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Production of l-asparaginase from *serratia marcescens* NCIM 2919 using citrus limetta pulp under solid state fermentation

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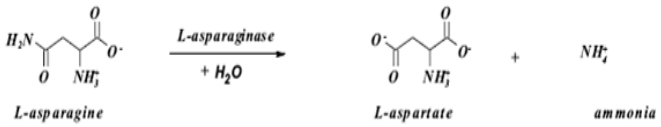
ABSTRACT

Intense interest in Asparaginase has resulted from the discovery of its ability to inhibit growth of tumors in mouse, rat and dog to suppress human leukamias in clinical trails. L-Asparaginase is an effective antineoplastic agent, used in the acute lymphoblastic leukemia.Asparaginase catalyzes the deamination of asparaginases into L-aspartic acid and ammonia. The aim of the present investigation was to study production of asparaginase from agricultural waste like citrus limetta pulp using solid state fermentation (SSF).Citrus limetta pulp used as a sole source for growth in SSF showed maximum enzymes production. Optimized process parameters like incubation time: 72 hrs; incubation temperature:28^{0}C; pH of the culture medium: 7.5; and moisture content: 60% v/w gave an overall yield of 101 U/g.

Introduction

The enzyme L-asparaginase was eventually identified as the anticancer agent. L-asparaginase was isolated and tested successfully on human leukemias. Eventually the enzyme asparaginase was also found and isolated from the bacteria, *E. coli* [3] L-asparaginase derived from bacterial and fungal sources have dominant application in pharmaceutical sector [8]. If the enzyme L-asparaginase is given to humans, various types of leukemias can be controlled. Tumor cells, more specifically lymphatic tumor cells, require huge amounts of asparagines to keep up with their rapid, malignant growth. This means they use both asparagine from the diet as well as what they can make themselves (which is limited) to satisfy their large asparagines demand.L-asparaginase is an enzyme that destroys asparagine external to the cell. Normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. The enzyme converts asparagine in the blood into aspartic acid by a deamination reaction. The leukemia cells are thus deprived of their supply of asparagine and will die.L-Asparaginase is used for treating acute lymphoblastic leukaemia, pancreatic carcinoma11, and bovine lymphomosarcoma. L-Asparaginase is produced throughout the world by submerged fermentation (SmF). This technique has many disadvantages, such as the low concentration production, and consequent handling, reduction, and disposal of large volumes of water during the downstream processing. Therefore, the SmF technique is a cost intensive, highly problematic, and poorly understood unit operation13. Solid-state fermentation (SSF) is a very effective technique as the yield of the product is many times higher when compared to that in SmF. Therefore L-asparaginase is an anti-neoplastic agent used in the lymphoblastic leukaemia chemotherapy. L-asparaginase belongs to an amidase group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonia



As L-Asparaginase has wide applications in Clinical Pharmacology [7], there is demand in the production of L-Asparaginase with a cheap substrate. To achieve good results we have chosen fruit pulp, which is cheaply available in huge quantities in various food and beverage industries. To achieve high productivity, attempt has been made to optimize the process parameters for the production of L-asparaginase using the microorganism *Serratia marcescens*. Currently, L-Asparaginase is mainly produced by submerged fermentation, but a very few works are carried on solid state fermentation (SSF). SSF provides an opportunity to exploit some value added products. The objective of the present study includes production and optimization of various process parameters like time, temperature, pH and moisture content under SSF.

Materials and methods:

Medium:

Medium used for maintenance of the microorganism is Nutrient Agar. Ingredients include Peptone(5g/l), Meat extract(1g/l), Yeast extract(2g/l), sodium chloride(5g/l), Agar(15g/l), and pH-7.0 ± 0.2. Suspend 28 grams in 1000 ml distilled water. Heat it to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring. Nutrient media thus prepared was a basic culture media used for maintaining microorganisms.

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Substrate:

Citrus limetta pulp is collected from local fruit juice shop around Visakhapatnam and dried naturally and powdered, packed and stored until further use. 200gms of Citrus limetta pulp is taken, dried for 3days and powdered.

Microorganism:

Serratia marcescens NCIM 2919 was procured from National Collection for Industrial Microorganism (NCIM), Pune. This organism is used for the production of L-Asparaginase enzyme using Citrus limetta pulp as substrate. Nutrient agar medium is used for the maintenance and sub culturing of the microorganism.

Preparation of Inoculum:

Streaking was done from the old cultures of *Serratia marcescens* NCIM 2919 on pure agar slants of nutrient agar medium and incubated at 30^0C for 3 days.

Development of Inoculum:

10ml of sterile distilled water was added to the cells from 3 day old slant; from that 1ml of suspension containing approximately 10^5-10^6 cells/ml is used as the inoculum to each flask.

Solid State Fermentation:

SSF was carried out in 250-mL conical flask by taking production medium containing (in g/L): Ingredients include Glucose (12.5g/l), NH_4NO_3 (2.6g/l), $FeSO_4.7H_2O$ (0.01g/l), L-asparagine (0.5 g/l), KCl (0.5 g/l), K_2HPO_4 (0.01g/l) and pH-6.8± 0.2.SSF was carried out by taking 5g of substrate in 250ml Erlenmeyer flask, moistening it with 2.5ml of production medium, mixed thoroughly and autoclaved at 15lb pressure, 121^0C for 15min. After cooling, the flasks are inoculated with 1ml of cell suspension. The contents are mixed thoroughly and incubated.

Standard graph:

Standard graph was prepared by treating 1ml of 0.25, 0.5, 0.75 and 1mM ammonium sulphate with trichloroacetic acid, NaOH and Nessler's reagent (Fig-1).

Stock solution of 10 mM:

It was prepared by dissolving 132 mg of ammonium sulphate in 100 ml distilled water in a volumetric flask.

Working solution of 1mM:

It is prepared by taking 5 ml of stock solution and made upto 50 ml with distilled water. From this 0.25 and 0.5 mM are prepared.

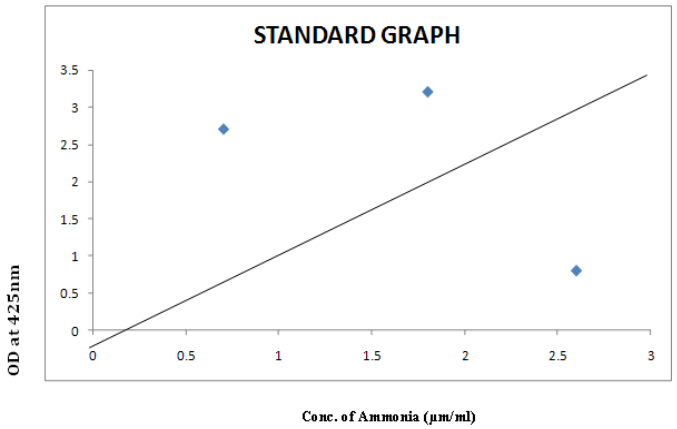

Fig-1 Standard graph for determination of Ammonium Sulphate

Determination of Enzyme Activity:

Enzyme Extraction:

The cultivation is carried out at a temperature of 30^0C for 24hrs interval. The solid state fermented material corresponding to one Erlenmeyer flask is mixed with 40ml of 0.1M Phosphate buffer and homogenized with constant stirring for 30min at 150rpm on rotary shaker, to extract the liquid from bacterial cells. The extract is filtered through Whatmann filter paper no1. The extracted solution is centrifuged at 8000 rpm for 15 min.

Enzyme Assay:

L-asparaginase activity is determined by measuring the amount of ammonia formed by nesslerization. 0.5 mL sample of crude enzyme, 1.0 mL of 0.1M sodium borate buffer (pH 8.5) and 0.5 mL of 0.04M L-asparagine solution are mixed and incubated for 10 min at 37^0C. The reaction is then stopped by the addition of 0.5 mL of 15% trichloroacetic acid. The precipitant is removed by centrifuging at room temperature at 10000rpm for 5 minutes, from this 1ml of supernatant liquid is collected and 1ml of Nessler's reagent is added. The liberated ammonia is determined by direct nesslerization [4]. Suitable blanks of substrate and enzyme containing samples are included in all assays. The yellow colour is read in a spectrophotometer at 500 nm. One unit (IU) of L-asparaginase is the amount of enzyme which liberates 1 µmole of ammonia in 1 min.

Results And Discussion:

The solid state fermentation process (SSF) process was observed to be less sensitive to contamination than submerged state fermentation**.** Solid state fermentation holds tremendous potential for the production of microbial enzymes**.** Solid substrate fermentation can be of special interest in those processes in which the crude fermented product may be directly used as enzyme source. In the present study substrate namely Citrus limetta pulp was used for the production of L-asparaginase using the strain of *Serratia marcescens* NCIM 2919.The physicochemical parameter like incubation time, temperature, pH and moisture content were optimized. The results of study were presented and discussed in the light of literature. The following table shows the obtained values of L-asparaginase activity.

Table 1: Production of L-asparaginase using *Serratia marcescens NCIM* 2919

Organism	L-asparaginase activity (U/ml)
Serratia marcescens NCIM 2919	35

Optimization:

Effect of Fermentation time on L-asparaginase production:

To study the influence of fermentation time on L-asparaginase production, 5 gm of substrate (autoclaved and moistened with 50 %v/w production medium), 20 %v/w inoculum (3 day old culture) is added and incubated at varying fermentation times ranging from 24 to 120 hrs with in interval of 24 hrs. The flasks were analyzed for every 24 hrs. The maximum yield of 69 U/ml of substrate obtained at 72 hrs of incubation time (Fig-2) and yield was slightly decreased with further increase in time period. The reduction in L-asparaginase activity after an optimum incubation was probably owing to a reduced growth rate resulted from fast depletion of nutrients available to the organism and also could be owing to the production of secondary metabolites resulting in lower enzyme activity. Maria *et al.*,2004 reported highest L-asparaginase activity level by *Aspergillus terres* at 48^{th} hour. Abha Mishra,

2006 presented many optimized parameters, all of them being optimized at 96th hour by *Aspergillus Niger*.

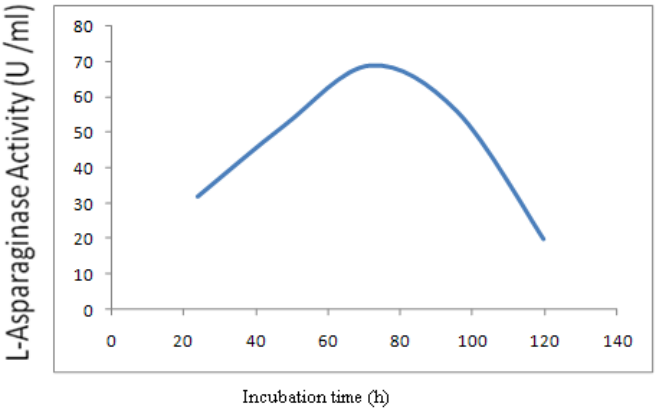

Fig 2: Effect of Incubation time

Effect of different incubation temperatures:

Different incubation temperatures, viz. 28, 32, 36 and 40^0 C, were tried to cultivate the culture for L-asparaginase production. The organism exhibited a better growth as well as enzyme production of 84 U/ml at 28^{o}C with *Serratia marcescens*, respectively as shown in (Fig-3). A decrease in the activity of L-asparaginase was observed when the incubation temperature was higher than the observed optimum incubation temperature. The significance of temperature in development of biological process is such that I could determine the effects of protein denaturation, enzyme inhibition, promotion or suppression of the production of a particular metabolite, cell viability and death. In SSF, during fermentation there is a general increase in the temperature of the fermenting mass due to respiration. Heat built-up is in fact a drawback in SSF system. Abha Mishra, 2006 reported the optimum yield at 30^{0}C with enzyme activity of 29± 3.14 U/gds, 27.6 ± 2.95 U/gds, 34.4 ± 3.32 U/gds by *Aspergillus Niger* respectively.

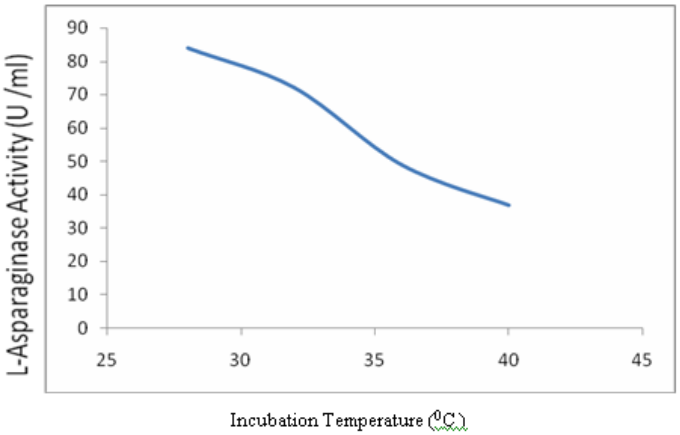

Fig: 3 Effect of temperature

Effect of pH:

pH strongly influences many enzymatic process and transport of various components across the cell membrane, which in turn support the cell growth and product production. The effect of initial pH of the substrate on L-asparaginase production was studied. The L-asparaginase productivity increased with increase in pH from 6.0 to 7.5. Maximum activity of L-asparaginase of 97 U/ml with *Serratia marcescens* was attained at pH 7.5, respectively. A further increase in pH resulted in decreased L-asparaginase production. The results are presented in (Fig- 4). As the metabolic activities of the microorganisms are very sensitive to changes in pH, L-asparaginase production is found to be affected in higher or lower pH level compared to the optimum value. [2] also obtained maximum enzyme production at pH 7.5 by a strain of *E.coli* B with phosphate buffer being more effective than carbonate or Tris for producing high enzyme levels. Mukherjee *et al*.,2000 reported an optimum asparaginase production at pH 8 by *Enterobacter aerogenes*.

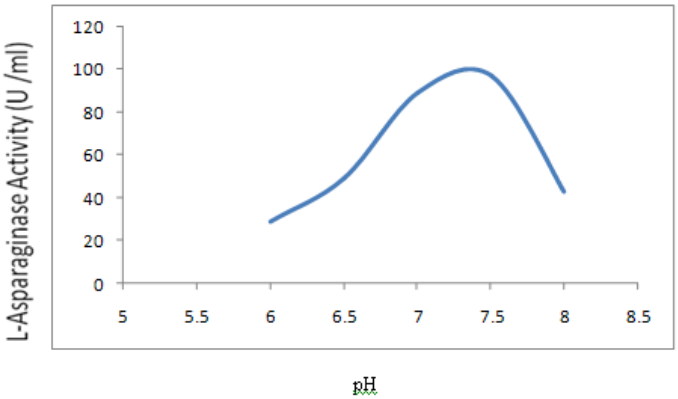

Fig: 4 Effect of pH

Effect of initial moisture content:

The effect of initial moisture content of the substrate (40, 50, 60,70,80,90 and100 % v/w) on L-activity have gradually increased with increase in initial moisture content of the substrate from 40 – 60 % v/w, but the high L-asparaginase titres of 101 U/ml with *Serratia marcescens* was attained when the initial moisture level was 60% in comparison with that at low or high moisture levels. The enzyme activity was lower when the substrate moisture was higher or lower than this level. The decrease in the enzyme activity with an increase in moisture might be attributed to the phenomenon of flooding of inter-particle space of the substrate, which causes decreased porosity and lowers oxygen transfer. Similarly, a moisture level lower than optimum leads to higher water tension, a lower degree of swelling, and a reduced solubility of nutrients of the solid substrate. When water is made available in a lower or higher quantity than what is optimally required, the productivity of the process is significantly affected. Moisture optimization can be used to regulate and to modify the metabolic activity of the microorganisms. Abha Mishra, 2006 reported only 70% moisture content with *Aspergillus Niger*.

Effect of moisture content

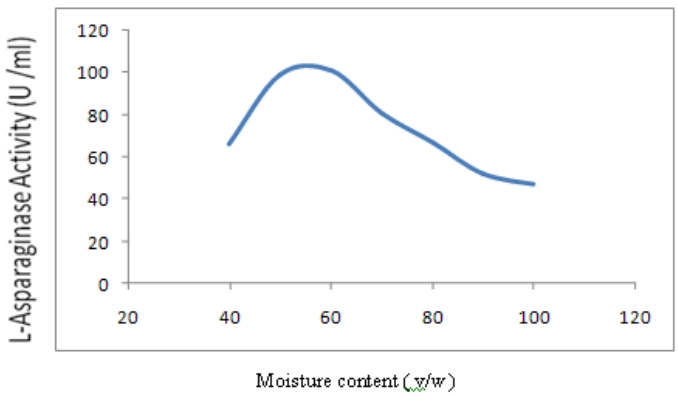

Fig: 5 Effect of moisture content

Conclusion:

The present study deals with the solid state fermentation by using Citrus limetta pulp for L-Asparaginase production by *Serratia marcescens* NCIM 2919. The production conditions were tried to enhance L-Asparaginase yield. Quantitative

determination of L-Asparaginase is carried out by using UV spectroscopy. Various cultural conditions like incubation time, temperature, pH and moisture content of the culture medium were optimized. The optimum time, temperature, pH and moisture content for the production of L-Asparaginase enzyme were 72hrs, 28^0c, 7.5 and 60%v/w respectively. Under these optimum conditions, maximum production of L-Asparaginase was found to be 101U/g. Thus our present study shows that *Serratia marcescens* NCIM 2919 can produce L-Asparaginase with relatively good yield and Citrus limetta is potential substrate for L-Asparaginase production.

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