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Improving the production of a thermostable amidase through optimising IPTG induction in a highly dense culture of recombinant *Escherichia coli*

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ABSTRACT

The production of a novel thermostable amidase (EC 3.5.1.4) from *Geobacillus pallidus* RAPc8 using recombinant *Escherichia coli* BL21 (DE3) was investigated. Volumetric and specific enzyme activities were investigated in relation to inducer concentration in a batch process using a defined medium with glucose as the carbon source. While IPTG is routinely used to induce expression of genes under the control of *lac* promoter, the impact of high biomass concentration on IPTG induction has not been reported rigorously. In this study, biomass production was unaffected by IPTG concentration across the range 0–1000 μM. Induction of recombinant protein expression by 400 μM IPTG at late lag phase of growth (3rd hour) inhibited cell growth while induction at early exponential phase of growth (5th hour) gave a 3 fold increase in volumetric amidase activity compared to induction at mid exponential phase (8th hour). Protein production increased by a factor of two with IPTG addition, independent of IPTG concentration in the range of 40–1000 μM. Amidase activity, measured on a volumetric basis and relative to protein and biomass concentrations, increased with increasing IPTG concentration up to 400 μM. While inducer concentrations are typically reported on a volumetric basis, their mode of action is consistent with a biomass dependence. Analysis of the data across a range of biomass concentration confirmed that induction was a function of inducer concentration per unit biomass. The amidase enzyme was predominantly soluble and cytoplasmic with less than 3% retained within the cell debris.

1. Introduction

Amidases (EC 3.5.1.4) catalyse the hydrolysis of carboxylic acid amides to free carboxylic acids and ammonia. Amidase activity has been described in many members of the bacterial kingdom including *Bacillus* [1], *Microbacterium* [2], *Arthrobacter* [3], *Rhodococcus* [4,5], *Geobacillus* [6], and *Pseudomonas* [7]. Amidases exhibit diverse substrate specificities and biological functions. They are generally involved in nitrogen turnover in prokaryotes [8] and may be stereoselective for aliphatic or aromatic substrates, while others hydrolyse amides of α- or β-amino acids [9].

Amidases are one of the most extensively used amide-hydrolysing enzymes in industry due to their capacity for the large scale production of optically pure organic acids such as *p*-aminobenzoic acid, nicotinic acid and acrylic acid [8,10]. They have also been applied in treatment of industrial effluents containing toxic amide compounds [11] and in the production of therapeutic agents such as antibiotics [12].

A thermostable amidase was previously identified in the thermophile *G. pallidus* [13]. The open reading frame (ORF) encoding a 348-amino acid amidase (38.6 kDa), located immediately downstream of ORFs encoding nitrile hydratase α and β subunits, was cloned and over-expressed in *Escherichia coli BL21* (*DE3*) [14]. Analysis of the biochemical properties of this amidase [6] showed temperature and pH activity optima at 50 °C and pH 7, respectively. The amidase exhibited moderate thermal stability at 50–60 °C with a half life in excess of 5 h at both temperatures. These properties were further enhanced on immobilisation on Eupergit C with the immobilised enzyme preparation showing optimal activity in a broadened pH range, with stability enhanced by 67% at 80 °C. These properties, coupled with its D-selectivity, suggest a possible role in the synthesis of pure carboxylic acids.

Bioprocess-based approaches for maximal volumetric productivities of recombinant proteins include growing *E. coli* to high concentration using a high cell density cultivation method (HCDC) and manipulation of inducer concentration and time of induction for effective transcription and translation of the recombinant gene. Moreover, HCDC has the advantage of increased cost effectiveness, improved space-time utilisation, enhanced downstream processing and minimised waste water generation [15]. In most recombinant systems, a range of inducer concentrations are used without a motivation for such. In most cases, the optimal inducer

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concentration to fully induce the *lac* promoter is chosen to balance the decreasing biomass productivity after induction and increasing protein expression by the cells [16]. A wide range of IPTG concentrations, ranging from 0 to 10 mM, has been reported by different authors [2,17–23], while the most frequently used concentration for *lac*-derived promoters is around 1 mM [16]. Nevertheless, most induction studies carried out are volume-based, with no thorough investigation of the impact of high cell density culture.

In the present study, the use of high cell density cultures of *E. coli* in the production of the recombinant *G. pallidus* amidase has been investigated. We demonstrate that inducer concentration (both on a volumetric and biomass basis), time of induction and biomass concentration are all important factors in optimising enzyme production.

2. Materials and methods

2.1. Bacterial strain

A recombinant strain of *E. coli* BL21 (DE3) expressing the *G. pallidus* amidase gene from plasmid PNH 223, described by Cameron et al. [14], was obtained from the Institute of Microbial Biotechnology and Metagenomics, University of Western Cape, South Africa. Ampicillin was used to maintain plasmid selectivity.

2.2. Precultures and medium preparation

A glucose based defined medium containing 25 g l^{-1} described by Korz et al. [24] was used as the growth medium. Luria broth (LB) medium (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1} and NaCl 10 g l^{-1}) was used to preculture the inocula for the defined medium. The pre-inoculum (30 ml LB in a 250 ml shake flask), inoculated from an agar plate culture of *E. coli* BL21 (DE3), was incubated for 12 h at 30 °C with agitation at 180 rpm. The culture was transferred into 270 ml volumes of defined culture medium containing 15 g l^{-1} glucose in a 2000 ml shake flask, and incubated for 12 h at 30 °C and 180 rpm. The pH of the medium was adjusted to pH 6.7 with aqueous ammonia (25%, w/w) prior to inoculation.

2.3. Batch operation of the bioreactor and control

E. coli BL21 (DE3) was cultured in a 7-litre Bioflo 110 bioreactor (New Brunswick, USA) or a series of 300 ml Sixfors bioreactors (Infors bioreactor, version 3.01, Switzerland), as specified. Online pH (6.7), dissolved oxygen and temperature (30 °C) were monitored and automatically controlled. A 10% (v/v) inoculum was used. The dissolved oxygen concentration was maintained at a minimum of 5% of air saturation by either increasing the agitation speed or air flow rate or both.

2.4. IPTG induction for maximum amidase expression

For determination of the optimal time of amidase induction, amidase production was induced at the third hour (end of lag phase), fifth hour (early exponential phase) and eighth hour (mid exponential phase) of cell cultivation using an IPTG concentration of 400 μM. The Sixfors bioreactor system was used for the IPTG induction studies to allow the use of identical inoculum across the 6 independent bioreactors (300 ml). The IPTG concentration was varied from 0 to 1000 μM on a volumetric basis (0–200 μmol g^{-1} biomass on a biomass basis), based on the ratio of IPTG to final cell concentration using data reported previously [20–22,25,26]. Amidase expression was induced at the 5th hour of cell culture.

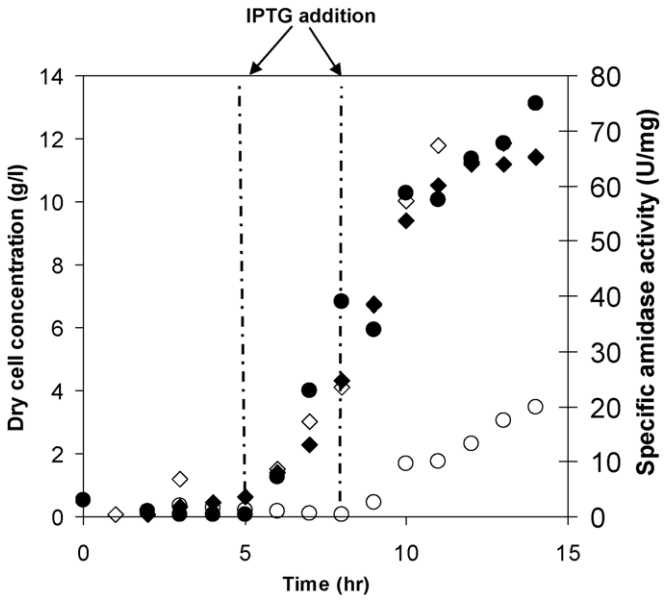

Fig. 1. Effect of time of inducer addition on formation of biomass and specific amidase activity the early (5th hour) and mid exponential phase (8th hour) using 400 μM IPTG in the New Brunswick Bioflo reactor. Early biomass concentration ♦, Mid biomass concentration ◊, Early specific amidase activity ●, Mid specific amidase activity ○.

2.5. Analytical methods

Biomass was harvested from suspension by centrifugation at 13,000 × *g* for 10 min. Cells were washed and re-suspended in potassium phosphate buffer solution (50 mM, pH 7), then disrupted by sonication on ice (Virsonic ultrasonic 100 cell disrupter, 50 W, 22.5 kHz, sonication time of 2 min divided into 15 s treatment followed by 30 s rest time). The crude extract was centrifuged to separate supernatant and debris, and both fractions subjected to protein and amidase assays. Biomass concentration was measured gravimetrically as cell dry weight and by absorbance at A_{660}. Residual glucose concentration was determined by a modified dinitrosalicylic acid colorimetric method [27]. The soluble protein concentration was determined by Bradford Coomassie Brilliant Blue G-250 assay [28]. Amidase activity was determined using a modified phenol-hypochlorite ammonia detection method developed by Weatherburn [29] and described in detail by Makhongela et al. [6]. One unit of activity is defined as the amount of enzyme required to produce one μmol of ammonia from acetamide per minute under standard assay conditions (pH 7.0 and 50 °C).

3. Results and discussion

3.1. Induction time in a batch process

The stage of cell cultivation suitable for optimal expression of recombinant amidase was determined by inducing expression with 400 μM IPTG at the 3rd, 5th and 8th hour, representing the lag, early and mid exponential phases of growth, respectively. While induction at early and mid exponential phase gave similar trends, with dry biomass concentration increasing to a final dry cell weight of about 11 g l^{-1} (Fig. 1), addition of inducer at the lag phase inhibited biomass production by up to 50% (Table 1). In consequence, induction for this system was studied only within the exponential phase of cell growth. Soluble protein concentration increased by a factor of approximately two with IPTG addition in early and mid exponential phase compared to the process without inducer. Conversely, little difference was observed in the protein concentration as a