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ORIGINAL PAPER

L(+)-Lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*

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Abstract Lactic acid is used as an additive in foods, pharmaceuticals, and cosmetics, and is also an industrial chemical. Optically pure lactic acid is increasingly used as a renewable bio-based product to replace petroleum-based plastics. However, current production of lactic acid depends on carbohydrate feedstocks that have alternate uses as foods. The use of non-food feedstocks by current commercial biocatalysts is limited by inefficient pathways for pentose utilization. *B. coagulans* strain 36D1 is a thermotolerant bacterium that can grow and efficiently ferment pentoses using the pentose-phosphate pathway and all other sugar constituents of lignocellulosic biomass at 50°C and pH 5.0, conditions that also favor simultaneous enzymatic saccharification and fermentation (SSF) of cellulose. Using this bacterial biocatalyst, high levels (150–180 g l^{-1}) of lactic acid were produced from xylose and glucose with minimal by-products in mineral salts medium. In a fed-batch SSF of crystalline cellulose with fungal enzymes and *B. coagulans*, lactic acid titer was 80 g l^{-1} and the yield was close to 80%. These results demonstrate that *B. coagulans* can effectively ferment non-food carbohydrates from lignocellulose to L(+)-lactic acid at sufficient concentrations for commercial application. The high temperature fermentation of pentoses and hexoses to lactic acid by *B. coagulans* has these additional advantages: reduction in cellulase loading in SSF of cellulose with a decrease in enzyme cost in the process and a reduction in contamination of large-scale fermentations.

Keywords *Bacillus coagulans* · Lactic acid · Fermentation · SSF · Cellulose · Microbial biocatalyst

Introduction

Due to the growing concern over the finite nature of fossil fuels and the environmental impact of the greenhouse gases released into the atmosphere by their use, there is a resurgence of interest in environmentally friendly renewable and sustainable sources of fuels and chemicals. Production of bio-based plastics starting with optically pure lactic acid as the precursor is gaining momentum as an alternative to petroleum-based plastics [3, 4, 31]. Lactic acid use in bio-based plastics is expected to outstrip other uses of lactic acid as a food additive, industrial chemical and pharmaceutical as well as in cosmetics provided the cost of the polylactide polymers can match the cost of polymers derived from petrochemicals. Fermentation of sugars by lactic acid bacteria supplies all the optically pure lactic acid produced world-wide [7]. Although lactic acid production by these bacteria is very efficient, further improvements in the process can help reduce the production cost of lactic acid to make this cost-competitive with petroleum-based polymers for bio-based plastics production [7, 19].

There are at least three critical areas of interest that can help reduce the production cost of lactic acid: the cost of sugars, the ability to use non-food sources as starting sugars for fermentation, and potential contamination of fermentations at temperatures lower than 40°C. Industrial lactic acid production by fermentation starts with glucose derived from starch or sucrose. Mandated increase in biofuel

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production worldwide consumes a larger share of available sugars, leading to an increase in grain price [18]. Increasing demand for these sugars and carbohydrates as food source due to a growing population combined with the use of these compounds as feed stocks for producing petroleum replacements, such as ethanol, has led to projections of more hunger, especially in food-importing poor nations of the world [29]. The food-vs-fuel argument could also abolish the use of food carbohydrates for production of bio-based plastics. To overcome these constraints, non-food sources of fermentable sugars need to be identified and appropriate microbial biocatalysts that can ferment these sugars to high lactic acid yield and titer need to be developed.

Lignocellulosic biomass is estimated to provide a cheaper source of sugars that can be fermented to various products including optically pure lactic acid [28, 32, 33] provided the cost of enzymes required for depolymerization of cellulose can be reduced from the present estimated $0.50 per gallon of ethanol produced [14]. In separate hydrolysis and fermentation (SHF), the cellulose fraction of biomass is hydrolyzed with fungal enzymes at the optimal temperature and pH (50°C and pH 5.0) and the released sugars are fermented by microbial biocatalyst at its own unique optimum temperature and pH [15, 35]. However, the known inhibition of cellulases by their products limits the amount of sugars that can be accumulated before the start of fermentation [8]. This inhibition by released sugars is addressed by simultaneous (enzyme) saccharification and fermentation (SSF) of cellulose in which the enzyme hydrolysis step is coupled to fermentation to eliminate accumulation of inhibitory sugars [5]. For optimum SSF process, the enzyme and the microbial biocatalyst are expected to have similar temperature and pH optima to lower the cost of cellulases, a significant cost component of biomass conversion to products. Furthermore, SSF in a single vessel is expected to also lower the overall process cost compared to SHF. An SSF process at 55°C that combines commercial fungal cellulases and a thermotolerant *Bacillus coagulans* required only about one-quarter of the amount of enzymes (5 FPU g cellulose^{-1}) needed for a similar SSF process with a mesophilic *Lactococcus lactis* (40°C) (20 FPU g glycan^{-1}) for the same volumetric productivity of lactic acid (1.2 g l^{-1} h^{-1}) from crystalline cellulose [21]. Thus, to lower the cost of production of lactic acid, a thermotolerant microbial biocatalyst is preferable over mesophilic lactic acid bacteria used by the industry.

Hemicellulose, a waste product of pulp and paper mills, offers a less expensive fermentable sugar stream for production of lactic acid, ethanol, etc. [9, 13, 17]. However, many of the lactic acid bacteria do not ferment pentose sugars, a major component of hemicellulose in biomass [7]. The lactic acid bacteria that do ferment pentoses utilize the phosphoketolase pathway, which is not conducive for fermentation of pentoses to a specific product [7, 23, 27]. Since phosphoketolase pathway leads to production of equimolar amounts of lactic acid and acetic acid from xylose or other pentoses, only 60% of the pentose carbon is recovered as lactic acid. By increasing the cost of purification of lactic acid from such fermentations, due to the co-production of acetic acid, the cost advantage of using hemicellulose streams or biomass as feedstock may not exist. Fungi, such as *Rhizopus oryzae*, are also known producers of optically pure lactic acid, but only during aerobic growth [16].

In order to economically harness the full potential of lignocellulose as a feedstock, it is necessary to identify microbial biocatalysts suitable for growth on and fermentation of the entire spectrum of hexoses and pentoses present in lignocellulose. Lactic acid bacteria have been engineered to produce lactic acid from pentoses using the pentose–phosphate pathway leading to homolactate production [20]. Reported lactic acid yield and titer of such strains have not reached the same high values of glucose fermentation. *Bacillus coagulans*, a sporogenic lactic acid bacterium grows and ferments the hexoses and pentoses in lignocellulosic biomass to L(+)-lactic acid [23]. Fermentation of pentose sugars by *B. coagulans* utilizes the pentose–phosphate pathway that maximizes the pentose carbon to lactic acid. The optimum growth and fermentation condition for *B. coagulans* is 50–55°C and pH 5.5 [22], a condition that match the activity optima for commercially developed fungal cellulases. Thus, using *B. coagulans* as bacterial biocatalyst offers an opportunity to reduce the cost of lactic acid for bio-based plastics production and replace food products as carbohydrate feedstocks with carbohydrates from lignocellulose. Fermentation at high temperature such as 50°C using thermotolerant/thermophilic bacteria such as *B. coagulans* provides another added advantage in minimizing contamination since most of the common contaminants do not grow at this temperature [1].

Batch fermentations with *B. coagulans* were found to be limited to a lactic acid yield of about 60 g l^{-1} in the broth even with pH control [23, 24]. This contrasts to lactic acid bacteria like *Lactobacillus delbrueckii* which produce concentrations higher than 100 g l^{-1} lactic acid in batch fermentations [7]. In order for *B. coagulans* to be competitive for industrial production of lactic acid, this apparent upper limit needs to be increased. One possibility is that the inherent metabolic ability of *B. coagulans* is limited to low yield of lactic acid. Alternatively, lactic acid is inhibiting growth and fermentation of *B. coagulans* as the concentration of lactic acid increase in the broth, a characteristic that is shared with several other bacteria [6, 11, 25]. Trapping the lactic acid produced during fermentation as calcium lactate by the addition of $CaCO_3$ to the fermentation would provide a means to evaluate the metabolic potential

of *B. coagulans* and compare it to other microbes of industrial interest.

The results presented in this communication show that *B. coagulans* strain 36D1 can produce over 100 g l^{-1} lactic acid in batch fermentations of glucose or xylose. In addition, fed-batch fermentation supported production of close to 200 g l^{-1} of lactic acid from glucose and over 80 g l^{-1} of lactic acid from crystalline cellulose. These results show that the fermentation capacity of *B. coagulans* is comparable to other industrially relevant microbes and can serve as an ideal bacterial biocatalyst for production of L(+)-lactic acid from sugars from non-food sources or cellulose.

Materials and methods

Organism, media, and growth conditions

B. coagulans strain 36D1 was described previously [23]. Media used for growing *B. coagulans* was L broth (LB; per liter: 10 g trypticase peptone, 5 g yeast extract, and 5 g NaCl). Mineral salts medium used in fermentations was described previously [22] and was supplemented with either corn steep liquor (0.25–0.75% dry weight) or yeast extract (1% w/v), as needed. For fermentation experiments, media was supplemented with glucose or xylose, as indicated. Culture pH was maintained at the set value by automatic addition of 2 N KOH. Solid $CaCO_3$ (Fisher Scientific, Pittsburgh, PA) was added at the beginning of fermentation, as needed, at a concentration of 7.5% (w/v) unless specified otherwise.

Fermentations

B. coagulans strain 36D1 was grown overnight in L broth with 2% (w/v) glucose at 50°C and pH 5.0 with pH control. Cells from this culture were collected by centrifugation at room temperature (6,000×*g*; 10 min) and resuspended at the original cell density in fresh medium of choice for fermentation. This culture was used to inoculate experimental fermentations at an initial cell density of 0.08 g l^{-1} (O.D. of 0.2 at 420 nm; Beckman DU640 spectrophotometer, Fullerton, CA). For fermentations containing calcium carbonate, $CaCO_3$ powder was sterilized by autoclave (121°C; 20 min) separately as dry powder and added to the medium immediately after inoculation at an initial concentration of 7.5% (w/v). Although the optimal pH for growth of strain 36D1 was between 5 and 5.5, the optimum pH for the highest volumetric productivity of lactic acid in the presence of $CaCO_3$ was found to be pH 6.0. This is probably due to the higher solubility of $CaCO_3$ at the lower pH that lowers the buffering ability of solid $CaCO_3$. All sugar fermentations in this study were carried out at 50°C and pH 6.0 with automatic addition of KOH.

Simultaneous saccharification and fermentation (SSF)

Simultaneous (enzyme) saccharification and fermentation of cellulose to lactic acid by *B. coagulans* strain 36D1 was performed as described previously [21] with modifications. Fermentations in 250 ml of mineral salts medium with 0.25% corn steep liquor (dry weight) and $CaCO_3$ (7.5%, w/v) in a 500-ml vessel were started with 8 g of Solka Floc and Biocellulase W (15 FPU per gram of cellulose; Kerry Biosciences, Cork, Ireland). At 48 and 96 h after initiation of the experiment, an additional 8 g of Solka Floc and cellulase (15 FPU per gram of glycan) was added to a total cellulose concentration of 24 g while maintaining the cellulases at 15 FPU per gram of glycan. Medium pH was maintained at 5.0 by addition of 2 N KOH and the temperature was 50°C. Samples were removed periodically for determination of fermentation profile.

Analytical methods

Concentration of sugars and fermentation products in the fermentation broth were determined by HPLC as described previously [30] using a Hewlett-Packard HP1090 equipped with Aminex HPX87-H column (Bio-Rad laboratories, Hercules, CA) and filter photometric and refractive index detectors in series (Agilent Technologies, Santa Clara, CA).

Results and discussion

Glucose and xylose fermentation in rich medium

In batch fermentation of glucose, *B. coagulans* strain 36D1 reached a maximum lactic acid concentration of about 60 g l^{-1} in the broth and a significant amount of the glucose was left unfermented (Fig. 1a). With xylose as the fermentable sugar, the lactic acid concentration in *B. coagulans* fermentation only reached about 40 g l^{-1} (Fig. 1b). Cessation of further sugar fermentation is apparently a result of the inhibitory effect of accumulating lactic acid. Several studies have included $CaCO_3$ in the fermentation medium to overcome this inhibition by sequestering the lactic acid produced by the bacterium as calcium lactate [2, 10]. If lactic acid produced from high concentration of lactate at pH 6.0 (above 40–60 g l^{-1}) is inhibitory to *B. coagulans* fermentation, then the addition of $CaCO_3$ to the fermentation may provide a simple method to alleviate this problem. Supplementation of fermentation broth with solid $CaCO_3$ increased the lactic acid yield in both glucose and xylose fermentations by *B. coagulans* strain 36D1 (Fig. 1). *B. coagulans* fermented 110 g l^{-1} of glucose or 120 g l^{-1} of xylose to over 100 g l^{-1} of lactic acid (Fig. 1; Table 1). The lactic acid yield was about 93 and 86% of the glucose and

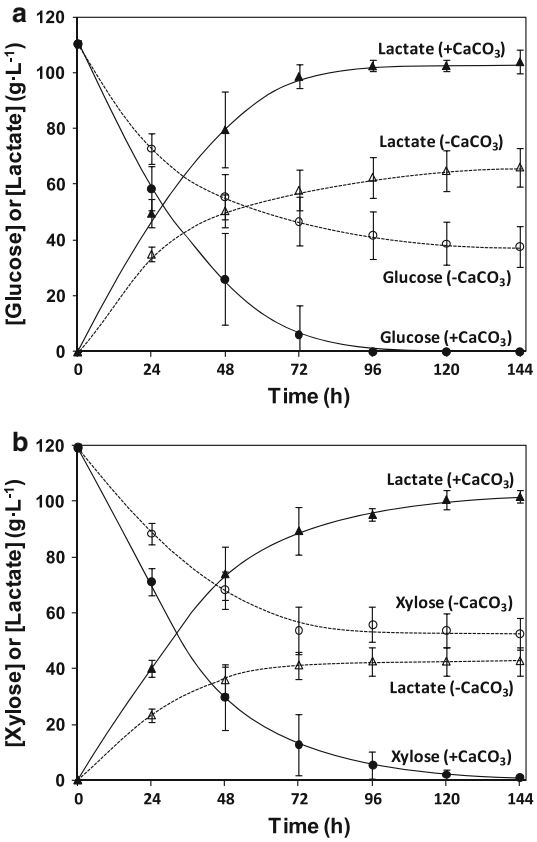

Fig. 1 Effect of $CaCO_3$ addition on fermentation of glucose or xylose to lactic acid by *B. coagulans* strain 36D1. Batch fermentations were performed in L broth with the indicated sugar concentration. See "Materials and methods" section for other details. **a** Glucose. **b** Xylose

xylose fermented, respectively. Less than 5 g l^{-1} of acetate, ethanol and formate accounted for the remaining sugar that was not fermented to lactic acid.

The maximum volumetric productivity of lactic acid during glucose fermentation with and without $CaCO_3$ addition was 2.6 ± 0.03 and 2.4 ± 0.06 g l^{-1} h^{-1}, respectively. These values are in the range of lactic acid productivity reported for various lactic acid bacteria fermenting glucose to lactic acid [7, 31]. The maximum volumetric productivity of lactic acid during xylose fermentation with $CaCO_3$ was about 1.7-fold higher than the rate of lactic acid production from xylose in the absence of $CaCO_3$ (1.3 ± 0.1 g l^{-1} h^{-1}). The high yield and high volumetric productivity of lactic acid from xylose makes *B. coagulans* an attractive organism for utilization of xylose as well as other pentose sugars as feedstock for lactic acid production.

Native microbial biocatalysts that can ferment pentoses to lactic acid had a significantly lower rate of xylose fermentation compared to *B. coagulans* [12, 16, 34]. Metabolic engineering of lactic acid bacteria that redirects carbon through the pentose–phosphate pathway and away from the phosphoketolase pathway was required to achieve fermentation rates of xylose to lactic acid that are comparable to *B. coagulans* [20].

Glucose and xylose fermentation in mineral salts medium

Lactic acid bacteria require complex medium for growth and fermentation that also contribute to the final cost of the product. *B. coagulans* grows in mineral salts medium with small amount of corn steep liquor (0.25–0.75% w/v), an inexpensive supplement [22]. Although glucose fermentation to lactic acid in such a mineral salts medium was effective at low sugar concentration or in SSF of cellulose to lactic acid [22, 23], increasing the glucose concentration to 100 g l^{-1} did lower the lactic acid titer and the highest lactic acid concentration in the broth was less than 50 g l^{-1} even with $CaCO_3$ addition. Replacing corn steep liquor with yeast extract increased the lactic acid yield to 95% of the 100 g l^{-1} glucose added (Fig. 2). In the mineral salts medium glucose was fermented at a lower rate than in rich medium.

Fermentation of 100 g l^{-1} of xylose in mineral salts medium was about two times faster than glucose fermentation in the same medium and all the added xylose was fermented in about 48 h to 89 g l^{-1} lactic acid (Fig. 2; Table 1). This high rate of xylose fermentation in mineral

Table 1 Fermentation profiles of *B. coagulans* strain 36D1 grown in batch or fed-batch mode

Medium	Sugar	Mode	Sugar utilized (g l^{-1})	Fermentation products (g l^{-1})				Yield (%)	
				Lactic acid	Acetate	Ethanol	Formate	Lactic acid	Total
LB	Glucose	Batch	111.5 ± 2.3	103.6 ± 3.5	1.0 ± 0.6	2.8 ± 0.2	0.7 ± 0.3	92.9 ± 4.5	100.3 ± 4.3
LB	Glucose	Fed-batch[a]	197.5 ± 10.5	182.2 ± 10.4	0.8 ± 0.4	5.5 ± 2.0	2.1 ± 0.7	92.3 ± 0.4	98.8 ± 1.5
LB	Xylose	Batch	118.4 ± 0.7	102.3 ± 2.3	0.7 ± 0.2	1.5 ± 0.5	0.3 ± 0.2	86.4 ± 2.2	91.5 ± 3.3
LB	Xylose	Fed-batch[a]	186.8 ± 1.3	163.0 ± 1.1	1.7 ± 0.5	2.3 ± 0.0	1.2 ± 0.3	87.3 ± 0.1	92.4 ± 0.7
MM	Glucose	Batch	98.2 ± 6.6	94.6 ± 3.1	0.4 ± 0.1	1.2 ± 0.3	0.1 ± 0.2	96.3 ± 3.4	100.5 ± 4.3
MM	Xylose	Batch	101.0 ± 1.0	89.1 ± 1.7	1.3 ± 0.2	2.4 ± 0.0	2.8 ± 0.3	88.2 ± 1.5	95.9 ± 1.5

[a] Fed-batch (FB) fermentations started with 100 g l^{-1} of sugar and were supplied with additional 50 g l^{-1} sugar at 48 h and 96 h to a total of 200 g l^{-1}

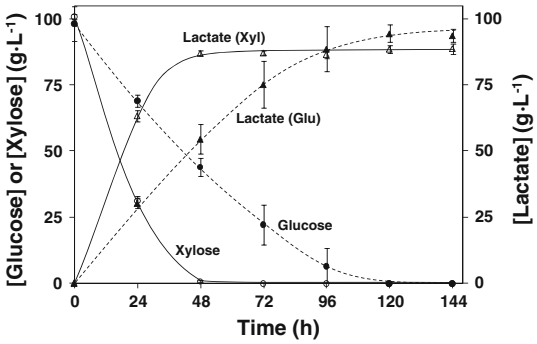

Fig. 2 Fermentation of 100 g l^{-1} glucose or xylose in mineral salts medium (+ yeast extract) to lactic acid by *B. coagulans* strain 36D1

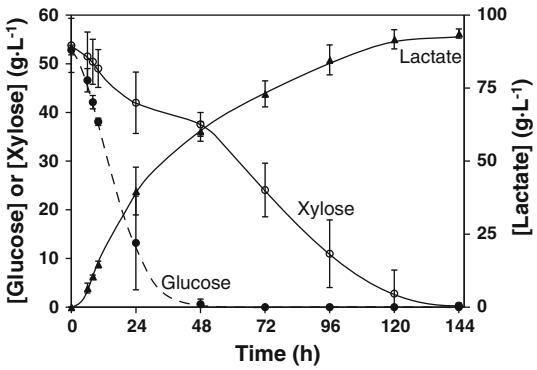

Fig. 3 Effect of glucose on xylose fermentation by *B. coagulans* strain 36D1. Mineral salts medium with yeast extract (1% w/v) contained 53 g l^{-1} of glucose and 54 g l^{-1} of xylose. pH of the culture was maintained at 6.0 by addition of KOH and the culture temperature was 50°C

salts medium with $CaCO_3$ is comparable to the rate of fermentation of glucose or xylose in rich medium. The lactic acid yield was 96.3 and 88.2% for glucose and xylose, respectively, in mineral salts medium (Table 1) and was not impacted by switching growth medium from rich medium to mineral salts medium with yeast extract (1% w/v) supplementation.

Although xylose was fermented more rapidly in mineral salts medium compared to glucose, and probably a preferred carbon source in this medium, glucose still exerted catabolite repression on the use of xylose when both glucose and xylose were present in the medium at about 50 g l^{-1} each (Fig. 3). This is not unexpected since glucose-mediated repression of xylose utilization genes are well established in *Bacillus* [26].

In the presence of glucose and xylose (53 and 54 g l^{-1} of glucose and xylose, respectively) glucose was completely fermented within 48 h (Fig. 3). During the same 48-h period, less than 20 g l^{-1} of xylose was fermented although the results presented in Fig. 2 show that *B. coagulans* strain 36D1 fermented xylose at a higher rate than glucose in a medium without added glucose. Xylose fermentation

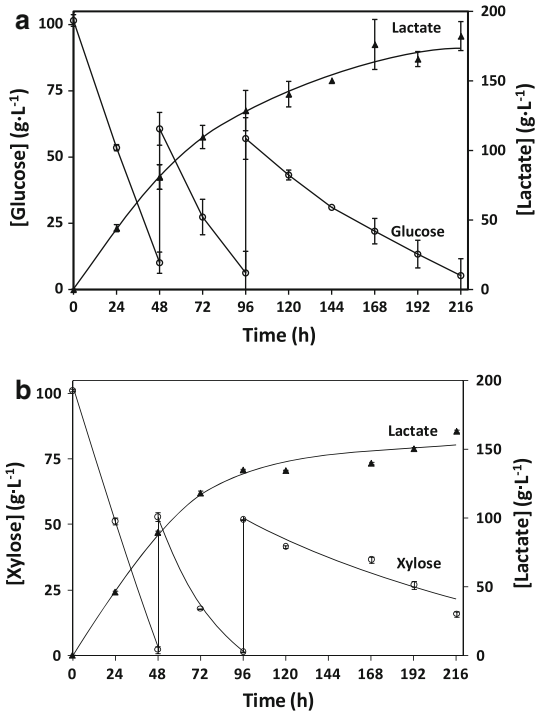

Fig. 4 Fed-batch fermentation of 200 g l^{-1} of glucose or xylose by *B. coagulans* strain 36D1. Fermentations in LB were initiated with 100 g l^{-1} sugar and additional 50 g l^{-1} of sugar was added at 48 h and at 96 h to a total of 200 g l^{-1}. See text for other details. **a** Glucose. **b** Xylose

increased to a higher rate after complete removal of glucose from the medium by fermentation but this rate was still lower than the rate of glucose consumption during the first 48 h. All the added sugars were fermented by about 144 h to 94 g l^{-1} of lactic acid at a yield of 88%.

Fed-batch fermentation of glucose and xylose

Increasing glucose concentration to 150 g l^{-1} decreased the rate of sugar consumption and volumetric lactic acid productivity even in rich medium compared to the values from 110 g l^{-1} glucose fermentation (data not presented). Osmotic effect of high sugar concentration may account for this decrease. Supplementation of the growth medium with betaine as an osmoprotectant did not significantly alter the rate of fermentation of 150 g l^{-1} of glucose. To overcome the osmotic effect of high glucose concentration, fed-batch fermentation was conducted to evaluate the highest amount of lactic acid that can be produced by *B. coagulans*. Fermentations were initiated with 100 g l^{-1} sugar and additional 50 g l^{-1} sugar was added at 48 h and at 96 h when the sugar concentration reached almost zero (Fig. 4a). Rate of fermentation was almost linear until about 125 g l^{-1} glucose was converted to about 110 g l^{-1} of lactic acid. The rate of fermentation of glucose beyond this point decreased continuously until a final lactic acid concentration of about

180 g l^{-1} was reached. This decline in productivity could be a result of decreasing metabolic potential of an aging microbial biocatalyst and/or the effect of accumulating calcium lactate.

In a similar fed-batch experiment, the rate of fermentation of xylose after about 72 h was found to be significantly lower than the initial rate and the highest lactic acid concentration in the broth was only about 160 g l^{-1}, a yield of about 80% (Fig. 4b). Although the rate of lactic acid production decreased over time with the accumulation of lactic acid, *B. coagulans* still produced higher than 160 g l^{-1} at 50°C from either glucose or xylose.

SSF of cellulose to lactic acid

In a fed-batch SSF experiment, *B. coagulans* converted about 80% of the glucose equivalent of the added cellulose to lactic acid to a concentration of 80 g l^{-1} (Fig. 5). Due to the presence of $CaCO_3$, the actual amount of cellulose that was hydrolyzed and fermented was not determined. In similar SSF experiments without $CaCO_3$, about 90–95% of the added cellulose was found to be converted to lactic acid by fungal cellulases and *B. coagulans* [21, 22]. Assuming that 90% of the added cellulose was also converted in this fed-batch SSF experiment (Fig. 5), the yield of lactic acid can be calculated to be about 88%. Ethanol was the only other major product of fermentation (less than 3 g l^{-1}) and mirrored that of lactic acid production until 96 h but at a significantly lower level. Small amounts of succinate and 2,3-butanediol were also produced during this SSF. These results confirm the potential of *B. coagulans* in the SSF of cellulose to lactic acid at high yield.

The high temperature fermentation of sugars to L(+)-lactic acid by *B. coagulans* has several advantages over conventional lactic acid bacteria used by the industry today. (1) The high temperature can lower the amount and associated cost of commercial fungal cellulases (one of the major cost components of biomass conversion) needed for SSF of biomass-derived cellulose to about one-quarter of a mesophilic SSF process to reduce the cost of lactic acid production [21]. (2) *B. coagulans* ferments pentoses, primary sugars in hemicellulose syrups, to lactic acid at high yield with minimal secondary products and without foreign genes [23]. (3) Fermentation of sugars to lactic acid can be realized in mineral salts medium with small amount of yeast extract (or other supplements such as yeast hydrolysate). (4) The growth temperature of 50–55°C for *B. coagulans* can minimize contamination of large-scale fermentations with a further potential reduction in process cost.

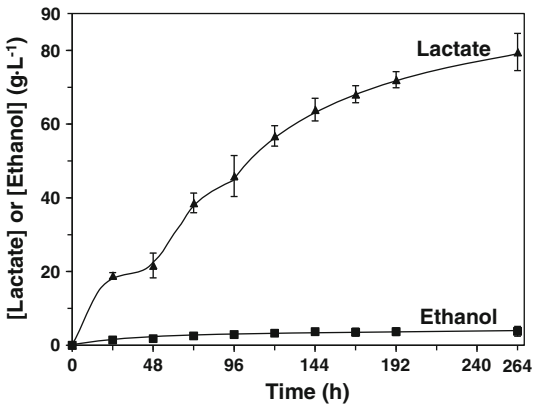

Fig. 5 Simultaneous saccharification and fermentation of cellulose to lactic acid by *B. coagulans* strain 36D1 in a fed-batch mode. Fermentations in mineral salts medium with corn steep liquor (0.25%, w/v) were started with 32 g l^{-1} of Solka Floc as crystalline cellulose and 15 FPU (g glycan)$^{-1}$ of Biocellulase W. At 48 and 96 h, additional cellulose was added at 32 g l^{-1} each to a final cellulose concentration of 96 g l^{-1}. With each cellulose addition, cellulase W was also added to maintain the cellulase to cellulose ratio at 15 FPU (g glycan)$^{-1}$

Conclusions

The results presented above show that *B. coagulans* has a rapid fermentation rate at 50–55°C leading to production of over 100 g l^{-1} of L(+)-lactic acid at a volumetric productivity of about 2.5 g l^{-1} h^{-1}. This titer can be increased to over 180 g l^{-1} of lactic acid in a fed-batch mode. These titers and yields are comparable to that of mesophilic lactic acid bacteria used by the industry. Native *B. coagulans* also fermented xylose, the main component of hemicelluloses, to lactic acid at a similar high rate and yield in mineral salts medium with minimal amount of supplement in contrast to the need for more complex medium for conventional lactic acid bacteria. In a typical SSF of crystalline cellulose, *B. coagulans,* in conjunction with fungal cellulases, converted about 80% of the added cellulose to lactic acid. These characteristics suggest that *B. coagulans* is an effective microbial biocatalyst for production of optically pure lactic acid from glucose, xylose, and crystalline cellulose for synthesis of bio-based plastics and other products derived from lactic acid from non-food sources of carbohydrates. In addition, fermentation of xylose-rich hemicellulose liquor from paper/pulp mills to lactic acid by *B. coagulans* can provide an additional value-added product from a resource that is not effectively used today.

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