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Soil as a Source for Potential Nitrilase Producer

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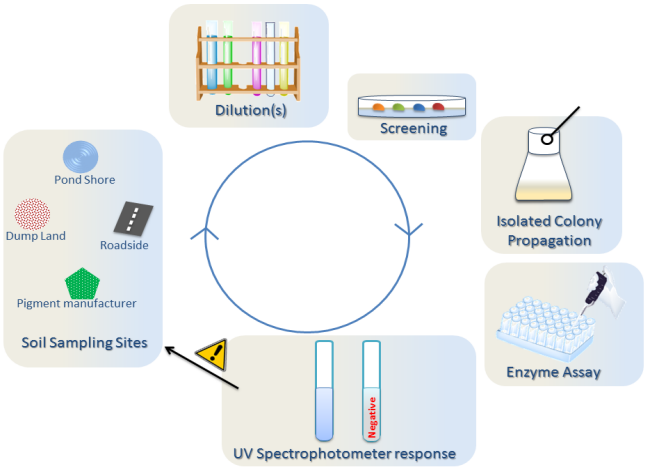
Graphical Abstract



Abstract: Enzymes have been identified as critical proteins for ages to carry out functions that spread across various industries. One of the recent applications of the enzymes is in the bioconversion schemes which ensure the formation of only one enantiomer product. The enzyme category hydrolase carries out the hydrolysis of C-N, C-C, C-O etc. bonds. Nitrilase(s) are a sub-category of hydrolase (EC 3.5.5.1) that has a huge potential in biocatalysis of nitriles. Nitrilase(s) carry out the hydrolysis of carbon-nitrogen bonds other than the amide bonds. Bioconversion schemes by nitrilases can generate chiral acids, which are generated otherwise from a very harsh and tedious chemical process. These chiral acids can be incorporated into various schemes of drug synthesis such as the production of chiral intermediates like nicotinic acid and mandelic acid etc.

Various ecological habitats have been explored in the past to screen nitrilases with activity over a broad temperature range. Soil is one such component in nature where the microorganisms can sustain for long periods. Soil sources like garden soil, Himalayan region soil etc. have been used to screen nitrilase in the past. Selecting a soil sampling site is very critical as it can significantly affect the final outcome(s) of the research objectives. The present work deals with screening of nitrilase producing microorganisms from four soil sampling sites, which are different from each other in respect with the annual temperature range, rainfall, and presence of industrial belt etc. Although this research work has provided many microbial isolates, none of these showed nitrilase activity.

Keywords: Biocatalysis, Bioconversion, Benzonitrile, Enzyme Assay, Minimal Media, Nitrilase

Introduction

Nitrilases are the enzymes that convert the nitriles into the corresponding carboxylic acids and ammonia. Unlike nitrile hydratases, nitrilase do not involve the formation of an amide intermediate.[1,2] Bacteria, fungi, and plants have been reported to harbor nitrilase(s).[3–5] Studies on nitrilase occurrence, mechanism of action, characteristics, substrate specificity, applicability, and gene cloning have been reported in the past.[6–8] With regard to the nitrile substrate nitrilases have been classified as aromatic nitrilase, aliphatic nitrilase and arylacetonitrilase. [9]

Nitrilases have been commercially utilized for the industrial production of acrylamide[10] and nicotinic acid.[11] According to the statistical data, there has been an increase in the reports on nitrilase research conducted at various institutes and universities. These research activities have led to isolation, identification and characterization of an array of microorganism harboring nitrilases from various places. Some of these research work(s) have already found application with reasonable success.[12]

Despite the identification of many nitrilase producing bacteria, fungi and plants, [5,13] the nitrilases from these sources have a limited application. This limited application is due to the properties relevant to enzyme activity, stability, substrate range etc.[14–16] There is a need for studies on screening nitrilase producing microorganisms that can produce nitrilase with industrially acceptable standards.

The present research work was aimed at screening the potential nitrilase producers from soil.

Table 1: Description of Soil Sampling Sites

Site Number	Details of Sampling Site	Location
1	Pond shore	Rohtak, Haryana INDIA
2	Dump Ground	Gurgaon, Haryana INDIA
3	Krima Sil Private Limited	Ankleshwar, Gujarat INDIA
4	Roadside GIDC area	Ankleshwar, Gujarat INDIA

Experimental

Culture, Reagent and Chemicals: *Alicagenes faecalis* NCIM 2949 was purchased from NCIM (National Collection of Industrial Microorganisms), Pune INDIA. All the media components and chemicals used in the studies were of analytical grade and purchased from Hi-media laboratory Pvt. Ltd., Merck Specialties Pvt. Ltd. INDIA, Fisher Scientific Qualigens INDIA, S. D. Fine Chemicals Pvt. Ltd. INDIA, and Loba Chemie Pvt. Ltd. INDIA.

Soil Sampling Sites: Four soil sampling sites as shown in **Figure 1** were identified to be screened for potential nitrilase producers (both bacterial and fungal strains). The soil sampling sites were representative of a broad annual temperature range from 5 °C to 45 °C, which could allow screening microorganisms with nitrilase activity over these temperature(s). **Site 1** has a microbial population coexistent with soil and water, **site 2** has a microbial population with waste catalyzing features, **site 3** is a green pigment manufacturing site, these pigments having nitrile groups in them and **site 4** is a roadside in the industrial belt used for material transportation. Samples were collected in sterile zipper polyethylene bags.

Physicochemical characterization of soil samples: The physicochemical parameters that were evaluated included color, temperature, pH, moisture content, total organic carbon and total organic content. Depth of soil sample collection has a critical role as the microbial population decreases with depth which may be due to limitations of available carbon, molecular oxygen etc. Temperature and color of the soil samples were noted on the spot. 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 72h in oven (Dolphin) and then the moisture content was calculated as follows: [17]

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

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

Carbon and Organic mass percent: In a 500mL conical flask, 1g soil sample was mixed with 10mL potassium dichromate (1N) and 20mL concentrated H_2SO_4. 200mL distilled water were added after 30min of incubation. The solutions were filtered and 10mL of 85% phosphoric acid was added. The contents were mixed and 1mL of diphenylamine was added. Titration was performed against Ammonium iron (II) Sulfate (0.5N) to a bright green end point.[18,19] Percent organic carbon (POC), Total organic

Carbon (TOC), and Percent Organic Matter (POM) were calculated using the following formula: [19,20]

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

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

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

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 the test site titration.

Screening - Minimal media composition

Isolation of the microorganisms was carried out by plating 10^{-5}, 10^{-6} and 10^{-7} dilutions of soil sample on minimal media containing 20mM benzonitrile as an inducer as well as the sole energy source. The minimal media was 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 chloride hexahydrate (0.001g/L), zinc sulphate (0.0067g/L) and agar (20g/L) prepared in distilled water. [21–23]

The plates were then incubated in the incubator (Meta Lab) at 25°C and 37°C to screen for fungal and bacterial strains respectively. The incubation temperature may be optimized at a later stage once a positive isolate is obtained. The isolated colonies were then propagated and analyzed regarding presence of nitrilase by modified Berthelot assay.[24] 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 isolates respectively. The incubation was carried for a minimum of 72h in an orbital shaker (Thermo-scientific).

Enzyme assay: Modified Berthelot assay was used for the determination of enzyme activity by tapping the generated ammonia. Benzonitrile was used as substrate.[25–27] *Alicagenes faecalis* has been reported to produce nitrilase and hence *Alicagenes faecalis* NCIM 2949 was used as the positive reference for the nitrilase assay.[28]

In the modified method, cells were harvested by centrifugation at 4000rpm/20°C/7min. Both the extracellular and intracellular components were analyzed. The intracellular enzyme examination involved cell lysis by ultra-sonication (TOSHCON) for 15min in cold conditions (0-4°C), followed by centrifugation at 6000rpm/20°C/7min. The respective intracellular components were re-suspended again in phosphate buffer pH 7.5 having 50mM benzonitrile and incubated at 37°C for 2h. After incubation the reaction was stopped by addition of 0.2mL hydrochloric acid (1N). A final centrifugation at 10000rpm/20°C/7min was done; and the supernatant of this stage was analyzed by the enzymatic assay.

0.08mL of phenol solution were added to 2mL of supernatant and vortexed gently. 0.08mL sodium nitroprusside and 0.2mL of oxidizing solution were added, mixed and incubated at room temperature for 1h followed by measurement of the absorbance at 630nm.

Results and Discussions

The research work reported through this paper was intended to screen potential nitrilase producers that could perform bioconversion of aromatic nitriles.

Bioconversion of aromatic nitriles to the corresponding chiral carboxylic acids is a valuable application as these acids could be utilized in drug intermediate and/or drug molecule synthesis. Soil samples were taken from regions with an annual temperature ranging from 5°C to 45°C, as these sites could provide nitrilase that can perform bioconversion for various processes. **Figure 1** shows the physical appearance of soils collected from different sites. Except for the dump site, which was taken from a 1 cm deep layer since the surface was a bit rocky, the soil samples were taken from the surface layer.

Basic physicochemical properties like pH, temperature and total organic carbon content of the soil were determined to assist in upstream optimization of the parameters related to incubation temperature and media pH of the screened microorganism. As shown in **Table 2,** it was observed that the soil from site 3 was more alkaline in nature than the other sites.

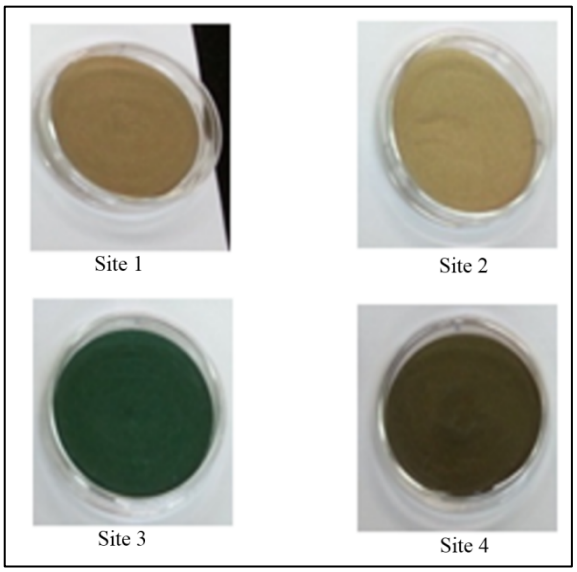


Figure 1: Soil from different sampling sites

was neutral. It was site 2 which contributed the maximum of 12 colonies in comparison with the other sites. A graphical representation of total organic carbon and pH is shown in **Figure 2**.

Screening for potential nitrilase producers was done on minimal media with trace elements, buffering components and benzonitrile, which acted as an inducer as well as nitrogen source. Growth of microorganisms on the minimal media revealed their potential as nitrilase producers. As shown in **Figure 3** the isolated strains were propagated with the objective of screening the most potent strain.

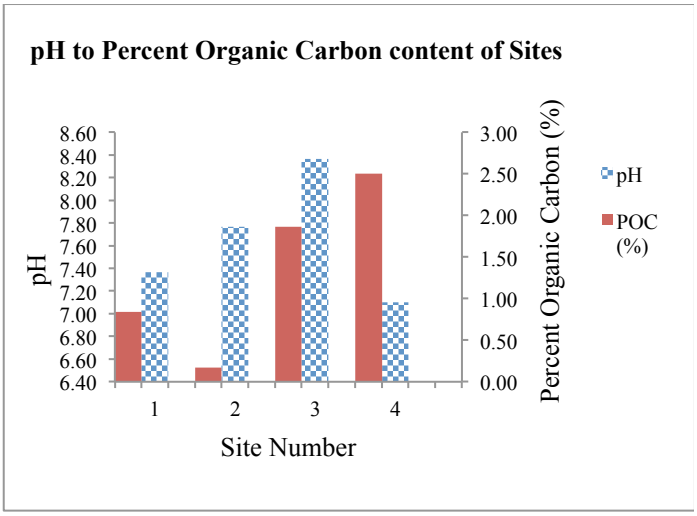


Figure 2: pH to organic carbon content relationship between different sites

After fermentation for 72h, the isolated colonies were evaluated for their nitrilase activity by the enzyme assay. **Table 3** shows the results for the screening exercise. The isolated strains were evaluated for their extracellular nitrilase activity. Extracellular nitrilase activity was not observed in any of the isolated strains. Enzyme assay for intracellular nitrilase activity was also negative for all 23 isolated strains. Various modifications of the method were attempted like increasing the enzyme-substrate (nitrilase-benzonitrile) incubation time (till 48h), substrate concentration (50mM-300mM), the ultrasonication time (5min,10min and 15min) and whole cell bioconversion, but none of the trials was positive. Other methods of screening based on pH change also reflected negative results.[29]

Table 1: Results of the Screening Exercise.

Parameter Evaluated	Result	Inference
Growth on Minimal Media	Yes	Isolates may be harboring nitrilase
Extracellular Nitrilase activity	Not Observed	No extracellular Nitrilase
Intracellular Nitrilase activity	Not Observed	No Intracellular Nitrilase

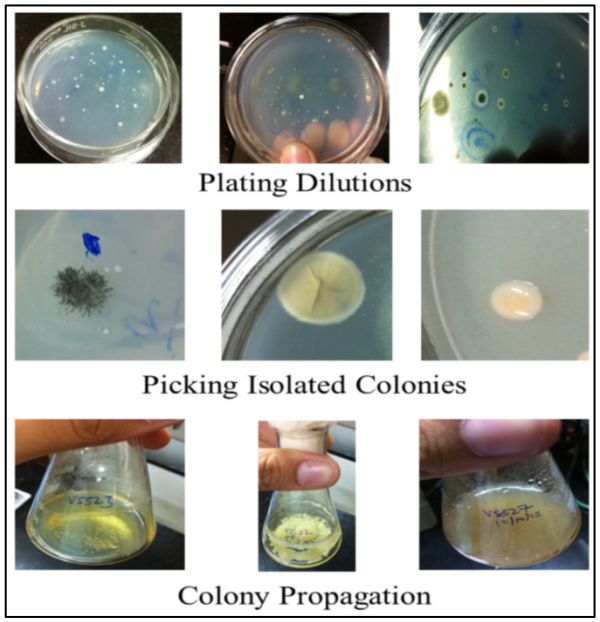


Figure 3: Various stages of the experiment

The negative results lead to the following conclusions:

- One of the parameters for selecting soil sampling sites was the broad annual temperature. This parameter may be co-included with other parameters like nitrile manufacturing sites to increase the probability of finding a potential nitrilase producer.
- Screening on minimal media needs to be complemented with some other screening approach like the inclusion of small amount of energy sources.
- Most of the colonies appeared after 4 days of incubation on minimal media which may be indicative of slow growth rate.
- Some of the plates showed the presence of fungal and bacterial colonies in the same plate; the role of other incubation temperature(s) like 20°C, 30°C etc. needs to be examined besides 25°C and 37°C.

- Benzonitrile was used as an inducer and a nitrogen source in the minimal media. Growth on minimal media with benzonitrile may also be due to presence of enzymes other than nitrilase.
- Ultrasonication was performed to release the intracellular nitrilase; it may also be possible that this step has affected the enzyme functionality.

The absence of nitrilase activity in all the microorganisms screened on the minimal media and understanding the possible reason(s) behind the negative results may be helpful in designing the research strategy for future screening exercises.

Acknowledgments

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Appendix- Table 2

Table 2: Physicochemical characterization of soil

Site No.	Color[30]	Temperature (°C)	Moisture Content (%)	pH	POC (%)	POM (%)	No. of Colonies isolated
1	Café au lait	38	0.50	7.37	0.84	1.93	09
2	Camel	37	0.89	7.77	0.17	0.40	12
3	CalPoly Pomona green	24	0.27	8.37	1.87	4.27	01
4	Café noir	24	0.14	7.10	2.51	5.73	01

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