Bioresource Technology 100 (2009) 4468–4474

Contents lists available at ScienceDirect

Bioresource Technology

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

Enhanced production of recombinant streptokinase in *Escherichia coli* using fed-batch culture

Deepika Goyal, Girish Sahni, Debendra K. Sahoo *

Institute of Microbial Technology, Council of Scientific and Industrial Research, Sector – 39A, Chandigarh 160036, India

ARTICLE INFO

Article history:
Received 23 November 2008
Received in revised form 30 March 2009
Accepted 3 April 2009
Available online 9 May 2009

Keywords:
Streptokinase
Recombinant *Escherichia coli*
Fed-batch culture
Dissolved oxygen concentration
Plasmid stability

ABSTRACT

Fed-batch culture strategy is often used for increasing production of heterologous recombinant proteins in *Escherichia coli*. This study was initiated to investigate the effects of dissolved oxygen concentration (DOC), complex nitrogen sources and pH control agents on cell growth and intracellular expression of streptokinase (SK) in recombinant *E. coli* BL21(DE3). Increase in DOC set point from 30% to 50% did not affect SK expression in batch culture where as similar increase in fed-batch cultivation led to a significant improvement in SK expression (from 188 to 720 mg l^{-1}). This increase in SK could be correlated with increase in plasmid segregational stability. Supplementation of production medium with yeast extract and tryptone and replacement of liquid ammonia with NaOH as pH control agent further enhanced SK expression without affecting cell growth. Overall, SK concentration of 1120 mg l^{-1} representing 14-fold increase in SK production on process scale-up from flask to bioreactor scale fed-batch culture is the highest reported concentration of SK to date.

1. Introduction

Streptokinase (SK), a 47 kD protein, naturally secreted by several strains of hemolytic *Streptococci*, is a clot-buster drug most widely used world-wide for the dissolution of blood clots in cardiovascular diseases like heart attack or stroke (Longstaff and Whitton, 2003). The increased prevalence of thrombo-embolic disorders has led to a tremendous rise in the demand for clot-dissolver drugs preferably at low price, especially in developing countries and to meet this, it is imperative to develop a production process with high yield (mg protein l^{-1}). The low SK production yields from natural host and its pathogenicity are the main reasons for exploration of recombinant DNA technology route for this important protein. *Escherichia coli* is the most commonly used host for heterologous protein production and the preferred method for increasing the concentration of heterologous recombinant proteins, which is proportional to both cell density (unit cell mass per unit volume) and specific cellular product yield (amount of product per unit cell mass), is the fed-batch strategy (Lee, 1996).

Often the transition from shake flask to bioreactor mode of cultivation decreases specific yield of the recombinant protein. In this regard, the importance of nutritional and metabolic parameters during bioreactor cultivation cannot be underestimated. These parameters become more significant in recombinant systems because the replication and gene expression of the plasmid depend on a number of factors including enzymes, energy and biosynthetic precursors available in the host cell. Oxygen is one such process parameter and the effect of oxygen on the cultivation process is generally viewed from two aspects: oxygen transfer rate and dissolved oxygen concentration (Sahoo and Agarwal, 2002). In practice, there is no unanimity on maintenance of a DOC and its control in the range of 10–50% is reported to yield optimal expression of different recombinant proteins during fed-batch cultivation of *E. coli* (Saraswat et al., 1999; Shin et al., 1997; Wang et al., 1998). Similarly the increase in recombinant protein expression levels with the addition of complex nitrogen sources has been reported in many instances in *E. coli* fermentations.

Although a fair amount of general literature is available on the large-scale production of heterologous proteins in *E. coli*, it should be realized that each expression system is unique in terms of promoter system, host–vector interactions, sequence and characteristics of recombinant product and the effect of the expressed foreign protein on host cell physiology. Hence the optimum requirements for growth and product formation also vary from case to case. The importance of growth parameters can be deduced from the fact that although parameters like stable maintenance of recombinant plasmid, plasmid copy number, protease degradation of the recombinant product and inclusion body formation are primarily a function of genetic makeup of the host and vector system, these are also known to be greatly affected by the cultivation conditions and media composition (Lee, 1996; Yee and Blanch, 1992; Zabriskie and Arcuri, 1986). Hence the production or upstream processing of any recombinant product from *E. coli* requires detailed

* Corresponding author. Tel.: +91 172 269 6680; fax: +91 172 269 0585.
E-mail address: debsahoo@imtech.res.in (D.K. Sahoo).

0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.04.008

study of the effect of different cultivation conditions and media constituents so that key parameters affecting product yield can be optimized.

The development of recombinant strain, *E. coli* BL21 (DE3) pET [NTM-CTD] containing the complete gene encoding for streptokinase from *Streptococcus equisimilis* H46A have been reported before (Chaudhary et al., 1999; Nihalani et al., 1998). Herein, the effects of process parameters like dissolved oxygen concentration (DOC) and nutritional parameters like addition of complex nitrogen sources and type of pH control agents on the overproduction of the life-saver protein drug, streptokinase (SK) in *E. coli* are described. This communication reports the development of a fed-batch process for SK production yielding highest concentration of SK to date, without noticeable change in specific yield on process scale-up of recombinant *E. coli* from flask level to bioreactor scale fed-batch culture.

2. Methods

2.1. Bacterial strain and plasmid

The strain used in this study was ampicillin-resistant recombinant *E. coli* BL21 (DE3) developed for over-expression of the gene encoding for SK protein of *S. equisimilis* H46A. The over-expression of cloned gene was under the regulation of T7 polymerase responsive promotor and a *lac* operator in a pET-series expression vector viz. pET-23d (Fig. 1). Also, the recombinant plasmid contained the ampicillin resistance gene for selection of plasmid containing bacterial clones. It was maintained in 30% sterile glycerol at −70 °C.

2.2. Cultivation medium

The medium used for initial batch cultivation contained (a) 13.3 g l^{-1} KH_2PO_4, 4 g l^{-1} $(NH_4)_2HPO_4$, 25 ml l^{-1} trace metal stock solution; (b) 10 g l^{-1} glucose; (c) 1.2 g l^{-1} $MgSO_4 \cdot 7H_2O$ solution (modified from Shin et al., 1997, 1999). Each component (a–c) of the medium was separately sterilized for 20 min at 121 °C and complete medium was reconstituted by mixing the sterile components aseptically. The feed medium used for fed-batch studies contained (a) 400 g l^{-1} glucose; (b) 25 g l^{-1} $MgSO_4 \cdot 7H_2O$. Filter-sterilized ampicillin was added at a final concentration of 100 mg l^{-1} in all cases. The inducer, isopropyl-β-D-thiogalactopyranoside (IPTG) was also filter-sterilized and added to the culture when required. The role of complex nutrients was studied by adding yeast extract in combination with tryptone, peptone and casein acid hydrolysate to the initial and the feed medium at glucose to organic nitrogen source ratio of 2:1.

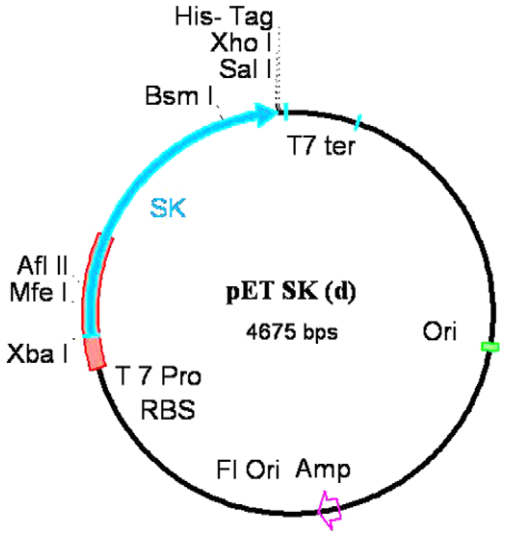

Fig. 1. Plasmid vector pET 23(d)-SK: The full-length SK gene was cloned under the regulation of T7 RNA polymerase responsive promoter in a pET-series expression vector viz. pET-23d (Chaudhary et al., 1999; Nihalani et al., 1998). The recombinant plasmid also contained the ampicillin resistance gene for selection of plasmid containing bacterial clones. The recombinant vector was then transformed into *E. coli* BL21 (DE3) competent cells.

2.3. Inoculum development

A loopful of frozen glycerol stock (kept at −70 °C) was streaked on a LB plate containing ampicillin, 50 μg ml^{-1} and incubated at 37 °C for 14–16 h. A single isolated colony was then transferred to LB medium incubated on a rotary shaker at 37 °C and 200 rpm for 6–8 h. This pre-inoculum was transferred at a rate of 5% (v/v) to the main inoculum medium and incubated for 12–14 h.

2.4. Shake flask cultivation

The cells were grown on a rotary shaker at 37 °C and 200 rpm and induced with 1 mM IPTG (final concentration) when the cell density (OD_{600}) reached 1.0–1.2 in ~2 h. The culture were further incubated for 4 h and then harvested.

2.5. Bioreactor cultivation

All batch and fed-batch culture experiments were conducted in a 5 l Bioflo 3000 laboratory bioreactor (New Brunswick Scientific Co., USA) at pH of 6.8 and 37 °C. 25% (v/v) ammonia or 2 N NaOH and 1 N H_2SO_4 were used to control pH. DOC was monitored using a polarographic steam sterilizable oxygen electrode (Mettler–Toledo International Inc., Switzerland), and reported as percentage of air saturation. The DOC was maintained at specified values by varying airflow and impeller speed and when necessary by enrichment of inlet air with pure oxygen and achieved by setting DOC, agitation and oxygen supplementation in cascading mode. The on-line data of various operating parameters (viz. temperature, DO concentration, pH, impeller speed and % oxygen enrichment) were recorded using Advanced Fermentation Software (AFS) of New Brunswick Scientific Co. Inc., USA. Shake flasks, inoculated with the same inoculum as that of bioreactor, were run simultaneously to check the quality of inoculum.

2.5.1. Batch cultivation

The batch culture were induced when cell density (OD_{600}) reached 3.8–4.2 (at 4 h) and then, further cultivated for 4 h before harvest. DO was maintained at 30% (unless otherwise stated) by cascading agitation rate (300–900 rpm) with DO concentration at constant aeration rate (1.25 vvm).

2.5.2. Fed-batch cultivation

Fed-batch cultivations were started as batch cultures and after the exhaustion of glucose, feeding was started with a glucose feeding rate of 4.5 g l^{-1} h^{-1} for 3 h. Induction was done when the OD_{600} reached around 30 and feeding was continued at the same rate for another 4 h. In un-induced run, same feed continued till harvest. DOC was maintained at the set point (30% or 50% or 60%) by cascading impeller speed and supplementation of air with pure oxygen (oxygen enrichment). Oxygen enrichment was recorded as percentage of pure oxygen added to the total air/gas (taken as 100%) supplied for aeration.

2.6. Analytical methods

The bacterial cell concentration was measured off-line by optical density at 600 nm (OD_{600}) in a spectrophotometer (Shimidzu,