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Research Article

Biodegradation of Mandelonitrile Using *Bacillus* sp.: A Potential Approach for Environmental Remediation

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ABSTRACT

Mandelonitrile, a cyanogenic compound, poses environmental risks due to its toxicity and potential to release hydrogen cyanide. The potential technique for removing mandelonitrile from polluted environments is biodegradation. In this study, we investigated the isolation and identification of bacterial strains from soil samples for the biodegradation of mandelonitrile. The selective enrichment culture technique was used for isolation, using mandelonitrile as the sole carbon and nitrogen source. The isolated strain was identified and named Bacillus sp based on morphological studies and biochemical analysis. MN1. Further degradation studies demonstrated the efficiency of the isolated strain in degrading mandelonitrile under optimum conditions. Bacillus sp. MN1 can degrade 0.5% v/v mandelonitrile completely into mandelic acid and ammonia under optimum conditions of 30 °C and pH 7.5 within five days of incubation. The enzyme assay and HPLC analysis show that mandelonitrile degradation by Bacillus sp. MN1 follows the Nitralase pathway, mandelic acid, and ammonia. This research study reveals the potential application of Bacillus sp. in the bioremediation of mandelonitrile-contaminated sites, highlighting the importance of microbial biodegradation in environmental management strategies.

Keywords: *Mandelonitrile, Bacillus sp., Biodegradation, Nitralase.*

INTRODUCTION

Nitriles are organo-cyanides with the general formula R C≡N. They are products, intermediates, byproducts, and waste products of the chemical, pharmaceutical, and agriculture industries and the processing of fossil fuels (Martinkova et al., 2009). These compounds cause severe health hazards as most of them are highly toxic and some are mutagenic and carcinogenic, whose exposure can lead to disorders of the cardiovascular, central nervous system, hepatic, gastrointestinal, and renal systems in mammals (Mukram et al., 2016; & Yu et al., 2019).

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Nitrile compounds can be degraded by microbiological processes or by chemicals. However, the chemical process needs a harsh environment and produces a lot of undesirable byproducts in addition to inorganic waste. For these reasons, biotransformation conversion with the aid of microorganisms has become a modern technology. The biological breakdown of nitriles yields fewer secondary byproducts and makes product purification simpler and more specific (Agarwal et al., 2012). The biotransformation of nitriles to carboxylic acids is of synthetic importance, as various carboxylic acids are being produced from nitriles, and their corresponding nitrile educts are easily accessible. Nitrile hydrolysis occurs through two enzymatic pathways (Egelkamp et al., 2019). One pathway involves the sequential hydrolysis of the nitrile molecule to its corresponding carboxylic acid and ammonia via an amide intermediate catalyzed by two different enzymes: nitrile hydratase and amidase. The other is the direct hydrolysis by nitrilase to corresponding carboxylic acid and ammonia (Mukram et al., 2016).

Mandelonitrile (2-hydroxy-2-phenylacetonitrile) is a cyanogenic glycoside. (R)-mandelic acid (MA) is an essential and practical chiral molecule for the synthesis of semi-synthetic penicillin and antibiotics like cephalosporins, as well as anti-obesity and anti-tumour agents (Lukito et al., 2021). (R)-MA is mainly synthesized chemically. The cyanide-based approach comprised two steps: first, benzaldehyde was cyanated using either NaCN or transition metal catalysts, such as vanadium or titanium complexes of chiral ligands; next, mandelonitrile was hydrolyzed with HCl to yield enantiopure (R)-MA (Corson et al., 2003). Numerous techniques for (R)-MA biosynthesis have been discovered recently. Nitrilase (NLases) is primarily used in the kinetic resolution of racemic mandelonitrile in the single-step biosynthesis of (R)-MA (He et al., 2008). The present study focused on isolating a bacterial strain from the soil sample, an isolated strain identified as *Bacillus* sp. MN1, which harbors nitrilase activity and could degrade mandelonitrile into mandelic acid and ammonia. Degradation conditions for maximum degradation of mandelonitrile were optimized, and the isolated strain can utilize various other nitriles. The isolated strain can be further used to treat toxic nitriles and nitrile derivatives in the environment.

MATERIALS AND METHODS

Screening for mandelonitrile degrading strains

The pour plate method was employed on the soil samples after serial dilution. The sole nitrogen and carbon source used in the selective enrichment culture approach was mandelonitrile supplied to a minimal salt medium (MM1). Individual bacterial colonies were isolated by plating serial dilutions of the cultures on agar plates containing mandelonitrile. The phenol red indicator plate approach was used for screening mandelonitrile-degrading microorganisms (Santoshkumar et al., 2010). The bacterial strain MN1 was isolated from the plate, and morphological and biochemical analyses were performed to identify the strain.

Microbial identification

The positive strain for mandelonitrile degradation, MN1, was identified by conducting various morphological and biochemical tests. Microscopic examinations such as Gram staining and motility of bacteria by the hanging drop method were done to identify the isolated organism. The biochemical tests were used to examine the ability of the organism to produce indole, catalase, and urease. Methyl Red - Voges Proskauer test, Citrate Utilization test, Casein, Starch hydrolysis tests, and ability of acid production in the presence of glucose, starch, and maltose for the identification of isolates.

Biodegradation of mandelonitrile:

Using the bacterial strain MN1, several batch experiments were carried out in 250 ml Erlenmeyer flasks to investigate the degradation of mandelonitrile. Each flask holds 50 millilitres of filter-sterilized mandelonitrile (0.5%) and autoclaved MM1 medium. The flasks were incubated at 30 °C,

whereas uninoculated flasks containing mandelonitrile and culture flasks without mandelonitrile were incubated simultaneously as controls. Every experiment was conducted in triplicate. Bacterial growth measured the OD at 540 nm to evaluate its growth. Samples taken at various intervals were centrifuged, and the culture filtrate was examined using high-performance liquid chromatography (HPLC) to identify the degradation of mandelonitrile to mandelic acid and estimated the released ammonia by Schar et al. method (Schar et al., 1986).

Enzyme assay:

The Nitrilase assay was carried out by mixing the substrate mandelonitrile (50 mM) with the enzyme in sodium phosphate buffer (100 mM, pH 7.2). The reaction mixture was incubated at 30 ℃, samples were taken at fixed time intervals, and the reactions were terminated by adding 100 µl HCl (2 M) (Zhang et al., 2010). The amount of ammonia released was calculated using the Schar et al. method (Schar et al., 1986). The nitrile hydratase and amidase activity were assessed (Santoshkumar et al., 2010). All experiments were repeated in triplicate. One unit of enzyme activity was defined as the amount of enzyme-catalyzed for the formation of 1 µmol of product per minute.

Utilization of other nitriles as Carbon and Nitrogen sources by MN1 strain

The utilization of aromatic (benzonitrile, 2-cyanopyridine, 4-cyanopyridine, and mandelonitrile) and aliphatic (acetonitrile and acrylonitrile) nitriles by MN1 was studied. Studies were carried out in 250 ml Erlenmeyer flasks containing 50 ml of autoclaved MM1 medium (pH 7.2) supplemented with different nitriles (0.5 %) as the sole carbon and nitrogen source. The flasks were inoculated with MN1 and incubated for seven days at 30 °C. Growth was assessed by measuring OD, and the estimation of ammonia confirmed degradation.

RESULTS

A bacterial strain capable of utilizing mandelonitrile as the sole source of nitrogen, carbon, and energy was isolated from the soil samples. Among the isolates that were obtained from various soil samples, the strain designated as MN1 showed substantial growth in the MM1 medium containing 0.5 % v/v mandelonitrile. The cultural morphological characters of isolates showed irregular, slightly raised, flat, and cream-coloured colonies (Fig. 1). In a microscopic examination by gram staining, the isolates were found to be gram-positive, rod-shaped bacteria. In contrast, in the motility test, the isolates showed motility, and the strain is a spore-forming bacteria. The biochemical characterizations of the isolate showed that the strain exhibited catalase activity (Table 1). The strain utilized casein and could not utilize citrate, starch, urea, and indole. The acid was produced with glucose and maltose. The strain does not produce the acid in the presence of starch. Based on biochemical tests and according to Bergey's manual of systematic bacteriology (Boone et al., 2001), the mandelonitrile degrading strain MN1 was identified as *Bacillus* sp. named as *Bacillus* sp. MN1.

Fig. 1: Morphological studies: MN1 strain growth on the LB agar plate

Table 1: Morphological and Biochemical characterization of strain MN1

Characteristics	**Result**
Cell shape	Rods
Gram staining	Gram positive
Motility	Motile
Endospore	Present
Starch hydrolysis	-
Casein hydrolysis	+
Indole production	-
Acid Production a. Glucose b. Maltose d. Starch	 + + -
MR-VP tests	+
Citrate utilization	-
Urease	-
Catalase test	+

Biodegradation of mandelonitrile:

The MN1 strain was grown in a mineral salt medium supplemented with 0.5 % v/v mandelonitrile as the sole carbon and nitrogen source. Maximum growth of the strain was observed at 98 hours of incubation. Organisms capable of utilizing mandelonitrile as a source of carbon and nitrogen result in the release of ammonia. This released ammonia causes an increase in the pH of the indicator plate, resulting in a color change of the indicator dye from red to pink (Santoshkumar et al., 2010). The degradation of mandelonitrile was noticed with an increase in the growth of the culture and an increase in the pH of the medium from 7.2 to 8.5. The bacterial growth (OD) is evident that the strain is capable of utilizing mandelonitrile as a carbon and nitrogen source. The change in the pH of the medium and the production of ammonia indicates mandelonitrile degradation, as shown in Fig. 2. The concentration of mandelonitrile in the media gradually decreases with the accumulation of the products mandelic acid and ammonia. Mandelonitrile was not detected in the 5th-day sample, which confirms that a complete 0.5 % mandelonitrile was degraded within five days of incubation.

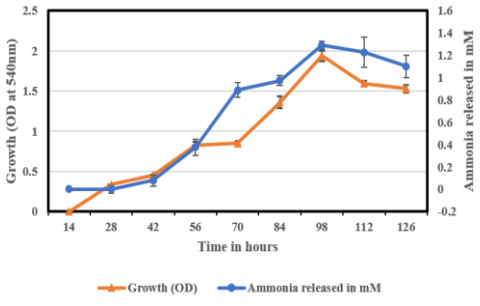

Fig 2: Growth of MN1 strain measured by absorbance at 540nm (filled ▲); the amount of ammonia released (filled ●).

Enzyme assay

The MN1 strain produced the nitrile-degrading enzyme Nitrilase (NLase) when cultured in an MM1 medium containing mandelonitrile, as evidenced by the mandelic acid synthesis in the media supernatant following incubation. Mandelonitrile-grown cells exhibit nitrilase activity of 49 units per mg wet cell weight. The Nitrilase activity, as well as the production of intermediates, confirms that the MN1 strain breaks down mandelonitrile along the subsequent route (Fig. 3).

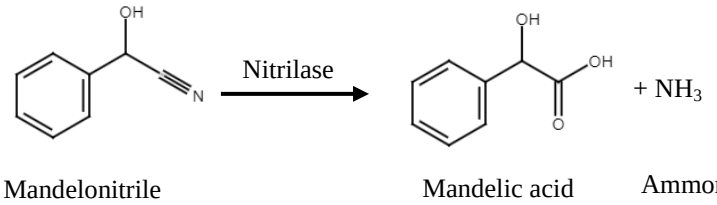

Fig 3: Degradation pathway of mandelonitrile by *Bacillus* sp. MN1

Utilization of various nitriles by MN1 strain:

The strain is capable of utilizing various nitriles, such as acetonitrile, mandelonitrile, acrylonitrile, and 2-cyanopyridine, but failed to utilize acrylonitrile, 4-cyanopyridine, and benzonitrile. The growth of MN1 strain in an MM1 medium supplemented with nitriles measured (Table 2), along with the ammonia estimation, confirmed that the MN1 strain shows broad specificity towards various nitriles.

Table 2: Utilization of other nitriles as substrates by *Bacillus* sp. MN1

Sl. No	Substrate	Growth (OD at 540 nm)
1.	Benzonitrile	0.2
2.	Mandelonitrile	1.67
3.	2 Cyanopyridine	0.65
4.	4 Cyanopyridine	0.2
5.	Acetonitrile	1.73
6.	Acrylonitrile	0.85

DISCUSSION

A bacterial strain was isolated from a soil sample by selective enrichment culture technique and identified as *Bacillus* sp. MN1 strain based on physiological, morphological, and biochemical tests. In general, enzymatic hydrolysis of nitrile compounds to their corresponding acids and ammonia takes place by two different pathways: nitrilase and a combination of nitrile hydratase and amidase. *Bacillus* sp. MN1 was shown here to have a nitrilase for mandelonitrile conversion to mandelic acid and ammonia. Good growth with high enzyme activity was obtained on 98 hours of incubation, along with maximum ammonium release. Nitrilases from two *Nocardia* species (rhodochrous group) (Harper, 1977), *Fusarium solani* (Harper, 1985) (Kaplan et al., 2006), *Nocardia globerula* NHB-2 (Raj et al., 2007), *Paraburkholderia graminis* CD41M (Fan et al., 2017) and *Rhodococcus rhodochrous* J-1 (Nagasawa et al., 1988) preferentially hydrolyze aromatic nitriles such as benzonitrile or bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) but not aliphatic nitriles. Nitrilases that have broad substrate specificity and that hydrolyze both aromatic and aliphatic nitriles have been isolated from *Acinetobacter* sp. strain AK226 (Yamamoto & Komatsu, 1991) and *Rhodococcus rhodochrous* K22 (Kobayashi et al., 1990). *Bacillus* sp. MN1 hydrolyzes mandelonitrile, along with other aromatic and aliphatic nitriles.

In the current research work, it was confirmed that the MN1 strain has more potential to degrade 0.5 % of mandelonitrile in five days, which was confirmed by observing the growth, increasing the pH, and estimating the amount of ammonia released. Further enzyme assays and metabolite identification by HPLC analysis confirmed the biodegradation pathway of mandelonitrile by the MN1 strain that follows a Nitrilase pathway. The isolated strain has more potential to be used for the treatment of mandelonitrile-contaminated sites and to synthesize mandelic acid.

CONCLUSION

In conclusion, the research conducted on the biodegradation of mandelonitrile using Bacillus sp. MN1 presents a promising approach for environmental remediation. Through experimentation and analysis, this study has demonstrated the ability of Bacillus sp. MN1 to effectively degrade mandelonitrile, a toxic compound, into less harmful and pharmaceutically useful mandelic acid catalyzed by the Nitrilase enzyme. The findings highlight the potential of utilizing microbial biodegradation processes as eco-friendly solutions for the remediation of nitrile-contaminated environments. The efficiency and specificity of Bacillus sp. MN1 in degrading mandelonitrile emphasizes its significance as a potential tool for justifying environmental pollution caused by cyanogenic compounds.

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Conflict of interest:

The authors declare that there is no conflict of interest among them.

Authors Contribution:

All authors have participated in critically revising and approving the final manuscript.

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