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Research article

Integration of whole-cell reaction and product isolation: Highly hydrophobic solvents promote *in situ* substrate supply and simplify extractive product isolation

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ABSTRACT

Product isolation from aqueous-organic reaction mixtures that contain high concentrations of whole cells constitutes a challenging task in bioprocessing. Stirring of the biphasic reaction media leads to the formation of solvent droplets coated by cells and other surface active components and an emulsion forms. We used an early focus on phase separation to simplify a whole-cell bioreduction. Octanol, heptanol, hexanol, hexane and dipropylether were tested as co-solvents in the *E. coli* catalyzed reduction of *o*-chloroacetophenone. All solvents showed very similar performance in bioreductions and highest yields were obtained with low organic-to-aqueous phase ratios. Reaction mixtures were directly investigated for organic-phase recovery. Phase separation was optimized in small-scale settling experiments and confirmed by the isolation of 20.4 g (*S*)-1-(2-chlorophenyl)ethanol from a 0.5 L batch reduction containing 40 g_{CDW}/L whole-cell catalyst. Solvent consumption during product isolation could be halved by the simple addition of sodium hydroxide prior to product extraction. Basification to pH 13.5 and three extraction steps with a total of 1.2 v/v hexane led to an isolated yield of 87% (97% reduction yield). A general emulsion destabilizing effect under harsh conditions, as extreme pH values and presence of toxic reactants, was observed.

1. Introduction

Application of a water immiscible, organic solvent as substrate reservoir and product sink is a common strategy to intensify processes. Fine dispersion of the reaction mixture promotes efficient substrate transfer to supply the biocatalyst in the aqueous phase. Accumulation of the biocatalyst (free enzymes or whole cells) at the organic-aqueous interface enhances mass transfer but stabilizes organic solvent drops. Product isolation subsequent to the biotransformation is hampered by emulsified reaction mixtures (*e.g.* Collins et al., 2015; Furtado et al., 2015; Heeres et al., 2015; Moreira et al., 2016). Information on separation behavior of reaction mixtures at an early stage in bioprocess development can preclude solvents that lead to complex product isolation. Utilization of the same solvent in the bioreaction as auxiliary phase and in the subsequent product isolation as extractant is a common strategy to simplify processes. However, dual use increases requirements imposed on the solvent. The prerequisite for solvent application in biotransformation and product isolation is a high extraction capability for the product (Bräutigam et al., 2009). The solvent's biocompatibility is an additional requirement for its suitability as a co-solvent in the bioreaction. Hydrophobic solvents show in general higher biocatalyst compatibility (Weber and de Bont, 1996). Boiling points of co-solvents (and products) are determining product isolation strategies. Low-boiling solvents (<100 °C) facilitate easy solvent removal by solvent evaporation, high-boiling solvents (>150 °C) or ionic liquids suggest distillation, pervaporation or back-extraction of the product (Dennewald et al., 2011; Park and Kazlauskas, 2003; Wriessnegger et al., 2014). Here, the most often exploited whole-cell biotransformation, the reduction of a ketone to the corresponding optically pure alcohol (Pollard and Woodley, 2007), was optimized by selection of

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one solvent for in situ substrate supply and subsequent product extraction. The bioreduction of *o*-chloroacetophenone to the corresponding *S*-alcohol with recombinant *E. coli* provides a model case for the conversion of a hydrophobic substrate with fast catalyst deactivation (Kratzer et al., 2011). Several water immiscible solvents were tested for extractions of *o*-chloroacetophenone and 1-(2-chlorophenyl)ethanol and as co-solvents in bioreductions. Obtained reaction mixtures were directly investigated for organic-phase recovery by filtration, gravity settling and centrifugation. Phase separation was optimized with regard to variation in pH and temperature, addition of salts, chaotrops or surfactants. Our results are generally applicable to whole-cell biotransformations that require a co-solvent and a high concentration of the whole-cell catalyst.

2. Materials and methods

2.1. Chemicals and strain

Ampicillin, D-xylose, guanidine hydrochloride (guanidine HCl), hexane (≥ 99%), NAD^+ (≥ 97.5% pure), Triton X-100, cetyltrimethylammonium chloride (CTABr) were purchased at Roth (Karlsruhe, Germany). Chloramphenicol, dipropylether (≥ 99%), hexanol (98%), kanamycin, *o*-chloroacetophenone, polymyxin B sulfate and sodium formate were obtained from Sigma-Aldrich (Vienna, Austria). Celite® 545 (particle size 20–45 μm) was from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS) was purchased at Serva (Heidelberg, Germany). B-Per® Reagent was from Pierce (Rockford, IL, USA) and 1-(2-chlorophenyl)ethanol from Alfa Aesar (Karlsruhe, Germany). All other chemicals were from Sigma–Aldrich/Fluka or Roth, and were of the highest purity available. The strain used was an *E. coli* Rosetta2 co-expressing *Candida tenuis* xylose reductase and *Candida boidinii* formate dehydrogenase. Cultivation was done in shaken flasks as described earlier (Schmölzer et al., 2012). Bioreactor cultivation was optimized by Eixelsberger et al. (2013) and is summarized in the Supplementary data.

2.2. Partition of substrate and product between buffer and co-solvents

The organic phases used were hexane, hexanol, heptanol, octanol and dipropylether, the aqueous phase was 100 mM potassium phosphate buffer, pH 6.2. Mixtures contained 50% v/v organic solvent in a total volume of 10 mL. 5 mM of *o*-chloroacetophenone was dissolved in the organic phase and 5 mM 1-(2-chlorophenyl)ethanol in the aqueous phase. The phases were combined, filled into 10 mL glass tubes (screw capped from Pyrex) and mixed at an end-over-end rotator (SB3 from Stuart) at 30 rpm and room temperature. Ketone and alcohol concentrations were measured in both phases after 2 h of stirring to ensure equilibrium conditions. HPLC analysis was performed as described previously (Schmölzer et al., 2012).

Partition coefficients ($P = \frac{c_{org}}{c_{aqu}}$) were calculated as the ratio of analyte concentration in the organic (c_{org}) to aqueous phase (c_{aqu}).

2.3. Biotransformations

2.3.1. Eppendorf tube-scale

Reductions were performed in a total volume of 1 mL incubated in 1.5 mL Eppendorf tubes (Vienna, Austria). Experiments were carried out as previously reported (Schmölzer et al., 2012). Phase compositions of bioreductions in 1 mL-scale are listed in the Supplementary data. Samples were diluted with 9 mL ethanol and remaining biomass was separated by centrifugation prior to HPLC analyses.

2.3.2. Bioreactor-scale

Total reaction volumes were 0.5 L. Reductions were performed in a Labfors III 3.6-L bench-top bioreactor from Infors AG (supplied from Bartelt, Graz, Austria) with a working volume of 1 L, a vessel diameter of 11.25 cm, equipped with a twin 6-blade disc turbine with a stirrer diameter of 4.5 cm. pH kept constant at 6.2 by addition of 1 M H_3PO_4 and 2 M KOH, temperature was kept at 25 °C (double jacket vessel) for 24 h (Eixelsberger et al., 2013). Phase compositions of bioreductions in 0.5 L-scale are listed in the Supplementary data. Samples (1 mL) were diluted with 9 mL ethanol and remaining biomass was separated by centrifugation prior to HPLC analyses. For determination of the partition coefficients, samples were taken, centrifuged for phase separation and the organic and the aqueous phases analyzed separately.

Product isolation at bioreactor-scale: The reaction mixture contained ~300 mM (*S*)-1-(2-chlorophenyl)ethanol with 20% v/v hexane as co-solvent. Further 200 mL of hexane were added and the pH was adjusted to 13.5. The mixture was incubated at 25 °C and 500 rpm in the bioreactor for 30 min. Centrifugation was done using a Sorvall RC–5 B centrifuge for 30 min at 9000 rpm (4400 g). The separated organic phase was collected by pipetting. Two further extraction steps were carried out using 200 mL of hexane each time with subsequent stirring and centrifugation. Solvent was removed using a rotary evaporator (Laborota 4000, Heidolph, Schwabach, Germany) at 40 °C and 100 mbar.

2.3.3. Analytics

HPLC analysis was performed as described previously (Schmölzer et al., 2012). Conversions were calculated as:

$$Conversion = \frac{[Product]}{[Product] + [Substrate]}$$

2.4. Filtration

A Schott DURAN Büchner filtering funnel with grade 4 frit (10–16 μm nominal max. pore size, Supplementary data), 50 mL capacity and 3.5 cm disc diameter was used. A vacuum of 10 mbar was applied using a vacuum pump (model MZ 2 CNT, ultimate vacuum 7 mbar, from Vacuubrand, Wertheim, Germany), equipped with a fine vacuum gauge (range 150-10–3 mbar) from ILMVAC (Ilmenau, Germany). For high filter aid loading 6 g Celite (Kieselghur) were suspended in 50 mL of water to precoat the filter. The suspension was subjected to filtration; the filtrate was collected and five times re-filtered. 10 g of Celite were mixed with 30 mL reaction mixture prior to filtration. For low filter aid loading 4 g Celite were used as precoat, and 2 g were suspended in 30 mL reaction mixture prior to filtration.

2.5. Settling and centrifugation

2.5.1. Settling cell

The settling cell consisted of a glass cylinder with two agitator shafts each equipped with 4 pitch 4-blade impellers and temperature control (double jacket vessel with a total volume of 1 L) (Henschke et al., 2002). A mechanical overhead stirrer (Heidolph RZR2021, Schwabach, Germany) with hand wheel for speed adjustment was used. Coaxial mixing with counter rotating mixing elements prevented spinning of the liquid after mixing was stopped. A photo of the cell and the cleaning procedure are given in the Supplementary data. The solvent mixture was filled into the cell and mixed for 5 min to saturate both phases. For the settling experiments the phases were mixed (800 rpm, 30 s, 30 °C) and