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A STUDY OF THE EFFECT OF SPECIFIC GROWTH RATE AND ACETATE ON RECOMBINANT PROTEIN PRODUCTION OF *ESCHERICHIA COLI* JM107

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ABSTRACT

The growth of *Escherichia coli* in fed-batch and continuous culture is examined and results show that α-amylase production is strongly dependent on specific growth rate (dilution rate) of the culture and production is greatest at an intermediate rate. Using continuous culture, it has also been found that the presence of acetate above a certain concentration reduces both μ_{max}, and the production of recombinant protein.

INTRODUCTION

Culture pH, temperature and dissolved oxygen are among the variables which affect growth and recombinant protein production in *Escherichia coli*; each is routinely measured and controlled to optimum values. Now that monitoring techniques are more advanced, other variables such as specific growth rate, and the concentrations of nutrient sugars and excreted metabolites can also be controlled. However, before implementing any control strategy, it is important to know what effects these variables might have on a fermentation system.

E. coli cells excrete acetate when they grow rapidly or when the medium contains an excess of sugar. Holms (1986) suggested that the production of acetate enables the cells to grow faster, by partially uncoupling glycolysis and the TCA cycle. Whatever the reason, acetate is an undesirable fermentation product. It disrupts the proton motive force, and inhibits the growth of the cells and their ability to produce recombinant protein (Sun *et al.* 1993; Luli *et al.* 1990; Yang 1992; Pan *et al.* 1987; George *et al.*1992). Furthermore, the effect of acetate on the growth of recombinant cells is more marked than on that of host cells (Koh *et al.* 1992). It is therefore important to devise a suitable control strategy, by limiting carbon source feeding, to keep acetate concentrations within acceptable limits.

Although the rate of growth and sugar concentration in the medium affect acetate production, they also have an effect on recombinant protein production which may be independent of the acetate concentration. Thus all the factors need to be examined together. A number of researchers have worked on the effect of specific growth rate (dilution rate) in continuous culture on the production of recombinant protein (Fu *et al.* 1993; Nancib *et al.* 1992; Brown *et al.* 1985; Park *et al.* 1990) and their results show that product yields were generally higher at

low dilution rates. In contrast, little has been reported on the control of specific growth rate in fed-batch culture.

This paper examines the role of specific growth rate and acetate concentration on the production of recombinant protein in *E. coli* fermentations, and demonstrates that the effect of these two variables is independent. The effect of acetate concentration on the maximum specific growth rate of the organism is also discussed.

MATERIALS AND METHODS

Organism:

The organism used in this study was a recombinant *Escherichia coli* JM107 carrying the high copy number plasmid pQR126 (Bahri and Ward, 1991). The latter contains the genes for α-amylase, which is secreted into the periplasm, and for resistance to kanamycin A, which was used for plasmid selection. The *lac* promoter which controls the α-amylase gene is subject to catabolite repression by glucose. For this reason, we used galactose as the carbon source in all fermentations.

Growth media

The basic medium used for all fermentations contained ($g.L^{-1}$):
$(NH_4)_2SO_4$, 10; Na_2HPO_4, 2.16; KH_2PO_4, 0.64; NaCl, 5; citric acid, 0.2; $FeSO_4.7H_2O$, 0.2; $MgSO_4.7H_2O$, 0.2; kanamycin, 0.01; thiamine, 0.1; trace element solution, $1mL.L^{-1}$.

The medium also contained the following trace elements ($g.L^{-1}$):
$CaCl_2$, 0.001; H_3BO_3, 0.004; $MnCl_2.4H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.002; $CuSO_4.5H_2O$, 0.0004; $CoCl_2.6H_2O$, 0.0004; $NaMoO_4.2H_2O$, 0.0002.

Galactose was added as described in the text.

All chemicals were obtained from the Sigma Chemical Company Ltd. The kanamycin A potency was a minimum of 750 $\mu g.mg^{-1}$. Thiamine was present as the hydrochloride.

Fermentation

Inoculum

An inoculum of 6.25% by volume, grown from plate colonies for 12 hours at 37°C, 200 rpm in an orbital incubator, was added to the fed-batch fermentations.

Fed-batch

Fed-batch fermentations were performed in a Chemap 14 L fermenter, with the monitoring and control of standard fermentation variables including pH to 7 using 3 M sodium hydroxide and temperature to 37°C. The culture was grown aerobically with dissolved oxygen tension (DOT) always greater than 20 %. Galactose (500 $g.L^{-1}$) was fed according to algorithms in a process control system described elsewhere (Gregory et al. 1993a, 1994).

Continuous culture

The continuous fermentation was performed in an LH 2000 series 2 litre fermenter. The fermentation medium used was that described above, supplemented with galactose (5 $g.L^{-1}$). The dilution rate was varied to set a number of different steady states allowing a minimum of 3-5 pot volumes before each steady state was sampled. At least three samples were taken at each steady state, and each sample was separated by at least half a pot volume ie. 750 mL diluent. In the experiment in which the effect of acetate on specific growth rate was determined, acetate was added as sodium acetate in concentrations 2, 5 and 8 $g.L^{-1}$ to the medium. In the experiment in which the effect of acetate on amylase production was determined, acetate was added as a single pulse of 7.4 $g.L^{-1}$ sodium acetate in 15 mL water.

Assay methods

Determination of maximum specific growth rate (μ_{max})

The dilution rate (D) was increased to approximately twice the expected value of μ_{max}, and the absorbance of the fermentation broth was measured at 600 nm every 15 minutes. If the natural logarithm of the optical density is plotted against time, the slope (m) is related to μ_{max} according to equation 1

$$\mu_{max} = D + m \quad (1)$$

Off-line monitoring and HPLC

Fermentations were sampled at intervals for measurement of their absorbance at 600 nm, their dry weight and their α-amylase activities. Galactose and acetate concentrations were measured in the samples after their separation on an HPLC system supplied by LDC and fitted with a refractive index detector. The analytes were eluted isocratically from a Biorad Aminex HPX-87H column with a mobile phase comprising 4mM H_2SO_4 at a flow rate of 0.65 $mL.min^{-1}$ at 50°C. The peak areas of acetic acid and galactose were compared to those from standard solutions.

On-line monitoring and control

Galactose and acetate were also measured automatically in the fed-batch fermentations using an on-line HPLC system which aseptically removes and centrifuges a sample before injecting it onto an HPLC column (Turner et al. 1993). The monitoring system was serially linked to a computer running LabView. This is a process control system which can program a feeding strategy and alter it in response to the on-line HPLC results (Gregory et al. 1993b). The system was programmed to feed galactose to cells during fed-batch fermentations at exponential rates to give constant specific growth rates of 0.1, 0.2 and 0.4 h^{-1}. Such rates can be obtained provided the cells are not overfed, the galactose input does not exceed the oxidative capacity of the cells, and the feed-pump is accurately calibrated and the initial biomass known. Galactose and acetate were monitored on-line throughout the experiments to ensure that the cells were not overfed. If either was detected then the algorithm was revised with a new lower estimate of biomass, thus reducing the galactose feed rate.

α-amylase assay

The assay for α-amylase was based on the decolourisation of starch-iodine complex. A sample (1 mL) of fermentation broth was centrifuged; the supernatant was assayed directly for any extracellular leakage of α-amylase, and the pellet, after treatment, was assayed for the bound periplasmic enzyme. The latter was released with 0.2 mL lysozyme buffer (sucrose 20% w/v, 1mM EDTA, 0.2 M tris buffer adjusted to pH7.5 and 0.5 $mg.mL^{-1}$ chicken egg white lysozyme obtained from Sigma) in which the pellet was resuspended at 22°C. After 10 minutes, 0.2 mL distilled water was added, and finally, after a further 10 minutes, the sample was centrifuged. The assays should be performed within 2 hours.

The α-amylase assay was adapted by Dr Carol French in the department of Biochemistry at UCL from the method described by Blanchin-Roland and Massen (1989). Samples of the supernatants were diluted to 0.5 mL with 15 mM.sodium phosphate buffer adjusted to pH 5.8, and were mixed with 0.5 mL of the same buffer containing 0.5 % soluble starch (obtained from Sigma). The experimental samples were incubated at 50°C. Portions (50 μL) were removed at timed intervals between 0.5 and 15 minutes and were mixed with 1 mL of a solution of iodine in potassium iodide (100 mL 2 % KI mixed with 0.2 mL 4.4 % KI containing 2 2 % I_2). The absorbances of the resulting mixtures were read at 620 nm.

A plot of A_{620} versus time will be linear if the correct sample dilution is used. Typical slopes range from 0.01 to 0.035 absorbance units per minute. One unit of α-amylase activity corresponds to a decrease in absorbance of 1 unit per minute.

RESULTS

The effect of specific growth rate on α-amylase production

Continuous culture

The rate at which recombinant *E. coli* cells produce α-amylase is dependent on the growth rate of the organism, which, in continuous culture, is equal to the dilution rate. The fastest production rate occurs at about 0.29 h^{-1} (figure 1) which is intermediate amongst the dilution rates tested. It is also well below the maximum specific growth rate (μ_{max}) above which washout occurs (0.53 h^{-1}). Indeed this latter value is increased to about 0.65 h^{-1} if the cells are grown at a higher dilution rate just below washout for an extended period.

Experiments at increasing dilution rates also show how fast the cells can grow without exceeding their oxidative capacity. As the rates are increased, this limit is characterised by their excretion of acetate when all the galactose fed to the culture is still consumed. When μ_{max} is 0.53 h^{-1} this limit is reached at a growth rate of about 0.5 h^{-1}.

Fed-batch culture

The galactose feed system was programmed with a control algorithm set to achieve specific growth rates of 0.1, 0.2 and 0.4 h^{-1} (see Methods). The actual specific growth rates estimated from optical density or dried biomass data, were held between 0.1 and 0.4 h^{-1} (table 1). The rate of production of amylase was greatest when the specific growth rate was controlled at 0.2

h^{-1} (table 1) and this high level of amylase production was maintained throughout the fermentation (figure 2).

It is difficult to compare rates of production in the fed-batch fermentations in which the cell concentration changes, with those in continuous culture where the concentration is fixed. However, at a biomass concentration in the fed-batch culture of about 2.5 $g.L^{-1}$, which is comparable with that in the continuous culture when it is fed with nutrients containing 5 $g.L^{-1}$ galactose, the maximum rates of amylase production appear to be similar in the two fermentations (compare figure 1 and table 1), although the precise specific growth rate at which the maximum occurs may be lower in the fed-batch fermentation.

The effect of acetate on cell growth

When a continuous culture of *E. coli* JM107 is diluted with feed at a rate sufficient to wash out the cells, the presence of acetate in the feed medium reduces the measured μ_{max}. When these experiments are conducted with normal medium, μ_{max} is about 0.65 h^{-1} (table 2). However, if the dilution medium contains 5 $g.L^{-1}$ acetate μ_{max} is reduced by about 12 % while at 8 $g.L^{-1}$, the reduction is over 50 %. A similar amount of sodium as sodium chloride had no effect, implying that the effects were due to the acetate alone.

The effect of acetate on α-amylase production

Acetate in high concentrations is known to reduce the production of recombinant proteins in *E. coli.* Since it is a metabolite which the organism tends to excrete at high growth rates it seemed that its effect might not be direct, but linked to growth. When some fed-batch fermentations of *E. coli* JM107 were fed an excess of galactose, the concentration of acetate rose as high as 7.4 $g.L^{-1}$. When this concentration was added as a single pulse to a continuous culture whose dilution rate was 0.3 h^{-1}, it unexpectedly caused an increase in the rate of cell growth, and after some 40 minutes, a low level of galactose (up to 0.1 $g.L^{-1}$) appeared in the medium (figure 3). The transitory fall in the growth rate which apparently occurs immediately after adding the acetate is probably due to the shock to the cells. It seems that then the cells temporarily used the acetate as a source of carbon for growth. After 5 hours, the acetate was either consumed or was diluted from the medium and the growth rate returned to its preset value (figure 3).

In contrast to its effect on growth, the added acetate almost completely inhibited the production of α-amylase; the amount of enzyme fell almost as rapidly as the theoretical rate of decline due to dilution of the culture (figure 4). Although the increase in growth rate to about 0.45 h^{-1} would decrease the rate of synthesis of the recombinant protein, it would still be some 35 % of synthesis at a growth rate of 0.3 h^{-1} (figure 1) and it might be more if the effect were only transient. Moreover, the production of α-amylase continues as before once the acetate concentration has fallen below about 1 g. L^{-1} even though the growth rate remains above 0.4 h^{-1} (figures 3 and 4). We believe that this demonstrates a direct effect of acetate on α-amylase production which is not mediated through its effect on the rate of cell growth.

	Controlled specific growth rate h^{-1}		
	0.1	0.2	0.4
Actual specific growth rate (optical density)	0.09	0.18	0.41
Actual specific growth rate (dry cell weight)	0.1	0.2	0.33
Specific production rate of α-amylase (units.mL^{-1}.min^{-1} .(gDCW.L^{-1})$^{-1}$.h^{-1})	0.09	3.4	1.2

Table 1. Specific growth rates set and those obtained based on dry biomass and optical density data in controlled fed-batch fermentations. Specific α-amylase production rates are given for each growth rate.

ACETATE CONCENTRATION ($g.L^{-1}$)	μ_{max} (h^{-1})	% REDUCTION IN μ_{max}
0	0.655	0
2	0.67	0
5	0.58	11.6
8	0.303	52.6

Table 2. Summary of experiment in which different concentrations of acetate (as sodium acetate) were added to the feed medium in washout experiments in continuous culture. In each case, cells were growing at μ_{max} when the medium was supplemented with acetate.

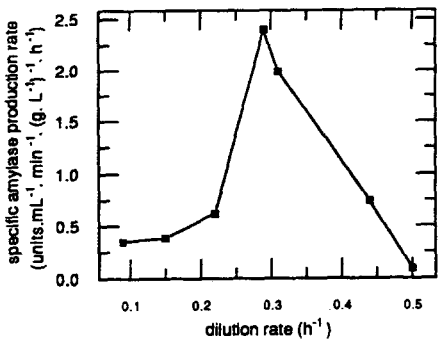

Figure 1. Specific amylase production rates of *E. coli* JM107 at different dilution rates for a continuous culture.

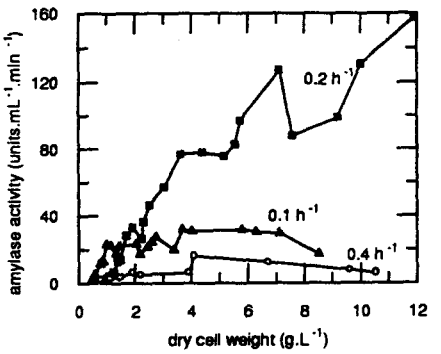

Figure 2. Amylase activity versus dry cell weight of samples taken during three fed-batch cultures. μs were controlled at 0.1, 0.2 and 0.4 h^{-1}.

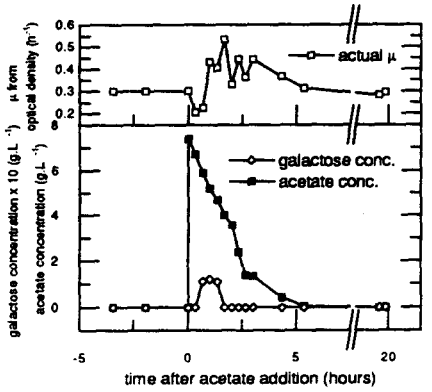

Figure 3. Acetate and galactose profiles of continuous culture after addition of acetate dose (7.4 $g.L^{-1}$). Plotted specific growth rate was calculated from the optical density increase and the known dilution rate of the culture.

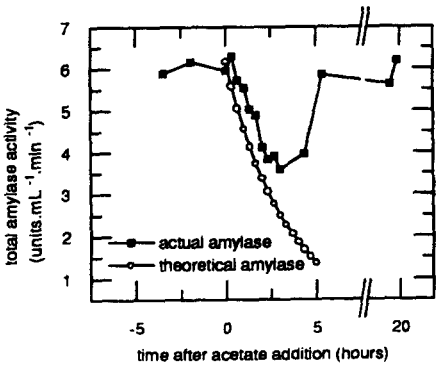

Figure 4. Amylase profile of a continuous culture after a dose of acetate (7.4 $g.L^{-1}$) had been added to the fermenter. Also plotted on graph is the amylase profile that would result due to dilution effects alone if no further amylase had been produced by the cells after the acetate addition.

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