

Studies on Nitrilase of *Gordonia Terrae* and its Application in Synthesis of Non-Proteinogenic Amino Acids

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ABSTRACT

Nitriles are potential pollutants of soil and industrial waste waters. The use of these toxic compounds, particularly as solvents and as pesticides, has led to their wide distribution in the environment. Nitrilase (EC 3.5.5.1) catalyzes the conversion of nitriles directly into the corresponding carboxylic acids and ammonia. Amino acids are the building blocks of proteins. All organic compounds which contain both amino (NH₂) and carboxylic (COOH) groups are called amino acids. All amino acids (except glycine) contain an asymmetric carbon atom and hence are optically active. Optically active amino acids are of two types namely L-amino acids and D-amino acids. The L- α amino acids are the building blocks of proteins in living organisms and are called proteinogenic amino acids. The rest of the amino acids either occurring naturally or synthesized in the laboratory are known as non-proteinogenic amino acids. They are also sometimes referred to as unnatural or rare amino acids. Due to their structural diversity and functional versatility they are widely used as chiral building blocks and important tools in modern drug discovery research. In the field of protein engineering, non-natural amino acids can be incorporated into proteins in order to study protein structure and function. They can also be used in order to create drugs or other bioactive molecules that will not be degraded as quickly as natural amino acids by protease enzymes.

The α -aminonitriles are intermediates in the synthesis of racemic amino acids. The enzyme nitrilase converts α -aminonitriles to corresponding α -amino acids. A number of α -amino acids are produced by the asymmetric hydrolysis of α -amino nitriles using nitrilase of *Rhodococcus rhodochrous* PA-34. An actinobacterium, *Gordonia terrae*, has been isolated in our laboratory that exhibited high nitrilase activity. Nitrilase of this organism has been reported to convert 3-cyanopyridine to nicotinic acid and some

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cyano group containing herbicides to corresponding acids. However, comparatively fewer efforts have been made in the past to explore potential of nitrilase to convert aminonitriles to amino acids. Therefore, the present research work entitled 'Studies on nitrilase of *G. terrae* and its application in synthesis of non-proteinogenic amino acids' has been undertaken with the following objectives: 1) To optimize conditions for production of nitrilase of *G. terrae* to catalyse the conversion of α -aminonitriles to optically active amino acids; 2) To optimize process parameters for bioconversion of α -aminonitriles to optically active amino acids by using resting cells of *G. terrae*; 3) To purify and characterize nitrilase of *G. terrae*; 4) To immobilize *G. terrae* whole cells in agar and use of these agar entrapped cells in biotransformation of α -aminonitriles to optically active amino acids; and 5) To clone, sequence and express nitrilase gene of *G. terrae* in *E. coli*.

G. terrae (previously *Rhodococcus* sp. NDB 1165) isolated in our laboratory is a nitrile metabolizing microorganism. Depending on the inducer present in the growth medium, this organism expresses nitrile metabolizing enzymes. In the present study the nitrilase of this organism has been explored for enzyme catalyzed production of non-proteinogenic amino acids from their corresponding nitriles. The culture conditions for *G. terrae* were optimized. It was revealed that *G. terrae* grows better in the medium M4 containing (g L^{-1}): 10 g glycerol, 5 g peptone, 3 g malt extract and 3 g yeast extract (pH 8.0). The organism grows best at 30°C. Nitrilase of *G. terrae* was an inducible enzyme and expressed in the presence of nitriles. The screening of inducers showed that isobutyronitrile was a better inducer for the expression of nitrilase in *G. terrae*. The inoculum size of 4% (v/v) was best suited for expression of nitrilase of *G. terrae*. The growth kinetic studies of *G. terrae* revealed that uninduced cells attained stationary phase in 18 h whereas induced cells entered the stationary phase in 30 h. No activity was expressed in absence of inducer (nitrile).

Multiple feeding of isobutyronitrile in the medium to increase its concentration exponentially resulted in high level expression of nitrilase ($1.72 \text{ U mg dcw}^{-1}$, 2.05 U ml^{-1}) in short incubation time (30 h). The specific activity increased by almost 54% and total activity increased by 2.8 times as compared to single induction with isobutyronitrile. The nitrilase of *G. terrae* showed higher specific activity towards following aminonitriles (phenylglycinonitrile > 3-aminobenzonitrile > 4-aminophenylacetoneitrile > 4-aminobenzonitrile). The reaction conditions were optimized for conversion of DL α -phenylglycinonitrile to D-phenylglycine and 3-aminobenzonitrile to 3-aminobenzoic acid. The best optimized reaction conditions for D-phenylglycine production are at pH 7.0 (0.1M potassium phosphate buffer) and 30°C. The K_m and V_{max} for D-phenylglycine were 7.6mM and 8.2 U mgdcw^{-1} . The highest nitrilase activity was with 50mM substrate. In case of 3-aminobenzoic acid production the optimized pH and temperature were pH 10.5 (0.1M carbonate buffer) and 30°C respectively. The K_m was 4.5 mM and V_{max} was $0.86 \text{ U mgdcw}^{-1}$.

The nitrilase of *G. terrae* was partially purified through ammonium sulphate precipitation and gel permeation chromatography to 8.3 fold with a specific activity of 12.5 U mg protein⁻¹. In SDS-PAGE analysis the molecular weight of the purified nitrilase corresponds to 40 kD. The partially purified nitrilase of *G. terrae* was active from pH 6.0 to 8.0 the optimum pH was 7.0. The temperature optimization studies revealed that the best activity was recorded at 30°C. The thermostability studies revealed that this enzyme had better stability below 30°C. MALDI-TOFF analysis of the purified nitrilase showed complete similarity of the peptide fragments with *R. rhodochrous* tg1-A6 nitrilase. The agar concentration of 1.5% w/v and cell concentration of 1 mgdcw ml⁻¹ were most appropriate for *G. terrae* whole cell immobilization. The pH of 7.0 (0.1 M potassium phosphate buffer) and 40°C was best suited for the immobilized cells. The agar discs showed maximum stability at 35°C. The immobilized cells were reusable for upto six cycles and retained their activity. The nitrilase gene was amplified using primers based on the nitrilase gene sequence of *R. rhodochrous* PA-34. The size of the PCR product was 1.2 kb. It was sequenced and it showed significant similarity with the previously reported nitrilases of *R. rhodochrous* J1 and *R. rhodochrous* tg1-A6. The PCR product was then used for cloning using Novagen kit with pET-46 Ek-LIC as cloning and expression vector. The PCR product was ligated with this vector and initially cloned in *E. coli* novagiga cells (Novagen). The plasmids were isolated from transformed cells and their analysis revealed an increase in size thereby indicating successful cloning of nitrilase gene in *E. coli*. This was further confirmed when these plasmids were used as templates for PCR amplification using nitrilase primers. Successful amplification of 1.2 kb segment indicated that the plasmids contained the gene of interest.

The sequence analysis of translated nucleotide sequence revealed that the nitrilase of *G. terrae* had approximately 365 amino acids. The deduced molecular weight using ExPASy tools is 40 kD. The nitrilase of *G. terrae* also has the conserved catalytic triad consisting of Glu-48, Cys-165 and Lys-131 which is present in all nitrilases. The other conserved amino acid residues are Ala -11 and 278; Gln- 14; Pro-47, 259 and 55; Glu-48, 106, 138 and 167; Tyr- 54 and 106; Arg-130, 139 and 300; Lys- 131; Gly- 103, 145 and 296; Cys-165; Trp-166; Val-222; Thr-135; Asp-281, 293 and 302; His- 297. All this data suggests that the nitrilase of *G. terrae* is both aliphatic and aromatic in nature. The nitrilase gene was then expressed in *E. coli* BL-21. The isolated plasmids from the cloning experiments were inserted in competent cells of *E. coli* BL-21 using heat shock method. IPTG was used as an inducer. The transformed cells on analysis for nitrilase activity showed positive results. A number of factors were optimized for best expression of nitrilase gene in the transformed cells and best activity (0.5 U mgdcw⁻¹) was reported at 25°C and with 1.0 mM IPTG concentration with an incubation period of 10 h. The results reported in the thesis will be immensely useful in developing enzymatic process for the production of optically active non-proteinogenic amino acids.