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Screening of Streptomyces Albus CN-4 For Enzyme Production and Optimization of L-Asparaginase

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Abstract- A total of ten actinomycete strains were isolated from the soil sample. All the strains were sub cultured as pure on Yeast extract, Malt extract and Dextrose agar and screened for the biologically active enzymes, and optimization of L.aspariginase enzyme was carried out by various parameters including pH, temperature, and carbon, nitrogen sources by growing the strain CN-4 in modified ISP-5 broth for 96 h. Among the seven carbon sources tested, lactose proved to be the best carbon source by supporting high yields of L-asparaginase enzyme. Influence of six nitrogen sources on L-asparaginase production and growth of the strain was tested. Yeast extract was found to enhance both growth and production of L-asparaginase. Once the preferable carbon and nitrogen sources were determined as lactose and yeast extract, efforts were made to identify their suitable concentrations. Lactose @ 0.5% and yeast extract @ 0.5% with 1% L-asparagine as optimized nutrient broth adjusted to pH 7.0 inoculated with *Streptomyces albus* CN-4 incubated at 30°C for 96 h improved the production of L-asparaginase.

Index Terms- Bioactive enzymes, Yeast extract, Nutrient broth, Optimization

I. Introduction

Actinomycetes are also the producers of a wide array of commercially and biotechnologically important enzymes. Enzymes are macromolecular biological catalysts. They are responsible for thousands of metabolic processes that sustain [1, 2] life like amylases, cellulases, chitinases, L-asparaginases, pectinases, proteases etc. Microbial L-asparaginase has been widely used in the treatment of certain human cancers. The clinical accomplishment of this enzyme is attributed to the reduction of L-asparagine; tumor cells incapable of synthesizing this amino acid are selectively killed by L-asparagine scarcity. Though several L-asparaginases of bacterial origin have been developed and their prospective usage in clinical trials has been deliberated to prevent the advancement of L-asparagine dependent tumors, mainly lymphosarcomas, the success hitherto has been rather limited. Actinomycetes strains isolated from soils such as *Amycolatopsis* sp[3], *Streptomyces gulbargensis*[4] and *S. albidoflavus*[5] have capabilities of producing detectable amounts of L-asparaginase.

Chitin as the second most copious insoluble natural source constitutes the structural component of fungal cell walls, crustacean cells and insect exoskeletons, its recycling is of considerable economic and environmental significance. Current techniques to transform chitin to functional carbohydrate products necessitate harsh chemical treatments that incur problems of undesirable byproducts [6]. Chitinase producing microorganisms directly or indirectly chitinase enzyme in purified form could also be used as potential biocontrol agents [7, 8]. Some chitooligosaccharides such as GlcNAc6 and GlcNAc7 have been reported to possess antitumor activity [9, 10].

Amylase and proteases catalyze the release of simple sugars from starch and control the protein levels respectively. Amylase from actinomycetes and other microbial sources are used in the food processing industries for making syrups[11] , baking processes and Proteases used in the manufacturing of biscuits to reduce the protein level in the flour. Cellulases used in biologically fabric conditioners. Catalase used in the rubber industry for generate oxygen from peroxide to convert latex into foam rubber along with all these enzymes some other enzymes are also playing a lead role in different industries. So the present study was focused on the production of biologically useful enzymes by using actinomycetes strains because of their abundant presence in nature.

II. MATERIALS AND METHODS

Sample collection: Laterite soil sample collected from Kandukuru, Prakasam (Dist) a depth of 5 – 8 cm was pretreated with calcium carbonate (1:1 w/w) and dried at 45oC for 1 h in order to reduce the aboundence of bacteria and fungi[12].

Isolation of actinomycete strains:

The two different media used for isolation and maintaining pure cultures are Yeast extract malt extract dextrose agar (YMD) and starch casein salts agar media were prepared, sterilized at 15 lbs pressure (120°C) for 15 min and poured into Petri plates under aseptic conditions. Both streptomycin (50 µg/ml) and nystatin (50 µg/ml) were added to the media just before pouring into Petri plate. Soil dilution plate technique was employed for isolation of actinomycetes strains [13]. The pretreated soil (1 g) sample was suspended in 100 ml of sterile distilled water. Serial dilutions were prepared and 0.1ml of 10^{-3} and 10^{-4} dilutions were plated on media with the help of a spreader. The inoculated

plates were incubated at 30°C for 7-14 days. After incubation, actinomycete colonies were isolated from soil. Streak plate method was used to purify the cultures of actinomycete strains. The colonies were picked with the loop according to the condition. The picked up specks of the colonies were streaked over YMD agar medium followed by incubation at 30oC for 7 days. Further, pure cultures were maintained on YMD agar slants and stored at 4oC for further studies [13].

Enzymatic profile of the strain:

Amylase:

Starch agar medium was used to test the strain for the production of amylase. The culture was inoculated in the Petri plate containing solidified starch agar medium. The Petri dish was incubated at 28-30°C for 48 h. After incubation the plate was flooded with Gram's iodine solution (2:1 of potassium iodide and iodine in 300 ml of distilled water). A clear zone around the colony against the blue background was taken as positive (Holding and collee, 1971).

Cellulase:

To detect the cellulolytic activity of the strains, modified ISP-4 medium with cellulose (1%) in place of starch was employed. Inoculated plates were incubated at 30°C for 7 days. Hydrolysis of cellulose was seen as clear yellow zone within a reddish brown background after flooding the plates with a mixture of hydrochloric acid and iodine solution, prepared by mixing 1 ml of 0.1 N HCl with 5ml of 2% KI [14].

L-asparaginase:

To determine the ability of the strain to secerete Lasparaginase that catalyzes the hydrolysis of L-asparaginase to Laspartic acid and ammonium ion. Modified ISP-5 medium with Lasparagine (1%) and phenol red (0.03%) with initial pH 7.0 was prepared. Sterilized medium was poured into Petri plates and allowed to solidify. The plates were inoculated with the strain and incubated at 30°C for 48-72 h. Change of dye color around the actinomycete colony from yellow to pink indicates the positive reaction while no color change interprets the test as negative[15].

Catalase:

Sterilized trypticase soy agar slants inoculated with the strain were incubated at 30°C for 48-72 h. After incubation, 3-4 drops of hydrogen peroxide was allowed to flow over the culture. The tubes were observed for the appearance or absence of air bubbles (Holding and Collee, 1971).

Chitinase:

Colloidal chitin was prepared according to the method of [16]. Chitin powder (5 mg) was added slowly to 90 ml of concentrated HCl under vigorous stirring for 2 h, followed by the addition of 1L distilled water. Thus obtained fine white precipitate was collected at 4°C by centrifugation (6,000 rpm) for 10 min. It was then washed repeatedly with distilled water until the colloidal chitin became neutral (pH 7) followed by drying at 50°C and used for further studies. Colloidal chitin (0.5%) was added to the rest of the components of Chitin Yeast extract Salts (CYS) broth. The plates were inoculated with the strain and observed for a zone of clearance around the actinomycetes colony after incubation at 30°C for 7 days.

Deoxyribonuclease (DNase):

Production of the enzyme capable of degrading deoxyribonucleic acid (DNA) was tested by inoculating the strain on DNA agar plates. After 3-5 days of incubation, plates were flooded with 1N HCl and the appearance of clear zone around the actinomycete colony infers the degradation of DNA in the medium [17].

Nitrate reductase:

Sterilized trypticase nitrate broth inoculated with the strain was incubated at 30°C for 6 days. After incubation, 5 drops of reagent A (8 g of sulphanilic acid in 1000 ml of 5 N acetic acid) and 5 drops of solution B (10 g of α-naphthalamine in 1000 ml of 5 N acetic acid) were added to the culture tubes and observed for color change. Appearance of cherry red color indicates the ability of the strain to produce nitrate reductase (Holding and Collee, 1971).

Protease:

Proteolytic activity of the strain was determined in terms of caseinolysis. Starch casein agar medium were prepared, autoclaved and poured into sterilized Petri plates. After solidification, the strain was inoculated and incubated at 30°C. After 3 days of incubation, appearance of clear zone around the actinomycete colony indicates the test as positive (Holding and Collee, 1971).

Urease:

To detect the urease activity of the strain, Christensen agar medium was prepared, sterilized and poured into sterile Petri plates. The strain was inoculated at the center of the agar medium and the plates were incubated at 30°C for 48 h and observed for color change from yellow to pink around the colonies indicating positive reaction (Holding and Collee, 1971).

Arginine hydrolase:

To test the ability of the strain to produce Arginine hydrolase, agar tubes containing arginine agar medium were prepare (L-arginine 1%, K2HPO4 0.03%, NaCl 5%, MgSO4 .7H2O 0.01%, phenol red 0.001%, agar 2%), sterilized and inoculated with the respective strains by the stab culture technique. After incubation at 37ºc for fivedays, the tubes were observed for color change. Formation of a bright magenta color indicates the presence of arginine hydrolase (Holding and Colle,1971).

L-asparaginase production by *Streptomyces albus* CN-4:

To screen the strain for the production of L-asparaginase, culture was inoculated into modified ISP-5 agar containing glycerol (1%), L-asparagine (1%), K2HPO4 (0.1%), trace salt solution (FeSo4.7H2O - 0.01%, MnCl2.7H2O - 0.01%, ZnSO4.7H2O - 0.01%) - 0.1% with initial pH 7.0. Phenol red (0.1%) was added to the Petri dish before inoculation. The inoculated Petri plates were incubated at 35ºC for 4 days and observed for the formation of red color around the colony.

Asparaginase assay:

The enzyme assay was performed as described by[18]. with minor modifications. Biomass was seperated by centrifuging the culture broth at 10,000 rpm for 15 min and was mixed with Tris - HCl buffer. It was again centrifuged and the cell free extract (0.2 ml) obtained was mixed with 0.8ml of 0.05M L-asparagine. After incubating the reaction mixture for 15 min at 37ºC in a waterbath shaker, the reaction was terminated by the addition of 0.5 ml of trichloroacetic acid (15% w/v). Precipitated proteins were removed by centrifuagation and the liberated ammonia was determined by U.V. spectrophotometre at 450nm by Nesslerization. Tubes kept at zero time incubation served as control. Enzyme production was determined on the basis of liberation of ammonia calculated with reference to a standard curve of ammonium sulfate. One L-asparaginase unit (U) equals to the amount of enzyme which releases 1µM of ammonia per minute at 37°C. Growth of the strain was also recorded by measuring the dry weight of the biomass obtained during centrifugation step.

Optimization of L-asparaginase production:

Influence of different cultural conditions such as initial pH, temperature, carbon, and nitrogen sources on the production of L-asparaginase by the strain CN-4 studied. Fermentation medium was initially adjusted to pH levels of 4-9 to determine the optimum pH for maximum L-asparaginase production. As the growth of the strain and production of L-asparaginase was maximum at pH 7.0. In the present study, the strain showed maximum L-asparaginase yields at 96 h with initial pH 7.0. Impact of temperature on the production of L-asparaginase by the strain was studied. As the production of L-asparaginase, as well as the growth of the strain was maximum at 30°C, it was recorded as the optimum temperature and used for further studies. Different carbon sources was employed to determine their influence on L-asparaginase production by strain CN-4 with modified ISP-5 media broth. The **c**ulture medium supplemented with lactose stimulated the production of L-asparaginase by *Streptomyces* sp. CN-4. Production of L-asparaginase was minimum when glucose was used as the carbon source. Influence of different concentrations of lactose on L-asparaginase production is recorded. Effect of six nitrogen sources on L-asparaginase production and growth of the strain CN-4 was tested. Yeast extract was found to be the best nitrogen source to enhance the growth and as well as the production of L-asparaginase. Once the preferable carbon and nitrogen sources were determined as lactose and yeast extract, efforts were made to identify their suitable concentrations.

Impact of pH on growth and L-asparaginase production by the strain:

Impact of pH on L-asparaginase production was examined by culturing the strain in modified ISP-5 broth adjusted to pH levels ranging from 4 to 9. Both the growth of the strain and enzyme activity was determined. The optimal pH was used for further study.

Influence of temperature on growth and enzyme production:

To determine the optimal temperature for growth and Lasparaginase production, the strain CN-4 inoculated in modified ISP-5 broth with optimal pH was incubated at different temperatures viz., 25ºC to 45ºC for 96 h.

Effect of carbon sources on growth and enzyme production:

To investigate the effect of carbon sources on L-asparaginase production by the strain, modified ISP-5 broth was amended with different carbon sources such as fructose, galactose, lactose, maltose, xylose, sucrose and starch each at a concentration of 0.5% (w/v). The carbon source which supports high yields of L-aparaginase production by the strain was chosen for further study. Impact of different concentrations of the best carbon source (0.1- 1.0%) among the other carbon sources tested on L-asparaginase production by the strain CN-4 was studied and the optimal level achieved was fixed for further studies.

Effect of nitrogen sources on growth and enzyme production:

The effect of nitrogen sources was determined by supplementing different inorganic and organic nitrogen sources viz. peptone, yeast extract, ammonium nitrate, sodium nitrate, L-proline and urea at the rate of 0.5% (w/v) to modified ISP-5 broth. The nitrogen source found to be good for optimal L-asparaginase productivity was selected for further study. Besides, the optimal concentration of nitrogen source (0.1-1.0% w/v) supporting good yields of L-asparaginase production by the strain was determined by maintaining all other conditions at optimal level.

Statistical Analysis: Data obtained on the bioactive metabolite production under different microbial culture conditions were statistically analyzed and expressed as mean ± standard error with one-way analysis of variance (ANOVA).

III. RESULTS

The enzymatic profile of the strain was interesting as it is a producer of a wide range of commercially important enzymes like amylase, cellulase, chitinase, L-asparaginse, protease, urease, lipase and DNase, but negative for pectinase, arginine hydrolase and nitrate reductase.

Table: 1 shows the production of enzymes by the strain *Streptomyces albus* CN-4

	Enzymatic activity		
Amylase	P	Cellulase	P
Chitinase	P	Pectinase	N

Lipase	P	Protease	P
DNase	P	L- Asperginase	P
Urease	P	Nitrate reductase	N

P – Positive; N – Negative

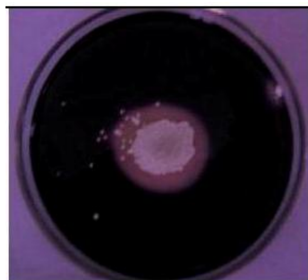

Fig: 1 Amylase

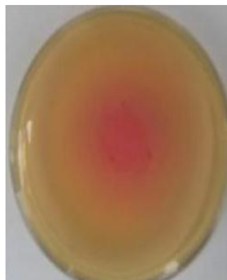

Fig: 2 Urease

Fig: 3 Dnase

Fig: 4 Cellulose

Fig: 5 L-asparaginase

Production of L-asparaginase by *Streptomyces albus* CN-4

The wide use of microbial L-asparaginse in the treatment of certain human cancers, the urgent need of L-asparaginase from various sources has been emerged. During screening of the strain for L-asparaginase production, pink color was formed around the colony inoculated in modified ISP-5 agar with 1% L-asparagine and 0.1% phenol red indicating positive result for the production of L-asparaginase. Hence attempts were made to study the effects of various conditions on L-asparaginase production by the strain by adopting one factor at a time method.

The strain was cultivated in modified ISP-5 broth and assayed for the production of L-asparaginase at every interval of 24 h up to 7 days to determine the optimum incubation period. Enzyme assay was based on ammonia liberated with reference to a standard curve of ammonium sulphate and expressed in terms of U/g of cell dry weight at 37ºC for 1 min. Dry weight of the cell was determined by weighing the dried biomass obtained during centrifugation step.

The production of L-asparaginase by the strain commenced after 48 h of cell growth and maximum production was observed after 96 h of incubation. A positive correlation between cell growth and L-asparaginase activity was reported in *S. karnatakensis* [19]. and *S. albidoflavus*

Optimization of L-asparaginase production

Influence of different nutritional and physiological conditions such as pH, temperature, carbon and nitrogen sources for the production of L-asparaginase by the strain.

Initial pH

Production medium was initially adjusted to pH levels of 5-9, used to determine the optimum pH for maximum L-asparaginase production. As the growth of the strain and production of L-asparaginase was maximum at pH 7.0, it was recorded as the optimal pH value. The results were parallel with the results of [20]. and *Streptomyces* sp. AQB VC67 [21]. [22]. also noted optimal L-asparaginase production by *S. aureofasciculus, S. chattanoogenesis, S. hawaiiensis, S. orientalis, S. canus* and *S. olivoviridis* at pH levels between 7 and 8. In the present study, the strain showed maximum L-asparaginase yields when cultured in modified ISP-5 broth for 96 h with initial pH 7.0.

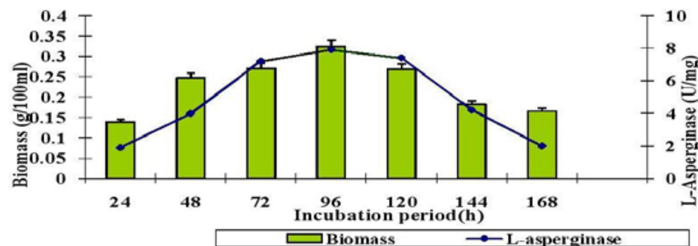

Fig: 6 Effect of incubation period on growth and L-asparaginase production by *Streptomyces albus* CN-4 (values are means of three replicates ±SD).

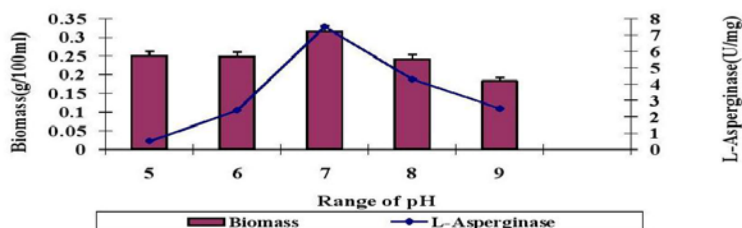

Fig: 7 Effect of pH on growth and L-asparaginase production by *Streptomyces albus* CN-4 (values are means of three replicates ±SD).

Temperature

Effect of temperature on the production of L-asparaginase by the strain is presented in figure 38. As the production of L-asparaginase as well as the growth of the strain was maximum at 30°C, it was recorded as the optimum temperature and thus used for further studies. Production of L-asparaginase was high at 30°C in *S.collinus* (Mostafa and Salma, 1979) and *S. longsporusflavus* F-15[23]. The optimum temperature for L-asparaginase production in *S. plicatus* was recorded as 29±2°C.

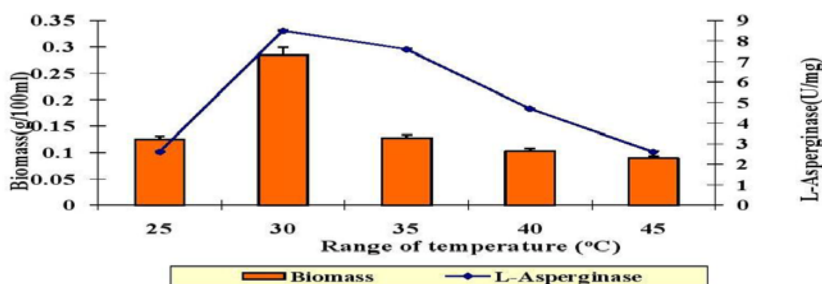

Fig: 9 Effect of temperature on growth and L-asparaginase production by *Streptomyces albus* CN-4 (values are means of three replicates ±SD).

Carbon Sources

Modified ISP-5 broth amended with different carbon sources was employed to determine their influence on L-asparaginase production by strain. The **c**ulture medium supplemented with lactose stimulated the production of L-asparaginase by *Streptomyces albus* CN-4. Production of L-asparaginase was minimum when glucose was used as the carbon source. [17]. also reported lactose as the best carbon source that enhanced the production yields of L-asparaginase by *Erwinia carotovora* while sucrose was reported as the suitable carbon source for the improved production of L-asparaginase by *Streptomyces tendae*[25] and *Streptomyces* sp. [26] Influence of different concentrations of lactose on L-asparaginase production is recorded in optimal yield of L-asparaginase by the strain was achieved in the medium amended with 0.5% lactose.

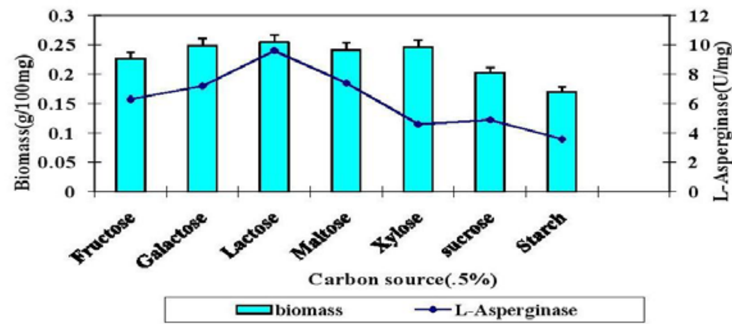

Fig: 10 Effect of Carbon sources on growth and L-asparaginase production by *Streptomyces albus* CN-4 (values are means of three replicates ±SD).

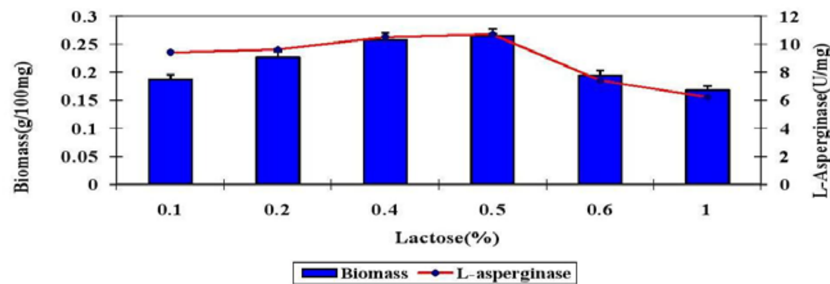

Fig: 11 Effect of concentration of optimized carbon source on growth and L-asparaginase production by *Streptomyces albus* CN-4 (values are means of three replicates ±SD).

Nitrogen sources

The effect of different nitrogen sources on the production of L- asparaginase by the strains was studied by incorporating different organic and inorganic nitrogen sources to modified ISP-5 broth containing 0.5% lactose. L-asparaginase production by the strain varied with different nitrogen compounds tested. Among them, yeast extract supported maximal L-asparaginase production by the strain followed by potassium nitrate. Yeast extract was reported as an excellent nitrogen source for L-asparaginase production by *Erwinia aroideae* [27], while only L-asparagine favoured high yields of L-asparaginase in *S.collinus* , *S.karnatakensis* and *S. venezuelae*. Tryptone and yeast extract served as good nitrogen sources for *E. caratovora* EC – 113 [28]. Optimization studies of Abdel-All revealed that glycerol-L-asparagine-yeast extract medium was suitable for the synthesis of L-asparaginase by *S. phaeochromogenes* FS-39. The optimal level of yeast extract was found to be 0.5% as it favored high yields of L-asparaginase by the strain. A gradual decline in L-asparaginase production was found with further rise in yeast extract levels. Yeast extract is essential for cell growth and L-asparaginase synthesis, but in high concentration it inhibits the production of L-asparaginase [29]. Narayana recorded 2% yeast extract as optimal concentration for L-asparaginase production by *S. albidoflavus*. In the present study, enhanced levels of L-asparaginase production by the strain was recorded in the modified ISP-5 broth containing 0.5% yeast extract and 0.5% lactose with initial pH 7.0 incubated at 30°C for 96 h.

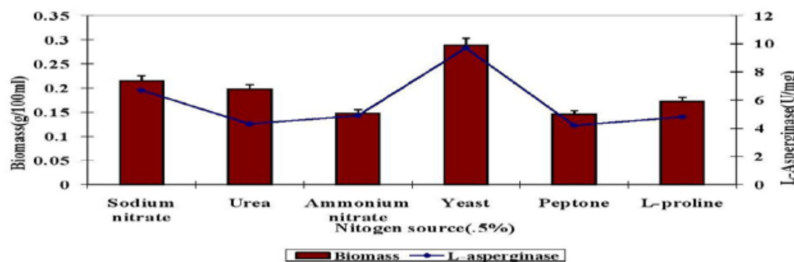

Fig: 12 Effect of nitrogen sources on growth and L-asparaginase production CN-4 . (values are means of three replicates ±SD).

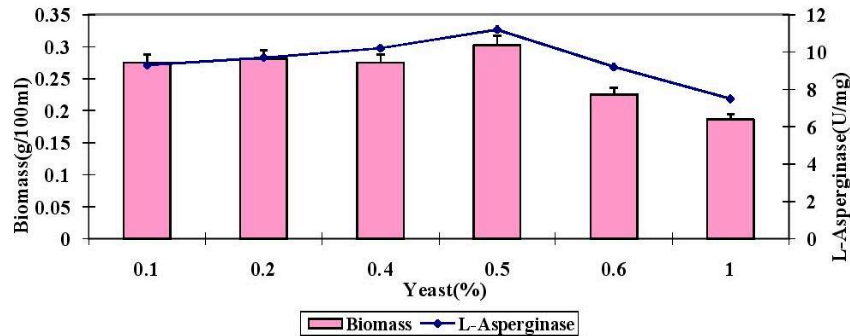

Fig: 13 Effect of nitrogen sources on growth and L-asparaginase production by the strain CN-4 . (values are means of three replicates ±SD).

IV. DISCUSSION

The strain has ample capacity to produce detectable amounts of a wide array of enzymes including amylase, lipase caseinase, L-asperginase, DNase cellulase, and urease. In addition to the bioactive metabolites, the strain also produced L-asparaginase enzyme in detectable amounts. The production of all the compounds had a marked increase after the optimization of cultural and physiological parameters.

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