

Production, Purification and Characterization of L-Asparaginase from Marine Endophytic *Aspergillus* sp. ALAA-2000 under Submerged and Solid State Fermentation

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Abstract

Among all endophytic fungi recovered from the marine soft sponge *Aplysina fistularis*, 72.2% were able to produce L-asparaginase. Among all obtained isolates, *Aspergillus* sp. ALAA-2000, the hyperactive producer for the anticancer agent, L-asparaginase, under submerged fermentation (SMF) and solid state fermentation (SSF) of different agriculture wastes was selected for the optimization of extraction process; optimization of physico-chemical parameters, which affecting the production of L-asparaginases in SSF and optimization of the purified L-asparaginases parameters. Maximum L-asparaginase activity 23.34 U/ml was recovered from soybean with hot water at 40 °C and 150 rpm for 30 min under SSF and 30.64 U/ml under submerged fermentation using glucose as carbon source and asparagine as nitrogen source. Two types of L-asparaginase (AYA-1 and AYA-2) were purified from the culture supernatant of *Aspergillus* sp. ALAA-2000 through ammonium sulfate precipitation and gel filtration chromatography (sephadex G-200). Molecular weights of the enzymes were 25 kDa (AYA-1) and 31 kDa (AYA-2). The parameters of purified L-asparaginase were optimized for AYA-1 (pH 6.0, stable at 30°C to 50°C for 60 min, reaction time 15 min, and substrate concentration 1.275 mg/ml) and AYA-2 enzyme (pH 10, stable at 30°C to 70°C for 60 min, reaction time 15 min, and substrate concentration 1.275 mg/ml). Whereas inhibitors of metalloproteases, chelating agents EDTA, had no effect on L-asparaginase. These finding suggest that L-asparaginase was not metalloproteases.

Keywords: Marine endophytic *Aspergillus* sp.; L-asparaginase; Solid state and submerged fermentation; Process parameters; Purification

Introduction

Chemotherapy is one of the principal modes of treatment for cancer. It is the process of using anticancer drugs to stop cells from dividing and multiplying but there are few curative chemotherapeutic drugs against cancer due to high toxicity, non selectivity, numerous side effects and low activity [1]. Therefore, searching and developing effective anticancer agents for these particular diseases have a great impact on morbidity and mortality of the patients and could therefore save many lives. Almost 60% of drug approved for cancer treatment are of natural origin. Although marine compounds are underrepresented in current pharmacopoeia, it is anticipated that the marine environment will become an invaluable source of novel compounds in the future, as it represents more than 70 % of the biosphere. The marine endophytic microflora in the coming decades will be the nature's best source of chemicals. Natural products metabolized from endophytic microorganisms represent desirable sources for effective therapeutic enzymes [2-4]. Marine fungi are rich profile of biologically active metabolites, especially from genera *Penicillium*, *Aspergillus* and *Fusarium* have been used aiming the development of novel therapies for treating cancer. Whereas the marine fungi are least studied than terrestrial counterparts and other ecological group, they have to study due to their production of new metabolites which are not found in terrestrial fungi [5]. Therapeutic enzymes are utilized in the treatment of diseases such as cancer, severe disorders like autism, chronic lung disease and multiple sclerosis but the major potential therapeutic application of enzymes is in the treatment of cancer. Interestingly, therapeutic enzymes have a particular ability to mediate high affinity interactions with unrelated proteins relevant in cancer conferring on it the property. The chemotherapeutic agents in miscellaneous include

therapeutically enzymes as L-asparaginase (E.C.3.5.1.1) which is degrades the particular amino acid L-asparagine and corticosteroid that act on specific cellular receptors to disrupts DNA in the majority of tumor cells [6]. The rationale behind L-asparaginase depends upon the fact that tumor cells are deficient in aspartate ammonia ligase activity which restricts their ability to synthesis the normally non-essential amino acid L-asparagine. These leukemic cells depend on circulating L-asparagine. The action of the L-asparaginase does not affected the functioning of normal cells which are able to synthesis enough for their own requirements but reduce the free exogenous concentration and so induces a state of fatal starvation in the susceptible tumor cells. Along with strain improvement by optimization of production parameters, the yield of L-asparaginase productivity was maximized.

The aim of this study is to isolate, identify and evaluate the potential of culturable marine endophytic fungi isolated from the internal healthy tissue of marine invertebrates for production of L-asparaginase as potent anticancer agents as well as develop an economically viable bioprocess for production of L-asparaginase by evaluating and

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optimizing process parameters through manipulating the nutritional and physical parameters using low cost substrates.

Materials and Methods

Isolation of marine endophytic fungi

The marine endophytic molds were maintained from the marine sponge as previously described by Holler et al. [7] and El-Bondkly and El-Gendy [8]. The healthy specimens of Egyptian sponge *Aplysina fistularis* were collected from Hurghada, Red Sea, Egypt, washed with distilled water and cut into small pieces of ca. 4 x 4 mm². Tissue pieces were rinsed in 0.1% Tween 20 for 1 min, then in 2.5% sodium hypochlorite for 15 min followed by washing in sterile distilled water for 5 min. Surface was sterilized with 75% ethanol for 5 min then rinsed in sterile water for three times. Finally the pieces were transferred to dishes of three different isolation media, potato dextrose agar, malt agar and peptone yeast extract glucose agar, which containing 100 µg/ml of oxytetracycline to inhibit the bacterial growth until the fungal mycelium or colony originated from the injury surface and incubated at 30 °C for two weeks. Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the sterilized segment surface. Single colonies were transferred periodically to the same medium and after 7 days at 30 °C. The obtained isolates were purified, maintained at 4 °C and examined for the production of L-asparaginase enzyme.

Screening of fungal isolates for L-asparaginase activity

Screening of fungal isolates for L-asparaginase activity was performed using the modified Czapek Dox medium (glucose 2 g, L-asparagine 10 g, KH₂PO₄ 1.52 g, KCl 0.52 g, MgSO₄·7H₂O 0.52 g, FeSO₄·7H₂O 0.01 g, agar 20 g, distilled water 1000 mL) for the plate assay. 3 mL of 2.5% stock solution of phenol red in ethanol (pH 6.2) was added to 1000 mL of Czapek Dox medium. After 72 h of incubation at 26 ± 1°C, the appearance of a pink zone around the fungal colony in an otherwise yellow medium indicated L-asparaginase activity [9].

Molecular identification of the hyper L-asparaginase producer strain

The selected hyper producer strain ALAA-2000 was identified according to its phenotypic and genotypic characteristics. The fungal strain ALAA-2000 was identified using internal transcribed spacer (ITS) sequences. The genomic DNA of this fungus was isolated and purified from protoplasts using a QIAGEN DNeasy Tissue Kit following the manufacturer's protocol for Gram-positive bacteria and animal tissue [10,11]. Amplification of ribosomal DNA was performed using puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences). The internal transcribed spacers (ITS) of the nuclear rDNA was identified according to the method described by Henry et al. [12] and White et al. [13] with some modifications. Next, polymerase chain reaction (PCR) was conducted using the ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [13], for amplification of the PCR product, 661 bp in size. The conditions for this PCR were: initial denaturation (5 min at 94°C) followed by 45 cycles of primer annealing (1 min at 55°C), primer extension (2 min at 72°C) and denaturation (1 min at 94°C), a final primer annealing (1 min at 42°C) and a final extension phase (5 min at 72°C) and then cooled to 4°C. PCR products were checked for correct length on a 1.5 % Tris-borate-EDTA TBE) agarose gel (1.5 % agarose, 8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA), stained with ethidium bromide and visualized under UV illumination. Sequence data were edited with Lasergene

Software SeqMan (DNASTar Inc.). Next relatives were determined by comparison to rRNA genes in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool, <http://www.ncbi.nlm.nih.gov> website) to create a matrix using MEGA6 and ClustalW programs. The tree topologies were evaluated by bootstrap analyses based on 1,000 replications with MEGA6 and phylogenetic tree were inferred using the neighbor-joining method. The rDNA sequence of hyper enzymes producing strain ALAA-2000 have been deposited in GenBank database under the following Accession number: KF877334.

L-Asparaginase assay

The activity of L-asparaginase was determined by estimating the amount of NH₃ liberated from asparagine during L-asparaginase catalysis using Nessler's reagent. For L-asparaginase determination the reaction mixture composed of 0.01 M L-asparagine and 0.05 M Tris-HCl buffer (pH 8.6), which incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 15% trichloroacetic acid solution and the liberated ammonia was coupled with Nessler's reagent and quantitatively determined at 480 nm. One international unit of L-asparaginase was defined as the amount of enzyme that liberates one micromole of ammonia from its substrate under the assay conditions [14].

Protein determination

Protein was determined by the absorbance at 280 nm using bovine serum albumin (BSA) as the standard [15]. All the sets have been performed in triplicates.

Some factors affecting production of L-asparaginase by *Aspergillus* sp. ALAA-2000 strain

Enzyme production under Solid state fermentation: The production of L-asparaginase by *Aspergillus* sp. ALAA-2000 under solid state fermentation was performed using different agro-industrial residues as *sugar cane bagasse*, *wheat bran*, *corn cobs*, *soy bean*, *kidney bean bran*, *wheat hay*, rice bran, rice straw and *corn casing*, which were collected from local suppliers and dried, grinded, sieved and preserved. Fermentation was conducted in 250 ml Erlenmeyer flasks containing 10 g of each solid substrate, separately. Each solid substrate was moistened with mineral salt solution composed of 10 ml of 0.01M phosphate buffer pH 6.2 [16].

Effect of different leaching agent: The leaching out of L-asparaginase from the fermented solids was carried out with different extractants such as hot water (40°C), ethanol, acetone, Tween 80 (1%), sodium chloride (0.1%), citrate buffer pH 3, phosphate buffer pH 7, glycine buffer pH 12, and at a ratio of 1:5 (w/v).

Enzyme production under submerged fermentation: For the production of L-asparaginase by *Aspergillus* sp. ALAA-2000, different fermentation media as a modified Czapek Dox medium, mineral salts and starch nitrate were applied [10]. As a result of this experiment, a selective medium for *Aspergillus* sp. ALAA-2000 strain was determined which favored higher yield of L-asparaginase production. *Aspergillus* sp. ALAA-2000 strain was incubated in 250 ml Erlenmeyer flasks containing 50 ml of Czapek Dox medium supplemented with L-asparagine and incubated at 27°C and 120 rpm on a rotary shaker for 4 days. At the end of fermentation period the clear supernatant after centrifugation at 4000 rpm for 20 min was used as enzyme preparations.

Effect of incubation period (typical production curve): *Aspergillus* sp. ALAA-2000 strain was grown in a modified Czapek Dox broth medium under shaking at different intervals of 2, 4, 6, 8, 10, 12 and 14 days. At each interval, final pH, L-asparaginase activities

were determined. As a result of this experiment, the suitable incubation period for each enzyme was determined and applied in the next experiments.

Effect of incubation temperatures and initial pH: The effect of different incubation temperatures ranged from 20°C to 37°C on L-asparaginase production by the marine *Aspergillus* sp. ALAA-2000 strain was studied in a modified Czapek Dox broth in shake culture. The initial pH of the growth medium (modified Czapek Dox broth) was adjusted to various pH values (3, 4, 6, 7 and 9) using 0.1 M of NaOH or HCl. The enzymatic activity of L-asparaginase at each initial pH was determined. The suitable initial pH, which support maximum enzymes production by *Aspergillus* sp. ALAA-2000 strain was determined and applied in the next experiments.

Effect of different nitrogen and carbon sources: Organic nitrogen sources; beef extract, yeast extract, peptone and urea as well as inorganic nitrogen sources; ammonium nitrate, ammonium sulfate, ammonium hydrogen citrate were examined for their ability separately to stimulate the enzyme production in modified Czapek Dox broth medium instead of L-asparagine as N-base. This study established the type of nitrogen source that stimulates the highest amounts of the enzyme production by the isolated marine *Aspergillus* sp. ALAA-2000 strain.

Effect of adding 10 g/L of various carbon sources separately in modified Czapek Dox broth medium instead of its carbon source (glucose) on L-asparaginase production was examined. These include raffinose, xylose, mannitol, mannose, sucrose, maltose, sorbose, lactose, galactose, fructose, starch, pectin, cellulose and dextrose. Carbon source which stimulates maximum enzymes production by *Aspergillus* sp. ALAA-2000 strain was used in the following studies.

Effect of some amino acids and element supplementations: Number of 10 amino acids including lysine, isoleucine, glycine, thiamine, arginine, treptophan, proline, glutamic acid, cysteine, and methionine were examined as a sole nitrogen source for L-asparaginase production. Each of them was added to the medium instead of its nitrogen source in such amount that the final concentration of N-base remained unchanged. Different concentrations (0.025%, 0.05%, 0.1%, 0.15% and 0.2%) of the most suitable amino acid source were tested for their ability to enhance the L-asparaginase production for *Aspergillus* sp. ALAA-2000 strain.

Some element supplementations (MgCl₂, LiCl, CaCl₂, K₂HPO₄, BaCl₂, and NaCl) were supplementation to the modified Czapek Dox broth medium to study their effects on L-asparaginase production by *Aspergillus* sp. ALAA-2000 strain. Different concentrations (0.025%, 0.05%, 0.1%, 0.15% and 0.2%) of the most suitable element supplementation were tested for their ability to enhance the enzyme production by *Aspergillus* sp. ALAA-2000 strain.

Purification and characterization of L-asparaginase produced by *Aspergillus* sp. ALAA-2000 strain

The concentrated enzyme was subjected to ammonium sulphate fractionation with concentration ranging between 20% and 100% according to the method of **Gomori** [17]. The precipitate of crude enzyme was dissolved in a minimum volume of 0.2 M phosphate buffer (pH 6.0) and dialyzed overnight in a dialysis bag against the same buffer at 4 °C. The dialyzed ammonium sulfate fraction was applied to a Sephadex G-200 column (Pharmacia, Sweden, 2.5 X 45 cm) that was pre-equilibrated with a 0.01 M Tris -HCl buffer pH 8.5. The protein elution was done with the same buffer at a flow rate of 5 ml/min [18]. The activity fractions of L-asparaginase were collected and pooled at 4

°C. It was assayed for protein at 280 nm as well as for L-asparaginase activity as described before.

Determination of the molecular weight of the purified enzyme

The molecular weights of the purified L-asparaginase were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by **Laemmli** [19].

Characterization of partially purified L-asparaginase

Optimum reaction time, pH and temperature: The optimum reaction time for the partially purified enzyme was estimated at different times 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 min for L-asparaginase and then assayed by the direct nesslerization method. The optimum pH was determined by measuring enzymatic activity at 37 °C using different buffers (0.1 M) with various pH values as: citrate-phosphate buffer (pH 3, 4, 5 and 6), phosphate buffer (pH 6 - 7), Tris-HCl buffer (pH 8 - 9) and glycine-NaOH buffer (pH 10 - 12). Optimum temperature was assayed by measuring activity with varying temperatures (27, 37, 47, 57, 67, 77 and 87 °C) at the optimum pH values.

Thermostability of partially purified L-asparaginase: Heat stability was determined by incubating the partially purified enzyme at various temperatures (20, 30, 40, 50, 60, 70, 80 and 90 °C) for 60 min and then the residual activity was determined at optimum pH and temperature.

Effect of substrate and enzyme concentrations: Partially purified L-asparaginase was incubated individually with different concentrations of L-asparagine in the reaction mixture (0.26, 0.64, 1.28, 2.55, 3.83 and 5.10 mg/ml) to find out the best substrate concentration for enzymatic assay under optimized assay conditions. Different concentrations (0.001, 0.03, 0.05, 0.1, 0.15 and 0.2 ml) of the partially purified L-asparaginase were incubated individually with the optimization concentration substrate solution in the reaction mixture to determine the best enzyme concentration under the optimized assay condition.

Effect of some chemical additives on enzyme activity: The effect of different metal ions including Na⁺, Ca²⁺, Mn²⁺, Mg²⁺, Co²⁺, Ba²⁺, and Ni²⁺ at a final concentration of 1.0 mM as well as EDTA at different concentrations (0.0001, 0.001 0.005, 0.01, 0.05 and 0.1 M) separately on purified L-asparaginase were studied. Residual activities in the presence of each chemical was assayed and compared with the control (without additions), which considered as 100% activity.

Results and Discussion

Isolation and screening of marine endophytic fungi for L-asparaginase activity

This study proved that Egyptian marine sponge *Aplysina fistularis* is a rich source of endophytic microorganisms. Whereas 18 fungal isolates were obtained on the isolation media peptone yeast extract glucose agar, potato dextrose agar and malt agar. All these marine endophytic fungi were evaluated as fruitful source of the therapeutic antitumor enzyme L-asparaginase. Overall, the activity of L-asparaginase was detected in most fungal strains. Among all isolates, the fungal strain under the isolation number ALAA-2000 was the hyper active producer of L-asparaginase. Therefore, this strain was selected for further studies. Endophytic fungi with different bioactive secondary metabolites are extremely distributed in all living organisms, whether terrestrial, marine plants corals and sponges [20-24]. Endophytes may be a novel and good producers of L-asparaginase. Of 82 endophyte isolates symptomless endophytic fungi isolated from seven green algae, six brown algae and

six red algae production of L-asparaginase enzyme was detected in 64 isolates and *Fusarium* sp. isolated from the thallus of *Sargassum wightii* and a sterile mycelial isolated from the thallus of *Chaetomorpha* sp. showed maximum activity of the enzyme [25].

Molecular identification of hyper L-asparaginase production strain ALAA-2000 through ITS region sequencing

The ITS region of hyperactive enzymatic strain ALAA-2000 was amplified, sequenced, and submitted to GenBank (accession no. KF877334). Amplification with primers ITS1 and ITS4 resulted in approximately 661 bp of fragment. The obtained sequences were compared with those in the National Center for Biotechnology Information (NCBI) Nucleotide Sequence Database by using the Basic Local Alignment Search Tool (BLAST) algorithm. A comparative analysis by MEGA6 and ClustalW software demonstrated that ITS region sequence from the producing strain ALAA-2000 had a significant identity to a number of *Aspergilli* (Figure 1). The comparison of the producing strain ALAA-2000 with sequences of the reference species of *Aspergillus* contained in genomic database banks exhibited a similarity of 99.52% with *Aspergillus fumigatus* M1, *Aspergillus sojae* JPDA1, *Aspergillus flavus* MJ49 and *Aspergillus parasiticus* DAOM 225948. According to the analysis genotypic together with phenotypic characteristics, the producing strain ALAA-2000 was identified as *Aspergillus* sp. and designated as *Aspergillus* sp. ALAA-2000.

Production of antitumor enzyme, L-asparaginase, under different fermentation modes

Production of L-asparaginase under solid state fermentation (SSF): Data in Table (1) indicated the impact of using different natural

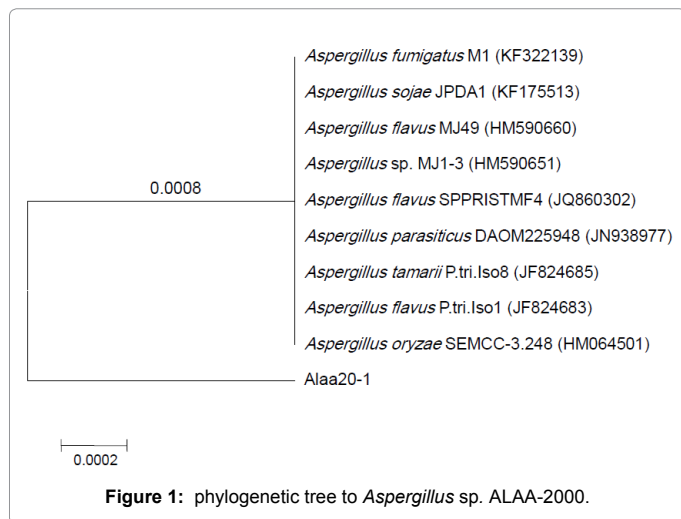


Figure 1: phylogenetic tree to *Aspergillus* sp. ALAA-2000.

Substrate	Leaching agent			
	Hot water 40 °C	Glycine	Tween 80	Citrate
Sugar cane bagasse	14.59	2.04	18.38	15.61
Wheat bran	10.21	0.15	22.76	15.03
Corn cobs	8.22	0.15	3.65	8.61
Soy bean	23.34	1.31	18.97	17.51
Kidney bean bran	9.33	1.02	13.57	22.03
Wheat hay	0.73	1.89	6.62	0.73
Corn casing	0.29	1.75	9.78	9.34
Rice bran	2.92	1.46	21.45	4.38

Table 1: Production of L-asparaginase under solid state fermentation (SSF) and extracted with different leaching agents.

sources as sugar cane bagasse, wheat bran, rice bran, corn cobs, soybean, kidney bean bran, wheat hay, corn casing and rice straw as a valuable and cheap energy sources for the production of this anticancer enzyme. On the other hand to optimize the enzyme yields leaching out from these fermented natural sources, each substrate was extracted with different leaching agents as hot water 40 °C, ethanol, acetone, sodium chloride (1%), Tween 80 (1%), citrate buffer (pH 3), phosphate buffer (pH 7) and glycine buffer (pH 12). Among these treatments, soybean as solid substrate extracted with hot water (40 °C) as leaching agent proved to be the best for achieving maximum L-asparaginase yield (23.34 U/ml) followed by wheat bran (22.76 U/ml), rice bran (21.45 U/ml), soybean (18.97 U/ml), sugar can bagasse (18.38 U/ml) after leaching out of the L-asparaginase enzyme from these fermented substrates by Tween 80 (1%) with the exception of kidney bean bran, which extracted with citrate buffer (pH 3). Consequently agro industrial residues proved to be promising cheap sources for the industrial production of the therapeutic enzyme, especially soybean. Moreover, among all leaching hot water gave the best results with soybean but, Tween 80 (1%), proved to be the fit leaching agent for L-asparaginase recovery from most fermented solid natural substrate under study followed by citrate buffer (pH 3).

The anticancer enzymes L-asparaginase was produced by a variety of fungi but the exclusive production of this enzyme in industry have been achieved mainly by *Aspergillus species* as *Aspergillus terreus* MTCC 1782 [26], *Aspergillus* sp. KUFS20 [27] and *Aspergillus fumigates* [28]. Soy bean was the best inducer for L-asparaginase production from *Fusarium quiseti* [29]. Negi and Banerjee [30] tested various parameters such as leaching agent selection, amount of leaching agent, soaking time, and temperature in order to determine optimum extraction conditions of enzymes produced by *Aspergillus awamari* nakazawa MTCC 6652 and optimum conditions were achieved in a 10% glycerol.

Production of L-asparaginase under submerged fermentation (SMF) versus typical course time: The effect of different three culture media named modified Czapek-Dox (M.Cz), mineral salts, and starch nitrate provided with 1% L-asparagine on L-asparaginase production by *Aspergillus* sp. ALAA-2000 was evaluated. Modified Dox medium showed the highest L-asparaginase activity (35 U/ml) while starch nitrate medium showed the lowest L-asparaginase productivity (17 U/ml). The activity of L-asparaginase increased with increasing the incubation period up to 10 days with maximal levels at the six day of fermentation under submerged fermentation. These data proved that SMF is preferred than SSF for L-asparaginase production from the marine endophytic fungus *Aspergillus* sp. ALAA-2000. Anamika et al. [31] and Elshafei et al. [32] reported the suitability of submerged fermentation for the production of L-asparaginase from *Aspergillus terreus* and *Penicillium brevicompactum* NRC 829, respectively but Siddalingeshwara and Lingappa [33] and Varalakshmi and Raju [26] favored SSF over SMF for the biosynthesis of L-asparaginase by *Aspergillus terreus* KLS2 and *Aspergillus* MTCC 1782, respectively. Many parameters that affect the submerged fermentation process were optimized. Typical time course of L-asparaginase production was an important criterion for assessment of potency of the producing strain *Aspergillus* sp. ALAA-2000. The highest biosyntheses of L-asparaginase was completed after 6 days of fermentation and thereafter decreased. These results are in accordance with the study conducted by Luhana et al. [34] on the production of L-asparaginase by *Aspergillus niger*, which reach its peak after 6 days of fermentation but differ from results obtained by Anamika et al. [31] and Patro and Gupta [35] for the maximum L-asparaginase production by *Aspergillus terreus* and *Penicillium* sp. after 3 and 10 days, respectively.

Enzyme extraction treatments from whole broth after submerged fermentation for different incubation period: The industrial development of the microbial bioactive secondary metabolites has been hampered by the difficulty and expense of the releasing and extracting of bioactive compounds from the mycelium in good yield. The amounts of L-asparaginase released from the mycelium of ALAA-2000 treated with toluene, ethyl acetate and butanol were detected to be 4.81, 9.19 and 4.67 U/ml, respectively compared to 13.71 detected in the cell free supernatant. These data clearly indicated that Most of the therapeutic enzyme L-asparaginase amount produces extracellular. Extracellular enzymes are of potent important in industrial application in the terms of minimize the cost of production process. Moreover L-asparaginase was reduced from 30.64 to 7.59, 7.29 and 6.13 U/ml at 10th day of incubation period of whole broth with solvents.

Effect of different pHs and temperatures on L-asparaginase production from *Aspergillus* sp. ALAA-2000: *Aspergillus* sp. ALAA-2000 was incubated at different pH values 3, 4, 6, 7, 8 and 9 with different incubation temperature 20, 27 and 37 °C for 6 days to determine the optimum temperature and pH, which supported the highest L-asparaginase production. Data in Table (2) showed that initial pH between pH 3 to pH 6 at 27 °C supported L-asparaginase production with a peak at pH 6 (32.54 U/ml) after 6 days of fermentation and then it was decreased gradually at higher temperature and pH values. Whereas maximum yields of L-asparaginase were achieved at the same incubation temperature (27 °C), they were elucidated at different with pH (6.0). In similar optimum incubation temperatures for L-asparaginase production from *Fusarium* sp was reported to be 27 °C but for *penicillium* sp., *Aspergillus niger*, *Aspergillus terreus* MTCC 1782 and *Aspergillus terreus* were found to be 30, 35, 30 and 30 °C, respectively, [25,26,35-37].

Effect of different substrate concentrations on L-asparaginase production:

L-asparagine is the inducer for L-asparaginase production. Different concentrations (0.5%, 1%, 1.5%, 2% and 2.5%) were added to the modified Dox medium individually to determine the optimum

concentration for the enzymes production. The maximum L-asparaginase production was achieved with 1% L-asparagine. On the other hand, higher L-asparagine concentrations (2% and 2.5%) did not increase the productivity (Table 3).

Effect of different nitrogen and carbon sources on L-asparaginase production: The influence of different organic nitrogen sources and inorganic sources were studied. Each one of these nitrogen sources was added to the modified Dox medium individually at concentration instead of the enzymatic inducer L-asparagine. Results indicated that the endophytic *Aspergillus* sp. ALAA-2000 strain was required the inducers L-asparagine for the best biosyntheses of L-asparaginase (33.85 U/ml). However urea and sodium nitrate showed lowest production of L-asparaginase (6.13 and 7.59 U/ml), respectively by *Aspergillus* sp. ALAA-2000 strain. Interestingly medium free nitrogen yielded 1.17 U/ml of L-asparaginase (Table 3). On the other hand, *Aspergillus* sp. ALAA-2000 strain was grown in modified czapek Dox medium supplemented with different carbon sources individually. From data in Table (3), it was obvious that galactose maximized L-asparaginase production by *Aspergillus* sp. ALAA-2000 strain to 32.83 U/ml followed by dextrose (31.08 U/ml) but mannose followed by mannitol were minimized its productivity to (17.65 and 14.14U/ml), respectively. Overall, all carbon sources did not exhibited much significant increase in enzyme production due to Dox.Cz modify medium free carbon relatively yielded a high amount of L-asparaginase (29.33 U/ml), this phenomenon in *Aspergillus* sp. ALAA-2000 strain is promising for cost effective of production process.

Carbon source represents the energy source that will be available for growth of the microorganism. *Aspergillus oryzae* produced the highest amounts of L-asparaginase under SMF using lactose as carbon source along with combination of tryptone 1% (w/v) and yeast extract 0.5% (w/v) as nitrogen source as well as L-asparagine as the best inducer [9]. Glucose as a carbon source in the medium inhibit L-asparaginase synthesis in fungi as *Fusarium* sp. and the enzyme activity increased with decreasing concentration of glucose in the growth medium [25]. Also, 0.5% Sucrose exhibited highest L-asparaginase activity (20.6 U/ml) by *Aspergillus tamari* [31] but glucose at a concentration of 1%, 1.5% and 2% resulted in the highest amount of L-asparaginase production from *Aspergillus terreus* MTCC 1782 and *Aspergillus terreus* [26,37].

Effect of different amino acids and element supplementations on L-asparaginase production: Data illustrated in Table (3) showed effect of different amino acids on L-asparaginase production. The highest L-asparaginase production (15% increases) was achieved with arginine, while isoleucine and cysteine strongly inhibited enzyme production by 69% and 72%, respectively. Moreover, Addition of arginine at a

pH value	Enzyme production (U/ml)		
	20 °C	27 °C	37 °C
3	4.82	17.79	18.38
4	27.72	31.37	27.72
6	21.89	32.54	26.26
7	21.16	29.47	27.28
9	16.78	28.01	18.53

Table 2: Effect of different pH values and temperatures on L-asparaginase production.

L-asparagine (%)	L-asparaginase (U/ml)	Nitrogen sources	L-asparaginase (U/ml)	Carbon source	L-asparaginase (U/ml)	Suppl. elements	L-asparaginase (U/ml)	Amino acids	L-asparaginase (U/ml)
0.5	17.51	Without nitrogen	1.17	Without carbon	29.33	Control	23.34	Control	23.10
1.0	39.39	Peptone	16.34	Raffinose	22.18	MgCl ₂	20.43	Lysine	23.19
1.6	35.75	Beef extract	9.48	Xylose	23.34	LiCl	16.92	Isoleucine	7.29
2.0	29.62	Yeast extract	17.51	Mannitol	14.15	CaCl ₂	18.53	Glycine	17.95
2.5	29.18	Urea	6.13	Mannose	17.65	K ₂ HPO ₄	19.99	Thiamine	19.69
		Ammonium sulphate	21.45	Sucrose	28.74	BaCl ₂	11.23	Arginine	35.16
		Diammonium citrate	18.24	Maltose	21.89	NaCl	18.82	Tryptophane	24.66
		Sodium nitrate	7.59	Sorbose	25.09			Proline	14.88
		Ammonium nitrate	20.72	Lactose	23.34			Glutamic acid	15.90
		L-asparagine	33.85	Galactose	32.83			Cystine	6.57
				Fructose	26.26			Methonine	25.53
				Starch	26.69				
				Pectin	28.45				
				Cellulose	17.79				
				Dextrose	31.08				

Table 3: Effect of different concentrations of substrate, nitrogen sources, carbon sources, supplementation elements and amino acids on L-asparaginase production.

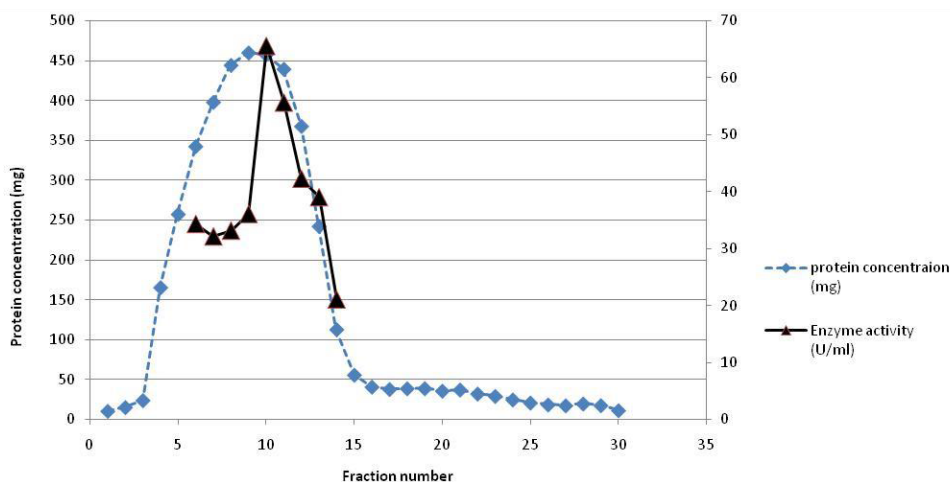


Figure 2: Elution diagram of L-asparaginase using Sephadex G-200 column chromatography.

concentration of 0.15% achieved the highest enzyme production (44.8 U/ml). The maximum L-asparaginase production was achieved with 1% L-asparagine, while lower or higher L-asparagine concentrations did not increase the productivity. On the other hand, all metal supplementations under study ($MgCl_2$, $LiCl$, $CaCl_2$, K_2HPO_4 , $BaCl_2$, and $NaCl$) resulted in a severe decrease in L-asparaginase productivity from 23.34 to 20.43, 16.92, 18.53, 19.99, 11.23 and 18.82 U/ml, respectively (Table 3).

Amino acids are common growth factor required for the biosynthesis of enzyme; hence the yield of L-asparaginase was varied according to the nature of amino acids. L-Asparagine and arginine stimulate L-asparaginase production in *Aspergillus* sp. ALAA-2000 strain to (33.85 and 35.16), respectively. Anamika et al. [31] reported L-asparagine as the best nitrogen source for L-asparaginase production by *Aspergillus terreus* in modified czapek Dox medium. None of the elements supplementations added to the fermentation medium enhanced the obtained yields of L-asparaginase from *Aspergillus* sp. ALAA-2000 strain. Varalakshmi and Raju [26] reported that L-asparaginase production by *Aspergillus terreus* MTCC 1782 was increased with adding magnesium sulphate to the production medium.

Purification of L-asparaginase: Crude extract of *Aspergillus* sp. ALAA-2000 strain of medium was purified of L-asparaginase by successive steps including, $(NH_4)_2SO_4$ fractionation and gel filtration chromatography (Sephadex G-200). The fraction obtained at saturation 80% of ammonium sulphate resulted in specific activity of (0.053 U/mg), (1.11-fold) purification folds, following Sephadex G-200 gel filtration, the purified L-asparaginase obtained exhibited (13.1 U/ml) of the total initial activity and there was 8.3-fold increase in purity with (0.4 U/mg) specific activity (Table 4 and Figure 2). By using molecular markers with known molecular weights (135.0, 100.0, 75.0, 63.0, 48.0, 35.0, 25.0 and 5.0 kDa), it was determined that the molecular weight of L-asparaginase from the strain *Aspergillus* sp. ALAA-2000 strain found to be two protein bands onn SDS-PAGE Figure (3) indicating the dimeric nature of enzymes with different molecular weights were 25 kDa (AYA-1) and 31 kDa (AYA-2). Patro and Gupta [35] recommended that ammonium sulphate precipitation at a concentration of 80% of L-asparaginase produced by *Penicillium* sp. and reported L-asparaginase with an apparent molecular weight of 66 kDa was purified with total activity 692.77 U, total protein 53.3 mg,

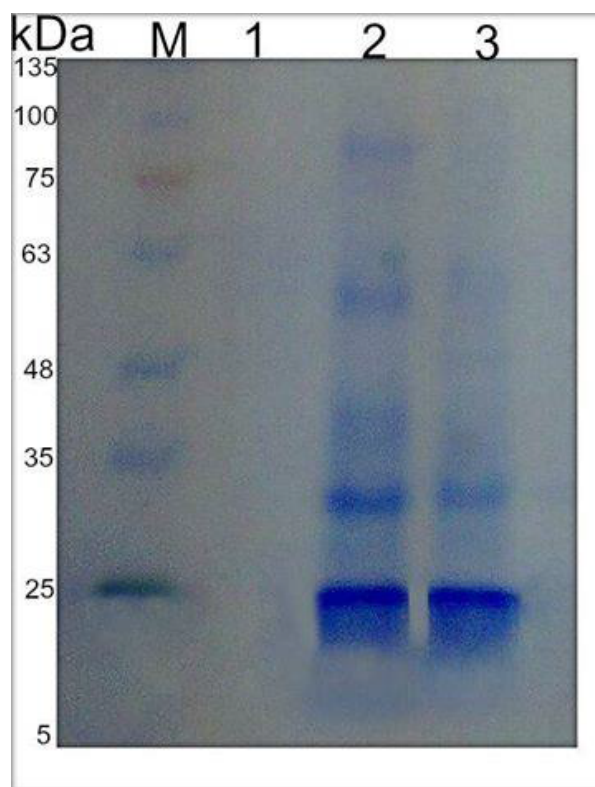


Figure 3: Electrophoretic analysis of L-asparaginase produced by *Aspergillus* sp. ALAA-2000 at various stages of purification. M, molecular weight markers; (135.0, 100.0, 75.0, 63.0, 48.0, 35.0, 25.0 and 5.0 kDa); lane 1, crude-enzyme extracts; lane 2, fractional precipitation by $(NH_4)_2SO_4$ 80%; and lane 3, partial purified L-asparaginase on Sephadex G-200.

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Filtrate	30	620	0.048	100	1
$(NH_4)_2SO_4$ precipitation	24.6	457.4	0.053	82	1.11
Sephadex G-200	13.1	32.3	0.4	43.6	8.3

Table 4: Summary of the purification steps of L-asparaginase.

specific activity 12.99 U/mg, purification 1.77-fold and yield 74.85% after Sephadex G-100 gel filtration. Akilandeswari et al. [36] showed the molecular weight of L-asparaginase produced by *A. niger* is 48kDa while Devi and Azmi [38] represented L-asparaginase dimeric with two distinct bands of molecular weights 40.2 and 39.8 kDa were purified from *Erwinia carotovora* MTCC 1428 by total activity 0.34 and total protein 0.91 mg.

Characterization of purified L-asparaginase: Due to the purification L-asparaginase showed two protein bands an SDS-PAGE indicating the dimeric nature of enzyme with different molecular weights of 25 kDa (AYA-1) and 31 kDa (AYA-2), these were two maximum peaks of enzyme activity at two different incubation temperatures. The first maximum activity peak was recorded at 47 °C and the other was noticed at 67 °C. However, both form of L-asparaginase activities were gradually decreased at the optimum incubation temperature. The thermo stability of L-asparaginase activities showed in Table (5). A wide range of L-asparaginase thermo stability from (30 to 60 °C) and higher than this temperature stability gradually decreased, maximum thermo stability of L-asparaginases at 30, 40 and 50 °C by 100%, 100% and 111%, respectively. L-Asparaginase activities were decreased (30%) after incubation at 90 °C for 60 min.

L-asparaginase activities at different pH values (3, 4, 5, 6, 7, 8, 9, 10, 11, and 12) were determined. Results in Table (5), indicated that L-asparaginase showed two-peak activities curve at two different pH values 6 and 10 with activities of 128% and 118%, respectively. Which indicated that marine endophytic *Aspergillus* sp. ALAA-2000 strain produced two types of L-asparaginase one thermo stable alkaline L-asparaginase at pH 10 and 67 °C (AYA-2) and other natural

Thermo stability	L-asparaginase relative activities (%)	pH	L-asparaginase relative activities (%)
20	70	3	72.9
30	100	4	78.7
40	100	5	82.9
50	111	6	128
60	93	7	95.2
70	83	8	100
80	50	9	117.3
90	30	10	118
		11	102.4
		12	80.5

Table 5: Effect of thermo stability and different pH values on L-asparaginase activities.

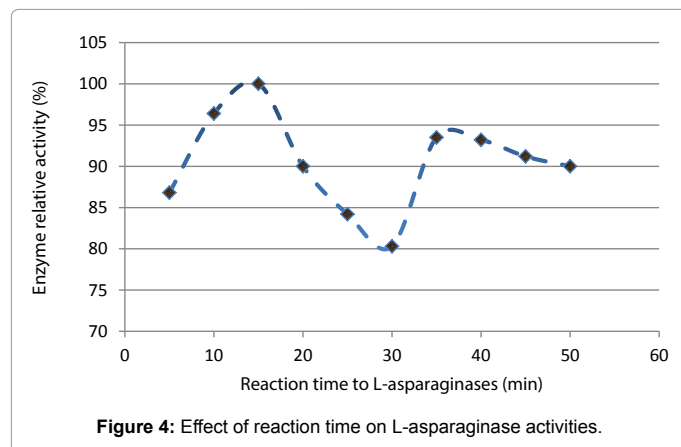


Figure 4: Effect of reaction time on L-asparaginase activities.

L-asparaginase, which exhibited its maximum activity at pH 6 and 47 °C (AYA-1). Moreover, Figure (4) showed that, the activity of partially purified L-asparaginases were recorded at 15 and 35 minutes, which increased up to 100% and 93%, respectively. These, results may be attributed to the production of dimeric form of L-asparaginases by marine *Aspergillus* sp. ALAA-2000 strain. On the other hand, the effect of different concentrations of L-asparagine 0.26, 0.64, 1.28, 2.55, 3.83 and 5.10 mg/ml, on the activity of L-asparaginase was detected. The activities of both L-asparaginases were increased with increasing substrate concentration to 1.28 mg/ml of L-asparagine. Different partially purified enzyme concentrations (0.01, 0.1, 0.15, 0.25, 0.5 and 0.2 ml) of L-asparaginase were attempted in the enzymatic reaction with asparagine at concentration of 0.85, the suitable enzyme concentration in the reaction mixture that supported the highest activity was 129.9 produced by 0.15 ml.

The effect of Ni²⁺, Ba²⁺, Ca²⁺, Na⁺, Co²⁺, Mn²⁺, and Mg²⁺ with final concentration of 0.5 mM of each cation on partially purified L-asparaginase activities were studied. Compared to control, only Ni²⁺ achieved increase in the L-asparaginase activity about 16%, however other cations inhibited enzyme activity. The highest inhibition was 22% achieved by Na⁺. The effect of different concentrations of EDTA (0.0001, 0.001, 0.005, 0.01, 0.05, and 0.1 M) on the activities of partially purified L-asparaginase, results indicated that different EDTA concentrations have no effect on L-asparaginase activities which means that enzyme activity is not dependent on di or trivalent ions. The parameters of purified L-asparaginase were optimized for AYA-1 and AYA-2 (for AYA-1 optimal pH 6, stable in pH range of 6.0 to 8.0, optimal temperature equal to 47 °C and thermal stability in the range of 30 to 60 °C for 60 min) and AYA-2 enzyme (optimal pH 10, stable in pH 10.0 - 11.0, optimal temperature equal to 67 °C and thermal stability in the range of 30 to 60 °C for 60 min). About 30% and 28% of L-asparaginase activities decline after incubation at 70 °C for 30 min and 80 °C for 5 min, respectively. Optimum incubation temperature for purified L-asparaginase from *Penicillium* sp. and *Aspergillus niger* was reported to be 37 and 35 °C, respectively [35,36]. The purified L-asparaginase from *Penicillium brevicompactum* NRC 829 was active at a wide range of temperature from 30 to 75 °C with an optimum at 37 °C and about 35% of its activity still present at 70 °C but it lost its activity at 90 °C and proved to be metalloenzyme due to the sever inhibition in its activity in the presence of EDTA [32]. On the other hand, Suchita et al. [39] and Kumar et al. [18] reported that L-asparaginase produced by *Erwinia carotovora* and *Streptomyces radiopugnans* MS1 non metalloenzymes. Akilandeswari et al. [36] reported the pH 8 is best value for maximum activity of L-asparaginase produced by *A. niger*.

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