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Production and screening of anticancer enzyme from marine Fungi

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Abstract

The Asparaginase has a great importance; because of its anti-cancer potential. This study aimed to produce and characterize L-asparaginase from *Emericella sp*. The parameters such as incubation period, temperature, pH, carbon and nitrogen sources, were studied to indicate a maximum production of L-asparaginase enzyme by *Emericella sp*. The specificity substrate was also studied. The results of this study showed that maximum activity was noted on the 6th day of incubation period with incubation temperature of 30°C and pH 7.0 and 120 rpm. Glucose (0.2%) was the best source of carbon. Among all the various nitrogen sources at concentrations ranging from 0.1% to 0.5%, ammonium nitrate (0.4%, 0.5%) showed a maximum production of L- asparaginase. L-asparagine was considered as a natural specific substrate to the L-asparaginase and retained 100% relative activity.

Keywords: *Emericella sp***,** L-asparaginase, optimum parameters, Substrate specificity.

Introduction

Fungi are the basic organisms in ecosystems, where the environment supports its growth anywhere, either on water or in land [(1)]. Special fungal diversity in the marine environment is still rare compared to many bacteria and viruses. The information that we have is based on research that was done in the laboratory, for this reason a selective group of very few fungal genera are being studied rigorously[(2)]. Recently, There has been a significant interest in the huge biodiversity of marine fungal communities and their high capacity to produce many biologically active compounds to balance marine environments [(3, 4)]. Fungi play significant roles in marine environments besides bacteria, which are among the most prevalent species in these environments and the largest contributor to the organic matter production. Many fungal communities can exist in the sea such as: aquatic fungi which grow permanently in seawater, optional marine fungus that grow in both sea water and fresh water, and terrestrial fungi that grow in sea water when conditions of growing are suitable [(5, 6)]. Many studies indicate that, many marine fungi

have the capability to produce L-asparaginase [7, 8] such as *Fusarium, Penicillium*, and *Aspergillus.* These mitosporic fungi, are commonly reported in several studies to produce asparaginase [9, 10]. L-Asparaginase is an enzyme that is widely used as an anti-tumour agent and acts on a substrate such as L-asparagine.

The L-Asparaginase enzyme has many advantages such as it is non-toxic, can be operated with complete ease at the specified location, cheap and biodegradable [11]. On the other hand, other anti-tumour agents are very expensive and very painful when administered to the patient. The present work focuses on the production of L- asparaginase from *Emericella sp* and optimization of cultural conditions to getting a maximum production.

Materials and Methods

Sample collection

Sixty-seven samples were collected from nine sites along Alexandria Eastern Harbor in Egypt. Each sample consisted of both water and sediment. Samples were collected in sterilized screw cap bottles 300ml. Collected samples are stored until they were processed during no longer than 24 hrs. later.

Fungal isolation

Sediment samples

Each sediment sample (10 gm) was added to 90 ml of sterilized distilled water. The resulted suspension (0.5ml) was spread onto potato dextrose agar (PDA) with 50 mg/L tetracycline to prevent the bacterial growth. Then incubated for seven days at 28^{o}C and the colonies were started to be observed after 4 days. After complete incubation, the colonies were identified morphologically.

Seawater samples

Each sea water sample (10 ml) was diluted in 90 ml of sterilized distilled water. The previously prepared dilution (1 ml) was further diluted in 9 ml of sterilized distilled water to achieve a dilution of 1^{-100} M. One milliliter of each of the prepared 1^{-100} M dilutions was spread onto potato dextrose agar medium with 50 mg/L tetracycline to inhibit the bacterial growth and also incubated for seven days at 28^{o}C. The resulted colonies were then identified morphologically.

Phenotypic characterization

All isolates grew equally well on potato dextrose agar plates at 28^{o}C. Most colonies grew up to 2.4–4.1 cm in diameter after 48 h of incubation. According to their morphology and

growth characteristics, the resulted well colonies were selected for more characterization and genetic identification [12].

Molecular identification of the fungal isolates

Fungal growth was suspended in 100 µl sterilized distilled water and boiled for 15 min. Amplification of ribosomal rRNA gene was done by using universal fungal primers ITS1 and ITS4. The PCR products were detected by using 1% agarose gel, then emerged in ethidium bromide solution overnight, and UV transilluminator was used for visualization. The resulted amplicons were cleaned using Gen Elute™ PCR clean-up kit according to instructions. BLAST analysis was carried out by using the NCBI database [13]. The resulted sequence then was submitted to NCBI Gen bank.

Determination of L-asparaginase activity from fungal isolates

Plate screening of L- asparaginase producing fungi

The isolated marine fungi were grown on Modified Czapek-Dox (MCD) agar medium. The incubation was carried out at 25^0C for 7 days. A positive result was detected by the appearance of a pink zone which indicates of the L-asparaginase production [14].

L-asparaginase production

The isolates were tested for L-asparaginase production by growing on the MCD medium for 3 days. Disc technique was carried out by inoculating a 5 mm disk into 100 ml of the MCD medium then incubated at 28°C for 7 days. After the incubation period, the broth was centrifuged at 10,000 rpm for 10 minutes at 4^oC. The resulted supernatant was obtained as a crude enzyme to estimate L-asparaginase activity.

Evaluation of L-asparaginase

To evaluate L-asparaginase, 0.5 ml of asparagine (0.04 M), as a substrate, 0.5 ml of buffer (0.5 M), 0.5 ml of enzyme and 0.5 ml of distilled water were added. The obtained mixture was incubated for 30 min. Then, 0.5 ml of 1.5 M TCA (Trichloroacetic acid) was added to stop the reaction. 0.1ml of the mixture was added to 3.7 ml distilled water and 0.2 ml of Nessler's reagent and incubated for 20 min. The OD of the result was estimated at 450 nm.

International unit

One IU of L-asparaginase activity is defined as the amount of enzyme which catalyses the formation of 1µmole of NH_3 per minute under the optimal assay conditions.

Statistical Analysis

The analysis of variance was carried out according to the rules of ANOVA. The significant differences between the means, were determined through Duncan's multiple range Test (DMRT).

$$\text{Units/ml enzyme} = \frac{(\mu\text{-mole of } NH_3 \text{ liberated})\ (2.5)}{(0.1)\ (30)\ (1)}$$

2.5 = Initial volume of enzyme mixture (ml)
0.1 = Final volume of enzyme mixture (ml)
30 = Incubation time (min)
1 = Volume of enzyme used (ml)

Characterization of L-asparaginase

L-asparaginase, after the fermentation process, were separated out by using filtration, centrifugation, and ammonium sulfate salt precipitation and then purified by chromatography techniques, this resulted enzyme was later used for further studies.

Optimization of parameters for production of L-asparaginase

The optimization of parameters for production of L-asparaginase were carried out, that by changing one parameter and keeping the others unaltered to giving the maximum production of L-asparaginase. The optimization of L-asparaginase by *Emericella sp* depends on several factors such as incubation period, temperature and pH. Also, different carbon and nitrogen sources on L-asparaginase production were studied.

Effect of incubation period

To **determine** the maximum L-asparaginase production at different incubation periods, 100ml of MCD containing 1% asparagine at pH 6 then inoculated and incubated at different growth intervals. The filtrate was incubated and observed from 1to8 day at 30^0C, and the clear supernatant was used to determine the enzyme activity.

Effect of pH on enzyme activity

To detect the effect of pH on the production of L- asparaginase by *Emericella sp,* the reaction medium was adjusted to various pH ranges of 3, 5, 7, 9and 11, then inoculated into the MCD medium with a 5mm culture disc and incubated for 7 days at 30^0C. The enzyme activity was determined.

Effect of temperature on enzyme activity

To determine the maximum production of L-asparaginase at different temperatures, the reaction medium was inoculated and incubated at different temperatures of 25ºC, 30ºC, 35ºC, 40ºC and 45ºC for 7days. The enzyme activity was determined.

Effect of carbon source

Five carbon sources; starch, lactose, maltose, sucrose, and glucose (0.2%) were used for producing a maximum production of asparaginase. The influence of different carbon sources on efficient production was assessed and incubated at 30^0C for 7days. The supernatant was utilized for determining the enzyme activity and protein content.

Effect of nitrogen source

The effect of different nitrogen sources; yeast extract, ammonium nitrate, peptone, sodium nitrate, and potassium nitrate were studied on L-asparaginase production in a range of concentrations from 0.1% to 0.5% and incubated at 30^0C for 7 days. The enzyme activity was obtained by utilizing the supernatant to measure the enzyme activity.

Substrate specificity

Mixtures that contained L-Asparaginase enzyme and buffer with different substrates; D-asparagine, glutamine, and L-asparagine were used under the present study [15, 16].

RESULTS AND DISCUSSION

Production of L-Asparaginase

In the present study, thirty-six strains were isolated and named serially from MF1 to MF36. Of the 36 fungal isolates, 23 isolates (63.8%) were screened positive for the L-asparaginase activity. The potential strains were selected on the presence of pink zone indicating the L-asparaginase production (Fig.1). Among these marine fungal isolates, MF30 was selected as a potential strain for the production of L-asparaginase. The isolate MF30 was selected for the optimization studies based on the initial screening by plate assay method. The isolate was identified as *Emericella sp.* and the resulted sequence was submitted to Gen bank with the accession no. AUMC 6937.An increase in pH due to ammonia accumulation in the medium results in the change of color from yellow to pink and is suggestive of the fact that the marine fungi are able to utilize the asparagine as a substrate by the secretion of the enzyme L-asparaginase. The results of the plate assay method procedure for L-asparaginase production are depicted in Table.1**.** The study

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indicated that the L-asparaginase production was maximum (6.05 IU) at the 7th day of the incubation period [17].

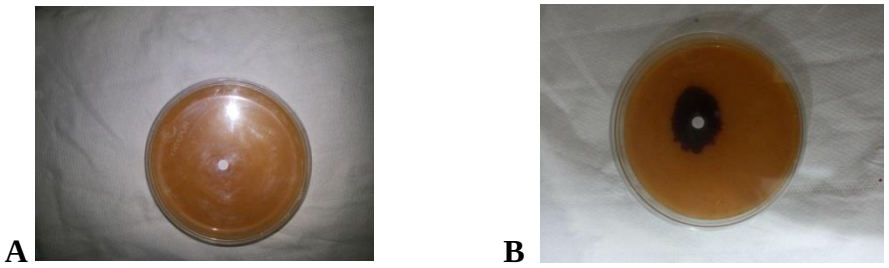

Fig.1: Fungal isolate on a Modified Czapekdox plate (MCD)
A) Asparaginase negative plate B) Asparaginase positive plate

Table.1: Screening of marine fungi for L-asparaginase production

MF.No	Zone diameter(in mm)
MF1	0
MF2	23.00±0.58
MF3	36.10±0.40
MF4	0
MF5	0
MF6	0
MF7	0
MF8	46.13±0.81
MF9	27.70±0.20
MF10	48.01±0.47
MF11	44.00±0.50

MF12	53.30±0.80
MF13	39.00±0.59
MF14	0
MF15	41.60±0.62
MF16	28.01±0.31
MF17	52.00±0.45
MF18	45.40±0.11
MF19	0
MF20	36.00±0.50
MF21	0
MF22	0
MF23	36.00±0.24
MF24	27.29±0.85
MF25	45.00±0.51
MF26	0
MF27	36.55±0.50
MF28	0
MF29	47.10±0.80
MF30	**66.00±0.51**
MF31	34.40±0.30
MF32	26.90±0.80
MF33	35.00±0.50

MF34	29.00±0.60
MF35	0
MF36	0

Optimization of culture parameters for L-asparaginase production

The culture parameters for obtaining a maximum production of L-asparagine have been studied. These parameters play an important role in the production of L-asparaginase, which necessitates conditions in which fungus can produce high yields of L-asparaginase. Chemical and physical parameters such as incubation period, temperature, pH, carbon, and nitrogen sources, have an essential role in improving L-asparaginase secretion.

Effect of incubation period

In the present study, the effect of incubation period was estimated to obtain the maximum production of L-asparaginase by *Emericella sp*. The activity of L-asparaginase was determined from 1st to 8th day to estimate the optimum incubation period for the maximum production of L-asparaginase. It was observed that the maximum production of L- asparaginase was estimated on the ^{6th} day of incubation period with an activity of 0.65 U/ml **(**Figure 2). Further increase in the incubation periods after the 6th day showed a gradual decrease in L- asparaginase production. The result obtained may be due to depletion of nutrients in the media or the production of metabolites which may result in a change in pH or growth inhibition [(18)]. Some reports indicated that only two days of incubation period were required to produce of L-asparaginase enzyme by *Fusarium* sp. [(19, 20)]. Other studies reflect on the shorter incubation period required for L- asparaginase production [(21, 22, 23)]. On the other hand, similar reports showed that the maximum incubation period to produce of L-asparaginase was on the 6th day of the incubation [(24)] which supported our results.

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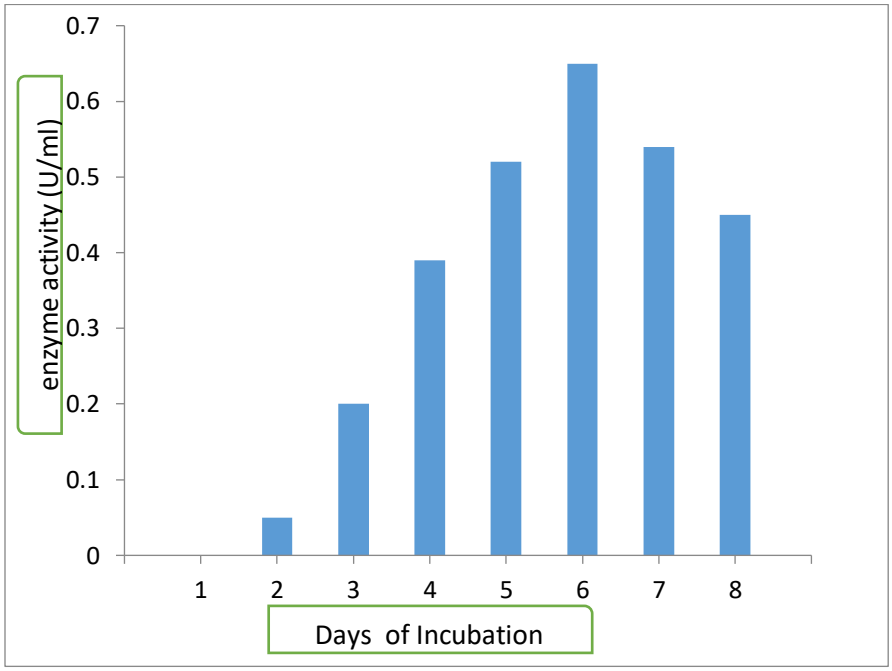

Figure 2: Effect of incubation period on L-asparaginase production

Effect of pH

Enzyme activity is significantly affected by pH change which can be inhibited or enhanced. thus, can affect the growth of microorganisms [25]. It was observed that the maximum enzyme activity was obtained at pH 7.0 with an activity of 0.671 U/ml. A gradual decrease in the enzyme activity observed lower and higher pH 7 may be due to the inhibitory effect on the growth and enzyme production by acidic and alkaline pH **(**Figure 3). Also, any variation in pH will result in a change in the properties and form of the enzyme or the substrate. thus, will prevent binding of the enzyme to the substrate [26]. Some reports, which closes to our results, indicates a maximum enzyme activity lower than pH 7[27 ,28 , 29].

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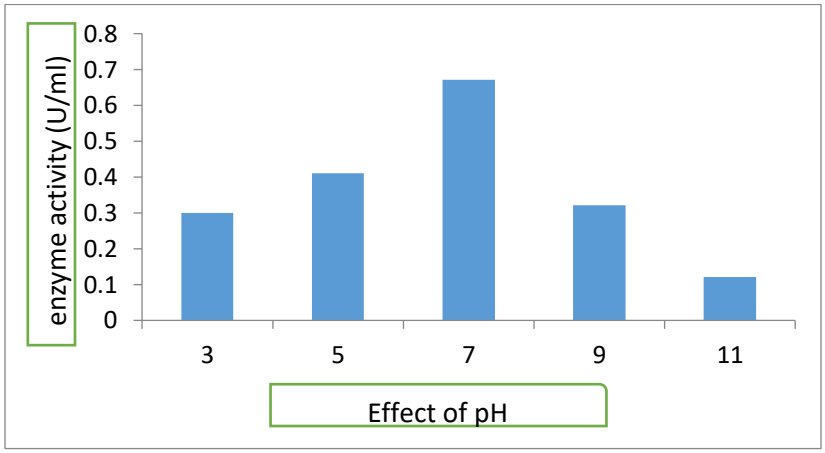

Fig 4: Effect of pH on L-asparaginase production

Effect of Temperature:

Temperature is the most important environmental factor for L-asparaginase production; because it regulates the growth of the microbes and the enzyme secretion. Also, Temperature affects the activity rate of the enzyme; because it affects the rate of its chemical reaction. The maximum enzyme production was observed at 30^0C with an activity of 0.415 U/ml **(**Figure 5). The enzyme activity started to decrease at the higher temperature of 30^0C due to the denaturation of partial enzyme leading to a change in the metabolic activities of the microbe. Similar reports support the same results on other marine fungi [30, 31, 32]. On the other hand, some reports indicate that the optimum temperature for L-asparaginase was found of 37 and 45 ^{0}C [20, 22].

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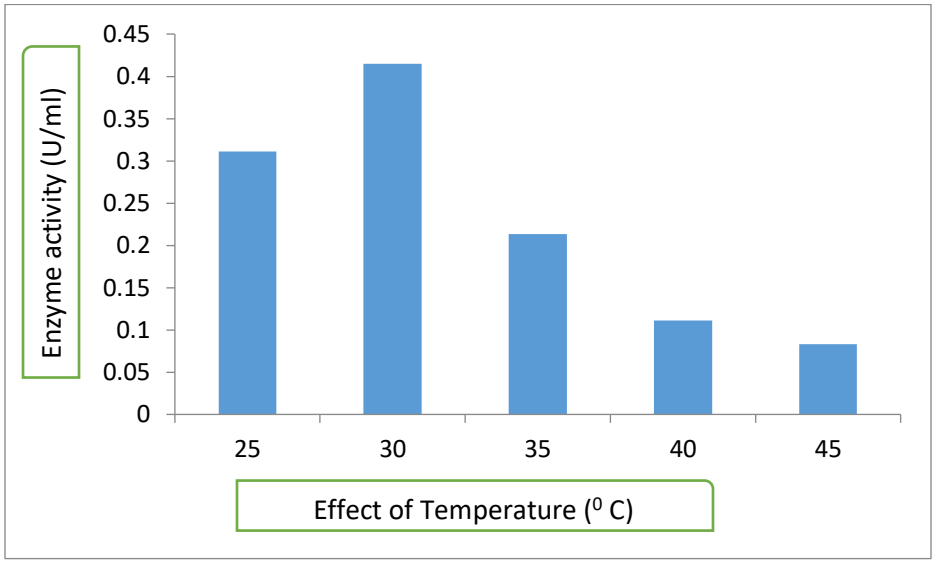

Fig.5: Effect of Temperature on L-asparaginase production

Effect of Carbon sources

Carbon is the main component of microbial cells due to its structure and function. Most of the fungi prefer simple sugars as a source of carbon other than carbon compounds [(27)]. In this study, five different sugars were studied for their ability to L- asparaginase production. The present study reveals that the maximum production of L-asparaginase when Glucose (0.2%) is used as the highest carbon source compared to other sugars with enzyme activity of 0.163U/ml (Figure 6) [(33)]. Similar studies have reported glucose as a good carbon source to induce L- asparaginase production by marine fungi [(34)]. A study on L- asparaginase reveals glucose at (0.4%) concentration as the best carbon source [(31, 35)].

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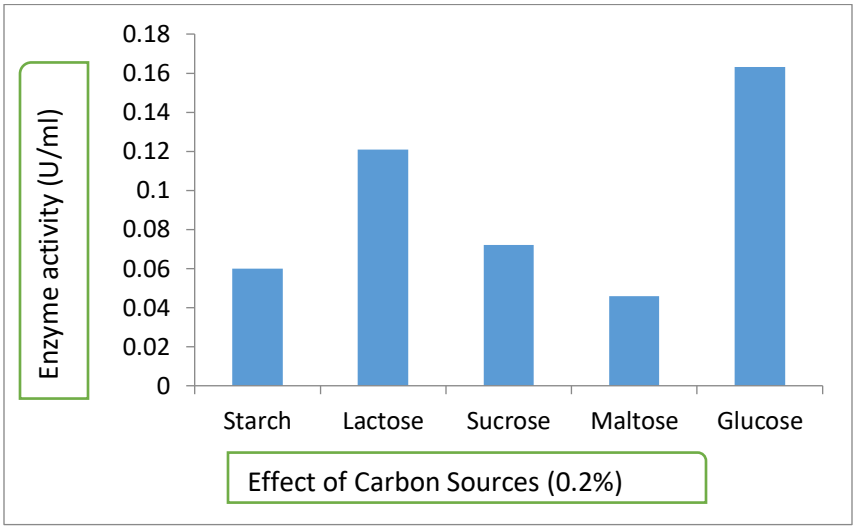

Figure.6: Effect of different Carbon sources on L-asparaginase production

Effect of Nitrogen sources

In this study, five different nitrogen sources were studied for their ability to L-asparaginase production. Each one of these nitrogen sources was added to the MCD medium individually at concentrations ranging from 0.1% to 0.5% for L- asparaginase production then supplemented into the reaction medium. The results indicated that ammonium nitrate at concentrations of 0.4% and 0.5% indicates a maximal L-asparaginase production (Figure 7). On the other hand, sodium nitrate, yeast extract, peptone and potassium nitrate showed the lowest production of L-asparaginase. The Similar report indicated that sodium nitrate showed the lowest production of L-asparaginase [(36)].

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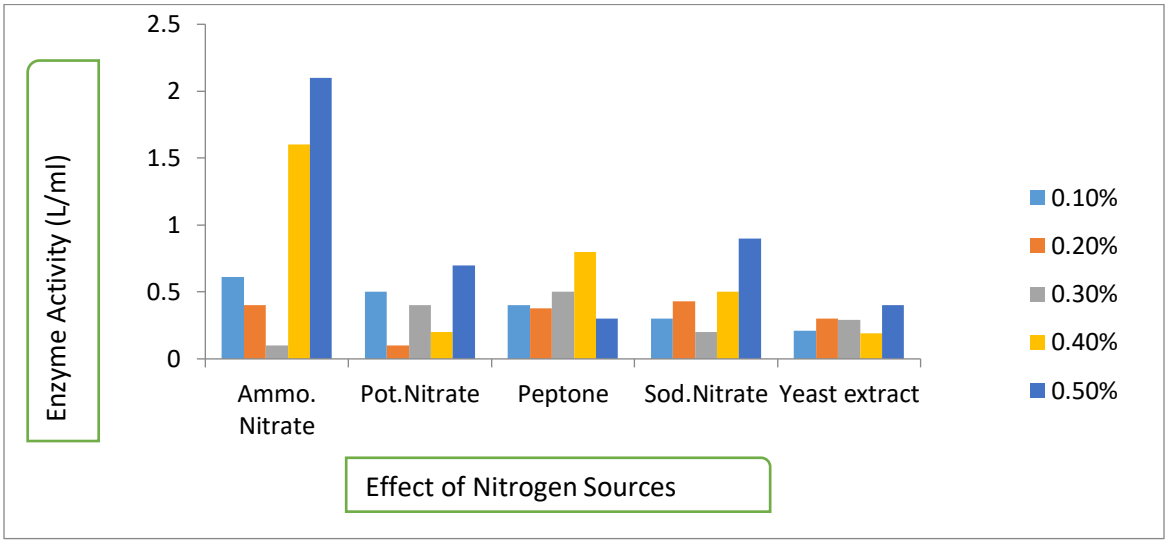

Figure .7: Effect of different nitrogen sources on L-asparaginase production

Substrate specificity of enzyme

The results indicated that the enzyme was 100%, 3% and 2% active on L-asparagine, D-asparagine, and L-glutamine respectively (Table. 2). This characteristic of the enzyme is very necessary to treat patients requiring incomplete removal of asparagine. Similar studies indicated the same results [37, 38].

Table.2: Substrate specificity of enzyme

Substrate	Concentration(mM)	Relative activity (%)
L-Asparagine	10	100
D-Asparagine	10	3
L-Glutamine	10	2

CONCLUSION

The present study indicated that the L-asparaginase production from marine fungi. Among 23 isolates, the marine fungus FM30 (*Emericella sp.*) exhibited a maximum L-asparaginase production. It was observed that maximum enzyme activity was recorded on the 6th day of incubation period at pH 7.0 with an incubation temperature of 30°C at 120 rpm. the glucose (0.2%) was the best carbon source. Also, the ammonium nitrate (0.4%, 0.5%) showed a maximum production of L- asparaginase. L-asparagine was used as a specific substrate to the L-asparaginase with 100% relative activity.

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REFERENCES

1. Pang, K.-L., Mitchell Julian, I., 2005. Molecular approaches for assessing fungal diversity in marine substrata. Bot. Mar. 48, 332.

2. Anderson, I.C., Cairney, J.W.G., 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ. Microbiol. 6 (8), 769–779.

3. Antunes, A., Ngugi, D.K., Stingl, U., 2011. Microbiology of the Red Sea (and other) deep-sea anoxic brine lakes. Environ. Microbiol. Rep. 3 (4), 416–433.

4. Masuma, R., Yamaguchi, Y., Noumi, M., Omura, S., Namikoshi, M., 2001. Effect of sea water concentration on hyphal growth and antimicrobial metabolite production in marine fungi. Mycoscience 42 (5), 455–459.

5. Hyde, K.D., Jones, E.B.G., Lean˜ o, E., Pointing, S.B., Poonyth, A.D., Vrijmoed, L.L.P., 1998. Role of fungi in marine ecosystems. Biodivers. Conserv. 7 (9), 1147–1161.

6. Jones, E.B.G., Pang, K.L., 2012. Marine fungi: and fungal-like organisms. De Gruyter.

7. Wade H.E., Robinson H.K., and Philips B.W., Asparaginase and Glutaminase a ctivities of

bacteria. J. Gen. Microbiol, 1971, 69, 299-312

8. Pinheiro I.O., Araujo J.M., Ximenes E.C.P.A., Pinto J.C.S., Alves T. L. M, of Lasparaginase

by *Zymomonas mobilis* strain CP4.Biometerial and Diagnostic BD06, 2001, 243-244

9. De-Angeli L.C. Pucchiari F. Russi S. Tonolo A. Zurita V.E.Cialanti E.and Perin A., Nature, 1970, 225, 550.

10. Arima K. Igarasi S. Nakahama K. and Isonom M., Production of extracellular L- from microorganisms.Agricul. Biol.Chem,1972,36, 356-361.

11. Imada A. Igarasi S., Nakahama K, and Isono M., Lasparaginase and glutaminase activities of

Microorganisms, Journal of General Microbiology, 1973, 76, 85-99.

12. H. L. Barnett, B. Barry Hunter, Illustrated genera of imperfect fungi, fourth edition. APS Press: St. Paul, Minnesota. ISBN: 0-89054-192-2, 1998.

13. Cui JL, Guo SX, Xiao PG, Journal of Zhejiang University Science B., 2011, 12: 385-392.

14. Dhevagi P, Poorani E, *Ind J Biotechnol*., 2006, 5: 514-520.

15. Imada A. Igarasi S., Nakahama K, and Isono M., Lasparaginase and glutaminase activities of

Microorganisms, Journal of General Microbiology, 1973, 76, 85-99.

16. Mannan. S., Sinha A., Sadhukhan R., Chakrabarty S L.,Purification, characterization and antitumor activity of Lasparaginase isolated from Pseudomonas stutzeri MB-405. Curr Microbiol., 1995, 30, 291-298.

17. Sutthinan Khamna, Akira Yokota, and Saisamorn Lumyong, L-Asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soil, 2009, of Integrative Biology, Vol. 6, No.1, 22-26.

18. Hosamani R, Kaliwal BB, *Int J Microbiol Res.*, 2011, 3:108-119.

19. Thirunavukkarasu N, Suryanarayanan TS, Murali TS, Ravishankar IP, Gummadi SN, *Mycosphere*, 2011, 2(2):147-155.

20. Chandrasekhar AP, *Int J Res Chem Environ*, 2012, 2:38-43.

21. Siddalingeshwara KG, Lingappa K, *Int J Pharm Tech Res*., 2011, 3:314-319.

22. Gulati R, Saxena RK, Gupta R, *Lett Appl Microbiol.*, 1997, 24:23-26.

23. Mushtaq MS, Siddalingeshwara KG, Karthic J, Sunil DPLNSN, Naveen M, Prathiba KS, *Int J Res Pharmacol Pharm.*, 2012, 1:147-150.

24. Abbas Ahmed MM, Nageh Abo Dahab F, Taher Taha M, Fareed Hassan SM, *J Microb Biochem Technol*, 2015, 7:165-172.

25. Jalgaonwala RE, Mahajan RT, *Euro J Exp Biol*, 2014, 4(3):36-43.

26. Khalaf ZA, Al-Ani NK, Jasim HM, Iran J Plant Physiol., 2012, 2:517-521.

27. Niharika Yadav, Supriya Sarkar, Intl J Pharmaceutical Science Invention, 2014, 3(6):32-40.

28. Thirunavukkarasu N, Suryanarayanan TS, Murali TS, Ravishankar IP, Gummadi SN, *Mycosphere*, 2011, 2(2):147-155.

29. Mohsin SM, Sunil Dutt PLNSN, Siddalingeshwara KG, Karthik J, Jayaramu M, Naveen M, Vishwanatha T, Prathiba KS, J Acad Ind Res., 2012, 1(4):180-182.

30. Kotra SR, Prudvi N, SadaSai KRA, Mannava KK, Peravali JB, Kumar A, Sambasiva Rao KRS, Pulicherla KK, Mintage J Pharmaceutical Med Sci. 2013, 2(1):45-50.

31. Monica T, Lynette L, Niyonzima FN, Sunil SM, Biocatal Biotransform., 2013, 2(2):1-9.

32. Balasubramanian K, Ambikapathy V, Panneerselvam A. Intl J Adv Pharmaceu Res., 2012, 3(2): 778 –783.

33. Soniyamby AR, Lalitha S, Praveesh BV, Priyadarshini V, *Int J Microbiol*., 2011, 2:38–42.

34. Radhika Tippani, Girisham Sivadevuni, *Afr J Biotechnol*., 2012, 11(15):3692-3696.

35. Muhammad AZ, Rabia B, Ishtiaq A, Tehreema I, *J Tecknologia.*, 2013, 62(2):47-51.

36. Makky EA, Jian OJ, Rezaul MK, Lee CM, *Afr J Biotechnol*., 2013, 12(19):2654-2658.

37. Campbel, H.A. Mashburn, L.T., Boyse., E.A. and Old, L.J, Biochemistry, 1967, 6, 721.

38. Mannan. S., Sinha A., Sadhukhan R., Chakrabarty S L.,Purification, characterization and antitumor activity of Lasparaginase isolated from 1995, 30, 291-298