Bioresource Technology 142 (2013) 320–328

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Nitrile, amide and temperature effects on amidase-kinetics during acrylonitrile bioconversion by nitrile-hydratase/amidase *in situ* cascade system

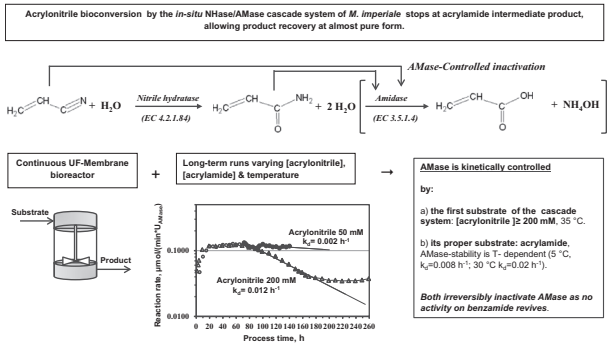
Laura Cantarella [b], Alberto Gallifuoco [a], Agata Spera [a], Maria Cantarella [a,*]

[a] Department of Industrial and Information Engineering and Economics, University of l'Aquila, Via Giovanni Gronchi n.18- Nucleo industriale di Pile, 67100 L'Aquila, Italy
[b] Department of Civil and Mechanical Engineering, University of Cassino and of Lazio Meridionale, Via Di Biasio 43, 03043 Cassino (FR), Italy

HIGHLIGHTS

- Acrylonitrile conversion by *in situ* NHase/AMase-enzymatic system stops at the amide.
- Controlled amidase inactivation avoids enzyme purification to obtain desired product.
- The intermediate amide of the two-step reaction is recovered in almost a pure form.
- Membrane bioreactor runs show AMase-inactivation by acrylamide, nitrile and temperature.
- Membrane bioreactor studies show benzamide protection against AMase-inactivation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form 29 April 2013
Accepted 30 April 2013
Available online 9 May 2013

Keywords:
Amidase kinetics
UF-membrane bioreactor
Temperature dependence
Nitrile inactivation
Amide inactivation

ABSTRACT

In this study the amidase kinetics of an *in situ* NHase/AMase cascade system was explored as a function of operational parameters such as temperature, substrate concentration and product formation. The results indicated that controlling amidase inactivation, during acrylonitrile bioconversion, makes it possible to recover the intermediate product of the two-step reaction in almost a pure form, without using purified enzyme. It has been demonstrated, in long-term experiments performed in continuous stirred UF-membrane bioreactors, that amidase is kinetically controlled by its proper substrate, depending on the structure, and by acrylonitrile. Using acrylamide, AMase-stability is temperature dependent (5 °C, $k_d = 0.008\ h^{-1}$; 30 °C $k_d = 0.023\ h^{-1}$). Using benzamide, amidase is thermally stable up to 50 °C and no substrate inhibition/inactivation occurs. With acrylonitrile, AMase-activity and -stability remain unchanged at concentrations <200 mM but at 200 mM, 35 °C, after 70 h process, 90% irreversible inactivation occurs as no AMase-activity on benzamide revives.

1. Introduction

The bioconversion of nitriles into higher value compounds, the corresponding amides or acids, has been intensively investigated recently and the ability of several microorganisms and fungi to degrade nitriles has been proved (Banerjee et al., 2002; Martínková and Křen, 2010; Singh et al., 2006; Thuku et al., 2009). In nature, the bioconversion of nitriles follows two pathways: one, catalyzed by nitrilase, directly converts the nitrile into the corresponding acid and ammonia; the other, involves a two-sequential reactions, catalyzed by nitrile hydratase and amidase, NHase/AMase, that transform the nitrile into acid and ammonia with the amide as intermediate product.

* Corresponding author. Tel.: +39 0862434215.
E-mail address: maria.cantarella@univaq.it (M. Cantarella).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.04.126

Since the discovery of these nitrile converting enzymes, the biotechnological potential of several groups of microorganisms to degrade nitrile has been ascertained (Martínková et al., 2009; Thomas et al., 2002). Some important industrial processes, based on these routes, have been already developed advantageously using mild pH and temperature conditions compared to those of chemical processes. Among these, the production of acrylamide (Nitto Chemical Industry) of nicotinamide and/or nicotinic acid, and of 5-cyanovaleramide (DuPont). *Pseudomonas chlororaphis* B23 has also been employed in the first step in the manufacture of a new herbicide, and other examples of biocatalytic synthesis of pharmaceuticals and their intermediates, agrochemicals, food and feed additives are reported (Kobayashi et al., 1992; Lievano et al., 2012; Yamada and Kobayashi, 1996). Their extensive use in organic synthesis is foreseen also because of the stereo-, regio- and chemoselectivity (Martínková and Křen, 2002; Naik et al., 2008).

On the other hand, the accumulation in the environment of a great number of aromatic and aliphatic nitriles as intermediates, products, or waste products from chemical and pharmaceutical industrial processes and from intensive agriculture has to be faced with dedicated processes (Cantarella et al., 2006a; Chand et al., 2004; Kohyama et al., 2006; Li et al., 2013). These compounds dispersed in nature may cause a wide range of toxicological effects on living organisms (Balbuena and Llorens, 2001). Hence biotechnological processes strategic for the environmental protection are called, even though they are often hampered by economics and not yet applied intensively, for nitrile biodegradation in their less toxic amide or carboxylic acid that can be further metabolized by several microbial strains present in nature (DeVito, 1996).

Among nitrile bioconverters, *Microbacterium imperiale* CBS 498-74, represents an interesting and efficient strain that follows a two-step reaction. The use of resting whole cells, containing the naturally expressed *in situ* nitrile hydratase/amidase cascade system, used as biocatalyst, were extensively investigated in previous papers. Mostly, these papers (Cantarella et al., 1998, 2004, 2006b, 2010) aimed to show that, in peculiar operational conditions (low temperature, high substrate concentration, appropriate enzyme loading and residence time) the reaction that was carried out by the adopted reactor configuration, the continuous stirred UF-membrane reactor (CSMR), was arrested at the intermediate product, and in other cases high yield in acid was reached (Cantarella et al., 2011). In the bioconversion of acrylonitrile and propionitrile, AMase-activity was insignificant, or at least the corresponding acid formed was not detectable. To overcome NHase-inactivation by acrylonitrile during its bioconversion and reach satisfactory yield in acrylamide, a multi-cycle bioconversion in a UF-membrane bioreactor was suggested. Besides, recent studies (Cantarella et al., 2012) on the effect of 3-cyanopyridine on AMase-activity have demonstrated the capacity of this substrate to depress the activity at high temperature and concentration. These findings suggested to face a more in depth investigation for determining the role played by both substrates, acrylonitrile and acrylamide, involved in the cascade reaction, on the control, through reversible inhibition and/or inactivation, of the AMase-activity. Since the use of multiple enzymes is of special interest (Xue and Woodley, 2012) this investigation could help to understand how some cascade catalyzed reactions are more easily controlled and the intermediate product, amide, is obtained while others give directly the final product, i.e. the acid.

This paper explores the effects of acrylonitrile and acrylamide effects on AMase activity of *M. imperiale* CBS 498-74 resting cells that contains in the cultural conditions adopted a activity ratio of 8.34 (U_{NHase}/U_{AMase}) (Cantarella et al., 2010). The presence of course, of AMase could represent a serious drawback whenever the process has to be stopped at the amide stage, unless an inhibiting or inactivating effect of the substrates on AMase-activity holds. The reversibility or irreversibility of the acrylonitrile and/or acrylamide "adverse effect" on the AMase-activity was rather difficult to define and an auxiliary reaction, chosen as a reference reaction, was used. Benzamide amidase catalyzed biotransformation which exhibit a different stability, was proved to be appropriated to evaluate the AMase-activity after exposure to these compounds. A continuous stirred UF-membrane reactor (CSMR) was used and proved to be, once again, a valuable laboratory tool for obtaining data in controlled operational conditions. Interestingly the temperature dependence of AMase-activity and stability of catalyzed reactions of benzamide and acrylamide is rather different. In our reasoning, since in long-term runs AMase, in presence of benzamide as substrate, was highly stable and active, by changing the reactor feed (acrylamide *versus* benzamide) and restoring AMase-activity with benzamide could prove the reversibility of the phenomenon.

2. Methods

2.1. Chemicals

All reagents used in the experiments discussed below were purchased from Sigma-Aldrich (USA). Media components were obtained from Oxoid (England).

2.2. Microorganism, growth, enzyme production

M. imperiale CBS 498-74 was routinely maintained at 4 °C on YMPG-agar plates (in g L^{-1} of distilled water, agar 20.0, yeast extract 3.0, malt extract 3.0, bacteriological peptone 5.0, glucose 20.0; pH 7.0). *Pre-culture*: 100 mL of sterile YMP-culture medium (YMPG medium without glucose), was inoculated with a colony from a YMPG-agar plate and incubated for 24 h. *Culture*: 10 mL of the pre-culture was aseptically transferred into 90 mL of sterile YMP-culture medium supplemented with 5 g L^{-1} of glucose (Cantarella et al., 2002). Both pre-culture and culture were grown in 500 mL Erlenmeyer flasks kept for 24 h in a New Brunswick G 25 Shaker Incubator (NJ, USA) set at 28 °C and 220 rpm. The NHase activity was maximal after 20–24 h in both steps, and the cell concentration achieved was approx. 1.1 and 3.0 mg_{DCW} mL^{-1} in the pre-culture and culture, respectively. Cells were harvested by centrifugation (11,400 rpm for 15 min at 4 °C), washed three times with Na-phosphate buffer (50 mM, pH 7.0) and resuspended in the same buffer to give a final concentration corresponding to 10 OD_{610}. The harvested cells were used immediately or kept at −18 °C until use. Culture growth was assessed by spectrophotometrical measurements of the optical density at 610 nm (OD_{610}) using a Perkin Elmer lambda 2 spectrophotometer. A concentration of 0.26 mg_{DCW} mL^{-1} was equivalent to 1.0 unit of OD_{610}. The resting cell preparation contained approx. 0.26 mg_{DCW} mL^{-1}, 0.76 U_{NHase} mg_{DCW}^{-1} and 0.09 U_{AMase} mg_{DCW}^{-1} (Cantarella et al., 2010). All experiments were performed in duplicate and the relative standard deviations were <5%.

2.3. Enzyme assays for nitrile hydratase and amidase

The activity of nitrile hydratase and amidase in reaction mixture were determined using acrylonitrile and acrylamide as substrate, respectively. The assays were performed in a 2 mL reaction mixture of 50 mM sodium phosphate buffer, pH 7.0, containing acrylonitrile or acrylamide (50 mM each) and an appropriate amount of whole cells (ranging from 1 to 5 mg_{DCW}). The reaction was carried out at 20 °C under stirring (250 rpm) and stopped after 20 min by the addition of HCl (1 mL, 0.5 M) followed by centrifugation (10 min at 10,000 rpm). One unit of NHase or AMase was defined as the amount of enzyme releasing 1 μmol min^{-1} of total product (acrylamide and acrylic acid) or acrylic acid, respectively under the assay conditions.