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Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor

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Abstract High-cell-density cultivations of *Escherichia coli* K12 in a dialysis reactor with controlled levels of dissolved oxygen were carried out with different carbon sources: glucose and glycerol. Extremely high cell concentrations of 190 g/l and 180 g/l dry cell weight were obtained in glucose medium and in glycerol medium respectively. Different behaviour was observed in the formation of acetic acid in these cultivations. In glucose medium, acetic acid was formed during the earlier phase of cultivation. However, in glycerol medium, acetic acid formation started later and was particularly rapid at the end of the cultivation. In order to estimate the influence of acetic acid during these high-cell-density cultivations, the inhibitory effect of acetic acid on cell growth was investigated under different culture conditions. It was found that the inhibition of cell growth by acetic acid in the fermentor was much less than that in a shaker culture. On the basis of the results obtained in these investigations of the inhibitory effect of acetic acid, and the mathematical predictions of cell growth in a dialysis reactor, the influence of acetic acid on high-cell-density cultivation is discussed.

Key words *Escherichia coli* · High-cell-density cultivation · Acetic acid · Inhibition · Dialysis reactor

Introduction

The productivity of microbial processes can be improved significantly by high-cell-density cultivation. *Escherichia coli* is the microorganism used most widely as a host for producing recombinant proteins. Various attempts have been made to achieve high-cell-density cultivation of recombinant *E. coli* (Lee 1996). It is well known that acetic acid produced during *E. coli* cultivation inhibits cell growth (Sakamoto et al. 1994; Yee and Blanch 1993; Kwon et al. 1996; Lui and Strohl 1990; Konstantinov et al. 1990; Turner et al. 1994; Shimizu et al. 1992). In addition, the ability to produce recombinant proteins is significantly affected by acetic acid (Sakamoto et al. 1994; Shimizu et al. 1992; Jensen and Carlsen 1990; Macdonald and Neway 1990), hence many reports have dealt with the mechanisms of acetic acid formation and attempts to suppress its formation.

Acetic acid is considered to be produced at a high specific growth rate when there are excess carbon source or oxygen-limited conditions (Sakamoto et al. 1994; Konstantinov et al. 1990; Korz et al. 1995; Shimizu et al. 1988; Yoon et al. 1994). It has also been reported that the formation of acetic acid depends on the nature of the carbon source (Lee 1996; Kwon et al. 1996; Korz et al. 1995). The physiological background of such acetic acid formation is explained well by imbalances between carbon flux into the central metabolic pathway and the biosynthetic demands or the capacity for energy generation within the cell (Lee 1996). The formation of acetic acid as well as its inhibitory activity have been successfully avoided by controlling the concentration of the carbon source in the culture broth at a very low level by an exponential feeding strategy (Yoon et al. 1994), by controlling the dissolved oxygen level (Konstantinov et al. 1990) and by an on-line system monitoring the carbon source (Sakamoto et al. 1994; Lui and Strohl 1990; Shimizu et al. 1992) or acetic acid (Sakamoto et al. 1994; Turner et al. 1994; Shimizu et al. 1988). Under carbon source limitation, not only is the formation of acetic acid suppressed but the cells also assimilate acetic acid and convert it into cell mass, resulting in a low acetic acid concentration (Turner et al. 1994; Shimizu et al. 1992, 1988).

We have developed a dialysis reactor and its application in high-cell-density cultivations of *E. coli* has been

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Table 1 Medium composition used for pre-culture, batch culture and the feed for fed-batch culture of *Escherichia coli* K12

Component	Pre-culture	Batch culture	Feed A	Feed B
Glycerol	10.00 g/l	40.00 g/l	1066.00 g/l	–
(Glucose monohydrate)	(7.72 g/l)	(38.62 g/l)	(700.00 g/l)	(–)
Citric acid	3.00 g/l	3.00 g/l	3.00 g/l	6.00 g/l
KH_2PO_4	1.11 g/l	5.55 g/l	5.55 g/l	11.1 g/l
$K_2HPO_4 \cdot 3H_2O$	1.12 g/l	5.60 g/l	5.60 g/l	11.2 g/l
$NaH_2PO_4 \cdot H_2O$	0.45 g/l	2.25 g/l	2.25 g/l	4.50 g/l
$(NH_4)_2SO_4$	0.20 g/l	1.00 g/l	1.00 g/l	2.00 g/l
NH_4Cl	0.02 g/l	0.10 g/l	0.10 g/l	0.2 g/l
$MgCl_2 \cdot 6H_2O$	0.25 g/l	1.25 g/l	1.25 g/l	2.50 g/l
$FeSO_4 \cdot 7H_2O$	40.00 mg/l	0.02 mg/l	0.02 mg/l	0.40 mg/l
$CaCl_2 \cdot 2H_2O$	40.00 mg/l	0.02 g/l	0.02 g/l	0.04 g/l
$MnSO_4 \cdot H_2O$	2.58 mg/l	12.90 mg/l	12.90 mg/l	25.8 mg/l
$ZnSO_4 \cdot 7H_2O$	1.74 mg/l	8.70 mg/l	8.70 mg/l	17.4 mg/l
$CoCl_2 \cdot 6H_2O$	1.29 mg/l	6.45 mg/l	6.45 mg/l	12.9 mg/l
$CuCl_2 \cdot 2H_2O$	0.64 mg/l	3.20 mg/l	3.20 mg/l	6.4 mg/l
$Na_2MoO_4 \cdot 2H_2O$	0.54 mg/l	2.70 mg/l	2.70 mg/l	5.4 mg/l
$AlCl_3$	0.16 mg/l	0.80 mg/l	0.80 mg/l	1.6 mg/l
H_3BO_3	0.10 mg/l	0.50 mg/l	0.50 mg/l	1.0 mg/l
Thiamine-HCl	5.00 mg/l	5.00 mg/l	–	–

investigated (Märkl et al. 1993; Ogbonna and Märkl 1993). Our dialysis reactor is composed of two cylindrical chambers, the inner chamber being formed and separated from the outer chamber by a dialysis membrane. This structure enables low-molecular-mass substances, like acetic acid, to be dialysed out of the cell broth without exposing the organism to different conditions of temperature and pH or to deficient concentrations of nutrients and oxygen, which are caused by external cell recycling or dialysis systems. In the present work, a DO-stat strategy, was used to control the feed of the carbon source at the fed-batch phase. Glucose and glycerol were compared as carbon sources for high-cell-density cultivation of *E. coli*. In order to estimate the influence of acetic acid generated during these high-cell-density cultivations, its inhibitory effect on cell growth was investigated in a shaker culture and in a batch culture with a conventional fermentor. On the basis of the results obtained in these investigations of the inhibitory effect of acetic acid and the mathematical predictions of cell growth in a dialysis reactor, the influence of acetic acid on high-cell-density cultivation is discussed.

Materials and methods

Bacterial strain and culture media

Escherichia coli K12 ATCC 27325, obtained from Hoechst AG (Germany), was used in this work. After the strain had been grown in a nutrient broth (Merck KGaA, Germany), a portion of the culture broth was mixed with the same volume of 99.5% (v/v) glycerol and kept in a freezer at −80 °C as stock culture.

The medium composition for the pre-culture, batch culture and the feed for the fed-batch culture used in this work is shown in Table 1. During the fed-batch phase, carbon source (feed A) and salt components (feed B) were fed separately into the inner chamber and the outer chamber respectively.

Seed culture and pre-culture

The seed culture was prepared in a 500-ml conical flask containing 100 ml nutrient broth; after the inoculation of 1 ml melted stock culture, it was incubated at 37 °C for 20 h on a rotary shaker working at 180 rpm. Then 5 ml seed culture broth was transferred to a 500-ml conical flask containing 100 ml pre-culture medium and incubated for 9 h under the same conditions as the seed culture.

Batch and fed-batch culture in the dialysis reactor

The dialysis reactor used in this work is shown in Fig. 1. This reactor is composed of two cylindrical chambers. The inner chamber is set inside the outer chamber, formed and separated from the outer chamber by a cuprophan membrane (Akzo Nobel Faser AG Wuppertal, Germany) with a molecular mass cut-off of 10 kDa. A detailed description of this reactor has been reported in our previous publication (Märkl et al. 1990).

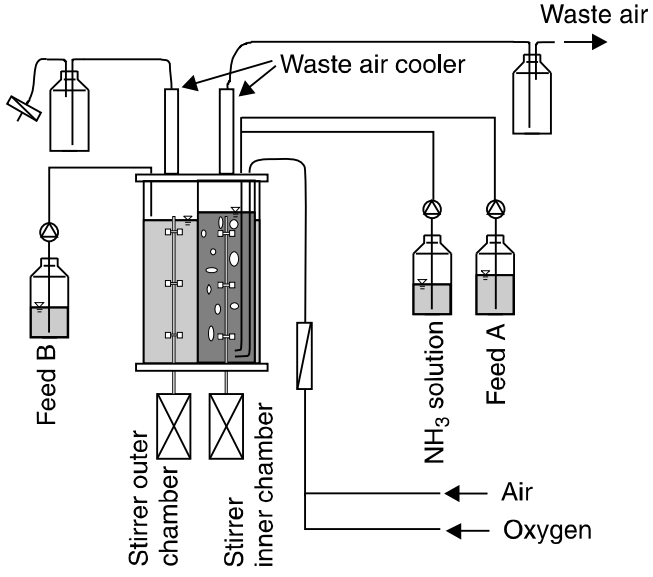

Fig. 1 Schematic diagram of the dialysis cultivation system

The inner and outer chambers were filled with the batch medium without thiamine hydrochloride, and the pH was adjusted to 3.5 with 25% ammonium solution. Sterilization was carried out in situ as described previously (Märkl et al. 1990). After cooling, thiamine hydrochloride, sterilized by filtration, was added and the pH was adjusted with ammonium solution to an optimum value of 7.0. A mixture of air and oxygen was supplied to the inner chamber with a flow rate of 1 vvm. The agitation speed of both chambers was set at 800 rpm. A 10-ml inoculum of pre-culture broth was added to the inner chamber.

The fermentation was started with working volumes in the inner and outer chamber of 1 l and 3.5 l respectively. The pH was automatically controlled by feeding 25% ammonium solution to the inner chamber, and the temperature was maintained at 37 °C during the fermentation. The agitation speed in the inner chamber, the proportion of oxygen in the gas mixture supplied or the flow rate of aeration were increased gradually to accomodate the increased rate of oxygen consumption accompanying cell growth. The ranges of agitation speed and aeration flow rate were 800–2300 rpm and 1–3 vvm respectively. By this means, the dissolved oxygen concentration could be maintained above 5% saturation before the cell concentration reached around 150 g/l, after which the feed rate of the carbon source was reduced to avoid the dissolved oxygen concentration becoming zero.

The fed-batch phase was started when the carbon source in the inner chamber was completely consumed. This was indicated by a rapid increase of the dissolved oxygen concentration. If this phenomenon was observed during the fed-batch phase, it was an indication of a lack of substrate and the feed rate of the carbon source was increased gradually. In order to keep the carbon source concentration of the inner chamber as small as possible, the increase of the feed rate was carried out gradually. Silicone anti-foam emulsion M-30 (Serva AG, Germany) was used for foam control if necessary.

The membrane cannot be used if there is appreciable difference in the levels in the inner and outer chambers. Feeding the carbon source and ammonium solution into the cell broth caused the liquid level in the inner chamber to rise. The glucose medium can not be used in as concentrated a form as the glycerol medium, so that the amount of feed volume added during cultivation could not be neglected. It was therefore necessary to take out 100–200 ml of the cell broth during the fed-batch phase to keep the level in the inner and outer chambers approximately constant. In addition, a 1.0 l salt solution (feed B) was temporarily fed into the outer chamber to increase the liquid level outside. Feed B was given in small quantities (100–200 ml) in order to avoid excess nutrient feeding. The time schedule and the amount of feed B that was supplied depended on the level inside and differed among the fermentations described.

Analyses

The biomass concentration was determined by measuring the dry weight of the cells. The cellulose acetate filter-paper (pore size: 0.2 μm, Sartorius AG, Germany), which was washed with 0.1 M HCl after the culture sample had been filtered, was dried at 105 °C for 12 h and cooled in a desiccator prior to weighing. The concentrations of glycerol and acetic acid were analysed with test kits (Boehringer-Mannheim GmbH, Germany). Glucose analyser YSI 2700 select (Yellow Springs Instruments Co. Inc., USA) was used to determine the glucose concentration.

Results

High-cell-density cultivation of *E. coli* in the dialysis reactor

Figure 2 shows the results of a high-cell-density cultivation of *E. coli* K12 in the dialysis reactor with glucose as the sole carbon source. In this experiment, the fed-batch phase was started after 17.5 h and the feed rate of glucose was increased gradually if a rapid increase of the dissolved oxygen concentration was observed. As a result, the glucose concentration of the inner chamber was kept below 0.5 g/l throughout the fed-batch phase. These glucose-limited conditions caused the cells to assimilate acetic acid. From batch to the early fed-batch phase, the acetic acid concentration increased to 2.0 g/l. However, it subsequently decreased during the fed-batch phase as it was assimilated by the cells. The measurements show that its concentration remained at less than 1 g/l even at high cell concentrations. It was not clear why the acetic acid increased after the start of fed-batch feeding even though the glucose level was below 0.5 g/l. It may be due to a delay in the change of metabolism from production to consumption of acetic acid. The cell concentration increased and reached the maximum value of 190 g/l after 30 h of fermentation.

The results of a high-cell-density cultivation of *E. coli* K12 in glycerol medium are shown in Fig. 3. In this experiment, the fed-batch phase was started after 16 h and the glycerol concentration was again kept at a very low level throughout this phase with a similar strategy to that used with glucose medium. However, different behaviour was observed in the formation of acetic acid. Acetic acid formation was quite slow in the batch and early fed-batch phases, compared with the cultivation using glucose, even though there was about the same amount of glycerol as sole carbon source. However, in the glycerol medium, a rapid increase of acetic acid concentration was observed just before the cell

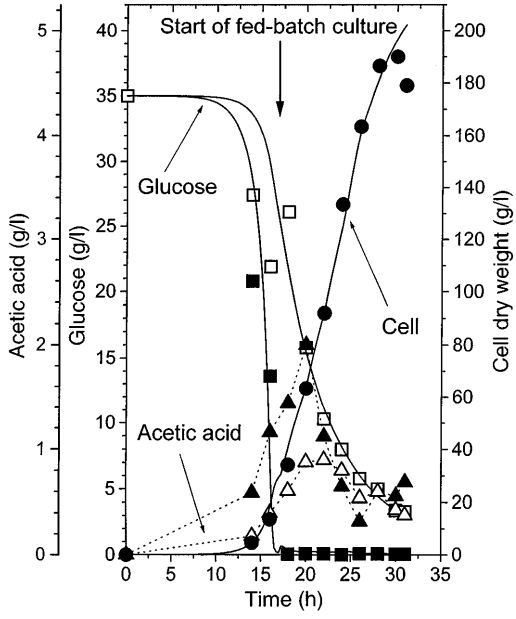

Fig. 2 High-cell-density cultivation of *Escherichia coli* K12 using glucose medium in a dialysis reactor. ● Cells, □ ■ glucose, ▲ △ acetic acid. ■ ▲ Experimental values in the inner chamber, □ △ experimental values in the outer chamber. *Solid lines* results of mathematical predictions

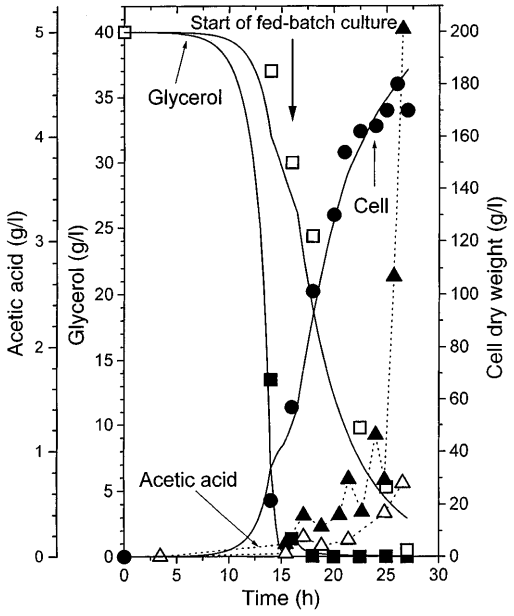

Fig. 3 High-cell-density cultivation of *E. coli* K12 using glycerol medium in a dialysis reactor. ● Cells, ■ □ glycerol, ▲ △ acetic acid. ■ ▲ Experimental values in the inner chamber, □ △ experimental values in the outer chamber. *Solid lines* results of mathematical predictions

concentration reached the maximum value of 180 g/l, although the glycerol concentration was always kept low. This different behaviour in glucose and glycerol media was also shown by Korz et al. (1995).

Influence of acetic acid on cell growth of *E. coli* K12

In order to estimate the influence of acetic acid on high-cell-density cultivation, the inhibitory effect of acetic acid on cell growth was investigated. Shaker cultures were used to compare the different carbon sources glucose and glycerol. The compositions of these media were the same as in the batch medium described in Materials and methods. After autoclaving and addition of various amounts of acetic acid, the pH of the medium was aseptically adjusted to 7.0 with ammonium solution prior to inoculation. The culture conditions were the same as in the pre-culture, which is described in Materials and methods. Figure 4 shows the influence of the acetic acid concentration on the specific growth rate of *E. coli* K12 in the shaker cultures. Because the pH of the shaker culture medium is reduced if the organisms are growing, the specific growth rate was determined from the early exponential phase, where the change of the pH was negligible. If the organisms did not grow, the pH was stable. In the shaker culture using glycerol medium, acetic acid started to inhibit cell growth at concentrations over 1 g/l and no cell growth was observed at an acetic acid concentration over 4 g/l. On the other hand, in the shaker culture using glucose medium, acetic acid started to inhibit the cell growth at a concentration

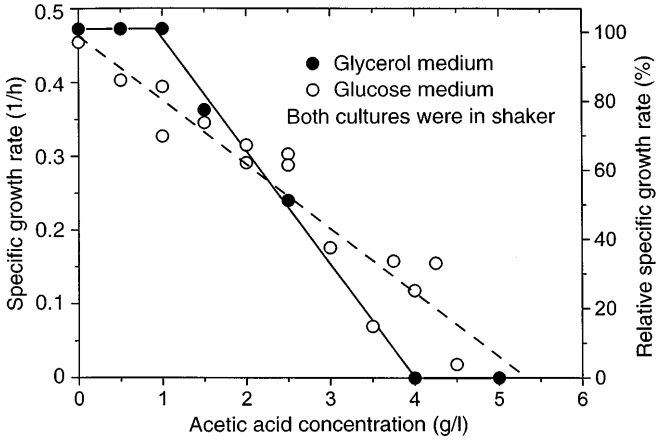

Fig. 4 Influence of the acetic acid added exogenously to the medium on the specific growth rate of *E. coli* K12 in shaker culture using glucose or glycerol as carbon source

of 0.5 g/l and the growth rate decreased linearly with increasing acetic acid concentration. These differences between glucose and glycerol medium were not significant, as shown in Fig. 4.

The influence of acetic acid on cell growth of *E. coli* K12 was also investigated by batch culture in a conventional fermentor without a dialysis membrane and compared with the results in the shaker culture. The cells were cultured in the fermentor with glucose medium and various amounts of acetic acid were added at the mid-exponential growth phase. In this experiment, the pH and the dissolved oxygen concentration were kept constant. The comparison of the influence of acetic acid on cell growth in the shaker culture with that in the batch fermentor is shown in Fig. 5. The specific growth rate in the batch culture using a fermentor also fell linearly as the acetic acid concentration increased, as it did in the shaker cultures. However, the inhibitory effect of acetic acid on the cell growth in batch cultures in the

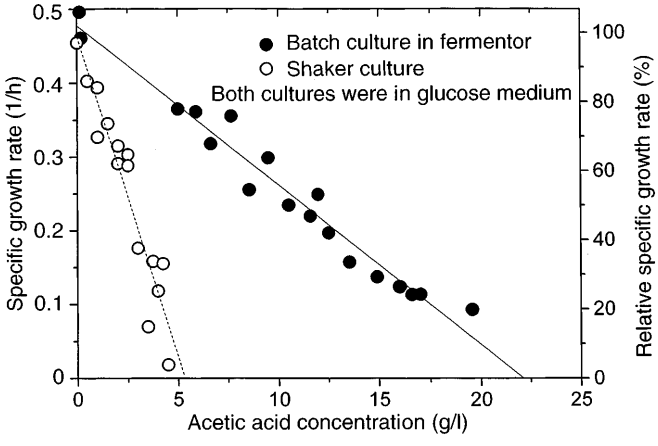

Fig. 5 Comparison of the influence of the acetic acid added exogenously to the medium on the specific growth rate of *E. coli* K12 in shaker culture and in batch culture in a fermentor. Both cultivations were carried out in glucose medium

fermentor was much less than in the shaker culture. As shown in Fig. 5, the cell growth in the shaker culture almost stopped at an acetic acid concentration of around 5 g/l, whereas the same acetic acid concentration in the fermentor caused a decrease in cell growth of only about 20%. These results indicate that the influence of acetic acid on cell growth depends on the culture conditions, especially the dissolved oxygen concentration, and that results in shaker culture can not be used to evaluate cultivation in the fermentor.

Discussion

The results of the experiment on the inhibition of cell growth by acetic acid in a fermentor (Fig. 5) suggest that the acetic acid concentration in the high-cell-density cultivation using glucose medium (Fig. 2) was too low to have any serious effect. Although the acetic acid concentration at the end of the high-cell-density cultivation using glycerol medium (Fig. 3) was higher than in the cultivation using glucose medium, it may still not be high enough to affect cell growth seriously. This assumption is confirmed by a mathematical model described in our previous work (Ogbonna and Märkl 1993). Good agreement between the experimental cell concentration and the model calculation was obtained, as shown in Figs. 2 and 3. As this model prediction did not take account of any inhibitory effects, these results show that there was no serious growth inhibition in these high-cell-density cultivations. Consequently the cell concentration in both cultivations reached the theoretically maximal attainable value (160–200 g/l), as calculated in our previous work (Märkl et al. 1993) and this would seem to be the reason why the increase of cell concentration stopped.

However, the generation of acetic acid at the end of the cultivation on glycerol medium was particularly rapid, so that it could not be moderated by dialysis as shown in Fig. 3. Although the attainable cell concentration seemed not to be affected by acetic acid in this case, such rapid formation of acetic acid at the end of the cultivation could be a problem if the induction of a recombinant protein were initiated after a high cell concentration had been reached. From this point of view, glycerol may be not the appropriate carbon source for a microbial production induced after the maximal cell concentration of the fermentation has been attained. However, if the induction is initiated earlier, the production of proteins should not be disturbed by acetic acid. Whether or not glycerol is a suitable carbon source depends on the point of induction.

The formation of acetic acid was different in glucose and glycerol medium; however, there was no significant difference in the cell concentration attainable by dialysis cultivation using a DO-stat strategy. Such a culture of *E. coli* could avoid growth inhibition by acetic acid effectively and achieve the extremely high cell concentrations of 190 g/l and 180 g/l dry cell weight in glucose and glycerol medium respectively. Some unidentified by-products that cause the cell-free culture broth to turn brown at high cell concentration have also been considered to inhibit cell growth (Shimizu et al. 1992; Ogbonna and Märkl 1993). However, such extracellular products were diluted in this dialysis reactor, which might allow the cell concentration to approach the theoretical maximum.

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