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Optimizing the Production of Glutaminase-Free L-asparaginase by Halotolerant *Penicillium* sp. Isolated from Halophyte Cogongrass Rhizosphere

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KEY WORDS

L-asparaginase free from glutaminase. Optimization. Halotolerant fungi. *Penicillium* sp. *Imperata cylindrical*.

ABSTRACT

L-asparaginase is an enzyme included in the treatment of acute lymphoblastic leukemia (ALL). Its therapeutic mechanism is the hydrolyzes of L-asparagine, which is an essential amino acid for neoplastic cells while it can be synthesized by normal cells. The problem of L-asparaginase from microbial origin represented in its association with glutaminase activity. The search for new eukaryotic L-asparaginase-producers among microorganisms with low glutaminase affinity is important. This study investigated the biotechnological potential of filamentous fungi isolated from the cogongrass (*Imperata cylindrical*) rhizosphere soil. Fifteen fungal species were isolated from 12 environmental samples, and enzyme production was measured using a plate assay, followed by cell disruption to determine L-asparaginase release after submerged fermentation. L-asparaginase free of glutaminase-producing fungus was identified as *Penicillium* sp. using molecular based methods by PCR (18S rRNA). According to optimization results for enzyme production, asparagine at 15 g/L^{-1} and sucrose at 5 g/L^{-1} were the best sources of C and N, respectively, along with pH 7, 35°C, 75 g/L^{-1} of NaCl, and 0.1 g/L^{-1} of Fe^{2+} under shaking conditions at 150 rpm for 4 days.

Introduction

The most prevalent cause of cancer-related death in children is leukemia, with acute leukemialastic, where leukemia being the most frequently diagnosed form in those under the age of 14.

L-asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) is an important enzyme in the pharmaceutical field and still used for Acute Lymphoblastic Leukaemia (ALL) treatment in children since 1970 **(Darwesh *et al*., 2022; Højfeldt *et al*., 2023).** It is designated by the World Health Organization (WHO) as an essential drug. Because neoplastic cells lack l-asparagine synthetase, the anti-leukemic potential of L-asparaginase depends on the hydrolysis of l-asparagine and depriving cancer cells of it. Additionally, glutamine can serve as a raw material for L-asparaginase and asparagine synthetase: Asparagine synthetase uses glutamine to generate the amine and attaches to aspartate to produce asparagine **(Cachumba *et al*., 2016)**. L-asparaginase can be found in various plants, Microorganisms and animal tissues, including fish, mammals, and birds. Microorganisms like bacteria and fungi were discovered to be effective and affordable sources of L-asparaginase because of their high rate of multiplication and the possibility to consumption of low cost substrate **(Bahreini *et al*., 2014; Lopes *et al*., 2015)**. This is accurate even though different organisms have different enzyme properties and tumor- inhibtors. The production of L-asparaginase goes through two stages, upstream and downstream processing. Upstream process includes choosing the microbial producer, media component, fermintaton method and bioreactor conditions as pH and temperature in addition to optimization process, while downstream processing involves all the stages required for enzyme purification **(Brumano *et al*., 2019)**. L-asparaginase from *E. coli* and *Erwinia carotovora* are examples of L-asparaginase formulations that are commercially available. However, when *E. coli* L-asparaginase is used, side effects as minor allergic reactions or anaphylactic shock have been reported in about 5–50% of the patients. In responsive but antigenically sensitive patients, *Erwinia asparaginase* is given instead of *E. coli* asparaginase because it has equal anticancer activity but a different antigenic structure **(Maese *et al*., 2021)**. The administration of asparaginases from *E. coli* and Erwinia reported to cause allergic reactions in children suffering from leukemia while this side effects can represent fatal risks like lymphoma and venous thrombosis in adults **(Caruso *et al*., 2007)**. Additionally, when administering anti-Erwinia asparaginase intravenously and intramuscularly antibodies were

developed, which then affected the pharmacokinetics of *Erwinia* asparaginase. The peak frequency of hyperammonemia in children with ALL treated with PEG-asparaginase may be a result of the hydrolysis of both asparagine and glutamine in plasma into ammonia, despite the extension of half-life of asparaginase through the PEG-ylation technique **(Heitink-Polle *et al.*, 2013).** Degradation of L-asparaginase by both normal and cancer cells through lysosomal proteases induces an immune response. This potentiates the processing of the antigen which is the key cause of the discontinuation of L-asparaginase therapy **(Fonseca *et al.*, 2021).** In this case, extra microbial sources, like eukaryotic microbes, may lead to the finding of an enzyme possessing better compatibility with the immune system of human **(Doriya and Kumar, 2016)**, and may have an enzyme that results in fewer adverse effects. Asparaginase has a glutaminase activity which is another element that influences its hazardous side effects. Additionally, glutaminase's action may cause some body cells to become sensitive to a glutamine deficit. The amount of glutaminase activity in *E. coli* asparaginases is quite low, compared to the other two asparaginases that have received US Food and Drug Administration approval; however, asparaginase from *Erwinia* posess higher glutaminase moiety, which makes KM and VMAX better options for deaminating glutamine **(Avramis, 2012).** Investigating of microorganisms isolated from harsh environments for L-asparaginase offers promising breakthrough therapeutic uses that have fewer side effects **(Souza *et al.*, 2017; Kumar *et al.*, 2022).** Microorganisms that are halophilic or halotolerant tend to produce enzymes that are more tolerant to high levels of salt, temperatures, pH values and organic solvents **(de Lourdes *et al.*, 2013).** L-asparaginase toxicity is partially caused by glutaminase activity **(Howard and Carpenter, 1972).** L-glutamine is involved in numerous metabolic processes, including the synthesis of L-asparagine by L-asparagine synthetase. The glutaminase activity may restrict the body's ability to tolerate a therapeutic dosage of glutamine. Most research nowadays concentrates on employing microbial systems to make L-asparaginase without glutaminase. It has been demonstrated that L-asparaginases with low affinity to glutamine got a high affinity to L-asparagine pose fewer difficulties when administrated as anticancer therapy. Finding halophilic/halotolerant microorganisms that can produce L-asparaginases with potential applications was the goal of this study.

Thus, efforts have been made to choose microorganisms with potential biotechnological use in pharmaceutical

interest in L-asparaginase production that lack glutaminase activity and may be less immunogenic, as well as to improve upstream processing for selected fungi to produce more L-asparaginase.

Materials and methods

Collection of soil and water samples

Water and soil samples were collected from various hyper-saline locations in Egypt, (Borg El-Arab City, Upper Egypt, and Wadi Alnatroon, Sakha, Kafr Elshikh from the rhizosphere soil of cogongrass (*Imperata cylindrical*) grown in hyper-saline soil), in a sterile container, transferred to the lab in the ice box, where they were kept at 4°C until analysis.

Fungi isolation from samples collected

Fungi were isolated from the collected samples using the dilution plate assay method **(Johnson *et al.*, 1959)**. In a sterile 250 conical flask, 10 grams of soil sample were dissolved in 90 ml of sterilized hypersaline solution, 15% (w/v) of sodium chloride. The flasks were shaken at 150 rpm for 30 minutes. Then ten ml of the suspension were pipetted into a sterilized Erlenmeyer flask with a capacity of 250 ml and 90 ml of sterile 15% (w/v) sodium chloride solution and shaken for 30 min. In the same way, consecutive dilutions were prepared until 10^{-3} dilution, which was found suitable for plating. One milliliter of a 10^{-3} dilution was relocated and distributed over the surface of the plate containing the appropriate Potato Dextrose Agar medium (PDA). Its components g/l: Potato infusion 200, Dextrose 20, Agar 20, Sodium chloride 150 (PH 7.0).The plates were incubated for 7 days at 30ºC. Following incubation, the colonies were purified and kept on PDA slants with 150 g/l of sodium chloride added.

Inoculum preparation of the fungus isolate

Spore suspension of each fungus isolate was made by incorporating 1 ml of 15% saline solution containing a few drops of Tween -80 to the 7 days old culture grown on Modified Czapek Dox's agar medium, and the culture was scraped gently with an inoculation loop under aseptic conditions. The spores were diluted with the same solution and then counted with a hemocytometer. The inoculum was a volume of 1ml of spore suspension containing 10^5 spores/ml.

Qualitative screening of L-asparaginase-producing fungi

The solid-state method was used for the qualitative screening of L-asparaginase-producing microorganisms **(Gulati *et al.*, 1997)** using a modified Czapek Dox medium (MCD). Czapek Dox's modified medium **(Saxena and Sinha, 1981)** containing (g/l): glucose 2.0, L-asparagine 10.0, KH_2PO_4 1.52, KCl 0.52, $MgSO_4.7H_2O$ 0.52, $CuNO_3.3H_2O$ trace, $ZnSO_4.7H_2O$ trace, $FeSO_4.7H_2O$ trace, Sodium chloride 150 and agar 20.0, pH was

changed to 7.0. The medium (100 ml) was supplemented with 0.3 ml of phenol red dye (stock solution 2.5%). Control plates replaced l-asparagine with sodium nitrate as a nitrogen source. The plates were incubated at 30°C for 7 days to get pink colonies.

The semi-quantitative screening for L-asparaginase-producing fungi

According to **Kaur and Saxena (2014)**, the agar disk diffusion method was used for the semi-quantitative screening with a few modifications. A glass rod was used to evenly distribute 1 ml of spore suspension, which contained 10^5 spores per milliliter, onto the modified Czapek Dox's agar medium-coated Petri dishes. The dishes were then incubated at 30°C for 7 days. The fungal disks (5 mm in diameter) were aseptically punched out from the growing edge of each fungal isolate. Disks were transferred to Modified Czapek Dox's agar medium surface and incubated at 30 ºC for 7 days. The color of the zone around the fungal growth was observed and using the following formula, the zone index was determined:

Zone Index = Zone diameter/ Colony diameter

Screening with semi-quantitative and qualitative glutaminase-free L-asparaginase activity

The isolates that showed L- asparaginase activity were evaluated for glutaminase production using the plate assay method and the agar well diffusion method **(Gulati *et al.*, 1997; Kaur and Saxena, 2014)**. All steps are conducted as in the case of detecting asparaginase activity, but asparagine was replaced by glutamine.

Preservation of the isolated fungi

The fungal isolates showing free glutaminase L-asparagine activity were maintained in PDA agar slants supplemented with 15% NaCl at 4ºC ±1º C.

Culture preparation for quantitative screening and enzyme extraction from fungal isolates

Fungal isolates were cultured in 250 ml Erlenmeyer conical flasks containing 50 ml of Modified Czapek Dox's broth medium. All experiments for quantitative screening were done in triplicate, and data were expressed as average values. Flasks were incubated on the shaker (150 rpm) at 30ºC for 7 days. The broth culture was filtered using Whatman no. 1 filter paper. Culture filtrate was centrifuged at 4°C and 10000 rpm for 20 min using a cooling centrifuge **(Theantana *et al.*, 2007).** The produced supernatant was termed crude enzyme preparation and used for quantitative assay of extracellular L-asparaginase while the produced mycelia were lysed and tested for intracellular enzyme activity. To prepare crude cell lysate, the mycelia resulting from the filtration step were re-suspended in the

same buffer after being twice washed with 50 mM Tris-HCl (pH 8). Then, while the cells were being cooled to 4°C, they were broken up by sonication over three periods of 15s each, separated by 45s. Centrifugation at 15,000 rpm and 4°C for 15 minutes was used to remove unbroken cells and cellular debris. The supernatant was then collected to measure the intracellular enzyme activity **(Kalimuthu *et al*., 2008).**

Quantitative assay of L-asparaginase and L-glutaminase

Using Nessler's reagent, the L-asparaginase activity was quantified by **Imada *et al*., (1973).** The reaction mixture containing 0.5 ml of crude sample, 0.5 ml of 0.5 M buffer (phosphate buffer, pH 8), 0.5 ml of 0.05 M asparagine, and 0.5 ml of distilled water was added to make the volume up to 2.0 ml, and incubate for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic acid (TCA). The mixture was then centrifuged at 10,000 rpm for 5 min, 0.1 ml of the supernatant was taken, and 3.7 ml of distilled water was added; 0.2 ml of Nessler's reagent was added to the reaction tube. The color reaction was developed over 10 min, and the absorbance was measured at 450 nm using a special trophotometer. The ammonia liberated was extrapolated from a curve derived with ammonia sulfate as the standard curve. One IU of L-asparaginase is the amount of enzyme that liberates 1 mol of ammonia per ml per minute [1 mole/ml/min]. The glutaminase activity was determined by using a similar method of Nesslerization which was used to determine the activity of L-asparaginase. The mixture containing L-asparagine was replaced with l-glutamine, and the same procedure described above was used to determine the enzyme activity.

Molecular identification of the most potent isolate

Fungal DNA was extracted using Omega fungal DNA kit (Omega Bio-tek, Inc., Norcross, GA, USA). 18S rRNA gene fragments were amplified by PCR using fungal primers (combinations either EF4: 5′GGAAGGGRTGTATTTATTAG-3′ and FUNG5: 5′-GTAAAAGTCCTGGTTCCCC-3′ **(Smits *et al*., 1999)**. PCR was conducted in a thermocycler (Eppendorf Mastercycler) where 25-μL reactions mixture containing 0.2 μM of each primer, 1 U of ExTaq DNA polymerase, and associated master mix (Takara) in addition to 5μL of crude genomic extract. DNA was denatured at 95°C for 2 min, followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final 5-min extension at 72°C. PCR amplification products were sequenced using the suitable forward primer. 18S rRNA gene sequences were identified in GenBank using BLAST. The

sequences appear in GenBank with the accession numbers MW876244.

Optimizing the culture conditions for the production of L-asparaginase free of L-glutaminase

The production of the enzyme was subjected to more detailed experiments to find out the factors which induce the highest growth and the highest production of the enzyme using the one factor at time method (3 replicate flasks per treatment). The inoculum for each flask containing modified Czapek Dox's broth medium was 1 ml of spore suspension containing 10^5 spores/ml. After incubation, As previously mentioned, a quantitative assessment of L-asparaginase activity was made. Fungal dry weight was measured utilizing Whatman No. 1 filter paper to filter the mycelia, the mycelia were washed twice with distilled water and then used to determine the dry weight after being dried in an oven at 70^{o}C until constant weight. The examined factors included the incubation period (1-7 days), and *Penicillium* sp. was grown in MCD broth medium at various pH levels between 5.0 and 9.0. For the best temperature, the fungus was incubated at various temperatures (25, 30, 35, 40, and 45°C). We added various carbon sources to the MCD broth in the same amount of molecular weight. L-asparaginase production was then examined. Carbon compounds, including 0.2% by weight of (glucose, sucrose, mannitol, malic acid, fructose, starch, galactose, and sodium acetate) followed by carbon source concentrations (0.2, 0.5, 1.0, 1.5% w/v). Various nitrogen sources were included at equivalent weight, including 0.5% w/v of (peptone, yeast, beef, asparagine, urea, ammonium chloride, ammonium acetate, and sodium nitrate), followed by nitrogen source concentrations (0.2, 0.5, 1.0, 1.5, 2.0% w/v). NaCl concentrations were (0, 2.5, 5.0, 7.5, 10, 15, 20, and 25%). In addition, the impact of metal ions like Co^{2+}, Mn^{2+}, Fe^{2+} $Cu^{2+,}$ and Se^{2+} was studied in different concentrations (0.005, 0.01, 0.03% w/v). Also, the effect of incubation under shaking or static conditions was determined.

Statistical analysis

The measured data were subjected to the analysis of variance (ANOVA) using a one-way, completely randomized design in triplicates, and the mean values with standard deviation were calculated. Using the CoStat statistical software, the critical difference at a 5% level of probability was compared with the significant differences between treatments using Duncan's test.

Results

The isolated Fungi

Halophilic and halotolerant fungi were targeted by isolation from localities containing a high of sodium chloride as sources of halophilic microorganisms (Borg

El-Arab City, Upper Egypt, El Behira and WadiAlnatroon, Sakha, Kafr Elshikh from the rhizosphere soil of cogongrass (***Imperata cylindrical)*** grown in hypersaline soil). Species were isolated from the samples using Potato Dextrose Agar (PDA). 15 fungal species were obtained and isolated from 12 samples (Fig.1)

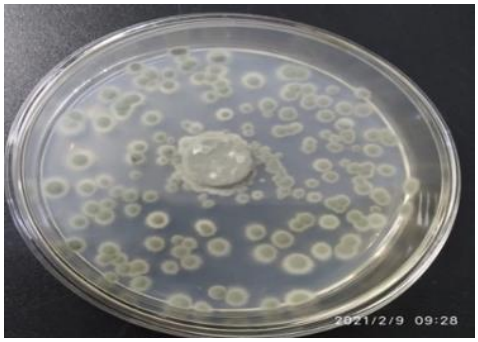

Fig. (1): Growth of *penicillium* sp. in Potato Dextrose Agar (PDA).

Primary screening analysis using phenol red indicator

The plate assay is an efficient and dependable technique that allows for the immediate observation of L-asparaginase production. The L-asparaginase is free of glutaminase-producing fungi initially screened from marine water and soil samples. The results obtained showed pink color. One fungal isolate out of the fifteen fungal isolates can produce the lL-asparaginase free of glutaminase after 7 days. The isolate that showed growth in asparagine media was chosen to complete the study.

Semi-quantitative assay

The development of the pink zone around the isolates' wells was a sign that L-asparaginase free of glutaminase was being produced. Appearing of the colored zones around the colonies indicating the pH elevation caused by ammonia build up in the media because of the production of L-asparaginase free of glutaminase. The value of the zone index obtained using phenol red was 13.57. The fungal isolate showed no growth in glutamine media (Fig. 2).

L-asparaginase quantitative assay

This experiment was carried out to investigate the potentiality of the different isolated species to produce L-asparaginase free of glutaminase in their culture filtrate. The productivity of the L-asparaginase enzyme in the filtrates was measured as the amount of ammonia released because of L-asparagine degradation. The chosen isolate in this study was capable of producing L-asparaginase intracellularly. Results of a quantitative assay obtained demonstrated that the examined culture produced no glutaminase activity and 11.91U/ml of L-asparaginase activity.

(a) (b) (c)

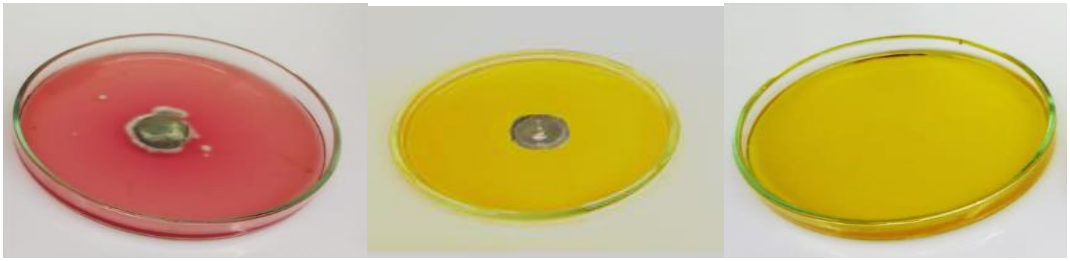

Fig. (2): Semi-quantitative assay of L-asparaginase: (a) Appearance of pink color in asparagine media. (b) No pink color in glutamine media. (c) Control.

Identification

100% similarity to *Penicillium* sp. was found in the strain homology of the 18S RNA gene sequence. sp. The Phylogenetic relationships based on the 18S gene sequence are represented in Fig. (3).

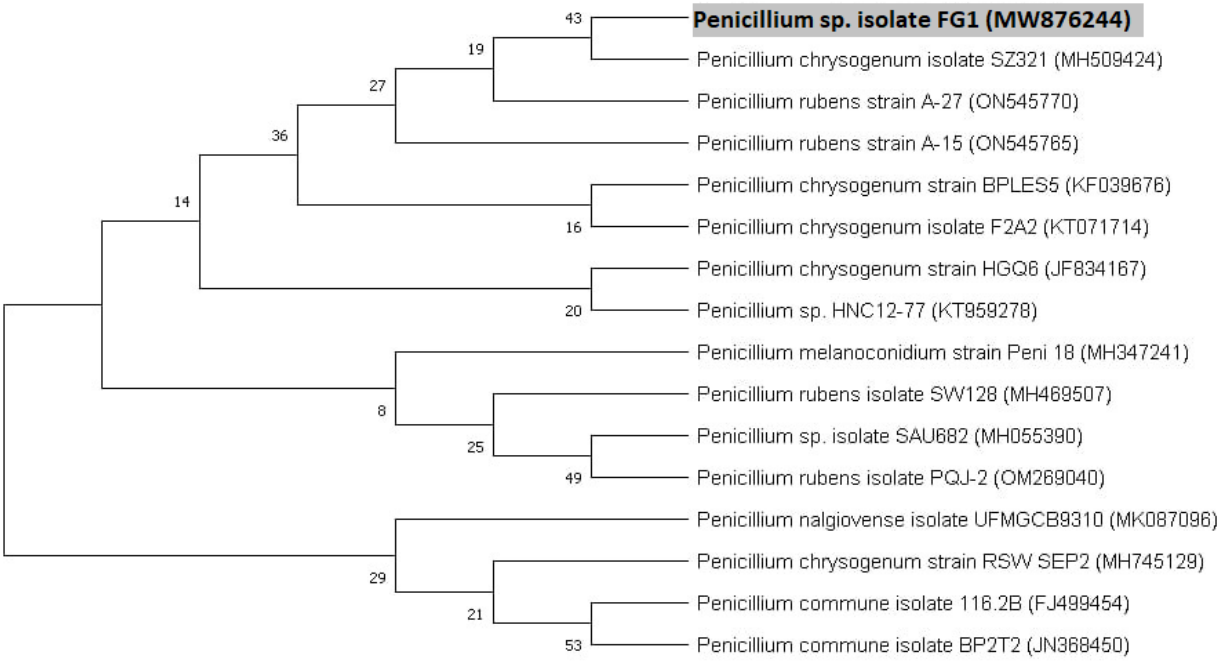

Fig. (3): Phylogenetic tree based on 18S gene sequence of a strain producing L-asparaginase.

Optimization of L-asparaginase production

To increase the microbial production of L-asparaginase by the chosen fungal isolates, ten cultivation conditions were adopted. Optimizing medium and cultural conditions were conducted at various incubation periods, temperatures, pH values, carbon and nitrogen sources, essential elements, trace elements....etc.

The incubation period's impact

The findings demonstrated that after 4 days of incubation, the fungal L-asparaginase activity peaked at 11.30 U/ml. In contrast, dry weight (0.24 g/20ml) was recorded at the end of 6 days. The results also showed that L-asparaginase activity and dry weight were decreased gradually with a further extension of the incubation periods to 7 days, Fig. (4).

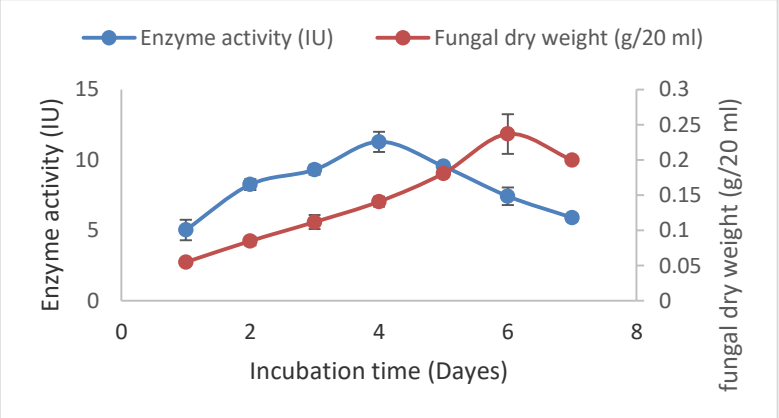

Fig. (4): Effect of various incubation times on the L-asparaginase activity and fungal dry weight from *Penicillium* sp.

Effect of PH values

The pH value of the culture medium is essential in improving L-asparaginase production efficacy. The influence of five initial medium pH values (5, 6, 7, 8, and 9) on L-asparaginase production was studied. The fungal results showed that pH 7.0 was optimal for L-asparaginase activity (11.28 U/ml). Above and below pH 7 the activity of L-asparaginase was substantially lower and the optimal dry weight (0.35 g/20ml) was at pH 6.0. The experimental fungus can develop and generate intracellular L-asparaginase at a variety of pH levels (Fig. 5).

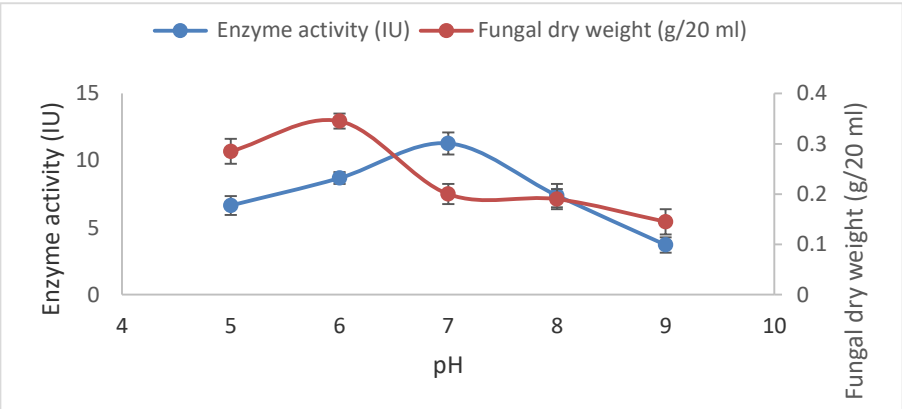

Fig.(5): Impact of various pH levels on the fungal dry weight and L-asparaginase activity from *Penicillium* sp.

Impact of aeration

The maximum fungal enzyme activity under shaking at 150 rpm (30.79 U/ml) was 1.6 times greater than under stationary (19.27 U/ml). The ideal dry weight (0.05 g/20 ml) was measured while the shaker was spinning at 150 rpm Table (1).

Table (1): The impact of aeration on the fungal dry weight and L-asparaginase activity from *Penicillium* sp.

Shaking or static condition	Enzyme activity (IU)	Fungal dry weight (g/20 ml)
Shaking	30.789(±0.630)a	0.055 (±0.005)a
Static	19.265(±0.276)b	0.022(±0.003)b

Effect of NaCl concentrations

The outcomes depicted in Fig. 6 showed that L-asparaginase activity and dry weight were obtained at different concentrations of sodium chloride, where the maximum enzyme activity was 16.43 U/ ml at 75 gL^{-1} of NaCl and the dry weight was 0.06 g/20ml at 25 gL^{-1} of NaCl. Further increasing or decreasing in sodium chloride concentration led to a reduction in dry weight and L-asparaginase activity (Fig. 6).

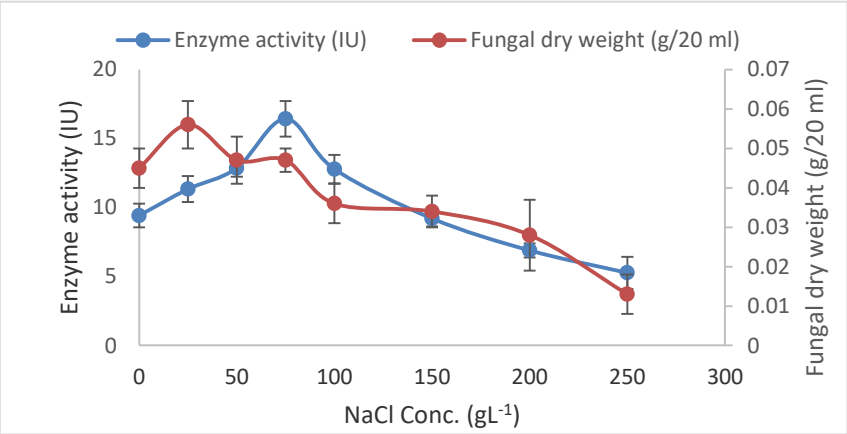

Fig. (6): The impact of various NaCl concentrations on the fungal dry weight and L-asparaginase activity from *Penicillium* sp.

Impact of various carbon sources

Production of L-asparaginase is dependent on the presence of both carbon and nitrogen sources in the medium. Both influence how enzyme synthesis is regulated. According to the findings in Fig. (7), sucrose was the most efficient carbon source for the activity of the fungus L-asparaginase. (35.78U/ml), and the maximum fungal dry weight was

produced using glucose as a carbon source. While L-asparaginase activity was present in the other carbon sources as well, they did not produce as much as sucrose did (Fig. 7).

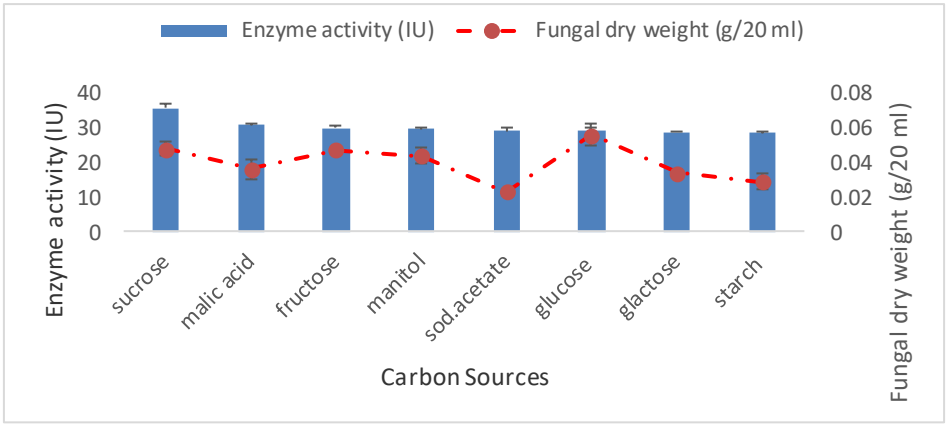

Fig. (7): Effect of various carbon sources on the fungal dry weight and L-asparaginase activity from *Penicillium* sp.

Effect of different sucrose concentrations

The effects of various sucrose concentrations (0.5, 1, 1.5, and 2% w/v) were investigated because sucrose was the best carbon source for L-asparaginase activity. The maximum enzyme activity was at 0.5% of sucrose concentration (41.71 U/ml), and the activity decreased significantly as the sucrose concentration rose. The enzyme activity reached 20.58 U/ml at a concentration of 2% w/v. Fungal dry weight is affected by different sucrose concentrations and exhibits optimum value at concentration 1.5% w/v but around this concentration, the fungal dry weight decrease (Fig. 8).

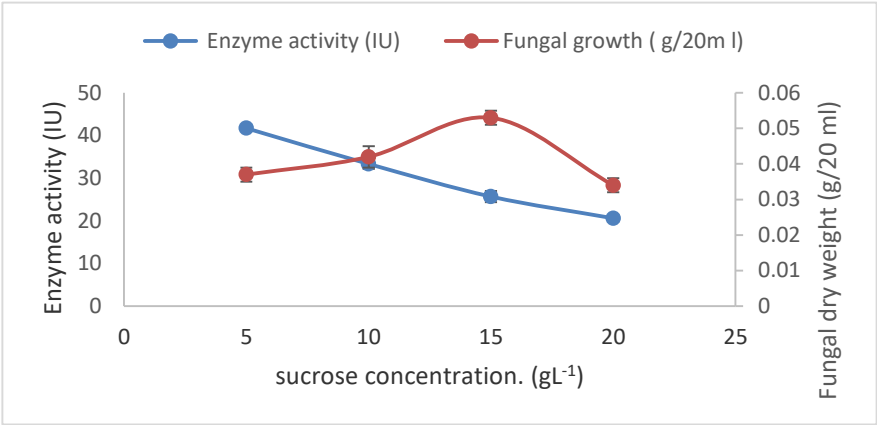

Fig. (8): Effect of various sucrose concentrations on the fungal dry weight and L-asparaginase activity from *Penicillium* sp.

Effect of heavy metals

Some enzymes may require the presence of specific metals for their activation or for increasing their activity. Five metal-containing compounds were used at three different concentrations (0.005, 0.01, and 0.03% w/v) of the metal testing their effect on fungal dry weight and enzyme productivity. The L-asparaginase activity was enhanced considerably by the presence of Fe (31.66 U/ml) (0.01% w/v) followed by Cu (0.03% w/v), a remarkable inhibition in both L-asparaginase activity and dry weight were obtained with using of 0.03 % Si. The best dry weight was obtained at 0.03% w/v Mn.

Effect of nitrogen sources

Nitrogen source as the organic and inorganic form is the second nutrition-related factor that affects the outcome of enzymes and performance. To investigate how nitrogen sources affect L-asparaginase production, asparagine as the primary nitrogen source was exchanged for beef, peptone, yeast, urea, ammonium acetate, ammonium chloride, and sodium nitrate. Figure (9) showed that not every nitrogen source has the same inductive effect on the amido hydrolytic enzyme. L-asparagine was used to produce the enzyme with the highest activity, followed by ammonium acetate, yeast extract, peptone, and beef, where L-asparaginase activities were 40.21, 33.67, 31.47, and 28.01 U/ml, respectively. However, minimal L-asparaginase activity was seen when urea, ammonium chloride, and sodium nitrate were used where L-asparaginase activities were 25.77, 21.39, and 19.18 U/ml, respectively. Maximum fungal dry weight was obtained using yeast extract, peptone, and beef: there is no significant value between these nitrogen sources. The minimum fungal dry weight was sodium nitrate (0.24 g/ 20 ml) (Fig. 9).

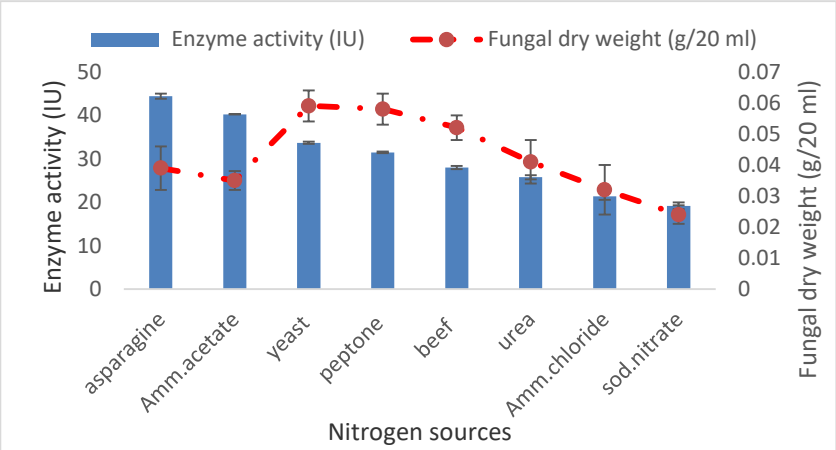

Fig. (9): Effect of various nitrogen sources on the L-asparaginase activity and fungal dry weight from *Penicillium* sp.

The effect of different L- asparagine concentrations

L-asparagine was added to the medium in concentrations ranging from 0.2% to 2.0% (w/v). Fig. (10) revealed that the greatest activity of L-asparaginase (48.95 U/ml) became available at a concentration of 1.5 % of L-asparagine. By decreasing or increasing the concentration of L-asparagine, the enzyme activity decreased. Also, the concentration of 1.5% w/v exhibited the highest fungal dry weight (0.055g). Around this concentration, the fungal dry weight decreased (Fig.10).

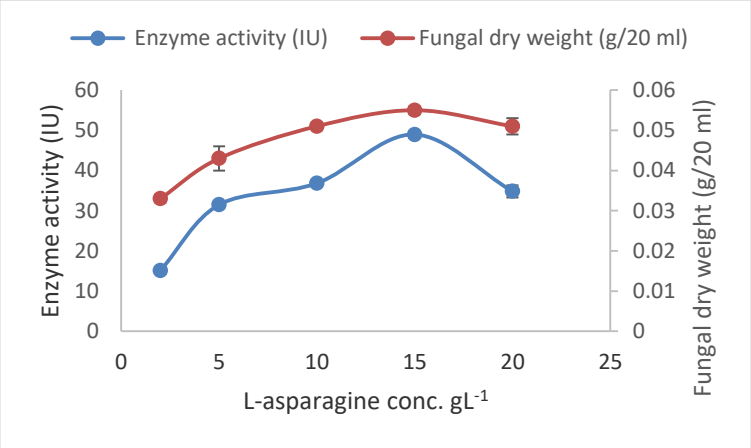

Fig. (10): Effect of different concentrations of L- asparagine on the fungal dry weight and L-asparaginase activity from *Penicillium* sp.

Effect of incubation temperature

The effect of temperatures ranging from 25-45^{o}C was studied on l-asparaginase productivity and fungal development. The results showed that the L-asparaginase production increased as the incubation temperature was raised, with the maximum enzyme productivity occurring at 35°C (26.28 U/ml). The L-asparaginase activity was significantly lower both above and below this temperature. Maximum dry weight was obtained at 35°C and 30°C; there is no significant value between the two degrees. Around these degrees, the fungal dry weight decreased (Fig. 11).

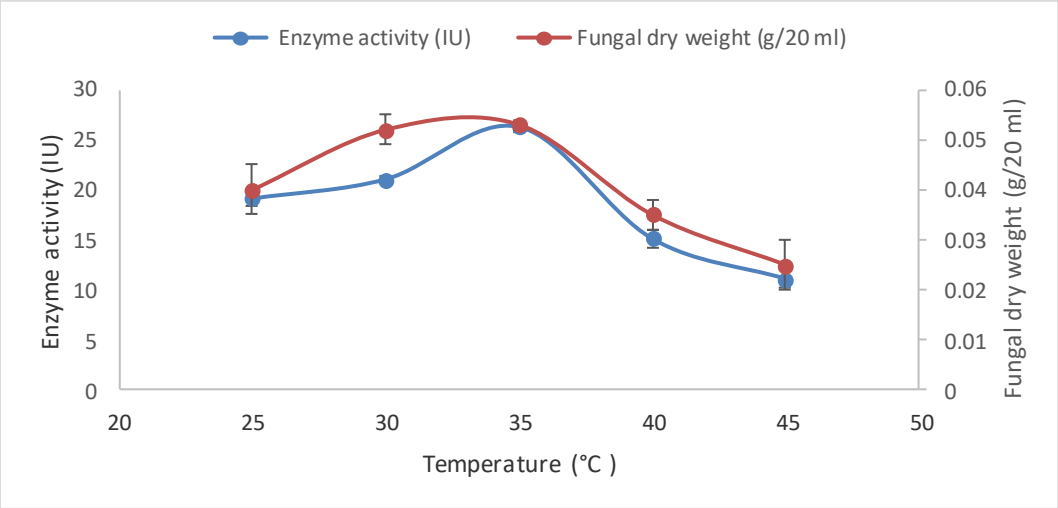

Fig. (11). Effect of various incubation temperatures on the L-asparaginase activity and fungal dry weight from *Penicillium* sp.

Discussion

pH is considering an indicator for L-asparaginase activity because it breakdown asparagine to aspartic acid and ammonia, which lead to alkalinization of the culture. So the qualitative detection of L-asparaginase depends on the incorporation of pH indicator as phenol red to the media component which turns to pink in alkaline medium. The diameter of pink zone can be used to classify and ordering the tested isolates for asparaginase production potential. This is similar to the report of **Devi and Ramanjaneyulu (2016)**, who came to the conclusion that strains exhibiting a diameter zone above 9 mm are known as good L-asparaginase producers, while those with a diameter zone of 6–9 mm and those with below 6 mm diameter zone may be referred to as moderate and poor L–asparaginase producers, respectively. In this connection, *Trichosporon asahii* IBBLA1 isolate from Antarctica had a maximal zone index value of 5.8 **(Ashok *et al.*, 2019)** After incubation for 72 h, *Rhizopus* sp. W3, *Curvularia* sp. S3.4, *Aspergillus* sp. C3, *Rhizopus* sp. W5, and *Aspergillus* sp. MTCC 1782 isolates from Indian soils showed zone indices fluctuating from 1.0 to 2.4. In cultures inoculated with conidia, *Penicillium* sp. T8.3, *Penicillium* sp. T6.2 and *Fusarium* sp. T22.2 exhibited a zone index more than 1.0 after 72 h; also, *Penicillium* sp. T9.1 showed a zone index greater than 1.0 after incubation for 168 hours, and *Penicillium* sp. T6.1 exhibited a zone index of 0.88 after incubation for 168 hours using bromothymol blue as indicator **(Gonçalves *et al.*, 2016)**. The results of the current study indicated that the fungus produces L-asparaginase at its

highest levels after 96 hours of incubation. The results also demonstrated that as the incubation periods were extended to 7 days, L-asparaginase activity gradually decreased. This might be because there would have been an exponential phase for the fungus. The decrease in enzyme activity after 4 days of incubation, the metabolic activity, inhibition, and denaturation of the enzyme, as explained, may be caused by lowering the nutrient level of the medium. The data also showed no correlation between L-asparaginase production and fungal dry weight. Maximum dry weight was recorded at the end of 6 days. **Thirunavukkarasu *et al.*, (2011)** reported that a shorter incubation period lowers the cost of submerged fermentation and lessens the possibility of proteolytic enzymes degrading L-asparaginase **(Rani *et al.*, 2012)**. A crucial environmental factor for the production of L-asparaginase by microbes is the incubation temperature because it controls microbial growth and, as a result, enzyme secretion **(Chaloupka, 1985)**. The represented data showed that L-asparaginase activity and dry weight of Penicillium increased with increasing incubation temperature where the maximum enzyme productivity and dry weight was at 35°C. The L-asparaginase activity was significantly lower both above and below this temperature. Microbial activity is known to be sensitive to environmental temperature, and the internal temperature of the microorganism must be equal to that of its environment **(Lapmak *et al.*, 2010)**. The majority of L-asparaginase-producing fungal species reported an optimal temperature range of 30 to 37°C **(Jayaramu *et al.*, 2010; Rani *et al.*, 2012)**. According to the present research, L-asparaginase activity is best at a pH of 7.0. The activity of L-asparaginase was significantly reduced at pH levels above and below 7. The optimal dry weight was at pH 6.0. The experimental fungus can thrive and produce L-asparaginase in a wide range of pH conditions. Our findings corroborated those of **Abdel Fattah and Olama (2002)**, who discovered that *Pseudomonas aeruginosa* produced the most biomass and L-asparaginase at a pH range of 7.0-7.5. Additionally, our findings partially concur with other researchers' reports on the production of enzymes. **(Geckil *et al.*, 2004; El-Sayed, 2008)** where demonstrated that the optimal L-asparaginase activity was found at neutral and alkaline pH values (6.5-9.5). **Sivakumar *et al.*, (2006)** have documented how pH is a key factor in the regulation of growth, metabolism, and the production of enzymes. The pH

optimal range varies among microorganisms, and any change in this range could reduce the activity of their enzymes **(Adinarayana and Ellaiah, 2002)**. The maximum L-asparaginase yield was also achieved by *Fusarium equiseti* at an initial pH of 7.0 **(Hosamani and Kaliwal, 2011).** In the majority of cases, carbon sources for microbial fermentation processes come from carbohydrates. During industrial fermentation, the desired microorganism's growth is powered by either light or the oxidation of medium components **(Stanbury *et al.*, 1995)**. Carbon sources in the media formulation promote growth and increase the production of enzymes, which is what is typically seen in the synthesis of primary metabolites like enzymes **(El-Hefnawy *et al.*, 2015)**. According to reports, catabolic repression prevented the microbial synthesis of L-asparaginase, which required a lower carbon source **(Baskar and Ranganathan, 2009)**. Data in this study showed that the most efficient carbon source for L-asparaginase activity was sucrose. When compared to other carbon sources, sucrose has the inductive effect of being an infinite carbon source, which results in a significant amount of enzyme production. It also aids in keeping the enzyme stable **(Soniyamby *et al.*, 2011)**. Sucrose influenced L-asparaginase synthesis by *Streptomyces* sp. TA22, which has been reported by **Mohana *et al.*, (2011)**. Our results illustrated that 0.5% sucrose after four days of incubation at 35°C, led to the peak of the L-asparaginase activity. The data showed that the kind of carbon substrates provided and the amount of sucrose present in the medium were both sensitive factors that affected the L-asparaginase activity and dry weight. Both the type of carbon substrates provided and the amount of sucrose in the medium were delicate variables that impacted the dry weight and L-asparaginase activity; around this concentration, the fungal dry weight decreased. While the lowest L-asparaginase activity and dry weight were recorded at a concentration of 2% sucrose. Our result agreed with **Anamika *et al.*, (2013).** The production of enzymes by microorganisms is strongly influenced by nitrogen. The presence of nitrogen sources promoted growth and subsequent enzyme production **(Mukherjee *et al.*, 2000)**. In fact, in a nitrogen-free medium, the microorganism exhibits very minor variations in its growth pattern **(Lapmak *et al.*, 2010)**. Using both organic and inorganic nitrogen sources, the impact of various nitrogen sources on the production of L-asparaginase was examined. Our presented data indicated

that the amido hydrolytic enzyme is not inductively affected by all nitrogen sources in the same way. The L-asparagine compound produced the highest fungal L-asparaginase activity. The use of sodium nitrate, on the other hand, was suggested to guarantee minimal L-asparaginase activity. Maximum fungal dry weight was obtained using yeast extract, peptone, and beef there is no significant value between these nitrogen sources. The minimum fungal dry weight was sodium nitrate. The same result was obtained by **Gulati *et al*., (1997),** they claimed that the presence of l-asparagine led to the highest level of L-asparaginase produced by *A. oryzae*, *Penicillium* in a fermentation medium, while there is no detectable activity of L-asparaginase generated by these examined microorganisms in the presence of sodium nitrate as a nitrogen source in their bioprocess. Additionally, L-asparagine was discovered to be the best inductive nitrogen source for *S. cerevisiae* L-asparaginase productivity **(Oliveira *et al*., 2003)**. It was discovered after examining the effects of various concentrations of the nitrogen source present in L-asparagine that 15 gm L^{-1} was the best concentration which produced the most L-asparaginase and fungal dry weight. Above and below this optimal concentration of l-asparagine, L-asparaginase activity, and dry weight are almost reduced this observation wasn't equivalent to that had been found by **Prakasham *et al*., (2007)** for *Staphylococcus* sp. **Amena *et al*., (2010)** found 2 gm L^{-1} (0.2%) was the best concentration for *S. glbargensis*. The lowest L-asparaginase production at a higher level of l-asparagine may be due to their negative effects on L-asparaginase gene expressions or downregulation of nitrogenous compounds availability **(Sabu *et al*., 2000)**. The metals play an important role as cofactors in enzymes. It is necessary to modify the medium's composition to prevent the inhibitory effects brought on when these cations are present in toxic concentrations because the optimal concentrations of these ions, Mn^{2+}, Zn^{2+}, Mg^{2+}, and Cu^{2+}, vary so widely with the different strains under study **(Raha *et al*., 1990)**. Additionally, some metallic ions may have an inhibitory effect on the production of enzymes and biomass due to interactions with the biosynthetic pools of the enzymes or with certain amino acids at the active sites of the enzyme **(Mansour, 2001)**. Investigations were done into how certain trace elements in the fermentation medium affected growth and L-asparaginase activity. The current findings demonstrated that the presence of Fe^{2+} ions significantly increased both L-

asparaginase activity and dry weight as recommended by **Anamika *et al*., (2013)**. **Nawaz *et al*., (1998)** reported an improvement impact of $Fe^{2+}$and Co^{2+}on the production of *E. cloacae* L-asparaginase, while an inhibitory effect of Cu^{2+}, $Mn^{2+}$and Zn^{2+} on the enzyme productivity. **Ferrara *et al*., (2006)** found that the ideal quantity of L-asparaginase produced by *Pichia pastori* was obtained using the fermentation media supplemented with Cu^{2+}, Mn^{2+}, Na^{+}, Zn^{2+}, $Fe^{2+}$and Co^{2+}.

Conclusion

L-asparaginase is crucial in medical applications because it has potent anti-tumor properties, especially in the treatment of acute lymphoblastic leukemia. For the current study, 15 fungi strains were isolated from the Egyptian marine environment. When testing to produce fungus-derived L-asparaginase, only one fungus strain, *Penicillium* sp., was found to produce the enzyme without exhibiting glutaminase activity. Fermentation performed best at pH 7.0 and 35°C on the fourth day, which was also the day with the highest enzyme productivity. Using sucrose and asparagine as carbon and nitrogen sources also resulted in a high yield. Our current data encourages formulating plans for the large-scale, glutaminase-free production of L-asparaginase, a project that is currently underway in our lab. Because both fungi and humans are eukaryotic organisms, fungi can mimic the characteristics of human cells, which facilitate the use of L-asparaginase from fungi in treating ALL.

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تحسين إنتاج ل- أسباراجينيز الخالي من الجلوتامينيز بواسطة فطر البنيسليوم المحب للملوحة معزول من من تربة الجذور (إمبيراتا الأسطوانية)

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ل- أسباراجينيز هو إنزيم يستخدم في علاج سرطان الدم الليمفاوي الحاد. آليته العلاجية هي التحلل المائي لل- أسباراجين ، وهو حمض أميني أساسي للخلايا الورمية بينما يمكن تصنيعه بواسطة الخلايا الطبيعية. تتمثل مشكلة ل- أسباراجينيز في ارتباطه بإنزيم الجلوتامينيز . لذلك من المهم البحث عن منتجي جدد لل- أسباراجينيز الخالى من إنزيم الجلوتامينيز بين الكائنات الحية الدقيقة حقيقية النواة. بحثت هذه الدراسة في الإمكانات التكنولوجية الحيوية للفطريات الخيطية المعزولة من تربة الجذور (إمبيراتا الأسطوانية). تم عزل خمسة عشر نوعًا فطريًا من إثنا عشر عينة بيئية ، . تم اختبار العينات على قدرتها على إفراز الإنزيم الخالى من الجلوتامينيز ، متبوعًا بتفجير خلايا الفطر لتحديد وقياس إنزيم ل- أسباراجينيز بعد عمليه التخمير. تم التعرف على نوع من الفطريات يقوم بإنتاج إنزيم الل- اسباراجينيز الخالي من الجلوتامينيز باستخدام الطرق الجزيئية على أنه فطر البنيسليوم. وفقًا لنتائج التحسين لإنتاج الإنزيم ، كان الأسباراجين عند ١٥ جم / لتر والسكروز عند ٥ جم / لتر أفضل مصادر للنيتروجين والكربون على التوالى. وتم تحقيق أفضل إنتاجية للإنزيم عند الرقم الهيدروجيني ٧ ، ٣٥ درجة مئوية ، ٧٥جم / لتر من كلوريد الصوديوم و تركيز ٠.٠١ جم / لتر من أيون الحديد الثنائى تحت ظروف الاهتزاز عند ١٥٠دورة في الدقيقة لمدة ٤ أيام.