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Optimization of Some Physical and Nutritional Parameters for the Production of L-asparaginase by Isolated Thermophilic *Pseudomonas aeruginosa* strain F1

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The effect of some physical and nutritional parameters were studied for the optimum production of extracellular enzyme L-asparaginase employing isolated thermophilic *Pseudomonas aeruginosa strain F1* by submerged fermentation. The effects of incubation period, initial pH, incubation temperature, age of inoculums, inoculums level, agitation speed and effect of aeration were studied. The maximum enzymatic activity was obtained with incubation period of 25h, initial pH 6.5, incubation temperature 40°C, inoculums age 16 h, inoculums level 6%, agitation speed 150rpm and 50 ml medium in 250 ml flask. The effect of different carbon and nitrogen sources were studied. The results indicated that lactose is the best carbon source in combination with L-asparagine and yeast extract in combination with beef extract were found to be the best nitrogen sources.

Key words: L-asparaginase, *Pseudomonas aeruginosa* strain F1, Enzymatic assay & Leukemia.

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is a clinically acceptable anticancer agent, for the effective treatment of certain lymphomas and leukemias in both experimental animals and humans. L-asparaginase has been used in combination with other agents in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma chemotherapy, pancreatic carcinoma, melanosarcoma, reteclesarcoma[1,2] and inhibited the growth of the two human cell lines including hepatocellular and colon carcinoma[3]. L-asparaginase is known commercially as Oncaspar, Colaspase, Crasnitin, Kidrolase, Erwinase and Elspar. L-asparaginase is used in food industry by effectively reducing the level of acrylamide (a potent carcinogen) up to 90%, in a range of starchy foods[4]. L-asparaginase is a model enzyme for the development of new drug delivery system[5] and L-asparagine biosensor for leukemia[6].

L-asparaginase is an amidohydrolase that catalyzes the hydrolysis of the amino acid L-asparagine to aspartic acid and ammonia. The tumor cells have a compromised ability to generate L-asparagine endogenously, either due to low expression levels of asparagine synthetase or insufficient amount of its substrates, aspartate or glutamine[7]. Because of their dependence on exogenous L-asparagine, the cancerous Acute Lymphoblastic Leukemia cells, but not normal cells, can be starved and eliminated by L-asparaginase

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treatment which depletes the levels of L-asparaginase in circulating pools.

Based upon the therapeutic importance of L-asparaginase the present work was undertaken to optimize enzyme production parameters like the effect of incubation period, initial pH, incubation temperature, age of inoculums, inoculums level, agitation speed and aeration employing *Pseudomonas aeruginosa strain F1* by submerged fermentation. The effect of carbon sources, nitrogen sources on enzyme production was also evaluated.

MATERIALS AND METHODS

Microorganisms

The thermophilic bacterial species isolated from Taptapani hotspring of Odisha, India i.e. TPS-5 (wild strain) that Identified as *Pseudomonas aeruginosa strain F1* by 16S rDNA sequencing, produces L-asparaginase was employed in the present study. The slants were sub cultured at monthly intervals and stored at 4^0C in the refrigerator.

Cultivation medium and cultural conditions

The composition of initial cultivation medium was (g/l): L-asparagine - 4.0, Glucose - 2.0, $Na_2HPO_4.2H_2O$ - 6.0, KH_2PO_4 - 3.0, NaCl - 0.5, $MgSO_4.7H_2O$ - 0.5, $CaCl_2.2H_2O$ - 0.015, yeast extract - 1 .0, peptone - 1.0 and initial pH was maintained at 6.5[8]. The inoculum was prepared by adding loopful of selected isolates into sterilized medium specified above in a 250 ml Erlenmeyer (EM) flasks. The flasks were agitated at 150 rpm and incubated at 37°C for 12 h ($O.D._{600}$ nm=0.6-0.8) in an orbital shaker incubator[8]. The seed culture (2 %) was added into 50 ml of the medium in 250 ml EM flasks in an orbital shaker incubated at 37°C and 150 rpm for 36 h and the cells were then removed by centrifugation at 6000 × g for 10 min at 4°C. The supernatant collected was subjected to assay of extracellular L-asparaginase production.

Enzymatic Assay

L-asparaginase activity was measured by direct Nesslerization of ammonia. The activity of L-asparaginase was measured employing the modified method of Wriston[9]. The L-asparaginase catalyzes L-asparagine to Laspartic acid and ammonia and the latter react with the Nessler's reagent to produce an orange colored product. The enzyme assay mixture consisted of 100 µl of freshly prepared L-asparagine (189 mM) in Tris–HCl buffer (pH 8.6) and 100 µl of crude extract of the enzyme. The reaction mixture was incubated at 37^0C for 30 min and the reaction was stopped by adding 100 µl of 15% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 6,000×g for 5 min at 4^0C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 500 µl Nessler's reagent into the sample containing 200 µl supernatant and 4.3 ml distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min, scanned for λ_{max}. OD was measured at λ_{396} nm against the blanks that received TCA before the addition of crude enzyme. The ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One unit (U) of L-asparaginase activity was defined as the amount of the enzyme that liberates 1 µM of ammonia/ min at 37^0C.

Optimization of physical parameters

Effect of incubation period

To study the impact of incubation period on enzyme production the initial production media (*p*H 6.5) was inoculated and incubated. The samples were withdrawn at regular interval of 5 h and assayed for enzyme production. The optimum incubation period obtained in this experiment was used in the subsequent studies.

Influence of initial pH

To investigate the influence of initial pH on enzyme production by the organisms, the selected production medium was adjusted to various levels of pH (5.0-9.0). Fermentation was conducted and samples were assayed for L-asparaginase production pattern and biomass content. The optimum pH obtained in this study was used in the subsequent studies.

Effect of incubation temperature

To study the impact of incubation temperature on enzyme production and cell growth exhibited, the production medium was inoculated and incubated at various temperatures ranging from 20°C to 60°C. The samples were withdrawn after optimized period of incubation and assayed enzymatic activity. The optimal incubation temperature obtained at this level was used for further studies.

Effect of inoculums age

To study the effect of age of inoculum, 8, 12, 16, 20, 24 and 28 h old inocula were added at 2% w/v level (1 ml) to the flasks with production medium 49 ml. The fermentations and assays are conducted as per the general procedure. The optimum age of inoculums obtained was used in the subsequent studies.

Effect of Inoculums level

The flasks with the basal production medium were inoculated with 0.1, 1, 2, 4, 6, 8 and 10% inoculums and incubated and 5ml samples were withdrawn at 12 h intervals and examined for biomass (mg/ml) and enzyme activity. The optimal level of inoculums obtained was used in further experiments.

Effect of rate of agitation (rpm)

The effect of agitation (rpm) on L-asparaginase production was investigated by conducting the fermentations at different agitation speeds of 60, 90, 120, 150, 180 and 210 rpm at 40^0C for 25h. The optimal rate of agitation obtained in this study was used in further experiments.

Effect of aeration (medium volume to air-space)

To study the effect of aeration on L-asparaginase production, different volumes (25, 50, 75 and 100 ml) of production medium were taken in 250 ml EM flasks and fermentation was conducted and tested for L-asparaginase activity. The optimal medium volume to air-space in this study was used in further experiments.

Optimization of nutritional parameters

Effect of various carbon sources

To select the most suitable carbon source for maximizing L-asparaginase production, the isolated strain TPS-5, was transferred to EM flasks (250 ml) containing 50 ml basal semisynthetic broth medium[8], replacing one of the carbon source glucose (2 g/L), with other selected carbon sources (fructose, glucose, mannitol, sorbitol, sucrose, maltose, lactose, starch potato soluble, dextrin, molasses, methyl cellulose and ethyl cellulose) keeping the concentration of L-asparagine (4.0 g/L) unchanged. A control set was also run in parallel. The fermentations were conducted as per the conditions specified above. The optimized carbon source found in this study was used in further experiments.

Effect of nitrogen sources

The selections of best nitrogen sources was carried by measuring the enzyme activity by growing the microorganism in two sets of experiment in the basal semisynthetic broth medium[8] containing yeast extract and peptone, each 1 g/L respectively. In the first set of experiment peptone is used as fixed component and yeast extract was substituted and in the second set yeast extract remains unchanged and peptone was replaced with different nitrogen sources viz., para amino benzoic acid (PABA), gelatin, beef extract, casein enzyme hydrolysate and tryptone. A control set was also run in parallel. Fermentation was conducted and enzymatic activity was assayed. The optimized nitrogen sources found in this study was used in further experiments.

RESULTS AND DISCUSSION

The effect of Incubation period on enzyme production profile was evaluated in *Pseudomonas aeruginosa strain F1* is presented in Fig. 1. Optimum enzyme production was detected after 25 h of incubation (28.58 IU/ml). After that the L-asparaginase production decreased gradually with increased incubation periods.

The effect of initial medium *p*H on enzyme production profile at different *p*H values was evaluated in *Pseudomonas aeruginosa strain F1* is presented in Fig. 2. Optimum enzyme production was detected at *p*H 6.5 (35.2 IU/ml). The organism has produced less amounts of L-asparaginase in highly acidic and alkaline conditions.

The results on incubation temperature (Fig. 3) indicated that the optimum enzyme activity (38.96 IU/ml) was obtained at 40°C, while a gradual decrease in enzyme activity was observed later.

The effect of inoculums age on L-asparaginase production is indicated in Fig. 4. The highest enzyme activity (39.78 IU/mL) was recorded with 16h inoculum age.

The impact of inoculums size on L-asparaginase production is indicated in Fig. 5. The highest enzyme activity (41 IU/ml) was recorded with 6 % w/v inoculums level. There was a gradual decrease in the yield by increasing and decreasing the inoculums level.

The impact of agitation speed on L-asparaginase production is indicated in Fig. 6. The highest enzyme activity (41.65 IU/ml) was obtained with an agitation speed of 150 rpm. The decrease

in L-asparaginase activity was observed at both lower (60, 90, 120) and higher agitation speeds (180, 210) respectively.

The impact of medium volume to air-space on L-asparaginase production is indicated in Fig. 7. The results indicated that the maximum L-asparaginase activity (41.89 IU/ml) was obtained with 50 ml of production medium in 250 ml EM flask. This is 80% air space in flask.

Optimization of nutritional parameters

Effect of various carbon sources

The results indicated lactose to be the best carbon source followed by L-asparagine, glucose and molasses (44.02, 42.9, 41.76 and 36.9) exhibiting enzyme stimulation by the isolate. The other carbon sources sorbitol, mannitol, starch

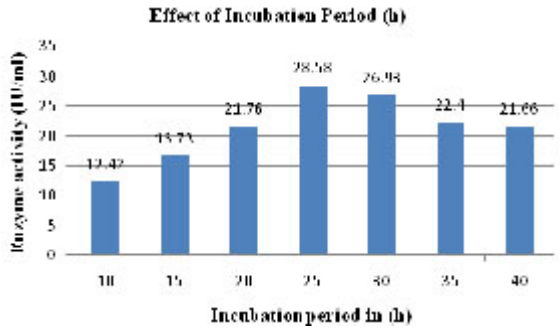

Fig. 1. Effect of Incubation period on enzyme production

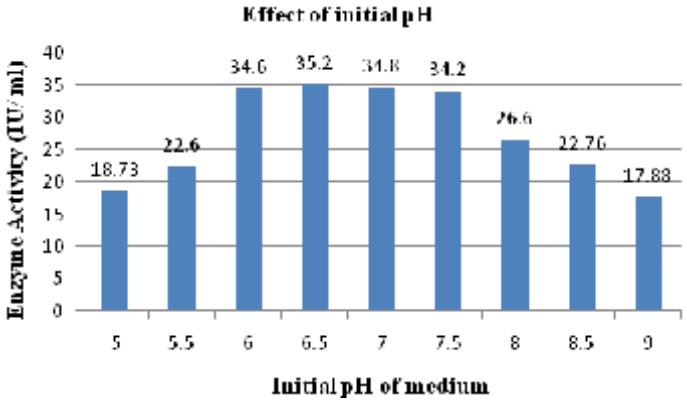

Fig. 2. Effect of initial medium pH

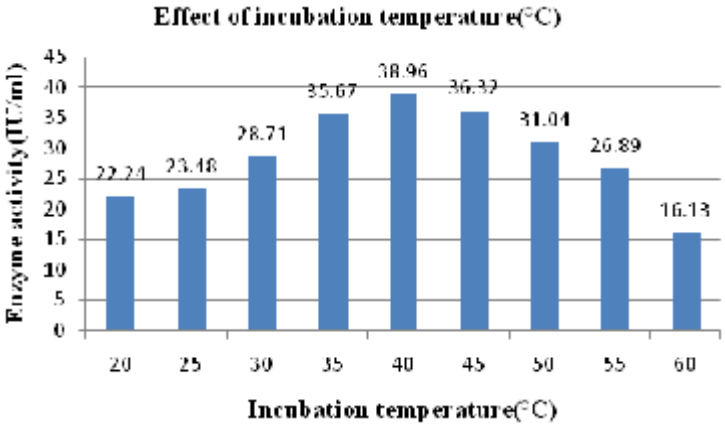

Fig. 3. Effect of incubation temperature

potato soluble, fructose, dextrin, sucrose, maltose, ethyl cellulose and methyl cellulose (27.48, 26.06, 25.3, 24.5, 23.9, 22.81, 21.74, 16.5 and 13.93) did not influence the enzymatic yield.

L-asparaginase is inducible enzyme and is generally induced in the presence of glucose (0.2%) as carbon source. The effect of glucose on optimum enzyme production has been reported by many workers[10]. Glucose was also regarded as a repressor for L-asparaginase production in bacteria[11, 12]. However the findings of the present investigation revealed lactose to be the best carbon source in combination with L-asparagine to develop an economically viable process. The production of the enzyme is significantly influenced by L-asparagine as one of the chief carbon

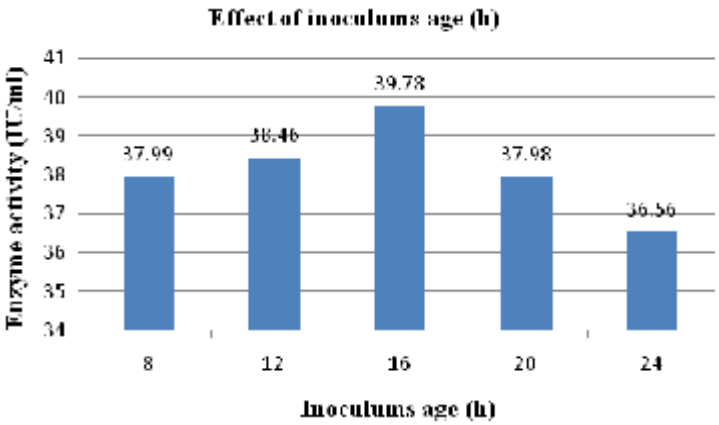

Fig. 4. Effect of inoculums age

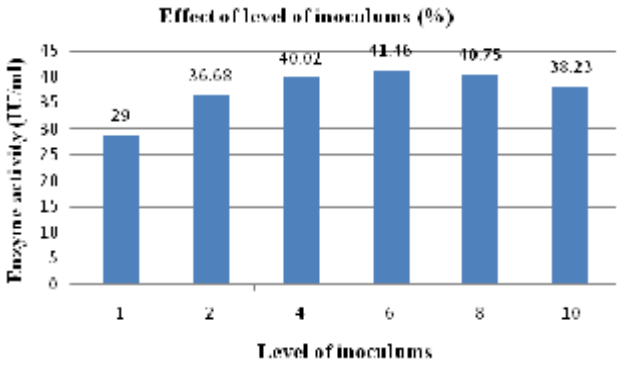

Fig. 5. Effect of Inoculums level

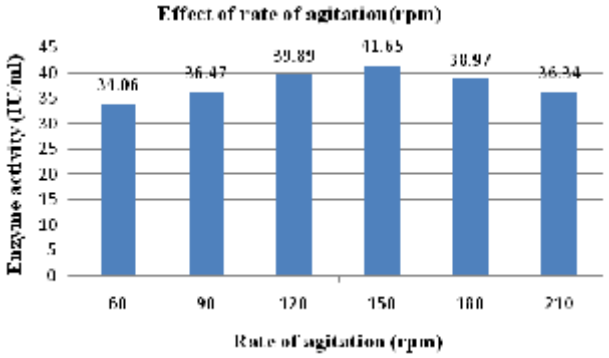

Fig. 6. Effect of rate of agitation (rpm)

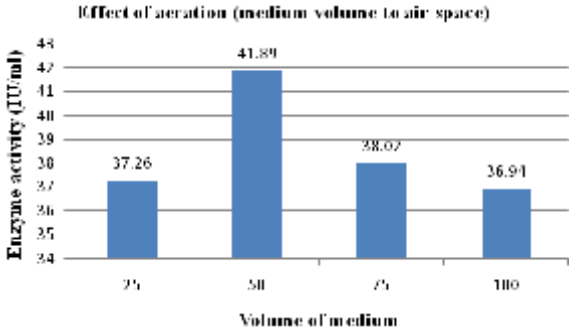

Fig. 7. Effect of aeration (medium volume to air-space).

components in the medium as evidenced by our isolate and other reported microorganisms[2,8,13].

Effect of various nitrogen sources

The findings of the first set of fermentation experiments keeping peptone (1g/l) as fixed component and substituting yeast extract (1g/l) with selected nitrogen sources, did not register any enhancement of enzymatic activity (IU/ml), rather the activity was inhibited, with marked inhibition shown by peptone (9.8) alone followed by the combined regimen peptone + beet extract (18.9), peptone + gelatin (20.16), peptone + casein hydrolysate (20.60), peptone + tryptone (22.6) peptone + beef extract (24.07) and peptone + PABA (26.12) respectively.

The second set of fermentation experiments was run by unultering yeast extract (1g/l) and substituting peptone (1g/l) with selected nitrogen sources, registered marked stimulation of enzymatic activity. The highest enzymatic activity (IU/ml) was exhibited by the combined regimen of yeast extract + beef extract (43.4) followed by yeast extract + tryptone (40.2), yeast extract + beet extract(39.03), yeast extract + casein hydrolysate (38.5), yeast extract + PABA (37), yeast extract + gelatin (36.7) and yeast extract (34.4).

The findings of the fermentation experiments revealed that yeast extract alone or in combination with selected nitrogen sources (substituting peptone in the basal semisynthetic medium), registered marked stimulation of enzymatic activity. However L-asparaginase activity was not influenced by peptone alone or in combination of peptone and other selected nitrogen sources, replacing yeast extract. The production of the enzyme is reported to be influenced by alternate nitrogen sources viz., yeast extract, tryptone, peptone, casein, soyabean meal and monosodium glutamate into the medium[14, 15] but yeast extract in combination with beef extract were found to be the best nitrogen sources for L-asparaginase production by our isolate.

CONCLUSION

The isolated *Pseudomonas aeruginosa strain F1* was found to be a potential producer of enzyme L-asparaginase. The results indicated that the enzyme production is influenced by the physiological conditions and nutritional parameters. The maximum enzymatic activity was obtained after 25 h of incubation (28.58 IU/ml) with an initial pH 6.5 (35.2 IU/ml), incubation temperature 40°C (38.96 IU/ml), inoculums age of 16h (39.78 IU/ml), inoculums size of 6 % w/v (41 IU/ml), an agitation speed of 150 rpm (41.65 IU/ml) and 80% air space (41.89 IU/ml). Lactose is the best carbon source in combination with L-asparagine to develop an economically viable process. Yeast extract in combination with beef extract were found to be the best nitrogen sources for L-asparaginase production by our isolate.

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