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Characterization of the impact of acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*

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ABSTRACT

Ethanolic fermentation of simple sugars is an important step in the production of bioethanol as a renewable fuel. Significant levels of organic acids, which are generally considered inhibitory to microbial metabolism, could be accumulated during ethanolic fermentation, either as a fermentation product or as a by-product generated from pre-treatment steps. To study the impact of elevated concentrations of organic acids on ethanol production, varying levels of exogenous acetate or lactate were added into cultures of *Thermoanaerobacter ethanolicus* strain 39E with glucose, xylose or cellobiose as the sole fermentation substrate. Our results found that lactate was in general inhibitory to ethanolic fermentation by strain 39E. However, the addition of acetate showed an unexpected stimulatory effect on ethanolic fermentation of sugars by strain 39E, enhancing ethanol production by up to 394%. Similar stimulatory effects of acetate were also evident in two other ethanologens tested, *T. ethanolicus* X514, and *Clostridium thermocellum* ATCC 27405, suggesting the potentially broad occurrence of acetate stimulation of ethanolic fermentation. Analysis of fermentation end product profiles further indicated that the uptake of exogenous acetate as a carbon source might contribute to the improved ethanol yield when 0.1% (w/v) yeast extract was added as a nutrient supplement. In contrast, when yeast extract was omitted, increases in sugar utilization appeared to be the likely cause of higher ethanol yields, suggesting that the characteristics of acetate stimulation were growth condition-dependent. Further understanding of the physiological and metabolic basis of the acetate stimulation effect is warranted for its potential application in improving bioethanol fermentation processes.

1. Introduction

The unsustainable consumption of petroleum-based fuels requires the production of renewable and carbon neutral transport fuels (Towler et al., 2004). Bioethanol has been the focus of technology development for renewable fuel production with its compatibility with gasoline and existing infrastructure (Agarwal, 2007; Demirbas, 2007). Currently, bioethanol can be produced from two major source materials, food stock, such as corn, and lignocellulose, which is the most abundant organic compound in nature (Kuhad and Singh, 1993). Food stock-based bioethanol is considered unsustainable with the growing competition between energy feedstock supply versus food supply to utilize the same existing farmland and water resources (Chakravortya et al., 2008). In comparison, the abundance of lignocellulosic biomass makes cellulosic bioethanol the more economically feasible alternative.

Current technologies for the conversion of lignocellulose to bioethanol require pre-treatment steps for the hydrolysis of lignocellulosic materials into fermentable sugars, such as glucose and xylose, prior to microbial fermentation, where ethanol is generated as an end product along with other fermentation by-products (Lynd et al., 1991, 2002). These pre-treatment procedures may result in a host of inhibitory by-products, including organic acids such as acetic acid, in lignocellulosic hydrolysates at concentrations that may significantly reduce ethanol yields and productivity during ethanolic fermentation (Palmqvist and Hahn-Hagerdal, 2000). Of these inhibitors, organic acids are of particular concern, since these compounds are also produced in considerable amounts in ethanolic fermentation as fermentation end products (Kumar et al., 2008). Considering the occurrence of organic acids in the production processes of bioethanol from lignocellulose, it is important to investigate the impact of these potentially inhibitory compounds on ethanolic fermentation.

Thermophilic fermenting microorganisms have been extensively studied for their potential uses in carbohydrate fermentation and ethanol production because of their higher fermentation efficiency and advantages in process control at elevated temperature

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(Demain et al., 2005; Zeikus, 1979). *Thermoanaerobacter ethanolicus* strain 39E has been a model for Gram-positive, thermophilic anaerobes suitable for bioethanol production with its ability to rapidly ferment both hexose and pentose sugars into ethanol at temperatures above 60 °C (Lee et al., 1993; Zeikus et al., 1980). Given the fact that strain 39E also produces acetate and lactate as major fermentation end products (Lacis and Lawford, 1991; Ng et al., 1981), it is important to study the impact of these compounds on ethanolic fermentation.

Therefore, the objective of this work is to characterize the impact of acetate and lactate as representative organic acids on ethanolic sugar fermentation using *T. ethanolicus* strain 39E as a model thermophilic ethanologen. It was found that the presence of acetate provided significant enhancement, instead of inhibition, for both growth and ethanol production in cultures of strain 39E during sugar fermentation. This stimulatory effect was further demonstrated in another *Thermoanaerobacter* strain X514 (Roh et al., 2002), as well as the more distantly related thermophilic ethanologen *Clostridium thermocellum* (McBee, 1948), suggesting that the stimulatory effect of elevated acetate on ethanol production might be widely present in ethanologenic microorganisms.

2. Methods

2.1. Microorganisms

Thermoanaerobacter ethanolicus strain 39E (ATCC 33323) and *C. thermocellum*, (ATCC 27405) were obtained from the American Type Culture Collection (Manassas, Virginia, USA). *T. ethanolicus* strain X514 is a novel isolate from the deep subsurface in the Piceance Basin, Colorado, USA and is maintained in our laboratory culture collection (Roh et al., 2002).

2.2. Media formulation and preparation

An anaerobic mineral basal medium modified from a previously described formula (He and Sanford, 2004a) was used throughout this study. The medium contained the following (per liter): 10.0 g NaCl, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2 \cdot 2H_2O$, 1 ml trace element solution, 1 ml selenium–tungsten solution, 10 ml vitamin solution, 2.52 g $NaHCO_3$, and 0.05 mg resazurin. The trace element solution contained the following (per liter): 1.5 g $FeCl_2 \cdot 4H_2O$, 0.19 g $CoCl_2 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg $Na_2MoO_4 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, and 2 mg $CuCl_2 \cdot 2H_2O$. The selenium–tungsten solution contained 6 mg $Na_2SeO_3 \cdot 5H_2O$ per liter, 8 mg $Na_2WO_4 \cdot 2H_2O$ per liter, and 0.54 of NaOH per liter. The vitamin solution contained the following (per liter): 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine hydrochloride, 50 mg riboflavin, 50 mg thiamine, 50 mg nicotinic acid, 50 mg pantothenic acid, 1 mg vitamin B_{12}, 50 mg *p*-aminobenzoic acid, and 50 mg thioctic acid.

Sulfide (0.048 g $Na_2S \cdot 9H_2O$) and cysteine (0.031 g L-cysteine) were added as reductants to the medium after it had been boiled and cooled to room temperature. The headspace of the medium container was continuously flushed with an oxygen-free nitrogen/CO_2 gas mix, and the pH was adjusted to 7.2 ± 0.1 by varying the CO_2 composition of the mix. The vitamin solution and fermentation substrates, i.e. glucose, xylose or cellobiose, were added from sterile anaerobic stock solutions after autoclaving.

2.3. Experimental design and growth conditions

All cultures were routinely grown in 125-ml serum bottles with 50 ml of boiled degassed medium or in 30-ml anaerobic culture tubes with 10 ml of medium and closed with butyl rubber stoppers and aluminum seals. Cultures were grown in the dark at 65 °C with occasional mixing. For routine cultivation, 0.5% (w/v) cellobiose was added as the fermenting substrate. The cultures were maintained by transferring a 1% (v/v) inoculum to fresh medium after cellobiose was depleted and growth had stopped. Strict anaerobic techniques were used throughout in experimental manipulations. Sterile syringes and needles, used for substrate addition and sampling, were flushed with N_2 prior to use.

To evaluate the impact of acetate or lactate on the fermentation of sugars, varying concentrations of sodium acetate or sodium lactate ranging from 10 to 150 mM were added into the mineral basal medium along with 0.5% (w/v), unless otherwise stated, of one of the three sugar substrates, glucose, xylose, or cellobiose. Fermentation was started with 1% (v/v) inoculation from a late log-phase culture of *T. ethanolicus* strain 39E, strain X514, or *C. thermocellum*. Cultures with no addition of sugar substrates, cells, acetate, or lactate were also set up as controls. All cultures were incubated at 65 °C with growth and fermentation products monitored periodically. All experiments were performed in triplicate cultures.

To test the influence of yeast extract on ethanolic fermentation, the above experimental procedure was repeated with the exception that 0.1% (w/v) Bacto™ yeast extract (BD Diagnostics, Sparks, Maryland USA) was added during medium preparation. All other experimental conditions were identical.

2.4. Analytical methods

To monitor the production of fermentation end products, samples (1 ml) from the cultures were taken periodically via degassed sterile syringes and filtered through Millipore GSWP 0.20-μm filters prior to quantification by chromatography. Ethanol was quantified with a Hewlett Packard 5890 Gas Chromatography (GC) equipped with a flame ionization detector (FID) and a DB-Wax column (Agilent Technologies, Santa Clara, California USA) using a previously described method (Zhang et al., 2007). Organic acids produced in fermentation were analyzed with a Hewlett Packard 1090 High Performance Liquid Chromatography (HPLC), which was equipped with a Chemstation analysis software package and a BioRad Aminex HPX-87H ion exclusion column. HPLC analysis was conducted using 0.005 N H_2SO_4 as the eluent as previously described (He and Sanford, 2004b). Previously filtered samples were acidified to 0.2 N H_2SO_4 by adding 100 μl of 2 N H_2SO_4 to 900 μl of sample. Eluent was pumped at 0.6 ml/min and detection of organic acids was at 210 nm by a UV detector. Sugar consumption was determined by the changes in sugar concentrations measured in filtered culture samples using the phenol–sulfuric acid assay (Daniels et al., 1994).

Culture growth was monitored spectrophotometrically as optical density at 600 nm wavelength. To measure the protein content of the cultures, samples of cell suspension were heated with an equal volume of 2 N NaOH in a water bath at 90 °C for 15 min. The protein concentration was determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as the reference. The pH of culture broth at the end of fermentation was measured with a pH meter after the culture was cooled down to room temperature.

2.5. Statistical analysis

To evaluate the impact of the treatment of acetate or lactate supplementation on ethanolic fermentation, differences in biomass growth and ethanol yields between cultures supplemented with varying levels of acetate/lactate were analyzed using the one-way analysis of variance (ANOVA). If significant differences in biomass growth or ethanol yields were found between different levels

of acetate/lactate treatment as indicated by a probability value less than 0.05 in ANOVA analysis, post hoc comparisons were performed using Tukey's test to further identify the treatment pairs with significant difference in biomass growth or ethanol yield as indicated by a probability value less than 0.05. For statistical analysis, the peak optical densities and ethanol yields in triplicate cultures were compared under five treatment levels of acetate or lactate: 0 (control), 10, 25, 75, and 150 mM. Details of experimental design are described in Section 2.3. Statistical analyses were performed with JMP 7.0.1 for Windows (SAS Institute Inc., Cary, North Carolina, USA).

3. Results

To test the impact of short-chain organic acids as metabolic end products during the fermentation of simple sugars on ethanol production, various concentrations of acetate or lactate were added into batch cultures of *T. ethanolicus* strain 39E with glucose, xylose, or cellobiose as the sugar substrate. Glucose and xylose were selected as representative monosaccharides of hexose and pentose, both major components of the hydrolysates of lignocellulosic biomass (Zaldivar et al., 2001). On the other hand, cellobiose was selected for testing as a potentially important disaccharide intermediate in the metabolism of cellulose, with a β-1,4-glycosidic bond that is characteristic of the linkage between the monosaccharide units in cellulose (Schwarz, 2001). The impact of organic acids was further compared between two fermentation conditions: with or without 0.1% (w/v) yeast extract as a nutrient supplement. Subsequently, the relevance of the results obtained from strain 39E was assessed in other thermophilic ethanologenic bacteria, *T. ethanolicus* strain X514 and *C. thermocellum* ATCC 27405.

3.1. Impact of acetate on ethanolic fermentation by T. ethanolicus strain 39E

In strain 39E cultures supplied with 0.5% (w/v) xylose as the fermentation substrate, initial addition of acetate ranging from 10 to 150 mM consistently resulted in enhanced growth as measured by OD_{600} and increased production of ethanol, which was accompanied by more rapid utilization of xylose (Fig. 1), which is apparently contrary to the potential inhibitory effects normally expected for elevated levels of fermentation end products (Moulin et al., 1984). The stimulatory effect of acetate also appears to strengthen with increases in the concentrations of acetate added initially, as the best cell growth and highest ethanol yield were observed at the highest acetate concentration tested (Fig. 1). Indeed, peak cell density during xylose fermentation more than doubled from less than 0.1 (OD_{600}) in control cultures without acetate addition to greater than 0.2 (OD_{600}) in strain 39E cultures exposed to 150 mM acetate (Fig. 1A). Similarly, ethanol yields increased from 4.6 mM in the control cultures to approximately 14 mM at 150 mM acetate, an increase greater than 300% (Fig. 1B).

A similar pattern was also observed in the fermentation of glucose by strain 39E, with both growth and ethanol production significantly improved when subjected to acetate addition as shown by ANOVA analysis (Fig. 2). However, in contrast to ethanol production in xylose fermentation, where the highest ethanol yield was found at 150 mM acetate (Fig. 1B), the largest increases in ethanol production and growth during glucose fermentation were seen at 75 mM of acetate, instead of 150 mM (Fig. 2B). Nonetheless, the stimulatory effect of acetate was evident.

In a separate experiment testing yet another sugar substrate cellobiose, both the growth and ethanol production by strain 39E were significantly enhanced by the exposure to elevated acetate levels as shown by ANOVA analysis (Fig. 3). Again, the stimulatory effect of acetate increased with higher acetate concentration, which is consistent with the observed stimulation of growth and ethanol production in the fermentation of xylose and glucose (Figs. 1 and 2). The consistent increases in both growth and ethanol production due to acetate addition in all three sugar substrates tested evidently demonstrated the significance of the stimulatory effects of acetate on ethanolic fermentation by strain 39E.

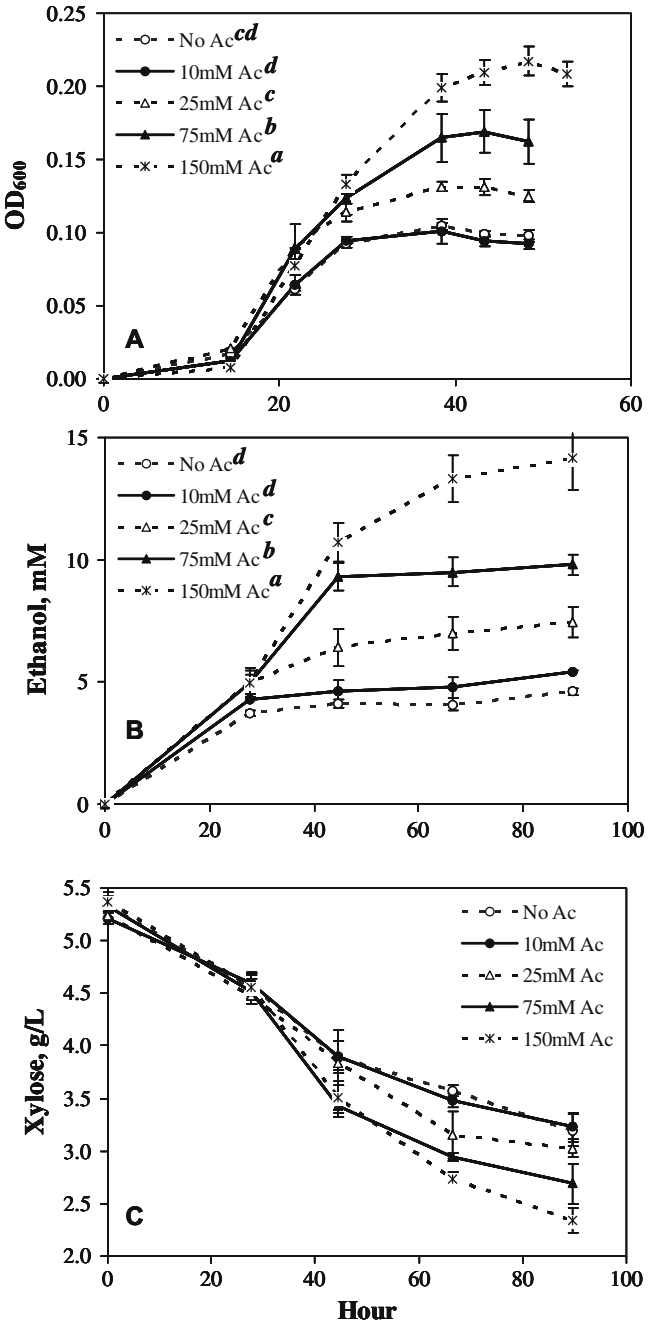

Fig. 1. Influence of exogenous acetate (Ac) on ethanol production from xylose fermentation by strain *T. ethanolicus* 39E in defined medium. (A) Growth as indicated by optical density at 600 nm; (B) production of ethanol; and (C) utilization of xylose. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were means of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

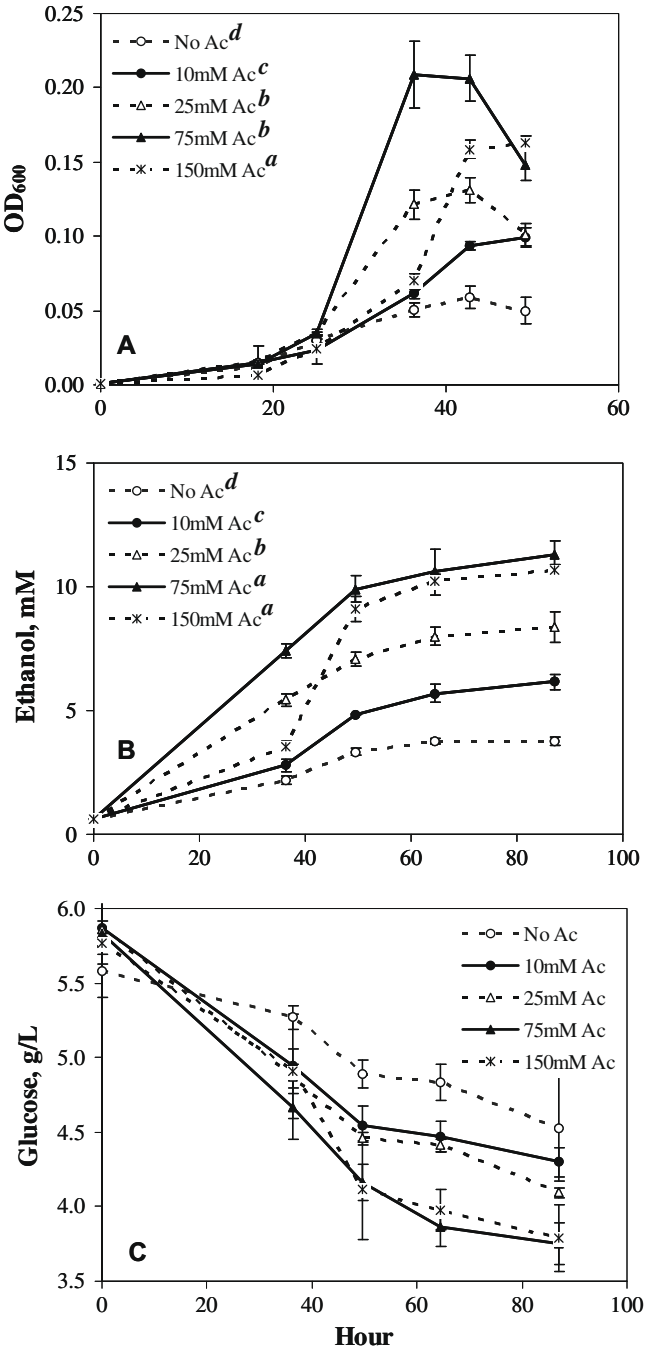

Fig. 2. Influence of exogenous acetate (Ac) on ethanol production from glucose fermentation by *T. ethanolicus* strain 39E in defined medium. (A) Growth as indicated by optical density at 600 nm; (B) production of ethanol; and (C) utilization of glucose. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were means of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

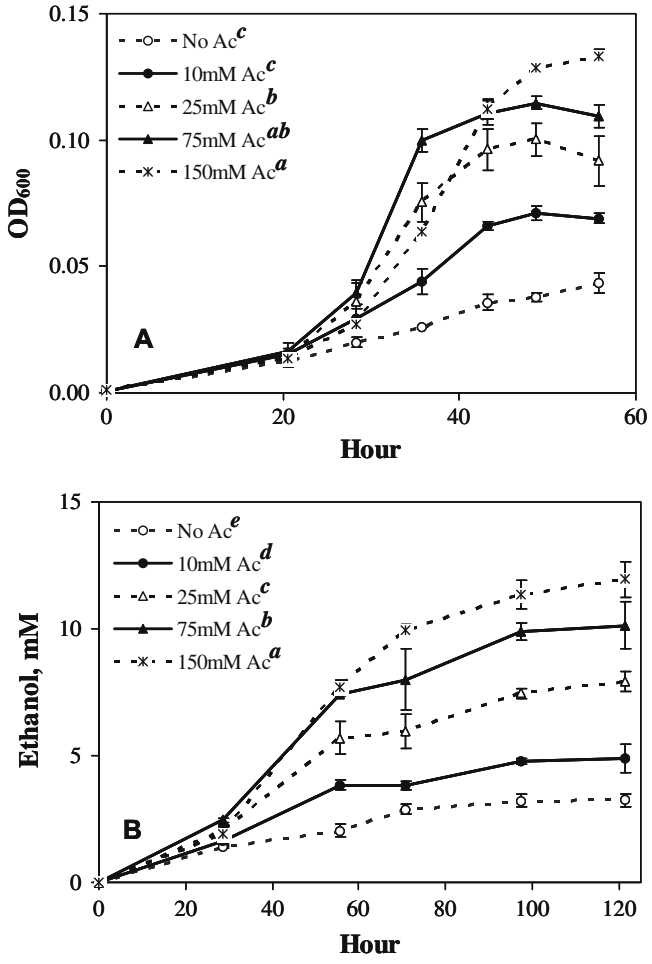

Fig. 3. Influence of exogenous acetate (Ac) on ethanol production from cellobiose fermentation by *T. ethanolicus* strain 39E in defined medium. (A) Growth as indicated by optical density at 600 nm; and (B) production of ethanol. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were means of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

3.2. Impact of lactate on ethanolic fermentation by T. ethanolicus strain 39E

Given that both lactate and acetate are major end products of sugar fermentation, whether the stimulatory effect of acetate could be extended to lactate would provide clues to the potential mechanisms involved in the seemingly unanticipated stimulatory effect observed for acetate (Figs. 1–3). In strain 39E cultures supplied with 0.5% (w/v) glucose as the fermentation substrate, initial addition of lactate ranging from 10 to 150 mM led to reductions in growth as measured by optical density (Fig. 4A). While the reductions in cell growth by lactate addition were significant at higher concentrations ($\geqslant$25 mM) as compared to those in the controls with no lactate addition, the magnitude of these reductions were albeit small, with the largest reductions occurring at the highest lactate level tested (150 mM). The differences in ethanol production between cultures exposed to elevated lactate and control cultures with no lactate were even smaller than those in cell growth (Fig. 4B), indicating a rather weak inhibitory effect by lactate on ethanolic fermentation. A similarly weak inhibitory effect by lactate was also observed in the fermentation of xylose (data not shown).

The differences in the impact on sugar fermentation and ethanol production between acetate and lactate point to the possibility that the stimulatory effect of acetate might be unique among organic acids as fermentation end products, which also suggests the stimulatory effect of acetate might be specifically related to certain unknown characteristics of acetate metabolism.

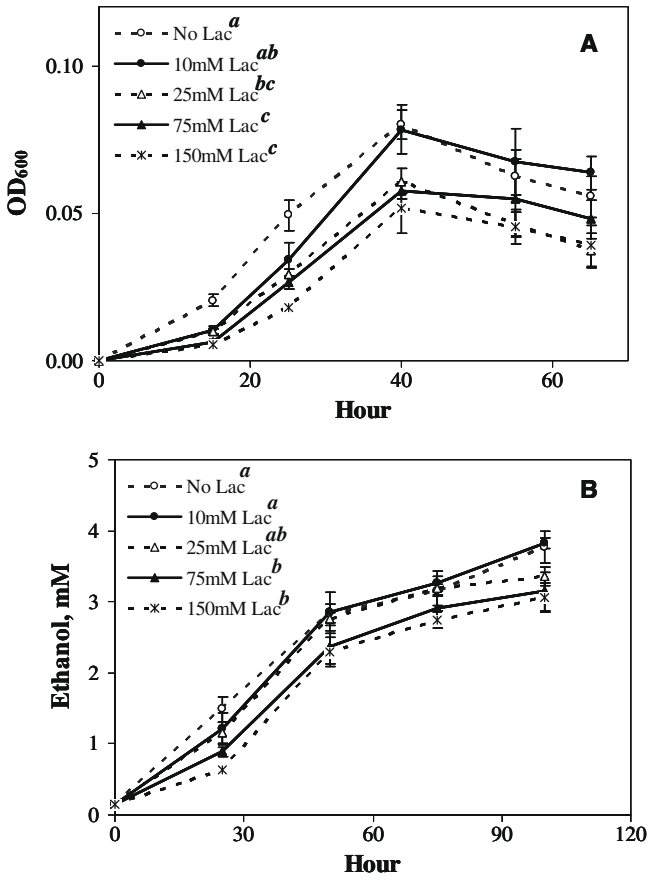

Fig. 4. Influence of exogenous lactate (Lac) on ethanol production from glucose fermentation by *T. ethanolicus* strain 39E in defined medium. (A) Growth as indicated by optical density at 600 nm; and (B) production of ethanol. Exogenous lactate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were means of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in lactate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

3.3. Significance of yeast extract as a nutrient supplement

The impact of elevated acetate and lactate on ethanolic fermentation so far in this work were studied in defined medium. An important observation during sugar fermentation in this defined medium is that the utilization of sugar was incomplete even after prolonged incubation when cell growth ceased (Figs. 1 and 2). This observation was most pronounced in control cultures with no acetate addition, where more than 60% of the sugar remained unutilized in a prolonged 90-h incubation.

The incomplete utilization of sugar substrate in cultures grown on defined medium is generally considered to be caused by nutrient limitation (Sato et al., 1992). Subsequently, a common practice to achieve more complete fermentation is the addition of yeast extract as a nutrient supplement. Since the addition of yeast extract is known to significantly influence carbohydrate metabolism in microorganisms (Hild et al., 2003; Lacis and Lawford, 1991), it is important to investigate how this common practice influences the impact of acetate and lactate on ethanolic fermentation. Therefore, the impact of varying concentrations of acetate and lactate was further tested in strain 39E cultures grown in defined medium supplemented with 0.1% (w/v) yeast extract.

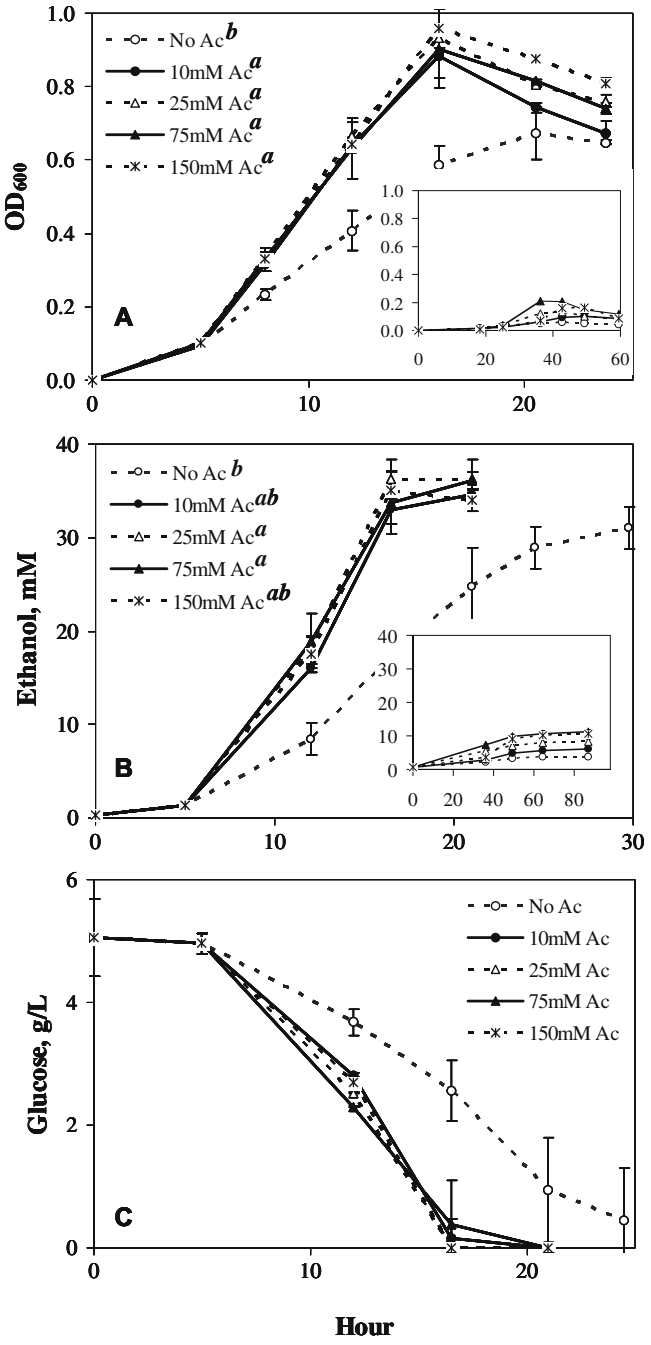

Fig. 5. Influence of exogenous acetate (Ac) on ethanol production from glucose fermentation by *T. ethanolicus* strain 39E in defined medium supplemented with 0.1% (w/v) yeast extract. (A) Growth as indicated by optical density at 600 nm; (B) production of ethanol; and (C) utilization of glucose. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were means of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$). For comparison, the insets with the same scales show growth and ethanol production by strain 39E in defined medium under the same conditions except the omission of yeast extract.

3.3.1. The effect of acetate on ethanolic fermentation by strain 39E when supplemented with yeast extract

The supplementation with a small amount of yeast extract resulted in dramatic improvements in the fermentation of glucose and xylose (Figs. 5 and 6). For example, ethanol yields in control

cultures amended with 0.1% (w/v) yeast extract increased by more than six times to more than 30 mM, as compared to the approximately 5 mM ethanol produced in control cultures receiving no yeast extract addition (Figs. 1 and 2). In the same strain 39E control cultures, the peak optical cell density increased from less than 0.1 to above 0.6 (OD_{600}), as compared to control cultures grown without yeast extract (Figs. 1 and 2). More importantly, the presence of yeast extract led to the nearly complete utilization of the sugar substrates in 25 h. In comparison, only less than 40% of the sugar substrates were consumed in 90 h by strain 39E when yeast extract was not added (Figs. 1 and 2). It appeared that ethanolic fermentation by strain 39E in defined medium was limited by nutrients which could be supplied by yeast extract.

While the addition of yeast extract resulted in remarkable enhancement of sugar fermentation, exposure to acetate further stimulated ethanol production, culture growth, and substrate utilization during the fermentation of 0.5% (w/v) glucose as compared to the controls with no acetate (Fig. 5). However, the extent of stimulation was not associated with the concentration of acetate, since no significant differences in growth, ethanol production, and glucose utilization were found between the acetate concentrations tested (ranging from 10 to 150 mM), which is in sharp contrast to the increased stimulatory effect with increasing acetate concentration observed in strain 39E cultures grown with no yeast extract addition (Fig. 2). Similarly, the supplementation of 0.1% (w/v) yeast extract did not change acetate stimulation in xylose fermentation (Fig. 6): the presence of acetate improved culture growth and ethanol production; and as in glucose fermentation, the concentration of acetate did not significantly affect the extent of the stimulation of growth and ethanol production. It is noted that the presence of yeast extract alone could not explain the stimulatory effect observed in this experiment (Figs. 5 and 6), as no detectable growth or ethanol production occurred in medium containing 0.1% (w/v) yeast extract but no glucose or xylose (data not shown). These results indicate that the presence of yeast extract masked the concentration effect of acetate stimulation.

3.3.2. The effect of lactate on ethanolic fermentation by strain 39E when supplemented with yeast extract

The inhibitory effect of lactate on ethanol fermentation observed in defined medium without yeast extract (Fig. 4) was not altered with the presence of 0.1% (w/v) yeast extract, as both growth and ethanol production by strain 39E were nonetheless reduced by

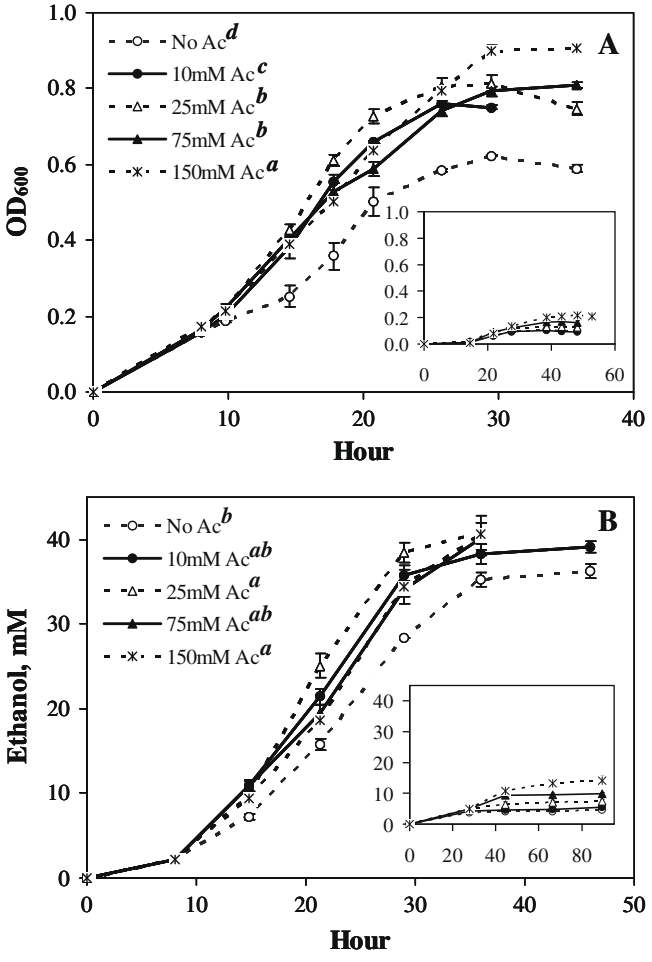

Fig. 6. Influence of exogenous acetate (Ac) on ethanol production from xylose fermentation by *T. ethanolicus* strain 39E in defined medium supplemented with 0.1% (w/v) yeast extract. (A) Growth as indicated by optical density at 600 nm; and (B) production of ethanol. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were means of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$). For comparison, the insets with the same scales show growth and ethanol production by strain 39E in defined medium under the same conditions except the omission of yeast extract.

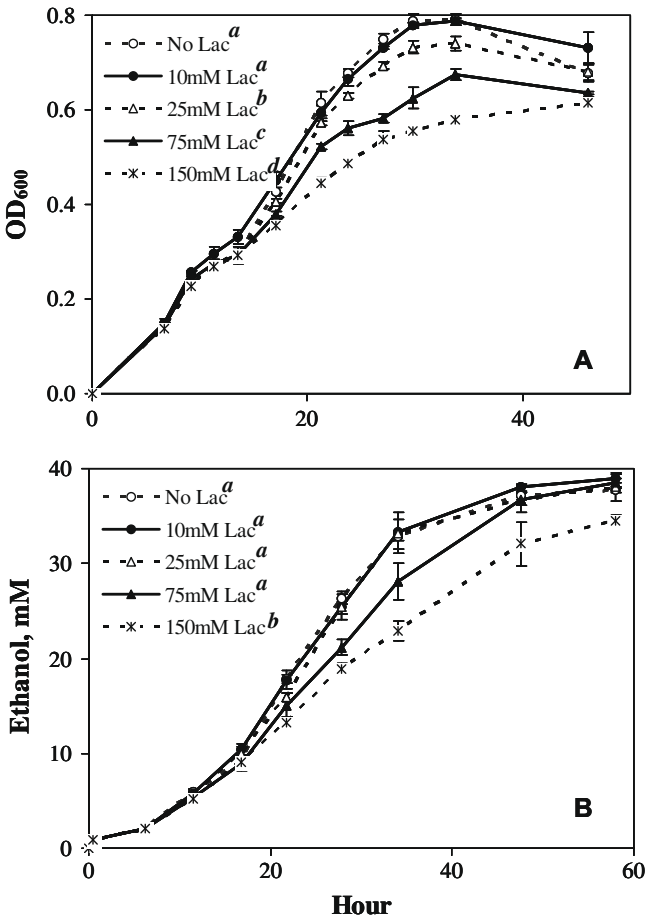

Fig. 7. Influence of exogenous lactate (Lac) on ethanol production from xylose fermentation by *T. ethanolicus* strain 39E in defined medium supplemented with 0.1% (w/v) yeast extract. (A) Growth as indicated by optical density at 600 nm; and (B) production of ethanol. Exogenous lactate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were averages of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in lactate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

lactate during xylose fermentation, even with the presence of 0.1% (w/v) yeast extract (Fig. 7). The inhibition was most significant at the highest lactate concentration tested (150 mM), observed with the largest decline in cell growth and ethanol production.

The same inhibitory effect of lactate was also found in glucose fermentation with the presence of yeast extract (Fig. 8). While the reduction in ethanol yields and growth by lactate appeared minimal, inhibition by lactate at 150 mM, albeit small, was still statistically significant according to ANOVA analysis.

These results indicate that while yeast extract could considerably change the metabolism of sugar fermentation, the stimulatory effect of acetate and the inhibitory effect of lactate remained the same. Since acetate is a common product in both lignocellulose pre-treatment and sugar fermentation, its stimulatory effect on ethanolic fermentation might have implications in improving bioethanol production processes.

3.4. Stimulatory effect of acetate on sugar fermentation by other ethanologenic thermophilic bacteria

While the stimulatory effect of acetate was evident in ethanolic fermentation by strain 39E as shown above, the significance of this effect needs to be tested in other ethanologenic bacteria. Therefore, the stimulatory effect of acetate on ethanol fermentation was further studied in two additional thermophilic ethanologenic bacteria, *T. ethanolicus* strain X514 and *C. thermocellum* ATCC 27405. Strain X514 was selected as a phylogenetically closely related relative to strain 39E with similar physiology (Roh et al., 2002). In contrast, *C. thermocellum* is distantly related to strain 39E with considerably different metabolic traits, such as cellulolytic capacity (Johnson

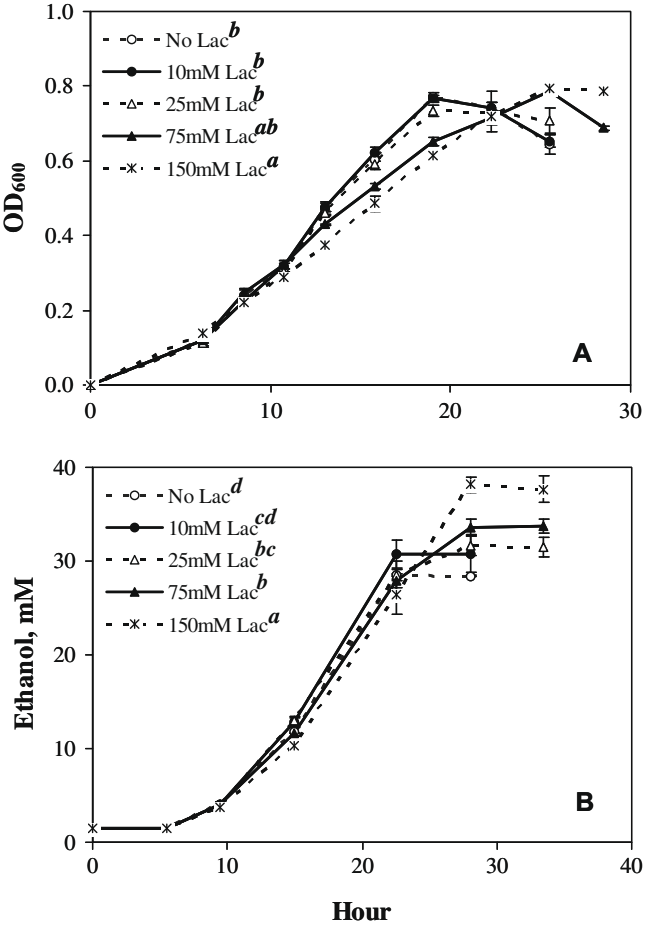

Fig. 8. Influence of exogenous lactate (Lac) on ethanol production from glucose fermentation by *T. ethanolicus* strain 39E in defined medium supplemented with 0.1% (w/v) yeast extract. (A) Growth as indicated by optical density at 600 nm; and (B) production of ethanol. Exogenous lactate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were averages of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in lactate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

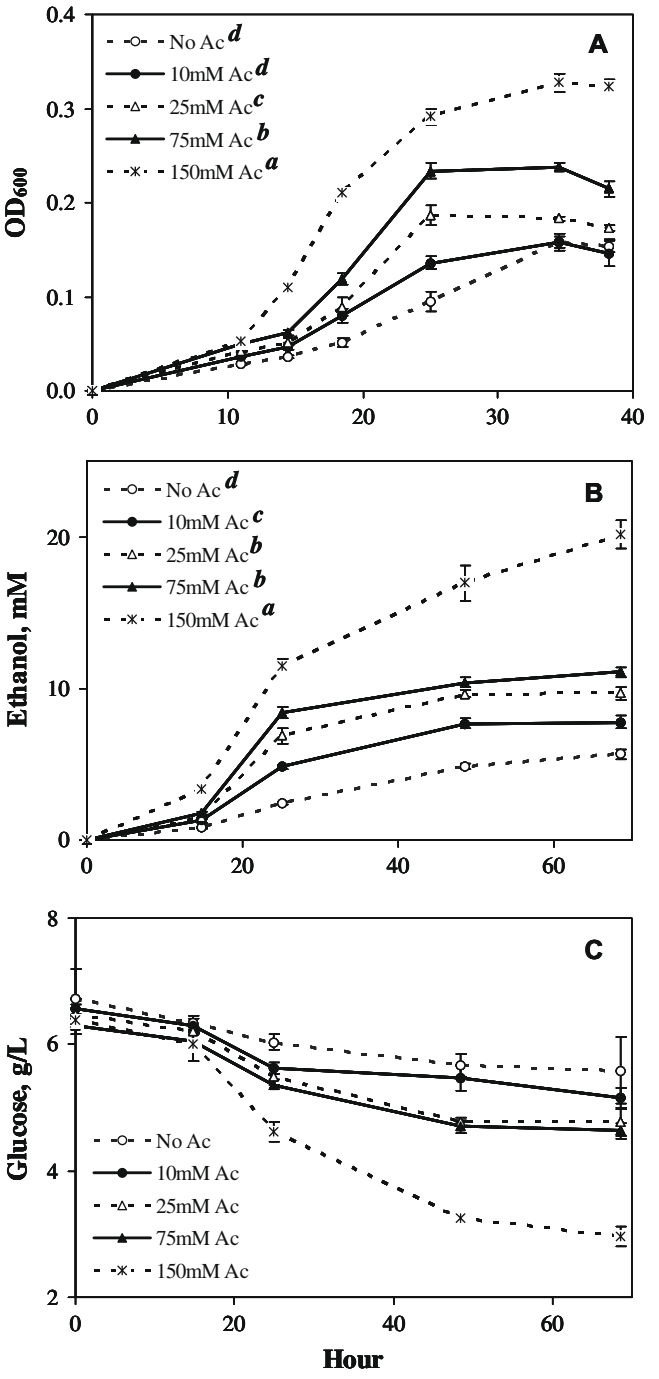

Fig. 9. Influence of exogenous acetate (Ac) on ethanol production from glucose fermentation by *T. ethanolicus* strain X514 in defined medium. (A) Growth as indicated by optical density at 600 nm; (B) production of ethanol; and (C) utilization of glucose. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were averages of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

et al., 1982). But all three strains are Gram-positive thermophiles capable of ethanologenic fermentation.

3.4.1. Stimulatory effect of acetate on T. ethanolicus strain X514

The impact of acetate on strain X514 was tested in ethanolic fermentation of 0.65% (w/v) glucose in defined medium without yeast extract. The stimulatory effect of acetate was shown clearly as increasing levels of acetate resulted in greater improvements in culture growth, ethanol production, and glucose utilization (Fig. 9). Strain X514 cultures exposed to 150 mM acetate nearly doubled peak optical density to 0.33 from approximately 0.15 (OD_{600}), accompanied by a 3.5-fold increase in ethanol production as compared to controls without acetate addition, which is consistent with the stimulatory effect observed in glucose fermentation by strain 39E (Fig. 2).

3.4.2. Stimulatory effect of acetate on C. thermocellum

While acetate was shown to stimulate sugar fermentation in both *T. ethanolicus* strains 39E and X514, its influence on ethanolic fermentation was further tested on *C. thermocellum* using cellobiose as the substrate, which was known to support better growth than glucose and xylose (Ng and Zeikus, 1982; Strobel, 1995). In defined medium with no yeast extract, cultures of *C. thermocellum* exhibited enhanced growth and ethanol production in all acetate concentrations tested, ranging from 10 to 150 mM (Fig. 10). In general, higher levels of acetate resulted in greater stimulation of growth and ethanol production. However, the acetate concentration effect diminished at concentrations over 75 mM, as cell growth, ethanol yield, and cellobiose utilization were identical at acetate concentrations of 75 and 150 mM, where the highest cell density and ethanol yields were detected. At both acetate levels (75 and 150 mM), optical density (OD_{600}) more than doubled from 0.21 to 0.46 as compared to controls receiving no acetate addition, which was accompanied by an average increase of 3.7 times in ethanol yields. Evidently, the stimulatory effect of acetate is also significant in *C. thermocellum*.

3.5. Analysis of ethanolic fermentation under the impact of acetate

Results shown above suggest a potentially broad distribution of the stimulatory effect of acetate on sugar fermentation among ethanologens, even though the occurrence of this stimulatory effect has not been tested in mesophilic strains. To gain insight into the mechanisms involved in the acetate stimulation of ethanolic fermentation, efforts were made to look into the relationship between substrate utilization and the end product profiles during fermentation by strain 39E under the influence of varying levels of acetate.

3.5.1. Profiles of end products in xylose fermentation

There were considerable differences in the end product profiles between cultures grown with or without 0.1% (w/v) yeast extract (Table 1). In the case in which yeast extract was added as a nutrient supplement, complete xylose utilization was achieved in all strain 39E cultures including the control cultures. The presence of acetate only moderately improved ethanol yields, since the largest increase was 4.5 mM or 12.4% as compared to the control cultures without acetate addition (Table 1). In the meantime, the production of acetate decreased significantly with increasing concentrations of acetate, and even became net losses at acetate concentrations above 25 mM, with the largest net loss of 18.8 mM acetate found in cultures amended with 150 mM acetate, suggesting the possibility of active uptake of acetate in these cultures.

It is tempting to postulate that the loss of acetate from medium was responsible for the rise in ethanol yields at elevated acetate concentrations. However, the fate of acetate lost from medium could not be completely explained by the increases in ethanol production, since the acetate losses were much greater than the increases in ethanol yield (e.g. 18.8 mM vs 4.5 mM). Further analyses of the relationship between ethanol yields and xylose utilization when xylose was omitted indicate that the specific ethanol yields were not statistically different in cultures amended with different concentrations of acetate as shown by ANOVA analysis (Fig. 11),

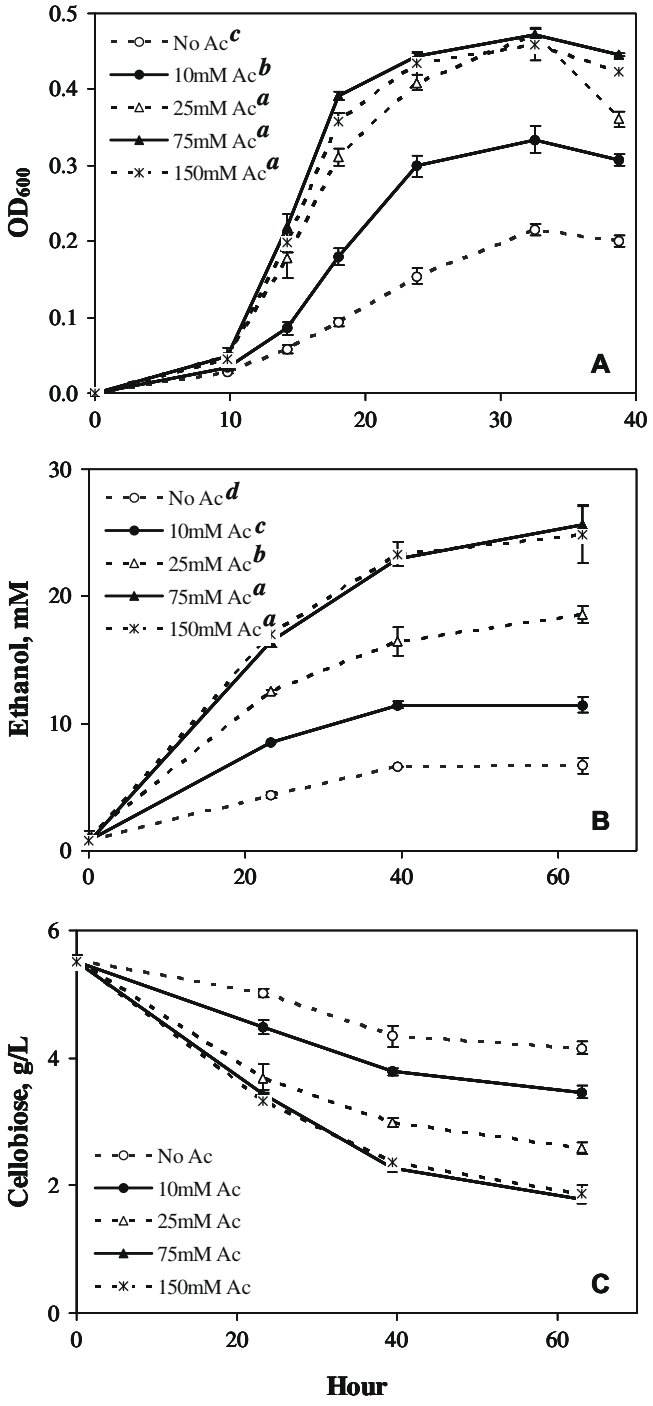

Fig. 10. Influence of exogenous acetate (Ac) on ethanol production from cellobiose fermentation by *C. thermocellum* ATCC 27405 in defined medium. (A) Growth as indicated by optical density at 600 nm; (B) production of ethanol; and (C) utilization of cellobiose. Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Data points were averages of triplicate cultures with the error bars showing standard deviations. Peak optical densities or ethanol yields are not significantly different from each other in acetate treatments labeled with the same lowercase superscript letters in the figure legend (ANOVA, Tukey's test, $p < 0.05$).

Table 1
Impact of acetate on the profiles of end products in xylose fermentation by *T. ethanolicus* strain 39E.[a]

Acetate level (mM)	Xylose consumed (g/L)	Ethanol produced (mM)	Acetate produced[b] (mM)	Lactate produced (mM)
Xylose with 0.1% yeast extract				
0	5.2 ± 0.1^{A}	36.2 ± 0.9^{B}	7.0 ± 0.3^{A}	9.2 ± 1.0^{A}
10	5.2 ± 0.1^{A}	39.2 ± 0.7^{AB}	1.9 ± 0.8^{B}	8.1 ± 0.8^{AB}
25	5.2 ± 0.1^{A}	40.9 ± 2.0^{A}	-0.7 ± 1.9^{B}	7.5 ± 0.4^{AB}
75	5.2 ± 0.1^{A}	40.1 ± 1.9^{AB}	-11.8 ± 2.9^{C}	7.5 ± 0.4^{AB}
150	5.2 ± 0.1^{A}	40.7 ± 1.2^{A}	-18.8 ± 1.2^{D}	7.0 ± 0.3^{B}
Xylose without 0.1% yeast extract				
0	2.1 ± 0.1^{C}	4.6 ± 0.1^{D}	7.7 ± 0.3^{A}	4.6 ± 0.3^{A}
10	2.0 ± 0.1^{C}	5.4 ± 0.1^{D}	4.3 ± 1.4^{B}	3.7 ± 0.1^{B}
25	2.2 ± 0.1^{C}	7.4 ± 0.6^{C}	3.3 ± 0.7^{BC}	4.4 ± 0.1^{A}
75	2.6 ± 0.1^{B}	9.8 ± 0.4^{B}	-0.1 ± 1.7^{C}	4.2 ± 0.4^{AB}
150	3.0 ± 0.1^{A}	14.2 ± 1.3^{A}	3.5 ± 1.3^{B}	4.4 ± 0.1^{A}

[a] Varying levels of acetate (mM) were added into the culture medium prior to the start of fermentation of 0.5% (w/v) xylose as the sole substrate. The values are the means of triplicate experiments ± standard deviation. Means with the same superscript letters are not significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).
[b] Data shown are net changes in acetate concentration calculated as the difference between the actual acetate concentration in the culture medium and the concentration of exogenous acetate added. Positive values indicate net acetate production and negative values indicate acetate consumption during ethanolic fermentation.

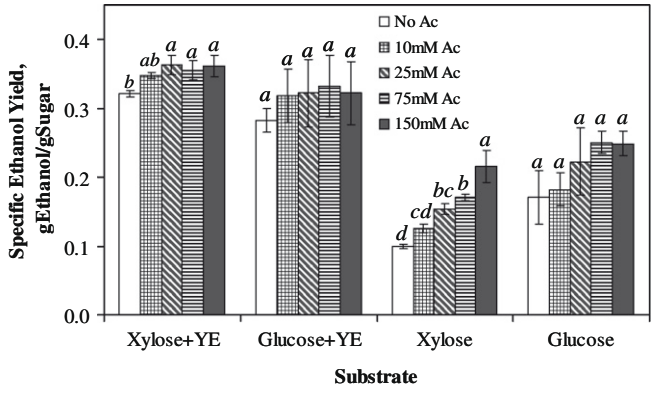

Fig. 11. Specific ethanol yields from sugar fermentation by *T. ethanolicus* strain 39E under various levels of exogenous acetate (Ac). Specific ethanol yield was calculated as the amount of ethanol produced (g) per sugar consumed (g). Fermentation medium supplemented with 0.1% (w/v) yeast extract was labeled with "YE". Exogenous acetate was added to concentrations of 10, 25, 75, and 150 mM, respectively, prior to initiation of fermentation. Results were means of triplicate cultures with the error bars showing standard deviations. The means are not significantly different from each other in columns labeled with the same italicized lowercase letters (ANOVA, Tukey's test, $p < 0.05$).

even though the amounts of acetate generated or taken up by the cells were vastly different, suggesting that the fate of acetate might be influenced by other metabolic routes. Since culture growth was also stimulated by elevated acetate, it is thus possible that the uptake of acetate might also contribute to growth enhancement.

In the case in which yeast extract was not supplemented and substrate utilization was not complete, the addition of acetate promoted the utilization of xylose from 2.1 g/L in the control cultures up to 3.0 g/L with 150 mM acetate, representing an increase of 143% (Table 1). Accordingly, ethanol yield showed an increase of 9.6 mM or 394% as compared to the controls receiving no acetate addition. Acetate production declined with increasing acetate levels; however, net loss of acetate from the medium was not apparent. In the meantime, changes in the production of lactate were mostly not significant between different acetate levels according to ANOVA analysis (Table 1). Therefore, it could be hypothesized that the increases in growth and ethanol production could be primarily attributed to the enhanced utilization of xylose as a result of the presence of elevated acetate. It is noted, however, that the specific ethanol yields increased with increasing acetate levels (Fig. 11), suggesting that the increases in ethanol production could not be solely attributed to the increases in xylose utilization, and other unknown mechanisms were likely involved.

3.5.2. Profiles of end products in glucose fermentation

Similar to xylose fermentation, the end product profiles of glucose fermentation by strain 39E were markedly different in cultures with or without yeast extract. The addition of 0.1% (w/v) yeast extract resulted in complete fermentation of 0.5% (w/v) glucose. Exposure to elevated acetate led to moderately enhanced ethanol yields and reductions in the production of lactate and acetate (Table 2). The reduction in acetate production increased with increasing acetate levels and turned into net losses when acetate reached above 25 mM, which is consistent with the effect of high levels of acetate on xylose fermentation (Table 1).

When strain 39E was grown in medium not amended with yeast extract, glucose utilization was not complete in all cultures but an increasing fraction of the glucose was converted with higher acetate concentration in the medium (Fig. 2). Notably, the increases in glucose utilization coincided with increases in ethanol yields in the same cultures (Table 2). In the meantime, changes in the production of acetate and lactate from glucose fermentation were insignificant. These results suggest that increased glucose utilization may be a major contributor to the greater ethanol yields at elevated acetate levels. However, similar to xylose fermentation, specific ethanol yields increased with increasing concentrations of acetate added into the medium (Fig. 11), pointing to the importance of other unknown mechanisms involved in the stimulation of ethanolic fermentation of sugars.

4. Discussion

Ethanolic fermentation of simple sugars is an important step in the production of bioethanol as a renewable fuel (Demain et al., 2005). Organic acids, generated as by-products during sugar fermentation and the pre-treatment of lignocellulotic feedstock, have been considered to be inhibitory to ethanolic fermentation (Palmqvist and Hahn-Hagerdal, 2000). The focus of this work was to test the impact of added acetate and lactate on the efficiency of thermophilic ethanolic fermentation of sugars by *T. ethanolicus* strain 39E. Our results show that lactate was indeed inhibitory to ethanolic fermentation under certain growth conditions (Fig. 7). However, acetate showed an unexpected stimulatory effect on ethanolic fermentation of sugars by strain 39E (Fig. 11). More importantly, the addition of acetate to culture medium also stimulated the sugar fermentation by two other ethanolgens, *T. ethanolicus* strain X514 and *C. thermocellum* ATCC 27405 (Figs. 9 and 10), suggesting a potentially broad significance of this stimulatory effect in ethanolic fermentation.

In fact, few studies in the literature investigated the effects of elevated acetate on ethanolic fermentation by bacteria. Previous

Table 2
Impact of acetate on the profiles of end products in glucose fermentation by *T. ethanolicus* strain 39E.[a]

Acetate level (mM)	Glucose consumed (g/L)	Ethanol produced (mM)	Acetate produced[b] (mM)	Lactate produced (mM)
Glucose with 0.1% yeast extract				
0	5.1 ± 0.6^{A}	31.0 ± 2.2^{B}	4.9 ± 0.6^{A}	9.9 ± 1.8^{A}
10	5.1 ± 0.6^{A}	34.6 ± 0.5^{AB}	-2.1 ± 0.9^{A}	10.0 ± 1.1^{A}
25	5.1 ± 0.6^{A}	36.2 ± 0.0^{A}	-4.9 ± 1.9^{A}	7.9 ± 4.4^{A}
75	5.1 ± 0.6^{A}	36.1 ± 0.9^{A}	-19.7 ± 8.1^{B}	7.6 ± 1.1^{A}
150	5.1 ± 0.6^{A}	35.1 ± 2.2^{AB}	-23.9 ± 2.4^{B}	6.7 ± 1.7^{A}
Glucose without 0.1% yeast extract				
0	1.0 ± 0.2^{B}	3.8 ± 0.2^{D}	3.2 ± 1.3^{A}	2.0 ± 0.2^{B}
10	1.6 ± 0.2^{AB}	6.2 ± 0.3^{C}	2.8 ± 0.5^{A}	1.9 ± 0.1^{B}
25	1.8 ± 0.3^{A}	8.4 ± 0.6^{B}	3.5 ± 0.7^{A}	2.4 ± 0.2^{AB}
75	2.1 ± 0.2^{A}	11.3 ± 0.6^{A}	4.0 ± 0.5^{A}	2.6 ± 0.0^{A}
150	2.0 ± 0.2^{A}	10.7 ± 0.3^{A}	3.6 ± 0.1^{A}	2.3 ± 0.4^{AB}

[a] Varying levels of acetate (mM) were added into the culture medium prior to the start of fermentation of 0.5% (w/v) glucose as the sole substrate. The values are the means of triplicate experiments ± standard deviation. Means with the same superscript letters are not significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).
[b] Data shown are net changes in acetate concentration calculated as the difference between the actual acetate concentration in the culture medium and the concentration of exogenous acetate added. Positive values indicate net acetate production and negative values indicate acetate consumption during ethanolic fermentation.

work done on the yeast *Saccharomyces cerevisiae* shows that the addition of acetate to culture medium resulted in a stimulatory effect on ethanol production (Narendranath et al., 2001; Taherzadeh et al., 1997). It is suggested that acetate may disrupt energy metabolism by acting as an uncoupler, which stimulates energy production through accelerated fermentation (Russell, 1992). Indeed, this hypothesis is very much consistent with the increased production of ethanol and reduced biomass yield observed in ethanol fermentation by yeast cultures subjected to elevated acetate levels (Taherzadeh et al., 1997). However, the uncoupling effect of acetate could not explain the increases in both ethanol production and cell growth throughout sugar fermentation by strain 39E, in contrast to the expected decrease in cell growth according to the potential uncoupling effect (Figs. 1 and 2). It is thus likely that mechanisms different from those proposed for *S. cerevisiae* were responsible for the stimulatory effect of acetate during fermentation by thermophilic ethanologenic bacteria.

Given the phenomenological nature of this study, the definitive identification of the mechanisms underlying the stimulatory effect may not be possible. However, the profiling of fermentation end products did provide implication for the potential mechanisms involved. In strain 39E cultures grown in medium supplemented with yeast extract, the addition of acetate to the growth medium significantly reduced the production of acetate as a fermentation product, and ultimately turned the production of acetate into net consumption of added acetate when the concentration of acetate rose above 25 mM (Tables 1 and 2). Apparently, the presence of acetate altered the organic metabolism in strain 39E, and as a result acetate might be used as a carbon source and contribute to the enhancement in ethanol production and growth.

However, the consumption of acetate observed in strain 39E cultures supplemented with yeast extract was not evident in cultures with no yeast extract (Tables 1 and 2). Instead, the increased ethanol production and growth appeared to a large extent to be the result of the improved sugar utilization when acetate was added into the medium. It is thus likely that the characteristics of the stimulatory effect of acetate is growth condition-dependent, and the potential application of this stimulatory effect in improving bioethanol fermentation processes requires further elucidation of the physiological and metabolic causes involved.

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