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A Novel Approach for Detection, Confirmation and Optimization of L-Asparaginase from *Emericella Nidulans*

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Abstract
L-Asparaginase is an important component in the treatment of acute lymphoblastic leukemia in children. The manufacture of L-asparaginase by *Emericella nidulans* were carried out by novel flask method for screening. Using modified Czapek Dox both containing L-asparagine and phenol red as indicator. L-asparaginase productions were detected by change into pink colour in flask. Further confirmations of L-asparaginase synthesis were carried out by thin layer chromatograph. Production of L-asparaginase was carried out by using pH, temperature and inoculums sizes for optimization process. The pH 6, temperature 30 ^{0}C and 0.75 ml of inoculums were used.

Keywords
L-asparaginase, Flask method, optimization, *Emericella nidulans*.

Introduction
L-Asparaginase has received increased awareness in current years for its ant carcinogenic potential. Cancer cells distinguish themselves from normal cells in diminished expression of l-asparagine[1,2]. Its antineoplastic activity is associated with the property of depleting the circulating pool of L-asparagine by the asparaginase catalytic activity. Malignant cells with low L-asparagine levels are killed due to lack of an exogenous supply of this amino acid combined with an impaired protein synthesis mechanism[3]. However, normal cells are protected from L-asparagine-starvation due to their ability to produce this amino acid[4]. Based on this, L-asparaginase has also been included in most contemporary, multi-agent regimens for adult acute lymphoblastic leukemia (ALL). Several microbial strains like *Aspergillus tamari, Aspergillus terreus*[5,6], *E. coli*[7,8], *Erwinia aroideae*[9], *Pseudomonas stutzeri* [10], *Pseudomonas aeruginosa*[11], *Serratia marcescens* (*Vibrio succinogenes*)[12], and *Staphylococcus* sp.[13] having potential for L-asparaginase production have been isolated and studied in detail. Aim of the present study is that develop a simple, novel, rapid method for detection and confirmation of L-asparaginase production from *Emericella nidulans* by qualitative flask assay method and Thin layer chromatography. It also describes the optimization of fermentation parameters like pH, temperature and inoculums size. This is the first report on L-asparaginase from *Emericella nidulans*. Therefore we made an attempt on screen, conform and production.

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Microorganism
The *Emericella nidulans* strains were isolated from different soils. Soils are taken from different region from Tumkur university campus.

Medium
The organisms were grown and kept on slants of solid modified Czapek Dox's medium containing (g/L distilled water) glucose, 2; L-glutamine 10; KH2PO4, 1.52; KCl, 0.52; MgSO4.7H2O, 0.52; CuNO3.3H2O, trace; ZnSO4.7H20, trace FeSO4, trace; agar, 20.0 and pH 6.2[14].Modified Czapek Dox's broth was supplemented with different concentrations of the dye. A 2.5% stock of the dye was prepared in ethanol and the pH was adjusted to 7.0 using 1 mol L^{-1} NaOH. The stock solution of the dye ranging from 0.04 ml to 0.3ml was added to 100

ml of modified Czapek Dox's broth, giving final dye concentration of 0.001-0.009% with a final pH of 7.0. The media were autoclaved and plates prepared. Control plates were modified Czapek Dox's broth (i) without dye and (ii) without asparagine (instead containing NaNO3). The plates were then inoculated with 96 hr cultures of *Emericella nidulans* for rapid screening of L-asparaginase. The pink colour was observed after 48 hr.

Rapid confirmation of L-asparaginase by thin layer chromatography method

Primarily screened strains were subjected to thin layer chromatography (TLC) for the confirmation of L-asparaginase production. Here the separation and identification of amino acids were carried out by thin layer chromatography technique as per lingappa and Siddalingeshwara[15] by using silica gel G and saturated phenol with water used as a solvent system. The enzyme activity, or an amount of aspartic acid produced was roughly estimated by redness of the spot developed by ninhydrin reagent.

Fermentation studies

The production of L-asparaginase was carried out by using 100 ml of production medium under submerged fermentation. The production medium was inoculated 1 ml of inoculum ($1x10^7$spores/ml). The content of the flask were mixed thoroughly and kept at 35 ^{0}C for 3-5 days. The pH 4.5 was maintained throughout the fermentation process.

Production Medium

The production medium containing (g/L distilled water) glucose, 20; Malt extract, 10; Yeast extract, 4; K2HPO4, 2; MgSO4, 0.1 and pH.6 for the synthesis of L-asparaginase.

Effect of initial pH on L-asparaginase production

The flasks containing 100 ml of the production medium were mixed with acid/alkali solution to obtain required pH. The pH was adjusted in the range of 3-7 with increments of 0.5. Thus prepared flasks were cotton plugged and autoclaved at 121^{0}C for 15 min.

Effect of initial temperature on L-asparaginase production

100 ml of the production medium were taken separately in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. Thus prepared flasks were incubated at different temperatures like 25, 30, 35 and 40^{0}C.

Effect of inoculum size on L-asparaginase production

The inoculum was prepared separately at different levels i.e., 0.25, 0.50, 0.75, 1.0 ml and 1.25ml were used to carry out the fermentation studies.

Extraction of L-asparaginase from fermented medium

The samples were withdrawn periodically at 24 hrs in aseptic condition 1 ml of extract was filtered through Whatman filter No.1. The clear extract was centrifuged. The supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for L-asparaginase assay.

Assay of L-asparaginase from crude extract

Assay of enzyme was carried out as per Imad et al[16]., 0.5 ml of 0.04 M asparagine was taken in a test tube, to which 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml Nessler's reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in International unit.

International Unit (IU)

One IU of L-asparaginase is the amount of enzyme which liberates 1 μmol of ammonia per minute per ml [μ mole/ml/min].

Results and Discussion

Emericella nidulans were isolated from the soil samples from Tumkur University Campus. Further confirmation was done at Agarkar Research Institute, Pune. A new approach was made to screen by flask method. The modified Czepek Dox's broth was showed pink coloration (fig.1) after 48 hrs of incubation period. This indicates the hydrolysis of L-Asparagine into aspartic acid and ammonia were released by L-asparaginase synthesized by *Emericella nidulans*. This is purely based on change in initial pH from acidic to basic due to release of ammonia. It is generally observed that L-asparginase production is accompanied by an increase in pH of the culture filtrates[17].The flask assay was devised using this principle by

incorporating the pH indicator phenol red in medium containing asparagines(sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns to pink, thus pink coloration is formed in flask containing *Emericella nidulans* producing L-asparaginase.

Fig 1: Rapid flask assay for screening of L-asparaginase production.

Further confirmation of L-asparaginase synthesis was carried by using thin layer chromatography (TLC) method. The results on the confirmation of L-asparaginase production from the *Emericella nidulans* using TLC are presented in Plate-1 and Table-1. To ensure whether the pink coloration exhibited by *Emericella nidulans* strains on rapid qualitative flask assay method was due to L-asparaginase activity, the enzyme extract was subjected to TLC for rapid confirmation. The TLC technique was used for the separation and identification of glutamic acid produced by *Emericella nidulans* strains were roughly estimated by redness of the spot developed by spraying Ninhydrin reagent. The aspartic acid is a compound produced, after the hydrolysis of asparagines by L-asparaginase enzyme synthesized by the *Emericella nidulans* strain. In this study, the compound produced by the isolates exhibited similar Rf values (0.866) as that of standard aspartic acid (0.86) (Table.1). These results were similar to that observed with filamentous fungi by Lingappa and Siddalingeshwara[15]. To our knowledge this is the first attempt to confirm L-asparaginase production by *Emericella nidulans* strains by thin layer chromatography.

L-asparaginase formation has been shown to have a firm link to active cell growth[18,19] found maximum L-asparaginase activity of *Streptomyces plicatus* at pH 7.0 while[20] reported maximum L-asparaginase production of *S.albidoflavus* at pH 7.5. Gulati et al[21] have reported 6.2 was the optimum pH for L-asparaginase producing *Aspergillus nidulans* strain. In our study the data revealed that the pH of 6.0 was found as suitable for maximum production of L-asparaginase with *Emericella nidulans* strain on production medium under submerged fermentation. Thus from the above studies it clearly indicates that the production of L-asparaginase mainly depends on the strains employed during the fermentation. As such our findings are in close agreement with the findings of Gulati et al[21].The effect of different temperatures on L-asparaginase production by *Emericella nidulans* were represented in Table 3. The data indicate the L-asparaginase production was increased with increase in temperature from 25^0C-30^0C. The decrease in L-asparaginase production was observed above 30^0C in all days of fermentation period. The maximum enzyme production of 0.96 IU was observed at 30^0C, whereas lowest enzyme production of 0.56 IU was observed at 40^0C after 48 hrs of fermentation period. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperature[22,23]. Sarquis et al[5] reported 30^0C is the suitable for L-asparaginase production through submerged fermentation by using *A.terreus* and *A.tamarii*. Sutthinan Khamna et al (2009) reported that Amycolatopsis CMU-H002 was showed 30^0C optimum for L-asparaginase synthesis. *S.albidoflavus* produced high amount of L-asparaginase at 28^0C-30^0C when grown at 35^0C[20]. Our results are close agreement with[24].The data revealed that the production of L-asparaginase by *Emericella nidulans* increased as the inoculum size increased up to 1 ml for 48 hrs fermentation periods was represented in Table.4. Further increase in the inoculum size has not yielded significant increase in the production of L-asparaginase. The inoculum size of 1 ml showed maximum production of L-asparaginase 1.11 IU at 48 hrs fermentation period.

Sl. No.	Sample	Rf Values
1	Standard aspartic acid	0.86
2	Aspartic acid produced- Asparagine hydrolysis by *Emericella nidulans* L-asparaginase	0/866

Table 1: Confirmation of L-asparaginase production from *Emericella nidulans* by TLC method.

Sl. No	pH	Enzyme Activity (IU)
1	3.0	0.45
2	4.0	0.61
3	5.0	0.68
4	6.0	0.83
5	7.0	0.71

Table 2: Effect of different pH on L-asparaginase production by *Emericella nidulans*

Sl. No	Temperature (^{0}C)	Enzyme Activity (IU)
1	25	0.65
2	30	0.96
3	35	0.78
4	40	0.56

Table 3: Effect of temperature on production of L-asparaginase by *Emericella nidulans*.

Sl. No	Inoculum Size(in ml)	Enzyme Activity (IU)
1	0.25	0.56
2	0.50	0.79
3	0.75	0.98
4	1.0	1.11
5	1.25	0.80

Table 4: Effect of inoculum size on production of L-asparaginase by *Emericella nidulans*

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