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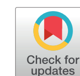
A simple, efficient and rapid screening technique for differentiating nitrile hydratase and nitrilase producing bacteria



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ABSTRACT

Nitrile hydrolyzing enzymes catalyze the hydration of nitrile compounds to corresponding amides and acids. Bacteria, isolated from soil samples were screened for nitrile hydrolyzing enzymes by simple dye based 96 well plate and nesslerization method. Bromothymol blue was used as an indicator for the detection of amides and acids based on colour change of the indicator dye from blue to dark green or yellow. The screening assay also differentiates between nitrile hydratase (NHase) and nitrilase producing bacteria. Among the 108 bacterial strains screened for enzyme activity, six strains were positive for NHase activity and eleven strains were positive for nitrilase activity based on their ability to degrade acrylonitrile into products. The strain showing maximum NHase activity in quantitative assay was identified as *Rhodococcus rhodochrous*. The modified method developed by us would be useful for rapid screening of nitrile degrading bacteria potent for acrylamide/acrylic acid production when acrylonitrile is supplied as substrate.

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1. Introduction

Nitrile compounds (i.e. organic cyanides) are cyanide substituted carboxylic acids with general formula R-CN and are widespread in the environment [1]. These compounds are naturally produced by plants and occur as cyanoglycosides, cyanolipids, ricinine, phenylacetonitrile and β-cyanoalanine, nitriles also occur as intermediates of microbial metabolism [2]. Nitrile compounds are of synthetic importance and are extensively used in the manufacture of a variety of chemicals, amides, carboxylic acids, heterocyclic compounds, pharmaceuticals, pesticides and polymers [3]. Although, most of the nitriles are useful, they are highly toxic, mutagenic, carcinogenic and teratogenic because of their cyano group [4,5]. Therefore, the extensive use of these compounds may lead to environmental problems [6]. In order to alleviate this problem, chemical hydrolysis of nitriles to their corresponding acids and amides are generally followed. However, the whole process produces unwanted byproducts, including large amounts of inorganic salts as wastes [7]. Microorganisms can degrade and detoxify a wide variety of nitriles and use it as a source of carbon or nitrogen for their growth [8]. The microbial metabolism for removing toxic nitriles from industrial waste proceeds through two distinct enzymatic pathways [9] (Fig. 1). In the first pathway, nitrilase (EC 3.5.5.1) catalyzes the direct conversion of nitriles to corresponding carboxylic acids and ammonia and in the second pathway nitrile hydratase (EC 4.2.1.84) catalyzes the hydration of nitriles to corresponding amides, which is subsequently hydrolyzed to carboxylic acids and ammonia by amidases (EC 3.5.1.4) [10,11] (Fig. 1).

Recently, nitrile hydrolyzing enzymes from microbes have been used for remediation of nitrile contaminated soil and water as well as for the conversion of nitriles to useful chemical products [12,13]. The hydrolysis of nitriles by microbial nitrile hydratase has also been exploited for the commercial production of acrylamide [14]. Therefore, screening for nitrile hydrolyzing enzymes from microorganisms is gaining much attention in recent years [15–17]. Among the microorganisms, bacteria are potent producers of nitrile hydrolyzing enzymes. A number of screening techniques like HPLC, GC, UV spectrophotometer have been reported in the past, to identify microorganisms producing nitrile hydrolyzing enzymes [18,19]. However, these techniques are tedious, time consuming and expensive. Any successful microbial screening requires a rapid, sensitive, reliable, reproducible and relatively inexpensive screening assay. Therefore, the aim of the present study was to screen for nitrile hydrolyzing enzymes with a simple high-throughput dye based 96 well plate screening assay which differentiates between nitrile hydratase and nitrilase producing bacterial isolates.

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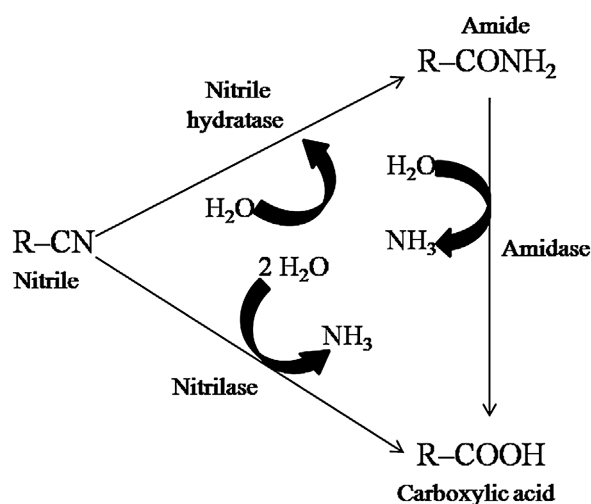

Fig. 1. The two different pathways of enzymatic conversion of nitrile compounds into amides and carboxylic acids.

2. Materials and methods

2.1. Chemicals and reagents

All the reagents used in the experiment were of analytical or HPLC grade. Acrylonitrile, bromothymol blue, sodium phenolate and sodium nitropruside were purchased from Sigma-Aldrich (St Louis, USA). The pure enzymes nitrile hydratase and nitrilase were also procured from Sigma-Aldrich (St Louis, USA). Acrylamide and acetonitrile were purchased from Merck (Germany). All other media components were purchased from Himedia, (Mumbai, India).

2.2. Sample collection and Isolation of nitrile metabolizing bacteria

Soil samples for the isolation of nitrile metabolizing bacteria were collected aseptically in screw cap glass tubes from different parts of Karnataka and other states of India. One gram of each soil sample was suspended in 10 mL of sterile distilled water, serially diluted (10^{-6}) and plated on to nutrient agar medium and incubated at 37 °C for 24–48 h. Individual bacterial colonies were further purified and maintained on nutrient agar slants, and stored at 4 °C. Nitrile metabolizing bacteria was also isolated from soil samples by enrichment culture technique [20].

2.3. Microorganisms and culture conditions

The strains obtained from the culture collection center (MTCC) and the strains isolated by us laboratory were cultivated in 100 mL of basal medium containing glucose (10.0 g L^{-1}), yeast extract (0.2 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}) and $FeSO_4.7H_2O$ (0.01 g L^{-1}), $CoCl_2$ (0.01 g L^{-1}), (pH 7.2) and incubated at 30 °C, 160 rpm in an incubator shaker for 24 h to prepare preculture. Two milliliter (2 mL) of pre culture was inoculated in above growth medium supplemented with 1 % acrylonitrile and incubated at 30 °C and 160 rpm for 36 h. The cells were harvested by centrifugation at 8000 × *g* for 5 min at 4 °C. The cell pellets were washed thoroughly twice with 100 mM potassium phosphate buffer (pH 7.2) and the pellets were re-suspended in the same buffer and stored at 4 °C until further use.

2.4. Screening for nitrile hydrolyzing enzymes

The colorimetric assay to screen for nitrile hydrolyzing enzymes was performed in 96 well microtiter plates using pH indicator dye (bromothymol blue). The screening assay was based on change in the pH occurring during hydrolysis of substrate (acrylonitrile) into amides or carboxylic acids and ammonia. The reaction mixture consisting of 201 μL of 10 mM phosphate buffer containing 0.01 % bromothymol blue (pH 7.4), 5.75 μL of 500 mM acrylonitrile and 23.25 μL of cell suspension (200 mg/mL) was used. The plates were incubated at 30 °C for 3–12 h. The assay was performed with *Rhodococcus rhodochrous* (MTCC-291), as positive control for nitrilase and *Rhodococcus erythropolis* (MTCC-1526), as positive control for nitrile hydratase. The strain *Escherichia coli* (MTCC-729) was used as a negative control, as it does not produce nitrile hydrolyzing enzymes. The control experiments were also performed without cells, substrate, products (acrylamide and acrylic acid) and dye in the reaction mixture [21]. The detection errors of the pH indicator dyes were maintained at ±0.05 %. The principle behind the experiment is the colour change of the pH indicator from blue to greenish yellow indicating the formation of amide (acrylamide) and thus positive for nitrile hydratase. A colour change of the pH indicator dye from blue to yellow indicates the formation of carboxylic acids (acrylic acids) and therefore positive for nitrilase. This colour change was used as an indicator to differentiate between nitrile hydratase and nitrilase producing bacterial strains. One hundred and eight (108) bacterial strains isolated by us from soil samples were screened for the production of nitrile hydrolyzing enzymes by this method.

2.5. Determination of ammonia

Further experiments were performed to distinguish between nitrile hydratase and nitrilase producing bacterial strains by determination of ammonia by nesslerization method [22]. The assay was performed in 96 well microtiter plate, with reaction mixture consisting of 201 μL of 10 mM phosphate buffer (pH 7.4), 5.75 μL of 500 mM acrylonitrile and 23.25 μL of cell suspension (200 mg/mL). The plates were incubated at 30 °C for 3–12 h. The assay was performed with *Rhodococcus rhodochrous* (MTCC-291), as positive control for nitrilase and *Rhodococcus erythropolis* (MTCC-1526), as positive control for nitrile hydratase. The strain *Escherichia coli* (MTCC-729) was used as a negative control. Control experiments were also performed without cells and substrate in the reaction mixture. The amount of ammonia liberated in the reaction was detected by addition of 50 μL of Nessler's reagent.

2.6. Quantitative screening for nitrile hydratase and nitrilase activity

The bacterial strains which showed positive for the presence of nitrile hydratase and nitrilase in the initial screening assay were chosen for further evaluation in the liquid medium to quantify nitrile hydratase and nitrilase by spectrophotometric assay [23,24]. Each of the selected strains were inoculated in 50 mL of minimal medium containing glycerol (10.0 g L^{-1}), peptone (5.0 g L^{-1}), malt extract (3.0 g L^{-1}), yeast extract (3.0 g L^{-1}), pH 7 and incubated at 30 °C, 160 rpm in an incubator shaker for 24 h to prepare preculture. Two milliliter (2 mL) of pre culture was inoculated in 50 mL of production medium supplemented with 1 % acrylonitrile and incubated at 30 °C and 160 rpm for 36 h. The cells were harvested by centrifugation at 8000 × *g* for 5 min at 4 °C. The cell pellets were washed thoroughly twice with 100 mM potassium phosphate buffer (pH 7.2) and the pellets were re-suspended in the same buffer and used for enzyme assay.

2.7. Estimation of nitrile hydratase activity

Nitrile hydratase activity was assayed in a reaction mixture (1 mL) containing 100 mM phosphate buffer (pH 7.0), bacterial cells (1 mg dcw/mL) and acrylonitrile (8 %, w/v, i.e. 1500 mM). The reactions were carried out at 10 °C for 1 h with shaking. The reactions were stopped by the addition of equal volume of 0.1 N chilled HCl [25]. The amount of acrylamide formed in the reaction mixture was determined spectrophotometrically by measuring the absorbance at 235 nm [26]. One unit of nitrile hydratase activity was defined as an amount of enzyme required to convert 1 μmol of acrylonitrile to acrylamide per minute under the standard assay conditions.

The end product of the reaction i.e. acrylamide was also determined using Shimadzu LC10ATVP series HPLC system equipped with C18 column (250 mm × 4.6 mm × 5 I.D micro meter) and diode array detector set at isocratic conditions with 97 % acetonitrile and 3 % 5 mM acetic acid as mobile phase at 1 mL min^{-1} flow rate and detection carried out at 210 nm at 28 °C [25].

2.8. Estimation of nitrilase activity

Nitrilase activity was determined by analyzing the amount of ammonia released during the reaction by Bertholet reaction [24]. The assay was performed in a reaction mixture (1.0 mL) containing 0.1 M potassium phosphate buffer, 50 mM acrylonitrile and 200 μg of resting cells. The heat denatured cells were used in control experiments. The reaction was carried out at 30 °C for 30 min and the reaction was stopped by adding 0.1 mL each of 0.33 M sodium phenolate, 0.02 M sodium hypochlorite and 0.01 % (w/v) sodium nitroprusside. The mixture was kept in boiling water bath for 2 min and the sample was diluted with 0.6 mL of water. The amount of ammonia liberated in the reaction was determined spectrophotometrically by measuring the absorbance at 640 nm. The experiments were calibrated with known concentrations of NH_4Cl solutions. One unit of nitrilase activity was defined as an amount of enzyme required to produce 1 μmol of ammonia by the hydrolysis of acrylonitrile per minute under the standard assay conditions.

2.9. Morphological and Molecular identification of selected bacterial strains

The morphological identification of the strain RS-6 was performed by Gram staining and biochemical tests. The identification was performed according to Bergey's manual of determinative bacteriology. The bacterial strain RS-6 was grown in 100 mL Nutrient Broth (NB) for 24–48 h at 37 °C. The total genomic DNA was extracted by cetyl trimethylammonium bromide (cTAB) method [27]. The purity of the extracted DNA was confirmed by Agarose Gel Electrophoresis. The 16S rDNA region of the purified DNA was amplified by PCR experiments using universal primers, 785 F primer 5′- GGATTAGATACCCTGGTA-3′ and 907 R 5′ - CCGTCAATTCMTTTRAGTTT-3′ [28]. The sequences obtained were compared with that of sequence provided in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) database using the Basic Local Alignment Search Tool (BLAST). Top hit sequences were selected for further phylogenetic analysis. Multiple sequence alignments were performed by using the CLUSTAL-X2 software version 2.1 (based on the algorithm of Waterman) [29]. Phylogenetic tree was constructed using Phylogenetic tree prediction GeneBee software and the alignment data was analyzed by neighbor-joining (NJ) method. The sequence was submitted to GenBank and the accession numbers were obtained.

2.10. Statistical analysis

All the experiments were carried out in triplicates and the results were expressed as mean ± SD using SPSS software (version 20.0).

3. Results and discussion

3.1. Screening for nitrile hydrolyzing enzymes

Nitrile hydrolyzing enzymes are potentially used in industries as desired biocatalysts for the production of amides and carboxylic acids. Several screening assays were documented for nitrile-converting enzymes based on continuous and blocked methods [18,19]. Recent techniques have been developed for the determination of ammonia by colorimetric and fluorometric methods [15,30].

One hundred and eight (108) bacterial strains capable of utilizing nitriles as sole source of carbon and nitrogen were isolated from soil samples collected from tropical areas of different parts of Karnataka and other states of India by serial dilution and enrichment culture technique [31]. The bacterial isolates were screened for activity of nitrile hydrolyzing enzyme by dye based method using acrylonitrile as substrate. This method was based on the principle of change in the pH of the medium, which results in the colour change of the pH indicator dye. Since the optimal pH for

Table 1
The pH of the growth medium before and after the reaction.

Sl no.	Culture code	Initial pH of the medium	Final pH of the medium (after 3 h)	Final pH of the medium (after 6 h)
1.	RS-1	7.40	6.93 ± 0.01	6.92 ± 0.01
2.	RS-2	7.40	6.93 ± 0.01	6.91 ± 0.02
3.	RS-3	7.40	6.89 ± 0.01	6.89 ± 0.01
4.	RS-4	7.40	6.86 ± 0.01	6.85 ± 0.01
5.	RS-5	7.40	6.92 ± 0.01	6.91 ± 0.01
6.	RS-6	7.40	6.82 ± 0.01	6.80 ± 0.02
7.	RR-1	7.40	6.12 ± 0.01	6.11 ± 0.01
8.	RR-2	7.40	6.06 ± 0.00	6.06 ± 0.01
9.	RR-3	7.40	6.15 ± 0.01	6.14 ± 0.01
10.	RR-4	7.40	6.10 ± 0.01	6.10 ± 0.00
11.	RR-5	7.40	6.02 ± 0.01	6.02 ± 0.01
12.	RR-6	7.40	6.07 ± 0.01	6.06 ± 0.01
13.	RR-7	7.40	6.10 ± 0.01	6.10 ± 0.01
14.	RR-8	7.40	6.07 ± 0.01	6.07 ± 0.01
15.	RR-9	7.40	5.96 ± 0.00	5.96 ± 0.00
16.	RR-10	7.40	6.10 ± 0.01	6.09 ± 0.01
17.	RR-11	7.40	6.13 ± 0.02	6.12 ± 0.01

RS and RR represents culture codes.

nitrile hydrolyzing enzymes lies between pH 7.0 and 7.5, bromothymol blue was used as pH indicator dye, which is blue in colour at pH 7.4. Due to the catalytic activity of nitrile hydratase and nitrilase, the nitriles were degraded into corresponding amides and carboxylic acids which changes the pH of the medium, and therefore change in colour from blue to dark green indicating the accumulation of amides by nitrile hydratase activity and the change in colour from blue to yellow indicating the accumulation of carboxylic acids by nitrilase activity. The results were validated by testing the pH of the medium before and after the reaction (Table 1). The change of colour is directly proportional to the amount of amides and carboxylic acids released into the culture medium and therefore the intensity of colour is directly proportional to the activity of enzyme.

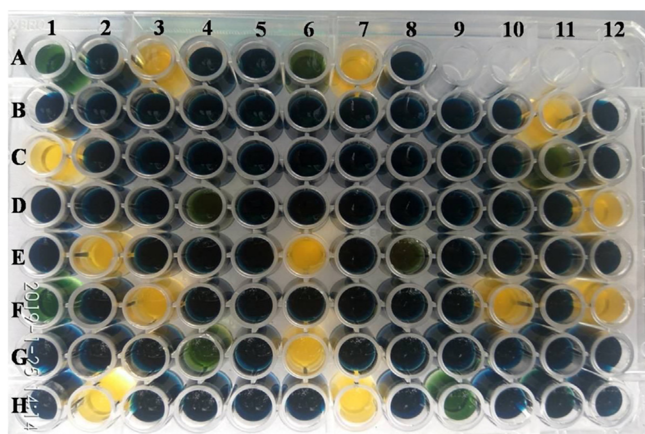

Fig. 2. Plate screening for nitrile hydratase and nitrilase producing bacterial strains using acrylonitrile as substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Row A: Control experiments: A1: Nitrile hydratase positive (colour change from blue to dark green), A2: Nitrile hydratase negative (no colour change), A3: Nitrilase positive(colour change from blue to yellow), A4: Nitrilase negative (no colour change), A5: Acrylonitrile control, A6: Acrylamide control, A7: Acrylic acid control, A8: Dye control. Rows B, C, D, E, F, G and H: Different bacterial strains tested for enzyme production. The bacterial strains in rows C-11, D-4, E-8, F-1, G-4 and H-9 were positive for nitrile hydratase activity and the bacterial strains in rows B-11, C-1, D-12, E-2, E-6, F-3, F-10, F-12, G-6, H-2 and H-7 positive for nitrilase activity.

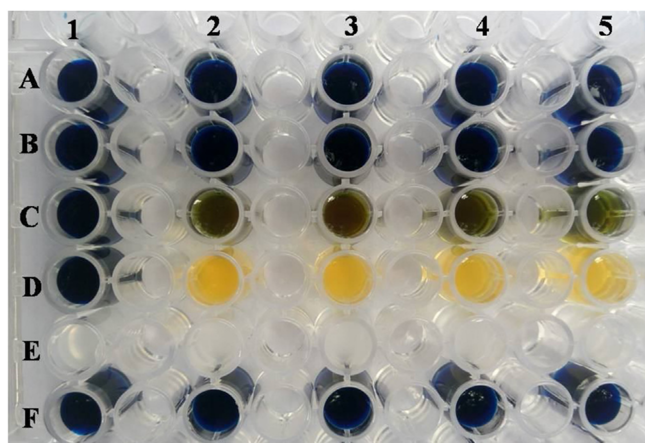

Fig. 3. Nitrile hydratase and nitrilase activity using whole cells in 96 well microtiter plate in different time intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Row A: Enzyme blank, Row B: Substrate blank, Row C: Nitrile hydratase positive (colour change from blue to dark green), Row D: Nitrilase positive (colour change from blue to yellow), Row E: Dye blank, Row F: Negative control.
Column 1: Zero hour, Column 2, 3, 4 and 5: 3, 6, 9 and 12 h respectively.

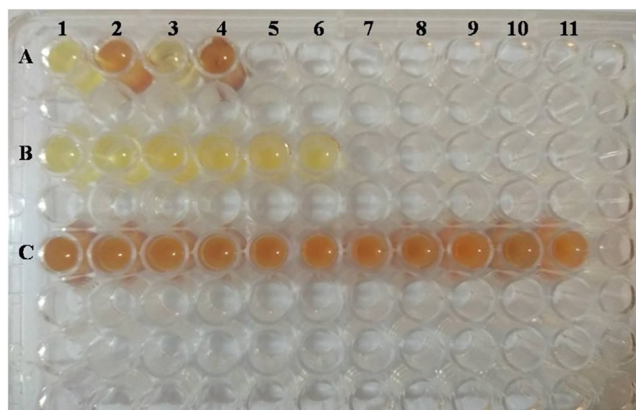

Fig. 4. Plate screening for nitrile hydratase and nitrilase producing bacterial strains by determination of ammonia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Row A: Control experiments: A1: Nitrile hydratase positive (No colour change), A2: Nitrilase positive (colour change from colourless to orange), A3: Acrylamide control, A4: Ammonia control.
Rows B1 to B6: Nitrile hydratase positive cultures (No colour change).
Rows C1 to C11: Nitrilase positive cultures (colour change from colourless to orange).

The positive control for nitrile hydratase i.e. *Rhodococcus erythropolis* (MTCC-1526) showed change in colour of the indicator dye from blue to dark green indicating the accumulation of amides (acrylamide) whereas the positive control for nitrilase i.e. *Rhodococcus rhodochrous* (MTCC-291) showed change in colour of the indicator dye from blue to yellow indicating the accumulation of carboxylic acids. The negative control for nitrile hydratase and nitrilase i.e. *Escherichia coli* (MTCC-729) did not show any colour change of the indicator dye. Control experiments were also performed with acrylonitrile, acrylamide, acrylic acids and dye (bromothymol blue) (Fig. 2). The results were also validated using commercially available nitrile hydratase and nitrilase enzymes. Preliminary experiments were performed to confirm that indicator dye do not have any inhibitory effect on enzyme activity and also on the growth of bacteria. Experiments were also performed without enzyme, substrate and dye (bromothymol blue) and proved that

Table 2
Quantitative screening of bacterial strains for nitrile hydratase activity.

Sl no.	Culture code	Nitrile hydratase activity (U mL^{-1})
1.	RS-1	0.12 ± 0.01
2.	RS-2	0.11 ± 0.01
3.	RS-3	0.46 ± 0.04
4.	RS-4	0.96 ± 0.04
5.	RS-5	0.17 ± 0.03
6.	RS-6	1.40 ± 0.12

Table 3
Quantitative screening of bacterial strains for nitrilase activity.

Sl no.	Culture code	Nitrilase activity (U mL^{-1})
1.	RR-1	0.08 ± 0.0
2.	RR-2	0.26 ± 0.01
3.	RR-3	0.03 ± 0.01
4.	RR-4	0.17 ± 0.03
5.	RR-5	0.45 ± 0.04
6.	RR-6	0.29 ± 0.01
7.	RR-7	0.22 ± 0.01
8.	RR-8	0.24 ± 0.03
9.	RR-9	0.64 ± 0.05
10	RR-10	0.12 ± 0.01
11.	RR-11	0.04 ± 0.01

colour change of the indicator dye was not due to the buffer salts or the cells themselves (Fig. 3). Experiments were also performed with addition of acrylamide and acrylic acids as positive control. Among the 108 bacterial strains screened for nitrile hydratase and nitrilase activity, six (6) strains were positive for nitrile hydratase activity and eleven (11) strains were positive for nitrilase activity (Fig. 2).

3.2. Determination of ammonia

The amount of ammonia liberated during nitrile degradation was determined by Nesslerization method [22]. The detection of the level of ammonia released was an additional confirmation of the efficacy of our screening for distinguishing between nitrile hydratase and nitrilase activity. Bacteria possessing nitrilase activity generally degrade nitriles and release carboxylic acids and ammonia into the medium. The ammonia released in the reaction was then estimated by adding Nessler's reagent. The intensity of the colour change (from colorless to orange) is directly proportional to the amount of ammonia released which in turn proportional to nitrilase production. Whereas, bacteria possessing nitrile hydratase activity, would degrade nitriles into amides and therefore no ammonia will be released into the medium. Hence, there was no colour change of the medium after the addition of Nessler's reagent. Seventeen (17) bacterial strains which showed positive for nitrilase and nitrile hydratase activity in the dye based screening method were tested for liberation of ammonia using acrylonitrile as substrate. Six (06) bacterial strains positive for nitrilase hydratase did not liberate ammonia as the end product in the reaction mixture and therefore, negative for nitrilase (Fig. 4). Whereas, eleven (11) bacterial strains positive for nitrilase liberated ammonia and showed colour change from colourless to orange in addition of Nessler's reagent (Fig. 4). Therefore, the results categorically indicate that ammonia was released into the medium when bacteria produce nitrilase to degrade nitriles.

3.3. Quantitative screening for nitrile hydratase and nitrilase activity

The quantitative screening for six (6) positive nitrile hydratase producing strains (coded as RS-1 to RS-6 and eleven (11) positive nitrilase producing strains (coded as RR-1 to RR-11) was carried

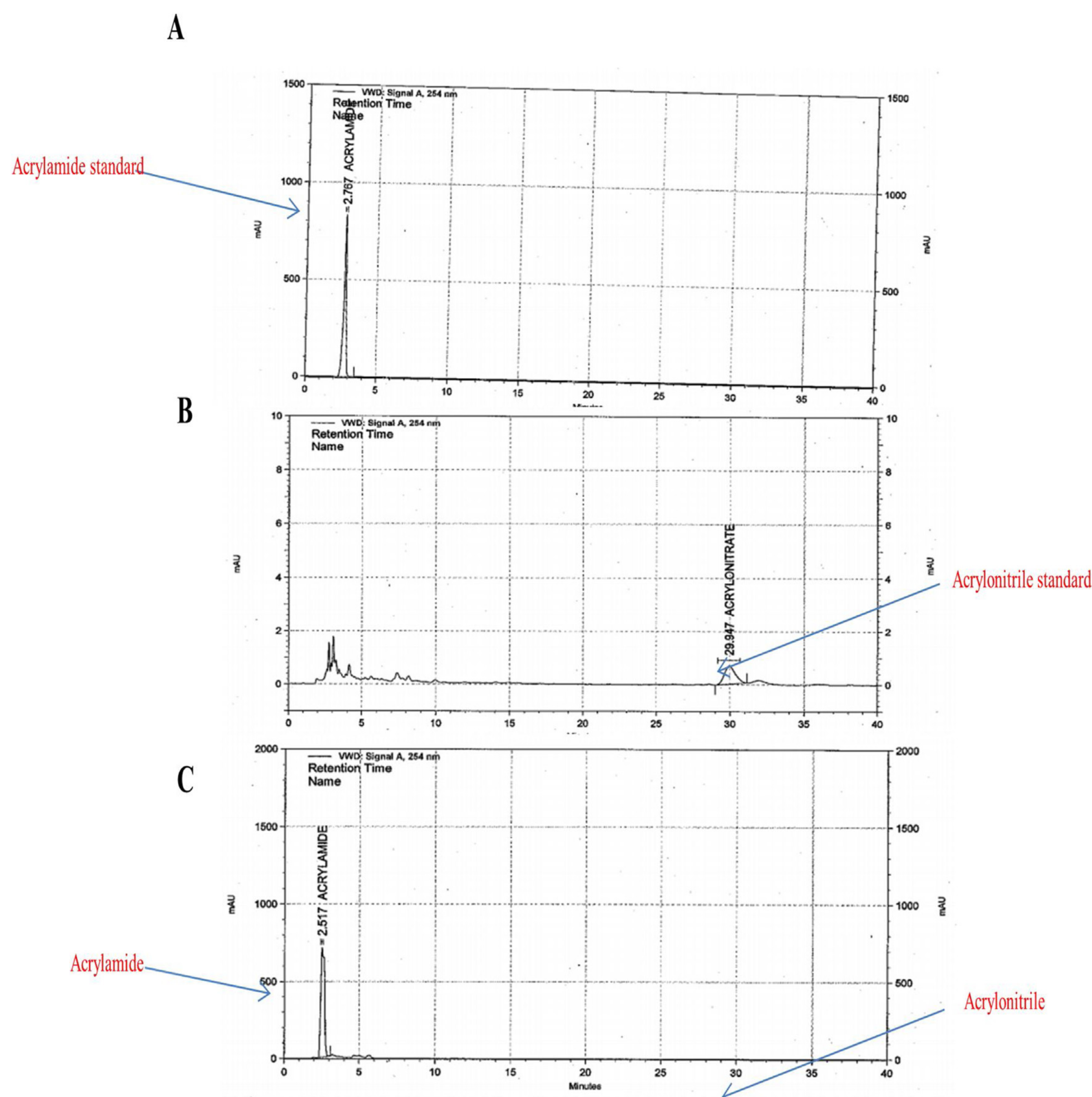

Fig. 5. HPLC analysis of bioconversion of acrylonitrile to acrylamide by *Rhodococcus rhodochrous* (RS-6). (A) Acrylamide standard (2.517 RT) (B) Acrylonitrile standard (29.497 RT) (C) Bioconversion of acrylonitrile to acrylamide.

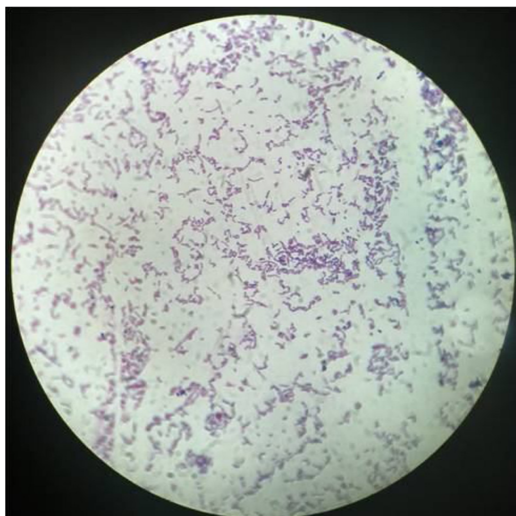

Fig. 6. Microscopic observation of Gram stained *Rhodococcus rhodochrous* (RS-6).

Table 4
Morphological and biochemical characteristics of *Rhodococcus rhodochrous* RS-6.

Morphological and Biochemical test	Results
Shape	Rod
Colour of the colony	Orange
Gram staining	Positive
Motility	Non motile
Sporulation	Non sporulating
Catalase	Positive
Oxidase	Negative

out by spectrophotometric analysis [23]. Among the six bacterial strains RS-6 showed maximum nitrile hydratase activity (Table 2) and the strain RR-9 showed maximum nitrilase activity (Table 3). The strain RS-6 was used for further study i.e. production of acrylamide from the substrate acrylonitrile and the end product i.e. acrylamide was confirmed by HPLC analysis (Fig. 5). The strain RS-6 was subjected to molecular level identification.

3.4. Molecular identification of selected bacterial strains

Morphologically the strain (RS-6) was identified as Gram positive, rod shaped, non-motile, non sporulating, aerobic and mesophilic bacteria (Fig. 6 & Table 4). The strain RS-6 was catalase positive and oxidase negative. The molecular identification of the potential NHase producing bacteria (RS-6) was done by sequencing and characterizing the gene encoding for 16S rDNA. The sequence was queried in nucleotide BLAST search from National Center for Biotechnology Information (NCBI) to find out the homology with the existing species of *Rhodococcus*. The length of the nucleotide sequence obtained was 1455 base pairs, which showed 99 % identity with *Rhodococcus rhodochrous* and subsequently coded as *Rhodococcus rhodochrous* (RS-6). The nucleotide sequence data were deposited to GenBank and obtained the accession number *Rhodococcus rhodochrous* RS-6 (MN365073.1). The phylogenetic tree was constructed by obtaining the ITS sequences of closely related taxa of the organisms collected from Genbank database (Fig. 7).

There are two potent enzymes which play an important role in nitrile metabolism: a) Nitrile hydratase and b) Nitrilase. Nitrile hydratase converts the nitrile compound into its corresponding amide while nitrilase to carboxylic acid with the release of ammonia. The liberated ammonia is used by bacteria as a source of nitrogen and excess ammonia gets released into the medium. Therefore, the amount of ammonia liberated is directly proportional to nitrilase activity. The substrate acrylonitrile used in the present study, is a potent inhibitor of amidase an enzyme responsible for the conversion of amide to carboxylic acid and ammonia [32] and therefore, resulted in the accumulation of amide i.e. acrylamide.

Owing to the importance of the above nitrile hydrolyzing enzymes in bioconversion of nitriles into useful carboxylic acids and amides, there has been a constant search in recent years for microorganisms capable of producing nitrile degrading enzymes. Both nitrile hydratase and nitrilase have great potential as industrial biocatalysts. Nitrile hydratase catalyzes the conversions of acrylonitrile to acrylamide [26], 3-cyanopyridine to nicotinamide [33], and phenylglycinonitrile to phenylglycinamide [34] and butyronitrile to butyramide [35]. Nitrilase are used in the industrial production of nicotinic acid [36], (*R*)-(-)-mandelic acid [37], (*R*)-4-cyano-3-hydroxybutyric acid [38] and glycolic acid [39].

A number of screening methods have been explored in recent years to find nitrile hydratase and nitrilase producing bacterial strains. Our aim was to screen wild bacterial strains isolated from their natural habitat for their ability to utilize by degrading nitrile compounds with nitrilase and nitrile hydratase or both. Though a rapid high-throughput colorimetric method for screening nitrilase-producing microorganisms using pH sensitive indicators was developed by Banerjee et al. [21], the method has the lacuna of

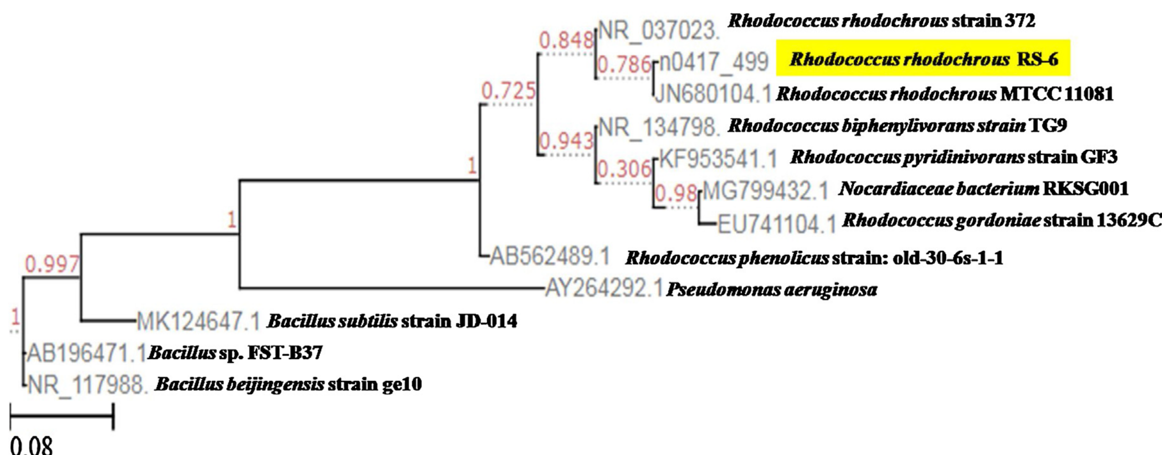

Fig. 7. Phylogenetic dendrogram based on the 16S rDNA sequence of *Rhodococcus rhodochrous* (RS-6). Number in parenthesis are accession numbers of published sequences. Bootstrap values were based on 100 replicates.

differentiating between nitrilase and nitrile hydratase in bacteria. Screening for both the enzymes simultaneously under dye based method and Nesslarization method have not been reported so far and a first report is presented here where a simple and rapid pH sensitive indicators are used to evaluate the activity of nitrile hydrolyzing enzymes by exploiting the sensitivity of amidase to the substrate acrylonitrile [40]. Detection of ammonia released due to nitrilase activity by Nesslarization method directly determines the activity of nitrilase, an additional confirmatory method. This enzymatic assay is capable of distinguishing between nitrile hydratases from nitrilases, but it is difficult to differentiate those bacterial strains with dual enzyme activity and get conclusive results.

4. Conclusion

The method used in the present study takes advantage of the change in the colour of the medium brought about by change in the pH due to the hydration of nitriles into amides and carboxylic acids, within the shortest period of time. The method identifies the end product of nitrile hydratase and nitrilase activity and thus enabling the identification and differentiation of bacterial strains producing these enzymes. Also, acrylonitrile plays dual role both as substrate as well as an amidase inhibitor.

Declaration of Competing Interest

None.

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