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SCREENING, ISOLATION AND IDENTIFICATION OF NITRILASE PRODUCING BACTERIA FROM SOIL

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ABSTRACT: Enzymes are identified as key catalysts in various processes occurring within the living cells. The role of various enzymes is constantly investigated to find their novel applications. Nitrilase is one such enzyme which carries out the hydrolysis of carbon-nitrogen bonds except for amides. This catalytic property of nitrilase is exploited to generate corresponding chiral carboxylic acids. These chiral molecules can be employed in various drug synthesis schemes. Nitrilase have been reported from various microorganisms with variable catalytic properties. With an objective to explore microorganisms which produce nitrilases and able to catalyze aromatic nitriles, screening of microorganisms from soil was carried out. Out of five soil sampling sites, screening from the soil sample taken from benzonitrile manufacturing industry (Anami Organics Ltd. Valsad, Gujarat-India) resulted in isolation of microorganism which was able to use benzonitrile as substrate and was positive for nitrilase enzyme assay which was done by modified indophenol method. The microorganism was later identified to be *Bacillus thuringiensis* using 16s rRNA and biochemical analysis. Nitrilase is present as an intracellular protein in this species.

INTRODUCTION: Hydrolysis of nitriles to corresponding acids and ammonia is carried out by nitrilase. These enzymes differ from nitrile hydratases as they avoid amide formation [1, 2]. Studies on nitrilases for its occurrence, characteristics, substrate specificity, mechanism of action and gene cloning from various sources like bacteria, fungi and plants have been reported [3–5]. Nitrile bioconversion by nitrilase favor economic and environmental acceptance and thus have found their applicability in chemical manufacturing [6–12]

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Depending on the nitrile structure the nitrilases can be categorized as aromatic nitrilase, aliphatic nitrilase and arylacetonitrilase [13]. Nitrilases have been commercially utilized for the industrial production of acrylamide [14] and nicotinic acid [15]. One of the key products of nitrilase bioconversion is (R)-(-) Mandelic acid, which acts as a key intermediate for the production of semi-synthetic cephalosporins and penicillins [16, 17].

It has been also reported for utility as a chiral resolving agent [18] and chiral synthon for the synthesis of anti-tumor agents [19], anti-obesity agents [20] etc. Studies have also reported production methods for chiral alpha hydroxy carboxylic acids using enzyme other than nitrilase like lipase [21], esterase [22], glyoxalase [23], mandelate dehydrogenase [24] and mandelateracemase [25], these methods were

not used for industrial scale of production due the parameters which include, expensive substrate, co-factor requirement, process stability etc.[26]

Some of the microorganisms which have been reported as nitrilase producer include- *Exophialaoligo sperma* R1 [27] *Rhodococcus*, *Acinetobacter*, *Pseudomonas*, *Acidovorax*, *Streptomyces* [28–32], *Rhodococcusrhodochorus* J1 [33], *Alcaligenesfaecalis* ATCC 8750 [34], *Pseudomonas putida* [35], *Acidovorax facilis72W* [36, 37], *Streptomyces sp. MTCC 7546* [32, 38] etc.

This paper reports the screening of microorganisms from soil sample(s) to isolate strains with nitrilase activity for aromatic nitrile(s).

MATERIAL AND METHODS:

Reagent and Chemicals:

Analytical grade media components and chemicals were used throughout the study and were purchased different suppliers like Hi-Media laboratory Pvt. Ltd., Merck Specialties Pvt. Ltd., Qualigens Fine Chemicals, Thermo Fisher Scientific India Pvt. Ltd., S.D. Fine Chemicals Pvt. Ltd., and Loba Chemie Pvt. Ltd.

Soil Sampling:

Soil for screening the microorganisms was collected from five different sites one of which was from outside Anami Organics Limited, District. Valsad- Gujarat, India. This site is a benzonitrile manufacturing unit and hence should provide positive strain(s) for nitrilase production. Samples were collected in sterile zipper polyethylene bags.

Physicochemical characterization of soil samples:

The soil sample was evaluated for parameters like color, temperature at the time of sampling, pH, moisture content, total organic carbon and total organic mass. Temperature and color of the soil samples were noted during sampling itself. Moisture content, pH, organic carbon and organic matter were determined according to following procedure:

Moisture content:

10g of soil samples were dried for constant weight at 60°C for 72hrs in oven (Dolphin) and then the moisture content was calculated. Percent moisture was calculated as follows:

$$Moisture\ (\%) = \left[\frac{Initial\ Weight - Final\ Weight}{100}\right] * 100$$

pH of soil sample:

Soil samples were dried at 60°C for 72hrs, mashed in pestle and mortar and filtered through ASTM sieve number 18 and 20 (Filterwel Test Sieves). The sieved soil was dissolved in demineralized water (40%w/v) mixed thoroughly and allowed to stand for 30minutes and then pH was measured by digital pH meter (Control Dynamics) [39].

Percent organic Carbon and Organic mass:

In a 500ml conical flask 1g soil sample was mixed with 10ml potassium dichromate (1N) and 20ml concentrated H_2SO_4. After 30 minutes of incubation 200ml distilled water was added followed by filtration. Post filtration 10ml of 85% phosphoric acid wad added followed by shaking to mix the contents. Added 1ml of diphenylamine and performed titration against Ammonium iron (II) Sulfate (0.5N) to bright green end point [39-41]. Percent organic carbon (POC), Total organic Carbon (TOC), and Percent Organic Matter (POM) were calculated from the following formula [39, 40, 42]:

$$POC(\%) = 10 * \left[\frac{B - T}{B}\right] * 0.003 * \left[\frac{100}{Wt\ of\ Soil\ Sample}\right]$$

$$TOC\ (\%) = POC\ (\%) * 1.3$$

$$POM\ (\%) = TOC(\%) * 1.724$$

Whereas, B= Volume (ml) of 0.5N Ammonium iron (II) Sulfate utilized in blank titration and T= Volume (ml) of 0.5N Ammonium iron (II) Sulfate used in test site titration.

Screening - Minimal media Composition:

Screening of the microorganisms was carried out by plating 10^{-5}, 10^{-6} and 10^{-7} dilutions of soil sample on minimal media which had 20mM benzonitrile as an inducer as well as the sole energy source. The minimal media composed of sodium nitrate (3.0g/L), di-potassium hydrogen phosphate (1.0g/L), potassium dihydrogen phosphate (1.35g/L), sodium chloride (5g/L), ferric chloride (1.25mg/L), cobaltous chloridehexahydrate (0.001g/L), zinc sulphate (0.0067g/L) and agar (20g/L) prepared in distilled water [43–45].

After plating the dilutions, the plates were incubated (Meta Lab) at 25°C and 37°C to screen for fungal and bacterial strains respectively. The colonies which appeared post incubation were propagated and analyzed for the presence of nitrilase by modified Berthelot assay [46]. Nutrient broth (at 37°C/ 200rpm) and Czapek Dox broth (at 25°C/ 200rpm) along with 50mM benzonitrile were used for propagation of bacterial and fungal strains respectively. The incubation was carried for 72hrs in Orbital Shaker (Thermo-scientific).

Statistical Design:

Design-Expert v7.0.0 was used to optimize the parameters for nitrilase enzyme assay conditions using *Alicagenesfaecalis* NCIM 2949 (purchased from NCIM, Pune) as a positive nitrilase strain [47]. Two statistical studies were carried, a four factors trial and three factor trial to optimize parameters like sonication time, Substrate (benzonitrile) concentration, volume of crude enzyme and incubation time as shown in **Table 1**and **Table 2.**

TABLE 1: DESIGN-EXPERT STUDIES FOR FOUR FACTORS AT TWO LEVELS TO ACHIEVE OPTIMIZATION OF ENZYME ASSAY PARAMETERS

Design Summary				
Study Type : Factorial				
Initial Design : 2 Level Factorial				
Design Model : 4FI				
Factor	**Name**	**Units**	**Low Actual**	**High Actual**
A	Sonication	Mins	5.00	15.00
B	Substrate	mM	50.00	100.00
C	Enzyme	µl	500.00	1000.00
D	Incubation	Min	20.00	60.00
Run Composition				
Run	**Sonication time (Minutes)**	**Substrate Concentration (mM)**	**Crude Enzyme µl)**	**Incubation Time (Minutes)**
1	15.00	100.00	1000.00	20.00
2	15.00	50.00	500.00	20.00
3	15.00	100.00	500.00	60.00
4	15.00	50.00	1000.00	20.00
5	15.00	100.00	1000.00	60.00
6	5.00	100.00	500.00	60.00
7	15.00	100.00	500.00	20.00
8	5.00	50.00	1000.00	60.00
9	5.00	50.00	1000.00	20.00
10	15.00	50.00	500.00	60.00
11	5.00	100.00	1000.00	20.00
12	5.00	50.00	500.00	60.00
13	5.00	100.00	1000.00	60.00
14	15.00	50.00	1000.00	60.00
15	5.00	100.00	500.00	20.00
16	5.00	50.00	500.00	20.00

TABLE 2: DESIGN-EXPERT STUDIES FOR THREE FACTORS AT THREE LEVELS TO ACHIEVE OPTIMIZATION OF ENZYME ASSAY PARAMETERS

Design Summary					
Study Type : Factorial					
Design Model : 2FI					
Factor	**Name**	**Units**	**Low**	**High**	**Levels**
A	Substrate	mM	100	200	3
B	Enzyme	µl	800	1600	3
C	Incubation	Mins	60	100	3
Run	**Benzonitrile (mM)**	**Crude Enzyme (µl)**		**Incubation Time (Minutes)**	
1	150	800		100	
2	150	800		80	
3	200	1200		60	
4	200	1200		80	
5	100	800		100	

6	100	1200	100
7	100	1200	60
8	150	1200	80
9	100	800	80
10	100	1200	80
11	200	1600	100
12	150	1600	80
13	100	1600	100
14	100	1600	80
15	200	800	80
16	200	800	60
17	150	1200	100
18	200	800	100
19	200	1200	100
20	150	1600	60
21	150	1600	100
22	200	1600	60
23	150	800	60
24	200	1600	80
25	100	800	60
26	100	1600	60
27	150	1200	60

Enzyme assay:

Modified Berthelot assay was used for the determination of enzyme assay by tapping the ammonia generated. Benzonitrile was used as the substrate [48–50]. *Alicagenesfaecalis* has been reported for production of nitrilase and hence *Alicagenesfaecalis* NCIM 2949 was kept as positive reference for nitrilase assay [51].

In the modified method, cells were harvested by centrifugation at 4000rpm/20°C/7minutes. Both the extracellular and intracellular components were analyzed. The intracellular enzyme examination involved cell lysis by ultra-sonication (TOSHCON) for 15minutes in cold conditions, followed by centrifugation at 6000rpm/20°C/7minutes. 800µl of the supernatant post ultrasonication was incubated in 2ml phosphate buffer pH 7.5 having 150mM benzonitrile at 37°C for 100minutes. Post incubation the reaction was stopped by addition of 0.2ml hydrochloric acid (1N). A final centrifugation at 10000rpm/20°C/7minutes was done and the supernatant was analyzed for enzyme assay.

To 2ml of supernatant, 0.08ml of phenol solution was added and vortexed gently. Subsequent to this0.08ml sodium nitroprusside and 0.2ml of oxidizing solution were added, mixed and incubated at room temperature for 1hr followed by measurement of absorbance at 630nm.[39]

Microbial Identification:

The positive strain for nitrilase was given an in-house identification number as VSS6001. Microorganism identification was outsourced to Yaazh Xenomics for Biochemical tests and 16s rRNA. The primers used for the analysis were; forward primer-AGAGTTTGATCMTGGCTCAG and reverse primer TACGGYTACCTTGTTACGA CT T.

RESULTS AND DISCUSSION:

Analysis of soil is a critical parameter in microbial screening exercise. The soil sample was analyzed for various parameters as shown in **Table 3**.

TABLE 3: PHYSICOCHEMICAL CHARACTERIZATION OF SOIL

S.No	Parameters	Results
1	pH	6.93
2	Color	Dark Brown
3	Temperature (°C)	20
4	Moisture Content (%)	0.63
5	Total Organic Carbon (%)	3.85
6	Organic matter (%)	6.63

Screening of the soil sample for presence nitrilase positive strains was first done on the minimal media. The colonies from the minimal media were then grown and analyzed for nitrilase activity. As *Alicagenesfaecalis* NCIM 2949 was taken as the

positive culture for nitrilase assay, there was a need to optimize the enzyme assay conditions which was done using design-expert platform. In the first factorial design as shown in **Table 1** run 5 was the only trial which provided a blue end point for enzyme assay, but it also presented a scope for further optimization of assay conditions and hence another factorial design as shown in **Table 2** was done. Run 1 of this factorial design gave maximum absorbance of 0.2AU at 630nm for enzyme assay and hence conditions of this trial were finalized for all the subsequent enzyme assays.

The nitrilase assay for VSS6001 gave an absorbance value equal to the positive culture. Biochemical tests reported the culture as gram positive, results for other parameters are as shown in Error! Reference source not found. On the basis of biochemical tests the strain VSS6001was identified as one of the two probable culture; Bacillus thuringiensis and Bacillus cereus. **Fig. 1** shows the assembled sequence for VSS6001 after 16s rRNA. The phylogenic tree for VSS6001 as shown in **Fig. 2** identifies the strain VSS6001 as Bacillus thuringiensis.

TABLE 4: RESULTS OF BIOCHEMICAL TESTS FOR VSS6001

S. No.	Parameter	Result
1	Urea	+
2	ß-D-Ribofuranoside	+
3	ß-D Glucopyranoside	-
4	ß-D Mannopyranoside	-
5	α-D-Glucopyranoside	+
6	Methionine	+
7	Serine	-
8	Sorbitol	-
9	Aesculin	-
10	Rhamnose	-
11	Trehalose	+
12	Ornithine	+
13	Cysteine	-
14	Glucose	+
15	B-Methyl Glucoside	+
16	Tyrosine	+
17	Arginine	+
18	Alanine	+
19	D-Alanine	+
20	Arginine	+
21	Glycerol	+
22	Sucrose	+
23	Citruline	+
24	Leucine	+
25	ß-D Galactopyranoside	-
26	Mannitol	+
27	Maltose	+
28	ß-D-Glucuronide	-
29	Threonine	-
30	Proline	-
31	Pyroglutamate	+
32	Valine	+

>VSS6001
GGGTGACGGTTATCCGGCATTATTGCGGCGTAAGCGCGCGCAGGTGGTTTCTTAAGTCTG
ATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAG
AAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCA
GTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
TTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGG
GCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTT
GGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCA
TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAA
GACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACT
CGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCACATGCCGGGGGAAAAAACCTT
TCCCGGGCCCTTGTACACACCGCCCGTCACACCACGGAGAGTTTGTAACACCCGAAGTCC
GGTGGGGTAACCTTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGTGACAAA
AAAAAACCCCCCCCGCCCCCACACACACCAAAAAAGAGGTCGAGAAAGGCGTCATTTCGC
GCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTC
TACGCATTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAG
TTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCT
GCGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCT
GCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCA
ACTAGCACTTGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCAC
GCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGT
AGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGC
ATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCAT
AAGTGACAGCCGAAGCCGCCTTTCAATTTCGAACCATGCGGTTCAAAATGTTATCCGGTA
TTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCA
CCCGTCCGCCGCTAACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCGACTTGCATGTA
TTAGGCACGCCGCCAGCGTTCATCCTGAGCGACAAAAAAAACCTTAAT

FIG.1: ASSEMBLED SEQUENCE OF VSS6001 AFTER 16s rRNA

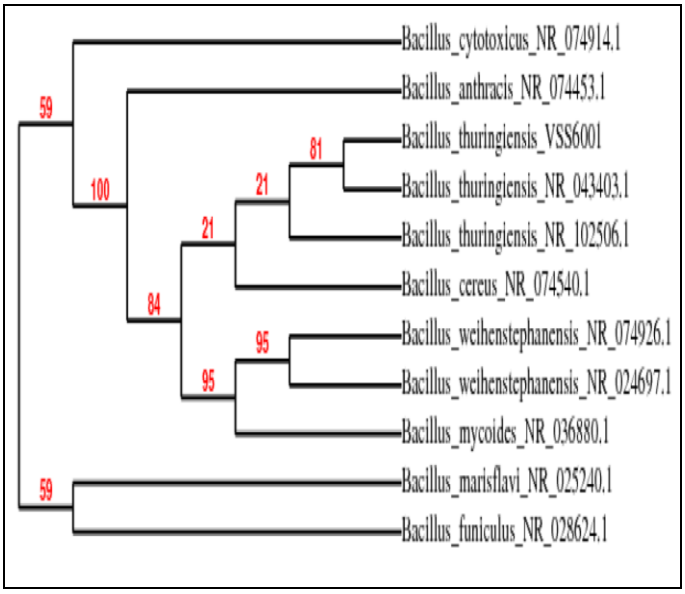

FIG. 2: PHYLOGENETIC TREE FOR VSS6001

CONCLUSION: Soil is one the ecological niche which harbors a large number of microorganisms.

The research work reported through this paper was planned to screen microorganisms harboring nitrilases that can catalyze aromatic nitriles. These aromatic nitrilase may then be employed in green synthesis of corresponding acids. Five soil sampling sites were selected for screening of the nitrilase producer microorganisms, out of which four sites provided no positive culture (Manuscript communicated as negative results, under review).

The presence of colonies on minimal media identifies them as a potential nitrilase producer strain(s). These strain(s) were further propagated and evaluated for nitrilase enzyme assay to select the most potent colony. It was the soil sample from outside Anami Organics Limited, District. Valsad-Gujarat, India which gave one potent positive strain, labeled as VSS6001 later identified as *Bacillus thuringiensis*. This strain has been reported for the production of bio pesticide [52–55].

Post identification of VSS6001, the Protein Data Bank (PDB) when searched with key words *Bacillus thuringiensis* showed 61 structure hits (As on 08-July-2014), which account for 16 enzyme structures, most of which are lactone hydrolase. Literature survey over various database(s) like PubMed, Science Directetc. provided no reports on *Bacillus thuringiensis* as a nitrilase producer. These reports of nitrilase from *Bacillus thuringiensis* make the future studies exciting as to how nitrilase from this species is different from the already existing nitrilases with respect to nitrile bioconversions and also whether this enzyme functions as a nitrile hydratases or nitrilase.

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