J. Simmonds · G. K. Robinson

Formation of benzaldehyde by Pseudomonas putida ATCC 12633

Received: 16 March 1998 / Received revision: 20 May 1998 / Accepted: 21 May 1998

Abstract Aromatic and heterocyclic aldehydes may be produced by the mandelate pathway of Pseudomonas putida ATCC 12633 via the biotransformation of benzoyl formate and substrate analogues. Under optimised biotransformation conditions (37 °C, pH 5.4) and with benzoyl formate as a substrate, benzaldehyde may be accumulated with yields above 85%. Benzaldehyde is toxic to P. putida ATCC 12633; levels above 0.5 g/l (5 mM) reduce the biotransformation activity. Total activity loss occurs at an aldehyde concentration of 2.1 g/l (20 mM). To overcome this limitation, the rapid removal of the aldehyde is desirable via in situ product removal. The biotransformation of benzoyl formate (working volume 1 l) without in situ product removal accumulates 2.1 g/l benzaldehyde. Benzaldehyde removal by gas stripping produces a total of 3.5 g/l before inhibition. However, the most efficient method is solid-phase adsorption using activated charcoal as the sorbant, this allows the production of over 4.1 g/l benzaldehyde. Addition of bisulphite as a complexing agent causes inhibition of the biotransformation and bisulphite is therefore is not suitable for in situ product removal.

Introduction Aromatic aldehydes are commercially significant compounds with applications in the fragrance, flavour and pharmaceutical industries. Biotransformations to produce these compounds are of interest as the products may be described as natural and exist in a relatively pure form, because of the high specificity of enzyme reactions. The production of aromatic aldehydes is limited by the toxicity of the products, therefore the rapid removal or sequestration of the aldehyde by in situ product removal (Freeman et al. 1993) is desirable. Such techniques previously used for aldehyde removal include gas stripping (Wecker and Zall 1987), pervaporation (Lamer et al. 1996), solid-phase adsorption (Berger 1995), aqueous-organic two-phase systems (Duff and Murr 1989) and condensation reactions (Shacher-Nishri and Freeman 1993). In situ product removal reduces the exposure of the bacteria to the aldehyde product and therefore decreases the toxic effect to the organism.

This paper discusses benzaldehyde production by Pseudomonas putida ATCC 12633 with a bench-scale bioreactor for the production of benzaldehyde and indicates the problems encountered when this method is used, including toxicity of the product and methods of product removal.

Materials and methods

Growth and maintenance of bacteria

Pseudomonas putida ATCC 12633 (NCIMB 9494), also designated P. fluorescens A.3.12 (Stanier 1947), was maintained on nutrient agar plates and subcultured bi-weekly. Pseudomonas minimal medium (PMM), as described by Hegeman (1966), was used for the liquid culture of the bacteria. Sodium succinate or glucose (5 mM) was used as a carbon and energy source for uninduced bacteria (no active mandelate pathway). For bacteria with an active pathway, racemic mandelic acid (5 mM) was used as the growth substrate. Appropriately induced bacteria (overnight seed culture grown in 50 ml PMM + 0.5% yeast extract + 5 mM carbon source) were grown for biotransformation in 2-l flasks containing PMM (500 ml) and mandelic acid (5 mM) and incubated at 30 °C, 200 rpm, to mid-exponential phase (3 h).

Whole-cell biotransformation protocol

Washed bacteria were resuspended to 0.5 g l⁻¹ in McIlvaine (citrate/phosphate) buffer (0.1 M, pH 5.4) and equilibrated to 37 °C before the addition of benzoyl formate, or an alternative substrate. Cells were reciprocally shaken (100 rpm) and samples were taken
over 3 h, the bacteria were removed by centrifugation (10 000 rpm, 2 min) and the resulting supernatant was analysed by HPLC.

Benzaldehyde was removed from the biotransformations at this scale (250 ml, working volume 150 ml) by gas stripping with an aeration rate of 2.6 vvm. It was reclaimed by passage through two hexane solvent traps (100 ml). Polytetrafluoroethylene tubing was used throughout, as benzaldehyde was shown to be adsorbed by rubber and silicone.

Large-scale biotransformations

*P. putida* ATCC 12633 was grown in an Applikon AD1-1012 1.5-l aerated fermentation vessel (working volume 1 l). The vessel was heated by an infra-red lamp, connected to a Systag TC1-88L temperature controller, and pH was monitored by a Mostec pH regulator. PMM was autoclaved within the vessel with 0.5 ml polypropylene glycol as antifoam; mandelic acid (30 mM) was added separately. An additional 20 ml minerals supplement was included with 0.5 ml polypropylene glycol as antifoam; mandelic acid (30 mM) was sterilized separately. An additional 20 ml minerals supplement (Hegeman 1966) was also included. The seed culture was grown for 15 h (30 °C, 200 rpm) with yeast extract (0.5%) and mandelic acid (30 mM) before inoculation with an overnight seed culture. *P. putida* ATCC 12633 was grown under these conditions for 15 h. Biotransformations (1 l) were performed in the growth medium. The accumulation of benzaldehyde in these large-scale biotransformations was initiated by adjustment of conditions to pH 5.4, 37 °C, 0.2 vvm aeration and 200 rpm agitation. The organism was allowed to equilibrate to this environment for 30 min before the addition of substrate.

A number of different methods for benzaldehyde removal were tested.

**Gas stripping**

Enhanced aeration at the 1 l scale was generated by aeration of the vessel at 0.8 vvm with agitation at 750 rpm.

**Bisulphite addition**

Sodium pyrosulphite was added directly to the reactor at intervals after the formation of 5 mM benzaldehyde (below toxic levels). A benzaldehyde-bisulphite complex was formed immediately, therefore no free bisulphite remained in the reactor. The reaction was equilibrated to 30 °C, pH 6.8, agitaiton 500 rpm, aeration 0.75 vvm, before inoculation with an overnight seed culture. *P. putida* ATCC 12633 was grown under these conditions for 15 h. Biotransformations (1 l) were performed in the growth medium. The accumulation of benzaldehyde in these large-scale biotransformations was initiated by adjustment of conditions to pH 5.4, 37 °C, 0.2 vvm aeration and 200 rpm agitation. The organism was allowed to equilibrate to this environment for 30 min before the addition of substrate.

**Solid-phase adsorption**

Granular activated charcoal, mesh 4-8 mm (Fluka) was added directly to the reactor (25 g l⁻¹), prior to the addition of the biotransformation substrate. Sufficient stirring of the vessel was achieved by 0.2 vvm aeration, therefore agitation was discontinued to prevent attrition of the charcoal pieces. The benzaldehyde was extracted from the adsorbant with ethyl acetate.

**HPLC analysis**

A Shimadzu LC-6A HPLC with a UV spectrophotometric detector, 10-μl loop and a Shimadzu CRGA Chromatopac integrator was used for all analyses in combination with a 4.6 × 250-mm Spherisorb ODS2 column with 5 μm packing (Jones Chromatography, Mid Glamorgan). The mobile phase was 47% acetonitrile, 0.2% orthophosphoric acid in water, with a flow rate of 1 ml/min. The detection wavelength was 235 nm. All samples were compared to authentic standards to calculate concentrations.

Relative toxicity of benzaldehyde

A method was developed to compare the toxicity of a range of aldehydes. *P. putida* ATCC 12633 was grown on a number of concentrations of different aromatic aldehydes in the presence of yeast extract (0.5%). The critical concentration was defined as the aldehyde concentration at which the cell density at the stationary phase was half that achieved when the organism was grown on yeast extract alone.

**Measurement of short-term toxicity**

1. The effect of benzaldehyde on the benzaldehyde dehydrogenase isoenzymes was measured at pH 7.0 as these enzymes were not active at pH 5.4. Bacteria were exposed to different benzaldehyde concentrations for 10 min in NaCl (0.9%) at pH 7.0 and 37 °C. The benzaldehyde dehydrogenase activity was measured by calculating the rate of benzoic acid production, using a Mettler DL21 titrator.

2. The effect of benzaldehyde on the benzoylformate decarboxylase enzyme was then measured. Conditions in the bioreactor were mimicked by incubating a suspension of bacteria with different concentrations of benzaldehyde at pH 5.4 and 37 °C. Benzoyl formate was added after 5 min and decarboxylase activity was measured by the rate of substrate utilisation.

**Results**

**Toxicity of aldehydes**

The ability of *P. putida* ATCC 12633 to grow on benzaldehyde was used as an indication of long-term toxicity. Growth was in PMM at pH 7.0, as at pH 5.4 dehydrogenase activity was reduced and this would prevent utilization of the carbon source. The organism was unable to grow with benzaldehyde as the sole carbon and energy source at concentrations above 4 mM. This toxic effect was shown to be related to inhibition of the pathway by which benzaldehyde was metabolised by growth on yeast extract (0.5%) in the presence of benzaldehyde. Toxic effects, i.e. a slower growth rate and a reduction in final cell density, were observed above 5 mM; there was no growth on yeast extract (0.5%) in the presence of 8 mM benzaldehyde.

Under biotransformation conditions, low levels of benzaldehyde (below 5 mM) caused an increase in activity of the benzaldehyde dehydrogenase enzymes as previously indicated (Simmonds and Robinson 1997). Exposure to concentrations between 10 mM and 30 mM for 10 min had little effect on the biotransformation ability of *P. putida* ATCC 12633 (Fig. 1a). However, at concentrations of benzaldehyde above >35 mM there was no further conversion after 10 min exposure.

The toxicity of benzaldehyde relative to that of other aldehydes was measured. A range of aromatic and heterocyclic aldehydes were tested (Table 1) and indicated that the toxicity of benzaldehyde was within the range of other aromatic aldehydes.

Benzoylformate decarboxylase activity was increased by 50% in *P. putida* ATCC 12633 exposed to 20 mM benzaldehyde, compared to the control, which had no