AUTOMATIC LABORATORY-SCALE FED-BATCH PROCEDURE FOR PRODUCTION OF RECOMBINANT PROTEINS USING INDUCIBLE EXPRESSION SYSTEMS OF ESCHERICHIA COLI

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SUMMARY
An automatic fed-batch procedure for the production of recombinant proteins in \textit{Escherichia coli} was developed. Using glycerol as carbon source and by controlling the growth rate by using feed-forward algorithm, enabled high specific expression level (10-20 \% of total cell protein) at high cell densities (20 g dry wt/l) to be achieved: rat and human soluble catechol-O-methyltransferase, calf prochymosin, and human troponin C were expressed with nearly 50-fold higher volumetric yield compared to the conventional (batch) procedures.

INTRODUCTION
Usually the recombinant proteins are expressed according to the procedure involving induction of the low density (0.2-0.5 g dry wt/l) exponentially growing culture on rich medium. The method suits for both, shaking flasks and fermenters, and enables to produce up to 1 g target protein using cultivation capacities (about 5-10 l) of an average laboratory. Often, however, higher amounts of recombinant proteins are required. An alternative to scaling up the volumetric fermentation capacity is to increase the cell density. The maximal cell density depends on the availability of substrates and on the accumulation of by-products. In batch culture the cell density is limited to about 12 g dry wt/l (Landwall and Holme, 1977; Luli and Strohl, 1990). The inhibition of growth together with the decrease of expression level (Bech Jensen and Carlsen, 1990; Brown et al., 1985), however, may even start at considerably lower culture densities. Inhibitory effect of acetate, one of the by-products, on the expression has been shown (Sun et al., 1993). Different feeding strategies (e.g., feeding with constant rate (Bech Jensen and Carlsen, 1990; Dalboge et al., 1989), carbon limited feeding using \textit{pO}_2 feed-back control (Riesenberg et al., 1990), exponential feeding (Yee and Blanch, 1993), two-stage cyclic (Curless et al., 1991) and two-stage continuous cultivation (Miao and Kompala, 1993)) have been used for expression of recombinant proteins at high cell densities. Some of these methods are quite complicated for the use in the laboratory not specialized in fermentation technology. Also, it is difficult to adjust these procedures to the conventional working day cycle. Using mineral media the by-product formation can be avoided and high cell densities (>50 g dry wt/l) can be achieved by means of substrate limited fed-batch cultivation techniques using feed-forward feeding algorithm (Paalme et al., 1990). Nearly 100-fold increase in the volumetric productivity of recombinant proteins could be theoretically achieved compared to the conventional laboratory expression protocol (Maniatis et al., 1989) using feeding strategy developed. In this paper we describe a simple fully automatic fed-batch laboratory cultivation procedure developed for the production of large amounts (1-50 g) of recombinant proteins.
MATERIALS AND METHODS

Strains and plasmids. E. coli strain BL21(DE3) (Studier and Moffat, 1986) was used for expression of rat and human soluble catechol-O-methyltransferase (S-COMT). JM109(DE3) (Yansch-Perron et al., 1985) (Promega, USA) for human troponin C fusion protein (gst-troponin C) and calf prochymosin. Rat and human S-COMT cDNAs were both cloned into the expression vector pEXK14 (Lundstrom et al., 1992), human troponin C cDNA into GST gene fusion system (pGEX-2T vector, Pharmacia, Sweden). Prochymosin cDNA was previously cloned (Ord et al., 1987) and subcloned into pEX2 vector, constructed by Peranen (Peranen, 1990).

Growth media. LB medium supplemented with ampicillin (50 mg/l) was used for stock glycerol cultures and cultures on Petri dishes. M9 medium (Maniatis et al., 1989) was modified (2 g glycerol/l, 50 mg ampicillin/l and 0.5 mg thiamin/l were added) and used for shaking flask cultures as well as for the fermentation inoculum. The following media were used for fed-batch cultivations: starting media (g/l): KH2PO4 8.0, MgSO4·7H2O 2.0, glycerol 2.0, NH4Cl 1.0, sodium ampicillin 50 (mg/l), thiamin 0.5 (mg/l). pH was adjusted to 6.8 by titration with 4M NH4OH, feeding media (mg/l): KH2PO4 150, CaCl2·2H2O 300, MnSO4·5H2O 50, CoCl2·6H2O 10, ZnSO4·7H2O 50, CuCl2·2H2O 10, Na2MoO4·2H2O 10, glycerol 300 (g/l), sodium ampicillin 250, thiamin 25. The cultivation temperature was 37°C, the dissolved oxygen concentration PO2>10%.

The desired specific growth rate \( \mu_s \) in the growth phase was 0.2 h⁻¹, 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) was used for the induction.

Expression procedures. In reference experiments cells were grown on LB or glycerol/minimal salts medium on the rotary shaker till 0.2-0.4 g dry wt/l or diluted with minimal medium to the same density (diluted control) and induced with 0.5 mM IPTG for 3-5 h.

SDS-PAGE. Expressed recombinant proteins were identified by SDS-PAGE (10% gel) by staining with Coomassie brilliant blue (Maniatis et al., 1989). Relative amount of recombinant proteins to the total bacterial protein, or to the diluted control was estimated by scanning of stained gels on a LKB Ultrascan XL laser densitometer.

HPLC. Concentration of glycerol and the by-products in the culture broth was estimated with HPLC by isocratic elution (0 6 ml/min) of Bio-Rad HPX-87H column with 0 009 N H2SO4 on a Bruker LC-31 system with UV (at 206 nm) and refractive index detectors. The content of amino acids was measured using a Biochrom amino acid analyzer.

Cultivation systems. Two different computer controlled cultivation systems were used. (1) 4 litre Ultraferm 1601 fermenter (LKFB, Sweden) supported with gas analyzers and 300 ml BioFlo fermenters (New Brunswick, USA) (2) 10 l BioEngineering fermenters and five Biostrat M (B Brauns, Melsungen, Germany) fermenters (1 litre). All fermenters were equipped with pH and temperature control and dissolved oxygen PO2 monitoring system "FermExpert" cultivation software (A/S BioExpert, Estonia) was used for process control (Vunster et al., 1992). The control algorithm (Paalme et al., 1990) was used to start the fed-batch mode and to control the growth rate at desired value. Depending on the experiment the culture was either pumped into separate fermenter(s) or directly induced by IPTG. The IPTG was added to the culture only after the oxygen consumption rate of the culture was stabilized.

The fed-batch expression procedure.

The following automatic laboratory-scale fed-batch procedure for production of recombinant proteins using inducible expression systems of E. coli was developed.

Batch phase. The overnight culture grown either on LB medium or mineral medium with glycerol in shaking flasks was inoculated (1:10) into the fermenter containing starting medium. In the beginning of the logarithmic phase the approximate time of glycerol exhaustion (\( t_{ex} \)) was calculated:

\[
t_{ex} = \ln(Y_{Sx}/S_x)/\mu_{max},
\]

where \( Y_{Sx} \) is the growth yield, \( S_x \) - the substrate concentration in starting medium, \( X \) - biomass concentration, \( \mu_{max} \) - the maximal growth rate.

Fed batch phase. The feeding algorithm was automatically started usually at 1-2 h before the expected glycerol exhaustion time \( t_{ex} \). Alternatively, the moment to start the feeding was automatically identified by simultaneous increase of dissolved oxygen or decrease of flash and oxygen consumption (Paalme et al. 1990). The current feeding rate of the carbon source \( F_c(t) \) was calculated as:

\[
F_c(t) = \mu_S S_c(t),
\]

where \( \mu_S \) is the chosen growth rate, and \( S_c(t) \) the total amount of carbon added into the fermenter up to calculation of new \( F_c(t) \) value. The \( \mu_S \) less than \( \mu_{max} \) should be chosen to avoid formation of by-products (Paalme et al. 1990).

Induction phase. The culture was induced using 0.5 mM IPTG after the desired culture density (10-15 g dry wt/l, oxygen was required for oxygenation at higher cell densities) was reached. Different feeding algorithms were used for feeding. For routine operation the constant feeding rate (\( F_c = F_c(t_{ind}) \), where \( t_{ind} \) is the moment of induction) was used.

RESULTS

The fed-batch experiments. The curves of a typical fed-batch cultivation of recombinant E. coli with controlled growth rate and the synthesis of rat S-COMT are shown on Fig. 1. Four hours after inoculation the expected time of glycerol exhaustion was calculated and automatic cultivation procedure was switched on. Culture was grown overnight under the control of "FermExpert" cultivation program. The (exponential)