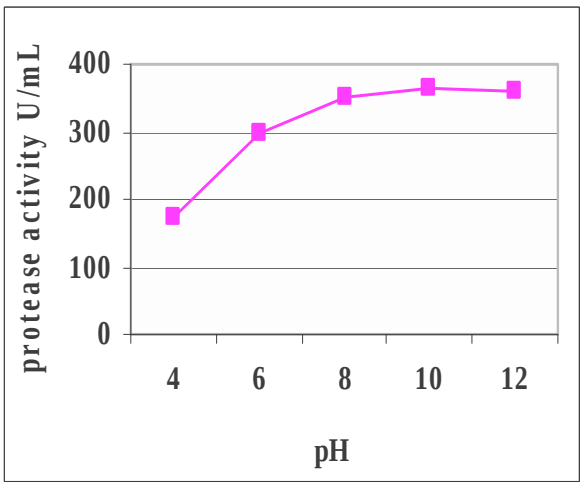

Figure 5: Effect of pH on enzyme activity.

Effect of incubation temperature on protease production.

To study the effect of various temperatures on the growth and alkaline protease production, different temperatures ranges (25, 30, 35,40,45,50 and 55 °C) were used. The fermentation and assays were carried out in triplicate as described earlier the results are shown in (Fig.6). The results indicate that the alkaline protease activity increased with increasing temperature from 25°C to 40°C, since the activity of 355U/ml at 25°C increasing to 368U/ml, 386U/ml at 30°C and 35°C respectively. Maximum protease activity was observed at 40°C (405 U/mL) but the activity decreased to120U/ml at 55°C we have agreed the results with the study reported by Kiranmayee *et al.*[19]. While Coral *et al.*[21] ,Srinubabu *et al.*[11] found that the optimum temperature for the produced protease from *A.niger* was 30°C. Towhid *et al*[20]reported that optimum temperature was 27°C to produced protease by *A.funiculosus*.

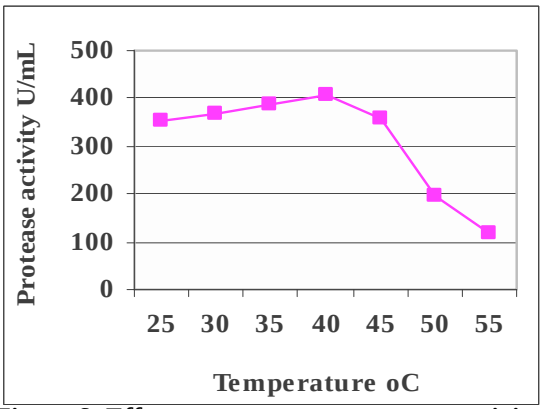

Figure 6: Effect temperature on enzyme activity.

Optimum incubation period for protease production.

The optimum incubation time was studied to determine in which phase of fungal growth the protease was produced, so it had been used different times of incubation (1, 3, 5 and7 days). (Fig.7) was shown that the highest protease activity was after 5 days of incubation which was 435U/mL.While after 7days of incubation the production was decrease and the activity was 310U/mL .it was found that the alkaline protease was produced throughout the first 2 days and the activity was increased after 4 days until it reached to highest activity in 5 days .The decrease of protease activity after 5 days of incubation may be due to autolysis or may be due to change in culture condition during longer incubation which effect negatively on protease production ,this results similar with the results obtained by Coral *et al.*[21] when produced alkaline protease from *A.niger* isolate .Kiranmayee *et al.*[19] found that the maximum alkaline protease activity produced by *Bacillus firmus* 7728 was after 3days, Towhid *et al.*[20] reported highest activity of the enzyme produced by *A.funiculosus* was after 3days**.**

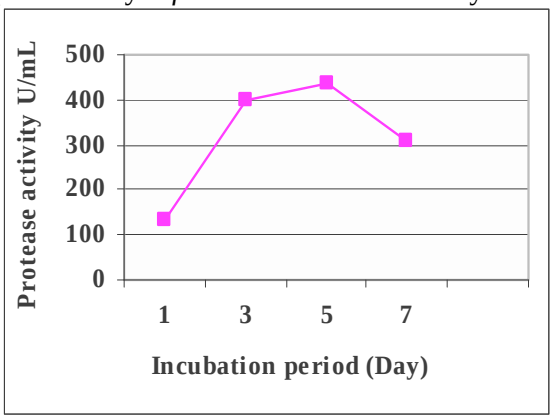

Figure 7: Effect of Incubation period on enzyme activity.

NaCl (sodium chloride) tolerance

The effect of salt on the *isolate A.niger was* studied by growing it in optimum (production) medium supplemented with varied concentrations of NaCl (1, 2, 3, 4, and 5%) at 40ºC in a shaker incubator at 140 rpm. Protease production was monitored at regular intervals. The maximum enzyme activity was 443 U/mL at 1% NaCl concentration was found to be optimum for this alkaline protease produced by *A.niger*. (Fig.8) Kiranmayee *et al.*[19] observed that the optimum alkaline protease activity produced by *Bacillus firmus* 7728 was at 2% Nacl concentration. While Abdulsahib[22] found that optimum protease production by *Bacillus cereus R29* was without using Nacl salt.

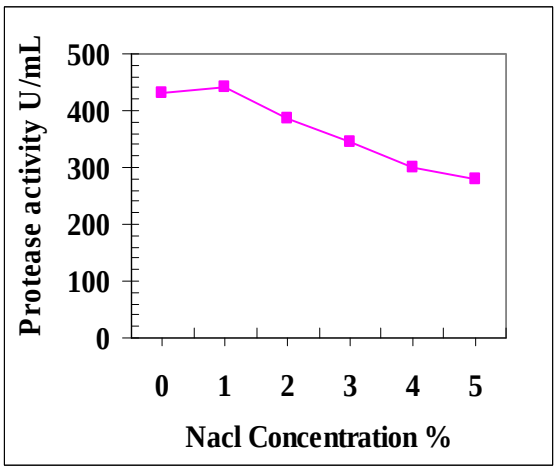

Figure 8: Effect of Nacl concentration on enzyme activity.

Conclusion

The extracellular alkaline protease produced by *A. niger* could have great potential in various industries such as detergent, degumming and several processes like silver recovery, bioremediation and protein hydrolysate production, as the isolate is capable of various functions like casein, gelatin and starch hydrolysis. The organism is active at mesophilic range of temperatures, which is the prerequisite for developing environment friendly technologies. The enzyme may have an important role to play in the food industry.

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