Characterisation of the Nitrile Biocatalytic Activity of *Rhodococcus Rhodochrous* ATCC BAA-870

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INTRODUCTION

A versatile nitrile-degrading bacterium, Rhodococcus rhodochrous ATCC BAA-870, was isolated through enrichment culturing of soil samples from Johannesburg, South Africa. Useful nitrile-metabolising activity of a potential biocatalyst, Rhodococcus rhodochrous ATCC BAA-870, was explored. The biocatalyst expressed a two enzyme system with sequential nitrile-converting activity: nitrile hydratase and amidase (Figure 1). This biocatalytic nitrile hydrolysis affords valuable applications in industry, including production of solvents, extractants, pharmaceuticals, drug intermediates (chiral synthons), and pesticides, as well as in the organic synthesis of amines, amides, esters, carboxylic acids, aldehydes, ketones and heterocyclic compounds. Applications of nitrile-hydrolysing enzymes include the production of amides and acids such as acrylamide, nicotinic acid and lactic acid from their corresponding nitriles, and the conversion of a–aminonitriles to optically active amino acids

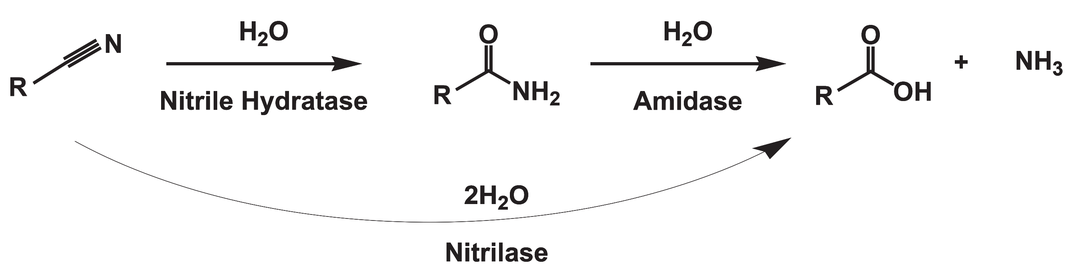


Figure 1: Nitrile hydrolysis catalysed by one of two distinct pathways

Enantioselective hydrolysis of a-hydroxynitriles (cyanohydrins) and the production of the optically active 2-arylpropionic acids, ibuprofen and naproxen, have also been achieved using nitrile-converting biocatalysts.

The development of appropriate activity assaying methods was investigated for the purpose of finding an assay specific enough for identifying nitrile hydrolysing activity in both whole cell extracts and purified enzyme reactions. The existing high performance liquid chromatography assay for identifying nitrile hydrolysis was developed and optimised for monitoring nitrile hydrolysis was against various substrates. The influence of time and protein concentration on nitrile hydrolysis was investigated. An enzyme preparation with industrial use need not necessarily be pure, but rather be one where specific activities can be easily separated in a one-step process. In this study, enzyme separation strategies were investigated and a simple, one-step separation protocol for preparation of biocatalytic enzyme was explored using chromatography methods.

HPLC assay development

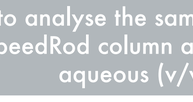
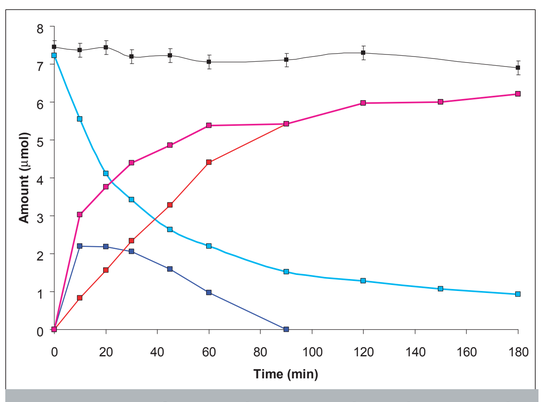
Compound	Structure	Absorbance Wavelength (nm)	Average Retention Time (min) +/- S.D.
Benzonitrile		224.1	5.55 ± 0.02 (30)
Benzamide		226.4	3.81 ± 0.01 (30)
Benzoic Acid		228.8	4.37 ± 0.01 (30)
3-Hydroxy-3-phenyl-propionitrile		206.5	4.15 ± 0.004 (30)
3-Hydroxy-3-phenyl-propionic Acid		206.5	3.78 ± 0.004 (30)
Hydro-cinnamo-nitrile		205.3	5.80 ± 0.01 (30)

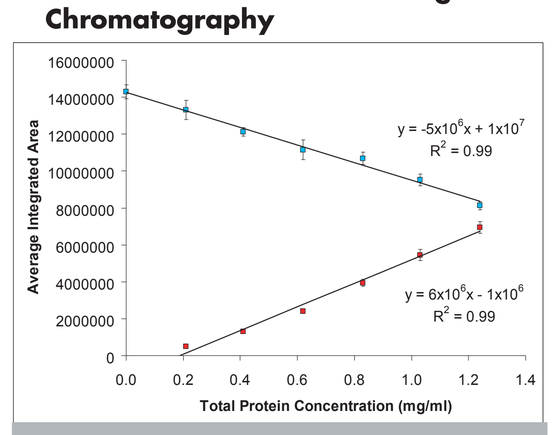
Figure 2: Compounds were prepared as 5 mM samples in methanol, and 100 ml added to 900 ml acidic eluent in 2 ml HPLC vials. A Waters 2690 Separations Module HPLC with 996 Photodiode Array Detector was used to analyse the samples. Compounds were eluted isocratically over 10 minutes from a Chromolith SpeedRod column at a flow rate of 0.5 ml/min using 40% acetonitrile:6% aqueous (v/v) trifluoracetic acid

Time and Protein Concentration Studies

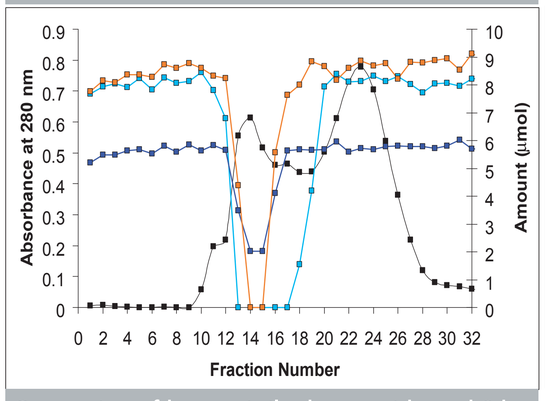


Conversion of 5 mM benzonitrile to benzamide and benzoic acid over three hours. Benzonitrile (■) was added to reaction mix containing Rhodococcus rhodochrous supernatant (at 30°C with stirring). The average integrated area of substrate remaining and benzamide (■) and benzoic acid (■) produced was measured by HPLC, and converted to amount. The additive amount of benzamide and benzoic acid was plotted on the same axes (■). Benzonitrile was added to control reactions in which no enzyme was present (■) thereby demonstrating the stability of the substrate under these reaction conditions.

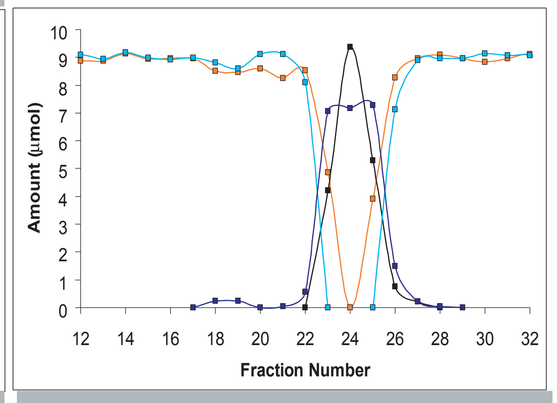
Gel Filtration and Ion Exchange Chromatography



Formation of benzoic acid from benzonitrile with increasing protein concentration. Different enzyme concentrations were incubated with benzonitrile for 20 min at 30 °C with shaking (200 rev./min). Disappearance of benzonitrile (■) and appearance of benzoic acid (■) was analysed in triplicate by HPLC and the average integrated area plotted ± S.D.



Conversion of benzonitrile, benzamide and 3-hydroxy-3-phenylpropionitrile in Sephacryl S-200 eluted fractions. Benzonitrile (■), benzamide (■) or 3-hydroxy-3-phenylpropionitrile (■) were reacted in duplicate with each fraction at 30 °C with shaking (200 rev./min) and the amount of substrate remaining analysed by HPLC. Benzonitrile reactions were reacted for 10 minutes, while benzamide and 3-hydroxy-3-phenylpropionitrile were reacted for 30 minutes. Elution of protein was monitored at 280 nm (■).



Amount of benzamide formed from benzonitrile, and conversion of 3-hydroxy-3-phenylpropionitrile in Toyopearl SuperQ 650M ion exchange eluted fractions. Benzonitrile (■) was reacted in duplicate with each fraction for 10 minutes at 30 °C with shaking (200 rev./min) and the amount of substrate remaining and benzamide (■) produced analysed by HPLC. 3-Hydroxy-3-phenylpropionitrile (■) was reacted in duplicate with each fraction for 30 minutes at 30 °C with shaking (200 rev./min) and the amount of substrate remaining and product produced (■) analysed by HPLC.

CONCLUSIONS

- Using a chosen nitrile substrate such as benzonitrile as the sole nitrogen source during simple enrichment culturing gives rise to bacterial enzyme systems that are adapted to metabolism of the substrate of interest.
- Rhodococcus rhodochrous ATCC BAA-870 converted benzonitrile to benzamide and benzoic acid, and also converted the b-hydroxynitrile, 3-hydroxy-3-phenylpropionitrile, to the corresponding amide and acid.
- Increasing the concentration of biocatalyst caused faster conversions, and extending the reaction time allowed complete conversion of all substrate to product without an equilibrium being reached. Activity against benzamide and 3-hydroxy-3-phenylpropionitrile was linear up to 1.3 mg total protein. R. rhodochrous ATCC BAA-870 was demonstrated to be a biocatalyst suitable for nitrile conversions over a wide pH range.
- Gel filtration chromatography has shown that both the nitrile hydratase and amidase enzymes in Rhodococcus rhodochrous ATCC BAA-870 are high molecular weight native structures, and are suited to conversions of mono-substituted aromatic nitriles such as benzonitrile and 3-hydroxy-3-phenylpropionitrile.
- Gel filtration chromatography indicated the enzymes responsible for nitrile-converting activity are functional proteins from 23 to 700 kDa. SDS-PAGE of gel filtration and ion exchange chromatography fractions indicated that a possible multimer-forming nitrile hydratase exists in R. rhodochrous ATCC BAA-870, and may be made up of subunits of approximately 22 kDa that form 48 kDa ab functional heterodimers, 84 kDa a2b2 tetramers, and possibly larger enzyme aggregates
- Ion exchange chromatography of the soluble protein extract from R. rhodochrous ATCC BAA-870 successfully separated nitrile hydratase from amidase activity.
- The enzymes appear to have a broad pH range, fast reaction times, and are relatively stable. Nitrile hydratase and amidase from Rhodococcus rhodochrous ATCC BAA-870 are potentially useful enzymes which could be applied to biocatalysis in industry.
- Rhodococcus rhodochrous (which converted 5 mM benzonitrile to benzamide in less than 10 minutes) is identified as an enzyme worthy of further investigation for practical applications in industry.

ACKNOWLEDGEMENTS

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