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PRODUCTION, OPTIMIZATION AND CHARACTERIZATION OF L-ASPARGINASE FROM *ASPERGILLUS FLAVUS*

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ABSTRACT

Five fungal isolates were investigated for the production of extra-cellular L-asparaginase. ***Aspergillus flavus*** showed to be the most potent for L-asparaginase production followed by ***Aspergillus subolivaceus, Ulocladium alternariae***, ***Fusarium oxysporum*** and ***Trichoderma viride*** in descending order. Maximal L-asparaginase productivity was attained after five days incubation period. The optimum conditions for enzyme production were pH 6.2 and at 30ºC. Fructose served as the best carbon source. On the other hand, the maximal enzyme yields on using L-asparagine as nitrogen source. The produced enzyme showed optimum activity after 10 minutes of incubation at 37**°C** andpH 8.2Mg^{2+} slightly activated the produced L-asparaginase activity.

Key words: *Aspergillus flavus*, L-asparaginase production, Optimization. Asparaginase activity,.

INTRODUCTION

L-asparaginase (EC 3.5.11) specifically catalyzes hydrolyzes of L-asparagine to L-aspartate and ammonia **(Bessoumy *et al*., 2001),** and plays an important roles both in metabolism of all living organisms as well as in pharmacology **(Borek & Jaskolski, 2001)**. The important application of L-asparaginase is in the treatment of acute lymphoblastic leukemia, Hodgkin disease, acute myelocytic leukemia, acute

myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma **(Verma *et al*., 2007).** In addition, L-asparaginase is the first enzyme with antitumor activity to be intensively studied in human beings. It is an enzyme drug choice for acute lymphoblastic leukemia in children used in combination therapy **(Schemer& Holcenberg, 1981).** L-asparaginase broadly distribute among the plants, animals and microorganisms. The microbes are the better source of L-asparaginase, because they can be cultured easily and the extraction and purification of enzyme from them is also convenient, facilitating the large-scale production **(Savitri *et al*., 2003).** The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine as in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, meanwhile, the normal cells are able to synthesize L-asparagine **(Narta *et al*., 2007)**.

L-asparaginase has also been found in variety of fungi, yeasts and algae. It has been observed that eukaryotic microorganisms like yeast and filamentous fungal genera such as ***Aspergillus, Penicillium*** and ***Fusarium*** are commonly reported in scientific literature to produce L-asparaginase with less adverse effects **(Sarquis *et al*., 2004).**

MATERIAL AND METHODS

The fungal cultures used in the present study were locally isolated from soil samples collected from Mansoura University garden. Fungal isolates were subjected to full identification using the most recent sophisticated facilities; an imaging analysis system using soft-imaging GbH software (analy SIS Pro ver.3.0) at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, EGYPT.The identified strains were subjected to modified Czapek Dox agar medium to make rapid plate assay for L-asparaginase. (Ref) The composition of this medium was: glucose 2 g, L-asparagine 10 g, KH_2PO_4 1.52 g, KCl 0.52 g, $MgSO_4.7H_2O$ 0.52 g, $FeSO_4.7H_2O$ 0.01 g, agar 20 g, distilled water 1000 mL.

Enzyme assay

From culture filtrate L-asparaginase was measured by the modified method of **Imada *et al*., (1973).** A reaction mixture containing

0.5 mL of 0.05 M tris HCl buffer (pH 8.2), 0.1 mL of 40 mM L-asparagine, 1.0 mL of suitably diluted enzyme source and 0.4 mL of distilled water (total volume of 2.0 mL) was incubated at 37°C for 30 min. The reaction was terminated by adding 0.5 mL of 1.5 M tricholoroacetic acid (TCA). Blank tubes were prepared by adding the enzyme source after the addition of TCA. After termination of the reaction, 3.7 mL volume of distilled water and 0.2 mL Nessler's reagent were added to 0.1 mL of the above reaction mixture and incubated for 20 min. The amount of ammonia released during the reaction was determined by measuring the absorbance at 450 nm.

One unit (U) of enzyme activity was defined as the amount of enzyme which librate one µmole of NH3 per min under the conditions of assay. **(Thirunavukkarasu *et al*., 2011)**.

Optimization studies:

The effect of incubation period for fermentation, initial culture pH values **(4.2-8.2),** incubation temperatures **(20-40°C),** equimolecular amounts of different carbon sources and different nitrogen sources were investigated to optimize the culture condition to maximize L-asparaginase production. The culture filtrate was separated by centrifugation at 5000 rpm and used as a source of L-asparaginase.

Characterization studies:

Effect of temperature and pH on enzyme activity were determined by changing individually the condition of activity assays: Temperatures from **(20ºC, 30ºC, 37ºC, 50ºC and 60ºC)** and at pH from **(4.2, 5.2, 6.2, 7.2, 8.2, 9.2 and 10.2)** using citrate buffer **pH4.2-6.2,** Tris HCl buffer **pH 7.2-8.2** and Glycine –NaOH buffer **pH9.2-10.2.** to determine the maximum L-asparaginase activity by ***Aspergillus flavus*** after **10** minutes incubation.

RESULTS AND DISCUSSION

Screening of L-asparaginase producing fungi:

Table 1 and Fig.1 showed L-asparaginase activity of five fungal isolates obtained from some local s oil samples and screened for their L-asparaginase activity using rapid assay method, after five days incubation at **30°C** and pH **6.2** under static submerged fermentation.

Table (1): Screening for the most active L-asparaginase producing fungi

No	fungal strain	Diameter of pink zone (cm)	L-asparaginase activity (U/ml
1	***Aspergillus flavus***	**6.3**	**37.615 ± 0.110**
2	***Aspergillus subolivaceus***	**2.2**	**20.821 ± 0.099**
3	***Ulocladium alternariae***	**4.5**	**13.440 ± 0.109**
4	***Fusarium oxysporum***	**4.6**	**11.194 ± 0.05**
5	***Trichoderma viride***	**3.2**	**3.813 ± 0.01**

When the concordance between agar plate assay and spectrophotometric method was examined, some isolates had small enzyme activity despite the presence of large zone radii. This result was similar to earlier work of **(Lee *et al.* 2005*)*** who suggested that the ability of fungi to produce enzyme was different in solid (plate assay) and liquid (spectrophotometric assay) state conditions and in this study it is also evident that some isolates had large enzyme activity despite the presence of small zone radii.. From the results obtained (Table: 1), screening indicate that ***Aspergillus flavus*** is the most active L-asparaginase producing fungus compared with other isolates. Therefore, ***Aspergillus flavus*** was used for further work of optimization and characterization studies.

Optimization of enzyme activity

Effect of incubation periods on L-asparaginase production by *A. flavus*

The maximum L-asparaginase activity is recorded after 120 h incubation on static submerged L-asparagine containing Czapek Dox medium Fig. 2. Similar results were reported by **Suresh and Jaya, (2013)** with *Aspergillus terreus* by using SSF method that showed increase in enzyme production till a certain period of 120 h and then gradually, enzyme production decreased.

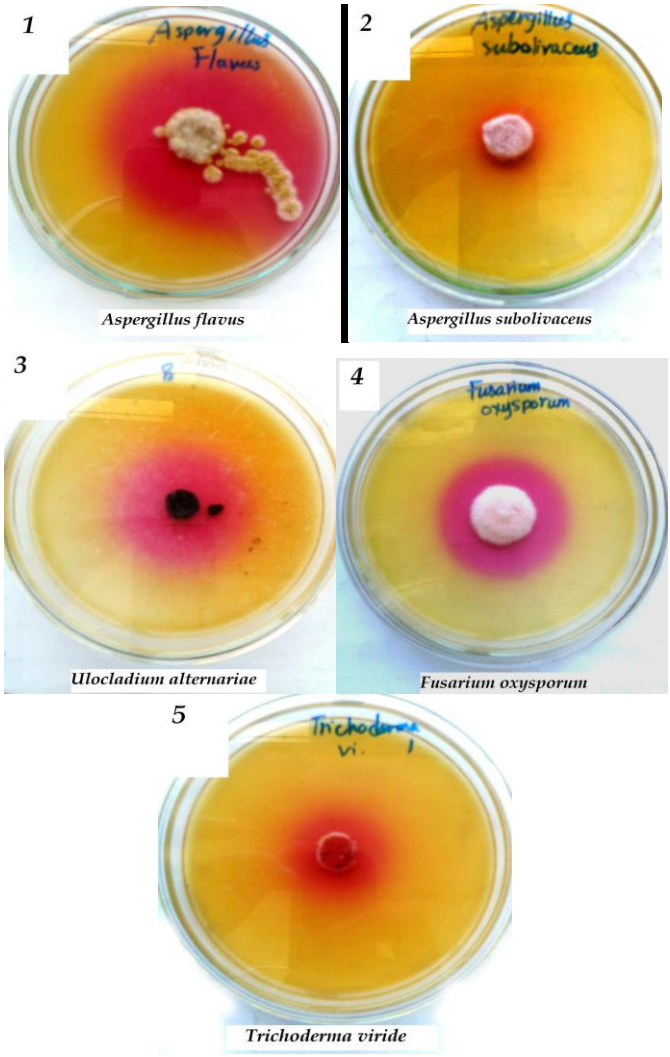

Fig. (1): Pink zone diameter for the most active L-asparaginase producing fungi by rapid plate assay method.

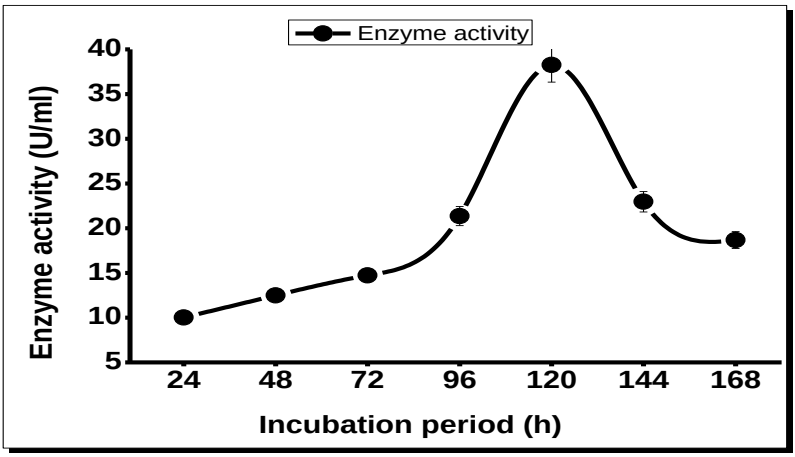

Fig. (2): Effect of different incubation periods on L-asparaginase production by *A. flavus*.

Effect of incubation temperature on L-asparaginase production by *A. flavus*

Fig.3 Showed the effect of different incubation temperature on enzyme production ,the maximum production of L-asparagenase (39.006 U/ml) was obtained at **30°C** for 30 min. This indicates that the enzyme production reduced gradually with further increase in incubation temperature. This incubating temperature is similar to that obtained for higher yield of L-asparaginase from *Penicillium sp*. **(Raju & Nibha, 2012).** While, **(Saranya *et al.*, 2012)** produced L-asparaginase at **35°C** and **(Suresh & Jaya, 2013)** produced L-asparaginase at **32°C** from ***Aspergillus terreus*.**

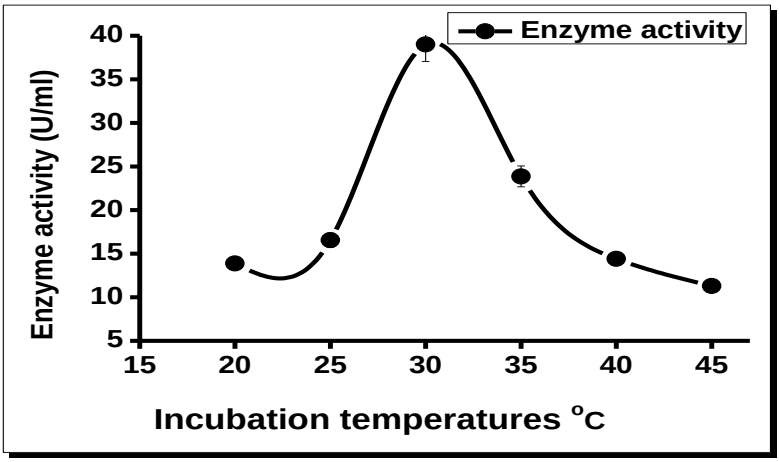

Fig. (3): Effect of different incubation temperatures on L-asparaginase production_by *A. flavus*

Effect of different initial pH values on L-asparaginase production by *A. flavus*:

Aspergillus flavus was grown in modified Czapek Dox medium amended with L-asparagine at different pH degrees (4.2, 5.2, 6.2, 7.2 and 8.2), and assayed for the enzyme activity at 37°C for 30 min. The results (Fig. 4) showed that the pH 6.2 was the optimum for L-asparaginase production by ***Aspergills flavus* 40.611** U/ml. This result is similar to that obtained at pH 6.2 by **Thirunavukkarasu *et al.*, (2011)** for maximum L-asparaginase production from ***Fusarium sp.*** However ***Aspergillus flavus*** was recorded most active at pH 7 by **Saranya *et al.*, (2012)**.

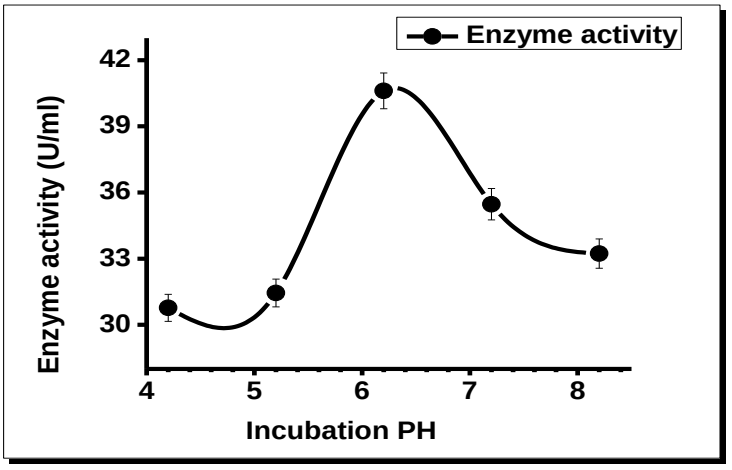

Fig. (4): Effect of different initial pH values on L-asparaginase production by *A. flavus*

Effect of different carbon sources on L-asparaginase production by *A. flavus*:

The effect of substitution of glucose in the basal medium by other carbon sources (fructose, lactose, galactose, maltose and sucrose) on an equimolecular carbon basis was investigated. The results (Fig. 5) indicated that the maximum L-asparaginase production **(52.356U/ml)** was recorded in presence of Fructose as carbon source. In this connection glucose was reported as the best carbon source under aerobic conditions for synthesis of L-asparaginase by *Serratia marcescens* **(Sukumaran *et al*., 1979)**. However**, (Venil and Lakshamanpalsmy 2009)** have reported decrease in L-asparaginase production when supplemented with fructose.

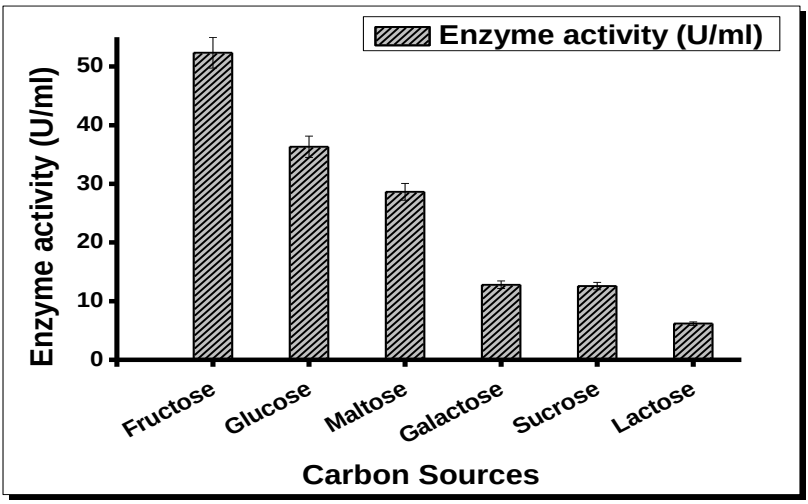

Fig. (5): Effect of different carbon sources on L-asparaginase production by *A. flavus*.

Effect of different nitrogen sources on L-asparaginase production by *A. flavus*:

The results in (Fig. 6) showed that L-asparagine was the most suitable nitrogen source for optimum L-asparaginase production by ***Aspergillus flavus*** with activity **(47.029 U/ml).** This result is similar with this obtained by **Nawaz *et al*., (1998)** that have described L-asparagine as the best nitrogen source. However, **Sreenivasulu *et al*., (2009)** observed that ammonium sulphate exhibited maximum activity.

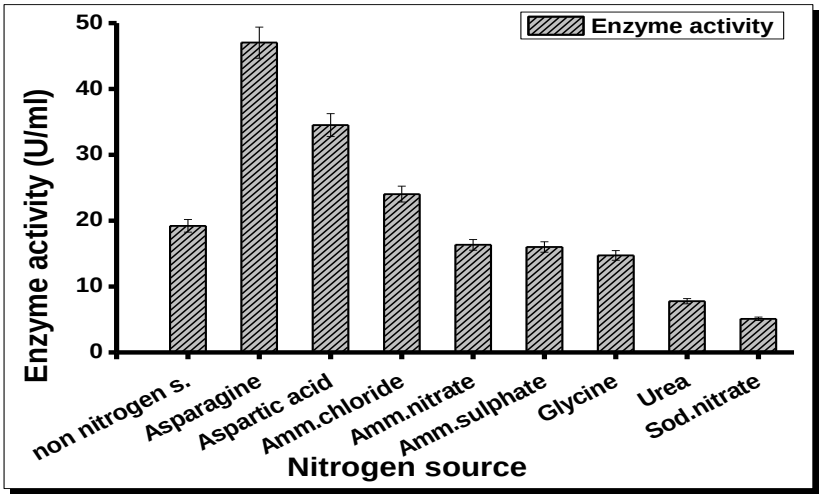

Fig. (6): Effect of different nitrogen sources on L-asparaginase production by *A. flavus*

Characterization of crude L-asparagenase

Effect of incubation time on L-asparaginase activity

The optimum incubation period of high relative enzymes activities, and the rate of substrate hydrolysis was investigated by incubating the enzyme for 1.0 hour with the corresponding substrate at the optimum conditions. The activity ($U\mu g^{-1}$) after interval times (10, 20, 30, 40, 50, and 60 min.) were determined. The result obtained in **(Fig. 7)** showed that

maximum asparaginase activity ($6.976 U\mu g^{1}$) was optained after incubation 10 min instead of 30 min. at 37 ^{o}C and pH 8.2 **.** and by increasing the incubation time above 10 min. the enzyme activity was decline. After 10 min a decline in the enzyme activity started. Other results showed maximum activity at incubation period of 3 min for *Aspergillus terreus* and *Aspergillus flavus* by **(Saranya *et al*., 2012).**

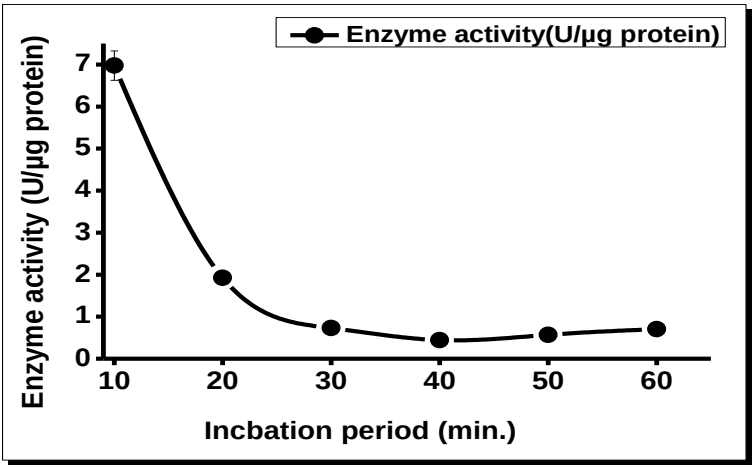

Fig. (7): Effect of different incubation periods on asparaginase activity

Effect of temperature on L-asparaginase activity:

Results illustrated in (**fig. 8)** indicated that L-asparaginase had maximum activity (**6.654 Uµg $^{-1}$**) at **37ºC** after **10** min. Similarly, **Siddalingeshwara & Lingappa, (2011)** recorded the optimum temperature **37ºC** for maximum enzyme activity from *A. terreus KLS2*. This property of enzyme makes most suitable for complete elimination of L-asparagine from the body when tumour patient treated with L-asparaginase in-vivo. **Mannan *et al.*, (1995)** found **37ºC** to be the optimum temperature for the enzyme activity by *Pseudomonas stutzeri* MB-405.

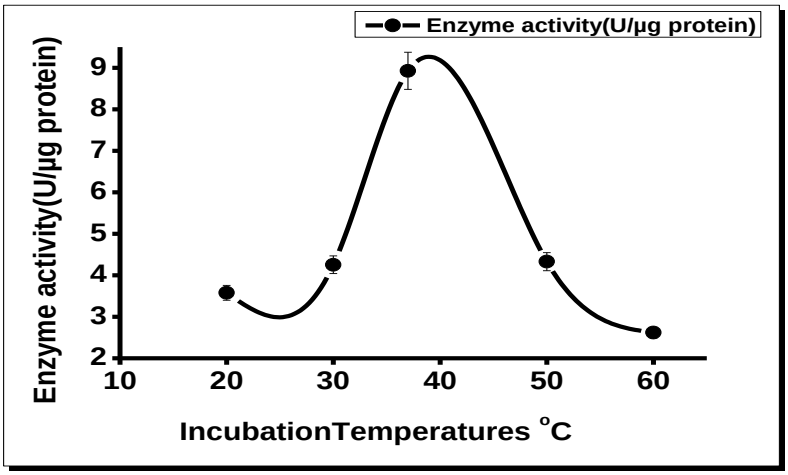

Fig. (8): Effect of different temperatures on L-asparaginase activity

Effect of pH on L-asparaginase activity:

Results (Fig.9) showed that ***Aspergillus flavus*** L-sparaginase was more active at pH **8.2** with activity of **7.484 Uμg $^{-1}$** at 37^{o}C after 10 min. reaction time. In this connection **Siddalingeshwara & Lingappa, (2011)** found that L-asparaginase from *Aspergillus terreus KLS2* was active over broad pH ranges **(4.0 -11.0)** with an optimum at pH of **9** and it was investigated that L-asparaginase activity below pH **8** would not be expected to be very effective for the treatment of tumour patients by **(Scheetz *et al*., 1971).**

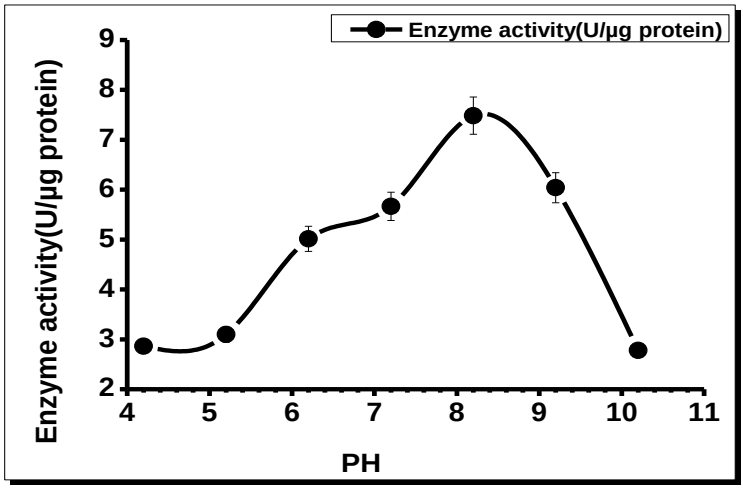

Fig. (9): Effect of different pH on L-asparaginase activity

Effect of different metal ions on L-asparaginase activity:

The effect of different metal ions include (Mg^{2+}, Ca^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and Fe^{2+}) were investigated by pre- incubation the crude enzyme with the tested ions at concentration (0.1%) dissolved in the buffer pH 8.2 used in assay method in absence of substrate for 30 min at room temperature, then the residual enzyme activity were detected through assaying enzyme at optimum conditions. The results obtained in **Fig. (10)** showed that the activity of crude L-asparaginase stimulated by pretreatment with (Mg^{2+}, Ca^{2+} and $Zn^{2+)}$ and slightly inhibited by pretreatment with Cu^{2+}, however the activity was highly inhibited by Fe^{2+} pretreatment. Similar results were obtained by **Joner *et al*., (1976)** referring that the divalent metal ions, Cu^{2+} inhibited the enzyme activity while Mg^{2+} enhanced the enzyme activity produced by *Acinetobacter calcoaceticus.*

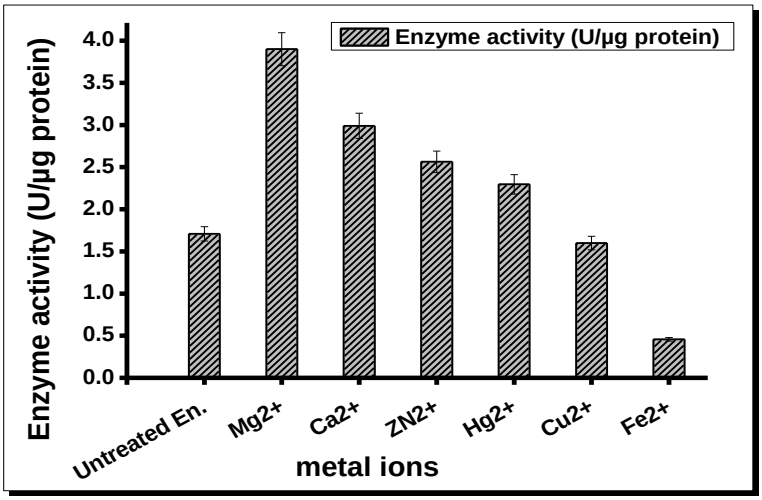

Fig. (10): Effect of different metal ions on L-asparaginase activity

CONCLUSION

In conclusion, maximal L-asparaginase productivity was attained at pH 6.2 and 30ºC with fructose as carbon source and L-asparagine as nitrogen source under fermentation after 5 days. L-asparaginase optimum activity was recorded at pH 8.2, 37°**C** and in presence of Mg^{2+} metal ion after 10 minutes incubation. These results make L-asparaginase near to physiological requirements which may suggest that enzyme may be used as therapeutic agent, because the physiological requirements is one of the prerequisite for antitumor activity **(Manna *et al*., 1995; Siddalingeshwara & Lingappa, 2011)**. Since, *A.flavus* could be used for L-asparaginase production on large scale in the future**.**

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إنتاج إنزيم الأسباراجينيز بواسطة فطرأسباراجيلس فلافس

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يهدف هذا البحث الى انتاج انزيم الاسباراجينيز المحلل للأسباراجين مع تحديد انسب الظروف المزرعية والفسيولوجية لانتاج اعلى معدل نشاط للانزيم وذلك ليتناسب مع استخدامه على مدى اوسع فى ظل ظروف اكثر ملائمه، وقد تم من خلال البحث التوصل الى مايلى:

١ – تم عزل وتعريف بعض السلالات الفطريه المحلله للأسباراجين الموجودة فى التربه المحليه وتشمل *Aspergillus flavus* , *Aspergillus subolivaceus, Ulocladium alternariae,* , *Fusarium oxysporum* and *Trichoderma viride.*

٢- تبين أن النشاط الانزيمى لفطر *Aspergillus flavus* النامى على بيئة الاسباراجين هو اكثر الفطريات المعزوله انتاجا لانزيم الاسباراجينيز.

٣- اوضحت الدراسه ان الظروف المزرعية والفسيولوجية المثلى لانتاج انزيم الاسباراجينيز بواسطة فطريات *Aspergillus flavus* كانت كالآتى:

* ظهرت اعلى انتاجية لانزيم الاسباراجينيز بعد اليوم الخامس من التحضين للفطر.
* عند درجة الحرارة ٣٠°م وهى درجة الحرارة المثلى و عند الرقم الهيدروجينى ٦.٢ pH ظهرت أعلى إنتاجيه للإنزيم كما أثبتت النتائج ان استخدام الفركتوز كمصدر كربونى هو الافضل لانتاج الإسباراجينيز.كما ان الاسباراجين هو انسب مصدر نيتروجينى لانتاج الاسباراجينيز.

٤- من خلال دراسة بعض الصفات الفيزيوكيميائية Physiochemical لانزيم الاسباراجينيز المنتج بواسطة فطر *Aspergillus flavus* ثبت أن اعلى نشاط للإنزيم كان عند درجة حرارة ٣٧ °م وان الرقم الهيدروجينى 8.2 pH هو افضل رقم هيدروجينى لنشاط إنزيم الاسباراجينيز وأن إضافه ايونات الماغنيسيوم و الكالسيوم لهما بينما كانت ايونات الحديد لها التأثير العكسى حيث قامت بتثبيط نشاط الانزيم.