Production of L-asparaginase By *Serratia Marcescens* in Solid State Fermentation

D. Praveen Kumar\(^{1*}\), B. Thangabalan\(^{1}\), M. Venkata Ramana\(^{1}\), Ch. Sandhya\(^{1}\)
S. Manohar Babu\(^{1}\) and D. Srinivasa Rao\(^{2}\)

1SIMS College of Pharmacy, Mangaldas Nagar, Guntur-522 001, India
2K. C. Reddy Institute of Pharmaceutical Sciences, Jangamgunta Palem Medikonduru Mandal, Guntur 522 348, India

**Abstract:** The present work deals the production of L-asparaginase using the Citrus limetta peel as substrate and the microorganism *Serratia marcescens*- NCIM 2919 in a solid state fermentation process. The L-asparaginase enzyme production conditions like incubation period, incubation temperature, particle size, pH, inoculum level and moisture content were optimized using Citrus peel as substrate. The fermentation time of 48hrs and the temperature of 28°C, particle size of 0.355mm, pH 7.0, inoculum level of 20% v/w and moisture content of 70% were observed optimum for the production of L-asparaginase. Five different carbon sources were screened for their influence on enzyme yield; they are glucose, sucrose, fructose, lactose, and maltose, used as supplements. Among this 1.5% w/w of sucrose gave better yield, 0.6% w/w of L-asparagine as nitrogen source was observed optimum for the production of L-asparaginase.

**Key words:** Citrus limetta peel, *Serratia marcescens*, solid-state fermentation.

**INTRODUCTION**

Solid-state fermentation (SSF) is defined as a process that occurs on a non-soluble material that acts both as support and a source of nutrients, with a reduced among of water, under the action of fermenting agent\(^{[1]}\). A large amount of research has been conducted upon the biosynthesis of L-asparaginase\(^{[2]}\) demonstrated antitumor activity. L-asparaginase is produced throughout the world by submerged fermentation (SF). 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 SF technique is a cost intensive, highly problematic, and poorly understood unit operation \(^{[3]}\). Solid-state fermentation is a very effective technique as the yield of the product is many times higher when compared to that in SF\(^{[4]}\). Microbial asparaginases have been particularly studied for their applications as therapeutic agents in the treatment of certain types of human cancer\(^{[5]}\). L-asparaginase from two bacterial sources (*E. coli* and *Erwinia carotovora*) is currently in clinical use for the treatment of acute lymphoblastic leukemia \(^{[6]}\). It is also used for the treatment of pancreatic carcinoma \(^{[7]}\) and bovine lymphomasarcoma \(^{[8]}\). Therefore, the aim of the present work is the discovery of a new L-asparaginase producer that is serologically different from the previously reported ones, but one that has similar therapeutic effects.

**MATERIAL AND METHOD**

**Substrate**

Citrus limetta peel is collected from local fruit juice shop and dried naturally and powdered, packed and stored until further use.
Microorganism

*Serratia marcescens* – NCIM 2919 was obtained from National Collection for Industrial Microorganism (NCIM), Pune. This organism was used for the production of asparaginase enzyme using citrus limetta peel as substrate. Nutrient agar medium was used for the maintenance and subculturing of the microorganism. The organism was subcultured at every 7 day interval. The culture was grown at 30 °C for 2 days after which, they were stored at 4 °C until further use.

Preparation of Inoculum

Streaking are done from the old cultures of *Serratia marcescens* NCIM 2919 on pure agar slants of nutrient agar medium and incubate them at 30°C for 3 days. Subcultures are made from this and are used for the production of an enzyme.

Development of Inoculum

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

Solid State Fermentation

SSF was carried out in 250-mL flat bottom shallow glass container by taking medium containing (in g/L): Glucose- 12.5 g, NH₄NO₃- 2.66g, FeSO₄.7H₂O - 0.01g, L-asparagine - 0.5g, KCl-0.5g, K₂HPO₄-1g. The pH was adjusted to 6.8. The medium was sterilized in autoclave at 15lb pressure; 121 °C for 20min. Solid state fermentation was carried out by taking 5g of substrate in 250ml Erlenmeyer flask, moistening it with 2.5ml of production medium solution, mixed thoroughly and autoclaved at 121 °C for 15min. After cooling, the flasks to room temperature, the flasks were inoculated with 1ml of cell suspension. The contents were mixed thoroughly and incubated at 30 °C temperature for the 24 hours.

DETERMINATION OF ENZYME ACTIVITY

Enzyme Extraction

The cultivation was carried out at a temperature of 30 °C for 1 day intervals. The solid state fermented material corresponding to one Erlenmeyer flask was 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 was filtered through whatmann filter paper no1. The extracted solution was centrifuged at 8000 rpm for 15 min.

Enzyme Assay

L-asparaginase activity was 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 were mixed and incubated for 10 min at 37 °C. The reaction was then stopped by the addition of 0.5 mL of 15% trichloroacetic acid. The precipitated was removed by centrifugation, from this 1ml of supernatant liquid is collected and to it add 1ml of Nessler’s reagent. The liberated ammonia was determined by direct nesslerization. Suitable blanks of substrate and enzyme containing samples were included in all assays. The yellow color was read in a spectrophotometer at 500 nm. One unit (U) of L-asparaginase was the amount of enzyme which liberates 1 µmole of ammonia in 1 min at 37 °C.

RESULTS AND DISCUSSION

L-asparaginase production at different time intervals is shown in the fig. 1. The maximum L-asparaginase activity was observed at 48hrs. After 48hrs, it was decreased due to depletion of nutrient materials. The maximum amount of L-asparaginase was produced at 28 °c temperature. To determine the optimum particle size of the substrate for the production of L-asparaginase, the production medium was prepared by taking 5gms of substrate which was passed through different sieve numbers of 10, 22, 44, 60, and 80. The maximum enzyme production was observed at sieve number 44 (36/44 that passed through no: 36 sieve but was retained by no: 44 sieve). To determine the effect of pH for the production of L-asparaginase, the bacterial nutrient medium
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adjusted with different pH ranges 6.0, 6.5, 7.0, 7.5 and 8.0 were taken along with 5g of citrus limetta peel in five 250ml conical flasks incubated at temperature of 28 °C for 48 hrs. After 48hrs the samples were assayed for L-asparaginase activity. The maximum production of L-asparaginase at pH 7.0 was recorded fig. 3. Different inoculum levels 16%, 18%, 20%, 22%, 24% v/w were studied for maximum L-asparaginase activity. The results indicate that maximum enzyme production was observed at 20% v/w of inoculum fig. 4. Further increase in inoculum level decreased the enzyme activity. Different moisture content 50%, 60%, 70%, 80%, 90%, 100% (v/w), were taken in each conical flask in the solid state fermentation and were incubated at 28 °C, pH 7.0, for 48 hrs. The maximum activity was observed at 70% (v/w) of the moisture content fig. 5. Five different carbon sources were screened for the production of L-asparaginase enzyme which includes glucose, sucrose, maltose, fructose and lactose. These are enriched with % w/w. The results indicate that sucrose supplementation gave marginally improved enzyme than other supplementations fig. 6. To determine the effect of sucrose concentration, the production medium was prepared by using 1.0%, 1.5%, 2.0%, 2.5% and 3.0% (w/w) sucrose in 250ml conical flasks and each flask was inoculated and was kept in an incubator. L-asparaginase production at different concentrations of sucrose levels was shown in the fig. 7. The results indicate that maximum enzyme production was observed at 1.5% w/w of sucrose concentration. To determine the effect of L-asparagine on the production of enzyme, the production medium was prepared with different concentrations of L-asparagine like 0.3%, 0.4%, 0.5%, 0.6% and 0.7% (w/w) were dispersed in 250ml conical flasks. After 48 hrs the enzyme activity was determined as shown in the fig 8. The result indicates that maximum enzyme production was observed at 0.6% w/w of L-asparagine concentration.

**CONCLUSIONS**

Finally we concluded that *Serratia marcescens* is a promising agent for industrial application since it gave a significant L-asparaginase (86.79 U/g) activity in citrus limetta peel under solid state conditions. As citrus limetta peel is low cost
substrate, easily available and showing suitability for solid state cultivation of microbes, it is suggested as a potential substrate for L-asparaginase production.

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