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Acetate production by *Clostridium thermoaceticum* in corn steep liquor media

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A low-cost nutrient medium based on corn steep liquor (CSL) was developed for the production of acetates by *Clostridium thermoaceticum*. Pre-treatment of CSL with dolime and vitamin supplementation increased the rate of acetate production. Adding excess nutrients in a fed-batch mode minimized by-product formation and increased final acetate concentration from 19 g L^{-1} to 40 g L^{-1} acetic acid. High yields of acetic acid (0.95 g g^{-1} glucose in fed-batch mode) was probably due to the conversion of the lactic acid in CSL into acetic acid by the organism.

Keywords: *Clostridium thermoaceticum*; fermentation; acetic acid; calcium magnesium acetate; corn steep liquor; nutrients

Introduction

The salts of acetic acid are important commodity chemicals. Sodium acetate is used as an acidulant and as a meat spray to inhibit microbial growth. Calcium magnesium acetate (CMA) has been identified by the US Federal Highway Administration as an environmentally safe and non-corrosive deicer for use on roads in winter [4], and potassium acetate could find use as a heat exchange fluid [6]. At present these products are made from petroleum-derived acetic acid at a cost of about $650 per ton [6]. Anaerobic fermentation of glucose by *Clostridium thermoaceticum* has the potential to produce these acetates at a lower cost [8], especially if high-producing mutant strains such as those developed in our laboratory are used [9,12]. However, one obstacle to the successful commercialization of this process is the expensive nutrients required in the medium. In addition to glucose, the medium contains yeast extract and various minerals, with cysteine as a reducing agent. A mutant strain of *C. thermoaceticum* requires a large excess of nutrients to produce acetic acid at high concentrations [12]. Since yeast extract is expensive, the cost of producing the acetates becomes prohibitively high. If a low-cost nutrient can be substituted for yeast extract, then it would be economically feasible to produce acetates by fermentation of agricultural raw materials.

One such nutrient is corn steep liquor [7], which is a by-product of the wet milling of corn. It is rich in amino acids, minerals and vitamins, and also contains other nitrogen compounds which may be useful for microbial growth [3]. It has been industrially used as a nutrient in ethanol fermentation and for production of pharmaceuticals. It has been identified as one of several alternate nutrients that could result in satisfactory fermentation [17]. This study was aimed at evaluating the effectiveness of corn steep liquor as a nitrogen and nutrient source for *C. thermoaceticum*. In particular, the relative efficiency of batch and fed-batch fermentation systems at different nutrient concentrations was evaluated in order to optimize the productivity and maximize the acetic acid concentration in the broth.

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Materials and methods

Microorganism

A mutant strain of *C. thermoaceticum* (ATCC 47909) was used in this study [9]. It was maintained in the active state by transferring it alternately to medium (described below) containing 6% sodium acetate and a medium without any acetate.

Medium

Corn steep liquor (CSL) was obtained from AE Staley Mfg Co, Decatur, IL, USA as a 50% (w/w) suspension. It was diluted to 5% and treated with dolime ($CaO{\cdot}MgO{\cdot}H_2O$, obtained from Carson Lime Company, Plymouth Meeting, PA, USA) to bring the pH to 7.0–7.5. This solution was then filtered using Whatman No. 3 filter paper.

The basic medium contained (g L^{-1}): glucose 20; buffering components $NaHCO_3$ 1.5, KH_2PO_4 1.4, K_2HPO_4 1.1; corn steep liquor 5 (on a dry weight basis); salts $(NH_4)_2SO_4$ 1, $MgSO_4{\cdot}7H_2O$ 0.25, $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ 0.04, $CoSO_4{\cdot}7.5H_2O$ 0.03, $Na_2WO_4{\cdot}2H_2O$ 0.0033, $NaMoO_4{\cdot}2H_2O$ 0.0024, $NiCl_2{\cdot}6H_2O$ 0.00024, $ZnSO_4{\cdot}7H_2O$ 0.00029, Na_2SeO_3 0.000017 and cysteine 0.25. The medium was supplemented with vitamins (mg L^{-1}): thiamin 0.15, riboflavin 0.35, pyridoxin 0.175, nicotinic acid 3 and pantothenic acid 1.

Table 1 shows the composition of media used in the experiments described in this paper. Concentrations as given above are referred to as × concentration. When concentrations of CSL and salts were two or three times the above concentration, they were referred to as 2× and 3× concentrations, respectively. In Experiments 8 and 9, there was no dolime pre-treatment of CSL nor any vitamin supplementation.

Fermentation

The procedure for preparing the media has been described previously [10,17]. All fermentation experiments were

Table 1 Experiment design and nutrient supply

Experiment	Fermentation process	Initial concentration			Total supply of nutrients		
		Glucose (g L^{-1})	CSL	Salts	Glucose (g L^{-1})	CSL	Salts
1	Batch	46	×	×	–	–	–
2	Batch	46	2×	2×	–	–	–
3	Fed-Batch	46	2×	2×	42	2.7×	2.7×
4	Fed-Batch	20	×	×	50	2.7×	2.7×
5	Fed-Batch	37	×	4×[a]	56	3×	6×
6	Batch	50	2.7×	2.7×	–	–	–
7	Fed-Batch	20	2×	2×	42	2×	2×
8	Fed-Batch[b]	20	2×	2×	38	2×	2×
9	Batch[b]	46	2×	2×	–	–	–

[a]Concentration of $(NH_4)_2SO_4$ was 2× initially and 3.4× overall
[b]No dolime pretreatment of CSL and no vitamin supplementation

carried out in a 2-L reactor with a 1.1-L working volume. Temperature was controlled at 60° C and pH was maintained between 6.8 and 7.0 by addition of 10 N NaOH. Agitation was 25–50 rpm, which was enough to keep the broth well-mixed. To ensure an anaerobic environment, the fermenter was maintained under a slight positive pressure by flowing filtered CO_2 in the overhead space in the fermenter. Fermentation was initiated by transferring 100 ml of 24-h old inoculum to 1 L of the medium. For batch experiments, initial glucose concentration was 46 g L^{-1}.

In fed-batch experiments, concentrated solutions of various nutrients were added during the fermentation to sustain the acetic acid production. The concentration of nutrients was varied in different experiments as shown in Table 1.

Analytical methods

Glucose and acetic acid were analyzed by HPLC using the Bio-Rad (Hercules, CA, USA) Aminex 87H column and a refractive index detector. Acetate concentrations are expressed in terms of acetic acid, with a molecular weight of 60. Thus, 1 g acetic acid = 1.375 g sodium acetate and 1.225 g CMA. Cell concentration was monitored by measuring optical density at 600 nm. A calibration curve was prepared to correlate optical density to dry cell weight. To determine dry cell weight, the sample was centrifuged and the supernatant phase was discarded. Cell pellet was washed with water three times and then dried in an oven at 80° C.

Results and discussion

Batch vs fed-batch fermentation

Figure 1 shows a typical fermentation profile with the normal level of nutrients (1×). The final acetic acid concentration was only 19 g L^{-1} and the cells reached a maximum density of 0.9 g L^{-1} before declining. This cell behavior was observed in almost all experiments and has been reported before [9–12,17]. A by-product of the fermentation, which has been identified as fructose [17], was observed after the cells stopped growing.

In Experiment 2, CSL and salts were added at 2× concentration (Figure 2). The fermentation was much better, resulting in 31 g L^{-1} acetic acid in 95 h of fermentation,

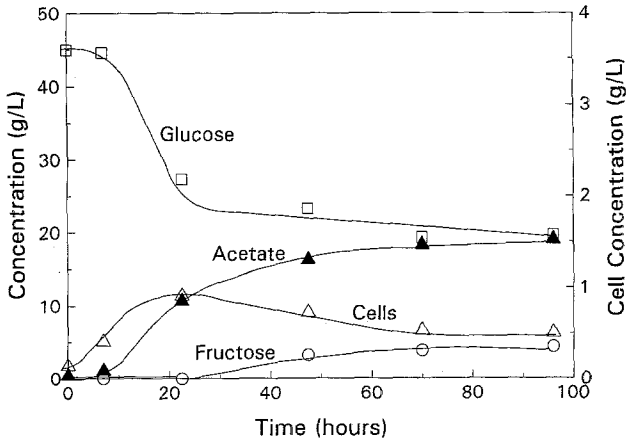

Figure 1 Batch fermentation profile of *C. thermoaceticum*. CSL and salts were at normal (1×) concentration

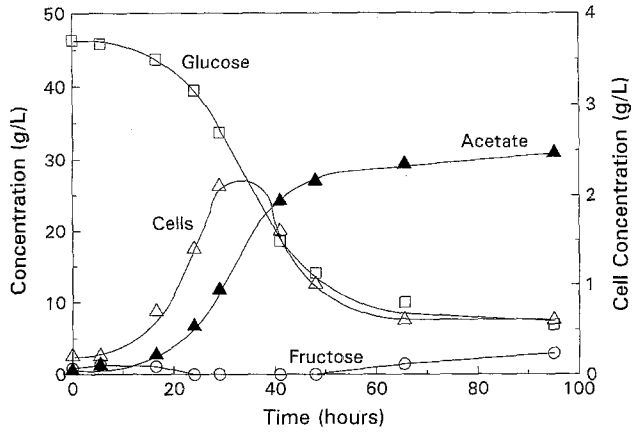

Figure 2 Batch fermentation profile with both CSL and salts at 2× concentration

and the maximum cell concentration was higher (2 g L^{-1}). Again fructose was produced towards the end of fermentation, although at a relatively low level. The maximum acetate produced with CSL was lower than when yeast extract was used at the same concentration [9,17]. Yields with CSL were only 0.7–0.8 g g^{-1} glucose compared to

Table 2 Fermentation parameters

Experiment	Time (h)	Acetate (g L^{-1})	Yield (g g^{-1})		Productivity (g acetate L^{-1} h^{-1})
			Acetate	Fructose	
1	96	19.0	0.78	0.18	0.20
2	95	30.8	0.82	0.08	0.32
3	156	37.9	0.97	0.01	0.24
4	140	39.1	0.95	0.01	0.28
5	210	39.9	0.92	0.01	0.19
6	125	38.5	0.84	0.06	0.31
7	94	31.1	0.86	0.08	0.33
8	143	29.7	0.93	0.00	0.21
9	106	25.9	0.77	0.14	0.24

0.85 g g^{-1} with yeast extract. The main reason for lower acetate yield in this experiment was the formation of fructose as a by-product (Table 2). Fructose is produced whenever the nutrient supply is inadequate [17]. At equal levels, yeast extract is a better source of nutrients, resulting in higher acetate yield and productivity, with little or no fructose [15,17].

Figures 3–5 show fed-batch fermentations with different rates of nutrient feeding. Experiment 3 was initiated with the same amount of nutrients as Experiment 2. When the cell concentration reached its peak value, fed-batch operation commenced, ie, more nutrients were added in small batches as indicated in Figure 3. By the end of the fermentation, the total amount of CSL and salts was equivalent to 2.7× concentration. However, although the maximum level and yield of acetate were higher (Table 2), the extra nutrients did not increase cell concentration. Acetic acid production continued for a longer time, reaching a final concentration of 38 g L^{-1}.

Fructose was also produced in this experiment during the death phase of the cells. However, when additional nutrients were supplied, it disappeared, apparently being converted into acetic acid (Figure 3). At the end of the fermentation, there was no fructose in the broth. The higher acetate yield (0.97 g g^{-1}) could be due to the lactic acid in CSL,

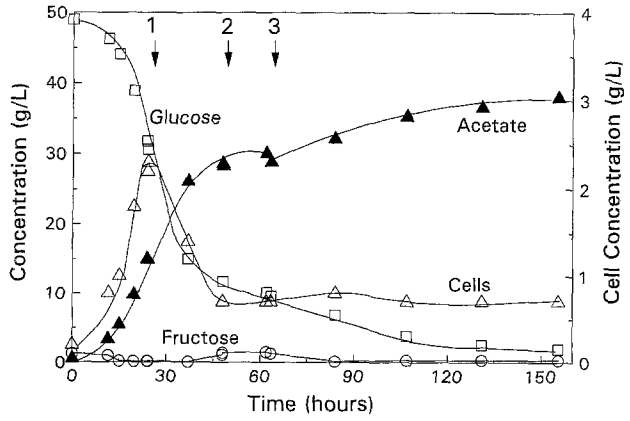

Figure 3 Fed-batch fermentation. Overall CSL level was 2.7× and salts was 2.7×. Arrows indicate the addition of nutrient solution containing CSL and salts (10× concentration) in the following amounts: 1 = 25 ml; 2 = 25 ml; 3 = 50 ml. Initial volume of broth = 1.1 L. Final volume = 1.2 L

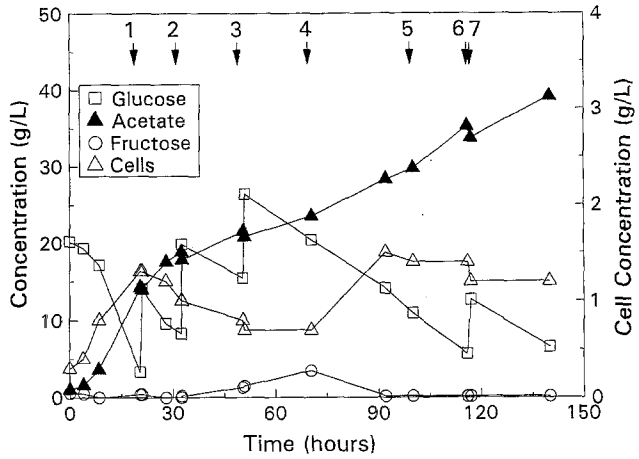

Figure 4 Fed-batch fermentation with 3× levels of CSL and salts. Arrows indicate the addition of the nutrient solutions in the following amounts: 1, 2 and 3 = 50 ml of solution A (CSL and salts 6.6×, glucose 260 g L^{-1}); 4 = 100 ml of solution B (CSL and salts 10×); 5 and 6 = 50 ml of solution B; 7 = 20 ml of solution C (glucose 500 g L^{-1}). Initial volume = 1.1 L, final volume = 1.47 L

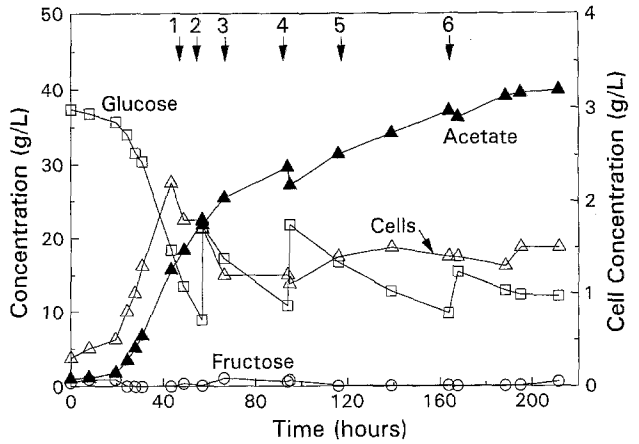

Figure 5 Fed-batch fermentation with 3× CSL, 3.4× $(NH_4)_2SO_4$ and 6× other salts. Arrows indicate addition of nutrient solutions as follows: 1 = 50 ml of solution D (CSL and $NH_4)_2SO_4$ 10×, other salts 20×); 2 = 30 ml solution C (glucose 500 g L^{-1}); 3 = 50 ml solution D; 4 = 50 ml of D, 30 ml of C and 50 ml solution E (20× salts; no $(NH_4)_2SO_4$); 5 = 50 ml solution F (20× CSL and $(NH_4)_2SO_4$); 6 = 40 ml solution G (20× CSL, 10× $(NH_4)_2SO_4$, 20× other salts and 250 g L^{-1} glucose). Initial volume = 1.1 L, final volume = 1.45 L

which was typically 20% (w/w) of the solids. The use of lactate as a carbon source by *C. thermoaceticum* has been reported before [1,5], and could account for the higher acetate yield with CSL.

In Experiment 4, the initial concentration of glucose was only 20 g L^{-1} while CSL and salts were at the normal concentration. Subsequently, more nutrients were added as shown in Figure 4. Overall, CSL and salts were supplied at 2.7× and glucose at 50 g L^{-1}. The final acetate concentration and yield were slightly better than Experiment 3. One noticeable difference was that the viability of the cells was much better with the fed-batch operation.

In Experiment 5 (Figure 5), concentrations of all salts except ammonium sulfate were at 4× level initially. Overall supply of salts, CSL and $(NH_4)_2SO_4$ was 6×, 3× and

3.4×, respectively. The improvement in fermentation parameters was marginal (Table 2) despite the higher supply of these nutrients. Fructose that was produced during the death phase of the cells disappeared after addition of excess nutrients. This also resulted in a partial restoration of the viability of the cells, in a manner similar to that of Experiment 4. However, the fermentation took longer (210 h to produce 40 g L^{-1} acetic acid). It appears that too high a level of salts can reduce acetate production. Ammonium sulfate was not supplied at a higher level, because ammonium ions are reportedly toxic to *C. thermoaceticum* [16].

Experiment 6 was conducted in a batch mode with overall nutrient supply the same as in Experiment 4. The only difference was that all the nutrients were added initially. As shown in Figure 6, although the maximum cell concentration was higher, the other fermentation parameters were not much better than Experiment 4 (Table 2). In addition, there was a higher amount of fructose produced at the end, probably due to exhaustion of some critical nutrients. Because of the higher density of cells, more nutrients were required for the formation of the cell mass, resulting in a lower yield of acetate (Table 2: compare Experiment 6 vs 3–5).

Effect of dolime treatment

Previous work in our laboratory [9,11,12] suggested that it was important to maintain a low level of the carbon source in the fermenter at all times to ensure viability of the cells. In Experiment 7, the initial concentration of glucose was 20 g L^{-1} and the nutrients and salts were initially 2×. When glucose was nearly depleted, a concentrated solution of nutrients and glucose was added continuously to the fermenter to maintain the glucose concentration between 3 and 7 g L^{-1} (Figure 7). Overall supply of CSL and salts amounted to 2× concentration. In 93 h of fermentation, acetic acid concentration reached 31 g L^{-1}.

Experiment 8 was carried out under the same conditions as the above experiment except that no dolime pre-treatment was given to the CSL and there was no vitamin supplementation. Productivity was lower than the previous experiment: it took 143 h to produce 29 g L^{-1} acetic acid (Figure 8). One reason for the slower production with untreated CSL could be the presence of inhibitory compounds such as phytic acid which are known to bind minerals and proteins [2] and perhaps make them unavailable to the organism.

To verify the effect of untreated CSL, Experiment 9 (Figure 9) was carried out in the batch mode with the same nutrient concentration as in Experiment 2 except that CSL was not pre-treated with dolime. In this run also acetic acid

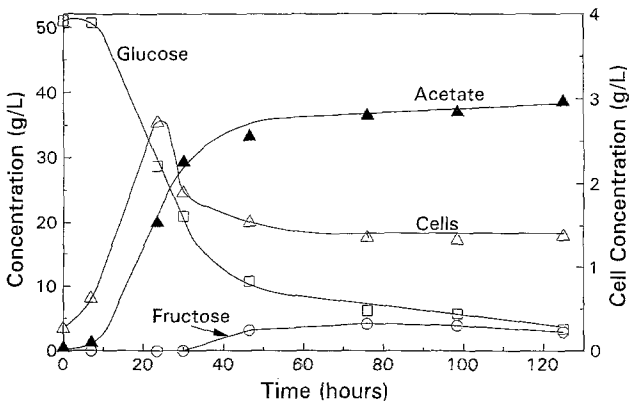

Figure 6 Batch fermentation with CSL and salts at 2.7×

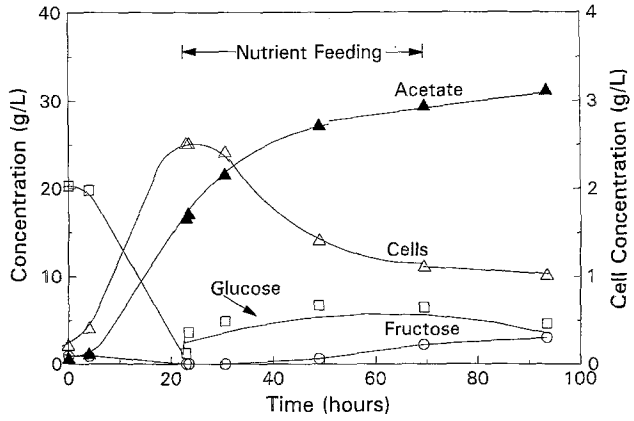

Figure 7 Fed-batch fermentation with CSL and salts at 2×. Initial volume = 1.1 L, final volume = 1.19 L

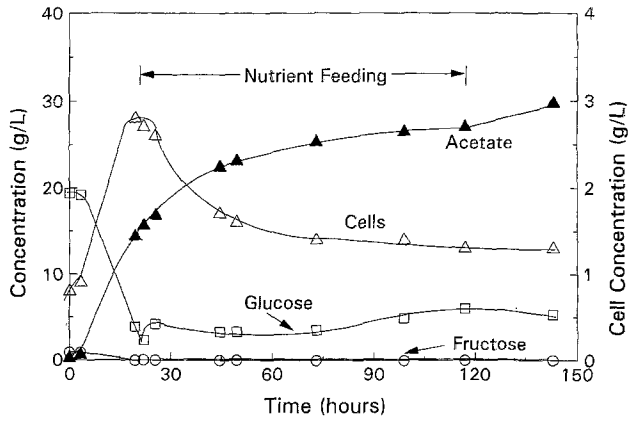

Figure 8 Fed-batch fermentation with CSL (untreated) and salts at 2×. Initial volume = 1.1 L, final volume = 1.17 L

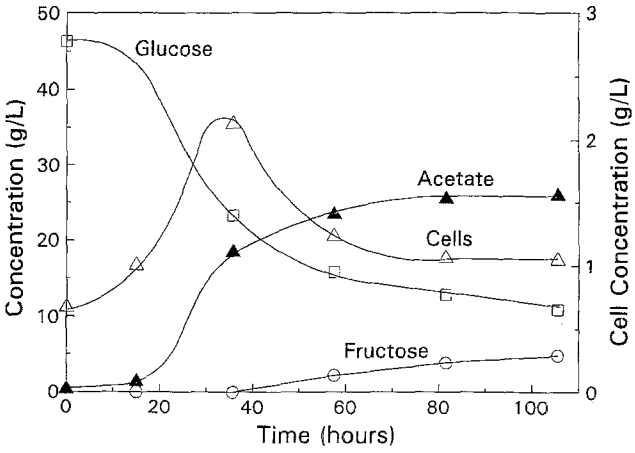

Figure 9 Batch fermentation with CSL (untreated) and salts at 2×

production rate was slower (Table 2). Thus, treatment of CSL with dolime and vitamin supplementation seems to boost the productivity in the fermentation.

Discussion

The data show a close relationship between growth and acetate production. A decrease in cell numbers resulted in a severe loss of productivity. *C. thermoaceticum* appears to require a continuous dose of nutrients to maintain its viability. Whenever the non-glucose nutrients were exhausted, it produced fructose in addition to acetate. If sufficient nutrients were supplied, all the glucose is converted to acetic acid; if any fructose is present, it also gets converted to acetate. To avoid producing fructose, some nutrients must be added during the death phase of the fermentation. The best manner to achieve the varying nutrient demands of this organism is the fed-batch mode of fermentation.

The importance of being able to use a low-cost nutrient such as corn steep liquor is reflected in its contribution to the cost of the acetate product. Commercial yeast extract in bulk costs \$10–11 per kg [13,14] while CSL costs only \$0.20 per kg on a dry weight basis. At the level suggested by our experiments, CSL requirements would be 0.25 kg CSL per kg acetate. This translates into a cost of \$50 per metric ton of acetate for CSL, compared to a yeast extract cost of \$2000–2200 per ton of acetate. However, although CSL is cheap and readily available from wet corn millers that produce glucose, it still represents a significant portion of the materials cost of a product like CMA. Further optimization of medium composition will be necessary to reduce the cost of fermentation-derived acetates to more economical levels.

Acknowledgements

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