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PARTIAL PURIFICATISON AND CHARACTERIZATION OF 3-CYANOPRIDINASE RESPONSIBLE FOR PRODUCTION OF NICOTINIC ACID

Tesnim Arfi, Lovely Singh, Dhirendra Prabhakar and V. K. Nigam*

Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi - 835215, Jharkhand, India.

Keywords:

Biotransformation, 3-Cyanopyridine, Nicotinic Acid, Nitrilase, Purification

Correspondence to Author:
Dr. V. K. Nigam

Department of Bio-Engineering
Birla Institute of Technology,
Mesra, Ranchi - 835215,
Jharkhand, India.

E-mail: vknigam@bitmesra.ac.in

ABSTRACT: A thermostable bacterial 3-cyanopyridinase enzyme has been identified that catalyze 3-cyanopyridine to nicotinic acid and ammonia. Nicotinic acid is mainly used in food, pharmaceutical and biochemical industries. This study was undertaken to optimize the purification of 3-cyanopyridinase enzyme and characterization of its various kinetic properties. The partial purification of enzyme has been carried out in five steps that results in approximately 2.3 fold increased purification with 46.5 % of enzyme recovery. The partially purified enzyme was observed approximately 43 kDa of maximum activity at 50 °C temperature and pH 7.0. The partially purified enzyme showed the K_m of 0.91 mM and maximum velocity (V_{max}) of 0.59 U. The half-life ($t_{1/2}$) of partially purified enzyme was about 60 min at optimal conditions. The enzyme has broad substrate specificity, hydrolyzing toxic compounds such as benzonitrile and propionitrile. Thus this novel enzyme has potential in bioremediation process.

INTRODUCTION: Nitrilases or the nitrile degrading enzymes are important class of hydrolytic enzymes that belongs to the nitrilase super family. They are found in prokaryotes as well as in eukaryotes, but mostly known nitrilases are isolated from bacteria, fungi and plants[1]. Nitrilases are commercially important enzymes because of their utilization in the production of large number of chemically important compounds such as acrylic acid, Indole-3-acetic acid, nicotinic acid, mandelic acid [2, 3, 4, 5] and bioremediation of toxic nitriles as well as in herbicide degradation etc. Nitrile compounds can be directly converted to carboxylic acids and ammonia by nitrilase enzyme expressed in certain microorganisms or it can be converted to amide (an intermediate product) through nitrile hydratase followed by synthesis of carboxylic acid and ammonia by enzyme amidase [6, 7].

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Fig. 1 represents different pathways involved in biotransformation of a cyanide group containing compound.

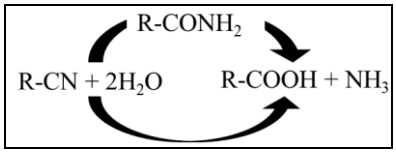

FIG. 1: THE REPRESENTATION OF TWO PATHWAYS INVOLVED IN THE DEGRADATION OF NITRILES

Nitrilases have broad range of substrate specificities which make them useful for hydrolysis of wide range of nitrile compounds. These enzymes are used in organic synthesis utilizing their enantio, regio- and chemo- selective properties. Therefore, due to their wider applicability in different perspective, they are considered as 'Green Catalyst [8, 9]. 3-cyanopyridine is a nitrile compound which hydrolyses into nicotinic acid (also known as vitamin B_3, niacin or vitamin PP) and ammonia in presence of 3-cyanopyridinase enzyme, a type of nitrilase enzyme [10, 11, 12]. Niacin is associated with a pandemic deficiency disease known as pellagra. The nicotinic acid is used in animal feed supplementation, in medicine and as a bio

stimulator for the formation of activated sludge and also as a deodorant for air and waste gases in control of pollution[13].

It has been reported that nitrilases produced from thermophilic sources have substantially higher thermal stability than those produced from mesophillic sources [14, 15]. The purification of nitrilase was carried by various protocols, since the enzyme is intracellular in nature and hence, the release of enzyme to the extra cellular environment with minimum activity loss is very crucial and that can be done using high pressure homogenizer [16] or an ultrasonicator [17].

The current research described the partial purification of 3-cyanopyridinase from *Bacillus* sp. MTCC 7545 and explored its physicochemical characteristics and potential biotransformation ability.

MATERIALS AND METHODS:

Microorganisms and culture conditions: The *Bacillus* sp. MTCC 7545, an 3-cynopyridinase producing bacteria was maintained on LB agar plates in presence of 10 mM adiponitrile as inducer, while the production of enzyme was carried in shake flasks containing mineral base media (1 g/L yeast extract, 1 ml/L trace elements, 200 ml/L mineral base, 10 ml/L glycerol) and 10 mM benzonitrile as inducer [18]. The bacterial cells were grown at pH 7.0 at 45 ˚C for 24 hours under agitation speed of 175 rpm.

Purification of Enzyme: The cells were harvested from production medium by centrifugation at 10,000 rpm at 4 ºC for 10 min and the cell pellet was washed twice with 0.1 M phosphate buffer (pH 7.0) containing 0.5 mM EDTA. Finally, cells were suspended in the same buffer and optical density of 2.0 at 600 nm was adjusted for carrying out enzyme activity and purification.

Step 1- Preparation of cell-free extract: In this process, 15 ml of cell suspension was disrupted at high frequency through ultrasonicator for different time durations (5 min, 7.5 min, 10 min and 15 min at pulse: 30 second on & off; amplitude: 20 kHz) for the preparation of cell-free extract. After sonication, the cell debris was removed by centrifugation (10000 rpm; 10 min.; 4˚C) and supernatant was tested for 3-cyanopyridinase activity.

Step 2- Concentration of protein: The supernatant obtained after centrifugation was concentrated through centrifugation at 5000 rpm at 4 ºC for 20 min by Amicon ultra filtration with the filtration cut off range of 10 kDa. The concentrated protein was used for the further analysis.

Step 3- Ammonium sulfate precipitation and dialysis: The concentrated supernatant was saturated fractionally by ammonium sulfate using the formula

$$\%\ saturation = \frac{533(S2 - S1)}{100 - (0.3 * S2)}\ g/L$$

Firstly, it was saturated in 0-20 % of ammonium sulfate and then different percentage range like 20-40%, 20-60 % and 40-80% was attempted for extracting maximum enzyme protein. The fraction with maximum precipitated enzyme protein was dialyzed overnight against 10 times diluted phosphate buffer (0.01M) on magnetic stirrer at 4 ºC.

Step 4- Sephacryl S-200 Column Chromato-graphy: 1 ml of dialyzed sample was passed through sephacryl S-200 column[19] which was pre-equilibrated with 100mM phosphate buffer and fractions were eluted at the rate of 1ml/ min using Phosphate buffer (pH 7.0) as eluting solvent. Different fractions collected were subjected to the enzyme assay as well as measuring protein concentration against bovine serum albumin standard plot. The active fractions were pooled, further dialyzed overnight against phosphate buffer 0.01 M (pH 7.0) at 4 ºC and further concentrated by amicon ultrafiltration.

Step 5- DEAE ion exchange column chromate -graphy: The concentrated fraction obtained from sephacryl S-200 column chromatography was then passed through the DEAE sepharose column equilibrated with 0.1M phosphate buffer containing 0.2M NaCl [20]. Fractions were eluted in phosphate buffer at a flow rate of 1ml/min. The active fractions were pooled, concentrated and stored at 4 ºC. Polyacrylamide gel electrophoresis was performed to determine the molecular weight of the purified enzyme.

Nitrilase assay: 3-cyanopyridinase assay was performed in 1 ml reaction mixture that includes 0.1 ml purified enzyme, 0.1 ml substrate (3-cynopyridine; final concentration 10mM) and 0.8 ml 0.1M phosphate buffer (pH 7.0). The enzyme-substrate reaction was incubated at 50 ºC for 2 h to monitor the nicotinic acid production and release of ammonia. The liberated ammonia was quantified by using Berthelot's reagent and confirmed spectrophotometrically at 640 nm [21]. Enzyme activity is expressed as "U" and is defined as millimoles of ammonia released per ml per minute under the optimal conditions.

Protein assay: The amount of protein was estimated according to Lowry's protocol by using Bovine Serum Albumin (BSA) as standard [22].

Characterization of purified enzyme: The partially purified 3-cyanopyridinase was characterized for its optimum temperature in the temperature range of 40 - 70 ºC keeping other parameters constant. Likewise, the optimum pH for the maximum activity was studied in the pH range of 4.0-10.0. Thermal stability, substrate specificity, effect of metal ions on the enzyme activity and other enzyme kinetics were also assessed.

Gel electrophoresis: To determine the molecular weight of 3-cyanopyridinase enzyme, 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis [23] was performed. Protein markers of 14-80 kDa range (BioLit SRL, Mumbai) along with different fractions of purified proteins were loaded in the lanes and protein bands were visualized by Coomassie Brilliant blue R-250 staining.

RESULTS:

Nitrilase purification: The purification of any enzyme requires the use of various chromatographic techniques. Whenever, the enzyme is intracellular, it needs to be released from the cells to exterior environment. The *Bacillus* sp. MTCC 7545 produced intracellular enzyme which is responsible for conversion of 3-cyanopyridine to nicotinic acid in a single step pathway. The table 1 describes the optimization of lysis time for maximum release of enzyme in terms of activity and proteins. The observation **Table 1** showed that the maximum enzyme activity was recorded when the cell suspension was lysed for 5 min duration.

TABLE 1: OPTIMIZATION OF CELL LYSIS TIME WITH ENZYME ACTIVITY

Lysis time	Enzyme Activity (U)	Protein Content (mg/ml)
0 min	2.485	--
5 min	3.36	0.395
7.5 min	1.26	0.410
10 min	0.41	0.428
15 min	0.005	0.472

Concentration of 3-cyanopyridinase and dialysis: The concentration of enzyme protein was performed by ammonium sulfate precipitation as shown in **Table 2**. It has been noticed that enzyme protein was precipitated to maximum extent in 20-60% ammonium sulfate saturation. The fractionated protein (20-60 %) was dialyzed using 0.01M phosphate buffer overnight on a magnetic stirrer at 4 ºC.

TABLE 2: OPTIMIZATION OF FRACTIONAL SATURATION OF AMMONIUM SULFATE

Ammonium sulfate	Protein concentration (mg/ml)
0-20%	0.003
20-40 %	0.024
20-60%	1.05
20- 80%	0.433

Gel filtration column chromatography: The dialyzed enzyme protein fraction was passed through gel filtration column chromatography (Sephacryl S-200) and fractions eluted with 0.01M phosphate buffer is presented in **Fig. 2**. Fractions with maximum 3-cyanopyridinase activity were pooled and concentrated for further purification.

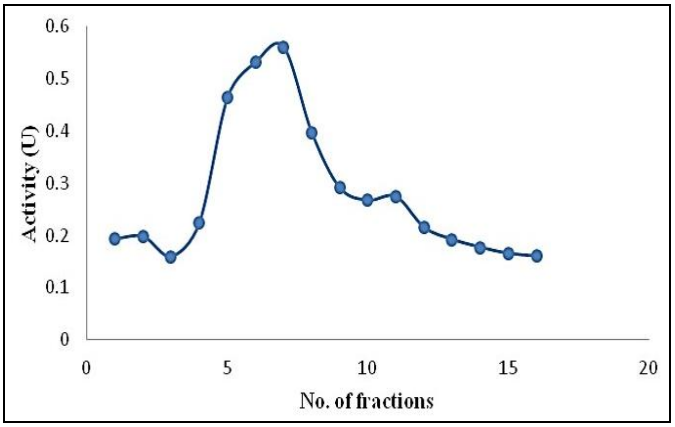

FIG. 2: ELUTION OF 3-CYANOPYRIDINASE ENZYME AND THEIR ENZYMATIC ACTIVITY. THE GRAPH SHOWS ENZYME ACTIVITY OF 16 FRACTIONS ELUTED THROUGH GEL FILTRATION (SEPHACRYL S-200) COLUMN CHROMATOGRAPHY

Diethyl amino ethyl Ion exchange chromate graphy: The 3-cyanopyridinase enzyme protein was further subjected to ion exchange chromatographic column equilibrated with phosphate buffer (0.1M) containing 0.2M NaCl and different fractions eluted were assayed for enzyme activity as shown in **Fig. 3**. The observation revealed that fraction number eleven showed highest nitrilase activity. The fraction was concentrated by amicon ultrafiltration as mentioned earlier and molecular weight of target enzyme was determined by polyacrylamide gel electrophoresis against protein ladder.

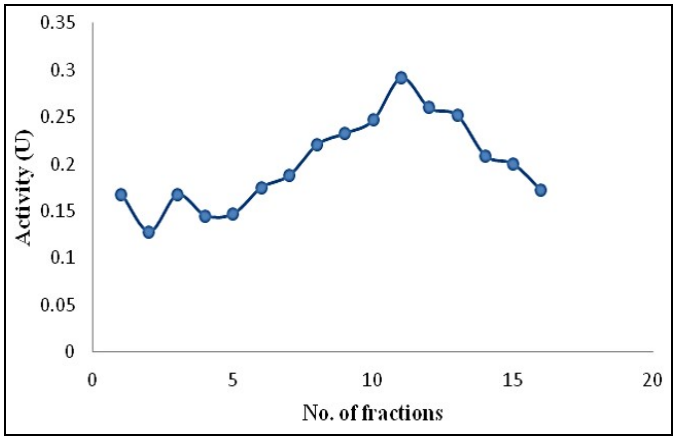

FIG. 3: ELUTION OF 3-CYANOPYRIDINASE ENZYME AND THEIR ENZYMATIC ACTIVITY. THE GRAPH SHOWS ENZYME ACTIVITY OF 16 FRACTIONS ELUTED THROUGH ION EXCHANGE (DEAE) COLUMN CHROMATOGRAPHY

Molecular mass determination: Gel electro phoresis was performed with different enzyme protein samples; like clarified supernatant of cell lysis, concentrated protein sample from amicon, ammonium sulfate saturated protein followed by and different fractions of column chromatography on 12 % SDS-polyacrylamide gel. After completion of electrophoresis, the bands were visualized by standard protocol and a molecular mass of about 43 kDa was found for 3-cyanopyridinase enzyme as shown in **Fig. 4.**

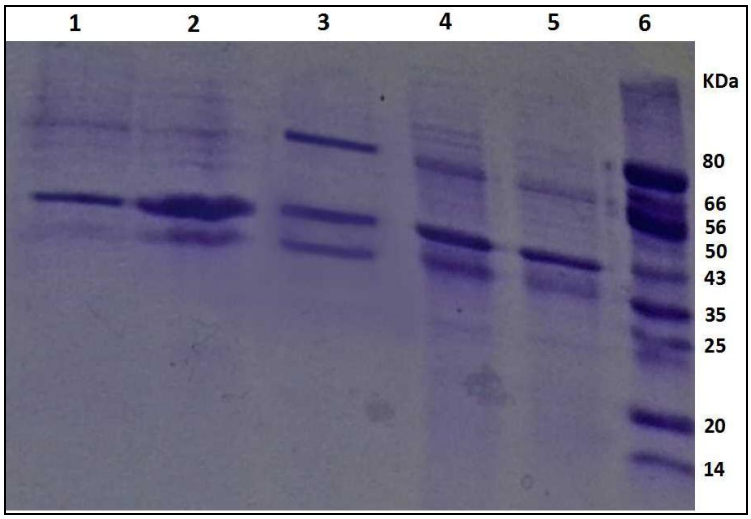

FIG. 4: GEL ELECTROPHORESIS OF 3-CYNOPYRIDINASE ENZYME. LANE 1: FRACTION OF DEAE ION EXCHANGE COLUMN, LANE 2: 7TH COLUMN FRACTION OF SEPHACRYL S-200, LANE 3: 20-60 % AMMONIUM SULPHATE PRECIPITATED AND DIALYZED FRACTION, LANE 4: AMICON CONCENTRATED CRUDE ENZYME, LANE 5: CRUDE ENZYME, AND LANE 6: PROTEIN MARKER.

The summary of enzyme protein purification (20ml) is shown in **Table 3** which resulted in 2.3 fold purification of protein with a recovery of 46.5%.

TABLE 3: SUMMARY OF PURIFICATION OF 3-CYANOPYRIDINASE

Sample	Activity (U)	Protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Crude lysate	138.0	8.40	16.63	1.0	100.00
Amicon concentrated	135.5	6.8	19.93	1.2	98.2
Ammonium sulphate precipitation and Dialysis	92.25	4.2	21.96	1.32	66.85
Sephacryl S-500	73.2	2.46	29.75	1.79	53.04
DEAE	64.2	1.63	39.38	2.30	46.5

Characterization of partially purified enzyme: After partial purification of enzyme protein, the different kinetic properties of 3-cyanopyridinase was studied such as effect of pH, temperature, stability, metal ions and affinity of enzyme towards substrates etc.

Effect of pH and temperature on enzyme activity: The enzyme substrate reaction mixture was prepared in buffer of different pH and placed for incubation at optimal conditions. The pH activity profile of 3-cyanopyridinase is given in **Fig. 5** which revealed an optimum pH of 7.0 is more suitable for maximum synthesis of nicotinic acid. Similarly, for temperature activity of enzyme, an optimum temperature of 50 ˚C is more suitable for product accumulation (**Fig. 6**).

Further, it was also noted that the enzyme is stable at higher temperature of activity profiling for the bio transformation study.

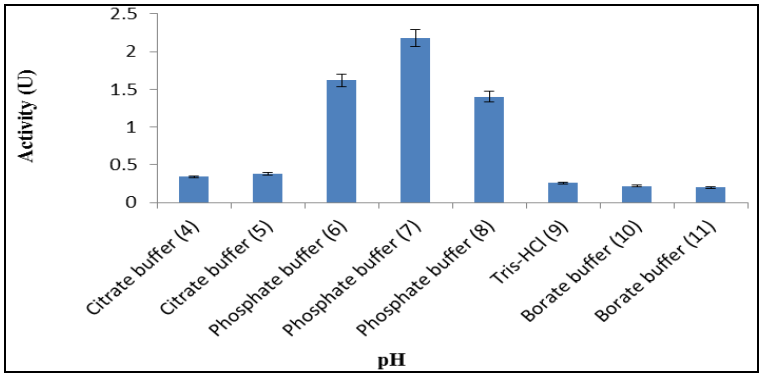

FIG. 5: EFFECT OF pH ON PARTIALLY PURIFIED 3-CYANOPYRIDINASE ENZYME ACTIVITY. THE MAXIMUM ACTIVITY OF ENZYME WAS FOUND AT pH 7.0.

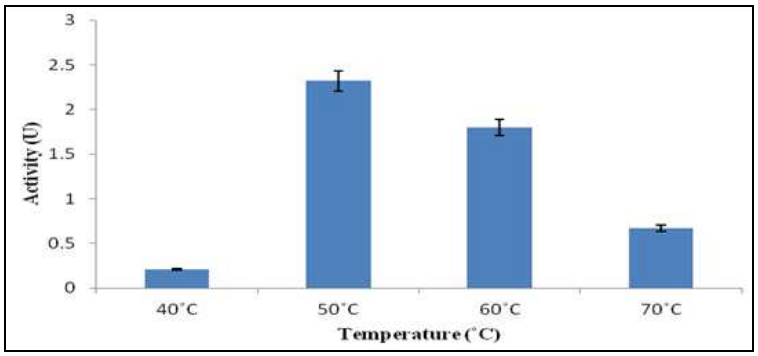

FIG. 6: EFFECT OF TEMPERATURE ON ENZYME ACTIVITY. THE MAXIMUM 3-CYANOPYRIDINASE ACTIVITY WAS FOUND AT 50 ºC

Thermal stability and enzyme activity: The enzyme 3-cynopyridinase was evaluated for stability at optimal temperature of 50 ˚C for 5h and the activity profile is shown in **Fig. 7**. The study of thermal profile inferred that the purified enzyme lost activity after 4h of incubation. The half-life ($t_{1/2}$) of enzyme was calculated as 1h at optimal conditions. The decreased in activity with increased incubation period is probably due to denaturation of enzyme protein.

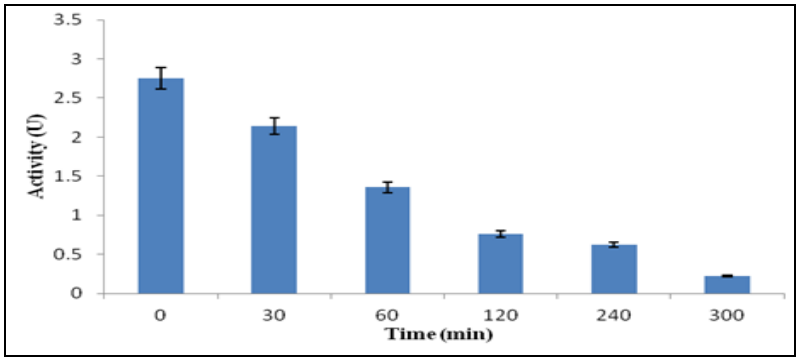

FIG. 7: THE GRAPHICAL ILLUSTRATION SHOWING THERMAL STABILITY OF PARTIALLY PURIFIED ENZYME 3-CYNOPYRIDINASE

Effect of metal ions on nitrilase activity: The effect of different metal ions was evaluated on the nitrilase activity at optimal conditions. The metal ions at a final concentration of 5 mM were added in the reaction mixture and results showed that $MgCl_2$ and $NiCl_2$ enhances the nitrilase activity almost twice of control whereas, $CuCl_2$, $CaCl_2$ and EDTA showed very little increase in the 3-cynopyridinase activity (**Fig. 8)**. It has been further observed that $FeSO_4$, $HgCl_2$, and $AgNO_3$ strongly inhibit the enzyme activity.

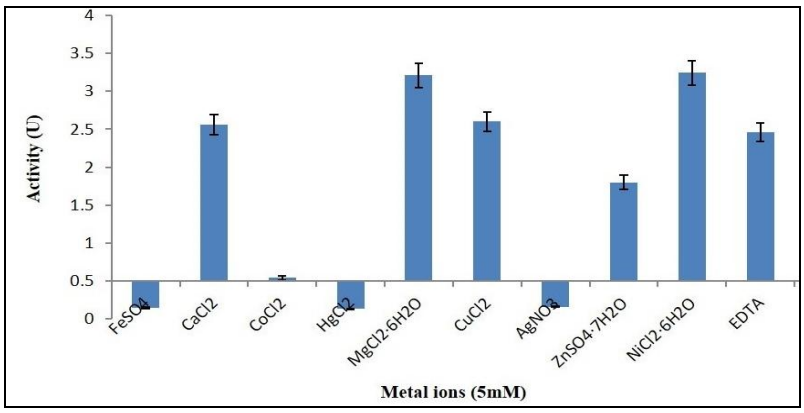

FIG. 8: EFFECT OF DIFFERENT METAL IONS ON 3-CYANOPYRIDINASE ACTIVITY

Substrate specificity of nitrilase enzyme: The substrate specificity of partially purified nitrilase was evaluated by using different nitrile and amide at a final concentration of 10 mM at operational conditions. The **Fig. 9** represents that the affinity of 3-cynopyridinase was very high for 3-cyanopyridine as substrate followed by propionamide and niacinamide. Benzonitrile and propionitrile were also hydrolyzed by the enzyme that is regarded as hazardous compounds thus enzyme has potential application in bioremediation of these toxicants.

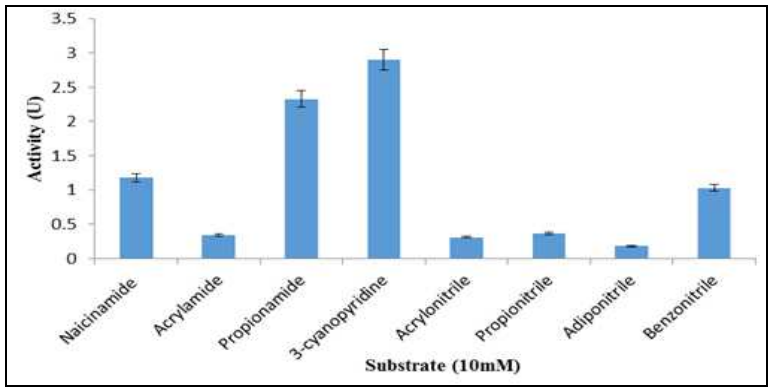

FIG. 9: THE GRAPH ILLUSTRATES THE ACTIVITY OF 3-CYANOPYRIDINASE ON DIFFERENT SUBSTRATE UNDER OPTIMUM CONDITIONS TO DEMONSTRATE ITS SUBSTRATE SPECIFICITY

Effect of 3-cyanopyridinase on different concentrations of 3-cyanopyridine: The partially purified 3-cyanopyridinase was allowed to act with different concentrations 3-cyanopyridine keeping all other conditions constant and the results of reaction is shown in **Fig. 10** (**a & b**). It was observed from **Fig. 10 (a)** that as the concentration of 3-cyanopyridine increased, the enzyme activity also increased up to certain level (70 mM) and then became stable up to 100 mM of substrate. The double reciprocal plot of enzyme catalyzed reactions was also calculated which showed a K_m (Michaelis-Menten Constant) of about 0.91 mM and V_{max} (maximum reaction velocity/activity) of 0.59 U respectively (**Fig.10 b**).

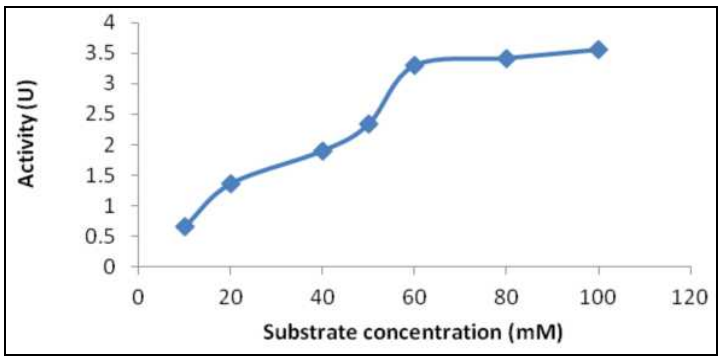

FIG. 10 (a)

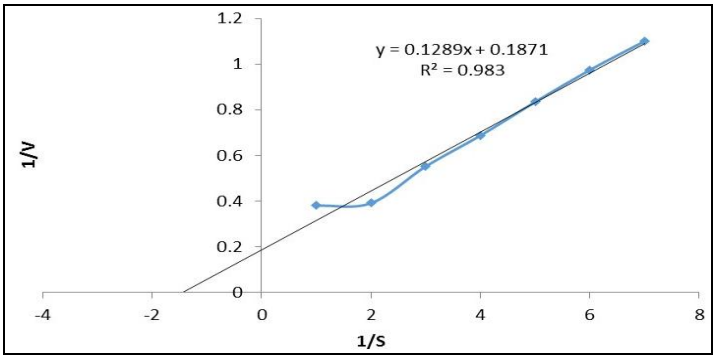

FIG. 10 (b)

FIG. 10: KINETIC CHARACTERIZATION OF PARTIALLY PURIFIED 3-CYANOPYRIDINASE ENZYME. a.) THE GRAPH PLOTTED TO SHOW THE ENZYME ACTIVITY FOR DIFFERENT SUBSTRATE CONCENTRATIONS. b.) THE DOUBLE-RECIPROCAL PLOT FOR DETERMINATION OF K_m AND V_{max}

DISCUSSION: Nitrilases are an important class of hydrolytic enzyme which hydrolyze nitrile compounds into valuable acids and amides. Nitrilases have attracted the interest as biocatalyst because of their varied applications in different industrial sectors. 3-cyanopyridinase, a type of nitrilase responsible for synthesis of nicotinic acid from bacteria *Bacillus* sp. MTCC 7545 was partially purified in five steps and molecular mass was determined by gel electrophoresis. The molecular mass was found to be about 43 kDa. Previous studies showed similar molecular mass of nitrilase isolated from *Rhodococcus rhodochrous* K22 and *Fusarium solani* IMI196840 [17, 24]. The 3-cyanopyridinase of selected microorganism was partially purified to 2.3 fold from cell-free extract with a recovery of 46.5 %.

The physiological properties of partially purified enzyme was compared to the reported nitrilases and it was found to be fairly thermostable enzyme. The optimum temperature for enzyme activity was found at 50 ºC in neutral environment. The half-life of the enzyme was observed to be 60 mins. Thermophilic nitrilase *Pyrococcus* sp. M24D13 showed half-life of 8 hr but the substrate is benzonitrile [25].

From the observation we observed that various compounds like $MgCl_2$, $NiCl_2$ $CuCl_2$, $CaCl_2$, EDTA, increased the 3-cynopyridinase activity while $FeSO_4$, $HgCl_2$, and $AgNO_3$ showed inhibitory action on the enzyme synthesized from *Bacillus* sp. MTCC 7545.

Fe^{2+} ions showed negative effect on nitrilase catalysis in present study, which is similar to the findings of nitrilase produced from *Nocardia* sp. NCIB 11216 and *Pseudomas putida* [26, 27].

Notably, the effect of $CuCl_2$ and EDTA was found contrary to the findings of present study. Nitrilase expressed in *Rhodococous rhodochrous* J1 showed the inhibitory effect of $CuCl_2$ whereas no influence of EDTA on the enzyme activity [20]. In *Fusarium proliferatum*, EDTA has enhanced the activity and $NiCl_2$ has inhibitory activity [28]. In certain studies, EDTA does not affect or very little influence on the activity of nitrilase protein indicating absence of metal ion or lack of requirement of metal ions as cofactors for enzyme catalysis [29, 30] which is quite similar to the present findings.

Enzyme kinetics studies revealed that the affinity of enzyme was higher towards 3-cyanopyridine followed by other nitriles and amides. The 3-cyanopyridinase of *Bacillus* sp. MTCC 7545 also degrade benzonitrile and propionitrile(carcinogenic substances) at high temperature though some reported studies showed hydrolysis at ambient temperature [31, 32]. From the double reciprocal plot, the calculated K_m and V_{max} were 0. 91 mM and 0.59 U against 3-cyanopyridine as substrate.

CONCLUSION: The 3-cyanopyridinase enzyme produced by *Bacillus* sp. MTCC 7545 hydrolyzes 3-cyanopyridine to nicotinic acid in a single step pathway. The present study was an attempt to develop an efficient process for the purification and characterization of the enzyme with minimum loss of activity because nitrilase mediated bio conversion are gaining more importance over chemical routes. Maximum 3-cyanopyridinase production was observed at temperature of 45 ˚C, 170 rpm for 24 h in presence of benzonitrile as inducer. The lysis time for maximum release of intracellular enzyme to the extracellular environment was optimized as 5 min. The precipitation of enzyme 3-cyanopyridinse was highest in 20-60 % saturation of ammonium sulfate. The enzyme was partially purified to 2.3 fold by size exclusion chromatography followed by ion exchange chromatography. The optimum temperature for partially purified enzyme was noted at 50 ˚C in the neutral pH. The partially purified enzyme also showed broad substrate specificity hydrolyzing not only 3-cyanopyridine but also catalyzes the bioconversion of different amides and nitriles like benzonitrile and propionitrile which are hazardous to the environment.

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CONFLICT OF INTEREST: The authors declare that they do not have any conflict of interest.

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