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Effect of Postinduction Nutrient Feed Composition and Use of Lactose as Inducer during Production of Thermostable Xylanase in *Escherichia coli* Glucose-Limited Fed-Batch Cultivations

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***Escherichia coli* is a microorganism routinely used in the production of heterologous proteins. The overexpression of a xylanase (Xyn10AΔNC), which originated from the thermophile *Rhodothermus marinus* cloned under the control of the strong T7/lac promoter in a defined medium (mAT) using a substrate-limited feed strategy, was however shown to impose a significant metabolic burden on host cells. This resulted in a decreased cell growth rate and ultimately also a decreased target protein production. The investigation hence centers on the effect of some selected nutrient feed additives (amino acid [Cys] or TCA-intermediates [citrate, succinate, malate]) used to relieve the metabolic burden imposed during the feeding and postinduction phases of these glucose-limited fed-batch cultivations. The use of either succinic acid or malic acid as feed-additives resulted in an increase in production of approximately 40% of the heterologous thermostable xylanase. Furthermore, use of lactose as an alternative inducer of the T7/lac promoter was also proven to be a suitable strategy that significantly prolonged the heterologous protein production phase as compared with induction using isopropyl β-D-thiogalactopyranoside (IPTG).**

[**Key words:** thermostable, xylanase, lactose, pulsed feeding strategy, nutrient feed]

Heterologous protein production in *Escherichia coli* is widely used to obtain large amounts of proteins that are not easily available from the wild type. The production is affected by numerous process factors, such as the cultivation mode, composition of the medium, time of induction (with respect to cell mass concentration) and duration of the production phase (1, 2). Due to the lack of a natural secretion mechanism in *E. coli*, the concentration of the produced protein is to a great extent proportional to cell concentration, and processes designed to yield high cell densities are therefore beneficial. In addition, other factors such as host cell-vector interactions, plasmid stability and cellular stress responses (3–5) need to be considered. As a result, research to optimize protein production is complex and encompasses the disciplines of both molecular biology and engineering, and numerous strategies are thus applied to maximize the production of recombinant proteins in *E. coli* (6–8).

Metabolic stress in *E. coli* is often proposed as a reason for decreased yields of a target protein during its gene expression, which can result in the redirection of cellular metabolism (9) and a decreased growth rate (6–10). This has in some cases been reported to occur due to the addition of the inducer IPTG (11, 12). However, it is also more generally observed during postinduction phases particularly when induction is initiated after a certain critical cell mass is attained, possibly due to the absence of necessary metabolites. It has for example previously been established that the depletion of certain amino acids in the medium significantly influenced the expression of recombinant proteins (2, 13). This lack of resources in the cell are likely due to the increased protein production, in combination with the finding that a heterologous protein often has an amino acid composition different from that of average *E. coli* proteins. This can result in limitations in certain key amino acids that hence act as bottlenecks and control protein production. Consequently, the addition of deficient amino acids to the growth medium has been reported to circumvent a decreased production of a recombinant protein in the late postinduction phase (2, 13). Such addition can also circumvent reduced tRNA levels during translation, a factor that has previously been observed during amino acid limitation (14), and results in conditions with an amplified proteolytic activity in cells to replenish amino acid resources (15). Released amino acids from nonessential proteins may be incorporated into essential proteins that are required for cell survival (16).

This investigation hence centers on the effect of nutrient additives from the tricarboxylic acid (TCA) cycle (present during the fed-batch and postinduction phases) on recombinant protein production in *E. coli* grown using a substrate-

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limited fed-batch strategy, designed to control the feed in order to maintain glucose below the critical level for overflow metabolism (17). By analyzing protease activity, a measure on the induction related stress response is also collected. In addition, the effect of an inducer (IPTG vs lactose) on both recombinant protein production and induction-related stress responses in cells grown by this fed-batch strategy is investigated. For these purposes, high-cell-density fed-batch cultures of *E. coli* producing thermostable xylanase (18), originating from the thermophilic bacterium *Rhodothermus marinus* (19), were analyzed. The results are expected to provide new complementary information regarding the protein-synthesizing machinery of the cell that may explain cell behavior as recombinant protein production proceeds.

MATERIALS AND METHODS

Bacterial strain, plasmid and inoculum preparation *E. coli* strain BL21(DE3) was used as the host microorganism. The plasmid was derived from the vector pET25b(+) (Novagen, Madison, WI, USA). The construction of the plasmid encoding xylanase (Xyn10AΔNC with previous nomenclature Xyn1AΔNC) is described by Nordberg Karlsson *et al*. (20). Xyn10AΔNC consists of the signal peptide (21aa) and the catalytic module (322aa) and has a short vector-derived stretch of 15aa in the C-terminus. Expression is in all cases under the control of the T7/lac promoter. The inoculum was prepared using 100 ml of the defined mAT medium (Table 1 [excluding IPTG and antifoam]). The salts, glucose and trace elements were sterilized separately at 121°C for 20 min and aseptically pooled into a 1 *l* baffled Erlenmeyer flask. Mid-log cultures (1 ml) in 20% glycerol (stored at −80°C) containing the *E. coli* clone were used to inoculate the flask, which was subsequently incubated at 30°C for 12 h on a rotary, water bath shaker at 125 rpm (Heto, Allerod, Denmark).

Experimental set-up and cultivation conditions Fed-batch cultivations were performed using a 3 *l* fermentor (Chemoferm FLC-B-3, Hägersten, Sweden) with an initial medium volume of 2 *l*. The salts were sterilized in the vessel at 121°C for 45 min; thereafter, sterile $MgSO_4$, glucose and trace elements were aseptically added. Ampicillin (see Table 1) was added by sterile filtration. The data capturing of dissolved oxygen concentration (%DO), feed-pump control, stirrer speed (rpm) and off-gas were conducted using the SattLine control system (Alfa Laval Automation AB, Malmö, Sweden). Cultivation temperature was controlled at 37°C and the pH was maintained at 7.0 by titration with 6.7 M aqueous ammonia. Dissolved oxygen concentration was measured using a polarographic electrode calibrated to 100% at 1100 rpm at 37°C and zeroed by sparging sterile nitrogen into the vessel. The DO was controlled at 30% saturation using a gain-scheduled PID controller connected to stirrer speed, as described by Akesson *et al*. (17). A pulse feeding strategy (21) was employed using various nutrient feed solutions listed in Table 1. A Tandem dual gas system (Adaptive Biosystems, Luton, UK) was used for off-gas analysis. Induction was carried out using IPTG or lactose. Fermentations and assay conditions were standardized to avoid any bias and for accurate and reproducible comparison. Furthermore, appropriate precursor supplementation concentrations were selected on the basis of the highest concentrations of organic acids obtained during uninduced control cultivations.

TABLE 1. Medium composition for batch and nutrient feed solutions

Ingredient	Concentration
$(NH_4)_2SO_4$	2 g l^{-1}
K_2HPO_4	14.6 g l^{-1}
$NaH_2PO_4 \cdot 2H_2O$	3.6 g l^{-1}
$(NH_4)_2$ H-citrate	0.5 g l^{-1}
Glucose	10 g l^{-1}
1 M $MgSO_4$	2 ml l^{-1}
Trace elements[a]	2 ml l^{-1}
Ampicillin	0.1 g l^{-1}
Antifoam (adekanol)	0.1 ml l^{-1}
Inducer	1 mM (IPTG) or 13 mmol/g CDW (lactose)
Feed (F)	
Glucose	500 g l^{-1}
1 M $MgSO_4$	50 ml l^{-1}
Trace elements	10 ml l^{-1}
Feed (F_{cit})	
Feed F+citric acid	2 g l^{-1}
Feed (F_{suc})	
Feed F+succinate	2 g l^{-1}
Feed (F_{mal})	
Feed F+malate	2 g l^{-1}
Feed (F_{cys})	
Feed F+cystein	2 g l^{-1}

[a] According to Holme *et al*. (35).

Sampling and sample treatment For intracellular enzyme activity, total protein estimation and SDS–PAGE, samples were centrifuged (11,000×*g*, 15 min, at room temperature) and the resulting cell pellets were dissolved to the original volume in 20 mM Tris–HCl (pH 7.5) and disrupted by sonication (90 s; cycle, 0.5) at a sound intensity of 230 W/cm^2 using a UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany), equipped with a 3-mm titanium probe. After sonication the samples were centrifuged (11,000×*g*, 15 min, at room temperature) and the supernatants were stored on ice or kept frozen (–20°C) until analysis.

For extracellular enzyme activity estimation (*i.e*., analysis of enzyme activity in the fermentation broth as a result of lysis or cell disruption), samples were centrifuged (11,000×*g*, 15 min, at room temperature) and the resulting supernatants were stored on ice or kept frozen (–20°C) until analysis.

For HPLC analysis of organic acids and glucose, samples were centrifuged (11,000×*g*, 5 min, at room temperature) and the resulting supernatants were filtered (0.2-μm) into vials and stored at –20°C until analysis.

Analytical methods

Optical density and cell dry weight (CDW) determination Optical density was determined at 620 nm. Samples exceeding an OD value of 0.5 were appropriately diluted with 0.9% (w/v) NaCl before determination. Triplicate CDW determinations (4 ml sample each) were carried out after drying of cells overnight at 105°C as described by Ramchuran *et al*. (13).

Enzyme activity Xylanase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method (22) under the conditions described by Ramchuran *et al*. (13). Xylanase activity was expressed in units (U), which is defined as the amount of xylanase required to liberate 1 μmol of reducing sugar equivalents per min under standard conditions.

Estimation of total protein Total protein concentration was determined using the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin (0.2–1.0 mg/ml) as a standard.

Organic acids The concentrations of organic acids were determined by HPLC using an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA). The column temperature was set at 30°C at a wavelength of 210 nm and a mobile phase (0.005 M H_2SO_4) using a flow rate of 0.5 ml/min. An organic acid standard (catalog no. 125-0586; Bio-Rad), which enables the quantification of citric acid, succinic acid, and malic acid, was used for calibration.