Biocatalytic amide reduction using *Clostridium sporogenes*

Olutosin Dipeolu¹, John Gardiner² & Gill Stephens¹,*

¹School of Chemical Engineering and Analytical Science, University of Manchester, P.O. Box 88, M60 1QD Manchester, UK
²School of Chemistry, University of Manchester, P.O. Box 88, M60 1QD Manchester, UK
*Author for correspondence (Fax: + 44-161-306-4399; E-mail: gill.stephens@manchester.ac.uk)

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**Abstract**

Washed cells of *Clostridium sporogenes* reduced benzamide (up to 20 mM) to benzylamine in yields up to 73% using H₂ as electron donor with less than 10 g biocatalyst/l over 24 h. Product formation exhibited complex kinetics, with a lag before benzylamine production began. Very little substrate was hydrolysed since the maximum yield of benzoic acid was only 9% of the substrate added. Boiled cells were inactivated thus confirming that amide reduction was enzyme-catalysed.

**Introduction**

Biocatalytic amide reduction could be extremely useful for preparation of chiral and achiral primary and secondary amines. Amidation can be achieved readily and provides a route to homologous chiral amines and amino acids, by introduction of an amide extended away from a chiral centre. However, subsequent reduction of the amide to amine requires the use of strong reducing agents such as LiAlH₄ or borane, and is thus intolerant of many other functionalities or multi-step routes around amide reduction. Therefore, development of a biocatalytic amide reduction is of high priority, to deliver the required selectivity under mild reaction conditions.

To our knowledge, anaerobic respiration is the only metabolic context in which amide reduction might be the favoured metabolic route. Anaerobic respiration occurs in various facultative and strictly anaerobic bacteria, and involves the reduction of inorganic or organic electron acceptors (e.g. NO₃⁻, SO₄²⁻, fumarate, DMSO) coupled to ATP synthesis, with no further metabolism of the reduced product (Ingledew & Poole 1984). The reductases acting on organic electron acceptors frequently have broad substrate ranges and may act on functionality unrelated to the natural substrate (e.g. Dolfing 1990; Valentine-Serano *et al.* 1991; Eck & Simon 1994;
This offers excellent prospects for cometabolism of amides during anaerobic respiration.

We decided to use *Clostridium sporogenes* in initial tests for amide reduction as it uses amino acids as respiratory electron acceptors via the Stickland reaction (Buehler *et al.* 1980; Giesel *et al.* 1981; Bader *et al.* 1982; Pitsch & Simon 1982; Bader & Simon 1983; Giesel & Simon 1983), and reduces aromatic and aliphatic nitro groups (Angermaier & Simon 1983a,b). Therefore, the substrate range for N-containing organics is very broad, making *C. sporogenes* an attractive choice for initial studies.

For this preliminary study, benzamide was chosen as substrate, to direct metabolism towards reduction rather than hydrolysis. Hydrolysis would yield benzoic acid which is very rarely used as an energy source by anaerobic bacteria (Harwood *et al.* 1999). Therefore, *C. sporogenes* should not derive any benefit from hydrolysing the amide functionality. We report that the choice of biocatalyst and substrate was successful, since we obtained reduction of benzamide to benzylamine in yields of up to 73%.

**Materials and methods**

**Organism and growth**

*Clostridium sporogenes* strain DSM 795 was grown at 30 °C in an anaerobic cabinet. Stock cultures were grown in anaerobic cooked meat medium slants (Oxoid cooked meat medium granules, 5 g/l; Difco-Bacto Agar, 50 g/l; KH₂PO₄, 5 g/l; L-cysteine·HCl, 0.5 g/l; resazurin, 10 mg) and sub-cultured every 2 weeks. Experimental cultures were grown in medium C (Giesel & Simon 1983) which was modified as follows. The basal medium solution (900 ml) contained proteose peptone (Difco; 20 g), yeast extract (Oxoid; 5 g) and resazurin (1 ml, 10 g/l) and was boiled, sparged with N₂ whilst cooling, autoclaved, cooled and transferred to the anaerobic cabinet. The following anaerobic, autoclaved solutions were then added: salt solution (20 ml/l containing K₂HPO₄, 131 g/l; KH₂PO₄, 34 g/l; NaSeO₃·H₂O; 0.021 g/l); sodium thiglycollate (10 ml/l, 30 g/l); trace element solution (10 ml/l; MgCl₂·6H₂O, 3.3 g/l; CaCl₂·2H₂O, 4 g/l; (NH₄)₂MnO₄·4H₂O, 1 g/l; FeSO₄·7H₂O, 2.9 g/l). Anaerobic, filter-sterilised solutions of vitamins (10 ml/l; p-aminobenzoic acid, 0.08 g/l; D-biotin, 0.004 g/l; riboflavin, 0.02 g/l) and phenylalanine (50 ml/l; 242 mM) were also added. Phenylalanine was dissolved in 1 M NaOH and then diluted with an equal volume of 1 M HCl to adjust the pH to 7.0. All medium components were made anaerobic by sparging with N₂ for 20 min before sterilization. Pre-cultures (2×10 ml) were inoculated with a single colony from a stock culture, grown for 18 h and then used to inoculate 250 ml experimental cultures.

**Biotransformations**

Cells were harvested as described by Li *et al.* (2004) except that they were resuspended in buffer to 20% of the culture volume. Reaction mixtures contained cell suspension (4 ml) and benzamide solution (1 ml). Benzamide was dissolved in 0.5% (v/v) aqueous ethanol, deoxygenated by sparging with N₂ for 5 min and added to the concentrations described in the text. The reactions were started by adding H₂ as described by Li *et al.* (2004).

**Analytical methods**

Cell growth was determined as described by Li *et al.* (2004). An OD of 1 = 0.39 g dry wt/l. Samples from biotransformations were prepared for analysis by centrifuging at 3000 × g for 5 min. Benzylamine and benzamide were analysed by GC, after basification of the supernatants with 2–3 drops of 0.1 M NaOH. Samples (10 μl) were analysed on a VF-MS 1 (Factor Four) capillary column (15 m×0.25 m×0.25 μm) with He as the carrier gas (1 ml/min). The column was held at 100 °C for 2 min, and the temperature was increased at 20 °C/min to 250 °C, which was maintained for 2 min. The FID was maintained at 280 °C. Benzoic acid was determined by HPLC by injecting samples (10 μl) onto a Hypersil HS C8 column (25 cm×4.6 mm) with water/acetonitrile (60:40 v/v) as the mobile phase at a flow rate of 1 ml/min. Products were detected at 254 nm. All analytical standards were dissolved in aqueous ethanol (0.5% v/v).