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Effects of Acetate on the Growth and Fermentation Performance of *Escherichia coli* KO11

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Abstract

Escherichia coli KO11, in which the genes *pdc* (pyruvate decarboxylase) and *adh* (alcohol dehydrogenase) encoding the ethanol pathway from *Zymomonas mobilis* were inserted into the chromosome, has been shown to metabolize all major sugars that are constituents of hemicellulosic hydrolysates to ethanol, in anaerobic conditions. However, the growth and fermentation performance of this recombinant bacteria may be affected by acetic acid, a potential inhibitor present in hemicellulose hydrolysates in a range of 2.0–15.0 g/L. It was observed that acetate affected the growth of *E. coli* KO11, prolonging the lag phase and inducing loss of biomass production and reduction of growth rate. At lower pH levels, the sensitivity to acetic acid was enhanced owing to the increased concentration of the protonated species. On the other hand, the recombinant bacteria showed a high tolerance to acetic acid regarding fermentative performance. In Luria broth medium with glucose or xylose as a single sugar source, it was observed that neither yield nor productivity was affected by the addition of acetate in a range of 2.0–12.0 g/L, suggesting some uncoupling of the growth vs ethanol production.

Index Entries: *Escherichia coli*; recombinant; ethanol; acetate; toxicity; biomass; fermentation.

Introduction

Ethanol may be used as an alternative fuel and it is believed to be beneficial with respect to environmental, social, and economical issues *(1,2)*.

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Industrial production of ethanol from sugar cane, corn, and sugar beet is largely established. In the near future, cheaper raw material, such as the abundant and renewable lignocellulose, will be used as a source of fermentable sugars. The utilization of lignocellulosic residues may largely contribute to reduced ethanol production costs, and technologies for the conversion of plant biomass to alcohol are available *(3,4)*.

Lignocellulose is a complex polymer consisting of cellulose bundles involved in a matrix of hemicellulose and lignin. The hydrolysis of the hemicellulosic fraction results in a mixture of pentoses and hexoses, and the complete bioconversion of all these sugars to ethanol is essential to increase the efficiency of the process and reduce the costs of the fuel ethanol *(4)*. Many authors have shown that *Escherichia coli* KO11 is capable of metabolizing a broad range of sugars, fermenting pentoses, and hexoses to ethanol with conversion efficiency near the theoretical maximum *(5,6)*. This recombinant strain also increases growth in liquid medium containing fermentable sugars, achieving final cell densities two to three times higher than that of the nontransformant control *(5)*.

Nevertheless, the fermentation of hemicellulosic hydrolysates is seriously limited owing to the presence of inhibitory substances to the growth and metabolism of the ethanologenic microorganisms *(7,8)*. Such substances are produced during the thermochemical processing of the biomass and include acetic acid, furfural, and hydroxymethylfurfural. Acetic acid is produced by the deacetylation of the acetylated groups present in the hemicellulosic fraction, and furfural and hydroxymethylfurfural are products of the degradation of carbohydrates. The inhibitory action depends on the type and concentration of the substrate, temperature, pH, concentration of oxygen, physiological condition of the cells, and cellular concentration *(9)*. The inactivation or removal of these toxic compounds is essential to improve the efficiency of the bioconversion process, and many strategies have been investigated, such as hydrolysate pretreatment, microorganism adaptation, and utilization of high cellular concentration inocula *(10–13)*.

It is known that acetic acid inhibits the growth of *E. coli* and causes the reduction of growth yield *(14)*. It is also known that the addition of acetic acid to the fermentation medium results in a decrease in the fermentation rate of *Saccharomyces cerevisiae* concomitantly with a reduction of internal pH *(15)*. The undissociated form of the acid has the ability to transverse the cell membrane freely; then, it dissociates intracellularly and causes the acidification of the cytoplasm. As a result, the gradient of protons through the membrane cannot be maintained, and the production of energy and the transport systems dependent on this gradient are discoupled *(14–16)*. Besides the decrease in the fermentative rate and growth yield, prolonged lag phase and inhibition of enzymes could be attributed to the acidification of the cytoplasm caused by acetic acid *(15)*. Acetic acid also induces an anion accumulation, resulting in increased internal osmotic pressure of *E. coli* cells *(17)*. It has been suggested that the cells deviate energy in order to reduce the intracellular concentration of acetate, inducing a transport

system, and, consequently, growth yield decreases. The inhibitory effect of acetic acid is pH dependent, because the concentration of the undissociated form is higher in lower pH levels *(14–16)*.

Nevertheless, it has been shown recently that recombinant *E. coli* is highly tolerant to acetic acid, and it has been suggested that setting and maintaining the pH of the fermentation broth at 7.0, when only 0.56% of the acetic acid is in the undissociated form, is an effective means of achieving sufficient acetate tolerance without significant losses in fermentation performance *(14,16)*. The purpose of this work was to observe the fermentation performance of recombinant *E. coli* as a function of acetic acid concentration with glucose or xylose as fermentable sugar, as well as to study the effect of acetic acid and pH on the growth of this genetic construct.

Materials and Methods

Microorganism

E. coli KO11, containing the *pdc* and *adh* genes of *Zymomonas mobilis* and genes for resistance to chloramphenicol, was used in this work *(18)*. Bacteria were cultured in solid Luria-Bertani broth medium (see next section for composition) containing 20.0 g/L of glucose and 40.0 mg/L of chloramphenicol, and incubated at 30°C for 24 h. Isolated colonies were transferred to liquid Luria broth medium containing 20.0 g/L of glucose and 40.0 mg/L of chloramphenicol, and incubated at 30°C for 6 h on a rotatory shaker. Stock cultures were prepared by diluting cultures with an equal volume of 80% glycerol and were kept at –20°C.

Culture Media

Liquid Luria broth medium contained the following: 10.0 g/L of tryptone, 5.0 g/L of yeast extract, and 5.0 g/L of sodium chloride *(19)*. Solid Luria broth medium contained, in addition, 12.0 g/L of agar. Luria broth medium was supplemented with glucose or xylose at the specified concentrations for each fermentation experiment; sodium acetate was added at a range of 2.0–12.0 g/L. Potassium phosphate buffer (pH 5.5–7.0) was prepared by mixing solutions of 0.8 *M* monobasic potassium phosphate and 0.8 *M* dibasic potassium phosphate. All media were sterilized by autoclaving at 121°C for 20 min. The fermentation broth was prepared by mixing liquid Luria broth medium, phosphate buffer solution, and sugar solution in a 1:1:2 ratio. Chloramphenicol was included in the fermentation broth at a final concentration of 40.0 µg/mL.

Preparation of Inocula for Fermentation and Growth Experiments

The bacteria were cultured in solid Luria broth medium containing 20.0 g/L of glucose and 40.0 mg/L of chloramphenicol, and incubated at 30°C for 24 h. Isolated colonies were transferred to liquid Luria broth medium containing 20.0 g/L of glucose and 40.0 mg/L of chloramphenicol, and

incubated at 30°C for 6 h, in a rotatory shaker. Resulting cultures were diluted 10-fold into flasks containing the fermentation broth.

Fermentation Experiments

All the fermentation experiments were conducted at 30°C and pH 7.0, on a rotatory shaker. One hundred–milliliter flasks containing 50 mL of the fermentation broth were capped with rubber stoppers drilled to allow the insertion of a sampling gage.

Growth Experiments

Cells were grown in liquid Luria broth medium containing 10.0 g/L of glucose or xylose, and sodium acetate in a range of 2.0–12.0 g/L. Tubes (18 × 180 mm) containing 10 mL of the culture medium were capped with appropriately drilled rubber stoppers. Cell density was measured with a Coleman spectrophotometer at 550 nm. At 1 OD, 1 mL contains approx 0.24 mg of cell protein/mL and 0.33 mg of cell dry wt/mL *(20)*.

Analytical Procedures

To measure the consumption of sugars and production of ethanol, samples were taken at appropriate intervals and clarified by centrifugation (10,000 rpm for 10 min) to remove cells. Supernatants were filtered in a 0.22-µm Millipore filter and stored at –20°C. Glucose, xylose, and acetic acid concentrations were measured using a Bio-Rad high-performance liquid chromatograph equipped with a refraction index detector, an electronic integrator, and an Aminex HPX-87H column at 65°C; five millimolar sulfuric acid at a flow rate of 0.8 mL/min was used as mobile phase. For ethanol analysis, supernatants were mixed with an equal volume of 20% isopropanol. Ethanol was determined using a Shimadzu gas chromatograph equipped with a Chromosorb 101 column at 120°C, detector at 210°C, and injector at 180°C.

Calculation of Fermentation Parameters

The maximum theoretical yield of ethanol from both hexose and pentose sugars is 0.51 g of ethanol/g of sugar. This value was used as a basis for the calculation of conversion efficiency (%). The volumetric productivity (Q_p) was calculated by dividing the maximum ethanol concentration by the time required to achieve such concentration (g/L·h). The ethanol yield ($Y_{P/S}$) was calculated as the maximum concentration of ethanol produced divided by the concentration of sugar initially present in the medium (g of ethanol/g of sugar).

Results and Discussion

To observe the tolerance of *E. coli* KO11 to acetic acid, defined concentrations of sodium acetate (range 2.0–15.0 g/L) were added to the fermen-

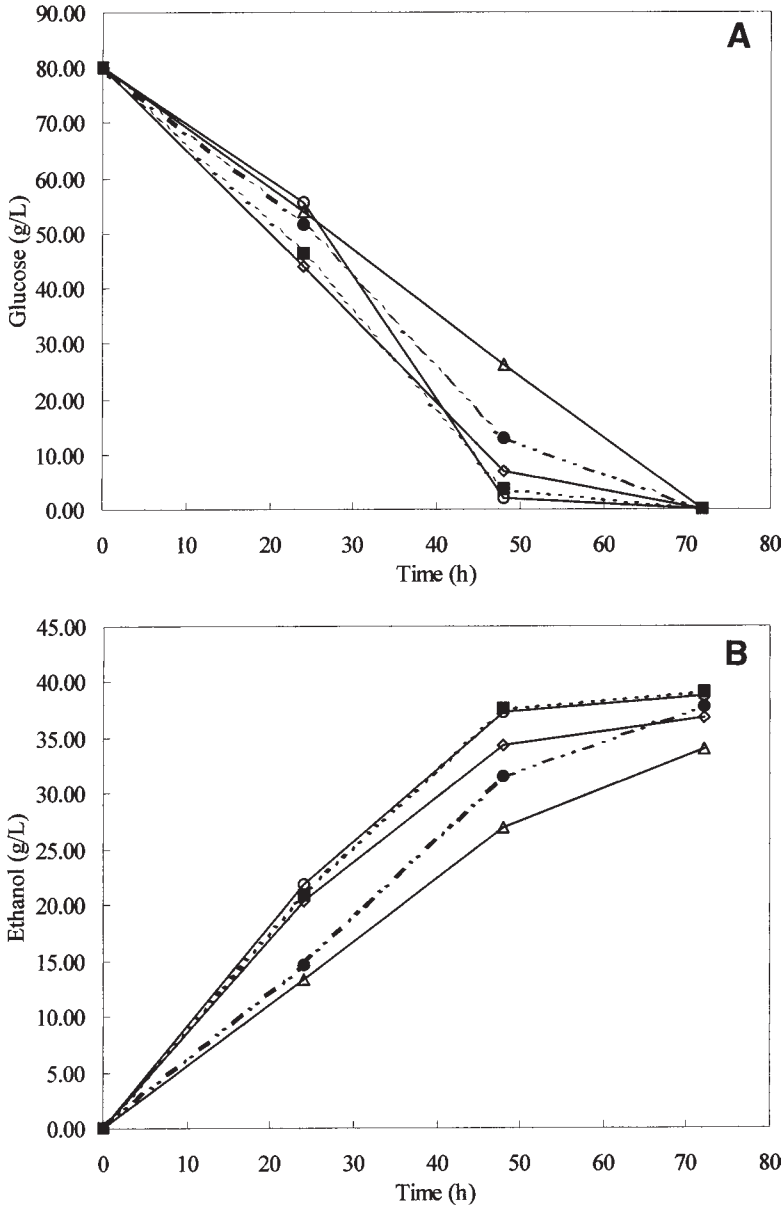

Fig. 1. Sugar consumption **(A)** and ethanol production **(B)** by *E. coli* strain KO11 in Luria broth medium containing 80.0 g/L of glucose and different initial sodium acetate concentrations. Fermentation was conducted at 30°C and initial pH of 7.0. (—◇—), reference; (– –■– –), 2.0 g/L acetate; (—○—), 10.0 g/L acetate; (– ·●· –), 12.0 g/L acetate; (—△—), 15.0 g/L acetate.

tation broth, and conversion of sugars to ethanol was observed in function of time.

Figure 1 shows the time course of glucose consumption and ethanol production in the different media. Almost all glucose was consumed within

Table 1
Effect of Different Initial Sodium Acetate Concentrations on Performance of *E. coli* KO11 in Luria Broth Medium Containing Glucose[a]

Sodium acetate (g/L)	Si (g/L)	Maximum EtOH (g/L)	Time (h)	$Y_{P/S}$ (g ethanol/g sugar)	Q_P (g ethanol/L · h)
0	80.00	36.81	72	0.46	0.51
2.0	80.00	39.08	72	0.49	0.54
5.0	80.00	39.57	72	0.49	0.55
8.0	80.00	39.15	48	0.49	0.82
10.0	80.00	38.77	72	0.48	0.54
12.0	80.00	37.78	72	0.47	0.52
15.0	80.00	34.06	72	0.42	0.47

[a]Si, initial sugar concentration; maximum EtOH, highest ethanol concentration achieved during fermentation; $Y_{P/S}$, ethanol yield (maximum ethanol concentration divided by initial sugar concentration); Q_P, volumetric productivity (maximum ethanol concentration divided by fermentation time). Luria broth medium = 10.0 g/L of tryptone, 5.0 g/L of yeast extract, and 5.0 g/L of sodium chloride.

48 h in media without acetate or containing 2.0–10.0 g/L of acetate. In media containing 12.0 and 15.0 g/L of acetate, about 16 and 33% of glucose was not consumed in 48 h, respectively. At first sight, ethanol yield and volumetric productivity were not affected in the presence of 2.0–12.0 g/L, in comparison with the control medium without acetate. Only in medium containing 15.0 g/L of acetate were both ethanol yield and volumetric productivity depressed (Table 1).

In Luria broth medium containing 50.0 g/L of xylose, fermentation was affected at all concentrations of acetate above 4.0 g/L (Fig. 2). After 24 h, about 77% of xylose was consumed in control medium without acetate and in medium containing 2.0 g/L of acetate. In the other media, xylose was consumed more slowly; in medium containing 15.0 g/L of acetate, about 55% of xylose was not consumed in 24 h, and ethanol production was adversely affected. Nevertheless, in the range of 2.0–10.0 g/L of acetate, fermentation pattern, in terms of ethanol yield and volumetric productivity, was not disturbed (Table 2). Many other investigators have also demonstrated the higher tolerance of this recombinant bacteria to acetic acid *(13,14,16,21)*.

The degree of acetate inhibition is interrelated with various environmental factors such as pH. It is known that the inhibitory effect of acetic acid is pH dependent, since the concentration of the toxic form of acetic acid decreases exponentially as the external pH increases *(14,15)*. Therefore, the control of pH is fundamental in order to maximize the tolerance of the microorganism to acetic acid. To define the best conditions in which *E. coli* KO11 shows high tolerance to acetate, we investigated the effect of different pH values on the fermentative performance of the bacteria grown in Luria broth medium containing 80.0 g/L of glucose and 5.0 g/L of sodium acetate.

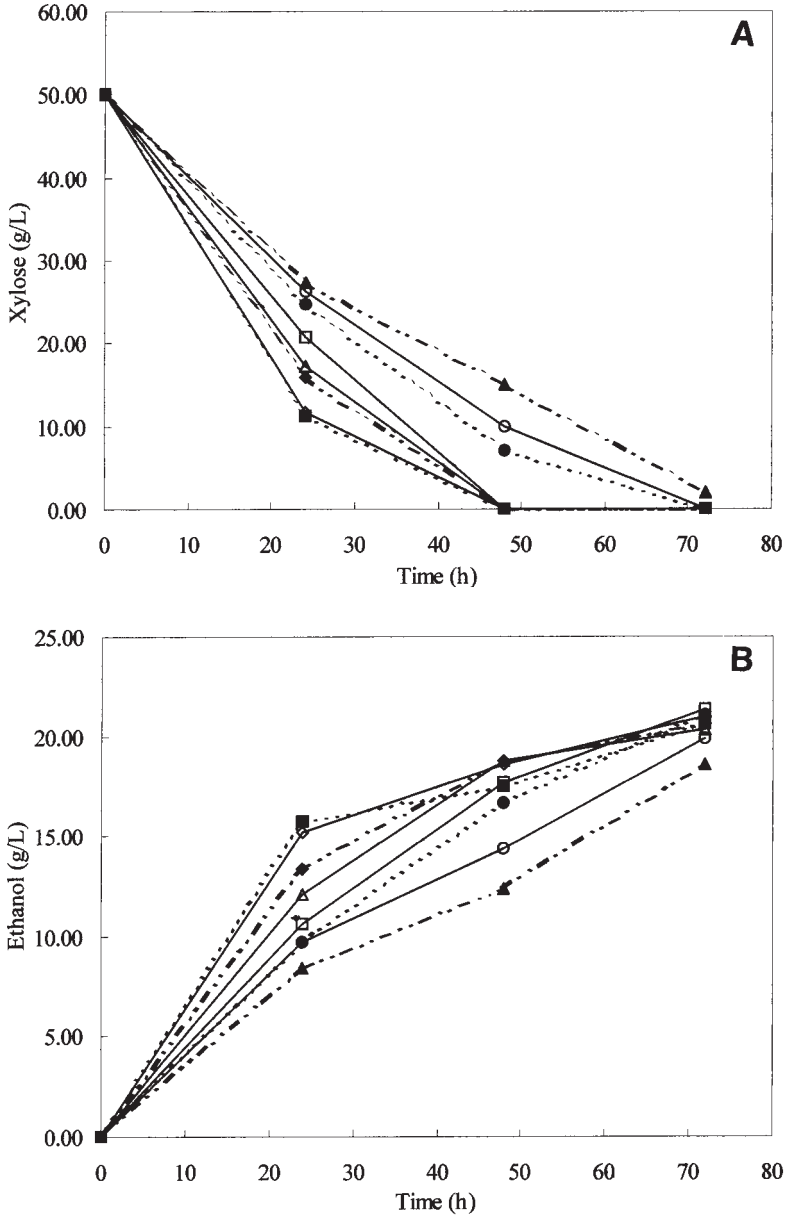

Fig. 2. Sugar consumption **(A)** and ethanol production **(B)** by *E. coli* strain KO11 in Luria broth medium containing 50.0 g/L of xylose and different initial sodium acetate concentrations. Fermentation was conducted at 30°C and initial pH of 7.0. (—◇—), reference; (– –■– –), 2.0 g/L acetate; (—△—), 4.0 g/L acetate; (– ·◆· –), 6.0 g/L acetate; (—□—), 8.0 g/L acetate; (– –●– –), 10.0 g/L acetate; (—○—), 12.0 g/L acetate; (– ·▲· –), 15.0 g/L acetate.

Analyzing glucose consumption and ethanol production in the first 24 h of fermentation, we observed that glucose consumption was affected especially at lower pH values (Fig. 3). At pH 5.5, 88% of glucose was not

Table 2
Effect of Different Initial Sodium Acetate Concentrations on Performance of *E. coli* KO11 in Luria Broth Medium Containing Xylose[a]

Sodium acetate (g/L)	Si (g/L)	Maximum EtOH (g/L)	Time (h)	$Y_{P/S}$ (g ethanol/g sugar)	Q_P (g ethanol/L · h)
0	50.00	20.93	72	0.42	0.29
2.0	50.00	20.64	72	0.41	0.29
4.0	50.00	20.30	72	0.41	0.28
6.0	50.00	20.58	72	0.41	0.28
8.0	50.00	21.30	72	0.43	0.29
10.0	50.00	21.04	72	0.42	0.29
12.0	50.00	19.88	72	0.40	0.28
15.0	50.00	18.59	72	0.39	0.26

[a]Si, initial sugar concentration; maximum EtOH, highest ethanol concentration achieved during fermentation; $Y_{P/S}$, ethanol yield (maximum ethanol concentration divided by initial sugar concentration); Q_P, volumetric productivity (maximum ethanol concentration divided by fermentation time). Luria broth medium = 10.0 g/L of tryptone, 5.0 g/L of yeast extract, and 5.0 g/L of sodium chloride.

consumed after 24 h, whereas at pH 7.0, almost 60% of glucose was fermented to ethanol. At pH 5.5, poor ethanol yield and slower fermentation were observed, as a result of the higher concentration of the toxic form of acetic acid. Furthermore, the lower pH level *per se* may also have an influence. The extent of acetate inhibition on the fermentation performance of a microorganism can be monitored by controlling the pH of the fermentation broth at levels that allow sufficient tolerance to acetic acid, without negative effects in terms of both yield and productivity.

However, acetic acid has a negative effect on the growth of this genetic construct *(14)*. Some investigators have observed that the energy derived from catabolism is utilized in order to maintain a constant value of cytoplasmic pH, thus reducing growth *(14)*.

To observe the effect of acetate on the growth of *E. coli* KO11, the bacteria were cultured in Luria broth medium containing glucose or xylose as sugar source and different concentrations of sodium acetate (2.0–12.0 g/L). A reference was made with no acetate. The pH of the medium was adjusted to values between 5.5 and 7.0.

All the acetate concentrations affected biomass production in medium containing glucose or xylose. Increasing acetate concentrations resulted in higher inhibition, as reflected in the reduction of growth rate. In Luria broth medium containing glucose, at pH 7.0, a cellular concentration of 0.31 mg/mL was reached after 6 h of growth, in the absence of acetate. This concentration was reduced to 0.25 mg/mL in medium containing 12.0 g/L of acetate. At lower pH values, growth was affected even more by the presence of acetate, and the highest inhibition was observed at pH 5.5: the presence of 12.0 g/L of acetate induced a 58.6% loss of biomass production. It was also

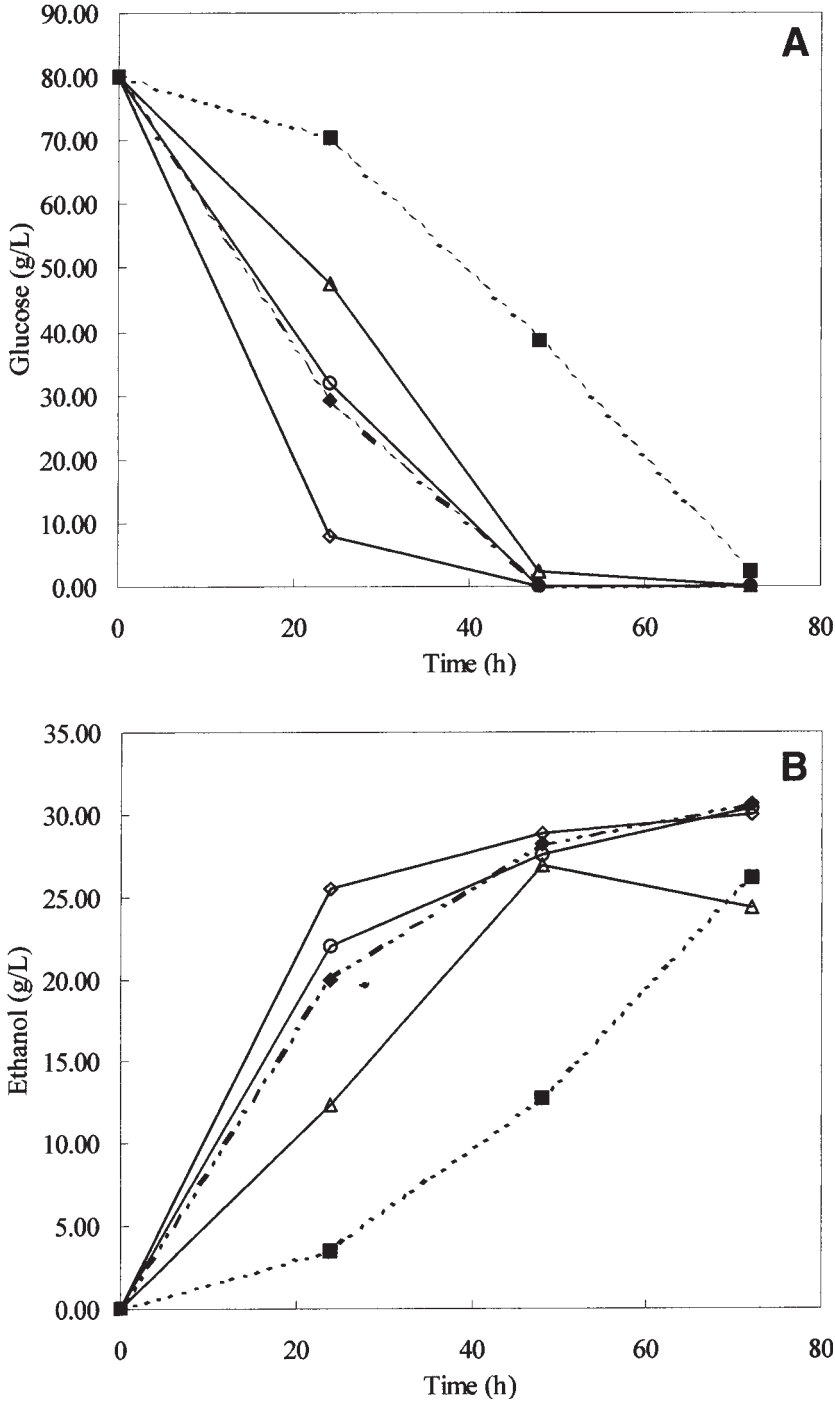

Fig. 3. Effect of different initial pH values on sugar consumption **(A)** and ethanol production **(B)** by *E. coli* strain KO11 in Luria broth medium containing 80.0 g/L of glucose and 5.0 g/L of acetate. Reference medium: without acetate. (—◇—): pH 7.0, reference; (– –■– –): pH 5.5, 5.0 g/L acetate; (—△—): pH 6.0, 5.0 g/L acetate; (– ·◆· –): pH 6.5, 5.0 g/L acetate; (—○—): pH 7.0, 5.0 g/L acetate.

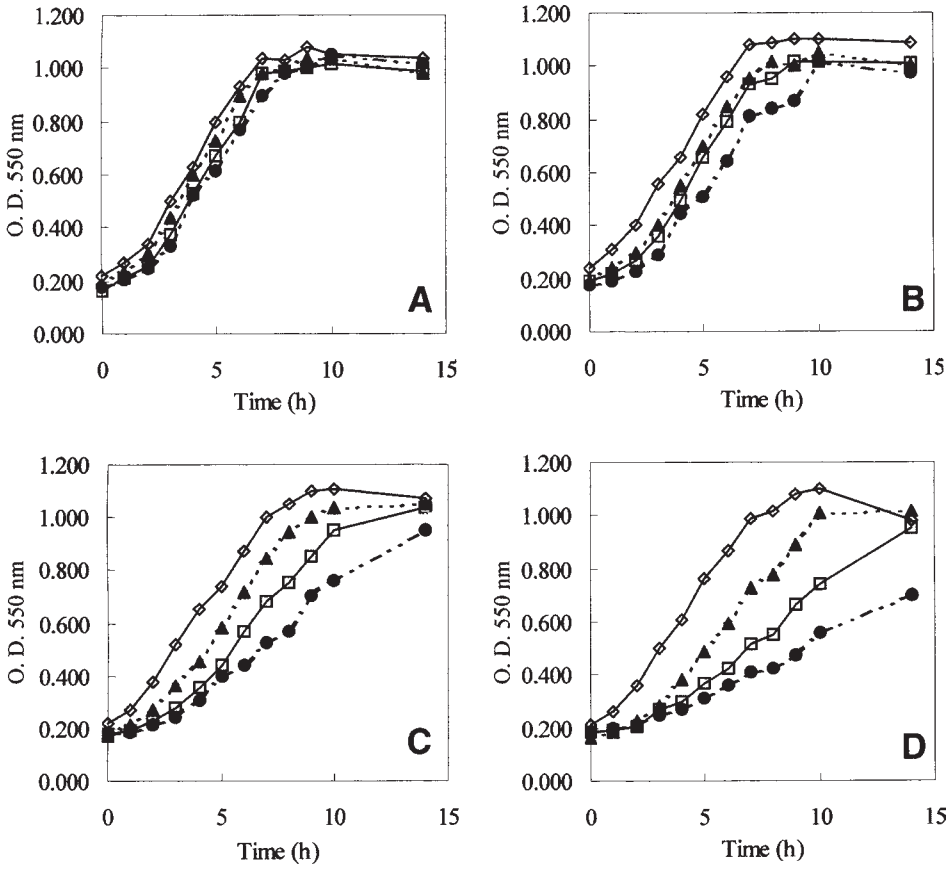

Fig. 4. Growth of *E. coli* strain KO11 with glucose as sole source of sugar and different initial contents of sodium acetate: **(A)** pH 7.0; **(B)** pH 6.5; **(C)** pH 6.0; and **(D)** pH 5.5. Essentially similar growth curves were observed with xylose as sole source of sugar. (—◇—), reference; (– –▲– –), 4.0 g/L acetate; (—□—), 8.0 g/L acetate; (– ·●· –), 12.0 g/L acetate.

observed that the higher the acetate concentration, the longer the lag phase in comparison with the control medium without acetate. In all the ranges of acetate concentrations, the reduction in the growth rate was higher at pH 5.5 (Fig. 4).

In Luria broth medium containing xylose, growth was also affected at all concentrations of acetate, and patterns of growth curves essentially similar to those of glucose were observed. Decreasing the pH value from 7.0 to 5.5 also caused the growth to decline further. At pH 7.0, a cellular concentration of 0.34 mg/mL was achieved in the control medium without acetate, after 10 h of growth. This concentration was reduced to 0.27 mg/mL in the presence of 12.0 g/L of acetate. At pH 5.5, this concentration of acetate reduced cellular concentration by 61.3%. Thus, the inhibitory effect of acetate on the growth of the bacteria was stronger at lower pH levels, as reflected in the decrease in growth rate. This finding confirms that sensibil-

ity to acetic acid is greater at lower pH values, owing to the higher concentration of the toxic protonated species of the acetic acid. Acetate also induced delayed growth, especially at lower pH values.

The results we have presented show that biomass production is reduced at all tested acetate concentrations whereas yields on ethanol are less or not affected, indicating some uncoupling of alcohol production from growth in the presence of acetate.

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