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SHORT COMMUNICATION

A plate method for screening of bacteria capable of degrading aliphatic nitriles

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Abstract A novel indicator plate method was developed for screening of aliphatic-nitrile-degrading bacteria. Isolated bacteria were tested for utilization of acetonitrile as sole source of carbon and nitrogen with the release of ammonia. The released ammonia causes increase of the pH of the medium. Phenol red indicator is used for detection of ammonia based on colour change of the indicator dye from red to pink. The liberation of ammonia from aliphatic-nitrile-utilizing bacteria is also studied in plates containing other indicators such as bromothymol blue and phenolphthalein. The usefulness of the indicator plate is demonstrated for bacteria that degrade certain aliphatic nitriles. Bacteria degrading nitriles as a nitrogen source can also be isolated with a medium containing additional carbon source. This plate method would be useful in isolation and screening of bacteria for degradation of aliphatic nitriles and also for production of nitrile-hydrolyzing enzymes.

Keywords Acetonitrile · Aliphatic nitriles · Biodegradation · *Paracoccus* sp. · Phenol red

Introduction

Nitrile compounds are cyanide-substituted carboxylic acids which are produced naturally and synthetically and that have the general structure R-CN. Naturally occurring nitriles are found in higher plants, bone oils, insects and microorganisms [5]. Chemical industries make extensive use of various nitrile compounds in the manufacture of a variety of polymers and other chemicals. They are also important for synthesis of amines, amides, carboxylic acids, esters, aldehydes, ketones and heterocyclic compounds [2]. The production or consumption of acetonitrile and acrylonitrile per annum is 40,000 and 4,000,000 tonnes, respectively [8]. Most nitriles are highly toxic and some are mutagenic and carcinogenic in nature [13]. The nitrile bond is very stable, and chemical hydrolysis of nitriles requires harsh conditions such as heating in acid (6 M HCl) or base (2 M NaOH) [17]. In addition, formation of byproducts such as toxic HCN or large amounts of salts are some drawbacks of chemical hydrolysis. Microbial degradation has been considered as an efficient way of removing highly toxic nitriles from the environment. Different enzymes are responsible for the metabolism of nitriles in microbes. Biological degradation of nitriles proceeds by two distinct routes: (a) nitrile hydratase hydrolyzes nitriles into amides, which are subsequently hydrolyzed to acids plus ammonia by an amidase, or (b) nitralase transforms nitriles directly into acids plus ammonia [7].

Because of the widespread presence of aliphatic nitriles in industrial effluents, the difficulty in their removal from these effluents, their toxicity, and the harsh conditions and slow rate of their degradation, considerable attention has been given to nitrile biodegradation. Many authors have reported degradation of nitriles by bacteria. The microorganisms used include *Nocardia rhodochrous* LL100-21 [5], *Arthobacter* sp. I-9 [18], *Pseudomonas* [14], *Klebsiella pneumoniae* [11] and *Rhodococcus* sp. [12]. Although nitrile-degrading bacteria have been isolated by enrichment culture technique, none of these studies employed a simple and rapid method for screening of bacteria capable of degrading nitriles. The high-performance liquid chromatography (HPLC)

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technique is the most commonly used method for analytical studies, with acetonitrile as one of the solvents. After product recovery, the spent solvent mixture contains acetonitrile, water and minor organic ingredients [6]. The present investigation was therefore undertaken to design a simple and rapid method for screening of bacteria capable of degrading aliphatic nitriles. We tested HPLC waste stream and solvent-enriched soil slurry as pollutants for newly isolated microorganism for possible industrial application. To our knowledge, this is the first report demonstrating the isolation and screening of aliphatic-nitrile-degrading bacteria using phenol red indicator plate, taking acetonitrile as an example for aliphatic nitrile degradation.

Materials and methods

Chemicals

The nitriles used in the present study were purchased from s.d. fine chemicals (India). The culture media ingredients were from Hi-Media (Mumbai, India). All other chemicals were of analytical grade and procured from various commercial sources.

Strains, media and culture conditions

Nitrile-degrading microorganisms, *Paracoccus* sp. SKG (FJ581419) and *Serratia marcescens strain* MSK1 (FJ581420), were isolated from chemical waste samples by enrichment culture technique and indicator plate method, respectively, and identified by biochemical characterization and 16S ribosomal DNA (rDNA) sequence [1]. *Ochrobactrum* sp. DGVK1 was previously isolated in our laboratory for its ability to degrade acetonitrile [16]. *Nocardia globerula* NHB-2 [3] was procured from the Microbial Type Culture Collection (MTCC) of the Institute of Microbial Technology, Chandigarh, India. Cultures were grown in mineral salts medium (MM1) for nitrile-degradation studies, which was devoid of carbon and nitrogen source and contained the following ingredients (in g l^{-1}): K_2HPO_4 6.8; KH_2PO_4 1.2; $MgSO_4{\cdot}7H_2O$ 0.1; $MnSO_4{\cdot}4H_2O$ 0.1; $CaCl_2{\cdot}2H_2O$ 0.1; $FeSO_4{\cdot}7H_2O$ 0.1; $Na_2MoO_7{\cdot}2H_2O$ 0.006. The pH of the medium was adjusted to 7.0 with 4 N NaOH or HCl and sterilized by autoclaving at 121°C for 15 min. The bacteria were propagated by using MM1 medium supplemented with 1% filtered sterile acetonitrile (500 mM) as sole source of carbon, nitrogen and energy. The flasks were inoculated and incubated at 32°C on an orbital shaker (B Braun, Germany) at 180 rpm. Indicator plates were prepared from MM1 medium with phenol red (0.02% w/v) and 2% agar, overlaid with 100 µl aliphatic nitriles. Additional carbon sources were added separately to MM1 medium at the following concentrations: glucose, 10 mM; citrate, 10 mM; acetate, 10 mM; succinate, 10 mM. The agar plates were incubated at 32°C in an incubator.

Slurry preparation

Soil collected from a garden was air-dried, sieved (2 mm) and stored at 4°C. One liter of slurry was prepared using 100 g soil and 500 ml distilled water, removing floating particles, then 500 ml 2× MM1 medium was added. The slurry was sterilized in an autoclave (121°C, 20 min) and mixed at 100 rpm by adding acetonitrile. HPLC waste stream was collected at a research laboratory, generated during the analysis of hydrolyzed product of soya milk oligosaccharides, containing acetonitrile:water (70:30 v/v).

Analytical methods

Growth of bacteria was determined spectrophotometrically at 600 nm. The concentration of liberated ammonia in the spent medium was measured by Nessler's method [17]. pH measurements were performed with a pH meter. Acetonitrile concentration in the spent medium was estimated by gas chromatography (GC) analysis.

Statistical analysis

In order to compare the degradation of different nitriles using *Paracoccus* sp., the concentration value of each experiment was normalized to the initial concentration. All results are presented as mean ± standard deviation (SD). The level of statistical significance was determined by analysis of variances (ANOVA) followed by Bonferroni's multiple-comparison *t*-test. For multiple comparisons $P < 0.001$ was considered statistically significant.

Results and discussion

Bacteria capable of utilizing aliphatic nitriles as sole source of carbon and nitrogen were isolated from chemical waste samples collected from industrial sites. Two isolates obtained from different samples were identified by biochemical characterization and 16S rDNA sequence. The shake-flask experiment was carried out with *Paracoccus* sp. in order to confirm acetonitrile degradation, tested in triplicate. Each flask containing 50 ml MM1 medium was autoclaved and supplemented with 1% acetonitrile (v/v). Flasks were inoculated with seed culture containing 6.5×10^9 colony-forming units (CFU)/ml to give initial absorbance of 0.05 at 600 nm. All flasks were kept on an orbital shaker at 32°C and 180 rpm for 3–4 days. Ten

millilitres of culture broth was removed from each flask at 12-h intervals. Growth of bacteria, pH of the culture broth, concentration of liberated ammonia and acetonitrile concentration in the spent medium were measured in triplicate.

The bacterial growth curve is shown in Fig. 1, from which it is evident that the maximum growth of the bacterium was observed after 48 h. The pH of the medium and production of ammonia by acetonitrile are shown in Fig. 2. Furthermore, it is clear that acetonitrile degradation initiates accumulation of ammonia in the culture medium. The liberated ammonia is also used by the bacterium as a source of nitrogen, and excess ammonia is released to the surrounding medium. This excess ammonia contributes to increase of pH of the growth medium from initial pH of 7.0–9.2. Degradation of acetonitrile by this bacterium decreased after 60 h of incubation; this may be attributed to the increase in pH of the medium, which might have suppressed its growth.

Based on the above results, a simple and rapid screening method was developed for the isolation and screening of bacteria capable of degrading aliphatic nitriles. Indicator plates were prepared by adding 0.02% phenol red and 2% agar to MM1 medium and overlaying 100 µl acetonitrile. Then the plates were divided into three sectors (Fig. 3). Sector 1 is a control where bacterium are not inoculated; sector 2 is streaked with a loopful of *Paracoccus* sp., which has the ability to degrade acetonitrile; sector 3 is streaked with a bacterium that is unable to degrade acetonitrile. The plates were incubated at 32°C for 2–3 days in an incubator.

Bacterial cultures capable of utilizing acetonitrile as source of carbon and nitrogen result in the release of ammonia. This released ammonia causes increase in the pH of the indicator plate, resulting in colour change of the indicator dye from red to pink (pH 7.0–9.2). There is no change in the colour of the indicator dye in sectors 1 and 3,

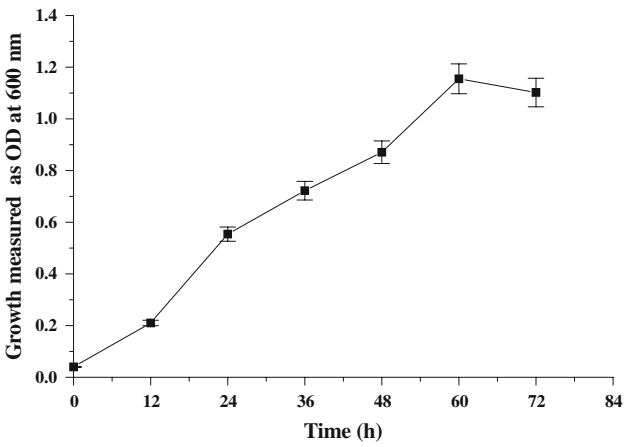

Fig. 1 Growth of *Paracoccus* sp. SKG in MM1 medium with 1% acetonitrile (v/v) as sole source of carbon and nitrogen (*Error bars* represent the deviation calculated from the mean of triplicates)

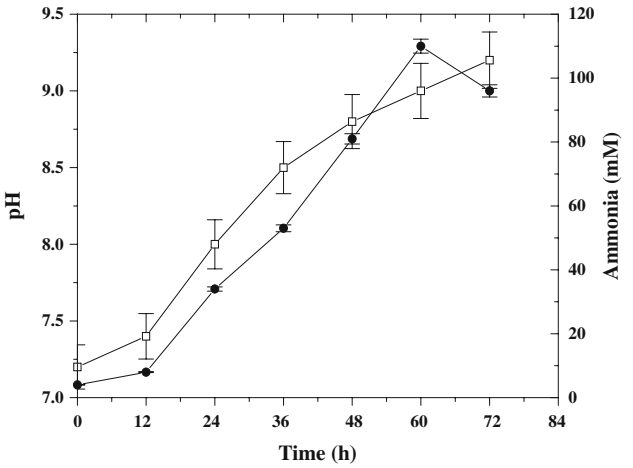

Fig. 2 pH of the growth medium (*open square*) and concentration of ammonia (*filled diamond*) liberated from acetonitrile degradation (*Error bars* represent the deviation calculated from the mean of triplicates)

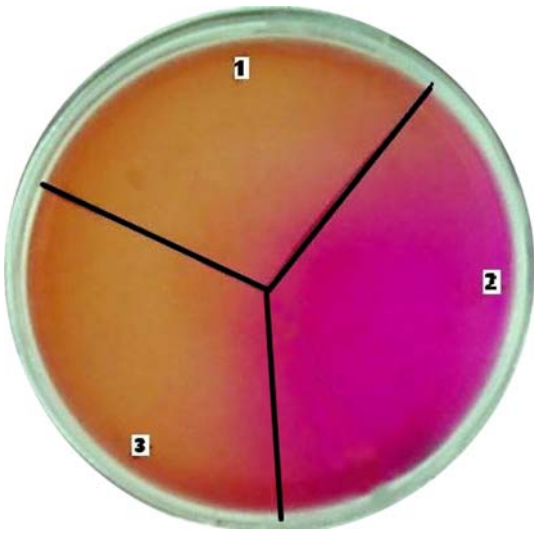

Fig. 3 Phenol red pH indicator plate showing degradation of acetonitrile. The plate was prepared as described in the text, being divided into three sectors. Sector 1: control (without bacterium). Sector 2: *Paracoccus* sp. SKG; appearance of *pink color* in sector 2 indicates the acetonitrile degradative ability of SKG. Sector 3: inoculated with bacterium which has no ability to degrade acetonitrile

whereas a colour change of the indicator dye in sector 2 from red to pink is observed. This is because sector 2 is inoculated with *Paracoccus* sp., which is capable of degrading acetonitrile with the release of ammonia. This liberated ammonia results in the colour change of the indicator dye. Further, this method was tested for different indicator dyes such as phenol red (0.02% w/v), bromothymol blue (0.02% w/v) and phenolphthalein (0.04% w/v). Phenol red shows good colour change from red to pink in the pH range from 7 to 7.8; other dyes take longer for the colour change to be discerned. Using this indicator plate

method, we screened the degradation ability of *Paracoccus* sp. on different aliphatic nitriles such as acetonitrile, propionitrile, valeronitrile and acrylonitrile. We applied this method to degrade HPLC waste stream and solvent-enriched soil slurry containing acetonitrile. The change in colour of the indicator shows the utilization of acetonitrile by bacteria. Using this indicator plate method we isolated the bacterium *Serratia marcescens* strain MSK1 from chemical waste samples collected from industrial sites for the degradation of acetonitrile. The growth of the bacteria and change in colour of the indicator plate containing various nitriles at different concentrations as carbon and nitrogen sources are presented in Table 1.

Acetonitrile seems to be a preferred carbon and nitrogen source when compared with other nitrile sources. Due to increasing pH of the medium acetonitrile degradation decreases, and optimal concentration seems to be 100–200 µl for optimal growth. Table 2 presents the different bacterial strains used in the indicator plate for acetonitrile degradation. All four strains were able to utilize acetonitrile as sole source of carbon and nitrogen.

The application of the indicator plate method has also been extended by making use of additional carbon sources. The growth of the degradative bacteria is usually slow if the nitrile serves as a sole source carbon and nitrogen, some times nitrile as a nitrogen source alone; during such period, additional carbon sources are necessary. It was reported that the use of additional carbon sources enhanced the degradation of xenobiotic compounds by bacteria [9, 15]. In this investigation the indicator plates were prepared with 10 mM of individual secondary carbon sources such as glucose, citrate, acetate and succinate, respectively. These plates were then spread with 100 µl acetonitrile as a model substrate and streaked with *Paracoccus* sp. This bacterium will not utilize the secondary carbon sources alone in MM1 medium. This is because nitrogen is a limiting factor and it has to be supplied through nitriles only. The results indicated that the plate supplemented with acetonitrile and no additional carbon sources formed pink colour in 2 days. However, indicator plates supplied with acetonitrile with acetate, succinate or citrate formed the pink colour within 1 day. This procedure enhances the growth rate of bacteria and also allows the selection of strains with the potential to degrade aliphatic nitriles. Using phenol red indicator we also tested various reported synthetic media, basal salt agar and minimal medium used for isolation of acetonitrile-utilizing microorganisms [4, 5, 10, 11]. Although these media appeared to be suitable for practical work, the MM1 medium containing phenol red is the most appropriate for good growth and colour change within a short period.

The liberation of ammonia during degradation of acetonitrile can be used as an indication for the activity of the bacteria in the growth medium. By using the bacteria *Paracoccus* sp. SKG, it could be demonstrated that the liberation of ammonia is proportional to the utilization of the acetonitrile. Under these conditions, a screening method was developed using pH indicators, for the isolation and screening of aliphatic-nitrile-degrading bacterial strains. The removal of nitriles from heavily contaminated soils required additional carbon sources. The pH indicator plate allows the selection of such strains. We also screened the bacterium *Serratia marcescens* MSK1, capable of

Table 1 Growth of *Paracoccus* sp. SKG on indicator plates containing various nitriles, and time taken to change the colour of an indicator

Growth of *Paracoccus* sp. on indicator plates spread with	Concentration (µl)	Time taken for colour change from red to pink (h)	
		Initiation of colour change	Final colour change
Acetonitrile	50	$23.33 \pm 1.76^{a\dagger\ddagger,b\S}$	–
	100	$22.3 \pm 1.86^{b\dagger\S\ddagger}$	$48.0 \pm 1.15^{b\dagger\S\ddagger}$
	150	$23.7 \pm 1.86^{a\dagger\S,b\ddagger}$	$47.0 \pm 1.20^{b\dagger\S\ddagger}$
	200	$25.7 \pm 0.88^{b\dagger\S\ddagger}$	$49.0 \pm 1.15^{b\dagger\S\ddagger}$
Propionitrile	50	33.7 ± 1.20	–
	100	34.7 ± 1.76	$74.3 \pm 1.20^{b\S\ddagger}$
	150	33.7 ± 1.45	$74.3 \pm 1.45^{b\S\ddagger}$
	200	37.0 ± 1.15	$79.7 \pm 1.45^{b\S\ddagger}$
Valeronitrile	50	37.3 ± 1.76	–
	100	36.7 ± 1.20	91.3 ± 2.03
	150	34.3 ± 0.88	92.0 ± 1.15
	200	38.3 ± 1.20	95.0 ± 1.15
Acrylonitrile	50	34.3 ± 1.20	–
	100	36.0 ± 1.15	92.3 ± 1.76
	150	35.7 ± 1.76	92.0 ± 2.31
	200	39.0 ± 0.57	96.3 ± 1.20
HPLC waste stream	50	$23.0 \pm 1.53^{a\dagger\ddagger,b\S}$	–
	100	$23.0 \pm 0.57^{b\dagger\S\ddagger}$	$48.3 \pm 1.45^{b\dagger\S\ddagger}$
	150	$19.7 \pm 1.20^{b\dagger\S\ddagger}$	$47.7 \pm 1.20^{b\dagger\S\ddagger}$
	200	$27.0 \pm 1.15^{b\dagger\S\ddagger}$	$49.0 \pm 2.0^{b\dagger\S\ddagger}$
Soil slurry	50	$24.0 \pm 0.57^{a\dagger,b\S,\ a\ddagger}$	–
	100	$23.7 \pm 0.88^{a\dagger,b\S\ddagger}$	$48.7 \pm 0.88^{b\dagger\S\ddagger}$
	150	$22.7 \pm 0.88^{a\dagger\S\ddagger}$	$48.7 \pm 1.76^{b\dagger\S\ddagger}$
	200	$26.0 \pm 0.57^{b\dagger\S\ddagger}$	$50.7 \pm 1.20^{b\dagger\S\ddagger}$

Values are mean ± standard deviation (SD) of triplicates. $^{a}P < 0.01$, $^{b}P < 0.001$

† When compared with propionitrile

§ When compared with valeronitrile

‡ When compared with acrylonitrile

(–) No complete colouration was observed

Comparison made between equal concentrations of different nitrile samples

Table 2 Bacterial strains used for degradation of acetonitrile on indicator plate

Strain	Time taken for colour change from red to pink (h)	Origin
Paracoccus sp. SKG	48	Own isolate
Serratia marcescens MSK1	72	Own isolate
Nocardia globerula NHB-2	60	Bhalla et al. [3]
Ochrobactrum sp. DGVK1	48	Veeranagouda et al. [16]

degrading acetonitrile. From the above results, it is clear that one can make use of this technique to screen a large number of microorganisms for their ability to degrade aliphatic nitriles within a short time. This may be a pre-requisite for their application in decontamination of the corresponding compounds.

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