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Glucose as a substrate in recombinant strain fermentation technology

By-product formation, degradation and intracellular accumulation of recombinant protein

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Summary. Glucose supplements to complex growth media of *Escherichia coli* affect the production of a recombinant model protein under the control of a temperature-sensitive expression system. The bacterial "Crabtree effect", which occurs in the presence of glucose under aerobic conditions, not only represses the formation of citric acid cycle enzymes, but also represses the formation of the plasmid-encoded product even though the synthesis of this protein is under the control of the temperature-inducible lambda P_R-promoter/cI857-repressor expression system. When the recombinant *E. coli* is grown at a moderate temperature (35° C) with protein hydrolysate and glucose as substrates, a biphasic growth and production pattern is observed. In the first phase, the cells grow with a high specific growth rate, utilizing glucose and forming glutamate as a by-product. The intracellular level of recombinant protein is very low in this phase. Later, glutamate is consumed, indicating an active citric acid cycle. The degradation of glutamate is accompanied by the intracellular accumulation of high amounts of recombinant protein.

Introduction

Genetic engineering methods are already in industrial use to produce many types of proteins using fast-growing microorganisms such as the bacterium *Escherichia coli*. For industrial use, it is important to have a recombinant bacterial strain that provides high productivity and sufficient genetic stability. For this purpose it is often convenient to choose expression systems in which cell growth and formation of recombinant protein appear in different cultivation phases. In order to separate growth and production phases, chemically inducible promoters, such as the *lac, tac,* or *trp* promoter, and temperature-inducible expression systems, such as the lambda promoter/lambda cI857-repressor system, are commonly used.

For the economical use of recombinant microorganisms that form intracellular products, it is important to utilize a fermentation process that results in a high intracellular level of product *and* a high cell concentration in the fermentor. An inexpensive and convenient substrate to obtain a high cell mass is a simple carbohydrate, such as glucose. Glucose feeding leads to a bacterial "Crabtree effect" under aerobic conditions, resulting in the formation of acetate and other metabolic by-products (Anderson and von Meyenburg 1980). To prevent accumulation of by-products, which reduce the growth yield, fed-batch strategies have been developed for non-recombinant *E. coli* (Gleiser and Bauer 1981; Mori et al. 1979). In cultivations of recombinant *E. coli* it was also observed that accumulation of acetate in the culture medium is concomitant with low levels of recombinant protein production (Brown et al. 1985; Meyer et al. 1984). Reducing the growth rate by decreasing the dilution rate in continuous culture or the substrate feed in fed-batch culture results in decreased acetate formation and in increased production of recombinant protein (Brown et al. 1985; Meyer et al. 1984; Zabriskie and Arcuri 1986). When complex (instead of defined) media are supplemented with glucose as an additional carbon source, a stronger reduction in growth rate is necessary to prevent the formation of acetate (Meyer et al. 1984).

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The development of a control-scheme process for use in recombinant strain fermentation technology requires knowledge about the connection between substrate utilization and product formation. Therefore, the objective of this work was to correlate substrate consumption, metabolic by-product formation, and by-product degradation with the intracellular accumulation of a recombinant model protein in *E. coli* grown in batch culture on complex medium supplemented with glucose.

We investigated an *E. coli* host in which the temperature-sensitive lambda cI857-repressor is formed by a chromosomally integrated, defective lambda lysogen. The formation of plasmid encoded product, a *cro-lac*Z fusion protein with β-galactosidase activity, is under the control of the lambda P_R-promoter (Zabeau and Stanley 1982). We observed not only that the temperature dependent interaction between promoter and repressor is responsible for the induction of product formation, but also that substrate supply and substrate-induced repression and derepression affect the extent of product formation.

Materials and methods

Bacterial strain and plasmid. Escherichia coli K12 MF (F^- trp^-_{am} lac^-_{am} Sm^R) with the chromosomal lambda lysogen (lambda Nam7 Nam53 cI857ΔHI) was used as a host (Castelazzi et al. 1972). The plasmid utilized was pCL47ΔY-T. This plasmid, carrying a *cro-lac*Z fusion under control of the lambda P_R-promoter, is described by Zabeau and Stanley (1982). Host and plasmid were kindly provided by K. Stanley (EMBL, Heidelberg, FRG).

Medium and culture conditions. LB medium, containing 10 g/l casein peptone (Difco, Detroit, Mich.), 5 g/l yeast extract (Sigma, St. Louis, Mo), and 9 g/l NaCl, was supplemented with 10 g/l glucose and 100 mg/l ampicillin. The cultivation was carried out in a 10-l stirred-tank reactor (Chemap, Volketswill). The aeration rate was 0.5 vvm at 500 rpm. The pH was kept constant at pH 7 with 4 *M* NaOH and 2 *M* H_2SO_4. An overnight culture (LB medium supplemented with 100 mg/l ampicillin) prepared from a 2-day-old, single colony was used as inoculum (1% v/v).

Electrophoresis of proteins. Sodium dodecyl sulphate (SDS)-polyacrylamde gel electrophoresis was performed in 7.5% polyacrylamide slab gels according to the method of Laemmli (1970). For sample preparation cells corresponding to 1 mg dry weight (DW) were recovered by centrifugation (7000 *g*, 5 min). Harvested cells were washed in 0.9% NaCl solution and centrifuged at 7000 *g* for 5 min. The remaining cell pellet was resuspended in 100 μl sample buffer. Samples were then boiled for 10 min. Insoluble material was removed by centrifugation. Supernatant (10 μl) was loaded onto the 7.5% SDS-polyacrylamide gel in the presence of 0.1% SDS. The composition of the sample buffer was as follows: 4% SDS, 10 m*M* dithiothreitol, 20% glycerol, 125 m*M* TRIS/hydrochloride (pH 6.8).

The gels were stained with Coomassie brilliant blue R 250. To estimate the amount of *cro-β*-galactosidase, the stained gels were scanned with a Camaq TLC scanner (Muttenz, CH).

β-Galactosidase activity. Samples (1 ml) removed from batch culture were centrifuged at 7000 *g* for 5 min, and the cells resuspended in 0.9% NaCl solution. The cells were disrupted with an ultrasonic disintegrator (Labsonic 1510, 100 watts, B. Braun, Melsungen, FRG) for 5 min on ice. Cell debris was removed by centrifugation at 7000 *g* for 5 min and β-galactosidase activity was assayed by measuring the rate of hydrolysis of *o*-nitrophenol-β-D-galactopyranoside (ONPG) according to the method of Miller (1982). A unit of activity was defined as 1 μmol *o*-nitrophenol liberated per minute. Specific activity of the cro-β-galactosidase was reported as total units of enzyme per gram of total DW of the recombinant cells.

Other methods. The growth curve was determined by measuring the turbidity of the culture medium at 600 nm against a blank of sterile culture medium and by obtaining the cells DW. Drying was performed at 105° C to constant weight.

Glucose concentration in the medium was analysed by HPLC using refractive index detection. A Shodex Ionpak S801 (8*500) column (Macherey Nagel, Düren, FRG) was employed at 75° C and 2 m*M* H_2SO_4 was used as the eluent at a flow rate of 1.0 ml/min.

The concentration of amino acids were determined by HPLC with the *o*-phthaldialdehyde/β-mercaptoethanol precolumn derivatization method (Qureshi et al. 1984).

The dissolved-oxygen probe was of the polarographic type (Ingold, Urdorf). The medium saturated with air or nitrogen was defined as 100% or 0% dissolved oxygen, respectively.

Results and discussion

The usual derepression condition of the lambda promoter/cI857 repressor system is a temperature shift from 30° C to 42° C. Our observation is that "inclusion body" formation is prevented, and that high amounts of soluble and biologically-active product are found when the lambda cI857 repressor is only partially inactivated, and thus the lambda P_R-promoter is not fully induced. A cultivation temperature of 35° C allows the formation of soluble and biologically-active *cro-lac*Z fusion protein (manuscript in preparation).

When LB medium is supplemented with glucose, the concentration of dissolved oxygen is crucial for the attainable intracellular product concentration. The following figures document the results of an aerobic batch fermentation at 35° C in LB medium supplemented with 1% glucose. Figure 1a shows the courses of cell growth, intracellular accumulation of product, and concentration of dissolved oxygen. The growth and production phases were separate. Specific product formation occurred in two steps. The first, smaller step occurred when the growth rate decreased, the concentration of dissolved oxygen

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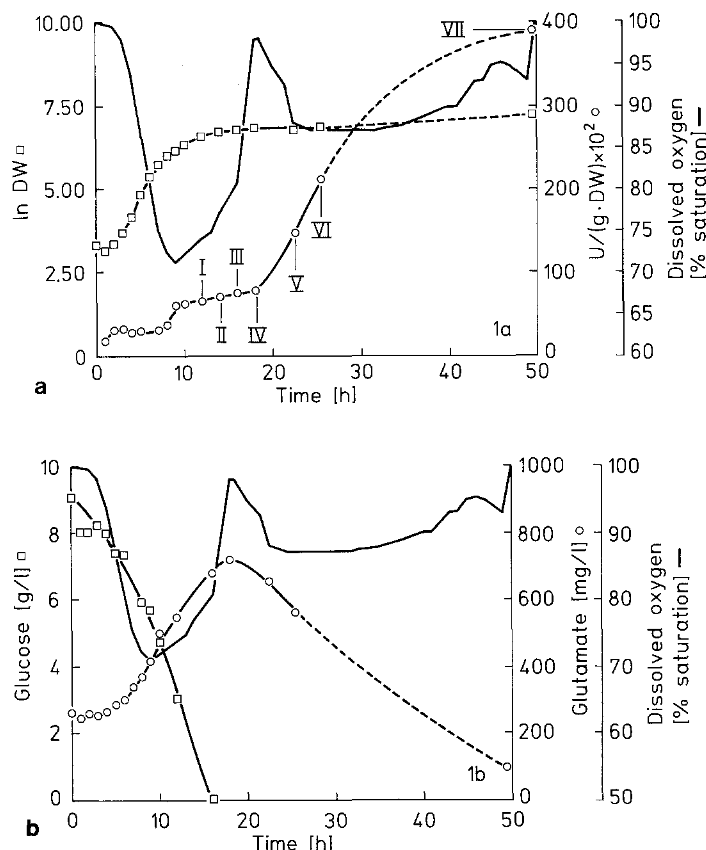

Fig. 1a, b. Growth, intracellular concentration of biologically active *cro-β*-galactosidase, and by-product formation and degradation of recombinant cells during aerobic growth on LB medium supplemented with 1% glucose. The *lines without symbols* represent the concentration of dissolved oxygen in the culture fluid (% saturation). **a.** Cell growth is given as the natural logarithm of the recombinant cells dry weigth (ln DW) —⊡—. Specific product concentration is shown as total units of *β*-galactosidase activity per total dry weight (U/g DW) —⊙—. *Roman numerals* correspond to the protein gel electrophoresis of total cell extracts in Fig. 3. **b.** The concentration of glucose (g/l) —⊡— and glutamate (mg/l) —⊙— in the culture fluid of the recombinant cells

reached a minimum, and amino acids, such as serine and aspartic acid had already been consumed (Fig. 2a, b). The second step occurred when the concentration of dissolved oxygen reached a maximum and the consumption of glutamic acid began (Fig. 1a, b).

Glutamic acid seems to be the key compound in this process. On the one hand, it is formed as a by-product of incomplete glucose oxidation, while on the other hand, it is used as a carbon source when substrates such as serine, aspartic acid, glucose, and threonine are already consumed (Figs. 1a, 2a, b). The beginning of glutamic acid consumption did not immediately follow the disappearance of glucose from the culture medium. Instead there was a delay of about 2 h (Fig. 1b), and the organisms did not start to degrade glutamic acid before the amino acid threonine was consumed (Fig. 2a).

The aerobic formation of glutamic acid during growth on glucose (in the presence of ammonium) indicates that the formation of 2-ketoglutarate dehydrogenase, the key enzyme of the citric acid cycle, was repressed (Yamada et al. 1972; Gray et al. 1966; Amarasingham and Bernhard 1965). As long as the organisms can generate sufficient amounts of ATP by substrate phosphorylation, and the citric acid cycle is not necessary for the synthesis of cell metabolites (since they are present as components of the complex medium), the organisms do not need an active Krebs cycle and the formation of these enzymes is repressed (Gray et al. 1966; Amarashingham and Bernhard 1965).

When the cell metabolism changed, and the Krebs cycle was activated (indicated by the onset of glutamic acid degradation), formation of recombinant protein increased significantly (Fig. 1a, b). The formation and degradation of acetate, another by-product of incomplete glucose oxidation, are correlated with the accumulation of the cro-*β*-galactosidase fusion protein in a similar manner (Kracke-Helm, unpublished data). Ace-

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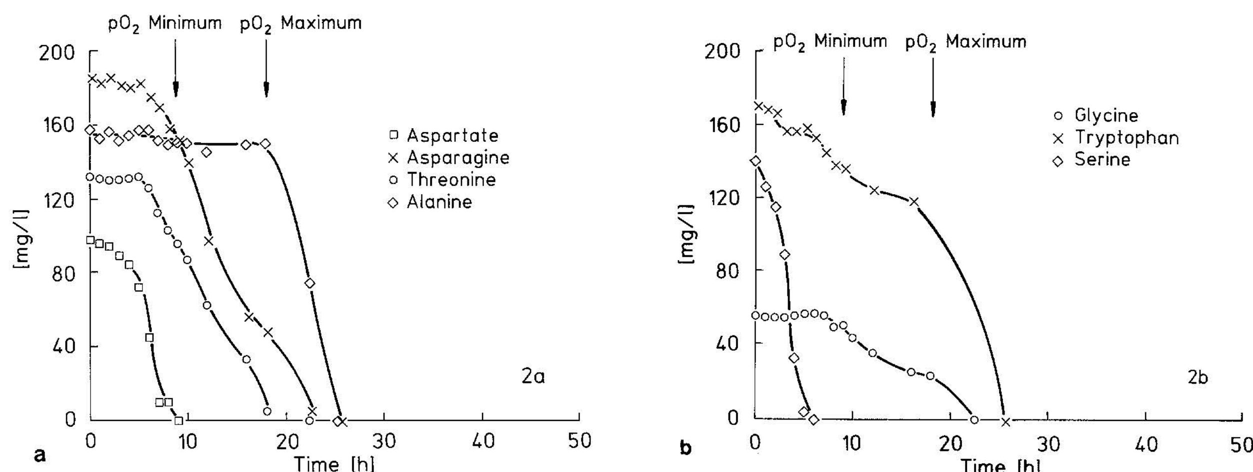

Fig. 2a, b. The concentration of amino acids in the culture fluid during aerobic growth of the recombinant cells on LB medium supplemented with 1% glucose. *Arrows* indicate the cultivation times when the concentration of dissolved oxygen reached minimum and maximum values. **a.** Aspartic acid, asparagine, threonine, and alanine. **b.** Glycine, tryptophan, and serine

tate, like glutamate, can only be degraded via the Krebs cycle.

When cells grow on substrates which can only be utilized via the citric acid cycle and respiration, it is evident that the oxygen supply to the cells becomes the critical variable in the fermentation process. Oxygen-limiting conditions result in an accumulation of metabolic by-products which cannot be degraded, and, consequently, in a low intracellular concentration of recombinant protein. When oxygen was not the limiting substrate, cells accumulated the product in quantities up to 25% of the total cell protein (Fig. 3), even though the lambda P_R-promoter was not fully induced.

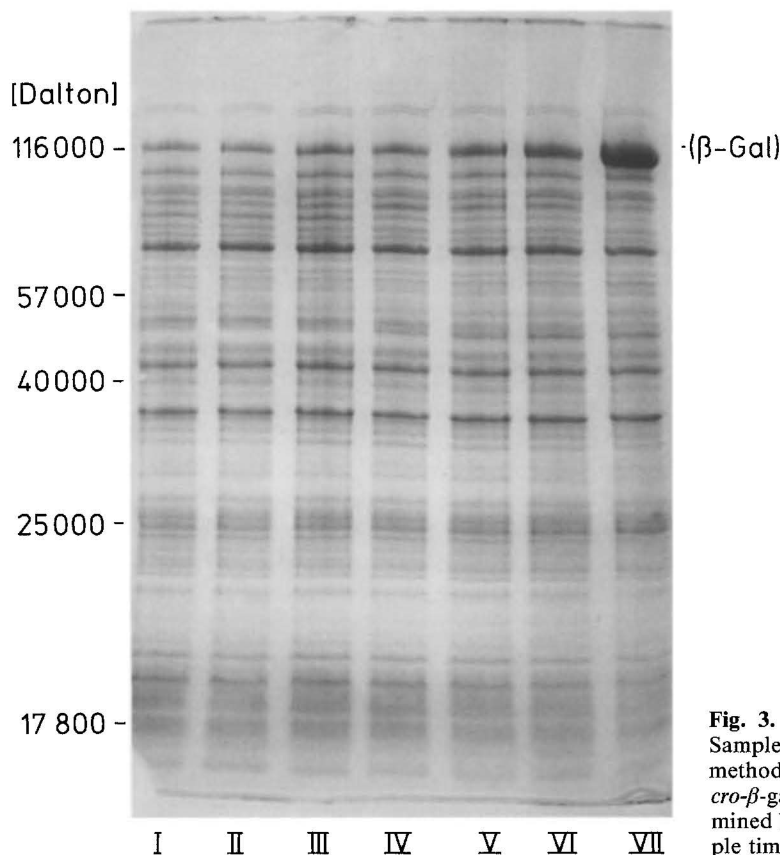

Fig. 3. Protein gel electrophoresis of total cell extracts. Samples were prepared as described in Materials and methods. After 50 h cultivation the intracellular level of *cro-β*-galactosidase reached 25% of total cell protein (determined by scanning). The *Roman numerals* refer to the sample times shown in Fig. 1a

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The intracellular accumulation of the recombinant *cro-β*-galactosidase in the stationary growth phase is in part explained by an increase in plasmid copy number with decreasing growth rate, although a simple correlation between plasmid copy number and product expression does not exist (Rinas 1987). Changes in the transcription level of the product and/or repressor gene and/or changes of the translation level of product and/or repressor mRNA may be other possible reasons to explain the observed effect.

In conclusion utilization of glucose as a substrate in recombinant DNA fermentations in order to achieve high amounts of cell mass *and* high intracellular product concentrations requires a fermentation strategy that facilitates the degradation of metabolic by-products that are formed by incomplete glucose oxidation. Oxygen-limiting conditions should be avoided, and, when controlling the glucose feed rate in fed-batch culture, the measurements of dissolved oxygen, glucose, and especially of by-product concentration should be used.

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