ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Screening and Isolation of Different Bacteria for Synthesis of Nicotinic Acid.

Tesnim Arfi, and Vinod Kumar Nigam*.

Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi - 831 215, India.

ABSTRACT

Thermostable nitrile metabolizing enzymes are getting more importance in the industrial and biotechnological field because of their role in the synthesis of various pharmaceutical products, in bioremediation of cyanide, in synthesis of plant hormone and nutrient assimilation etc. Based on the diverse habitat for presence of microorganisms, a number of soil samples have been used for the screening of bacteria producing nitrilases responsible for hydrolyzing 3-cyanopyridine to nicotinic acid (Vitamin B3) at high temperature. From a number of different bacterial isolates, few showed the capacity of synthesizing 3-cyanopyridinase responsible for converting 3-cyanopyridine to nicotinic acid at 50 $^{\circ}$C at neutral pH. The mineral base medium with glycerol as sole source of carbon was most suitable for synthesis of enzyme.

Keywords: 3-Cyanopyridine, Nicotinic acid, Nitrilases, Pellagra, Thermostable

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Nitriles compounds are the cyanide substituted carboxylic acid with their general formula R-CN. They are found almost everywhere in nature as they are synthesized naturally as well as xenobiotically [1]. Naturally, it is synthesized in the form of cyanolipids, glycolipids, phenylacetonitriles by plants etc. and chemically in polymer industries as byproducts of several chemical processes. They are important for synthesis of pharmaceutically important amines, amides, carboxylic acids, esters, aldehydes, ketones and heterocyclic compounds. Nitriles are toxic in nature therefore; their degradation is an issue since they are released from the different industries and accumulates in the environment causing serious threat to the living beings [2]. Nitriles are biologically degraded by class of enzymes, called nitrilases and also hydrolyzed in presence of chemicals that requires harsh conditions and thus biological hydrolysis of nitriles is gaining more preference over chemical process and hence nitrilases are being treated as "green catalyst". Nitrilases hydrolyze the nitrile compounds into their corresponding carboxylic acid and ammonia in a single step [3, 15] or in a two-step reaction. In single step, nitrile compound is directly converted into its corresponding carboxylic acid and ammonia whereas in two step reactions nitrile is first converted to amide in presence of nitrile hydratase followed by production of acid in presence of enzyme amidase (Figure 1).

$$\text{R-CN} + 2\text{H}_2\text{O} \xrightarrow{\textbf{E1}} \text{R-COOH} + \text{NH}_3$$

$$\text{R-CN} + 2\text{H}_2\text{O} \xrightarrow{\textbf{E2}} \text{R-CONH}_2 \xrightarrow{\textbf{E3}} \text{R-COOH} + \text{NH}_3$$

Figure 1: Pathway of nitrile hydrolysis, where E1, E2 and E3 are nitrilase, nitrile hydratase and amidase respectively.

Nitrilase belongs to nitrilase superfamily which is industrially important enzymes, found in prokaryotes as well as in eukaryotes. Nitrile hydrolyzing enzymes, namely nitrilases (EC 3.5.5.1), nitrile hydratases (EC 4.2.1.84) and amidases (EC 3.5.1.4) are highly selective and showed broad substrate specificity. Some of the literatures are available on the specific production of nitrilases hydrolyzed specific substrates to corresponding acids of commercial importance [4-6]. Most importantly, they can carry out the hydrolysis at ambient pressure and temperature, which are important properties for their development as potential industrial biocatalysts [7]. Thermostable nitrilases are highly specific and can resist extremely acidic and alkaline conditions, so they are gaining importance in industrial and biotechnological field. 3-cyanopyridinase is a type of bacterial nitrilase which hydrolyses 3-cyanopyridine into nicotinic acid and ammonia in single step pathway [3, 8]. Nicotinic acid is a pharmaceutical compound which is also known as Vitamin B3 and its deficiency causes pellagra (3D's). Nicotinic acid has important role in the growth & development of living organisms as its coenzyme NAD and NADP are involved in the energy metabolism, animal feed supplementation as well as in various pharmaceutical applications [9].

The main aim of present study was to isolate potential bacterial cultures producing thermostable nitrilase responsible for nicotinic acid production. Optimization of different process parameters have also been carried out for enhanced production as well catalysis.

MATERIALS AND METHODS

Media and growth conditions for isolation

Screening of nitrile hydrolyzing enzymes was carried in medium containing different types of inducers like benzonitrile, propionitrile, ε-caprolactum, and adiponitrile at a final concentration of 10 mM in presence of different media. Media 1 was mineral base medium used for the isolation of nitrilase producing bacteria [4]. The composition of medium 1 includes, yeast extract 1g/l, glycerol 10g/l, mineral base 200ml/l, trace element 1ml/l. The medium 2 includes starch 5g/l, peptone 5g/l, yeast extract 1.5g/l and NaCl 5g/l [10] and the different constituents of medium 3 are glycerol 15g/l, yeast extract 7.5g/l, KH_2PO_4 0.5g/l, K_2HPO_4 0.5g/l and $MgSO_4$ 0.5g/l [11]. All the three media were also used for evaluating their efficiency for 3-cyanopyridinase production. Samples for isolation of bacteria were collected from different areas like automobile workshops, sandy soil near the Swarnrekha river, medicinal gardens (Birla Institute of Technology, Mesra) Ranchi and

desert soil, Bikaner (Rajasthan) respectively. For the isolation of 3-cyanopyridinase producing bacteria from different samples, enrichment culture technique as well as indicator plate method were adapted. 1.0g of different soil samples in 50ml of each sterile media at pH 7.0 supplemented with the nitriles as inducers were kept for incubation at 45 ºC and 37 ºC at 175 rpm for 24 h. The well grown culture was serially diluted in the concentration range of 10^{-5}, 10^{-6}, 10^{-9} and 10^{-10} respectively and plated in triplicate on Luria broth agar (LB) plates containing respective inducer and phenol red (0.02% w/v) as pH indicator [12]. After the growth on the agar plate, average colony was counted as colony forming unit (CFU). The change in color of the plates from red to yellow confirmed as nitrilase producing bacterial isolates. After several streaking the pure colony was maintained on LB plates. Submerged cultivation of these nitrilase producers was also carried out to find out specific nitrilase (3-cyanopyridinase) that can synthesize nicotinic acid as secondary isolation.

Liquid culturing

The liquid culturing of the selected isolates was performed as batch mode of cultivation in all the three media in presence of respective inducers and incubated for 24h under agitation on an orbital shaker at 45 ºC and 37 ºC respectively. After 24h of growth, cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4 ºC and the cell pellet was washed twice with phosphate buffer (0.1M, pH 7.0). The concentration of cells was adjusted to an O.D. of 2.0 (600nm) for performing 3-cyanopyrinidase assay.

Enzyme assay

Enzyme assay was performed with whole cell suspension at 50 ºC for two hours in 1ml of reaction mixture containing 0.1ml of whole cells, 0.1ml of 3-cyanopyridine (10mM) as substrates and 0. 8ml of phosphate buffer (pH 7.0). The reaction was terminated by using fifty microliter of 0.1N HCl. The ammonia released during reaction was estimated at 640nm and the enzyme activity is expressed as IU which is defined the milli-mole of ammonia released per minute per ml. The production of nicotinic acid was confirmed by Thin Layer Chromatography (TLC) on pre-coated aluminium sheets using water, butanol, and acetone as mobile phase in the ratio of 50:45:5 and R_f value was calculated and compared with standard nicotinic acid as well as standard 3-cyanopyridine.

Optimization of different process parameters for production of nicotinic acid

The different physicochemical parameters affecting the production and characteristics of enzyme responsible for 3-cyanopyridine hydrolysis were investigated by observing the activity of 3-cyanopyridinase produced in three different media and also in presence of different inducers on synthesis of 3-cyanopyridinase in media-1. Similarly, the effect of different temperature on maximum activity of 3-cyanopyridinase was investigated in the range of 40 to 60 ºC. For pH profiling of enzyme activity, buffers of different pH (0.1M acetate buffer; pH 4 and 5), (0.1 M phosphate buffer; 6-8), (0.1 M tris-HCl buffer; pH 8-9), and (0.1 M carbonate buffer; pH 9-10) was used. The substrate specificity of 3-cyanopyridinase was evaluated by using different nitriles at a final concentration 10mM under optimal conditions.

RESULTS AND DISCUSSIONS

Screening and selection of nitrile metabolizing bacteria

From the preliminary screening on phenol agar plates during the isolation of nitrilases, 375 and 478 bacterial cultures were detected at 45 ºC and 37 ºC respectively in presence of different inducers from various soil sample (Table 1). Out of 853 different isolates, eighteen isolates showed change of color on the LB phenol plates confirming the presence of different nitrilases expressed in presence of different inducers (Table 2). Few of the bacterial isolates with prominent nitrilase activity on plate medium are presented in Figure 2.

ISSN: 0975-8585

Table 1: Different bacterial isolates on phenol agar plate (CFU)													
Soil sources	**Name of Inducers**	**45 ºC**						**37 ºC**					
		i	**ii**	**Iii**	**Average**	**Std. dev**	**Std. Error**	**i**	**Ii**	**iii**	**Average**	**Std. dev**	**Std. Error**
Soil from medicinal garden	Benzonitrile (10^{-5})	23	25	19	22.33	3.06	1.76	50	49	52	50.33	1.53	0.88
	Adiponitrile (10^{-6})	48	51	39	46.00	6.24	3.61	45	43	46	44.67	1.53	0.88
	Propionitrile (10^{-5})	05	07	10	7.33	2.52	1.45	13	17	11	13.67	3.06	1.76
	ε-Caprolactum (10^{-9})	04	04	03	3.67	0.58	0.33	10	03	02	5.00	4.36	2.52
Sandy soil nearby swarnrekha river	Benzonitrile (10^{-6})	28	29	28	28.33	0.58	0.33	20	23	21	21.33	1.53	0.88
	Adiponitrile (10^{-6})	34	37	33	34.67	2.08	1.20	45	56	57	52.67	6.66	3.84
	Propionitrile (10^{-5})	12	10	10	10.67	1.15	0.67	12	13	14	13.00	1.00	0.58
	ε-Caprolactum (10^{-9})	23	25	29	25.67	3.06	1.76	34	33	36	34.33	1.53	0.88
Soil from nearby Automobile shop	Benzonitrile (10^{-5})	45	50	51	48.67	3.21	1.86	65	67	64	65.33	1.53	0.88
	Adiponitrile (10^{-6})	39	34	34	35.67	2.89	1.67	36	39	41	38.67	2.52	1.45
	Propionitrile (10^{-10})	09	05	06	6.67	2.08	1.20	15	18	17	16.67	1.53	0.88
	ε-Caprolactum (10^{-9})	18	17	16	17.00	1.00	0.58	09	05	04	6.00	2.65	1.53
Desert soil from Rajasthan	Benzonitrile (10^{-5})	29	28	26	27.67	1.53	0.88	36	37	33	35.33	2.08	1.20
	Adiponitrile (10^{-6})	34	40	39	37.67	3.21	1.86	45	47	44	45.33	1.53	0.88
	Propionitrile (10^{-5})	03	00	01	1.33	1.53	0.88	00	03	01	1.33	1.53	0.88
	ε-Caprolactum (10^{-9})	26	28	25	22.33	3.06	1.76	31	33	39	34.33	4.16	2.40

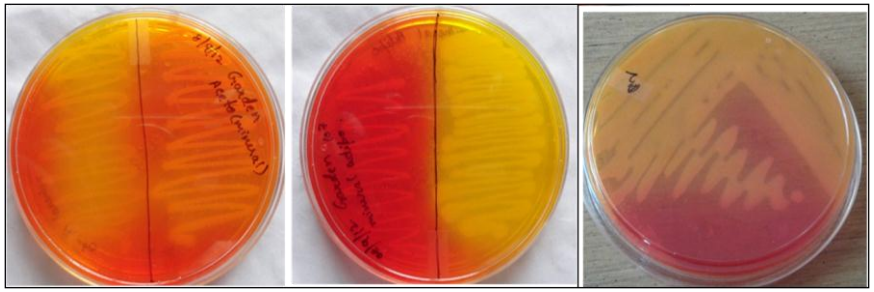

Figure 2: Bacterial isolates with positive results (nitrilase producers) on indicator plates.

ISSN: 0975-8585

Table 2: Activity of nitrilases from different bacterial isolates on phenol agar plates.

S. No.	Isolate	Inducer	Source	Positive on Plate
1	BIT NT001	Benzonitrile	Garden soil sample	++
2	BIT NT002	Adiponitrile	Garden soil sample	+++
3	BIT NT003	Benzonitrile	Garden soil sample	+
4	BIT NT004	Adiponitrile	Garden soil sample	+
5	BIT NT005	Adiponitrile	Garden soil sample	+
6	BIT NT006	Adiponitrile	Sandy soil	+
7	BIT NT007	Adiponitrile	Contaminated soil	+
8	BIT NT008	Adiponitrile	Garden soil	+
9	BIT NT009	Benzonitrile	Sandy soil	+
10	BIT NT010	ε-Caprolactum	Sandy soil	--
11	BIT NT011	ε-Caprolactum	Contaminated soil	+
12	BIT NT012	Adiponitrile	Desert soil	+++
13	BIT NT013	Adiponitrile	Desert soil	+++
14	BIT NT014	Propionitrile	Desert soil	--
15	BIT NT015	Benzonitrile	Desert soil	+
16	BIT NT016	ε-Caprolactum	Desert soil	--
17	BIT NT017	ε-Caprolactum	Contaminated soil	+
18	BIT NT018	Propionitrile	Contaminated soil	---

Out of 18 bacterial isolates, ten isolates namely BIT NT001, BIT NT002, BIT NT003, BIT NT004, BIT NT005, BIT NT006, BIT NT007, BIT NT008, BIT NT012 and BIT NT013 showed the higher nitrilase activity at 45 ºC and further selected for liquid culturing condition for 3-cyanopyridinase activity. Similarly, remaining eight isolates BIT NT009 BIT NT010, BIT NT011, BIT NT014, BIT NT015, BIT NT016, BIT NT017 and BIT NT018 of 37 ºC were also evaluated for 3-cyanopyridinase activity in liquid culturing condition by batch mode.

Liquid culturing

All the eighteen isolates were grown in media-I at 175 rpm for 24h at their respective optimal conditions and the whole cells of microorganisms were used as a source of 3-cyanopyridinase for monitoring the production of nicotinic acid at 50 ºC . The results of enzyme activity for production of nicotinic acid are given in Figure 3.

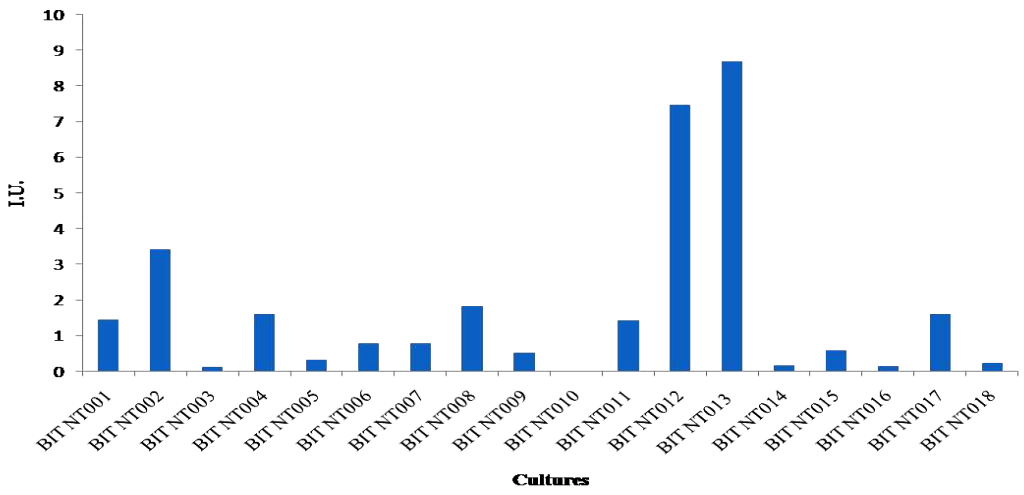

Figure 3: 3-cyanopyridinase activity of different bacterial cultures at 50 ºC.

Based on the observation of Figure 3, seven isolates viz. BIT NT001, BIT NT002, BIT NT004, BIT NT008, BIT NT12, BIT NT013 and BIT NT017 showed activity at higher temperature. As the main objective of the study was to screen the thermostable nitrilase suitable for the synthesis of nicotinic acid at high temperature, some of these bacteria was further evaluated for the synthesis of nicotinic acid. It has been also observed that the

activity of nitrilase is intracellular in nature as there was no hydrolysis of 3-cyanopyridine when supernatant was used as a source of enzyme. Finally, four isolates, BIT NT002, BIT NT008, BIT NT012 and BIT NT013 were used for the optimization studies toward nicotinic acid production. Based on the phenotypic and biochemical characterizations, these isolates belong to genus *Bacillus*.

Effect of different media on production of 3-cyanopyridinase

Three different media have been used for the maximum production of nitrilase by selected bacterial isolates and the results showed media- 1 is more favorable for 3-cyanopyrdinase synthesis with isolates BIT NT012, BIT NT013 and BIT NT008 respectively (Figure 4), whereas in case of BIT NT002 media 2 is favorable for growth but enzyme activity was too low. The medium 1 was selected for further optimization studies.

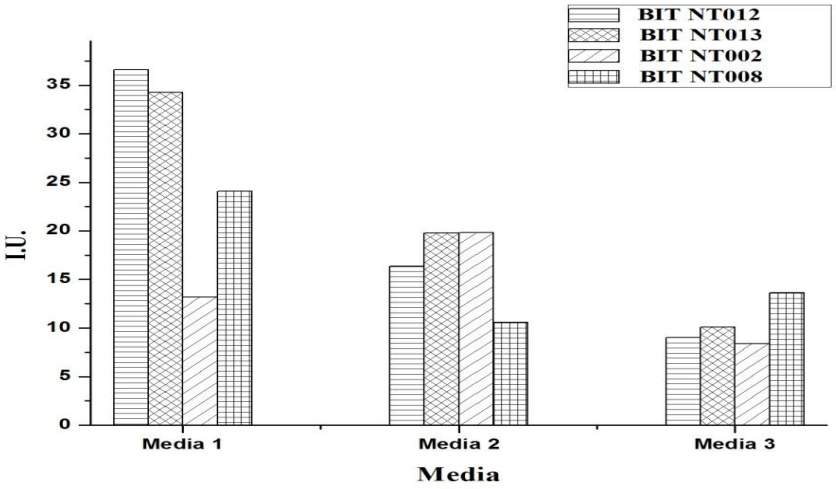

Figure 4: Effect of three different media on production of 3-cyanopyridinase

Effect of different inducers on production of 3-cyanopyridinase

The influence of different inducers (acetonitrile, adiponitrile, acrylonitrile, benzonitrile and ε-caprolactum) on the synthesis of enzyme by selected isolates were determined by measuring the enzyme activity and shown in Figure 5. The results showed that benzonitrile as inducer produced highest activity in case of bacterial isolate BIT NT012 BIT NT013 and BIT NT008 whereas incase of BIT NT002 adiponitrile was found as best inducer for enzyme production. Mathew et al. (1988) have also evaluated the role of inducers on expression of specific nitrilase responsible for hydrolyzing 3-cyanopyridinase [13].

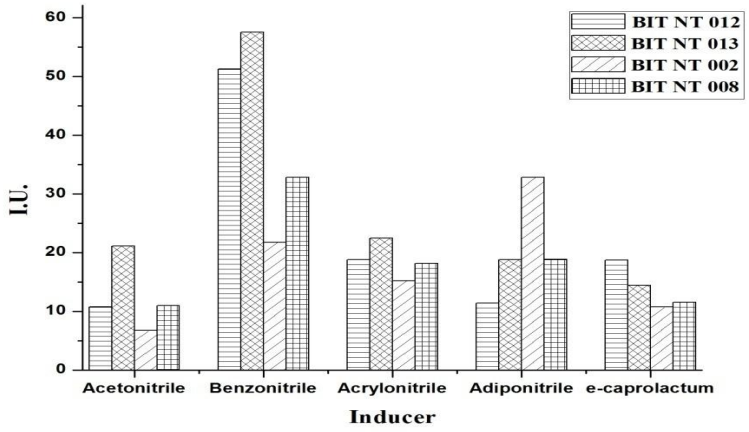

Figure 5: Effect of different inducers on production of 3-cyanopyridinase

ISSN: 0975-8585

Optimization of process parameters for production of nicotinic acid

Effect of Temperature

The temperature profiling for 3-cyanopyridinase activity responsible for synthesis of nicotinic acid from 3-cyanopyridine by all the four bacteria is given in Figure 6. It is clear from the figure that BIT NT012 showed higher activity (production of nicotinic acid) followed by BIT NT013. The least enzyme activity was recorded by the isolate BIT NT002 and BIT NT008. Bhalla et al. (2006) also noticed the nicotinic acid producing capability of nitrilase expressed in *Nocardia globerula* NHB-2 at 35 ºC [14]. Similar results were also noticed in case of 3-cyanopyridinase produced from *R. rhodochrous* and *Streptomyces* sp. [2].

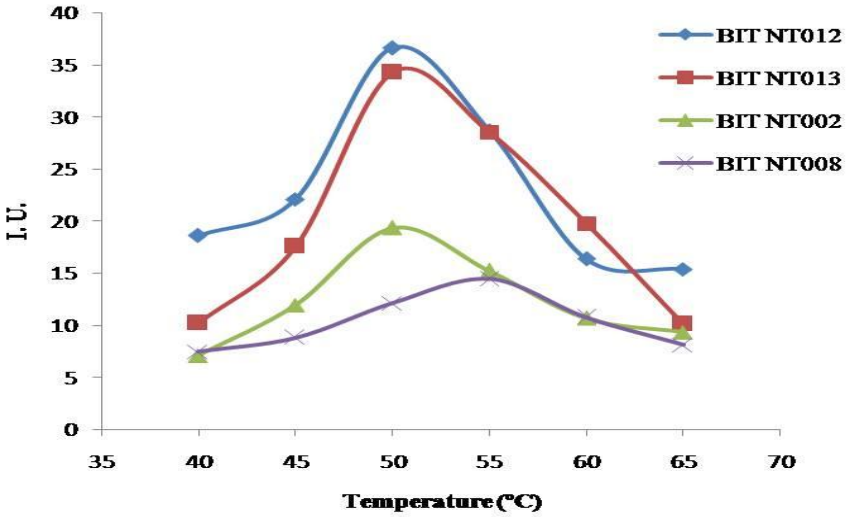

Figure 6: Effect of temperature

Effect of pH

The effect of pH on the 3-cyanopyridinase activity was carried out in the pH range of 4-10 and profiling of production of nicotinic acid is expressed in Figure 7. All the isolates showed higher activity at the neutral pH 7.0 (phosphate buffer), though the highest activity was observed with BIT NT012. Most of the nitrilases reported are stable in neutral and slightly alkaline environment.

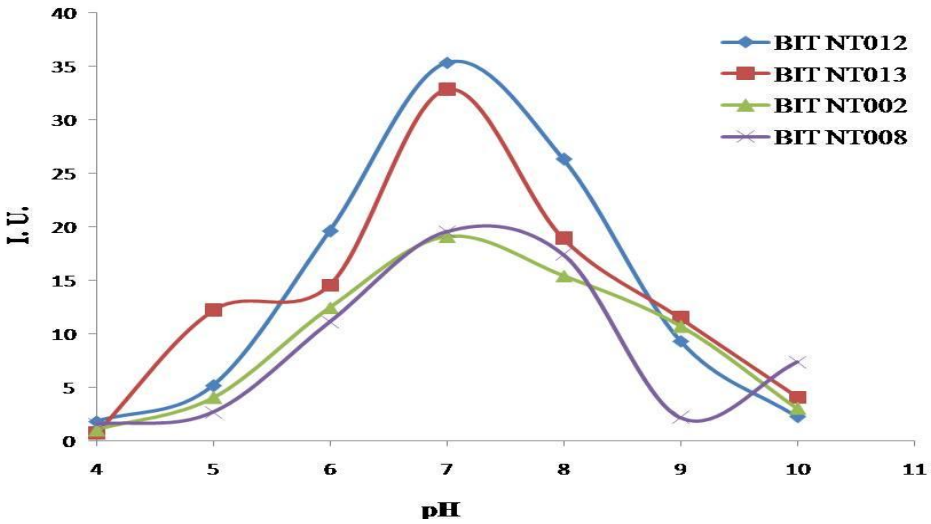

Figure 7: Effect of pH.

ISSN: 0975-8585

Effect of different nitriles (substrates) on activity of 3-cyanopyridinase

Different nitriles were used as substrate (10mM) to observe the catabolic property of 3-cyanopyridinase in phosphate buffer and the experimental results are shown in Figure 8. The findings showed that the affinity of the enzyme was higher towards 3-cyanopyridine followed by propionamide and acetamide. It is therefore confirmed that the isolates can be used for synthesis of nicotinic acid.

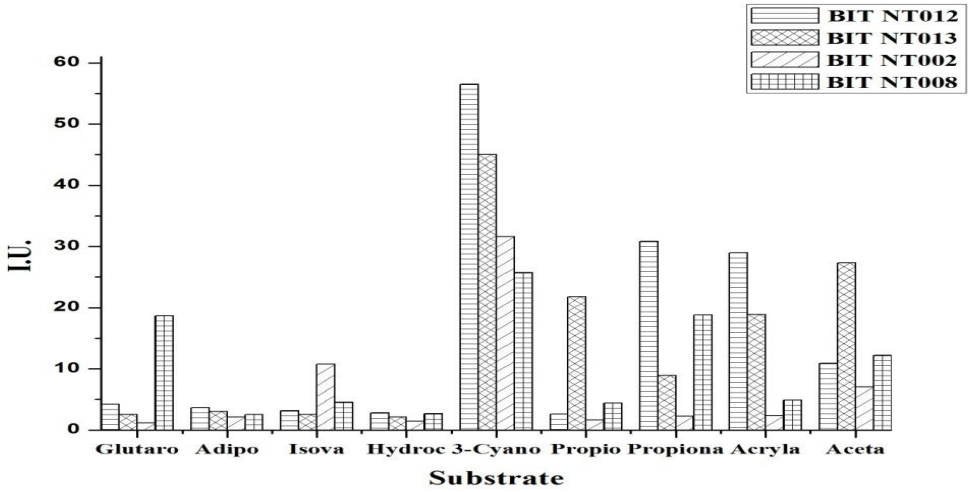

Figure 8: Effect of different substrates (10mM)

Product Analysis

Thin layer chromatography was used to detect and identify the bio-transformation of 3-cyanopyridine to nicotinic acid as product. The chromatogram was visualized under UV light at a wavelength of 254nm and R_f values of standards (3-cyanopyridine and nicotinic acid) and enzyme catalyzed reaction mixtures of all the four isolates were calculated (Figure 9). The R_f values of standard nicotinic acid and 3-cyanopyridine is 0.51and 0.87 respectively which is comparable to the R_f values of the reaction mixtures with substrate and hydrolyzed product.

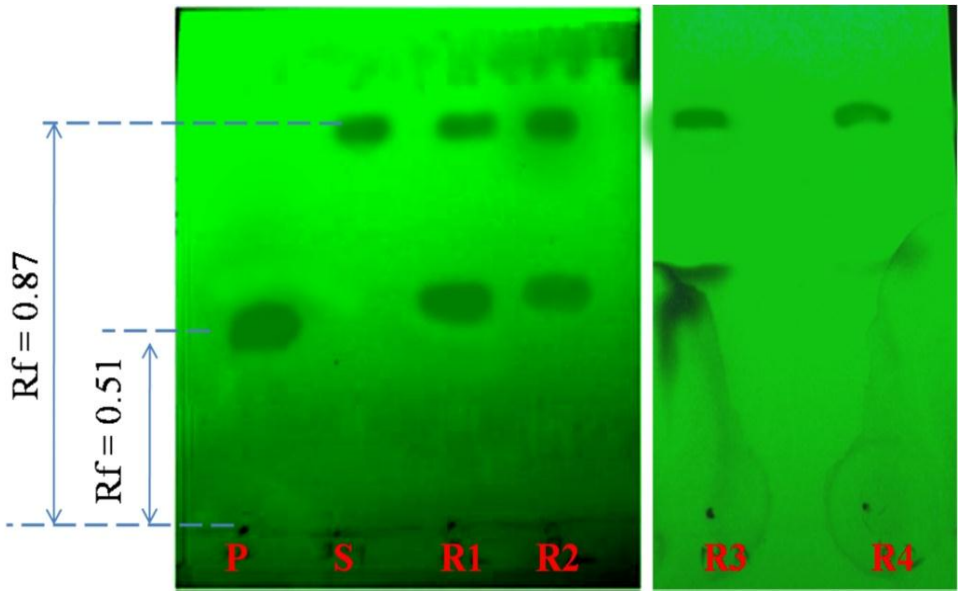

Figure 9: TLC chromatogram of standards and reaction mixtures. P is standard Nicotinic acid, S is standard 3-cyanopyridine, R1 is reaction mixture of BIT NT012, R2 is reaction mixture of BIT NT013, R3 is reaction mixture of BIT NT002 and R4 is reaction mixture of BIT NT008.

CONCLUSION

A number of different nitrile metabolizing bacteria have been isolated with different properties from the mesophilic sources. The enzymes produced from the mesophilic sources have low thermal stability where as the thermal stability of thermophiles is substantially higher. The present work focused on the isolation of thermostable nitrilase for nicotinic acid synthesis with better properties. Four bacterial isolates from two different soil sources, showed the ability of biotransformation of 3-cyanopyridine to nicotinic acid at 50 ºC and at neutral pH. Further optimizations are required for these isolates so that they can be exploited for production of nicotinic acid in high yield.

ACKNOWLEDGEMENT

One of the authors, Tesnim Arfi duly acknowledge the Senior Research Fellowship of Moulana Azad National Fellowship, U G C Government of India (F1-17.1/2012-13/MANF-2012-13-MUS-JHA-9035), New Delhi-110 002 for carrying out this work.

REFERENCES

[1] Agarwal A, Nigam VK, Vidyarthi AS. Int J Pharma Bio Scie 2012; 3:232-246.
[2] Kobayashi M, Nagasawa T and Yamada H. Appl Microbiol Biotechnol 1998; 29:231-233.
[3] Nigam VK, Agarwal A, Sharma M, Ghosh P and Choudhury B. Res J Biotechnol 2009; 41:33-36.
[4] Khandelwal AK, Nigam VK, Choudhary B, Ghosh P. J Chem Technol Biotechnol 2007; 82:646-651.
[5] Agarwal A, Nigam VK. Biocatal Agric Biotechnol 2014; 3:351-357.
[6] Nigam VK, Khandelwal AK, Gothwal RK, Mohan MK, Choudhury B, Vidyarthi AS and Ghosh P. J Biosci 2009; 34:21-26.
[7] Haki GD, Rakshit SK. Biores Technol 2003; 89:17-34.
[8] Arfi T, Agarwal A and Nigam VK. Int J Pharm Technol 2013; 5:2622-2631.
[9] Kumar S and Babu BV. Chem Biochem Engg 2009; 23:367–376.
[10] Banerjee A, Kaul P, Sharma R, Banerjee UC. J Biomol Screening 2003; 8:142-168
[11] Nagasawa T, Wieser M, Nakamura T, Iwahara H, Yoshida T, Gekko K. European J Biochem 2000; 267:138-144.
[12] Santosh M, Nayak AS, Anjaneya O, Karegoudar TB. J Industr Microbiol Biotechnol 2009; 37:111-115.
[13] Mathew CP, Nagasawa T, Kobayashi M, Yamada H. Appl Environ Microbiol 1988; 54:1030-1032.
[14] Bhalla TC, Sharma NN, Sharma M, Kumar H. Nocardia globerula NHB-2: Bench scale production of nicotinic acid. Proc Biochem 2006; 41:2078-2081.
[15] Nigam VK, Khandelwal AK, Agarwal A, Mohan MK and Vidyarthi AS. Int J Bio-Sci Bio-Technol 2012; 4:81-90.