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The effect of cultivation media and washing whole-cell biocatalysts on monoamine oxidase catalyzed oxidative desymmetrization of 3-azabicyclo[3,3,0]octane

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ABSTRACT

It is well known that washing whole-cells containing enzyme activities after fermentation, but prior to biocatalysis can improve their activity in the subsequent reaction. In this paper, we quantify the impact of both the fermentation media and cell washing on the performance of whole-cell biocatalysis. The results are illustrated using a recombinant monoamine oxidase (expressed in *Escherichia coli,* used in resting state) for the oxidative desymmetrization of 3-azabicyclo[3,3,0]octane. It was shown that the need for washing biocatalyst prior to use in a reaction is dependent upon growth medium. Unlike cells grown in LB medium, washing of the cells was essential for cells grown on TB medium. With TB media, washing the cells improved the final conversion by approximately a factor of two. Additionally, over 50-fold improvement was achieved in initial activity. A potential reason for this improvement in activity was identified to be the increase in transfer of substrates across the cell membrane as a result of cell washing.

1. Introduction

Today biocatalysis is well-established as a complement to synthetic chemistry in the fine chemical and pharmaceutical industry. Among the different biocatalyst formulations, whole-cell catalysis is often adopted in cases where co-factor regeneration is required or alternatively when a single use biocatalyst is desired. Additionally, the enzymes in whole-cells tend to be more stable because they are surrounded by their natural environment [1–4]. Several whole-cell oxidation reactions have been implemented at large scale, including oxidases, oxygenases and monooxygenases [5–7].

Whole-cell biocatalysts are typically produced by fermentation in complex media such as Luria–Bertani (LB) at lab scale. In order to ensure sufficient biocatalyst is produced, the growth media needs to include a carbon source (such as TB media). However, differences in growth media could cause changes in activity of the biocatalyst and in the physiochemical nature of the biocatalyst, consequently resulting in altered biocatalyst activity. Therefore, the effect of growth media on the activity per gram of cell was investigated.

There are two common modes of operating a whole-cell based biocatalytic reaction at scale. The first type combines fermentation and biocatalysis into a single step. In the second mode, fermentation is separated from biocatalysis and is generally referred to as a resting-cell process [8].

For resting-cell processes, various methods of handling cells downstream of fermentation have been adopted and reported in the scientific literature. Some investigations report the direct use of the cells after harvesting in the biocatalytic process [9–11], while in other cases, the cells have been washed in buffer prior to use [12–15]. The reason for washing cells prior to biocatalysis is often not stated. When it is mentioned, it is normally just to say that washing is necessary to improve biocatalytic activity by removing fermentation by-products (which acted as inhibitors) but rarely quantified [16]. In this manuscript we quantify the improvement in the biocatalytic activity as a result of washing the cells, particularly in relation to initial activity and reaction yield for a target whole-cell biocatalysis. Furthermore, the reason for improvement of activity by washing for this system has been identified.

The target reaction system used for this purpose involves the oxidative desymmetrization of 3-azabicyclo[3,3,0]octane using whole-cell *Escherichia coli* expressing monoamine oxidase (MAO) [E.C. 1.4.3.4] (Scheme 1). The enzyme is soluble and produced

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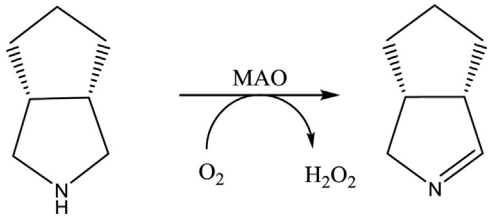

Scheme 1. Reaction scheme representing the oxidative desymmetrization of 3-azabicyclo[3,3,0]octane catalyzed by monoamine oxidase expressed in *E. coli*.

Table 1
Initial specific activities of biocatalysts in different growth media.

Growth media	Initial specific activity ($mg/g_{CDW}/h$)
LB	177.6
TB	3
LB (Washed)	120
TB (Washed)	285
TB crude extract	59

intracellularly in *E. coli* [17], and the reaction has been recently reported [18,19].

2. Materials and methods

All chemicals unless specified otherwise were used as purchased from Sigma Aldrich (Steinhiem, Germany). Solvents were GC grade and salts, analytical grade.

2.1. Fermentation media

2.1.1. Luria–Bertani (LB)-Amp broth

LB broth was made with 10 g/L tryptone (Nordic Biolabs AB, Täby, Sweden), 5 g/L yeast extract (Nordic Biolabs AB, Täby, Sweden) and 10 g/L sodium chloride. The broth was autoclaved and filter sterilised ampicillin (at a concentration of 100 μg ampicillin/mL media) was added prior to use.

2.1.2. Luria–Bertani (LB)-Amp plates

To LB broth, 2% (w/v) agar was added prior to autoclave. When the media had cooled down, filter-sterilised ampicillin (at a concentration of 100 μg ampicillin/mL media) was added and plated.

2.1.3. Terrific broth (TB)

TB media was prepared by mixing 12 g/L tryptone (Nordic biolabs AB, Täby, Sweden), 24 g/L yeast extract (Nordic biolabs AB, Täby, Sweden), 4 mL/L glycerol, 12.54 g/L potassium monohydrogen phosphate and 2.31 g/potassium di-hydrogen phosphate. TB media was autoclaved prior to use.

2.2. Biocatalyst

The plasmid (pET16b) containing MAO (variant D5) was kindly donated by Professor Nicholas J Turner (University of Manchester, UK) and was transformed into competent *E. coli* BL21 (DE3) cells by electroporation. To 50 μL of electro-competent cells, 0.5 μL of the plasmid was added and transferred into an electroporation cuvette (BIORAD, Copenhagen, Denmark) and exposed to a short pulse of high voltage. Following electroporation, 950 μL of LB media was added to the cuvette and incubated for 1 h at 37 °C. 50 μL of the cell suspension was plated onto a LB plate containing ampicillin. The cells produced thus were stored as glycerol stock until further use.

2.3. Biocatalyst production

2.3.1. Pre-culture

Cells were streaked from a glycerol stock onto LB plates containing ampicillin. The plate was incubated at 37 °C overnight. 5 mL of LB broth containing 100 μg/mL of ampicillin was inoculated with a colony from the LB-Amp plates. The culture tube was incubated at 37 °C in a shaker at 250 rpm for about 4 h (OD_{600} 0.6–1.0).

2.3.2. Fermentation

All fermentations were carried out in 500 mL un-baffled shake flasks and repeated in the same way for both LB and TB media. To 100 mL of media containing ampicillin, 1 mL of pre-culture was added (1% v/v inoculum). The flasks were incubated at 30 °C, 150 rpm. Cells were harvested after 24 h of growth by centrifugation (4000 rpm, 20 min, 4 °C).

2.4. Biocatalyst washing

Biocatalyst obtained from TB and LB media were re-suspended in 10 mL of 100 mM potassium phosphate buffer, pH 7.6 (25 °C). Subsequently, the cells were centrifuged again (4000 rpm, 20 min, 4 °C) and the supernatant discarded. The pellet was weighed and re-suspended in phosphate buffer to get a concentration of ~500 g_{CWW}/L (g cell wet weight per liter). Dry cell weight was measured at this point. Then, the concentrate was diluted for the biocatalysis to attain a final concentration of 5 g_{CDW}/L.

2.5. Dry cell weight measurements

500 μL of the cell suspension (obtained after washing and resuspension) was diluted ten-fold. 1 mL of the suspension was then vacuum-filtered with a 0.22 μm filter (pre-dried and weighed). The filter containing the cell pellet was dried in a microwave at 75 W for 20 min followed by drying in a dessicator overnight. The dried filter paper with cells was weighed and dry cell weight calculated. Triplicate measurements were made.

2.6. Biocatalysis

Biocatalytic reactions with whole-cells obtained from fermentations were carried out in baffled shake flasks with 20 mL working volume (substrate and biocatalyst suspended in 100 mM potassium phosphate buffer, pH 7.6) incubated at 37 °C and 150 rpm. The biocatalyst concentration was maintained at 5 g_{CDW}/L. Experiments with washed cells were conducted under identical conditions with the exception that washed cells were used for biocatalysis. The substrate, 3-azabicyclo[3,3,0]octane HCl was procured from AK Scientific (Union city, CA, USA). Substrate concentration in biocatalysis was ~2.96 g/L. The sampling frequency and duration varied for different experiments. Samples for gas chromatography were prepared and analysed as described in Ramesh and Woodley [18]. For biocatalysis with cells grown in LB and TB media, triplicates were made and used to obtain standard error. Specific activities of the biocatalysts based on initial reaction rates (refered to as initial specific activity in the later parts of the paper) have been calcuated by taking the slope of the linear region in a plot where conversion is represented as a function of cell concentration and time. To obtain the initial specific activities in mg/g/h the value of the slope is multiplied by the initial substrate concentration.