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# Thermophilic *Bacillus coagulans* Requires Less Cellulases for Simultaneous Saccharification and Fermentation of Cellulose to Products than Mesophilic Microbial Biocatalysts

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**Abstract** Ethanol production from lignocellulosic biomass depends on simultaneous saccharification of cellulose to glucose by fungal cellulases and fermentation of glucose to ethanol by microbial biocatalysts (SSF). The cost of cellulase enzymes represents a significant challenge for the commercial conversion of lignocellulosic biomass into renewable chemicals such as ethanol and monomers for plastics. The cellulase concentration for optimum SSF of crystalline cellulose with fungal enzymes and a moderate thermophile, *Bacillus coagulans*, was determined to be about 7.5 FPU $g^{-1}$ cellulose. This is about three times lower than the amount of cellulase required for SSF with *Saccharomyces cerevisiae*, *Zymomonas mobilis*, or *Lactococcus lactis* subsp. *lactis* whose growth and fermentation temperature optimum is significantly lower than that of the fungal cellulase activity. In addition, *B. coagulans* also converted about 80% of the theoretical yield of products from 40 g/L of crystalline cellulose in about 48 h of SSF with 10 FPU $g^{-1}$ cellulose while yeast, during the same period, only produced about 50% of the highest yield produced at end of 7 days of SSF. These results show that a match in the temperature optima for cellulase activity and fermentation is essential for decreasing the cost of cellulase in cellulosic ethanol production.

**Keywords** Cellulase · Cellulose SSF · *Bacillus coagulans* · *Saccharomyces* · *Zymomonas* · Lactic acid bacteria

## Introduction

The limited nature and rising costs of fossil fuels have provided the needed impetus to the use of sustainable and renewable sources of fuels and chemicals. Lignocellulosic biomass from plants is the only promising sustainable feedstock that can be converted to both fuels

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and chemicals without increasing greenhouse gas emissions [1]. Although the hemicellulose in the biomass can be readily hydrolyzed to fermentable sugars by mild acid treatment, bioconversion of cellulose in the biomass to glucose requires hydrolysis by cellulases before fermentation by microbial biocatalysts [2–7].

Fungal cellulases, such as those from *Trichoderma reesei*, dominate the industrial applications of cellulases and are one of the significant cost components of deriving sugars from cellulose for fermentation to ethanol and other chemicals [5–7]. One processing strategy in the production of cellulose-based fuels and chemicals involves separate hydrolysis of cellulose by enzymes followed by fermentation by microbial biocatalysts (SHF) [8]. Due to the low yield of glucose in the SHF process, a result of inhibition of cellulases by the hydrolysis products cellobiose and glucose [9], combined with inherent low specific activity of the fungal cellulases [10], the amount of cellulases required for optimal conversion of cellulose to ethanol is higher than desired for economical production of ethanol and other commodity chemicals. To overcome the product inhibition of cellulases, a strategy of simultaneous saccharification and fermentation (SSF) has been developed in which hydrolysis of cellulose is coupled with fermentation of the released sugars in the same reaction vessel [11]. In the SSF process, the fungal enzymes hydrolyzed the cellulose to sugars that were immediately fermented by the microbial biocatalyst to ethanol and/or chemicals. Because of this combination, hydrolysis of cellulose by the enzymes could not be optimized separately and the growth and fermentation optimum of the microbial biocatalyst also need to be considered in selecting an optimum temperature and pH for SSF of cellulose to products.

Although the SSF process minimized the product inhibition of cellulase activity, the relatively low specific activity of fungal cellulases in relation to bacterial-cell-associated cellulases is yet to be overcome [1, 8]. This is further magnified by the SSF process at a lower-than-optimum temperature for the enzyme activity (optimum of 50 °C and pH 5.0) [10, 12] due to the need for a lower temperature (30–35 °C) that is the growth and fermentation optimum for the current industrial biocatalysts, such as *Saccharomyces cerevisiae* [13] and *Zymomonas mobilis*. Lactic acid bacteria used by the industry for production of optically pure lactic acid, a potential renewable source of plastics, also suffers from the low temperature optimum (35–40 °C) for growth and fermentation compared to that of the fungal cellulase activity optimum [10, 12, 14–16]. Using these microbial biocatalysts in SSF of cellulose to products is expected to result in a mismatch in optima leading to either a higher requirement of fungal cellulases or an increase in the time required for SSF, both of which significantly increase the cost of bioconversion of cellulose to fuels and chemicals.

Our laboratory has described Gram-positive bacterial isolates that have growth and fermentation temperature optima which closely match those of fungal cellulases being developed for use in SSF of cellulose [12, 17]. These isolates, such as *Bacillus coagulans* strain 36D1, grow and ferment hexoses and pentoses at 50–55 °C and pH 5.0 producing L (+)-lactic acid as the primary fermentation product. Use of *B. coagulans* for optically pure lactic acid production for plastics industry or an engineered *B. coagulans* derivative for ethanol production at 50–55 °C is expected to reduce the amount of fungal cellulases required for optimum conversion of cellulose to products in the SSF process. However, no comparative experimental evidence exists establishing this possibility. In this communication, evidence is presented that coupling the microbial biocatalyst in the SSF process to the optimum temperature for fungal cellulases significantly reduces the amount of enzyme required for conversion of cellulose to products in comparison with yeast or lactic acid bacteria currently used by the industry as microbial biocatalysts. This reduction in the

amount of cellulase in SSF of cellulose to products is expected to reduce the cost of the process and final product.

## Materials and Methods

### Organisms, Media, and Growth Conditions

*B. coagulans* strain 36D1 was described previously [12, 17]. Media used in experiments with *B. coagulans* strain 36D1 contained, per liter: 6.25 g $Na_2HPO_4$, 0.75 g $KH_2PO_4$, 2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $Na_2MoO_4 \cdot H_2O$, 1 ml trace mineral solution [18], and 5 ml corn steep liquor (50% dry solids; Grain Processing Corp., Muscatine, IO), adjusted to pH 5.0 with $H_2SO_4$. Media used for *Lactococcus lactis* subsp. *lactis* (NRRL B-4449) SSF contained, per liter: 10 g yeast extract, 2 g $K_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot H_2O$, 2 g $(NH_4)_2SO_4$, adjusted to pH 5.5 with $H_2SO_4$. Media used for *S. cerevisiae* (NRRL Y-12632) SSF contained, per liter: 10 g yeast extract, 2 g $KH_2PO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, adjusted to pH 5.5 with $H_2SO_4$. Media used for *Z. mobilis* (ATCC 1050) SSF contained, per liter: 10 g yeast extract and 0.3 g $KH_2PO_4$, adjusted to pH 5 with $H_2SO_4$. These media were supplemented with glucose at 1% (*w*/*v*) for growth and fermentation without pH control and with 2% (*w*/*v*) in fermentations with pH control for preparation of inoculum for SSF experiments. Optimum temperature and pH for the growth of organisms were determined in batch fermentations without pH control as described previously [17], at various temperatures and initial culture pH.

### SSF of Cellulose

SSF of crystalline cellulose (Solka Floc) was carried out in the media described above containing 40 g $L^{-1}$ Solka Floc (International Fiber Corp., North Tonawanda, NY) and varying amounts of cellulase (GC220; Genencor International, Palo Alto, CA). Specific activity of the cellulase preparation as FPU/ml was determined before use as described previously [12]. SSF of crystalline cellulose was carried out at the temperature and pH that were found to be optimal for growth of the specific microbial biocatalyst (*B. coagulans* strain 36D1 at 50 °C and pH 5.0; *L. lactis* at 40 °C and pH 5.5; *S. cerevisiae* at 35 °C and pH 5.5; *Z. mobilis* at 35 °C and pH 5.0). Fermentation pH was maintained by automatic addition of 2 N KOH for strain 36D1 and *L. lactis* or 0.5 N KOH for *S. cerevisiae* and *Z. mobilis*. Sugar and fermentation products were determined using HPLC as described previously [19]. Inoculum for SSF experiments with crystalline cellulose was derived from pH-controlled fermentations in the same media but with 2% (*w*/*v*) glucose. Cells were collected by centrifugation at room temperature and resuspended in appropriate growth media before inoculation in the SSF medium at an initial O.D. at 420 nm of 0.2.

## Results and Discussion

In order to obtain the highest volumetric productivity for each organism, optimal temperature and pH for anaerobic growth and fermentation of glucose was determined for each of the four microbial biocatalysts in batch fermentations without pH control. Based on the results of these experiments, the optimal conditions for *B. coagulans* strain 36D1, *L.*

*lactis*, *S. cerevisiae*, and *Z. mobilis* were determined to be 50 °C and pH 5.0, 40 °C and pH 5.5, 35 °C and pH 5.5, 35 °C and pH 5.0, respectively. These conditions were subsequently used for SSF of cellulose by each organism such that growth and fermentation conditions of the microbial biocatalyst would not be limiting the rate of conversion of cellulose-derived glucose to products. Although industrial yeast fermentations of corn starch to ethanol are conducted at temperatures below 35 °C, this temperature was used in this study since the growth rate of *S. cerevisiae* NRRL Y-12632 at 35 °C was slightly higher than at 30 °C.

SSF of crystalline cellulose was carried out with varying concentrations of cellulase. For each organism, the fermentation profile was dominated by one major fermentation product: lactate produced by *B. coagulans* and *L. lactis* or ethanol produced by *S. cerevisiae* and *Z. mobilis*. The rate of product formation was linear with time and also the highest during the first 18 h; after this period, the rate of product formation continually declined, irrespective of the microbial biocatalyst or the cellulase concentration. Representative SSF profiles for *B. coagulans* and yeast are presented in Fig. 1. With *B. coagulans* as the microbial biocatalyst and the SSF at 50 °C, the initial rate of product formation was not significantly altered by increasing the cellulase concentration from 5 FPU to 20 FPU $g^{-1}$ of cellulose (Fig. 1a). Contrasting this observation, with yeast as the biocatalyst and SSF at 35 °C, the initial rate of ethanol production doubled with every twofold increase in cellulase concentration from 5–20 FPU $g^{-1}$ cellulose (Fig. 1b).

Using the initial high rate of product formation, maximum volumetric productivity for each cellulase concentration and microbial biocatalyst was calculated and presented in

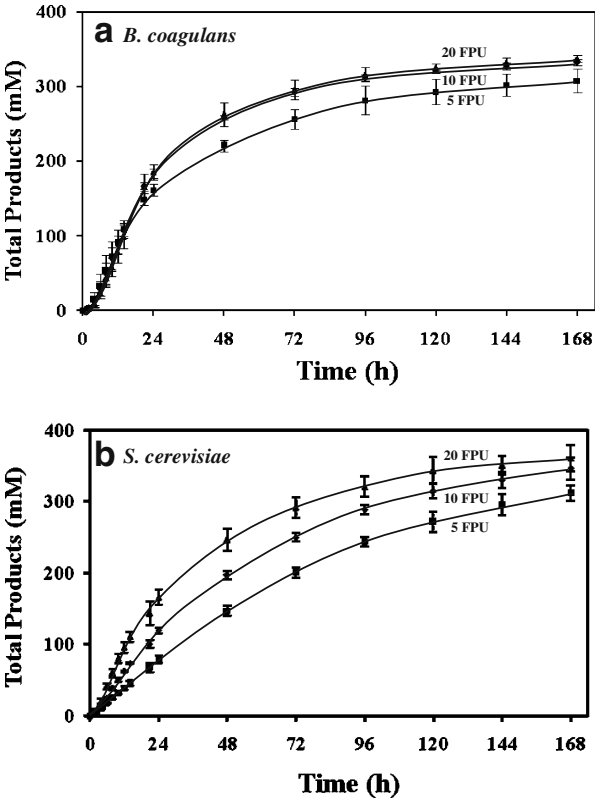


**Fig. 1** Time course of SSF of 40 g $L^{-1}$ crystalline cellulose (Solka Floc) with different concentrations of fungal cellulase (GC220; Genencor) **a** *B. coagulans* strain 36D1 at 50 °C and pH 5.0; **b** *S. cerevisiae* at 35 °C and pH 5.5

Fig. 2. Except for *L. lactis*, the volumetric productivity of the other three organisms increased with increasing cellulase concentration until the maximum value was reached. For *B. coagulans*, SSF at 50 °C, the cellulase concentration required for the highest volumetric productivity was between 5 and 7.5 FPU $g^{-1}$ cellulose. With yeast or *Z. mobilis* as the microbial biocatalyst, volumetric productivity continued to increase up to a cellulase concentration of 30 FPU $g^{-1}$ cellulose and, at this concentration of cellulase, the volumetric productivities of the ethanologens and *B. coagulans* at 50 °C were about the same. These results show that SSF at 50 °C can reduce the cellulase requirement by at least threefold without affecting volumetric productivity.

Increasing the SSF temperature to 55 °C with *B. coagulans* as the microbial biocatalyst, increased the volumetric productivity by about 40% without affecting the cellulase requirement (Fig. 2). *L. lactis*-based SSF also reached the same high volumetric productivity observed with *B. coagulans* at 55 °C but this required higher than 20 FPU $g^{-1}$ cellulose in contrast to *B. coagulans* at 55 °C that required only about 5 to 7.5 FPU $g^{-1}$ cellulose, a three- to fourfold reduction in cellulase requirement. At a cellulase concentration of 5 FPU $g^{-1}$ cellulose, the volumetric productivity with *B. coagulans* SSF at 55 °C was at least 2.5-fold higher than that of any of the other three microbial biocatalysts. This difference is apparently due to the higher activity of the enzyme at 55 °C [12] compared to by the optimum temperature for growth of the microbial biocatalysts.

Although the maximum volumetric productivity of yeast and *Z. mobilis* was lower in the presence of lower cellulase concentrations (7.5 FPU $g^{-1}$ cellulose) as compared to *B. coagulans*, all three fermentations produced about the same final yield of products at the end of 168 h of SSF (Table 1). At the end of 168 h of SSF and with 20 FPU $g^{-1}$ cellulose, all three microbial biocatalysts (except *L. lactis*) converted about 90–95% of the glucose equivalents of cellulose. For *B. coagulans*, the time required to reach this product yield was about 96 h while the other three microbial biocatalysts required at least 168 h to reach the same product yield. These results are in agreement that SSF with *B. coagulans* at 50 °C is more effective in converting cellulose to products than with yeast as the microbial biocatalyst even at its optimal growth and fermentation temperature.

The amount of product produced by the various microbial biocatalysts in the SSF of cellulose at the end of 48 h was determined to further evaluate the efficiency of the process. The results presented in Fig. 3 are the fraction of the major product of fermentation at 48 h as compared to the maximum yield obtained at the end of fermentation at 168 h. In an SSF

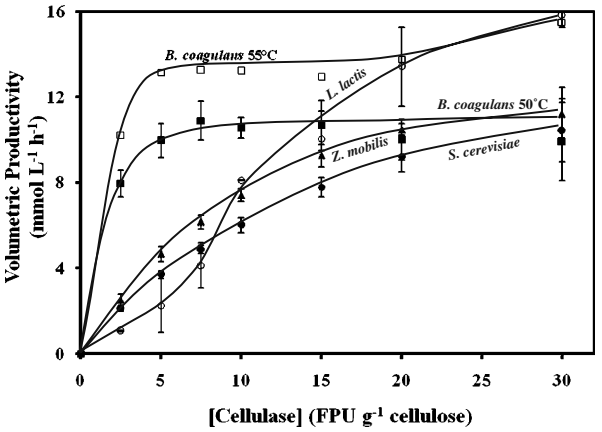


**Fig. 2** Maximum volumetric productivity of SSF of 40 g $L^{-1}$ cellulose (Solka Floc) by *B. coagulans* strain 36D1 (50 or 55 °C; pH 5.0), *L. lactis* (40 °C; pH 5.5), *S. cerevisiae* (35 °C; pH 5.5), and *Z. mobilis* (35 °C; pH 5.0) as a function of fungal cellulase concentration. Volumetric productivity was calculated as lactate produced by *B. coagulans* strain 36D1 and *L. lactis* and as ethanol produced by *S. cerevisiae* and *Z. mobilis*. See text for other details

**Table 1** Profile of SSF of crystalline cellulose by *B. coagulans*, *L. lactis*, *S. cerevisiae*, and *Z. mobilis*.

| Organism | Net production (mM) | | | | | Total product | Major product |
|---|---|---|---|---|---|---|---|
| | Lactate | Ethanol | Acetate | Succinate | Glycerol | Yield (%)[a] | Fraction (%)[b] |
| *B. coagulans* | 299.9±0.8 | 17.6±2.4 | 17.9±1.0 | 2.2±1.9 | 0.0 | 90.3±4.0 | 88.8±0.8 |
| *L. lactis* | 226.0±23.4 | 47.3±10.2 | 21.1±4.4 | 0.0 | 0.0 | 70.9±2.5 | 76.7±5.2 |
| *S. cerevisiae* | 0.0 | 349.8±16.7 | 2.9±3.4 | 0.8±1.4 | 7.2±4.8 | 93.2±11.4 | 97.0±1.0 |
| *Z. mobilis* | 0.7±0.7 | 373.7±17.4 | 16.5±15.2 | 0.0 | 0.8±0.7 | 94.4±0.9 | 95.4±3.0 |

SSF of 40 g $L^{-1}$ of crystalline cellulose (Solka Floc) was carried out at a cellulase concentration of 20 FPU $g^{-1}$ cellulose for 168 h. See "Materials and Methods" section for details. Growth temperature and culture pH for the various organisms are listed in the text. Reported average values were from three independent fermentations

[a] Total products yield was calculated as % ratio of all fermentation products listed in the table to theoretical expected yield based on the amount of cellulose consumed on a molar basis

[b] Major product fraction represents the % of major product in the total fermentation products

with *B. coagulans*, about 80% of the expected products were produced during the first 48 h of SSF at 50 °C with only about 10 FPU $g^{-1}$ of cellulose. Increasing the cellulase concentration did not influence this productivity. With the same amount of cellulase, both yeast and *Z. mobilis*-based SSFs yielded only about 50% of the ethanol during the first 48 h of SSF. The second 50% of ethanol production required an additional 120 h of SSF due to a continual decline in volumetric productivity. Even with 30 FPU $g^{-1}$ cellulose, these two microbial biocatalysts only yielded about 65% of the expected ethanol during the first 48 h of SSF. *L. lactis* reached the same 80% product yield as *B. coagulans* but this required slightly higher concentration of cellulase (15 FPU vs 10 FPU $g^{-1}$ cellulose). However, it should be noted that the final lactate yield is lower for *L. lactis* compared to *B. coagulans* (Table 1). These results show that SSF with an organism that can match the optimum conditions for fungal cellulase activity, such as *B. coagulans*, is a better choice of microbial biocatalyst to reduce the cost of fungal cellulase in SSF of cellulose to low-value commodity chemicals by decreasing the amount of enzyme needed and also the fermentation time, two significant areas of cost savings.

In conclusion, the temperature optimum for *B. coagulans* more closely match the optimum for fungal cellulases used in SSF than current generation biocatalysts used by the industry for producing ethanol or lactic acid, and this match leads to higher volumetric

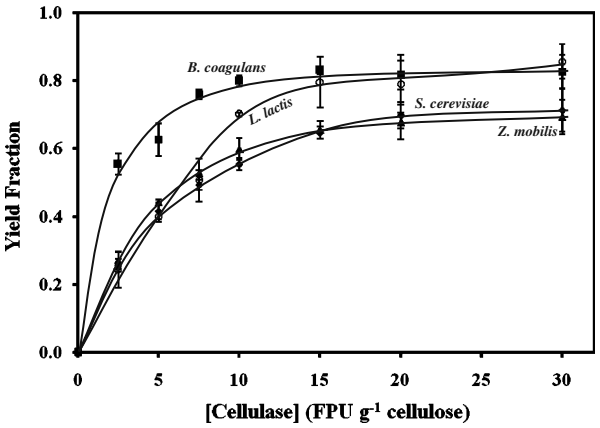


**Fig. 3** Yield fraction of the major fermentation product of SSF of 40 g $L^{-1}$ cellulose (Solka Floc) by *B. coagulans* strain 36D1, *L. lactis*, *S. cerevisiae*, and *Z. mobilis* at different fungal cellulase concentrations. Yield fraction was calculated as the ratio of major fermentation product (lactate for *B. coagulans* and *L. lactis*, ethanol for *S. cerevisiae* and *Z. mobilis*) produced at 48 h of SSF to that of the same product at the SSF endpoint of 168 h

productivity and more rapid progress to completion of SSF. This close match in temperature optima leads to a lower cellulase requirement for SSF by a factor of at least three for *B. coagulans* strain 36D1 versus the other microbial biocatalysts. The lower cellulase requirement and more rapid progress to the fermentation endpoint with *B. coagulans* in SSF is expected to lead to substantial cost savings in the amount of cellulase required for SSF. Metabolic engineering of *B. coagulans* and/or other thermotolerant microbial biocatalysts for production of ethanol as the main fermentation product should provide a new group of microbial biocatalysts that can contribute significantly in the conversion of lignocellulosic biomass to fuel ethanol in a cost-effective manner.

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