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Large-scale preparation of a nitrile-hydrolysing biocatalyst: *Rhodococcus* R 312 (CBS 717.73)

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Abstract

Lyophilized cells of *Rhodococcus* R 312 (CBS 717.73) can be employed as an easy-to-use biocatalyst for the biocatalytic hydrolysis of nitriles to furnish the corresponding carboxamides or acids on a preparative scale. The following practical aspects are advantageous: (i) Fermentation yields a high biomass ($\sim$ 10 g dry cell weight l^{-1}), (ii) enzyme induction is not required, (iii) a maximum of activity is obtained at the late exponential growth phase (25.7 μmol min^{-1} mg^{-1}), which can be monitored by a simple photometic assay and (iv) the cells can be stored at $+4$°C for several months without significant loss of activity.

Keywords: Nitrilase; Nitrile hydratase; Nitrile hydrolysis; *Rhodococcus* sp.

1. Introduction

The hydrolysis of a nitrile to yield the corresponding carboxylic acid is a synthetically useful transformation. However, its applicability is limited due to the harsh reaction conditions required, [1] which are often incompatible with other functionalities present in synthetically 'intelligent' target molecules [1]. In this context, the chemo-selective enzymatic hydrolysis of nitriles represents a valuable alternative because it occurs at ambient temperature and near physiological pH [2–8] Unfortunately, at present no biocatalyst for this purpose is commercially available, which limits the practical applicability of this useful technology. Several years ago, a crude immobilised enzyme preparation denoted as 'SP 361' or 'SP 409' derived from a *Rhodococcus* sp. was available from Novo Industri (DK) but its production has been stopped and is not to be resumed within the near future. [2] As a consequence, there is a need for a stable

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[1] The most widely employed procedures involve strongly acidic, basic and/or oxidising (H_2O_2) conditions.

[2] SP 361 and SP 409 were derived from the same *Rhodococcus* sp. strain which possesses a nitrile hydratase and an amidase, the enzyme preparations only differ by the carrier used for immobilisation. For applications see Ref. [9]

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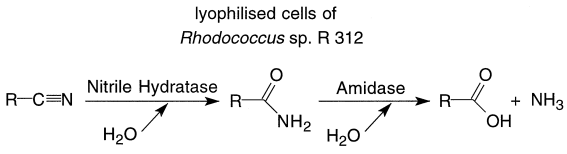

Fig. 1. Nitrile-hydrolysis catalyzed by *Rhodococcus* R 312.

and easy to prepare biocatalyst for the hydrolysis of nitriles (Fig. 1).

Due to the fact, that the enzymes involved in nitrile-hydrolysis—i.e., nitrile hydratases and/or nitrilases—are usually rather unstable in isolated form [10–12], the reactions are generally performed by using resting whole cells [7,13]. Among the various microorganisms employed—most commonly from the genus *Bacillus*, *Micrococcus*, *Bacteridium*, *Arthrobacter*, *Corynebacterium*, *Pseudomonas*, *Nocardia* or *Rhodococcus*—one strain can be recommended in particular: *Rhodococcus* R 312. [3]

2. Materials and methods

2.1. Preparation of lyophilised cells of Rhodococcus R 312

2.1.1. Medium

The following components of the medium were sterilised in separate groups: Group I (mineral salt medium): Citric acid (0.3 g/l), ammonium sulfate (6 g/l), $FeSO_4 \cdot 7H_2O$ (0.02 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), trace element solution SL6 (1 ml/l). Before addition of the trace element solution, the pH was adjusted to 7.0 using aqueous dil. NaOH. Group II (trace element solution): $ZnSO_4 \cdot 7H_2O$ (0.1 g/l), $MnCl_2 \cdot 2H_2O$ (25 mg/l), H_3BO_3 (0.3 g/l), $CoCl_2 \cdot 6H_2O$ (0.2 g/l), $CuSO_4 \cdot 5H_2O$ (10 mg/l), $NiCl_2 \cdot 6H_2O$ (20 mg/l), $Na_2MoO_4 \cdot 2H_2O$ (30 mg/l). Group III (phosphate buffer): NaH_2PO_4 (6 g/l), K_2HPO_4 (8.8 g/l). The pH of the latter solution was adjusted to 7.0 before use. Group IV (carbon source): Glucose (30 g/l).

2.1.2. Strain maintenance

Rhodococcus R 312 (CBS 717.73) was maintained on agar plates using the above described medium (15% agar). Sub-culturing was performed every 12 weeks and plates were stored at 4°C.

2.1.3. Shake-flask cultures

Cells for inoculum were precultured for 16 h at 30°C on a rotary shaker at 120 rpm in four 1 l baffled Erlenmeyer flasks using 250 ml of above described medium each.

2.1.4. Bioreactor cultivation

Large-scale growth was performed in a Biostat E bioreactor (Braun, Melsungen, Germany) with a stainless steel vessel ES-10 of 10 l working volume. Glucose was autoclaved in situ in the reactor vessel (120°C, 60 min), all other components were sterilised separately and added after cooling to room temperature. Cells were grown on 8 l of medium at 30°C, the oxygen electrode was calibrated by saturating the system with air and the pO_2 was kept at 70% using valve control with an aeration pressure of 1 bar (9 l/min) and a stirring speed of 250 rpm. The pH of 7.0 was controlled within ± 0.1 by automatic addition of acid/base (H_3PO_4 2 M, NaOH 8 M). Antifoam 289 (Sigma No. A-5551, 10 ml) in water (0.75 l) was used. For the inocolum, 1 l of a shake-flask preculture using the same medium as described above (30°C for 16 h, shaking speed 120 rpm) was used. During certain periods of the fermentation, foaming was a problem. During early stages of growth, the foam level was controlled via the automatic foam control, but after about 20 h, the direct addition of concentrated Antifoam 289 in portions of $\sim$1–2 ml via a syringe was necessary. The cell growth was monitored by analysing samples (20 ml each) for (i) cell dry weight (CDW) (ii) glucose concentration, (iii) ammonium concentration and (iv) nitrile hydratase activity. Fig. 2 shows the parallel con-

[3] For the reclassification of *Brevibacterium / Rhodococcus* sp. R 312 see Ref. [14].

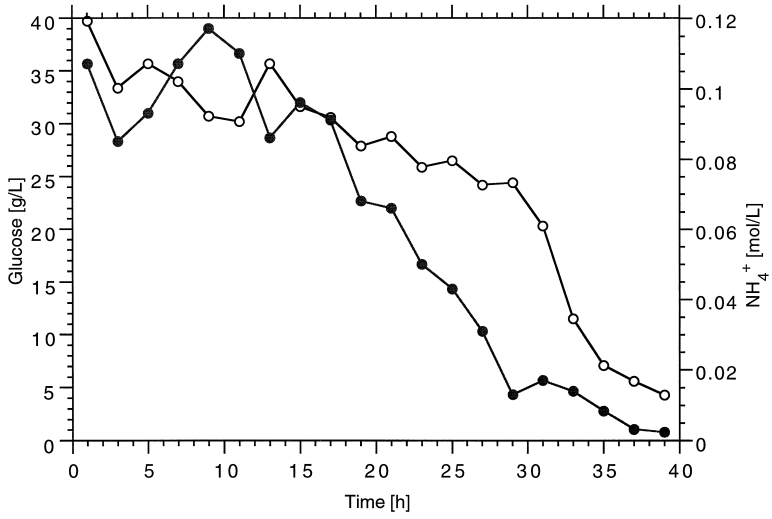

Fig. 2. Consumption of glucose (open symbols) and ammonium (filled symbols).

sumption of glucose and ammonium. At the late exponential growth phase, when glucose and ammonium were consumed and the biomass reached a maximum (after ~38 h), the cells were harvested. [4]

As may be seen from Fig. 3, the relative activity of the cells strongly depends on the growth stage. It reached a maximum during exponential growth and levelled off during a later stage. A detailed assay (see below) gave an impressive specific activity of 25.7 μmol min^{-1} mg^{-1} (corresponding to lyophilized cells) at the maximum. Although cells should be preferably harvested close to this point, even material from the late exponential growth phase is still active enough for practical applications, showing an activity of 1.62 μmol min^{-1} mg^{-1} (Fig. 3).

2.1.5. Determination of optical density

A culture sample (100 μl) was placed into a plastic cuvette (1.5 ml) and diluted with NaCl solution (0.9%, 900 μl). The optical density was measured using a Perkin Elmer UV/VIS-spectrometer (Lamda 2S) at 546 nm against NaCl solution (0.9%) as blank. The extinction (ε) should be below 1, if necessary, the dilution factor was increased. The OD_{546} value was obtained via the following formula: $OD_{546} = \varepsilon \cdot$ dilution factor.

2.1.6. Determination of cell dry weight

The CDW was determined on a thermobalance (Sartorius MA 30 moisture analyzer) at constant weight at 130°C.

2.1.7. Determination of glucose concentration

Glucose was monitored using a commercial glucose hexokinase test kit (DIPRO No. D590522) based on the hexokinase UV-method. A sample of clear filtrate from the CDW determination was diluted with NaCl (0.9%, 1:20) and 10 μl of this solution was added to 1 ml sample of Glucose-UV-Test solution and mixed well. After 20 min, the extinction at 340 nm was measured against Glucose-UV-Test as blank, the value should read below 1. Glucose concentration was calculated as follows: Glucose [g/l] $= \varepsilon \cdot$ dilution factor $\cdot 16 \cdot 0.18$.

2.1.8. Determination of ammonium concentration

Ammonium pH-adjusting 15A reagent (Orion No. 7972, 100 μl) was added to a sample of

[4] The corresponding values obtained via optical density nicely match the CDW.

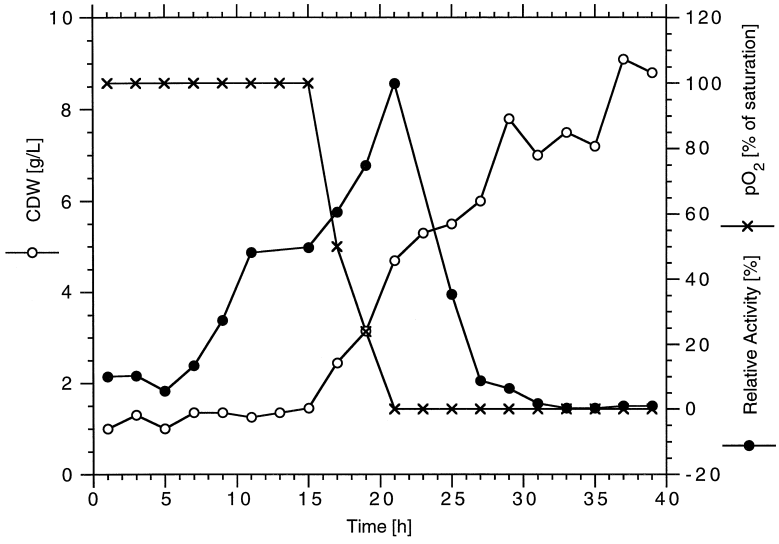

Fig. 3. Production of biomass (via CDW), relative activity [%] and pO_2 [% of saturation].

clear filtrate (2 ml) and thoroughly mixed. The ammonium concentration of this solution was determined using an NH_4^+-electrode (Orion 95-12).

2.1.9. Preparation of lyophilised cells

The cells were harvested by centrifugation ($300 \times g$, 15 min) and washed once by resuspending them in Tris-buffer (pH 7.5, 50 mM). Lyophilisation gave ~80 g of dry cells from a total volume of 8 l of culture. The cells can be stored at +4°C for several months without significant loss of activity.

2.2. Assay for activity

2.2.1. Exact assay for nitrile-hydrolysis

For an exact assay of nitrile hydratase based on the hydrolysis of acrylonitrile (which requires a cell-free extract) see Refs. [10,15]. This assay was modified for the analysis of samples from the cultivation as follows: To a series of six samples of cell suspension (50 μl each) in phosphate buffer (0.05 M, pH 7.0, 750 μl), acrylonitrile (5 μl) was added and the samples were agitated at 25°C with 180 rpm. In this series, the reaction was terminated by the addition of HCl (2 M, 495 μl) after 5, 10, 15, 20, 25 and 30 min, respectively. A blank sample was obtained by quenching the enzyme reaction through HCl-addition prior to substrate addition. In case of exceptional high enzyme activity, the dilution of the cell suspension sample with phosphate buffer was adapted. After vigorous shaking, the samples were centrifuged (13 000 $\times g$, 10 min) and the extinction of the clear supernatant at 235 nm was measured against the blank sample. If necessary, samples were further diluted. The relative activity (expressed as % of maximum) was obtained from the average variation of extinction per minute per amount of cell dry weight ($\delta E\ min^{-1}\ mg^{-1}$). From this value, the specific activity was obtained through exact determination of the acrylamide concentration using a calibration curve.

2.2.2. Quick assay

For a simple and rapid semi-quantitative activity test, benzonitrile was used as substrate: A sample of lyophilised cells (10 mg) was rehydrated in phosphate buffer (0.05 M, pH 7.0, 1 ml). After 30 min, a drop of benzonitrile was added (5 μl) and the sample was shaken with 130 rpm at r.t. The reaction was monitored by

TLC (Merck silica gel plates F_{254}) using petroleum ether/ethyl acetate (1/1) as the eluent. The disappearance of benzonitrile and the appearance of benzoic acid through the intermediate formation of benzamide (weak spot) was visible via UV: Benzonitrile ($R_f = 0.75$), benzoic acid ($R_f = 0.59$), benzamide ($R_f = 0.20$).

2.3. General procedure for preparative nitrile-hydrolysis

2.3.1. Screening

The acceptance of a substrate was most conveniently checked as follows: Lyophilised cells (20 mg) were rehydrated in phosphate buffer (0.05 M, pH 7.5, 0.5 ml) for ~30 min in an Eppendorff-vial. Then the nitrile (~5–10 mg) was added in one portion. Solid substrates were dissolved in a minimum amount of ethanol or DMSO (~100–150 μl). The vial was shaken at 20–30°C on a rotary shaker at 120 rpm. Samples were periodically withdrawn and analysed by TLC or HPLC. It is recommended to run a blank experiment (without biocatalyst) in order to detect spontaneous reactions and possible side products which might emerge from the cells. Ethyl acetate (+1% AcOH) was used as eluent for TLC and spots were visualised by spraying with the bromocresol green/bromphenol blue/$KMnO_4$ reagent. Carboxylic acids appear as light yellow stains on a blue background [16].

2.3.2. Preparative scale reactions

The reaction was performed as described for the screening by using ~1 g of substrate and 0.5–1 g of lyophilized cells in 25–50 ml of buffer. When an appropriate degree of conversion was reached (usually within 8–24 h), the mixture was acidified with 6 N HCl or dil. formic acid to a pH of < 3, saturated with NaCl or ammonium sulfate and the cells were centrifuged. Products were extracted from the supernatant using several portions of ethyl acetate, typical yields ranged from 60 to 80%. A second crop was recovered from the cells by treating the pellet with a small volume of acetone or ethanol. For highly polar compounds, extraction using the Extrelut system (Merck No. 1.13076. 1000) was advantageous. Thus, the clear (NaCl-saturated) supernatant was poured onto a short column filled with Extrelute (~15 g for 20 ml of solution). After the liquid was evenly distributed (~15–20 min), the organic material was eluted from the column with ethyl acetate (as monitored by TLC). Extrelute can be recycled by suspending it in excess water for several days. After filtration, the material was washed with several portions of acetone and dried at room temperature (12 Torr).

3. Results and discussion

Rhodococcus R 312, which was formerly denoted as *Brevibacterium* sp., has been isolated by Arnaud et al. in the mid-1970s [17,18]. Function and properties of its enzymes involved in nitrile-hydrolysis are well understood, which are (i) a nitrile-hydratase [19–21] and (ii) an amidase [22,23] (Fig. 1). [5] *Rhodococcus* R 312 is available from CBS (No. 717.73), [6] it is easy to grow and, in contrast to other strains, enzyme induction is not required to develop sufficient nitrile-hydrolysing activity. The range of nitrile substrates which are readily accepted is remarkable, not only saturated and unsaturated aliphatic nitriles, but also (hetero)aromatic compounds are easily hydrolysed. In addition, functionalised nitriles, in particular α-hydroxy- and α-aminonitriles have been hydrolysed to the corresponding α-hydroxy- and α-amino acids. Ali-

[5] Amidases are a subgroup of proteases which hydrolyse carboxamides to yield the corresponding carboxylic acids.

[6] Centraalbureau voor Schimmelcultures, p.o. box 273, NL-3740 AG Baarn, The Netherlands. ⟨http://www.cbs.knaw.nl/www.cbshome.html⟩. Strain CBS 717.73 is freely available, however, some granted patents still apply with respect to commercial applications: FR 73-33613, 1973; FR 74-41828, 1974; FR 79-01803, 1979; EP 0-298-796-A1, 1988; US 88/407296, 1988.

phatic and aromatic dinitriles are usually converted in a regioselective fashion, i.e., one nitrile group is transformed into the amide or acid functionality, whereas the other remains unaffected. It should be noted that in general the chiral recognition of the nitrile hydratase is low, and that any enantioselectivity is a consequence of the second step, i.e., the action of the amidase [13,24].

This contribution describes the large-scale preparation of lyophilised cells of *Rhodococcus* R 312 which can be used as a stable and easy-to-use biocatalyst for the hydrolysis of nitriles under mild conditions.

The following points should be considered beforehand: cyanide, which might be present in traces either from the chemical synthesis of the substrate or due to undesired decomposition of chemically labile nitriles—e.g., α-hydroxy- or α-aminonitriles—is a substrate for the *Rhodococcus* nitrile hydratase. Thus, enzyme inhibition by cyanide, which is not uncommon for nitrile-hydrolysing enzymes from other sources, is not a problem [25]. On the other hand, esterases from within the whole-cell preparation [26] may cause undesired side reactions, in case carboxyl ester functionalities are present in the substrate. This can be avoided by transforming them into sterically hindered esters, e.g., *tert*-butyl carboxylates or 2,2-dimethylpropionates of alcohols, since the latter are usually not accepted by esterases. Due to the fact that the reaction is performed in a simple phosphate buffer solution using resting cells, further side-effects, such as redox reactions or undesired metabolism of the substrate, are not to be expected.

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