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Thermophilic Biocatalysts for the Conversion of Cellulosic Substrates to Fuels and Chemicals

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Description: The primary objective of this study is to engineer a thermophilic bacterium *Bacillus coagulans* that grows optimally at 50-55 °C and pH 5.0, the optimum conditions for the activity of commercial fungal cellulases, for cost-effective depolymerization of cellulose to glucose for simultaneous fermentation to ethanol or other commodity chemicals as the sole fermentation product.

Budget: $192,000.00
Universities: UF

Progress Summary

Bacillus coagulans, a sporogenic lactic acid bacterium, grows optimally at 50-55 °C and produces lactic acid as the primary fermentation product from both hexoses and pentoses. Lactic acid is used as an additive in foods, pharmaceuticals and cosmetics as well as 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 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 was produced from xylose and glucose by trapping the lactic acid as calcium salt. In a fed-batch SSF of crystalline cellulose, $CaCO_3$ addition also improved lactic acid production by *B. coagulans* with a yield of near 80% based on a final titer of about 80 g L^{-1}. These results demonstrate that *B. coagulans* can effectively ferment non-food carbohydrates from lignocellulose to L(+)-lactic acid at sufficient concentrations for commercial application.

Ethanol is currently produced from corn starch and the food vs fuel debate is encouraging the use of non-food carbohydrates for fuel ethanol production. However, the use of lignocellulosic biomass requires pretreatment of biomass by both chemicals and cellulases to release the sugars before fermentation to ethanol. The amount of fungal cellulases required for simultaneous saccharification and fermentation (SSF) at 55°C was previously reported to be 3-4-times lower than for SSF at the optimum growth temperature for *Saccharomyces cerevisiae* of 35°C. An ethanologenic *B. coagulans* is expected to lower the cellulase loading and production cost of cellulosic ethanol due to SSF at 55°C. As a first step towards developing *B. coagulans* as an ethanologenic microbial biocatalyst, activity of the primary fermentation enzyme L-lactate dehydrogenase was removed by mutation (strain Suy27). Strain Suy27 produced ethanol as the main fermentation product from glucose during growth at pH 7.0 (0.33 g ethanol per g glucose fermented). Pyruvate dehydrogenase (PDH) and alcohol dehydrogenase (ADH) acting in series contributed to about 55% of the ethanol produced by this mutant while pyruvate formate-lyase and ADH were responsible for the remainder. Due to the absence of PDH activity in *B. coagulans* during fermentative growth at pH 5.0, the *l-ldh* mutant failed to grow anaerobically at pH 5.0. Strain Suy27-13, a derivative of the *l-ldh* mutant strain Suy27, that produced PDH activity during anaerobic growth at pH 5.0 grew at this pH and also produced ethanol as the fermentation product (0.39 g per g glucose). These results show that construction of an ethanologenic *B. coagulans* requires optimal expression of PDH activity in addition to the removal of the LDH activity to support growth and ethanol production.

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2010 Annual Report

Objectives

1. Engineer *B. coagulans* for production of ethanol or optically pure D(-)- or L(+)- lactic acid.
2. Eliminate competing metabolic pathways that drain both carbon and NADH away from the desired product.
3. Evaluate and optimize the SSF characteristics of the engineered microbial biocatalyst.

Ethanol producing *B. coagulans*: In order to generate a thermophilic bacterial biocatalyst, we chose B. coagulans as the platform for metabolic engineering. This bacterium produces lactic acid as the primary fermentation product and as the first step we mutated this activity and constructed a derivative that can not produce lactate. The *l-ldh* mutant of *B. coagulans*, strain Suy27, produced ethanol during fermentation of glucose at pH 7.0 and the ethanol yield from glucose was 0.32 (g ethanol per gram of glucose). About 55% of the ethanol was produced by the PDH/ADH pathway and the remainder from the PFL/ADH pathway. Coupling the 2,3-butanediol pathway to the PFL/ADH pathway appears to support redox balance while increasing the overall ethanol yield. Although this mutant grew aerobically at pH 5.0, it failed to grow fermentatively at this pH. This inability of the *l-ldh* mutant to grow anaerobically at pH 5.0 is probably due to the absence of PDH and PFL activities as seen with the parent strain 36D1 grown at pH 5.0. A derivative of the *l-ldh* mutant, strain Suy27-13, that produced PDH activity during anaerobic growth at pH 5.0, also produced ethanol as the main fermentation product from glucose during growth at pH 7.0 (0.39 g ethanol g^{-1} sugar) and pH 5.0 (0.26 g ethanol g^{-1} sugar). Although this ethanol yield of *B. coagulans* strain 27-13 at pH 7.0 is lower than the theoretical yield of 0.51 g per gram glucose fermented, this value is comparable to the published ethanol yield of batch cultures of other *Bacillus* and *Geobacillus* strains engineered for ethanol production by the PDH/ADH pathway; *B. stearothermophilus* strain LLD-15 (0.34 g per g sugar), *G. thermoglucosidasius* strain DL44 (0.42 g per g glucose), and a non-recombinant ethanologenic *E. coli* strain SE2378 (0.41 g per g glucose). Although a *B. subtilis* with *Z. mobilis* PDC activity (strain BS37) was reported to produce a significantly higher ethanol yield from glucose (0.45 g per g glucose), this strain required about 9 days to ferment 20 g L^{-1} glucose. Further metabolic engineering of strain Suy27-13 that deleted the competing 2,3-butanediol pathway and increased the PDH activity is expected to elevate the ethanol yield from glucose and pave the way for cellulosic ethanol production at 50-55 °C with a lower cellulase loading compared to SSF at 30-37 °C.

Increasing the level of L(+)-lactic acid production: The results obtained in this study show that *B. coagulans* has the ability to produce lactic acid at concentrations higher than 150 g L^{-1} from glucose or xylose in about 48 hours. This is comparable to lactic acid yields and productivity values reported for other lactic acid bacteria. This high lactic acid titer and yield of over 95% of the sugar fermented was obtained by trapping the lactic acid as calcium lactate. In the absence of calcium carbonate in the fermentation medium, the lactic acid titer did not increase beyond 50 g/L. The optical purity of the L(+)-lactic acid was close to 100% and the level of D(-)-lactic acid in the product was undetectable. In a SSF process of crystalline cellulose and commercial fungal cellulases, over 80 g/L titer was obtained at 50°C using the *B. coagulans* strains used in this study.

B. coagulans has the following advantages in optically pure lactic acid production over conventional lactic acid bacteria used by the industry today. (1) Native *B. coagulans* ferments pentoses to lactic acid at high yield with minimal secondary fermentation products. (2) *B. coagulans* grows at 50-55 °C, a temperature that minimizes contamination of large-scale fermentations. This is in contrast to mesophilic lactic acid bacteria that lack the ability to ferment pentoses to lactic acid as primary product and also has a potential for contamination by non-productive mesophilic microbes. (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) with *B. coagulans* in contrast to the need for rich medium for growth and fermentation of other lactic acid bacteria. (4) Due to the optimum growth and fermentation temperature of 50-55 °C, the amount of cellulases required for SSF of crystalline cellulose to lactic acid by *B. coagulans* can be significantly reduced with a projected lower product cost. Metabolic engineering to overcome the osmotic effect of high sugar and calcium lactate concentration in the fermentation by *B. coagulans* has the

potential to enhance the lactic acid yield to levels that are higher than what we have obtained so far. These advantages of *B. coagulans* based fermentation for lactic acid suggests that *B. coagulans* is as an effective microbial biocatalyst for production of optically pure lactic acid for synthesis of bio-based plastics and other products derived from lactic acid from non-food sources of carbohydrates.