European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 08, Issue 03 , 2021

CHARACTERIZATION AND OPTIMIZATION OF FERMENTATION CONDITIONS FOR INCREASED PRODUCTION OF L-ASPARAGINASE FROM MARINE FUNGI

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Abstract:

Present study details the isolation and screening of fungi from marine sediments for the selection of a high potential L-Asparaginase producing strain. The study includes characterization and identification by combination approach and optimization of the process parameters for maximization of L-Asparaginase production by the potential strain. Four fungal strains from twenty three isolates were isolated from different places of marine soils of adavuladeevi which show positive for L-Asparaginase production by producing characteristic pink coloration around the colony. Among isolated fungi tested by plate assay and antibacterial studies, the isolate αAW1- 9 from the marine sediment exhibited the highest zone of diameter (2.5cm) and maximum antibacterial activity against Vibrio cholera (22mm) were considered as the potent strain and were used for further studies. Based on it's morphological and microscopy characteristics as well as 18S rRNA sequence analysis, the isolate designated as αAW1- 9 were identified as novel Fusarium sporotrichioides strain MT232628. Findings made work hold immense importance for maximum production of L-Asparaginase enzyme after optimization of physico chemical parameters such as optimum incubation period for maximum biomass and L-Asparaginase production of αAW1- 9 were 120h and 48h (1.5g/100ml and 9.87/IU), temperature at 48°C (2.23g/100ml and 39.33/IU), pH 7.0 (2.14mg/100ml and 47.1/IU), lactose (1.92g/100ml and 52.35/IU), yeast extract (2.61mg/100ml and 97.75/IU) were considered to be the ideal conditions. Hence this study opens a new avenue for the researchers and pharmaceutics to pay a wider attention to the enzyme production from marine fungi.

Keywords: L-Asparaginase, Fusarium, optimization, antibacterial activity, cancer, enzyme

Introduction:

Cancer is a disease that cause unprecedented mortality. The occurring rate is usually mentioned as age standardized incidence rate (ASR) per 100,000 persons. As per

2018 statistic, the ASR was reported to be 218.6/182.6 (male/female) respectively [1]. Cancer could also be defined as unnecessary tissue growth that occur to an imbalance between cellular division and programmed cell death; caused by various genetic and epigenetic alterations. The precise explanation for cancer is elusive, which can be possibly attributed to viral genetics, chemical, radiations, environmental or immunological factors. The disease remains challenging despite of mammoth research efforts across the planet [2]. L-Asparaginase is very suitable for treatment of blood cancer as cancer cells are distributed throughout the body alongside the blood. L-Asparaginase is understood to act by hydrolyzing the Asparagine and causing deficiency of the amino alkanoic acid for cancer cells, whereby it limits the expansion of cancerous cell. L-Asparaginase may be an anticancer agent used with other chemotherapeutic agents L-Asparagine is a prime amino alkanoic acid for the expansion of tumor cells whereas the expansion of normal cell is independent of its requirement [3 & 4]. This amino alkanoic acid is often produced within the cell by an enzyme called Asparagine synthetase. Most of the native cells synthesize L-Asparagine in sufficient amounts for its metabolic needs but the tumor cells (especially Malignant and Carcinoma Cell) require external source of L-Asparagine for its growth and multiplication [5]. In the absence of L- Asparagine, the tumor cells are deprived of an important growth factor and they may fail to survive. Thus this enzyme is often used as a chemotherapeutic agent. The chemical reactions are catalyzed by enzymes which increase the speed of reactions. In enzymatic reactions, the molecules called substrates are converted into products. During a cell most biological processes need enzymes to catalyze reactions at eloquent rates. L-Asparaginase catalyses the conversion of L-Asparagine into L Aspartic acid and ammonium [6-10].Microbial systems have attracted significant attention for producing potential L-Asparaginase and it's supporting nature. a good range of microbes like bacteria, yeast and fungi showed potential source of L-Asparaginase. The bacterial L-Asparaginase (E.coli and Erwinia species) has been considered as an effective drug for the treatment of leukemia [11 & 12]. L-Asparaginase isolated from bacteria can cause allergies and side effects like diabetes, leucopenia and coagglutation abnormalities within the future use [13].This advances to discover a novel L-Asparaginase that are serologically different, but with similar therapeutic effects from eukaryotic microorganisms like yeast, fungi and the enzyme may have fewer side effects [14-17]. The target of this study is to isolate potential fungi from adavuladeevi, nizampatanam Mandal, marine sediments. Screening, characterization, optimization of enzyme production and biological applications at a cost effective mode to cater the social needs.

Materials and Methods:

Sample Collection:

Soil samples were collected from marine sediments at adavuladeevi shore area, Nizampatnam Mandal, Guntur district, Andhra Pradesh with coordinates 15.9153^{o}N 807720^{o}E, India, in a sterile stainless steel container. Then the samples were transported to the laboratory. Potato Dextrose Agar (PDA) was used for recovering the fungal isolates from soil samples [18].

Isolation of marine fungi:

Soil samples were partially dried and finely sieved to eliminate stones. About 1 g of fine soil sample was taken in to a 10ml of buffer solution (stock solution), from stock, serial dilutions were prepared from 10^{-2} to 10^{-5}. One ml of serially diluted water sample was plated on to the solidified potato dextrose agar medium. The plates were incubated at 25°C for 96-120 hours. Individual colonies were re grown on PDA at 25°C for obtaining pure culture. The pure cultures were maintained at 4-5°C and were sub cultured once in a month.

Screening of L-Asparaginase producing fungi:

Fungal isolates were screened for L-Asparaginase production by using modified protocol as described previously [19]. For this assay, A potato dextrose medium was used for plate assay. A 2.5% stock solution of phenol red was prepared in ethanol (pH 6.2) and 3 mL of this was added to 1000 ml of potato dextrose medium. A loopful of mycelia from the growing margin of the colony of an mother culture was placed in a petri dish containing 20 ml of PDB medium. After 96 h of incubation at 25±1°C, the appearance of a pink zone around the fungal colony indicates the production of L-Asparaginase enzyme.

Biomass yield (BMY):

Quantitative analysis of BMY was carried out in PD broth at 25^0C on rotator shaker at a speed of 250rpm. BMY was estimated by using dry cell weight method from broth culture. After 5 days of incubation, fungal mat was separated from broth by using pre weighed Whatmann No.1 filter paper. Fungal biomass was dried at 80^0C in hot air oven and was measured by using the formula [20].

$\mathbf{F_{bm} = W_F - W_P}$

(F_{bm} = Biomass of fungal mycelium (gm/100ml); $\mathbf{W_F}$ = Weight of filter paper; $\mathbf{W_P}$= Dry weight of filter paper with fungal mycelium).

L-Asparaginase (LAP):

LAP activity was estimated quantitatively by Nesslerization of ammonia method. The amount of ammonia liberated from asparagine was used to estimate the activity of L-Asparaginase. 5 days old incubation broth was filtered through Whatmann No.1 filter paper and centrifuged at 12,000 rpm for 15 min at 4^0C and supernatant was used as crude enzyme. About 0.5ml of crude enzyme was added to 1.5ml of reaction mixture **(R_m)** [0.5 ml of 0.5 M Tris-HCl buffer (pH 8.6), 0.5ml of 0.04M asparagine and 0.5ml of distilled water] and incubated at room temperature. After 30min of incubation, 0.5ml of 15% TCA was added to reaction mixture and centrifuged at 10,000 rpm for 10 min at 4^0C. Reaction mixture without crude enzyme was used as control. About 0.1ml of supernatant **(S)** was added to 4.9ml of Nesslerization mixture (3.7ml of distilled water, 1ml of 2N NaOH and 0.2ml of Nessler's reagent) and incubated for 20 min at room temperature. Development of orange color is positive test for ammonia production. O.D was measured at 450nm. Ammonia (µ mole) liberated was calculated. Enzyme activity was calculated by the formula [21].

$$\text{Enzymes activity} = \frac{NH_3 \text{ liberated X } R_m \quad \text{(IU /ml)}}{S \text{ X Incubation period}}$$

Screening for antibacterial activity:

The selected fungal isolates on the basis of screening were subjected to bioactive metabolite production in potato dextrose medium. A loopful of mycelia of 5 day old culture were inoculated in 100 ml pre-sterilized PDA broth in 250 ml of Erlenmeyer flask under aseptic conditions and were incubated at 25°C for 3-5 days. After the incubation, fungal mycelium was separated from broth through filtration using Whatman filter paper No.1 followed by centrifugation at 12,000 rpm for 15 minutes to get cell free supernatant. Supernatant of 50µl was loaded in to seeded agar well and then subjected to screening for antibacterial activity against the human pathogenic bacteria Vibrio cholerae, Enterococcus feacalis, S. pyogenes (ATCC 12344), S. aureus (ATCC 25923), S. typhimurium (ATCC14028), P. aeruginosa (ATCC 27853). Zone of inhibition was measured in mm and the test was done in triplicates [22].

Identification of fungal isolates:

The Isolate was inoculated and incubated at 25°C for 5 days. Colonies were compared for their overall color and color of conidia, reverse color, texture, zonation and sporulation. Further the isolate was also subjected to microscopic analysis for it characterization and identification. Genotypic identification was carried out by PCR amplification and partial sequencing of the rDNA for the confirmation of morphological identity. ITS (Intrinsic sequence) regions were amplified by PCR with primers forward (ITS1-5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4-5'-TCC TCC GCT TAT TGA TAT GC-3') primers. Comparative study of other rDNA sequences with rDNA sequence of isolates was done using BLAST algorithm at the website http://www.ncbi.nlm.nih.gov. The nucleotide sequence of isolated fungi has been assembled and submitted at the NCBI GenBank [23].

Optimization of fermentation parameters:

L-Asparaginase production was studied using potato dextrose medium for optimum incubation period (24, 48, 72, 96 and 120 h), temperature (10, 22, 28, 37 and 48°C), pH (4.0 to 8.0), carbon sources such as sucrose, lactose, maltose, mannitol, sorbitol, trehalose, galactose, d-ribose, xylose, rhamnose, fructose, dulcitol and dextrose and nitrogen sources such as yeast extract, sodium nitrate, ammonium sulphate, urea, potassium nitrite, tryptone, ammonium carbonate beef extract, peptone, cretinitne and ammonium borate. Enzyme assay was carried out as previously described and the optimum condition achieved was taken for further experiments and biomass was measured by gm/100ml [24]. Mean values are from analysis of triplicates with ± Standard deviation (SD).

Results:

Preliminary screening for L-Asparaginase production by plate assay method:

Twenty three fungal isolates were isolated from adavuladeevi marine soil **FIG. 1** and named as αaw1-9 to 23, and were examined for L-Asparaginase production through agar plate assay, out of 23 isolates, four isolates αaw1-9, 11, 17 and 21 showed pink zone around the colonies on potato agar containing phenol red, indicating the increase in pH which originated from ammonia accumulation in the medium. The dye indicator is yellow at acidic condition and turns to pink at alkaline condition. Out of four positive fungal isolates, αaw1 9 show the highest zone diameter (2.5 cm) and hence selected for further studies **Table. 1**.

FIG.1: Sample collection site of adavuladeevi shore area.

Table. 1: Colony and pink zone diameters after 5 days incubation

S. No.	Isolate	Zone Diameter	S. No.	Isolate	Zone around Colony
1.	αAW1-1	Absent	11.	αAW1-11	0.9±001
2.	αAW1-2	Absent	12.	αAW1-12	Absent
3.	αAW1-3	Absent	13.	αAW1-13	Absent
4.	αAW1-4	Absent	14.	αAW1-14	Absent
5.	αAW1-5	Absent	15.	αAW1-15	Absent
6.	αAW1-6	Absent	16.	αAW1-16	Absent
7.	αAW1-7	Absent	17.	αAW1-17	1.1±021
8.	αAW1-8	Absent	18.	αAW1-18	Absent
9.	αAW1-**9**	**2.5±010**	19.	αAW1-19	Absent
10.	αAW1-10	Absent	20.	αAW1-20	Absent
			21.	αAW1-21	0.4±011
			22.	αAW1-22	Absent
			23	αAW1-23	Absent

Screening of antibacterial activity:

L-Asparaginase positive strain αAW1-9 showed variation in their antibacterial activities. αAW1 9 showed highest inhibition against Vibrio cholerae 22mm, followed by Salmonella typhimurium 19mm, Streptococcus pyogenes 17mm, Pseudomonas aureginosa

15mm, Enterococcus feacalis 14mm and least inhibitory action shown on Staphylococcus aureus 10mm **FIG. 2**.

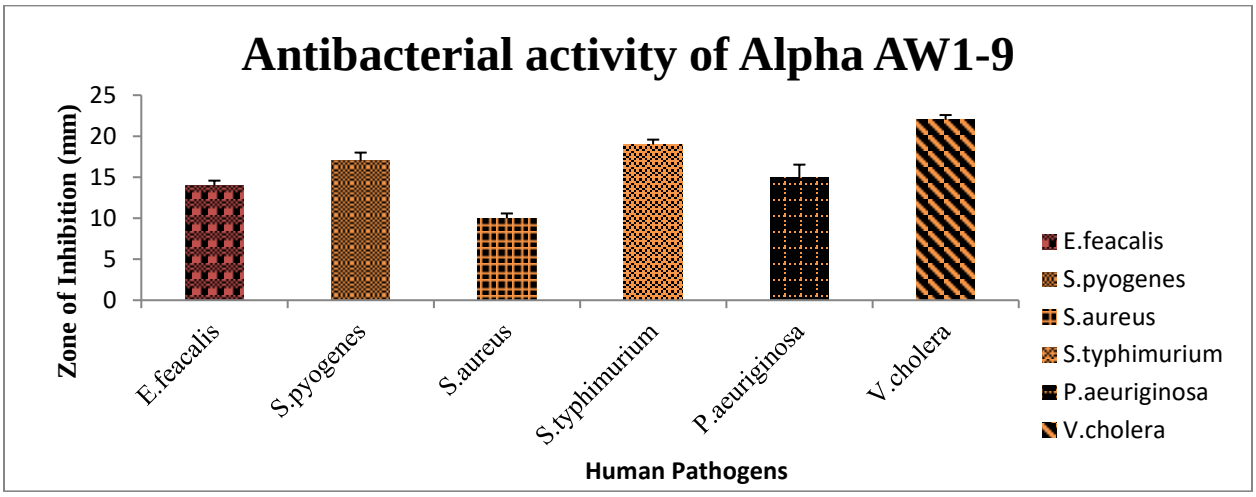

FIG. 2: Antibacterial activity of αAW1-9 against human pathogens

Identification of the fungal isolates:

Based on morphological and molecular analysis the isolate designated as αAW1-9 was identified as Fusarium sp. The sequence data showed that the isolate αAW1-9 has highest sequence similarity 99% with the genus Fusarium sporotrichoides. Hence, it is concluded that the isolated strains are Fusarium sporotrichoides strain MT232628 **FIG. 3**.

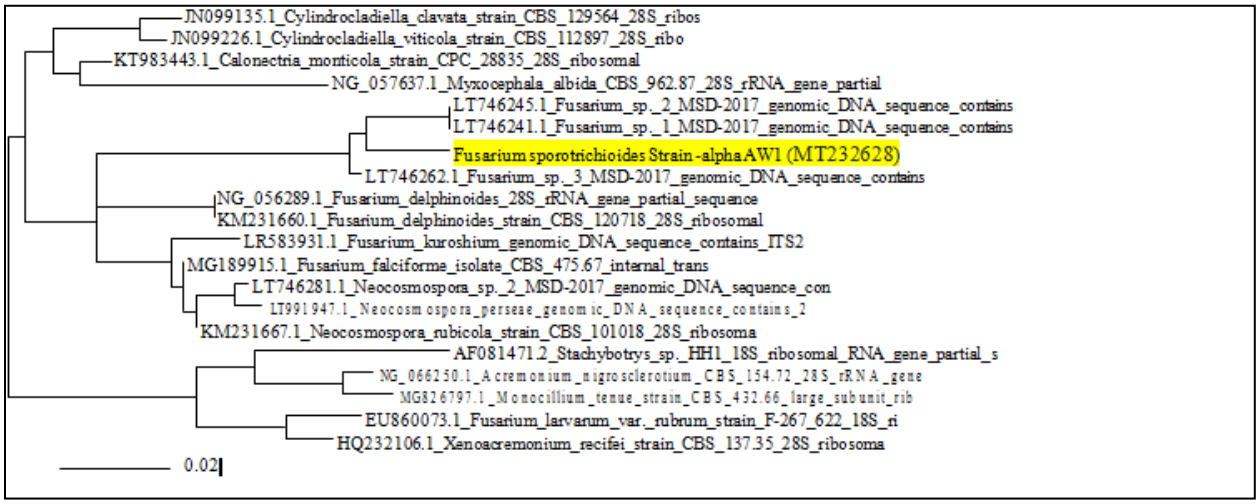

FIG. 3: Phylogenetic tree of fungal isolate αAW1-9 relationship among the selected strains based on sequencing analysis and the most closely related fungus species.

Optimization of culture conditions for the production of L Asparaginase
Effect of incubation period on the production of biomass and L- Asparaginase:

The production of biomass was reported maximum at 120h incubation (1.5g±0.15/100ml) followed by 96h (1.26±0.03g/100ml), 72h (1.13±0.01g/100ml). Whereas no growth was

observed at 24 and 48h incubation period. The production of L-Asparaginase was studied along 5 days of incubation when cultivated on potato dextrose medium with 1% L-Asparagine. Incubation period during the process fermentation is very much essential to study the optimum incubation time for maximum L-Asparaginase production of 9.87±0.14 IU/ml at 72h and further increase in the incubation period decreased the enzyme activity. Mean values are from analysis of triplicates with ± Standard deviation (SD) **FIG.4.**

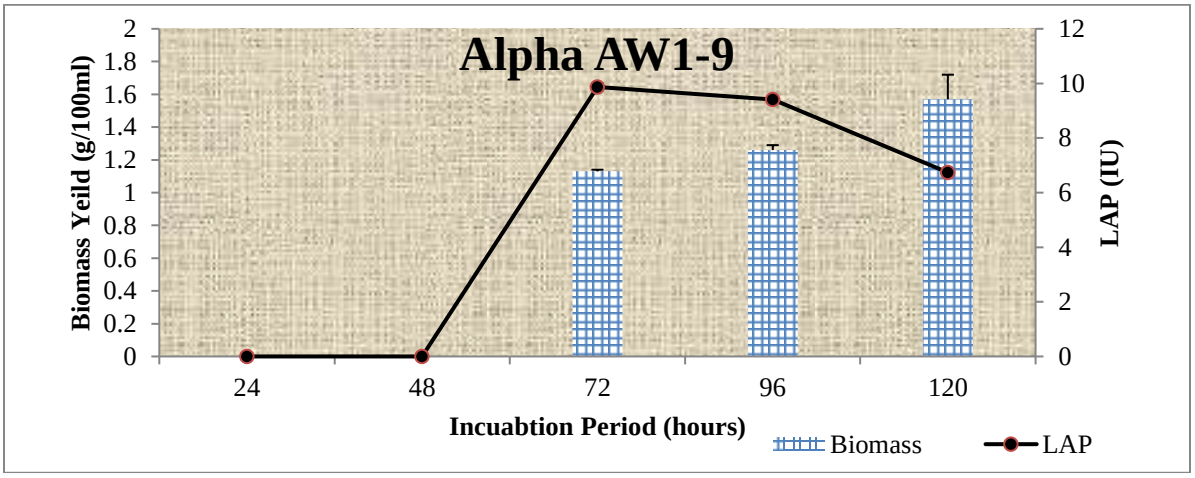

FIG. 4: Effect of incubation period on production of biomass and L-Asparaginase

Effect of temperature on the production of L-Asparaginase:

The production of biomass was reported maximum at 48^{o}C (2.23±0.1g/100ml) followed by 28^{o}C (2.12±0.01g/100ml), 37^{o}C (1.63±0.15g/100ml), 22^{o}C (0.83±0.12g/ml) were as no growth was observed at 10^{0}C. The production of L-Asparaginase was studied along 5 days of incubation when cultivated on potato dextrose medium with 1% L-Asparagine. Optimum temperature for maximum L-Asparaginase production of 39.33±0.1 IU/ml at 48^{o}C and further decrease in the temperature decreased the enzyme activity. Mean values are from analysis of triplicates with ± Standard deviation (SD) **FIG.5.**

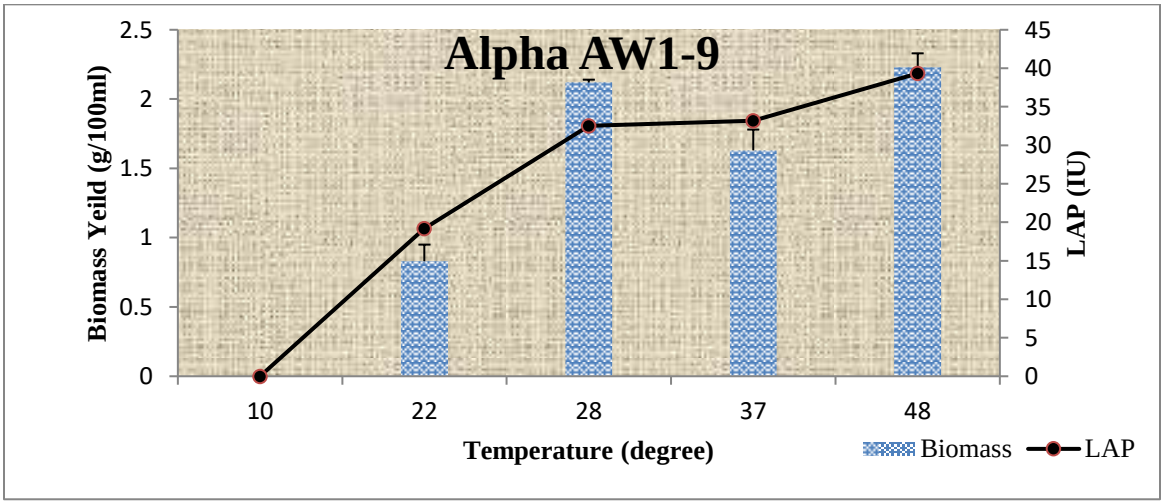

FIG. 5: Effect of temperature on biomass and L-Asparaginase production

Effect of pH on the production of L-Asparaginase:

The production of biomass was reported increased with increase of pH and reaches maximum at Ph 8 (2.31±0.05g/100ml) and decreased with increasing pH. The production of L-Asparaginase increases with increasing pH and reached maximum at pH 7 (47.10±0.1/IU) and further decreased with increasing pH. Mean values are from analysis of triplicates with ± Standard deviation (SD) **FIG.6.**

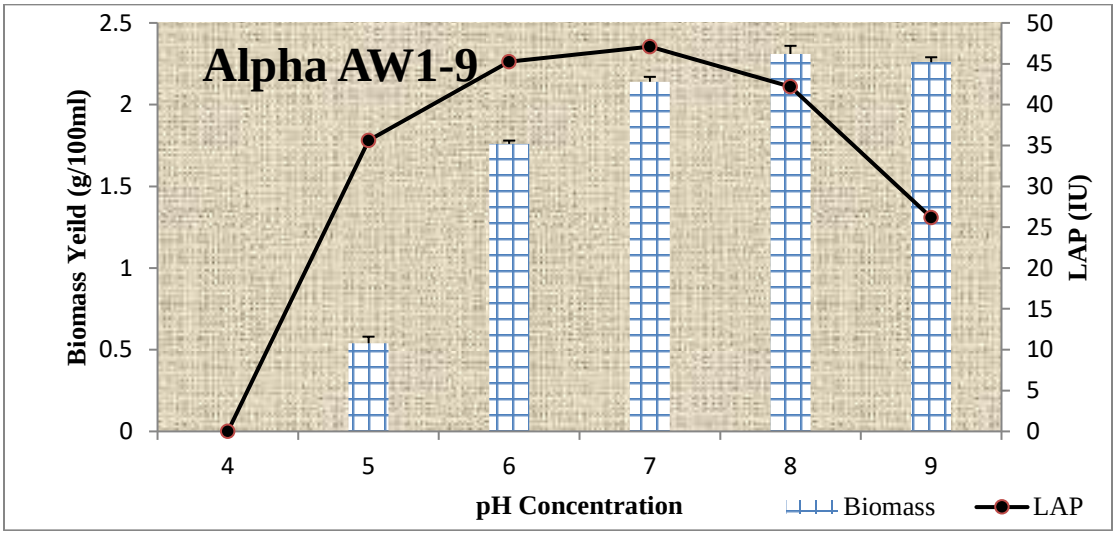

FIG. 6: Effect of pH on biomass and L-Asparaginase production

Effect of carbon source on the production of L-Asparaginase:

The production of biomass and L-Asparaginase was reported maximum in presence of lactose (1.93± 0.03g/100ml and 52.35±0.04/IU) when compared to other carbon sources such as sucrose, maltose, mannitol, sorbitol, D-ribose, trehalose, galactose, xylose, rhamnose, fructose, dulcitol, and dextrose. Mean values are from analysis of triplicates with ± Standard deviation (SD) **FIG.7.**

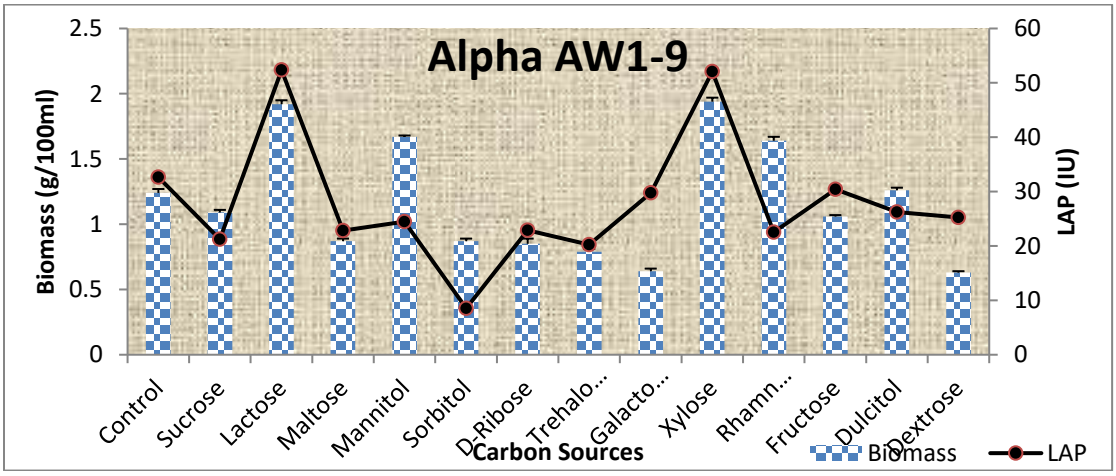

FIG 7: Effect of carbon source on the production of biomass and L-Asparaginase

Effect of nitrogen source on the production of L-Asparaginase:

The nitrogen source is the limiting factor and plays key role in the biomass and L-Asparaginase production. Most of the microorganisms utilize nitrogen source either inorganic or organic form or sometimes both. The results illustrated that the maximum biomass and L-Asparaginase production was observed in presence of yeast extract (2.61±0.03g/100ml and 97.71±0.04/IU). Thus, amongst all nitrogen sources provided for biomass and L-Asparaginase production yeast extract appears to be the good nitrogen source. Mean values are from analysis of triplicates with ± Standard deviation (SD)
FIG. 8.

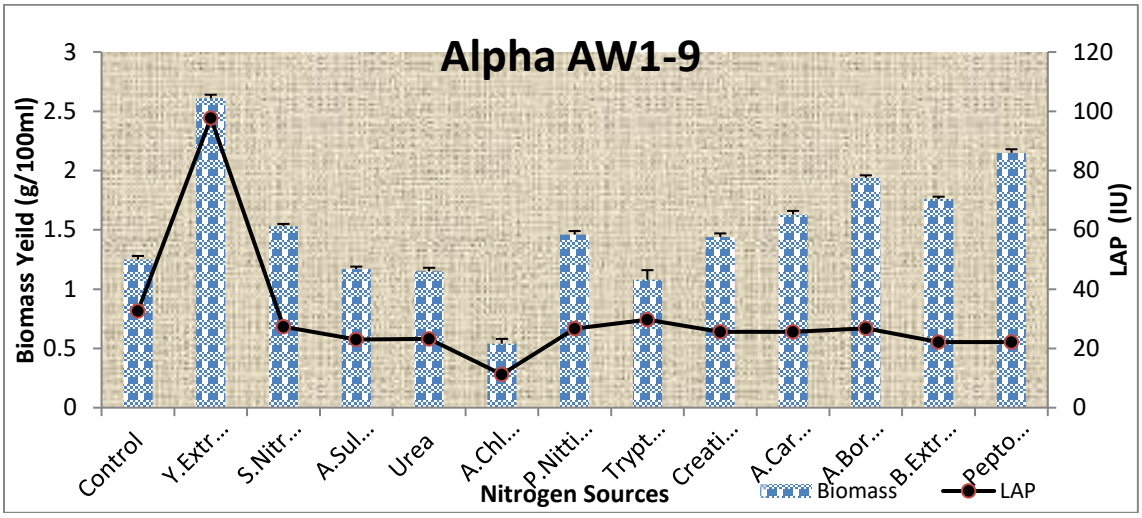

FIG. 8: Effect of nitrogen source on the production biomass and L-Asparaginase

Discussion:

In the present study, L-Asparaginase producing fungi were isolated from marine soils at various locations around adavuladeevi coastal area **FIG. 1** Guntur Andhra Pradesh. Among four isolated fungi tested by plate assay and antibacterial studies **FIG 2** the isolate αAW1-9 showed highest zone (diameter) for enzyme and antibacterial activity, were considered as the potential strain and were used for further studies **TABLE 1**. The positive L-Asparaginase producing fungus was identified morphologically and microscopically as Fusarium sp. The identity was further confirmed by Phylogenetic analysis based on 18S rRNA gene sequencing by National Collection of Industrial Organisms, Pune, India. Sequence data showed that the isolates αAW1-9 have highest sequence similarity (99%) with the genus Fusarium sporotrichioides **FIG 3**. Hence it is concluded that the strain αAW1-9 belongs to the genus Fusarium sporotrichioides with accession number MT232628. Various parameters influencing L-Asparaginase secretion were optimized. The L-Asparaginase producing fungi must be provided with optimum growth conditions in order to improve and increase the enzyme production without increasing the cost. A balance between various medium components is maintained, reducing the amount of unused nutrients after fermentation completion. Incubation period, temperature, initial pH, carbon source, and nitrogen sources were optimized. L-

Asparaginase production was greatly enhanced by the incubation temperature by fungi because its growth and enzyme secretion. In the present study, F. sporotrichioides production of L-Asparaginase started at 24 hours and reached maximum at 72 hours **FIG. 4** and it decreased significantly with increase in the incubation time. At longer incubation periods, the enzyme activity decreased which might be due to the depletion of nutrients, accumulation of toxic end products, and the change in pH of the medium, or loss of moisture. F. sporotrichoides was able to grow and produce the enzyme on all the temperatures evaluated with maximum production at 48°C, respectively although statistically at par with 37 °C. However a noticeable decrease in enzyme yield was seen at 48°C in **FIG. 5**. The partial enzyme denaturation resulted from a change in metabolic activities due to low enzyme activity value recorded at 45°C. The initial pH of the production medium is an important parameter affecting the enzyme production since it can indirectly act on the fungal growth by affecting the availability of medium nutrients. In order to find out there optimum pH for the L-asparaginse production, the initial pH of the fermentation medium was adjust to different levels and fermentation was carried out at 48°C. The maximum L-Asparaginase production was noted at an initial pH of 7.0; thereafter a decline in enzyme production was seen **FIG. 6**.

The influence of various carbon sources such as sucrose, lactose, maltose, mannitol, sorbitol, trehalose, galactose, d-ribose, xylose, rhamnose, fructose, dulcitol and dextrose were studied for L-Asparaginase production **FIG. 7**. Optimal activity of 52.35 IU for lactose and the least activity of 4.25 IU for sorbitol. It has been reported that the microbial synthesis of Asparaginase is under catabolic repression and requires less amount of carbon source. Therefore in the present context, the L-Asparaginase production was studied supplementing the nitrogen forms such as yeast extract, sodium nitrate, ammonium sulphate, ammonium sulphate, urea, potassium nitrite, tryptone, ammonium carbonate beef extract, peptone, cretinitne and ammonium borate gave the optimum activity **Fig. 8**. Hence, from the present findings it was clear that yeast extract were the best nitrogen source that can be used for L-Asparaginase production by F. sporotrichioides αAW1-9 MT232628. Our study clearly shows that marine soils can be a rich source of L-Asparaginase producing fungi when compared to other terrestrial soils and indicates Fusarium sp. (αAW1-9) isolated from the marine soil can be exploited as a potential source for large-scale production of L-Asparaginase enzyme to cope up the needs of industrial application and the demand of the global market.

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