Bioresource Technology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Optimization of high cell density fermentation process for recombinant nitrilase production in *E. coli*

Sujata Vijay Sohoni [a,b], Dhanaraj Nelapati [a], Sneha Sathe [a], Vaishali Javadekar-Subhedar [c], Raghavendra P. Gaikaiwari [c], Pramod P. Wangikar [a,b,d,*]

[a] Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India
[b] DBT-Pan IIT Center for Bioenergy, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India
[c] Hi Tech Biosciences India Ltd., C-2, 102/103, Saudamini Complex, Right Bhusari Colony, Paud Road, Kothrud, Pune 411038, India
[d] Wadhwani Research Center for Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India

HIGHLIGHTS

- Optimized batch process resulted in 5-folds increase in recombinant nitrilase.
- Fed-batch in shake flasks yielded fourfolds increase than optimized batch process.
- Glycerol–yeast extract feed was developed for improving nitrilase production.
- Scale up of fed-batch process to bioreactor resulted in further eightfolds increase.

ARTICLE INFO

Article history:
Received 29 November 2014
Received in revised form 9 February 2015
Accepted 10 February 2015
Available online xxxx

Keywords:
Nitrilase
High cell density fermentation
E. coli BL21 (DE3)

ABSTRACT

Nitrilases constitute an important class of biocatalysts for chiral synthesis. This work was undertaken with the aim to optimize nitrilase production in a host that is well-studied for protein production. Process parameters were optimized for high cell density fermentation, in batch and fed-batch modes, of *Escherichia coli* BL21 (DE3) expressing *Pseudomonas fluorescens* nitrilase with a T7 promoter based expression system. Effects of different substrates, temperature and isopropyl β-D-1-thiogalactopyranoside (IPTG) induction on nitrilase production were studied. Super optimal broth containing glycerol but without an inducer gave best results in batch mode with 32 °C as the optimal temperature. Use of IPTG led to insoluble protein and lower enzyme activity. Optimized fed-batch strategy resulted in significant improvement in specific activity as well as volumetric productivity of the enzyme. On a volumetric basis, the activity improved 40-fold compared to the unoptimized batch process.

1. Introduction

Nitrilases (EC 3.5.5.1) have potential as commercial biocatalysts for chiral synthesis of various drug intermediates. Nitrilases belong to nitrile hydrolase family and are involved in conversion of a variety of aromatic as well as aliphatic nitriles (R-CN) into carboxylic acids (R-COOH) and ammonia without producing intermediate amides (Pace and Brenner, 2001). Chiral carboxylic acid products of nitrilase reaction e.g., R-mandelic acid or 2-aryl propionic acid are precursors for synthesis of antibiotics and other pharmaceuticals (Brady et al., 2004; MartÍnková and Křen, 2002). Several nitrilase enzymes are reported that show high enantioselectivity toward compounds of commercial interest (Naik et al., 2008).

Enzymes are typically produced in smaller amounts in the native host. Once the enzyme is characterized for desired properties, large scale production is essential. Hence cloning and high level expression in heterologous hosts is generally helpful. *Escherichia coli* is greatly exploited as a host for production of recombinant proteins and enzymes. *E. coli* grows faster, has simple nutrient requirements and can be easily grown to high cell densities (Gopal and Kumar, 2013; Terpe, 2006). *E. coli* BL21 strain that is deficient in lon and ompT proteases is routinely used for larger scale protein production. A derivative of this strain *E. coli* BL21 (DE3) harbors T7 DNA polymerase copy under the control of lac UV5 promoter on the genome and is used for T7 driven protein expression (Studier and Moffatt, 1986; Zerbs et al., 2009). Synthetic, semi-synthetic as well as complex media and various feeding

* Corresponding author at: Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India. Tel.: +91 22 2576 72 32; fax: +91 22 2572 68 95.
E-mail address: wangikar@iitb.ac.in (P.P. Wangikar).

http://dx.doi.org/10.1016/j.biortech.2015.02.038
0960-8524/

strategies based on glucose have been used for production of recombinant proteins (Fass et al., 1991; Bae et al., 1997; Shin et al., 1997). Several reports available on high cell density fermentations (HCDF) for production of recombinant proteins focus on *E. coli* BL21 (DE3) as a host cell. Most of these reports demonstrate use of glucose feeding for protein production (Shin et al., 1997; Son et al., 2010). Grossman et al. (1998) reported that addition of glucose reduces basal level expression of the target gene. Hence glucose based feeding strategies are not ideal. There are a few reports that discuss optimization of culture conditions for *Pseudomonas putida* nitrilase expressed in *E. coli* (Banerjee et al., 2009; Naik et al., 2008; Nigam et al., 2012). However, these do not report enzyme activity on volumetric basis and hence it is difficult to estimate the production cost of biocatalyst. Liu et al. (2011) report nitrilase enzyme activity of ~19 units/ml with glycerol feeding in *E. coli* JM109. This study was initiated with the motivation of developing a feeding strategy based on favorable carbon and nitrogen substrate to achieve enzyme activity of greater than 400 units/ml of fermentation broth.

Codon optimized and previously characterized nitrilase from *Pseudomonas fluorescens* (Layh et al., 1992; Kiziak et al., 2005) was cloned in pET21a plasmid and expressed in *E. coli* BL21 (DE3). Effect of glucose on plasmid stability and thereby nitrilase production was also studied. Further, the effect of growth temperature and induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) was examined. Feeding strategy with combination of glycerol and yeast extract was developed for HCDF and maximizing nitrilase production.

2. Methods

2.1. Chemicals

All the chemicals used in this study were analytical grade unless otherwise stated. All solvents were HPLC grade. All the chemicals, solvents and racemic mixture of mandelonitrile were purchased from Merck (Massachusetts, USA). Antibiotics were purchased from Sigma Aldrich (Missouri, USA). Water (MQ) was purified with a Milli-Q-system (Millipore, Bedford, MA).

2.2. Bacterial strain, media and culture conditions

E. coli BL21 (DE3) is an all-purpose strain for high-level protein expression and easy induction. Synthesis of codon optimized nitrilase gene from *P. fluorescens* (PF) was outsourced to Biomatik Corporation (Ontario, Canada). It was cloned in pET21a plasmid at NdeI and BamHI sites and expressed in *E. coli* BL21 (DE3). The resulting strain *E. coli* BL21 (DE3)_PF nit was used to study recombinant nitrilase production in this study.

LB broth (HiMedia Laboratories) was used in initial studies. Super Optimal broth (SOB) medium (Tryptone 20 g/l, Yeast extract 5 g/l, NaCl 0.5 g/l, KCl 0.186 g/l) was used subsequently in the shake flask and reactor cultivations in this study. $MgCl_2$ (final concentration 10 mM) and carbenicillin (150 μg/ml) were added to all the media as well as feed after autoclaving. Feed used in fed-batch consisted of 50% yeast extract solution or 2.2 M glycerol solution. Feed used in the reactor for continuous feeding consisted of 200 g/l yeast extract and 67 g/l glycerol. Carbon substrates wherever applicable were also added to SOB medium after autoclaving at 1% final concentration for sugars or glycerol 30 mM. All the shake flask cultivations were carried out at 37 °C or 32 °C and shaking at 180 rpm.

2XYT agar (Tryptone 15 g/l, Yeast extract 10 g/l, NaCl 5 g/l, agar 15 g/l) with and without carbenicillin (150 μg/ml) was used for determining plasmid stability.

2.3. Seed conditions

Two seed stages namely, pre-seed and seed preceded the production. In the Pre-seed stage, 1 ml of glycerol stock culture was inoculated in 5 ml SOB broth. Incubation was at 37 °C or 32 °C for 9 h under static conditions. In the seed stage, 0.6 ml of Pre-seed culture was inoculated in 20 ml SOB in 100 ml shake flask. Incubation was at 37 °C or 32 °C, 180 rpm and for 12 h.

2.4. Protein production in shake flasks and bioreactor

3 ml of seed culture was inoculated in 100 ml SOB broth in 500 ml shake flask. Incubation was at 37 °C or 32 °C, 180 rpm and for 12–24 h. The nitrilase production was induced by the addition of IPTG to the final concentration of 1 mM in the production medium after 3 h of inoculation wherever applicable. Samples taken at different time points were analyzed for dry cell weight (DCW), Optical density (OD_{600nm}) and nitrilase activity.

Fed-batch cultivations in bioreactor were performed at 32 °C, pH 7.0, at the agitation rate of 800 rpm and aeration rate of 1 vvm (volume per volume per minute). Sartorius BIOSTAT bioreactors with 2 l working volume were used in this study (Sartorius AG, Gottingen, Germany). One l SOB containing 30 mM glycerol was inoculated with 30 ml of the seed. Solutions of 2 M NaOH and 2 M HCl were used to maintain the pH. Feed was initiated after 4 h of growth and at the constant flow of 22 ml/min. A BlueInOne Cell exit gas analyzer (Bluesens, Herten, Germany) was used to monitor the concentrations of CO_2 and O_2 in the exit gas. Samples taken from bioreactors at different time points were analyzed for DCW, OD_{600nm} and nitrilase activity.

2.5. Analytical methods

Samples taken from shake flasks and bioreactor were centrifuged at 8000 rpm for 10 min. Pellets were washed with 0.1 M Na-phosphate buffer (pH 7.5) and resuspended in the same buffer. Optical density was measured at 600 nm using a UV–vis spectrophotometer (V-540 Jasco, Tokyo, Japan). Supernatants from centrifugation of the fermentation broth were saved for estimation of glycerol wherever applicable.

Glycerol was estimated using HPLC with RI detector (Hitachi-Merck, Darmstadt, Germany) and HP-Aminex 87-H column (Bio-Rad, Hercules, CA). Samples were analyzed using 0.01 N H_2SO_4 as a mobile phase, 0.6 ml/min flow rate and column temperature of 50 °C. Free ammonia in the supernatants was analyzed using phenol-hypochlorite assay described by Weatherburn (1967), after suitable modifications.

2.6. Determination of nitrilase activity

The nitrilase activity in resuspended cell pellets was determined. The reaction mix (1 ml each) contained 50 mM Tris–HCl buffer (pH 7.5), 10 mM mandelonitrile and 50 μl cells. Mandelonitrile stock solution (100 mM) was prepared in methanol. The reaction mixtures with appropriate blanks were incubated at 37 °C for 30 min. The reactions were stopped by the addition of 500 μl methanol. The samples were centrifuged at 12,000 rpm for 5 min. The resulting supernatants were analyzed for ammonia released during the reaction using phenol-hypochlorite assay.

2.7. Testing plasmid stability

Plasmid stability testing was carried out at different stages of cultivation i.e. pre-seed (at 0 and 9 h), seed (at 12 h) and production medium (at 12 h) as described by Zhang et al. (2003). Two combinations were used at each stage, one containing only SOB

Please cite this article in press as: Sohoni, S.V., et al. Optimization of high cell density fermentation process for recombinant nitrilase production in *E. coli*. Bioresour. Technol. (2015), http://dx.doi.org/10.1016/j.biortech.2015.02.038