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EXTERACELLULAR L-ASPARAGINASE FROM LOCAL SOIL BACTERIA: PRODUCTION AND OPTIMIZATION OF PHYSIOLOGICAL CONDITION

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ABSTRACT

L-asparaginase produced by *Bacillushalotolerans* OHEM18 finds broad applications due to its hydrolysis activity so used as therapeutic agents in combination with additional medications in the cure of acute lymphoblastic leukemia and also in food processing. In the present study, L-asparaginase production was optimizedand the optimum factors for production under submerged fermentation (SMF) of the asparaginase were pH 7.0 after 28h using glucose and asparagine as carbon and nitrogen sources, respectively while bagasse was the best inducer for L-asparaginase production under SSF.

الاسباراجنيز من بكتريا *Bacillushalotolerans* OHEM18 له تطبيقات واسعة بسبب نشاط التحلل المائي حيث يستخدم كعامل علاجيى مع أدوية إضافية في علاج سرطان الدم الليمفاوي الحاد وأيضا في تصنيع الطعام .في هذه الدراسة ، تم تحسين إنتاج الاسباراجنيزوكانت العوامل المثلى للإنتاج الانزيم تحت تقنيه التخمر المغمور باستخدام الاس الهيدروجينى ٧,٠ و بعد ٢٨ساعه و باستخدام الجلوكوز والأسباراجين كمصادر للكربون والنتروجين ، على التوالي بينما كان تفل قصب السكر هو أفضل حافز لإنتاج الاسباراجنيز باستخدام تقنيه التخمر الصلب

Keywords: L-asparaginase; enzyme production; submerged fermentation and solid state fermentation.

INTRODUCTION

L-asparaginases are members of amidohydrolasewhich catalyze hydrolyzes of asparagine into its acids (aspartic) and ammonia and found in certain fungi, plants,actinomycetes, and a variety of bacteria (Meghavarnam & Janakiraman, 2015). Enzymes are very specific and selective catalytic proteins generated by living cells to accelerate, control and regulate biochemical process in the living cells. Susceptibility to genetic manipulation and extensive biochemical variety makes microorganisms considered as a main source of many therapeutic enzymes (Dhanam&Kannan, 2013).

L-asparaginases are among the most important industrial enzymes regarding to their application in pharmaceuticals. The first record of l-asparaginase as anti-tumor enzymes was reported in 1922 by Calimanti. Further studies demonstrate therapeutic effect of L-asparaginase in treatment of acute lymphocytic leukemia (ALL) (mainly in children), acute myelomonocytic leukemia, acute myelocytic leukemia, Hodgkin disease, chronic lymphocytic leukemia, reticulosarbom and melanosarcomalymphosarcoma treatment (Stecher*et al*., 1999).

According to hydrolysis ability of L-asparaginase; has an important role in food technology by reducing the amount of free asparagine in the beginning materials so decreased carcinogenic and neurotoxic acrylamide formation in food product (Tareke*et al*., 2002). Also used for the development of biosensor applications and have a role in the biosynthesis of fine-chemicals (Verma*et al*., 2012; Sinha*et al*., 2013). Enzymatic processes under industrial-scale are frequently achieved under harsh conditions as salinity or high temperature which cause deactivation to

enzymes extracted from terrestrial microorganisms. Halotolerant or halophilic microorganisms tend to expressed enzymes tolerate the extreme conditions such as high salt concentrations, the presence of organic solvents, high temperatures, non-physiological and pH values(Barati *et al.,* 2016).

Microbial intracellular L-asparaginase is a common in except little which is produced extracellular (Narayana*et al*., 2008). Extracellular L-asparaginase has more beneficial than intracellular one due to higher production of enzyme in culture broth at the normal conditions, stress-free extraction and downstream processing (Amena*et al*., 2010; El-Naggar*et al*., 2016). The aim of this study is concerned with L-asparaginase enzyme production besides optimization of the factors effecting on the production process.

MATERIALS AND METHODS

Isolation of L-asparaginase Producing bacteria

Different soil samples were collected from various localities of Egypt (Borg El-Arab City, Upper Egypt, El Behira and WadiAlnatroon). One gram of each soil sample was serially diluted using sterile saline solution and spread over modified Czapek-dox agar plate supplemented with 0.009% phenol red as pH marker (Gulati, *et al*., 1997). The plates were incubated at 34°C for 24 h and the isolates, which able to form pink color around their colonies were selected as apparent L-asparaginase producers. The selected bacterial isolates were then purified with sterile modified Czapek-doxplates and checked for their actual asparaginase production. The potent asparaginase producing bacterial isolate was preserved for further investigations.

Production of Extracellular L-asparaginase in Submerged Culture

The extracellular L-asparaginase was produced by potent bacteria isolate using a modified Czapek-dox (MCD) submerged culture medium. The production medium was prepared g/L; asparagine 10; glucose 2; KH_2PO_4 1.52; KCl 0.52;$MgSO_4$ 0.52the pH was adjusted to 7.0±0.2 (Gulati*et al*., 1997). For enzyme production by selected bacterial isolate; 4.0 mL ($8x10^8$ cells/ mL) of freshly prepared culture was inoculated into 250 mL Erlenmeyer flasks with final incubation conditions at 34°C and 150 rpm for 24-28 h. The bacterial cells were then removed by centrifugation at 3000 rpm for 30 min followed by filtration using Whatman filter papers, and the obtained supernatant was used for additional studies of the crude enzyme.

Assay of L-asparaginase Activity

The enzyme activity in the culture supernatant was tested according to the method of Imada*et al*.(1973). L-asparagine hydrolysis rate was determined by measuring the ammonia released using Nessler's reagent. A mixture of 1.5 ml of 0.04 M L-asparagine in 0.05 M tris-HCl (pH 8.2) and 0.5 mL of enzyme extract incubated in water bath 37℃ for 30 min and then 0.5ml of 1.5M trichloroacetic acid (TCA) added to stop the reaction, solution followed by centrifugation at 10000 rpm for 7 min. Then the ammonia released in the reaction was determined colorimetrically by adding 0.2 ml of Nessler's reagent into tubes containing 0.1 ml of supernatant and 3.7 ml of distilled water and incubated at room temperature for 20 min. The absorbance was read at 450 nm. The L-asparaginase enzyme activity was expressed as international unit (IU), where; 1 IU can be defined as the amount of enzyme required to produce 1μmol of ammonia per min under the conditions of the assay is defined as one international unit (IU) activity.

Optimization of L-asparaginase production

L-asparaginase activity was performed under two different fermentation modes; submerged fermentation (SMF) and solid state fermentation (SSF).In SMFthere are several factors such as incubation period, inoculum size, pH, temperature, different carbon and nitrogen sources on asparaginase production were investigated. The optimization of different process parameters were carried out by one factor at a time analysis keeping other factors

constant in production of L-asparaginase(Indira *et al*., 2015).

Effect of incubation period

The L-asparaginase production by *Bacillus halotolerans* OHEM18was carried out using 100ml of MCD (pH 6.5) containing 1% asparagine in 250ml Erlenmeyer flasks inoculated with 4.0 ml ($8x10^8$ cells/ mL) and wereincubated at different growth intervals. The culture filtrate wascentifugated and supernatant was assessed at different incubation periods (4 to 48 h) at 35℃ and the enzyme activity and protein content were determined.

Effect of inoculum size

To observe the effect of inoculum size on the production of L- asparaginase by *Bacillushalotolerans* OHEM18 the medium was inoculated from stock spore suspension ($8x10^8$ cells/ mL) by 2 to 10 ml incubated for 28 h at 35 ℃. The suitable concentrations which stimulate maximum enzyme productions were used for further studies.

Effect of pH

To determine the maximum production of L- asparaginase at a particular pH, the medium was adjusted to various pH ranges from2.0 to 11.0 using 1N NaOH and 1N HCl. The *Bacillus halotolerans* OHEM18(4.0 ml) wasinoculated into the MCD medium and the flasks were incubated for 28h at 35℃. The clear supernatant was used to determine the enzyme activity and protein content.

Effect of carbon source

The influence of different carbon sources on theproduction of L-asparaginase was assessed. Five carbon sources; glucose, sucrose, manitol, maltose, fructose, Lactose, galactose and rhaminose at 0.2% w/vwere utilized and incubated for 28 h at 35℃. The supernatant was utilized for determining the enzyme activity and protein content.

Effect of nitrogen source

The effect of different nitrogen sources (sodium nitrate, peptone, yeast, casein, valine, lysine, arginine, glycine, histidine, glutamine, asparagine, ammonium sulfate 1% w/v) on asparaginase production was determined, incubated for a period of 28h at 35℃. The enzyme activity was assessed by utilizing the supernatant in order to measure the L-asparaginase activity and protein content.

Production of L-asparaginase under SSF (solid state fermentation)

Influence of different natural sources were determined (rice bran, molasses, water hyacinth, coconut oil, corn bran, wheat bran and bagasse 1% w/v) inoculated with 4 ml andthe culture was incubated for 48 h at 35℃.

Estimation of protein

The concentration of protein was determined by Lowry method. The Blue color developed by the reduction of the Phosphomolybdic-Phosphotungastic components in the Folin-ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the color developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry method(Lowry *et al*., 1951).

Statistical analysis

The results obtained were analyzed statistically by using SigmaPlot 14 version software

RESULTS AND DISCUSSION

Isolation and Molecular Identification of L-asparaginase-producing bacterial Isolate

The L-asparaginase producing bacteria were preliminarily screened from soil samples; four bacterial isolates were screened and showed as productive source of the therapeutic anti- tumor enzymes L-asparaginase using modified CzapekDox agar medium supplemented with phenol red for L-asparaginase production. The activity of asparaginase producing microorganism alter pH of the medium by breaking down of amide bond in L-asparagine and liberated ammonia and so changed the medium color from yellow to pink. One isolate was chosen to complete the

study as it revealed a prevalent pink colored zone around its colony after 24 h.

The most potent isolated strain which exhibited maximum enzyme production was Gram-positive bacteriaidentified as *Bacillus halotolerans*OHEM18 (Figs. 1 & 2). For identifying the potent hyperactive L-asparaginase producing isolate, was amplified and sequenced 16S rRNA gene of the isolate and submitted to Gene Bank (NCBI Gene Bank) (Accession no. MH100769).

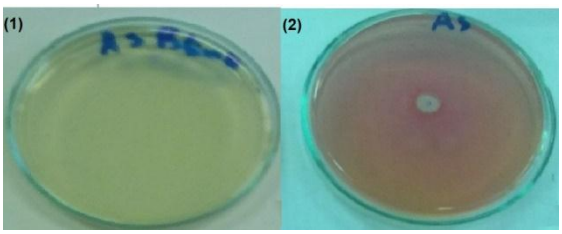

Figure 1. Plate Assay for Screening of L-Asparaginase (1) control, (2) after 24h.

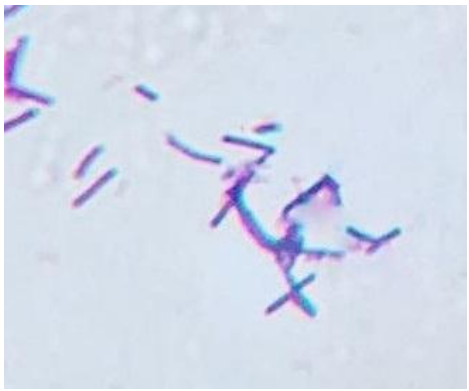

Figure 2.Rod-shaped Gram-positive bacteria.

Typical time course of L-asparaginase production under sub merged fermentation (SMF)

The resultsrevealed the activity of enzyme increased with increasing the incubation period up 28 h and then decreased with increasing time (Fig. 3). However the maximum production of L-asparaginase observed at 28 h with activity 120.345 U/mg and this disagreed with the result from Ebrahiminezhad*et al*. (2011) who reported that the optimum incubation period of L-asparaginase production by *Bacillus* sp. was after 48 h.

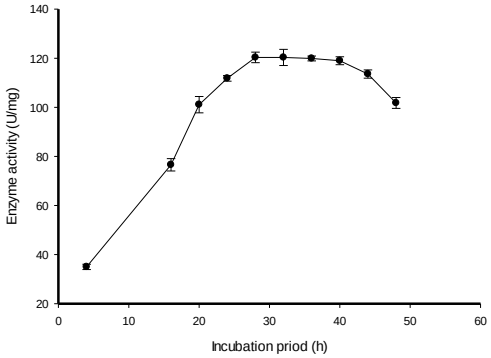

Figure 3. Effect of incubation period on L-asparaginase yield obtained from *Bacillus halotolerans* OHEM18 under SMF.

Effect of different inoculum size on L-asparaginase production

The results (Fig.4)observed that the activity of enzyme increased with increasing inoculum size and 4 % inoculum from $8x10^8$ cells/ mL promoted maximum enzyme production for L-asparaginase with activities110.13U/mg. Singh *et al*. (2013)reported that the optimal inoculum sizefor L-asparaginase from *Bacillus aryabhattai* ITBHU02 was 2 %**.**

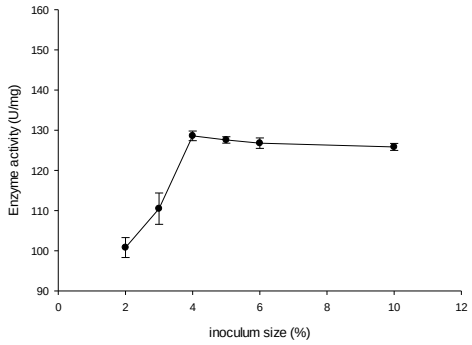

Figure 4. Effect of different inoculum size on L-asparaginase yield obtained from *Bacillushalotolerans* OHEM18 under SMF.

Effect of different pH values on L-asparaginase production

Figure 5 indicated that pH 7.0 (142.71 U/mg) support maximal enzyme production, although it could produce invariably considerable level of enzyme over a wide range of pH between 4.0 (48.69 U/mg)and pH 11.0 (39.12 U/mg). These observations also suggest that this bacterial strain have a wide range of pH.Jia*et al*. (2013) reported that the optimum pH was 7.5 from *B. subtilis* B11−06 while Erva (2018) showedthat theoptimum pH was 8.3.

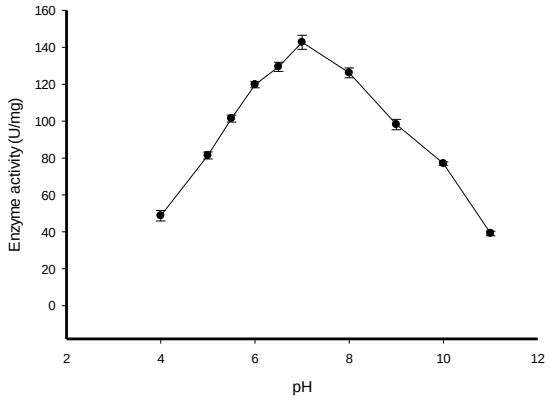

Figure 5. Effect of different pH values on L-asparaginase yield obtained from *Bacillushalotolerans* OHEM18 under SMF.

Effect of different carbon sources on L-asparaginase production

The results presented (Fig. 6)cleared that glucose maximizes the production of L-asparaginase about 86.58U/mg of enzyme. Fructose and lactose also enhanced L-asparaginase compared to control. Similarly to our study, was recorded by Vidhya *et al*. (2010) from *Bacillus* sp.

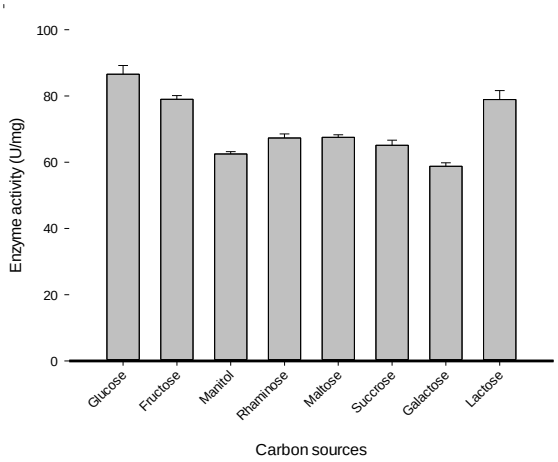

Figure 6. Effect of different carbon sources on L-asparaginase yield obtained from *Bacillushalotolerans* OHEM18 under SMF.

Effect of different nitrogen sources on L-asparaginase production

The results presented (Fig. 7) indicated very clearly that the maximum production was recorded with L-asparagine (68.58 U/mg) and flowed bypeptone, arginine and ammonium sulfate, with activity (60.38, 57.83 and 52.2 U/mg), respectively. This result agrees with Ebrahiminezhad*et al*. (2011).

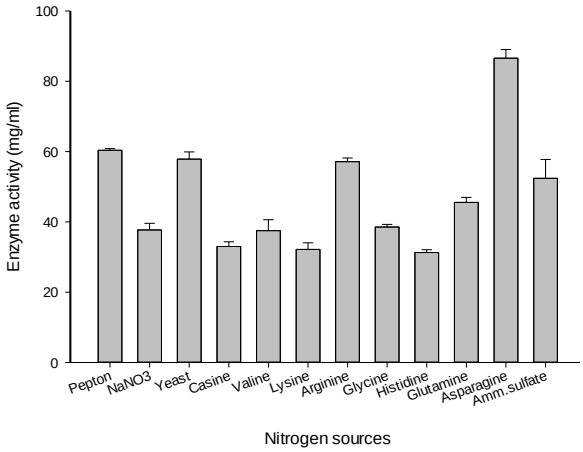

Figure 7. Effect of different nitrogen sources on L- asparaginase yield obtained from *Bacillushalotolerans* OHEM18 under SMF.

Production of L-asparaginase under SSF (solid state fermentation)

Different natural sources industrial and agricultural wastes such as wheat bran, corn bran, water hyacinth,rice bran, sugar can bagasse, coconut and also molasses were used as cheap carbon and nitrogen sources for the production of L-asparaginase by the most potent isolate (*Bacillus halotolerans* OHEM18). Form the provided results in figure 8 indicated that, bagasse(68.194U/mg) followed by Corn bran (62.752 U/mg) and Water hyacinth (60.434 U/mg) was exhibited the highest production among all the tested substrates. But, compared to the original carbon source and nitrogen source (glucose and L-asparagine)(control), it's still exhibited less productivity. But from the economical point of view, bagasse is very cheap and more studies must be carried out to maximize L-asparaginase production using bagasse.Consequently agro industrial residues proved to be a promising source for the industrial production of L-asparaginase, especially sugar cane bagasse.Venil*et al.* (2009) reported that rice bran was the best substrate for productionL-asparaginase from *Serratiamarcescens* SB08 with 79.84 U/gm.

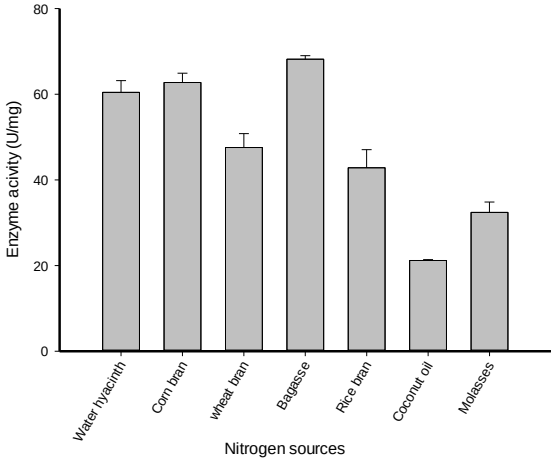

Figure 8. Effect of different natural sources on L-asparaginase yield obtained from *Bacillushalotolerans* OHEM18under SSF.

CONCLUSION

The observations made in this work hold great promise for maximum production of L-asparaginase enzyme after optimization of fermentation parameters such as incubation period, inoculum size pH, carbon source such as glucose, nitrogen source such as L-asparagine by *Bacillushalotolerans* OHEM18**.** This clearly indicates that the *Bacillushalotolerans* OHEM18is a potential strain for L-asparaginase production under submerged fermentation.This is proved the ability of L-asparaginase production under submerged cultivation condition by the investigated bacterium and can be utilizedas a potential antitumor agent, which has vast applications in healthcare and food industries.

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