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Full Length Research Paper

Purification and characterization of intracellular nitrilases from *Rhodococcus* sp. - potential role of periplasmic nitrilase

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Two bacterial species *Rhodococcus rhodochrous* and *Rhodococcus fascians* were evaluated for the production of nitrilase enzyme (EC 3.5.5.2) from benzonitrile. This is the first time report on comparative analyses of intracellular nitrilases in two different *Rhodococcus* species which showed the involvement of periplasmic nitrilases towards nitrile degradation rather than cytoplasmic nitrilases. The periplasmic nitrilases of *R. rhodochrous* showed nearly 50% more activity as compared to *R. fascians*. In comparison to *R. fascians*, *R. rhodochrous* was found to be better towards nitrile production and degradation as observed under different physiological conditions. The optimum nitrile degradation was recorded at 40˚C and at pH 8.0. The nitrilase enzyme produced by *R. rhodochrous* was found to be fairly thermostable, as an incubation temperature of 45˚C did not affect the activity of nitrilase enzyme produced by it adversely. The pre-incubation temperature resulted in a decrease of nitrilase activity. The substrate concentration of 40 mM was found to be optimum for hydrolyzing benzonitrile. These findings may prove beneficial from enzymology and industrial biotechnology point of view so as to render both the environment free from harmful nitrile compounds.

Key words: Benzonitrile, cytoplasmic, periplasmic, nitrilase, *Rhodococcus*.

INTRODUCTION

Nitriles are cyanide-substituted carboxylic acids with general structure as R-CN. The naturally occurring nitriles are found in higher plants, bone oils, insects and micro-organisms such as fungi, bacteria, sponges and algae. Nitrile compounds are also synthesized on a large scale and are used as solvents, in plastic industry, synthetic rubber and as a starting material for pharmaceuticals, herbicides and other industrially important chemicals. Antibiotics like toyamycin and treponemycin from *Streptomyces* are known to contain nitrile groups. Nitriles are known to be notorious and have been a cause of hazardous environmental pollution (Brandao and Bull, 2003). The wide spread use of herbicides containing the nitrile group such as 2, 6- dichloro-benzonitrile and bromoxynil (3,5-dibromo-4 hydroxy benzonitrile) has resulted in soil pollution due to their recalcitrant nature and toxicity. In addition, it has been shown that automobile exhaust contains 1 µg of hydrogen per ml and 100 µg of acetonitrile per ml (Methcohn and Wang, 1997).

Nitrile converting enzymes have attracted substantial interest as biocatalyst in preparative organic chemistry because of their ability to convert readily available nitriles into the corresponding higher value amides and acids under mild conditions without altering other labile reactive groups (Kaul et al., 2004; Banerjee et al., 2006). A wide variety of microorganisms, which hydrolyze nitriles to the corresponding carboxylic acid, have been investigated from a variety of prokaryotic and eukaryotic organisms. Various nitrilases that hydrolyse aromatic and aliphatic substrates have been described in *Rhodococcus, Acinetobacter, Pseudomonas, Acidovorax* etc with different

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properties (Rezende et al., 2003; Komeda et al., 2004). The nitrile hydrolyzing enzymes (nitrilase, nitrile hydratase and amidase) have emerged as potential catalysts in the production of commercially important chemicals and as agents in the degradation of environmental chemical pollutants (Yamada and Kobayashi, 1996). Nitrilases have been thought to hydrolyze aromatic substances while aliphatic nitriles have been believed to be degraded by a nitrile hydratase/amidase enzyme system (Wang, 2005).

Nitrile-converting biocatalysts have now been considerable industrial interest from the view points of treating toxic nitrile and cyanide-containing wastes and as agents for the synthesis of chemicals for a wide range of applications (Trott et al., 2001). Nitrilases are commercially important enzymes and are used in the production of pyrazinoic acid, an antimycobacterial agent, nicotinic acid, isotopically active amino acids from α-amino nitriles (Bhalla et al., 1992), Ibuprofen and in degradation of bromoxynil and ioxynil (Yamamoto et al., 1990). In industries, most nitriles are being converted chemically to corresponding high value acids and amides. However, chemical conversion requires either strongly acidic or basic media, high energy consumption or causes formation of unwanted toxic by-products. Thus, the application of enzymes to organic chemical processing is increasingly attracting attention. Nitrile hydratases were first investigated as biocatalysts for the manufacture of commodity chemicals e.g. acrylamide, acrylic acid nicotinamide (Nagasawa et al., 1993). Recently, bioproduction of glycolic acid from glyconitrile has been investigated from bacterial isolate of *Alcaligenes* sp. ECU0401 (He et al., 2009).

In an attempt to explore the possible use of the native flora for production of nitrilase, efforts were made to optimize the physiological conditions for nitrilase production by *Rhodococcus* species. A first time investigation was carried out to compare the periplasmic and cytoplasmic nitrilase activities towards nitrile degradation by *Rhodococcus rhodochrous* and *Rhodococcus fascians*.

MATERIALS AND METHODS

Materials

Benzonitrile used as substrate was procured from Sigma- Aldrich, India. Other chemicals were obtained from commercial sources and were molecular or reagent grade.

Microorganisms and their cultivation

Two bacterial species of *R. rhodochrous* (MTCC-291) and *R. fascians* (MTCC1531) used in the present study were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. The freeze-dried cultures of both species were revived by streaking on a solidified nutrient agar (NA) medium to get an isolated colony. Both *Rhodococcus* species were cultivated in the production medium containing: Glucose-10.0 g, $Na_2HPO_4.2H_2O$- 2.5 g, KH_2PO_4- 2.0 g, $MgSO_4.7H_2O$- 0.5 g, $CaCl_2.2H_2O$- 0.06 g, 0.1 g of yeast extract and 30 mM benzonitrile (Bhalla et al., 1992).

Isolation of periplasmic and cytoplasmic enzyme

The cell growth of *R. rhodochrous* (MTCC-291) was pinkish orange while the growth of *R. fascians* (MTCC-1531) was colourless. MTCC-291 was fast grower compared to MTCC-1531. Cells were harvested after every one hour growth till 24 h at 10,000 rpm and at 4˚C. The cells were washed twice in potassium phosphate buffer (pH 7.8). Lysozyme (20 mg/ml) was added to a 10 fold concentrated cell suspension in the same buffer and centrifuged for isolation of periplasmic enzyme. The contents were transferred to another centrifuge tube and stored at 4˚C for further use. Remaining pellets were crushed with the autoclaved sand (5 times of the pellet) and ample amount of buffer, so that cytoplasmic enzyme could be released. Paste was transferred to tubes and equal amount of phosphate buffer was added. The cells were again centrifuged and the supernatant was transferred to another centrifuge tube that contained the cytoplasmic enzyme.

Nitrilase assay

Periplsmic and cytoplasmic nitrilase activity of both *Rhodococcus* species were checked by the ammonia assay method as proposed by Fawcett and Scott (1960). The cell growth of *R. rhodochorus* and *R. fascians* was estimated turbidimetrically by means of dry cell weight calibration by absorbance at 640 nm with a Hitachi U-3210 spectrophotometer (Hitachi, Japan). A 0.75 mg dry cell weight per ml was found to be equivalent to 1.0 unit at 640 nm. The standard curve of ammonium chloride was prepared ranging from concentration 0.1 - 3 mM. Growth curve and nitrilase activity at different time intervals were measured spectrophotometrically at 640 nm. Cells growth and periplsmic and cytoplasmic enzyme activities in terms of nitrilase enzyme assays were evaluated for 24 h round the clock for each *Rhodococcus* species. One unit of nitrilase activity was defined as µmoles of ammonia released per ml per min under the assay conditions.

Effect of pre-incubation time

Both *Rhodococcus* species were exposed to a temperature of 45℃ for 6 h to test the stability of the enzyme in the free state. Cells of both *Rhodococcus* species were assayed once in every hour to check the nitrilase activity.

Optimization studies *Substrate concentration*

Both *Rhodococcus* species were assayed using various concentrations (5 - 50 mM) of benzonitrile to check the effect of concentration of substrate on the activity of the enzyme.

Time

Enzyme activities of both *Rhodococcus* species were assayed at different intervals (5 - 60 min) of time during the incubation at 45℃.

Buffer pH

Enzyme assay was carried out using phosphate buffer of different pH (6.5 - 9.5) using lysed cells of both *Rhodococcus* species.

Temperature

The activity of nitilase was observed for each *Rhodococcus* species at different temperatures ranging from (5 - 50°C) to evaluate the thermostability towards nitrilases.

Statistical analyses

The data recorded in triplicate for the parameters in both *Rhodococcus* species was subjected to ANOVA (analysis of variance) in accordance with the experimental design (completely randomized block design) using MSTAT-C statistical package to quantify and evaluate the sources of variation.

RESULTS AND DISCUSSION

Nitriles are xenobiotics responsible for polluting our environment, the major part being played by herbicides like Dichlobenil, Bromoxynil, Ioxynil etc. So, nitrilase production can be used as an economical, effective and biofriendly technology to degrade these chemical pollutants.

The evaluation studies of intracellular nitrilases activity of *Rhodococcus* cells and their application in bioconversion of benzonitrile to benzoic acid has a principal application in biodegradation of hazardous aromatic nitriles compounds to acids in order to render the environment pollution free. Nitrile metabolizing enzymes have the potential to perform valuable biotransformation such as the production of acids and amides of industrial importance. The chemical conversion of nitriles to acids and amides is feasible but it requires relatively harsh conditions of heat, acid or alkali. Most of the nitrile metabolizing enzymes (nitrilase or nitrile hydratase/ amidase) that have been explored are from mesophilic sources and especially from the rhodococcal system and this is widely used for the production of acrylamide, acrylic acid, and nicotinamide, etc. Members of the genus *Rhodococcus* are aerobic, gram positive, non motile nocardioform actinomycetes (Finnerty, 1992). These bacteria are common throughout nature, with only a few species possessing pathogenic properties (Goodfellow and Alderson, 1977). Their ability to use numerous compounds as carbon sources, even in the presence of less complex molecules, is consistent with findings that Rhodococci are ubiquitous in contaminated soils, and they lack catabolite repression mechanism. As a result, the *Rhodococcus* genus is an excellent candidate for numerous medical, industrial and environmental applications. A number of microbes have been reported in the literature but the optimization of different parameters for the maximum synthesis of enzymes has been scanty and there are very few reports on role of periplasmic nitrilase. Towards, this endeavour, the present investigation aims at the optimization of different physiological conditions that play an important role in the synthesis and characterization of periplasmic nitrilase produced from *Rhodococcus* species.

Comparison of growth rate and intracellular nitrilase production in both *Rhodococcus* species under different time intervals

The time dependent measurement of growth rate in both *Rhodococcus* species showed that *R. rhodochrous* was grown faster as compared to *R. fascians* (Figure 1a). Spectrophotometrically, measured value of OD_{660} 1.69 was obtained in 9 h in case of *R. rhodochrous*, whereas, for *R. fascians*, the same OD_{660} value was observed in 13 h. The first time, study conducted towards intracellular nitrilases (periplasmic and cytoplasmic) of each *Rhodococcus* species revealed very interesting results. Comparative values of periplasmic and cytoplasmic activity in each *Rhodococcus* species revealed that cytoplasmic nitrilase activity of both the tested species was far less compared to the periplasmic nitrilase activity (Figures 1b and 1c), as evaluated at different periodic intervals. Periplasmic activity was found to be higher in each *Rhodococcus* species which reflects that the cytoplasmic nitrilases may be restricted by some signal proteins. This information may have valuable application to understand the rate limiting pathways for nitrilase production in *Rhodococcus* sp. In comparison, *R. rhodochrous* was found to be better *Rhodococcus* species in terms of periplasmic nitrilase production. The previous studies indicated the presence of nitrilase activity under different phases of growth. For instance, Kumar et al. (2006) observed the maximum nitrilase activity against acetonitrile in the mid exponential phase in *Nocardia globerula* NHB-2 whereas Cramp et al. (1997) investigated the maximum nitrilase activity for acetonitrile in *Bacillus pallidus* between exponential to stationary phases. Dias et al. (2000) observed benzonitrile and acetonitrile degradation by intracellular nitrilase in resting cells (stationary phase) of *Candida guilliermondii* UFMG-Y65. However, in our study, a comparison of growth curves of both *Rhodococcus* species with their respective nitrilase activities highlighted the maximum production of nitrilase at stationary phase of growth. These observations showed that intracellular nitrilase may release at the stationary phase of growth.

Thermostability of *R. rhodochrous* nitrilase

In order to evaluate thermostability of periplasmic nitrilases, both *Rhodococcus* species were pre-incubated at 45˚C under different time intervals. A gradual decrease in benzonitrile hydrolyzing activity was observed in *R. rhodochrous* up to 4 h of pre-incubation, followed by no detectable enzyme activity (Figure 1d). The enzyme activity in *R. fascians* decreased up to 5 h with no further detectable enzyme activity. The decrease in enzyme activity may be due to denaturation of cells upon prolonged exposure to high temperature. Thus, the nitrilase produced by *R. rhodochrous* is fairly thermostable as it retains benzonitrile hydrolyzing activity even after

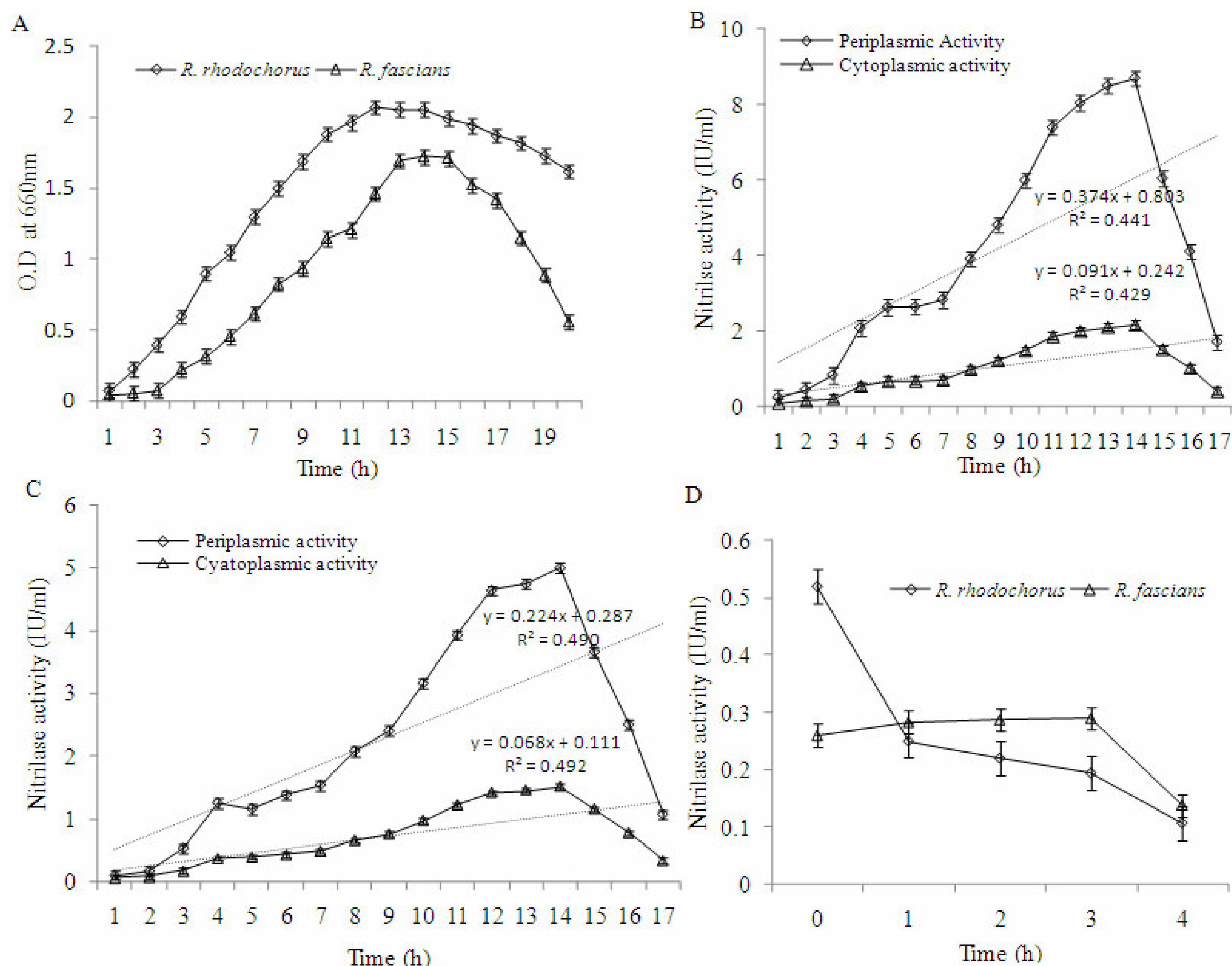

Figure 1. Comparative measurement of growth rate, nitrilases production, and pre-incubation temperature in *R. rhodochorus* and *R. fascians*. (A) Growth curve of both *Rhodococcus* species; (B-C) Periplasmic and cytoplasmic nitrilase activity from *R. rhodochorus* and *R. fascians*; (D) nitrilase activity of both *Rhodococcus* species at different time points and pre-incubation temperature (45 ℃). The differences in the mean were statistically significant at p<0.01 in one way ANOVA test".

pre-incubation at 45˚C for 4 h. This is also supported by the studies of Khandelwal et al. (2007) who also demonstrated thermostable nitrilase in *Streptomyces* sp. MTCC 7546.

Substrate concentration specificity

Production of most of the enzymes is often subjected to regulation by the readily metabolizable carbon sources and a decrease in the activity is noted after a certain concentration (catabolite repression). As far as carbon source is concerned not a single isolate producing nitrilase is characterized in terms of utilization of different carbon sources and the enzymatic activity (Khandelwal et al., 2007). Nitrilase produced from *Streptomyces* sp. MTCC 7546 utilized glucose, maltose, sucrose, lactose and glycerol and produced nitrilase with very similar trends in activity. The investigation of substrate preferences of nitrilase showed that nitrilase secreted from different organisms prefers different nitriles. For example *R. rhodochrous* K22 hydrolyze aliphatic nitriles but the concentraion of nitriles was very low (0.2 mmol L^{-1}) (Kobayashi et al., 1990). In our study, benzonitrile hydrolyzing activity increased with the increase in substrate concentration. It was found to be optimum at 40 mM substrate concentration in both *R. rhodochrous* and *R. fascians*. The maximum activity was 0.50 and 0.42 U/ml in *R. rhodochrous* and *R. fascians*, respectively. The further increase in substrate concentration did not result in proportionate increase in enzyme activity (Figure 2a).

Kumar et al. (2006) observed significant nitrilase activity in *Nocardia globerula* NHB-2 in the absence of acetonitrile in the medium. In our study, when the benzonitrile was absent in the medium, the corresponding nitrilase activity was lost in both *Rhodococcus*

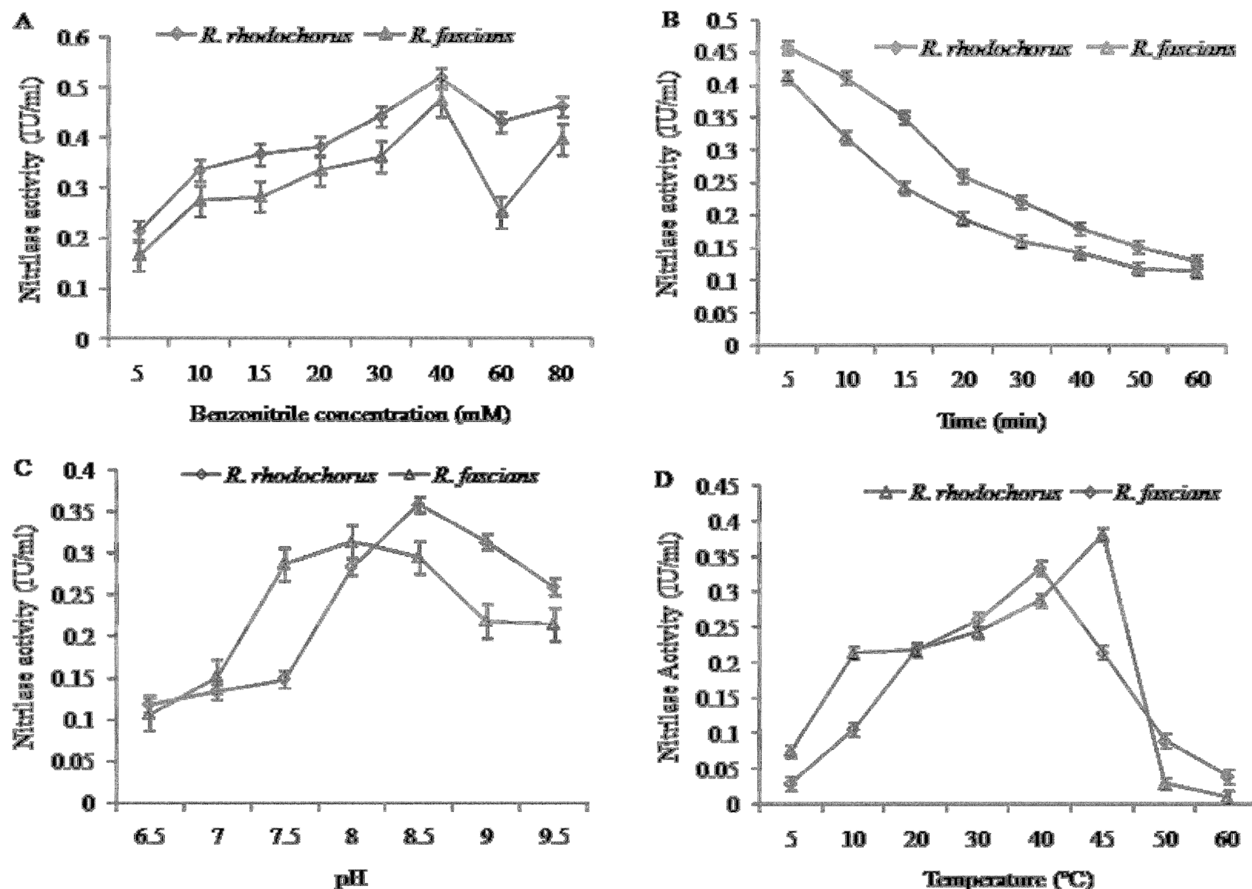

Figure 2. Optimization studies at different physiological conditions. (A) Growth of Rhodococcus species under different concentrations of benzonitrile; (B) time course of benzonitrile hydrolyzing activity with increasing time of incubation; (C-D) effect of pH and temperature on nitrilase activity. The differences in the mean were statistically significant at $p<0.01$ in one way ANOVA test.

species (data not shown). This signifies that, periplasmic nitrilase from both *Rhodococcus* species is substrate specific.

Effect of increase in time of incubation

The nitrilase activity was found to decrease with the increasing time of incubation. These results were in agreement with the findings of Michaelis Menten kinetics (1903) who reported enzyme activity that gradually decreased with the increase in time as the magnitude of product concentration was not directly proportional to the time period (Figure 2b).

Effect of pH and temperature

Nitrilase assay at different pH values revealed significant results. In *R. rhodochrous*, enzyme exhibited maximum activity (0.359 U/ml) at pH 8.5 and *R. fascians* exhibited maximum activity (0.314 U/ml) at pH 8.0. Both *Rhodococcus* species are stable over a wide range of pH and retained about 72 and 68% activities at pH 9.5, respectively (Figure 2c). On the other hand, the results of nitrilase activity at different incubation temperatures showed that the activity of *R. rhodochrous* increased gradually from 5 - 45˚C and decreased drastically at 50˚C. However, in case of *R. fascians*, nitrilase activity increased gradually from 5 - 40˚C only. No further increase in activity was recorded above 40˚C. This may be due to cell lysis and enzyme denaturation above 45 and 40˚C in *R. rhodochrous* and *R. fascians*, respectively (Figure 2d). The results of nitrilase activity of *R. rhodochrous* under different pH and temperature showed similarity with benzonitrilase A of *R. rhodochrous* J1 (Kobayashi et al., 1989; Nagasawa, 1988). Whereas, *R. fascians* showed similarity with benzonitrilase A from *Arhtrobacter* sp. strain J-1 (Bandyopadhyay, 1986) and nitrilase of *Fusarium oxysporum* f. sp. *Melonis* (Goldlust and Bohak, 1989).

In conclusions, *R. rhodochrous* was proved to be better

Table 1. Optimum values for various reaction parameters.

Parameters	*R. rhodochorus*	*R. fascians*
Maximum Growth	After 12 h	After 15 h
Periplasmic Nitrilase activity	8.71 (IU/ml)	4.74 (IU/ml)
Cytoplasmic Nitrilase activity	2.18 (IU/ml)	1.51 (IU/ml)
Buffer pH	8.5	8.0
Substrate concentration	40 mM	40 mM
Incubation temperature	45˚C	40˚C
Incubation period	15 min	15 min

Rhodococcus species as compared to *R. fascians*, as study under different physiological parameters (Table 1) revealed that *R. rhodochrous* grows faster, having high enzyme activity towards nitrile degrading compounds such as benzonitrile as compared to *R. fascians*. Apart from this, *R. rhodochrous* was found to be fairly thermostable and we have explored the potential role of periplasmic nitrilases towards benzonitrile degradation. The outcome of the current study is indeed quite valuable from applied enzymology and industrial biotechnology point of view.

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