Abstract

Aspartase producing bacterial strains were isolated from the effluent of a fertilizer industry. The effluent samples were collected periodically and studied for physico-chemical characteristics. The effluent was alkaline in nature and rich in nitrogenous contents. Heavy metals (zinc and mercury) and phenolic compounds were absent in the effluent samples. Biochemical oxygen demand and chemical oxygen demand were within the permissible limits for the discharge of industrial effluents in India. Aspartase producing bacterial strains were isolated by enrichment technique. Screening of isolated strains for aspartase activity has shown isolate NFB-5 to be the most efficient aspartase producer. This isolate showed higher aspartase activity (48.22±0.324 U/g wet wt) as compared to reference strains of *Escherichia coli* (18.83±0.326 U/g fresh biomass) and *Bacillus cereus* (17.94±0.416 U/g wet wt). The genus of bacterial isolate NFB-5 was identified by morphological, physiological and biochemical tests. Isolate NFB-5 was found to be Gram-negative, facultatively anaerobe, motile, non-spore-forming and rod-shaped. The isolate showed optimal growth at 37°C. It grew in a pH range of 5.0-11.0 and shown optimal growth at pH 6.8-7.0. The isolate was able to grow in the presence of lowest concentration (2.5%) of sodium chloride, beyond that no growth was observed. The isolate showed positive results for casein hydrolysis, starch hydrolysis, nitrate reduction, cytochrome oxidase, catalase test, gelatin hydrolysis test, arginine dihydrolase and growth on MacConkey agar. Out of the 19 carbohydrates tested for fermentation by isolate NFB-5, 13 carbohydrates yielded positive test results including cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, D-maltose, D-mannitol, D-melibiose, L-rhamnose, D-sucrose, D-trehalose, D-xylose and salicine. Species specific identification was done by
16S rDNA gene sequencing and phylogenetic analysis. Genomic DNA isolation protocol for *Aeromonas media* NFB-5 was standardized. Agarose gel electrophoresis showed satisfactory quantity and quality of DNA preparation. 16S rDNA gene sequence of the isolate NFB-5 was amplified with bacterial universal primers and nucleotide sequence of 1507 bp was obtained. The comparative analysis and homology (using nucleotide blast-NCBI) of the 16S rDNA gene sequence showed 98% similarity with the reference sequence of *Aeromonas media* (FJI68773). The phylogenetic analysis identified the isolate as *Aeromonas media* NFB-5, a motile member of aeromonads of family *Aeromonadaceae* which are ubiquitous inhabitants of fresh water and estuarine environments.

Aspartase gene (*aspA*) from *A. media* NFB-5 was amplified successfully and sequenced for nucleotides. The aspartase coding region was amplified in parts i.e. central conserved region, C-terminal region and N-terminal region. The central conserved region was amplified with degenerate primers while C-terminal region and N-terminal region was amplified with combinations of primers designed from *E. coli* aspartase and sequence obtained from central conserved region. The coding sequence of 1424 bp was amplified, cloned and sequenced successfully. Based on the nucleotide sequence obtained, primers were designed to amplify the whole length coding sequence by single pair of primers. The gene sequence of 1424 bp encoding a protein of 474 amino acid residues was obtained. The amino acid sequence of the enzyme showed a high degree of identity (91%) to aspartase from *E. coli* but differed from aspartases from other microorganisms. *Aeromonas media* aspartase contained 30 Ile (6.3%) residues as compared to aspartase from mesophiles like *E. coli* (32 residues; 6.69%) and *P. fluorescens* (31 residues; 6.49%). The Ile/Ile + Val + Leu ratio of *Aeromonas*
media (0.252) aspartase is comparable to that of *E. coli* (0.269), equivalent to *P. fluorescens* aspartase (0.252) and to some extent, lesser than aspartase from thermophilic *Bacillus* sp. YM55-1 (0.360). Aspartase gene (*aspA*) was cloned and expressed in *Escherichia coli* BL21 using pET21b(+) expression vector. Maximum production of aspartase was obtained at shake-flask after 5 h of IPTG (1.5 mM) induction and by supplementing the media with KH₂PO₄ (0.3%, w/v) and K₂HPO₄ (0.3%, w/v). Further production was investigated at a laboratory scale stirred tank reactor using response surface methodology (RSM). Agitation (130-270 rpm), aeration (0.30-1.70 vvm) and IPTG induction time (3-7 h) was optimized. Optimal levels of agitation (250 rpm), aeration (1.25 vvm) and induction time (6 h) were determined by statistical analysis of the experimental data. More than 7-fold increase in recombinant aspartase (1234 U/g wet weight) was observed than the parent strain (172 U/g wet wt).

The recombinant aspartase was purified by single-step purification technique using His-trap affinity column chromatography. The cloned gene (*aspA*) product revealed the molecular weight of approx 51 kDa by SDS-PAGE, which was in corroboration with the molecular weight calculated from putative amino acid sequence. The purified aspartase showed optimum pH of 8.5 and 8.0 in the presence and absence of magnesium ions, respectively. The enzyme was stable in pH range of 6.5-9.5 and temperature up to 45°C. The optimum temperature was determined to be 35°C. Temperature quotient for 20-30 °C and 25-35 °C was determined to be 1.315 and 1.247, respectively. Activation energy for aspartase was found 37.122 kJ/mol. At optimal temperature aspartase showed half-life of 12.03 min. The change in enthalpy, Gibbs free energy and entropy was found to be 34.561 kJ/mol, 63.961 and -95.45 J/mol/K, respectively. Apparent *Kₘ* and *Vₘₐₓ* values for L-aspartate were 2.01 mM and
114 U/mg, respectively. Divalent metal ion requirement of enzyme was efficiently fulfilled by $\text{Mg}^{2+}$, $\text{Mn}^{2+}$ and $\text{Ca}^{2+}$ ions.

Among the different agents screened for permeabilization of recombinant *E. coli* cells, Triton X-100 (0.2%, v/v) was found to be most efficient. Different solvents (isopropanol, ethanol and chloroform) were not found effective as Triton X-100. Optimal permeabilization was obtained by 40 min incubation at 37°C. Permeabilized free and homogenized immobilized cells were studied for L-aspartic acid synthesis. Production of L-aspartic acid was studied at 37°C for 60 min under shaking conditions. HPLC analysis of samples revealed higher production of L-aspartic acid (566 mg/g wet wt) from homogenized immobilized cells as compared to free cells (154 mg/g wet wt). Homogenized immobilized cells packed in a column resulted in lowest yield of L-aspartic acid (41.5 mg/g wet wt) which shows agitation has a significant impact on the production of aspartic acid from recombinant cells.

Limited reports are available on aspartase. Despite of limited information, aspartase is still considered as an industrially important enzyme. A new potential bacterial source (*Aeromonas media* NFB-5) of aspartase has been isolated and characterized. The aspartase gene was expressed in recombinant *E. coli* followed by its purification and characterization for the first time. The whole cells of recombinant *E. coli* have also been studied for production of L-aspartic acid that is important amino acid in food and pharmaceutical industries. Introduction of non-sugar or artificial sweeteners in food and medicinal preparations hikes the demand of L-aspartic acid. Aspartase has been found an efficient biocatalyst for cost effective production of L-aspartic acid and to be compatible with market.