Journal of Industrial Microbiology, 13 (1994) 225–232

Published by The Macmillan Press Ltd

# Glucose and acetate influences on the behavior of the recombinant strain *Escherichia coli* HB 101 (GAPDH)

A. Gschaedler, N. Thi Le and J. Boudrant

*Laboratoire des Sciences du Génie Chimique, CNRS, ENSAIA, 2 Avenue de la forêt de Haye, BP 172, F-54505 Vandoeuvre-les-Nancy, France*

(Received 6 December 1993; accepted 5 April 1994)

*Key words:* Fermentation; Recombinant *Escherichia coli*; Acetate inhibition; Glyceraldehyde-3-phosphate dehydrogenase

## SUMMARY

This study highlights data about the production of a recombinant protein (glyceraldehyde-3-phosphate dehydrogenase) by *E. coli* HB 101 (GAPDH) during batch and fed-batch fermentations in a complex medium. From a small number of experiments, this strain has been characterized in terms of protein production performance and glucose and acetate influences on growth and recombinant protein production. The present results show that this strain is suitable for recombinant protein production, in fed-batch culture 55 g $L^{-1}$ of biomass and 6 g $L^{-1}$ of GAPDH are obtained. However this strain, and especially GAPDH overproduction is sensitive to glucose availability. During fermentations, maximum yields of GAPDH production have been obtained in batch experiments for glucose concentration of 10 g $L^{-1}$, and in fed-batch experiments for glucose availability of 10 g $h^{-1}$ (initial volume 1.5 L). The growth of the strain and GAPDH overproduction are also inhibited by acetate. Moreover acetate has been noted as an activator of its own formation.

## INTRODUCTION

For many years the genetics of *Escherichia coli* has been extensively studied and vast knowledge has been accumulated. For this reason *E. coli* has become one of the most widely used microorganisms for expression of recombinant products. *E. coli* HB 101, obtained by Boyer and Roulland-Dussoix [1] during a study of the restriction and modification system in *E. coli*, is one of the first strains which has been able to accept a foreign DNA segment [1]. In the past this strain has been used quite extensively in both fundamental and applied research. For example Peretti et al. [23] used it in order to study the influence of the number of plasmid copies on its behavior. *E. coli* HB 101 was also used to produce recombinant proteins like β-galactosidase [25] and calf-prochymosine [14].

The recombinant strain used in this study, *E. coli* HB 101 (GAPDH), produces a homologous enzyme: glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In a similar system using *E. coli* C600galK as host, the biosynthesis of this enzyme was quite strong [7]. It remained soluble in the cell and had apparently no toxic effects. As in the above work, the carbon source used is glucose, whose metabolism in *E. coli* is well known [6]. In aerobic conditions with high glucose concentrations and high growth rate, organic acids are produced. These organic acids, mainly acetic acid, are growth inhibitors [16,17,22,24]. Koh et al. [15] studied acetate inhibition of several *E. coli* strains, recombinant or not, including among them *E. coli* HB 101. They showed that acetate inhibition is more significant on the recombinant strains, and in a defined medium, than in a complex medium. The production of recombinant protein might also be affected by acetate [3] or by glucose. However the effect of glucose on the biosynthesis rate of the recombinant has rarely been described. In this study our objectives were to examine the influence of the main carbon source, glucose, and product of the fermentation, acetate, on the behavior of the strain and especially on the production of the recombinant enzyme.

Correspondence to: J. Boudrant, Laboratoire des Sciences du Génie Chimique, CNRS, ENSAIA, 2 Avenue de la forêt de Haye, BP 172, F-54505 Vandoeuvre-les-Nancy, France.

## MATERIALS AND METHODS

### *Microorganism and plasmid*

*Escherichia coli* HB 101 ($pro^-$, $leu^-$, $thi^-$, $recA^-$) kindly provided by Dr C. Branlant (University of Nancy I, Vandoeuvre-les-Nancy, France) was used as host strain. This strain was transformed with the plasmid pBR Eco gap [2]. This plasmid derives from pBR 322 by insertion of the glyceraldehyde-3-phosphate dehydrogenase gene under the control of its own promoters into the tetracycline gene. So the genetically-modified strain is only ampicillin resistant. The strain was stored on LB (Luria Broth) medium containing glycerol (15%) at −80 °C. It was reactivated by subculture in LB medium in Erlenmeyer flasks. Subcultures and cultures were performed in the complex medium M2 described below.

### *Media*

Two fermentation media were used. LB medium containing bactopeptone (Biokar, Prolabo, Paris, France) 10 g $L^{-1}$, yeast extract (Fould Springer, Maison-Alfort, France) 5 g $L^{-1}$

and NaCl 5 g $L^{-1}$ and a complex medium M2 which contains bactotryptone (Biokar) 10 g $L^{-1}$, yeast extract 10 g $L^{-1}$, casaminoacids (Difco, OSI, Maurepas, France) 4 g $L^{-1}$, NaCl 5 g $L^{-1}$, $K_2HPO_4$ 4 g $L^{-1}$, $KH_2PO_4$ 1 g $L^{-1}$, $(NH_4)_2SO_4$ 2 g $L^{-1}$, $MgSO_4$ 0.1 g $L^{-1}$, $FeSO_4$ 0.05 g $L^{-1}$, histidine (Fluka, Mulhouse, France) 0.1 g $L^{-1}$, leucine (Fluka) 0.1 g $L^{-1}$, thiamine (Fluka) 0.1 g $L^{-1}$ and glucose at various concentrations.

The composition of the feeding solution for fed-batch fermentations is: yeast extract 100 g $L^{-1}$, casaminoacids 80 g $L^{-1}$, $KH_2PO_4$ 4 g $L^{-1}$, $K_2HPO_4$ 1 g $L^{-1}$, histidine 0.1 g $L^{-1}$, leucine 0.1 g $L^{-1}$, proline 0.1 g $L^{-1}$, thiamine 0.1 g $L^{-1}$ and glucose at various concentrations.

*Fermentations*

Batch and fed-batch fermentations were performed in a 3-L jar fermentor (Interscience, Saint-Nom-la-Bretèche, France) in the presence of different glucose concentrations with a 1.5-L working volume. The fermentor was equipped for control of pH, dissolved oxygen and temperature. The agitation speed was 600 r.p.m. and the aeration rate was 2 v.v.m. The pH was maintained at 7 by the addition of 3 N ammonium hydroxide and 4 N hydrochloric acid. The temperature was maintained at 37 °C and the percentage of dissolved oxygen at 40% of saturation. Inocula were prepared in 150-ml Erlenmeyer flasks (50 ml per flask).

In batch systems, the effect of initial glucose concentration (4 g $L^{-1}$, 10 g $L^{-1}$ and 20 g $L^{-1}$) was studied in the complex medium M2. Fed-batch fermentations were initiated batch-wise with the complex medium M2 containing 10 g $L^{-1}$ of glucose. The feeding was performed with complex solutions containing 200 g $L^{-1}$, 250 g $L^{-1}$ or 400 g $L^{-1}$ of glucose at a flow rate of 0.04 L $h^{-1}$. These feedings correspond to glucose additions of 8 g $h^{-1}$, 10 g $h^{-1}$ and 16 g $h^{-1}$.

Some experiments were performed in Erlenmeyer flasks, in studies of the effect of initial acetate concentration on growth and on acetate production. For this, *E. coli* HB 101 (GAPDH) was grown in M2 medium (10 g $L^{-1}$ glucose) containing different initial acetate concentrations (0 g $L^{-1}$, 5.9 g $L^{-1}$, 11.8 g $L^{-1}$ and 23.6 g $L^{-1}$).

All experiments were performed with a selection pressure (ampicillin 0.1 g $L^{-1}$). Batch and fed-batch fermentations were performed in each case in duplicate.

*Analysis*

Optical density determined with a Novaspec II spectrophotometer (LKB Pharmacia, Saint-Quentin-Yvelines, France) at 660 nm was converted to dry cell mass using a calibration curve. The glucose concentration was determined with a Technicon Autoanalyzer (Domont, France) using the hexokinase method. A gas chromatograph with a Porapack Q column (Intersmat, Courtry, France), equipped with a flame ionization detector (Delsi, Suresnes, France), was used for the determination of acetic acid. GAPDH activity was determined from the absorbance of NADH formed at 340 nm [8]. Enzymatic activities were determined in supernatant fluid obtained from centrifugation (20000 × *g*) of sonicated cells. Sonication was performed at 4 °C for 4 × 30 s. The amount of GAPDH in the supernatant fluid was calculated using the specific activity determined for pure GAPDH: 350 U $mg^{-1}$ at 37 °C [19].

## RESULTS

*Influence of initial glucose concentration*

*Kinetic behavior of* E. coli *HB 101 (GAPDH).* The changes of biomass, glucose, acetate and GAPDH concentrations observed in these fermentations are shown in Fig. 1. Growth profiles differed with initial glucose concentration in the medium. When this concentration was equal to 20 g $L^{-1}$, the initial growth rate was lower than for the two other concentrations. At 10 g $L^{-1}$ of glucose the growth rate was initially the highest, but decreased after 6 h of fermentation. At 4 g $L^{-1}$ of glucose, the stationary phase was reached after 8 h of culture.

The maximum specific growth rates at the different initial glucose concentrations (10 g $L^{-1}$, 4 g $L^{-1}$ and 20 g $L^{-1}$) are 0.8 $h^{-1}$, 0.6 $h^{-1}$ and 0.4 $h^{-1}$, respectively. These values show that the initial glucose concentration has a significant effect on the maximum specific growth rate.

The changes in acetate concentration indicate two different behaviors (Fig. 1). When the initial glucose concentration was below 20 g $L^{-1}$, a low biosynthesis, followed by a utilization after glucose exhaustion, was observed. Maximum acetate concentrations of 1.2 g $L^{-1}$ and 1.65 g $L^{-1}$ for 4 g $L^{-1}$ and 10 g $L^{-1}$ glucose concentration, respectively, were obtained after 6 h of fermentation. When the initial glucose concentration was 20 g $L^{-1}$, acetate production was higher and reached 6 g $L^{-1}$, no utilization of the acetate produced was observed at the end of the culture. As for acetate production, the recombinant GAPDH biosynthesis varied considerably with initial glucose concentration. At 4 g $L^{-1}$ initial glucose concentration, biosynthesis was growth-associated. At 10 g $L^{-1}$, a maximum of 300 mg $L^{-1}$ was produced after 7 h of culture, and a decrease of GAPDH concentration simultaneous with the decrease in growth rate was observed. When the initial glucose concentration was 20 g $L^{-1}$, a concentration of 50 mg $L^{-1}$ of GAPDH was obtained after 10 h of fermentation.

*Specific rates of glucose and acetate consumption and GAPDH overproduction.* The specific glucose consumption, acetate and GAPDH overproduction rates have been calculated and plotted with respect to time (Fig. 2). The specific glucose consumption rate profiles have similar shapes but the rates were higher for high glucose concentrations. As for the acetate production rate, two behaviors were observed. At the two lowest initial glucose concentrations (4 g $L^{-1}$ and 10 g $L^{-1}$), the rates decreased as a function of time. But, when this concentration was 20 g $L^{-1}$, this rate increased first sharply, reaching a maximum at 4 h and then decreased to zero at 8 h of fermentation. The specific rate of glucose consumption showed a shoulder when the specific acetate production rate was the highest. For the specific GAPDH production rate, the results were quite different. At 10 g $L^{-1}$

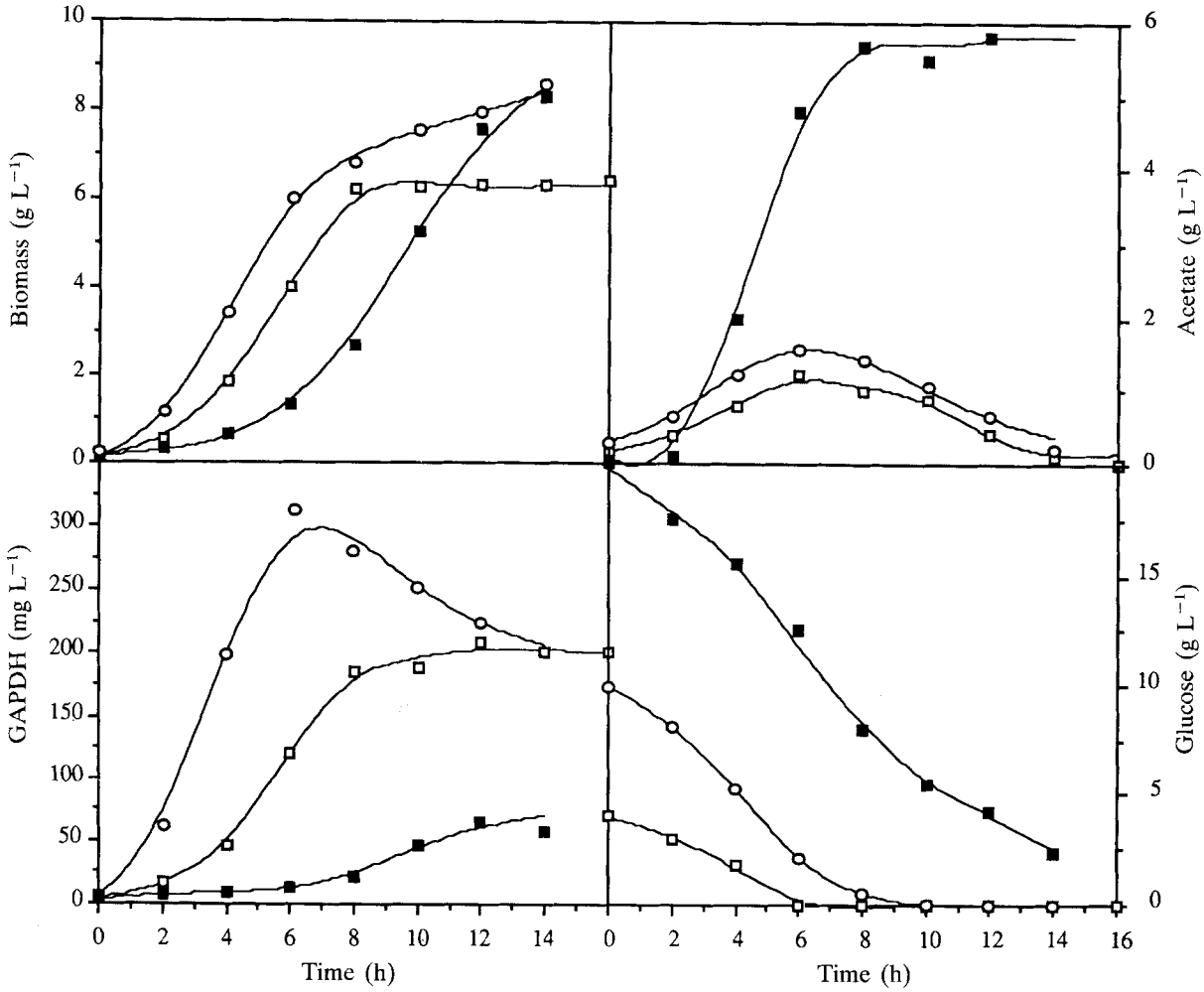


Fig. 1. Kinetics of growth, acetate and GAPDH production and glucose consumption of recombinant *E. coli* HB 101 (GAPDH) during batch fermentations performed in complex media containing different glucose concentrations. Glucose concentrations are: 4 g L$^{-1}$—□—, 10 g L$^{-1}$ —○— and 20 g L$^{-1}$ —■—.

initial glucose concentration, the specific GAPDH production rate was higher than that at 4 and 20 g L$^{-1}$ of glucose. At 4 and 20 g L$^{-1}$ of glucose, its specific production rate was low and almost stationary, whereas at 10 g L$^{-1}$ of glucose, this specific production rate started to decrease immediately. The values of the apparent maximum production yields of biomass, GAPDH and acetate on glucose ($R_{X/S}$, $R_{GAP/S}$, $R_{Ac/S}$) are given in Table 1. The apparent maximum production yields of GAPDH and acetate on biomass ($R_{GAP/X}$, $R_{Ac/X}$) are presented in the same table. $R_{GAP/S}$ sharply decreased when initial glucose concentration was greater than 10 g L$^{-1}$ and $R_{GAP/X}$ reached a maximum around this concentration.

*Influence of glucose availability during fed-batch fermentations*

Biomass, glucose, acetate and GAPDH kinetic evolutions are shown in Fig. 3. At the two lowest concentrations, (200 g L$^{-1}$ and 250 g L$^{-1}$) glucose was completely utilized during feeding and high biomass concentrations were obtained, 40 g L$^{-1}$ and 55 g L$^{-1}$, respectively. GAPDH biosynthesis was parallel to growth and at the end of the culture 4 g L$^{-1}$ and 6 g L$^{-1}$ of GAPDH were obtained for 200 g L$^{-1}$ and 250 g L$^{-1}$ of glucose, respectively, in the feed solution. At the highest glucose concentration (400 g L$^{-1}$), simultaneous glucose and acetate accumulations were observed in the culture medium. Very low biomass and GAPDH concentrations were obtained at the end of the fermentation.

The values of the apparent maximum production yields of GAPDH and biomass on glucose ($R_{X/S}$, $R_{GAP/S}$) and of the apparent maximum production yield of GAPDH on biomass ($R_{GAP/X}$) are given in Table 1. For these three yields, the highest values were obtained when glucose concentration in the feeding solution was 250 g L$^{-1}$. When it was 400 g L$^{-1}$ the yields decreased strongly.

*Influence of initial acetate concentration*

Growth decreased with increased acetate concentration (Fig. 4), and the maximum specific growth rate varied as indicated in Fig. 5, which shows a linear relationship between initial acetate concentration and this rate. The acetate produced during these fermentations was also measured.

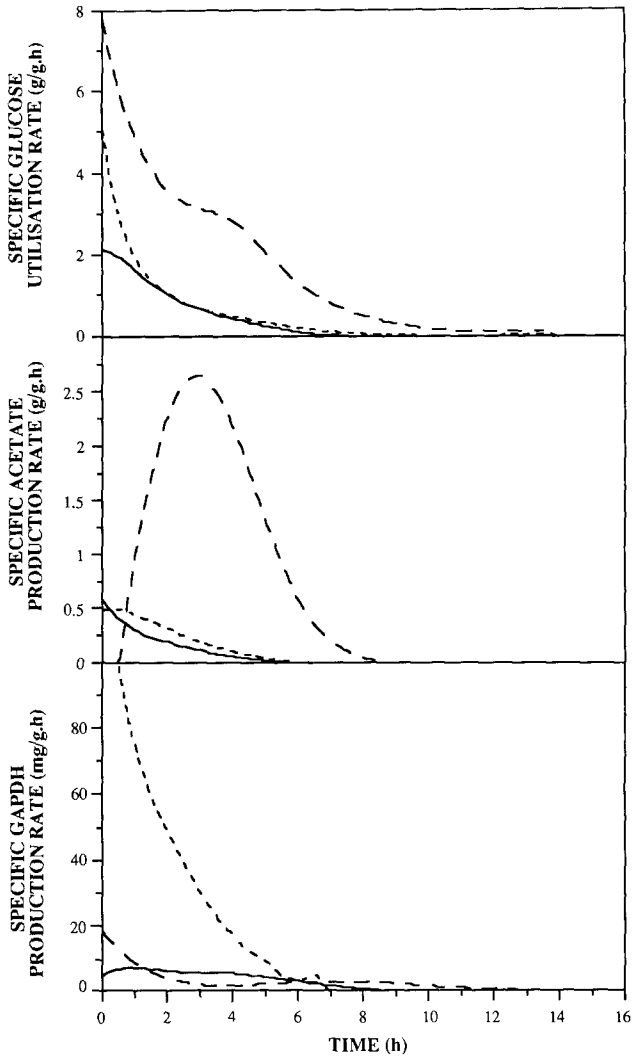


Fig. 2. Changes in specific glucose utilization rate, specific acetate production rate and specific GAPDH production rate of recombinant *E. coli* HB 101 (GAPDH) during batch fermentations performed in complex medium containing different glucose concentrations. Glucose concentrations are: 4 g $L^{-1}$ ——, 10 g $L^{-1}$ – – – and 20 g $L^{-1}$ — — —.

The results indicated in terms of acetate production yield on biomass ($R_{Ac/X}$) have been calculated and are indicated in Table 2. Although growth was considerably lowered, $R_{Ac/X}$ increased strongly: when initial acetate concentration was 6 g $L^{-1}$ and 12 g $L^{-1}$, the value of $R_{Ac/X}$ was twice and five times more than that obtained without acetate, respectively. These results were obtained in the partly oxygen-limited conditions corresponding to the use of Erlenmeyer flasks, which are more favorable to acetic acid biosynthesis than a fully aerated fermentor.

## DISCUSSION

Clearly, fed-batch cultures gave higher concentrations of biomass than batch cultures, while biomass production yields on glucose, were greater in the latter than in fed-batch fermentation. The amount of GAPDH produced in fed-batch cultures was high (6 g $L^{-1}$) and its production yields on glucose or on biomass were also greater than in batch cultures.

TABLE 1

Influence of initial glucose concentration in batch fermentations and of glucose concentration in the feeding medium during fed-batch fermentations on the maximum apparent yields of biomass production on glucose ($R_{X/S}$), of GAPDH on glucose ($R_{GAP/S}$), of GAPDH on biomass ($R_{GAP/X}$), of acetate on glucose ($R_{ACE/S}$) and of acetate on biomass ($R_{ACE/X}$). The different cultures were performed with the strain *E. coli* HB 101 (GAPDH)

| | Batch fermentation | | | Fed-batch fermentation | | |
|---|---|---|---|---|---|---|
| | Initial glucose concentration (g $L^{-1}$) | | | Glucose concentration in the feeding solution (g $L^{-1}$) | | |
| | 4 | 10 | 20 | 200 | 250 | 400 |
| $R_{X/S}$ (g $g^{-1}$) | 0.95 | 0.725 | 0.46 | 0.4 | 0.45 | 0.024 |
| $R_{GAP/S}$ (g $g^{-1}$) | 0.03 | 0.04 | 0.004 | 0.06 | 0.062 | 0.004 |
| $R_{GAP/X}$ (g $g^{-1}$) | 0.03 | 0.05 | 0.008 | 0.084 | 0.13 | 0.07 |
| $R_{ACE/S}$ (g $g^{-1}$) | 0.30 | 0.20 | 0.40 | ND[a] | ND | ND |
| $R_{ACE/X}$ (g $g^{-1}$) | 0.28 | 0.26 | 2.26 | ND | ND | ND |

[a]ND = not determined.

With simple culture conditions (without oxygen enrichment and with a constant feed rate) we obtained high biomass concentrations (55 g $L^{-1}$) and a large amount of GAPDH (6 g $L^{-1}$). These data are comparable with others mentioned in the literature [27]; for example Jung et al. [13], using recombinant *E. coli* K12 and *E. coli* B, obtained 55 g $L^{-1}$ of biomass and 2.2 g $L^{-1}$ of soluble recombinant protein (interleukin 1-$\beta$). However, in this case the air was enriched with oxygen and glucose feeding was modulated in order to maintain the acetate concentration below 0.4 g $L^{-1}$.

The influence of glucose is considered here only in the case of low acetate production (acetate concentrations less than 2 g $L^{-1}$). Indeed, with a high acetate concentration in the medium (>4 g $L^{-1}$), inhibition occurred as discussed below. In batch cultures with low initial glucose concentrations (4 g $L^{-1}$ and 10 g $L^{-1}$) acetate production was similar and the yields of acetate production on biomass were identical (Table 1). But the biosynthesis of GAPDH and its specific production rate were much higher at 10 g $L^{-1}$ than at 4 g $L^{-1}$ of initial glucose concentration (Figs 1 and 2). Production of the recombinant enzyme corresponds to a complementary energy burden used for plasmid replication, transcription and translation. From the results of batch fermentations indicated in Table 1, it is clear that a larger proportion of glucose was used for recombinant enzyme production when glucose concentration was increased. In fact the apparent biomass and GAPDH production yields on glucose were weaker and larger, respectively, at 10 g $L^{-1}$

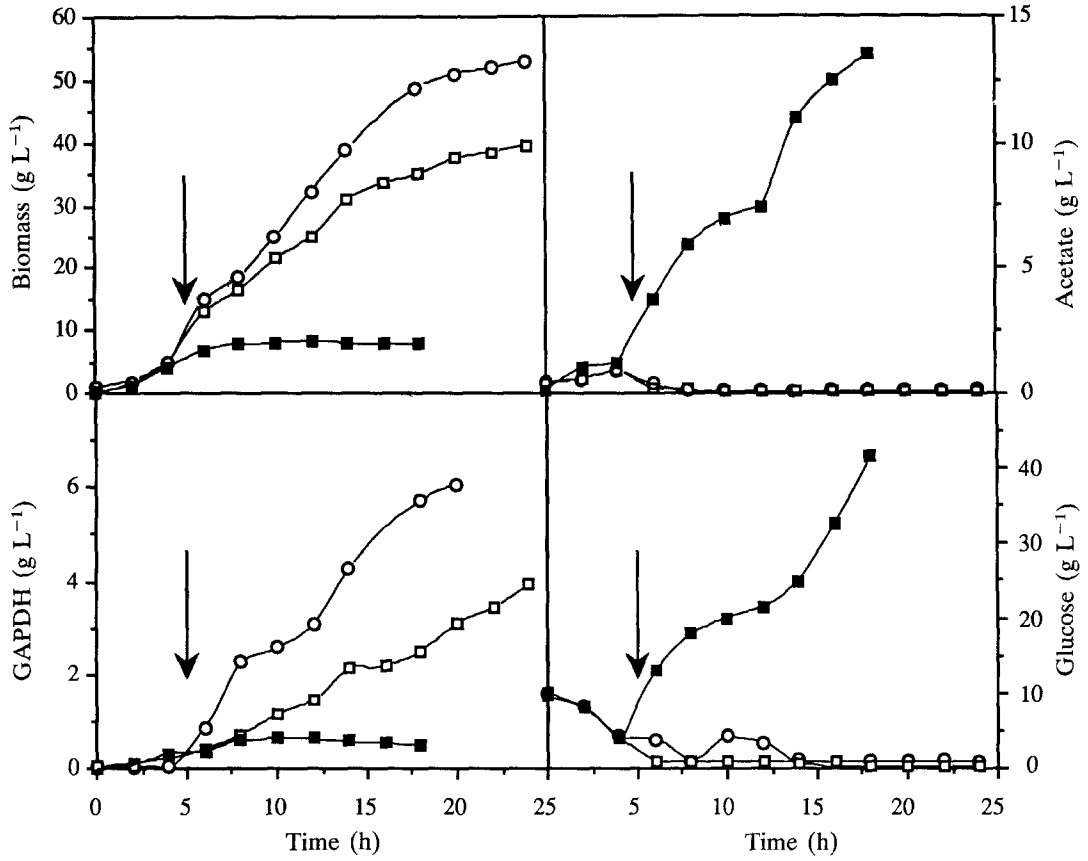


Fig. 3. Kinetics of growth, acetate and GAPDH production and glucose consumption of recombinant *E. coli* HB 101 (GAPDH) during fed-batch fermentations. The first phase was performed with 1.5 L of M2 medium (10 g $L^{-1}$ of glucose). The second phase (from the arrow) involved feeding M2 medium at a constant flow rate (0.04 L $h^{-1}$) with different glucose concentrations. Glucose concentrations are: 200 g $L^{-1}$ —□—, 250 g $L^{-1}$ —○— and 400 g $L^{-1}$ —■—.

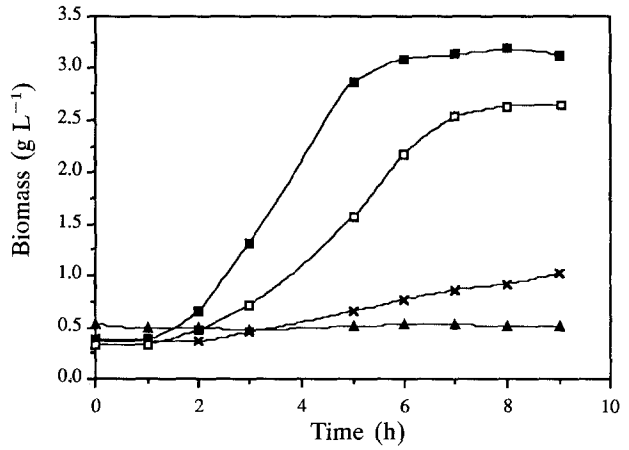


Fig. 4. Growth curves of *E. coli* HB 101 (GAPDH) during cultures in Erlenmeyer flasks at different initial acetate concentrations. Acetate concentrations are: 0 g $L^{-1}$ —■—, 5.9 g $L^{-1}$ —□—, 11.8 g $L^{-1}$ —×— and 23.6 g $L^{-1}$ —▲—.

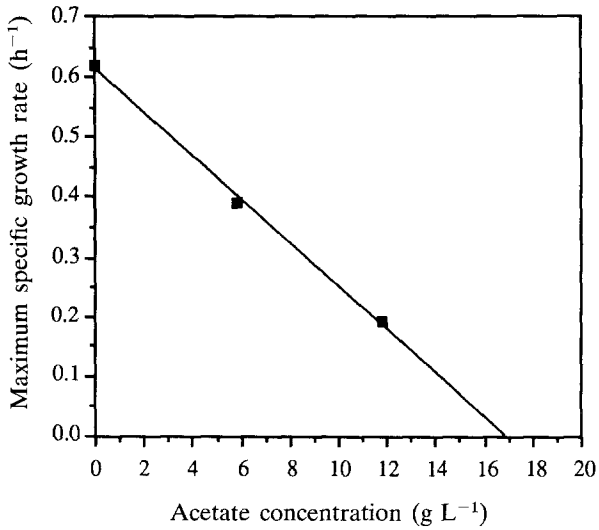


Fig. 5. Maximum specific growth rate as a function of acetate concentration for cultures in Erlenmeyer flasks with different initial acetate concentrations.

of glucose than at 4 g $L^{-1}$. This phenomenon is probably related to promoter utilization. In fact, Charpentier and Branlant [4] reported that the GAPDH gene can be transcribed from four different promoter sequences. The

TABLE 2

Influence of acetate initial concentration on the apparent yield of acetate production on biomass for cultures of the *E. coli* HB 101 (GAPDH) performed in Erlenmeyer flasks

| Acetate concentration (g $L^{-1}$) | 0 | 5.9 | 11.8 |
|---|---|---|---|
| $R_{ACE/X}$ (g $g^{-1}$) | 1.56 | 3 | 7.54 |

promoters are differently involved as a function of environmental conditions. We obtained similar results in experiments implying different glucose availabilities [Gschaedler et al., 1994, unpublished results]. For fed-batch fermentations, the GAPDH production yield on biomass also remained higher when using a 250 g $L^{-1}$ glucose feeding solution than a 200 g $L^{-1}$ solution. These results show the existence of an optimum glucose concentration allowing a higher production of the recombinant enzyme. This might be related to glucose influences on the promoters. However this kind of result does not appear general. Indeed Nancib et al. [21] carried out the same kind of studies in batch culture with the *E. coli* C600galK producing GAPDH. The apparent production yields obtained in this study are shown in Table 3. The apparent maximum production yields of GAPDH on biomass remained basically at the same value with the four different initial glucose concentrations used.

During batch fermentations, the highest acetate production corresponded to the highest initial glucose concentration (20 g $L^{-1}$) (Fig. 1). On the other hand, at 10 g $L^{-1}$ and 4 g $L^{-1}$ of initial glucose concentration, acetate production remained less than 2 g $L^{-1}$. Moreover, growth is not apparently slowed down (Fig. 1). During fed-batch fermentations, the influence of glucose concentration in the feeding solution is similar. When it is equal to 400 g $L^{-1}$, glucose accumulated in the medium, growth decreased and simultaneously acetate was produced in large quantities. High acetate concentration corresponded to a decrease of the maximum apparent yield of biomass noted in Table 1. This might be explained by acetate inhibition, shown clearly on the growth of *E. coli* HB 101 (GAPDH) in Fig. 5.

TABLE 3

Influence of initial glucose concentrations during batch fermentations on the maximum appparent yield of biomass production on glucose ($R_{X/S}$), of GAPDH on glucose ($R_{GAP/S}$), of GAPDH on biomass ($R_{GAP/X}$), of acetate on glucose ($R_{ACE/S}$) and of acetate on biomass ($R_{ACE/X}$). The different cultures were performed with the strain *E. coli* C600galK (GAPDH) on the complex medium in the same conditions as those described for *E. coli* HB 101 (GAPDH) (data from Nancib et al. [21])

| | Batch fermentation Initial glucose concentration (g $L^{-1}$) | | | |
|---|---|---|---|---|
| | 10.5 | 19.2 | 30 | 56.5 |
| $R_{X/S}$ (g $g^{-1}$) | 0.93 | 0.7 | 0.6 | 0.4 |
| $R_{GAP/S}$ (g $g^{-1}$) | 0.035 | 0.023 | 0.022 | 0.014 |
| $R_{GAP/X}$ (g $g^{-1}$) | 0.037 | 0.033 | 0.037 | 0.036 |
| $R_{ACE/S}$ (g $g^{-1}$) | 0.48 | 0.34 | 0.26 | 0.18 |
| $R_{ACE/X}$ (g $g^{-1}$) | 0.51 | 0.48 | 0.45 | 0.45 |

During batch and fed-batch fermentations, when acetate was present in large quantities in the culture medium, GAPDH production and its production yield on biomass and on glucose also decreased (Figs 1 and 3, Table 1). Brown et al. [3] also mentioned a decrease in productivity of interferon production by several recombinant *E. coli* strains due to the presence of acetate. Similarly in our case, acetate production affected both growth and production of the recombinant protein.

Acetate formation in aerobic cultures in the presence of an excess of glucose and at high growth rates (Crabtree effect) is well known [5,9,12]. Its production mechanism has been studied and several theories have been developed. The most recent, given by Han et al. [11] suggested a limitation of the tricarboxylic acid cycle, causing a transitory accumulation of acetyl CoA which is transformed into acetate. According to these authors acetic acid can generate more ATP and NADPH than other organic acids. An explanation of the inhibition mechanism of growth by acetate has been proposed by Smirnova and Oktyabr'skii [26]: above an intracellular acetate concentration threshold, the pH difference across the cell membrane decreases, leading to a decrease in the electrochemical gradient. The latter gradient is very important in many cell reactions (ATP synthesis, substrate transport, osmotic pressure regulation). Where high acetate concentrations are present these reactions slow down or stop completely. The results obtained here with *E. coli* HB 101 (GAPDH), compared with those already published with *E. coli* C600galK (GAPDH) [21] show that these two strains do not have the same behavior in regard to acetate inhibition. *E. coli* HB 101 is highly sensitive to acetate production, whereas *E. coli* C600galK, for which no decrease in the apparent production yields of GAPDH on glucose has been observed and for which growth inhibition is less important (Table 3), is less acetate sensitive. We can therefore make the assumption that there is a critical acetate concentration leading to inhibition which is different for each *E. coli* strain. Furthermore, in the case of *E. coli* HB 101, acetate is an activator of its own production (Tables 1 and 2). This can be explained by an inhibition of one or several enzymes of the tricarboxylic cycle. Where this metabolite is present, the tricarboxylic acid cycle would be less efficient and glucose flow in glycolysis would accelerate in order to provide for the cell's energy needs. This glucose excess would be transformed into acetate instead of providing energy. This hypothesis could explain the increase of the apparent yield of acetate production where acetate is present in the medium observed with *E. coli* HB 101 (GAPDH). A deeper investigation concerning that phenomenon would need complementary experiments performed in stable and well defined conditions of the cellular environment particularly expressed on glucose and acetate concentrations. This could be carried out in continuous cultures. Moreover note

that after glucose exhaustion, acetate is used up as in the case of *E. coli* C600galK (GAPDH) [21].

The fourth phenomenon that can be observed here (Fig. 1) is the decrease in enzymatic activity at the end of fermentation. A denaturation arises probably involving intracellular proteases. This has been previously shown by various authors. Intracellular proteases or stress proteins might be involved in this phenomenon [10,18]. In the present case the phenomenon was not analyzed further. We emphasize that in the system using *E. coli* C600galK as a host cell, such a decrease was avoided by supplementing the medium with amino acids for which the strain is auxotrophic [7], or with a protein hydrolyzate [20].

As for the regulation of the recombinant gene expression, in terms of promoters involved, no experimental data are given here. The utilization of the four different sequences mentioned above, is now under study in fermentor conditions. Preliminary results show that glucose and growth are factors affecting their utilization [4, Gschaedler et al., 1994, unpublished results].

*Escherichia coli* HB 101 (GAPDH) is suitable for the production of the recombinant enzyme GAPDH and does not require $O_2$ sparging in the fermentor for high yields. However during fermentation, acetate production should be controlled because it decreases recombinant protein production and growth. We have also demonstrated the existence of an optimal glucose concentration for production of GAPDH. In addition, the behavior of strain *E. coli* HB 101 (GAPDH) was compared with the behavior of strain *E. coli* C600galK (GAPDH) previously observed [21]. *E. coli* C600galK is less sensitive to inhibition by acetate. Further studies concerning the utilization of the four promoter sequences as a function of environmental conditions are in progress.

## ACKNOWLEDGEMENTS

Thanks are due to Dr C. Branlant from the University of Nancy for kindly providing facilities in her laboratory, for strain storage and maintenance as well as activity determination.

## NOTE ADDED IN PROOF

In a recent work, performed by Dr D. Ardaillon Simoes (INSA, Professor, G. Goma) at the Institut National Polytechnique de Toulouse, France, it has been also observed on *Escherichia coli*, that acetate is well an activator of its own production (Ph. D. thesis 'Croissance d'*Escherichia coli* à hautes concentrations cellulaires pour la production et l'excrétion d'une protéine hétérologue', April 1994).

## REFERENCES

1 Boyer, H.W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction modification of DNA in *Escherichia coli*. J. Mol. Biol. 41: 459–472.

2 Branlant, G., G. Flesh and C. Branlant. 1983. Molecular biology of glyceraldehyde-3-phosphate dehydrogenase genes of *Bacillus stearothermophilus* and *Escherichia coli*. Gene 25: 1–7.

3 Brown, S.W., H.-P. Meyer and A. Fiechter. 1985. Continuous production of human leukocyte interferon with *Escherichia coli* and continuous cell lysis in a two stages chemostat. Appl. Microbiol. Biotechnol. 23: 5–9.

4 Charpentier, B. and C. Branlant. 1994. The *Escherichia coli gapA* gene is transcribed by the vegetative RNA polymerase holoenzyme E$\sigma^{70}$ and the heat shock RNA polymerase E$\sigma^{32}$. J. Bacteriol. Feb 1994: 380–389.

5 Curless, C.E., P.D. Forrer, M.B. Mann, D.M. Fenton and L.B. Tsai. 1989. Chemostat study of kinetics of human lymphokine synthesis in recombinant *Escherichia coli*. Biotechnol. Bioeng. 34: 415–421.

6 Doelle, H.W., K.N. Ewing and N.W. Hollywood. 1982. Regulation of bacterial metabolism in bacterial systems. Adv. Biochem. Eng. 23: 1–35.

7 El Houtaia, N., N. Nancib, G. Branlant, C. Branlant and J. Boudrant. 1989. Production of glyceraldehyde-3-phosphate dehydrogenase using genetically engineered *Escherichia coli*. Biotechnol. Lett. 11: 775–778.

8 Ferdinand, W. 1964. Isolation and specific activity of rabbit muscle GAPDH. Biochem. J. 92: 1978–1985.

9 Fieschko, J. and T. Ritch. 1986. Production of human alpha consensus interferon to recombinant *Escherichia coli*. Chem. Eng. Commun. 45: 229–240.

10 Goldberg, A.L., K.H.S. Swamy, C.H. Chung and F.S. Larimore. 1981. Proteases in *Escherichia coli*. Methods Enzymol. 80: 680–702.

11 Han, K., H.C. Lim and J. Hong. 1992. Acetic acid formation in *Escherichia coli* fermentation. Biotechnol. Bioeng. 39: 663–671.

12 Ishikawa, Y., Y. Nonoyama and M. Shoda. 1981. Calorimetric analysis of *Escherichia coli* in batch culture. Biotechnol. Bioeng. 23: 2825–2856.

13 Jung, G., P. Denèfle, J. Becquart and J.F. Mayaux. 1988. High cell density fermentation studies of recombinant *Escherichia coli* expressing human interleukin-1$\beta$. Ann. Inst. Pasteur/Microbiol. 139: 129–146.

14 Kapralek, F., P. Jecmen, J. Sedlacek, M. Fabry and S. Zadrajil. 1991. Fermentation conditions for high level expression of the *tac* promoter controlled calf prochymosin cDNA in *E. coli* HB 101. Biotechnol. Bioeng. 37: 71–79.

15 Koh, B.T., U. Nakashimida, M. Pfeiffer and M.G.S. Yap. 1992. Comparison of acetate inhibition on growth of host and recombinant *E. coli* K12 strains. Biotechnol. Lett. 14: 1115–1118.

16 Konstantinov, K., M. Kishimoto, T. Sato and T. Yoshida. 1990. A balanced DO-Stat and its application to the control of acetic acid excretion by recombinant *Esherichia coli*. Biotechnol. Bioeng. 36: 750–758.

17 Lischke, H.H., L. Brandes, X. Wu and K. Schügerl. 1993. Influence of acetate on the growth of recombinant *Esherichia coli* JM103 and product formation. Bioproc. Eng. 9: 155–157.

18 Miller, C.G. 1987. Protein degradation and proteolytic modification. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, Vol. 1 (Neidhardt, F.C., J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, H.E. Umbarger, eds), pp. 680–691, American Society for Microbiology, Washington, DC.

19 Mougin, A.C., C. Corbier, A. Soukri, A. Wonacott, C. Branlant and G. Branlant. 1988. Use of site directed mutagenesis to probe the role of cystein 149 in the formation of charge transfer transmission. Prot. Eng. 2: 45–48.

20 Nancib, N., C. Branlant and J. Boudrant. 1991. Metabolic roles

of peptone and yeast extract culture of a recombinant strain of *Escherichia coli*. J. Ind. Microbiol. 8: 165–170.

21 Nancib, N., R. Mosrati and J. Boudrant. 1993. Modelling of batch fermentation of a recombinant *Escherichia coli* producing glyceraldehyde-3-phosphate dehydrogenase on a complex selective medium. Chem. Eng. J. 52: B35–B48.

22 Pan, J.G., J.S. Rhee and J.M. Lebeault. 1987. Physiological constraints in increasing biomass concentration of *Escherichia coli* in fed-batch culture. Biotechnol. Lett. 9: 89–94.

23 Peretti, S.W. and J.E. Bailey. 1987. Simultations of host–plasmid interactions in *Escherichia coli*: copy number, promoter strength, and ribosome binding site strength effects on metabolic activity and plasmid gene expression. Biotechnol. Bioeng. 29: 316–328.

24 Reiling, H.E., H. Laurila and A. Fiechter. 1985. Mass culture of *Escherichia coli*: medium development for low and high density cultivation of *Escherichia coli* B/r in minimal and complex medium. J. Biotechnol. 2: 191–206.

25 Shimizu, N., S. Fukuzono, K. Fujimori, N. Nishimura and Y. Odawara. 1988. Fed-batch culture of recombinant *Escherichia coli* with inhibitory substance concentration monitoring. J. Ferment. Technol. 66: 187–191.

26 Smirnova, G.V. and O.N. Oktyabr'skii. 1985. Influence of acetate on the growth of *Escherichia coli* under aerobic and anaerobic conditions. Microbiology 54: 205–209.

27 Yee, L. and H.W. Blanch. 1992. Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. Biotechnol. 10: 1550–1556.