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Metabolic roles of peptone and yeast extract for the culture of a recombinant strain of *Escherichia coli*

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SUMMARY

The influence of complex compounds on the growth of a recombinant strain of *Escherichia coli* containing the gene encoding glyceraldehyde 3-phosphate dehydrogenase, as well as the production of this enzyme have been studied. Batchwise cultures led to an accumulation of acetate, which was not utilized in a yeast extract-free medium. After glucose exhaustion, growth stopped and enzyme activity decreased. Whereas yeast extract allowed acetate assimilation and growth, peptone stabilized the enzymatic activity. The addition of both compounds resulted in optimal performances for enzyme production.

INTRODUCTION

In order to ensure microbial growth and effective metabolism, the medium composition must be chosen carefully. Indeed, interactions between medium substances can occur. However, the real reasons for the effects of the substances used are not well understood, mainly because they are complex. For example, rapid growth might induce acetate formation which may interfere with the metabolism. This could be very harmful, especially in the case of genetically engineered microorganisms, as has been shown for the production of interferon by a strain of *Escherichia coli* [11]. It is known that acetate formation depends on culture conditions: dissolved oxygen concentration [10] or the presence of other compounds such as metallic ions [14]. Its consumption might also be related to culture conditions.

The strain studied here synthesized an exceptionally high quantity of an homologous enzyme, and the importance of auxotrophic amino acids for the enzyme stabilization has been shown previously [7]. In the present study, we differentiated the roles of two complex compounds of the fermentation media, peptone and yeast extract, the latter influencing acetate utilization.

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MATERIALS AND METHODS

Strain

The strain used in this study, *E. coli* C600 gal K(GAPDH), is a genetically modified species of the strain *E. coli* C600 gal K, ATCC 23724 (lac$^-$, thr$^-$, leu$^-$, thia$^-$; [3] kindly provided by Rhône Poulenc Rorer, Paris, France, in which the gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified by introduction of the plasmid pBREco gap [4]. This plasmid is derived from pBR322 by insertion of the *E. coli* GAPDH gene into the tetracycline gene. The genetically modified strain is therefore only ampicillin-resistant (amp^r). The strain was stored on Luria broth (LB) medium containing glycerol (15%) at −80 °C. It was reactivated by preculture in Erlenmeyer flasks. Pre-cultures and cultures were performed in the same medium using a 10% inoculum.

Fermentation

Fermentations were performed in 3-l fermentor jars (Interscience, France) fitted with standard equipment: pH and temperature regulation, agitation (600 rpm) and aeration (about 2 vvm), and dissolved oxygen regulation (Ingold electrode). pH was regulated with ammonium hydroxide (3 N) or hydrochloric acid solutions (4 N). Temperature was regulated at 37 °C. Working volume was 1.5 l. Each run was performed twice. Inocula were prepared in 150 ml Erlenmeyer flasks (50 ml per flask). Agitation speed was 150 rpm (amplitude 2 cm).

Assays

Biomass was determined from absorbance at 660 nm with a Novaspec II spectrophotometer and also from dried cell weight. Glucose content was determined with a Technicon autoanalyser using the hexokinase method. Acetic acid concentration was determined using an Intersmat ICR 1B gas chromatograph with flame ionization detector (Delsi, Suresnes, France). GAPDH activity was determined from the absorbance of the formed NADH at 340 nm [8]. Enzymatic activities were determined in supernatants obtained from centrifugation (20 000 × *g*) of sonicated cells. Sonication was performed at 4 °C for 4 × 30 s. The amount of GAPDH in supernatants was determined using a specific activity determined for pure GAPDH: 350 U/mg at 37 °C [12].

Media

Two fermentation media were used: Luria broth (LB) medium which contains Bactopeptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, and a complex medium (CM) which contains peptone (Merck) 10 g/l, yeast extract (Prolabo) 15 g/l, NaCl 5 g/l, glucose 10 or 20 g/l, monopotassium phosphate 1 g/l, bipotassium phosphate 4 g/l, magnesium sulfate 0.5 g/l, thiamine 0.1 g/l, cysteine 1 g/l, leucine 0.3 g/l, threonine 0.3 g/l and 2.5 ml/l of trace elements in solution. This solution of trace elements contains $AlCl_3 \cdot 6\ H_2O$, 0.5 g/l; $CoCl_2 \cdot 6\ H_2O$, 4 mg/l; $CuCl_2 \cdot 2\ H_2O$, 1 mg/l; H_3BO_4, 0.5 g/l; $MnSo_4 \cdot H_2O$, 10 mg/l; $MoNa_2O_4 \cdot 2\ H_2O$, 2 mg/l; $ZnSO_4 \cdot 7\ H_2O$, 2 mg/l; and $CaCl_2$ 40 mg/l. Ampicillin was added to the media (100 mg/l) to assure strain stability.

Each component for the complex medium was autoclaved at 120 °C for 20 min and the complete growth medium was reconstituted by mixing shortly before inoculation. The medium for agar plates had the following composition: tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 5 g/l; and agar-agar, 20 g/l.

RESULTS

Batch and fed-batch runs were performed in a fermentor to characterize the behavior of the strain, and to study the influence of peptone and yeast extract added during fermentation.

Batch fermentations

Batch fermentations allowed characterization of the strain behavior. On Fig. 1A, the growth of the strain *E. coli* C600galK(GAPDH) on the complex medium CM containing 10 g/l of glucose shows two phases. The first phase, aerobic fermentation which lasted about 5 h, corresponds to the consumption of glucose and includes the exponential growth phase. The production of

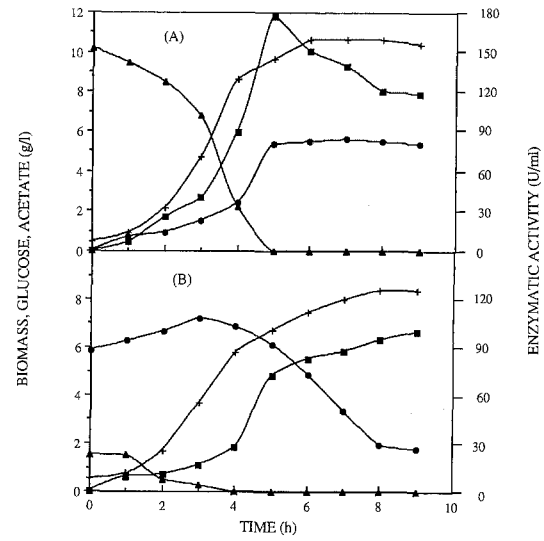

Fig. 1. Growth curves of *E. coli* C600galK(GAPDH) during batch fermentations performed in complex media (MC) containing either glucose (10 g/l) (curves A), or acetate (6 g/l) and glucose (1 g/l) (curves B). The assays were performed in a fermentor according to the conditions indicated in "Materials and Methods". Biomass (+————+), acetate (●————●), glucose (▲————▲), GAPDH activity (■————■).

GAPDH and acetate are then associated with the growth. The second phase, in which a decrease of growth rate, a loss of GAPDH activity and a stabilization of acetate concentration were observed, begins after glucose exhaustion; which is after approx. 5 h of fermentation. This acetate stabilization was further studied. According to the literature, acetate is normally assimilated by *E. coli* [5,6], but apparently the strain used in this study does not utilize acetate. In order to clarify this apparently different behavior, batch cultures using different initial acetic acid concentrations were performed (3.5, 6 and 11 g/l). Glucose was present in the fermentation medium (1 g/l) to initiate a rapid growth. Only those results concerning the intermediate acetate concentration (6 g/l) are represented on Fig. 1B. They show that (i) acetate is well utilized by the strain, (ii) growth occurs when this substrate is utilized, and (iii) there is a production of GAPDH. Regarding the observed acetate concentration stabilization during the first assay, it can be hypothesized that this could be due to a nutritional limitation. This hypothesis has been checked with fed-batch assays with continuous feeding of complex medium.

Fed-batch fermentations

Two experiments conducted with continuous feeding were performed. Their objectives were to check the effects of continuous feeding conditions on growth, acetate utili-

zation and enzymatic stability. In each case the fermentations were initiated with 1.5 l of CM medium containing 10 g/l of glucose. The continuous feeding, performed with the same medium but at a glucose concentration of 100 g/l, only began when glucose had reached zero. At the time (t = 5 h) continuous feeding was initiated, the acetate concentration was approx. 6 g/l. The concentration of acetate then decreased. The decrease in acetate (about 10% h^{-1}) was larger than the volume increase (1.3% h^{-1}) due to feeding, suggesting that acetate was being utilized. The different conditions in which the feedings were performed were the following: (i) continuous feed flow-rate 20 ml/h, and (ii) continuous feed flow-rate 20 ml/followed by a shift to 40 ml/h.

When the continuous feed flow-rate was 20 ml/h (corresponding to a supply of 2 g/h, which is the glucose consumption rate), glucose is the limiting substrate, and acetate utilization (aerobic respiration) began when glucose concentration reached zero. Final biomass concentration reached 24 g/l. However, a decrease in enzymatic activity is noted at the end of the biomass production (Fig. 2), indicating a possible nutritional limitation.

The second fed-batch was performed by anticipating the decrease of enzymatic activity with an increase of the continuous feed flow-rate from 20 ml/h to 40 ml/h (at 18 h on Fig. 3). In this condition enzymatic activity did not decrease further, clearly showing the effect of increasing the substrate inlet feeding.

The first fed-batch assay confirms the acetate utilization by the strain *E. coli* C600galK(GAPDH). The

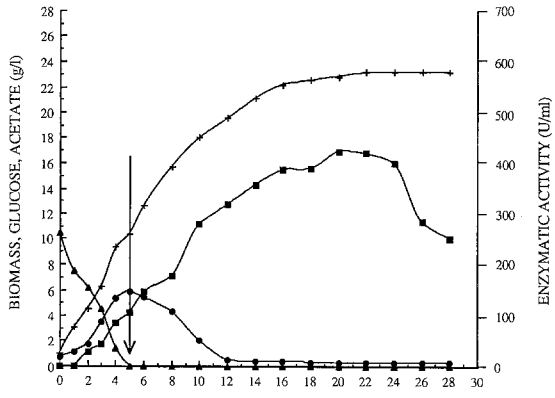

Fig. 2. Growth curve of *E. coli* C600galK(GAPDH) during a fed-batch fermentation. The first phase was performed with 1.5 liters of CM medium (glucose 10 g/l) according to the conditions indicated in "Materials and Methods". The second phase (from the arrow) was performed with feeding of CM medium (100 g/l glucose, 20 ml/h). Biomass (+————+), acetate (●————●), glucose (▲————▲) GAPDH activity (■————■).

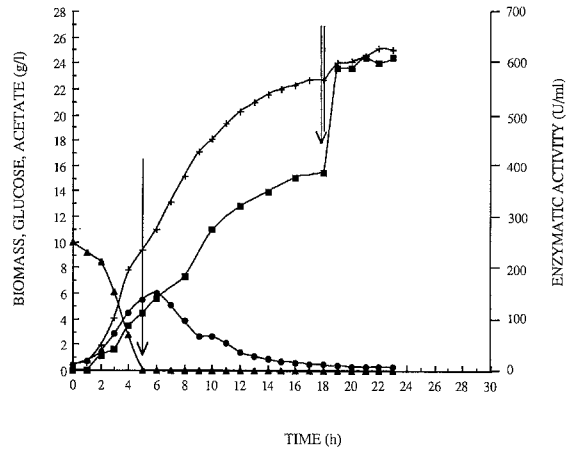

Fig. 3. Growth curve of *E. coli* C600galK(GAPDH) during a fed-batch fermentation. The first phase was performed with 1.5 liters of CM medium (glucose 10 g/l) according to the conditions indicated in "Materials and Methods". The second phase, with a feeding of CM medium (100 g/l of glucose) was separated into two parts: the first from the arrow → at 6 hours age with a feeding of 20 ml/h, the second from the arrow ⇒ at 18 hours age with a feeding of 40 mg/h. Biomass (+————+), acetate (●————●), glucose (▲————▲), GAPDH activity (■————■).

second fed-batch shows a limitation for maintaining the stability of enzymatic activity. Further assays were performed to clarify this limitation. The corresponding assays were performed to determine the role of the complex compounds of the medium, peptone and yeast extract.

Enrichment assays during batch fermentations

The influence of both complex compounds, peptone and yeast extract, was identified during batch fermentations (fermentation time 15 h) by adding, after approximately 6 h of fermentation, either peptone (quantity to get 10 g/l in the fermentation medium) or yeast extract (quantity to get 15 g/l in the fermentation medium) or both simultaneously after glucose exhaustion. Results of the corresponding assays are shown in Table 1. They indicate (i) biomass at 15 h, (ii) acetate at time t = 5 h, time required for maximum concentration, and at time t = 15 h, and (iii) enzymatic activities when it is maximum and at time t = 16 h. These results show that if peptone stabilized enzymatic activity, it did not allow acetate utilization. They also show that yeast extract favored growth and acetate utilization, but apparently did not have any positive effect on the enzyme stabilization observed with peptone. When added simultaneously, all positive effects are additive: acetate utilization, growth improvement and enzyme stabilization. This allowed an optimal performance with regard to enzyme production after a batch cycle.

TABLE 1

Separate and cumulative influences of peptone and yeast extract on the fermentation (batch) of *E. coli* C600galK(F61a). The assays were performed in a fermentor according to the conditions indicated in MATERIALS AND METHODS. Peptone and yeast extract were added at 6 hours age of fermentation alone or simultaneously at concentrations of 10 g/l and 15 g/l respectively.

Medium	Biomass (g/l)	Acetate ($g\,l^{-1}$)			Enzymatic activity ($U\,ml^{-1}$)		
	15 h	5 h	15 h	Consumption ($g\,l^{-1}\cdot h^{-1}$)	Maximum	16 h	Δ Enzymatic activity ($U\,ml^{-1}$)
Complex medium	10.5	5	5	0	150	120	−30
Complex medium + peptone	10.5	5.5	5.5	0	180	180	0
Complex medium + yeast extract	14.5	5.5	1.5	0.4	230	180	−50
Complex medium + peptone + yeast extract	15	5.5	1.5	0.4	230	230	0

Complementary assays

Since acetate is utilized in the presence of yeast extract, and not in the presence of peptone, it was suggested that the vitamin content of yeast extract might be responsible for this metabolization. Therefore, the influence of the vitamin mixture present in yeast extract (sterilized by filtration), except thiamine already present in the medium was studied in a run similar to the one carried out for the results obtained in Table 1 (As for thiamine, note that its concentration in the medium is 0.1 g/l and is probably in excess). The vitamins in question are pantothenic acid (2 mg/l), biotin (0.1 mg/l), nicotinic acid (30 mg/l) folic acid (0.1 mg/l), riboflavin (2 mg/l), pyridoxal (1 mg/l), cyanocobalamine (1 mg/l), (concentrations calculated from Merck information). In this instance, there was no acetate utilization, indicating that the vitamin content was not the sole reason for the consumption of acetate. It is thought that either other elements or synergistic effects are involved in that phenomenon.

DISCUSSION

These results demonstrate that acetate is used by the strain *E. coli* C600galK(GAPDH). This corresponds to what is normally observed with *E. coli* [9] which is observed in a shift from aerobic fermentation to aerobic respiration. But in this case, the complete utilization of acetate requires the presence of factors which are contained in yeast extract. Which element(s) of yeast extract is (or are) involved? We tried to answer this question by testing the effect of its vitamin content.

Vitamins normally contained in yeast extract which allow a better acetate utilization could have been pantothenic acid, a precursor of acetyl-CoA; biotin, which favors carboxylation reactions; and nicotinic acid, a precursor of NAD and NADP (which is present in large quantity in yeast extract). Since the strain *E. coli* C600galK is auxotrophic for thiamine [3], thiamine, already present in the medium at a concentration of 0.1 g/l, was not added. The results of the different runs performed showed that the addition of these vitamins in the described conditions did not by itself induce acetate utilization. This fact probably results from a more complex phenomenon which might be due to compounds other than vitamins, such as organic or mineral elements contained in yeast extract or synergistic interactions including vitamins ratio effects. Additional trials are required to identify the responsible parameters.

Studies concerning the effect of yeast extract on fermentation performance have recently been published. They concern normal strains of *Lactobacillus helveticus* for the production of lactic acid [1], and also genetically engineered strains of *E. coli* for the production of insulin-like growth factor [15] and β-galactosidase [16]. In this last case, the authors conclude that there is a complex action on the part of the yeast extract: it might affect regulatory processes concerning the initiation of transcription and translation and also synthesis of certain proteins.

The stability of the enzymatic activity appeared here to be under the control of peptone (Fig. 3 and Table 1). It also has been shown that stability can be obtained by adding the auxotrophic amino acids of the host cell into the medium [7]. Both peptone and auxotrophic amino acid effects probably indicate nutritional limitations. In fact peptone, which is a proteic hydrolyzate, contains

these amino acids in their free forms (7.5 g/l and 6.9 g/l for threonine and leucine, respectively). However, due to the fact that assays were performed at 37 °C, protease action should also be considered [15].

In this study, the other intermediate metabolites such as lactic, oxaloacetic, pyruvic and propionic acids have not been taken into account. This is because it is known that these are formed in lower amounts than acetic acid [2] and overall, our objectives of modeling the process has been reached by considering a model utilizing glucose, acetic acid, yeast extract and peptone [13].

This study examined the capacity of acetate utilization by the strain *E. coli* C600galK(GAPDH), permitted by yeast extract. The knowledge that yeast extract, combined with peptone, allows the stabilization of enzymatic activity, will be most useful in order to obtain high cell densities and high-quality protein production.

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REFERENCES

1 Aeschlimann, A. and U. von Stockar. 1990. The production of lactic acid from whey permeate by *Lactobacillus helveticus*. Appl. Microbiol. Biotechnol. 32: 398–402.

2 Anderson, K.W., E. Grulke and P. Gerhardt. 1984. Microfiltration culture process for enhanced production of rDNA receptor cells of *Escherichia coli*. Biotechnology 2: 891–896.

3 ATCC Catalogue of Bacteria and Bacteriophage. 1989. 17th Edn., (R. Gherna and P. Pienta, eds.), p. 87. Rockville, MD.

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

5 Clark, D.P. 1989. The fermentation pathway of *Escherichia coli*. FEMS Microbiol. Rev. 63: 223–234.

6 Cortay, J.C., F. Bleicher, B. Duclos, Y. Cenatiempo, C. Gautier, J.L. Prato and A.J. Cozzone. 1989. Utilization of acetate in *Escherichia coli*: structural organization and differential expression of the *ace* operon. Biochimie 71: 1043–1049.

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 Holms, W.H. and P.M. Bennett. 1971. Regulation of isocitrate dehydrogenase activity in *Escherichia coli* on adaptation to acetate. J. Gen. Microbiol. 65: 57–68.

10 Matsui, T. and H. Yokota and S. Sato. 1989. Pressurized culture of *Escherichia coli* for a high concentration. Agricult. Biol. Chem. 53: 3115–2120.

11 Meyer, H.P., C. Leist and A. Fiechter. 1984. Acetate formation in continuous culture of *Escherichia coli* K12D1 on defined and complex media. J. Biotechnol. 1: 355–358.

12 Mougin, A., 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.

13 Nancib, N., R. Mosrati and J. Boudrant. 1991. Modelling of the production of glyceraldehyde-3-phosphate dehydrogenase by a genetically engineered *Escherichia coli*. IVth World Congress of Chemical Engineering, Karlsruhe, F.R.G., 16–21 June 1991.

14 Reiling, H.E., H. Laurila and A. Fiechter. 1984. Mass culture of *Escherichia coli*: medium development for low and high density cultivation of *Escherichia coli* B/r in minimal and complex media. J. Biotechnol. 2: 191–206.

15 Tsai, L.B., M. Mann, F. Morris, C. Rotgers and D. Fenton. 1987. The effect of organic nitrogen and glucose on the production of recombinant human insulin-like growth factor in high cell density *Escherichia coli* fermentations. J. Industr. Microbiol. 2: 181–187.

16 Xiaoli, L., J.W. Robbins, K.B. Taylor. 1990. The production of recombinant beta-galactosidase in *Escherichia coli* in yeast extract enriched medium. J. Industr. Microbiol. 5: 85–94.