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BATCH AND FED BATCH CULTIVATIONS FOR THE TEMPERATURE INDUCED PRODUCTION OF A RECOMBINANT PROTEIN IN *ESCHERICHIA COLI*

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Summary

The temperature induced intracellular production of the fused protein SpA-ßgal (protein A/ß-galactosidase) with *E. coli* was compared in batch and fed batch culture. By introducing fed batch cultivation a final cell dry weight of 77.0 g/l was achieved, as compared to 16.4 g/l in batch cultivation. The concentration of SpA-ßgal in the fed batch cultivation was very high, 19.2 g/l. This corresponded to 25 % of the cell dry weight.

Introduction

In the production of recombinant proteins not only a high expression of the the target protein is important but also a high cell density, thereby improving productivity and final product concentrations. Non-recombinant *E. coli* has been grown to high cell densities, up to 125 g/l (Yano *et al.*, 1980), however reported cell densities for recombinant *E. coli* are usually much lower. Few papers report concentrations above 50 g/l with a concomitant expression of recombinant protein (Fieschko and Ritch, 1986; Jung *et al.*, 1988; Riesenberg *et al.*, 1990). Fed batch technique is usually employed in cultivations to obtain high final cell densities since it is necessary to restrict growth due to limitations in oxygen transfer as well as heat transfer. Oxygen limitation leads to the formation of fermentative by-products that will inhibit growth and decrease yields. It is also necessary to avoid restrictions in growth due to initially high concentrations of substrate or to the formation of inhibiting by-products, like acetic acid formed from excessive glucose consumption (Majewski and Domach, 1990; Reiling *et al.*, 1985).

We have earlier studied the influence of cultivation conditions on the formation of inclusion bodies in the temperature induced production of a fused protein with *E. coli* in batch cultures (Strandberg and Enfors, 1991). The protein consisted of the immunoglobulin binding regions from staphylococcal protein A fused to ß-galactosidase from *E. coli*. The fusion protein, hereafter called SpA-ßgal, can be used to label antibodies with ß-galactosidase activity. In this paper we report on the temperature induced production of the improved fusion protein in batch and fed batch cultivation.

Materials and Methods

E. coli KA197 *thi-1*, a K-12 derivate (Coli Genetic Stock Center), was co-transformed with pRIT1b and pRITcI857. pRIT1b encodes SpA-ßgal under the control of the p_R promoter as well as ampicillin resistance (Hellebust *et al.*, 1989). pRITcI857 encodes the temperature-sensitive repressor protein (cI857) for the p_R promoter as well as kanamycin resistance (Nilsson and Abrahmsén, 1990). Cultivations were performed in a Chemap SG7 bioreactor. The composition of the medium and feed solutions can be found in table 1, together with cultivation parameters. Cell dry weight, acetic acid, and glucose concentration were determined as described elsewhere (Förberg *et al.*, 1983). Cell growth was also followed by the optical density at 580 nm.

Table 1: Composition of the different solutions in g/l and cultivation parameters

	Batch	Fed batch	Feed solution (Σ 2 L)
Na_2SO_4	2.0	2.0	2.0
$(NH_4)_2SO_4$	----	----	3.0
NH_4Cl	0.5	0.5	0.5
K_2HPO_4	9.7	14.6	14.6
$NaH_2PO_4*H_2O$	2.4	3.6	3.6
$(NH_4)_2$-H-citrat	1.0	1.0	1.0
1M $MgSO_4$ (ml/l)	3.0	2.0	-----
Trace comp. (ml/l) (Holme *et al.*, 1970)	3.0	2.0	10.0
Glucose	30.0	25.0	600.0
Thiamine	0.1	0.1	0.1
Adecanol LG-109 (antifoam) (ml/l)	0.05	0.05	----
Ampicillin	0.07	0.07	0.07
Kanamycin	0.02	0.02	0.02
Initial volume (L)	5.0	3.5	
Stirrer speed	400-1250	400-1450	
Aeration rate (l/min)	0.5-5.0	0.5-5.0	
Back pressure (bar)	0.15	0.15 (0.3 after induction)	

In batch cultivation additional glucose (60 %) was added at 1.5 (160 ml) and 3 (90 ml) hrs after temperature induction.
In fed batch cultivation additional $MgSO_4$ was added to the bioreactor intermittently (Σ 40 ml) to avoid precipitation.
pH was controlled at 7.0 with 2 M H_2SO_4 or 25 % NH_3 (8 % in batch cultivation).
Additional antifoam was added when necessary.
Cell growth temperature = 30°C. Induction temperature = 40°C.

Viable count was performed on Tryptic Soy Broth plates with and without antibiotics (ampicillin 0.105 g/l and kanamycin 0.030 g/l). The plates were incubated at 30°C for approx. 24 h. Ammonia concentration was determined with an ammonia electrode (Kent Industrial Measurement, England). Ammonium and acetate is expressed as total concentration in cell free broth.

The feed solution was fed with a peristaltic pump with a predetermined exponential increase in feed rate from the initial 0.044 l/h to the final 0.100 l/h during three hours. After this time, the feed was kept constant. The accuracy of the feed rate was controlled by having the feed container on a balance and reading the output. The exponential increase in feed rate was manually adjusted every 15 minutes. O_2 was analyzed in the off-gas with

a paramagnetic analyser (Servomex Oxygen Analyser 540A, Sybron/Taylor, England) and CO_2 with an infrared analyser (Binos, Leybold-Hereaus, Germany), respectively. Dissolved oxygen tension was measured with a polarographic oxygen electrode (Ingold, Switzerland), saturation value set at 40°C. The dissolved oxygen tension was always maintained above 15 %. The amount of SpA-ßgal was determined after cell disintegration as ß-galactosidase activity (U) at 25°C with ONPG as substrate (Veide *et al.*, 1983) and expressed as U per ml cell broth or U per mg cell dry weight.

Results and Discussion

Data from a batch cultivation of the SpA-ßgal producing *E. coli* is shown in figure 1. The production of SpA-ßgal was induced at a cell dry weight of 5.2 g/l. The specific rate of SpA-ßgal formation was highest during the first hours after induction and thereafter declined. The final specific activity of SpA-ßgal, 153 U/mg, corresponds to approximately 38 % of the cell dry weight assuming SpA-ßgal has a specific activity of 400 U/mg protein (Strandberg and Enfors, 1991). The decline in the specific rate of SpA-ßgal formation might be due to the increasing concentrations of acetate and ammonium, figure 2, as well as the exhaustion of glucose 4.2 hours after temperature induction (not shown). After induction there was a sharp increase in the specific rate of acetate formation leading to a high concentration of acetate in the medium. This also led to an increasing ammonium concentration in the medium since ammonia was used for pH control. High levels of acetate (i.e. above 5 g/l, pH 7.0) reduces growth (Bech Jensen and Carlsen, 1990; Luli and Strohl, 1990; Yano *et al.*, 1980) as well as concentrations of ammonium above 170 mM (Thompson *et al.*, 1985). High acetate concentrations has also been shown to reduce the specific expression rate of hGH (Bech Jensen and Carlsen, 1990). Ammonia is

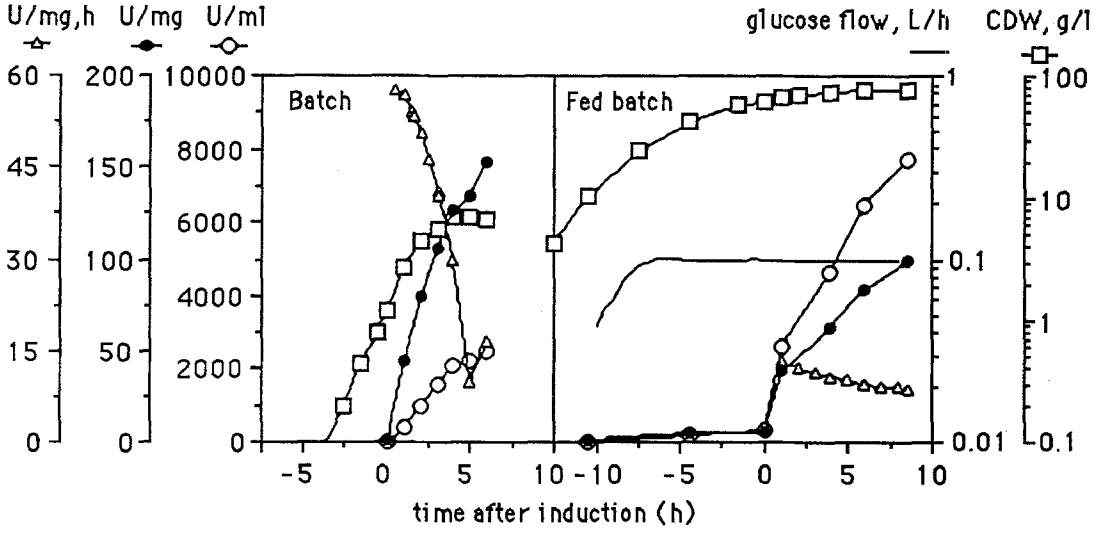

Figure 1. Cell dry weight (CDW), total and specific SpA-ßgal activity, and the specific production rate of SpA-ßgal during the different cultivations. Included in the figure is also the feed profile for glucose in the fed batch cultivation. The time scale relates to the time for temperature induction.

commonly used to control pH since the necessary ammonium for growth to high cell densities in a mineral medium can not be added initially due to the inhibiting effect. NaOH or KOH can been used instead for pH control. However, high salt concentrations can also reduce the expression rate of a recombinant protein (Bech Jensen and Carlsen, 1990). The specific CO_2 evolution rate, qCO_2, remained approximately constant up to the time for temperature induction and declined after a transient increase during the first hour after induction, figure 2. qO_2 showed the same pattern (not shown). The sharp increase in qCO_2 and qO_2 immediately after induction in batch culture probably reflects an increased energy demand during the heat shock.

In order to improve the process a fed-batch cultivation was performed, figure1. The feed was started at a cell dry weight of 11.6 g/l when the initial glucose was almost exhausted. The glucose feed increased exponentially during three hours thereafter the flow rate remained constant. The aim was to maintain the specific growth rate, μ, at 0.3 h^{-1} during the exponential feed period, however μ was 0.35 h^{-1} at the beginning and declined to 0.25 h^{-1} at the end of this period. During the linear feed μ declined from 0.25 h^{-1} down to 0.08 h^{-1} at the time for temperature induction. μ_{max} was in both cultivations 0.45 h^{-1}.

The initially formed acetate was consumed in the beginning of the feed period, figure 2. The expression of SpA-ßgal was induced when the cell dry weight was 61.6 g/l. Also in the fed batch there was a transient increase in qO_2 and qCO_2 during the first hours after temperature induction, though not as pronounced as in the batch cultivation. In order to avoid oxygen limitation during this period the back pressure was increased from 0.15 to

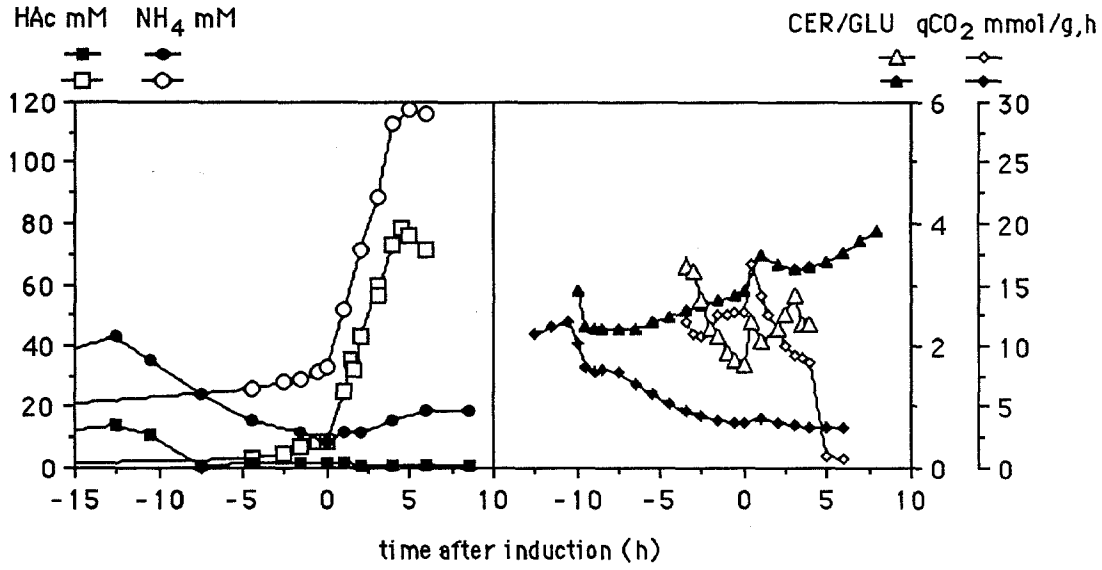

Figure 2. Total ammonium and acetic acid concentrations, specific CO_2 production rate (mmol/g cell dry weight,h), and the ratio of CO_2 evolution rate to glucose consumption rate (CER/GLU; mol,h/mol,h) during batch (open symbols) and fed batch (closed symbols) cultivation. The time scale relates to the time for temperature induction.

0.30 bar. The final total activity of SpA-ßgal after 8.5 hours at 40°C was 7700 U/ml with a specific activity of 100 U/mg cell dry weight. This corresponds to 25% of the cell dry weight or to 19.2 g SpA-ßgal/l. The specific activity was only 66% of the final specific activity in the batch cultivation. However, if the induction period had been prolonged it might have been possible to increase the specific activity since the specific rate of SpA-ßgal formation at the end still was 66% of the initial rate.

Plasmids were stably maintained both in batch and fed batch cultivations, figure 3, however the viable count declined after temperature induction in the fed batch cultivation. The decline in viable count was not followed by a similar decrease in product synthesis rate or respiration rate. We have also noted a similar decline in viable count in batch cultivations in the production of a similar protein (Strandberg *et al.*, 1991). A decline in viable count following the expression of recombinant proteins has been reported by others and assumed to be a consequence of excessive mRNA synthesis (Sugimoto *et al.*, 1987) or product accumulation (Betenbaugh *et al.*, 1989; Kaprálek *et al.*, 1991). The decline in viable count in the fed batch cultivation is probably not caused by the high intracellular concentration of SpA-ßgal since the level in the batch cultivation

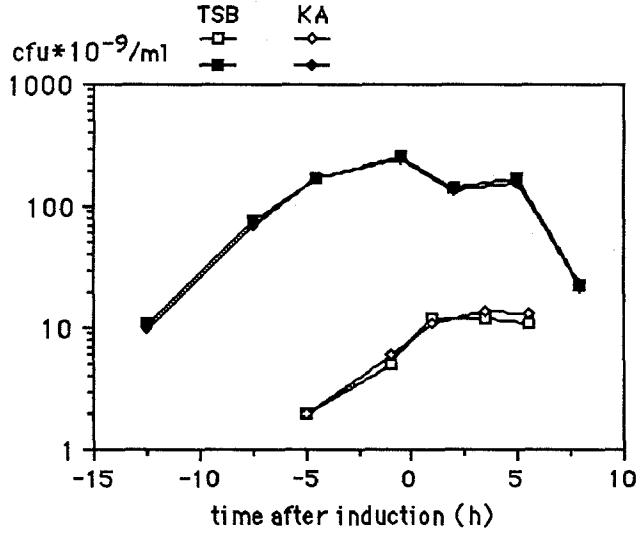

Figure 3. Viable count, expressed as colony forming units (cfu)/ml, on plates with (KA) and without (TSB) antibiotics. Open symbols refer to batch cultivation and closed symbols to fed batch. The time scale relates to the time for temperature induction.

was approximately 50 % higher. We have also observed declining viable counts with time when running fed batch cultivations of the untransformed strain (results not shown). The decline may rather be a consequence of prolonged starvation or a lack of carbon available for non-energy generating processes during the fed batch. As can be calculated from figure 2, approximately 33 % of the added carbon was liberated as CO_2 at the beginning of the feed period. This quotient increased to 66 % at the end of the cultivation.

In conclusion, by using fed batch cultivation we could very much improve the production process of SpA-ßgal. We could cultivate *E. coli* to a high final cell density and, despite energy limitation, produce SpA-ßgal in high total and specific concentrations. The cultivations were performed in a standard reactor with standard equipment and oxygen sparging was not necessary. The protein was also produced in a soluble and active form with no visible degradation pattern according to electrophoresis gels (not shown).

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