

Short Communication

A high-throughput screening assay for distinguishing nitrile hydratases from nitrilases

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Abstract

A modified colorimetric high-throughput screen based on pH changes combined with an amidase inhibitor capable of distinguishing between nitrilases and nitrile hydratases. This enzymatic screening is based on a binary response and is suitable for the first step of hierarchical screening projects.

Key words: nitrile hydratase, amidase, nitrilase, amidase inhibitor, high-throughput screening.

Nitrile metabolizing enzymes are of outstanding synthetic importance because of their ability to afford amides, carboxylic acids, amines and cyanohydrins, compounds used as building blocks in the pharma and chemical industries (van Pelt 2011, Chen 2009, Lin 2012, Yamada 2001). They can also be potent biocatalysts for detoxification of anthropogenic toxic nitrile pollutants that are widespread throughout the world (Vesela 2012, Vesela 2010). Among them, nitrile hydratase is an interesting commercial enzyme already used successfully on a ton scale for the production of commodities and pharmaceuticals such as acrylamide and nicotinamide (Prasad 2010, Kobayashi 2000, Yamada 2001). Nitrile hydratase was first discovered in 1980 by Yasano and collaborators and it is believed that for the most part, they are involved in a cascade reaction with amidases, affording carboxylic acids from nitriles passing through an amide intermediate (Yasano 1980). Nitrilases are also present in many different species and afford a carboxylic acid directly from a nitrile compound (Prasad 2010) (Figure 1).

A number of screening assays for nitrile-converting enzymes based on continuous and stopped methods are well documented in the literature (Asano 2002, Martinkova 2008; Reisinger 2006, Santoshkumar 2010; He 2011; Zheng 2011, Yazbeck 2006, Wang 2012). However, as nitrilases and nitrile hydratase-amidases afford the same fi-

nal product, it is important to design a screening assay able to distinguish between the two enzymatic pathways.

Herein, we describe a colorimetric high-throughput screening assay based on pH changes coupled with the use of an amidase inhibitor. This screen is based on a binary response allowing differentiation between nitrilases and nitrile hydratase-amidases enzymatic systems and is suitable for the first step of hierarchical screening projects.

A Banerjee-modified colorimetric and pH sensitive assay coupled with an amidase inhibitor was performed for screening nitrilase and nitrile hydratase-amidase enzymes. Commercially available microorganisms potentially containing the nitrile hydratase and nitrilase enzymatic systems were used as positive controls. All nitriles and their corresponding amides and carboxylic acids and an amidase inhibitor were evaluated to detect any possible color change interferences within the enzymatic assay system. It was assumed that the strains that did not accumulate the amide during nitrile degradation indicated nitrilase activity. The intermediate accumulation of the corresponding amide during nitrile metabolism combined with carboxylic acid formation was taken as an indication of the existence of a nitrile hydratase-amidase system (Layh 1997). The expression of nitrile hydratases was induced by acetonitrile or benzonitrile for aliphatic and aromatic nitriles, respectively. Mandelonitrile was too toxic to the microorganisms

Table 1 - Enzymatic assay results for detection of nitrilase, nitrile hydratase and amidase activities.

| Microorganism | Microbial control | Assay without DEPA | Assay with DEPA |
|--|-------------------|--------------------|-----------------|
| <i>Pseudomonas putida</i> CCT 2357 | blue | yellow | yellow |
| <i>Pseudomonas fluorescens</i> CCT 3178 | blue | yellow | yellow |
| <i>Arthrobacter</i> sp CCT 1875 | yellow | yellow | yellow |
| <i>Nocardia brasiliensis</i> CCT 3439 | blue | blue | yellow |
| <i>Nocardia simplex</i> CCT 3022 | blue | yellow | greenish |
| <i>Rhodococcus erythropolis</i> CCT 1878 | blue | blue | yellow |
| <i>Rhodococcus equi</i> CCT 0541 | blue | blue | yellow |
| <i>Rhodococcus ruber</i> CCT 1879 | blue | blue | greenish |

The control experiments with acetonitrile, DEPA and acetamide compounds as well as with the microbial cultures alone are blue. Acetic acid and other carboxylic acids are yellow. A greenish tint to the blue or yellow indicates a small drop in pH due to formation of acetic acid.

positive hits were detected, of which 12 were nitrilase producers, 3 were NHase-amidase producers and 5 produced both enzymatic systems.

Interestingly, all microbial cultures which afforded positive hits came from nitrile-impacted areas. These results suggest the importance of natural microbial acclimation and also suggest a strategy for enzyme identification. The enzymatic assay was capable of detecting and distinguishing nitrile hydratases from nitrilases when only one was produced in a bacterial strain. The enzymatic assay was validated by GC-FID monitoring. Nitrile maximum inhibitory concentrations (MIC) were determined using a MTT assay. The MIC values varied for each tested nitrile and microbial culture. The wild type microorganisms are currently being subjected to 16S RNA identification and show promise as whole cell biocatalysts.

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Supplementary Material

Figure S1. Chromatogram of acetonitrile (rt 1.90 min), acetamide (rt 9.38 min) and acetic acid (rt 5.60 min). Temperature program: 35 °C (4 min) 25 °C/min to 280 °C (3 min). Split ratio 1: 100.

Figure S2. Chromatogram of mandelonitrile (rt 7.45 min), mandelamide (rt 8.60 min) and methyl mandelate (rt 7.21 min). Temperature program: 80 °C (3 min) 25 °C/min to 300 °C (3 min). Split ratio 1: 100.

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