Product Selectivity Shifts in *Clostridium thermocellum* in the Presence of Compressed Solvents

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Organic solvents have been shown to affect the activity and selectivity of enzymes and whole cells in biphasic and nearly anhydrous systems. The choice of supercritical solvents and operating conditions may be used to vary the rates and selectivities of enzymes. Experiments were conducted to assess the impact of compressed nitrogen, ethane, and propane on the product selectivity of whole cell biocatalysts. The anaerobic thermophilic bacterium *Clostridium thermocellum*, which produces ethanol, acetate, and lactate, was used in the biphasic incubations. Compressed solvents lead to an increase in the ratio of ethanol to acetate produced by the organism. Furthermore, lactate formation was decreased in the presence of compressed and liquid solvents. The reduction in lactate formation is associated with a simultaneous reduction in the rate of cellobiose uptake by the microorganism. These results support the claim that incompatible solvents may affect the membrane and membrane transport systems of the microorganism. Finally, this work shows the potential for the manipulation of the product selectivity of whole cell biocatalysts by tuning solvent properties.

Introduction

Organic solvents are increasingly used in bioprocessing applications involving poorly water-soluble or toxic compounds. The benefits of in situ extraction using organic solvents include the possibility of diminishing substrate and product inhibition and of integrating bioconversion and product recovery in a single unit operation.[1] Supercritical fluids are of increasing interest as organic solvent replacements for bioprocessing applications because they are generally less toxic than traditional solvents and leave behind solvent-free product streams.[2] Numerous examples of enzyme processing using organic solvents or supercritical fluids exist.[3,4] However, bioconversions using whole cells lag the current technology in nonaqueous systems. Whole cells have significant advantages over purified enzymes for bioconversions, including their relative inexpensiveness and ability to perform complex, multienzymatic reactions.[1] The potential applications of whole cells in immobilized, anhydrous, and biphasic systems for bioconversions and biosensors have not been realized in supercritical fluids.

Previous attempts to conduct supercritical fluid extractive fermentation have been hindered by the toxicity of the commonly used extractive solvent, supercritical CO_2, to the bacteria and yeasts investigated.[5,6] We have recently demonstrated the ability of the anaerobic thermophilic bacteria *Clostridium thermocellum* to metabolize carbohydrate while incubated in contact with compressed or supercritical alkane solvents.[7,8] Fermentation by *C. thermocellum* and related bacteria is of particular interest because they convert complex carbohydrates, such as biomass, to ethanol.[9] However, many thermophilic organisms are inhibited by low levels of ethanol. Extractive fermentation using compressed solvents may be used to prevent this toxicity, increasing the rate of fermentation and the conversion of biomass.

Organic solvents have been shown to affect the activity and selectivity of enzymes and whole cells in biphasic and nearly anhydrous systems.[1,10] Choice of the supercritical fluid and operating pressure may be used to vary the enzyme rate and selectivity and to control product enantioselectivity in a single process fluid.[3,4,11] In the described work, experiments were conducted to assess the impact of several compressed and supercritical fluids on the metabolism of *C. thermocellum*, which ferments cellobiose to ethanol, acetate, and lactate (Figure 1). The major goal was to determine if the selectivity of whole cell product formation may be tuned by incubation in the presence of various supercritical and compressed solvents.

Experimental Methods

Materials. Nitrogen (ultrahigh purity) and ethane (chemically pure grade) were obtained from MG Industries (Malvern, PA). Propane (chemically pure grade) was obtained from Scott Specialty Gases (Plumsteadville, PA). Pentane, hexane, heptane, and decane were obtained from Fisher Scientific and were of 98% or greater purity. Dodecane (laboratory grade) was purchased from Fisher Scientific. All other reagents were obtained from commercial sources.

Cell Growth and Resting Cell Preparation. *C. thermocellum* ATCC 31549 was obtained from the American Type Culture Collection (Rockville, MD) and grown as previously described.[12] Nongrowing (resting) cell suspensions were prepared from growing cultures

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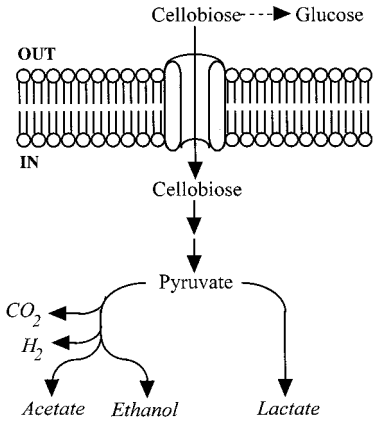

Figure 1. Simplified metabolic pathways of *C. thermocellum* illustrating major fermentation products. An enzymatic hydrolysis of cellobiose to glucose can occur outside the cell.

(1 L) that had been initially provided with 11.7 mM cellobiose. Cultures were harvested during the latter portion of exponential growth by centrifugation (10000*g*, 20 min, 25 °C), and the cell pellets were resuspended with media which did not contain yeast extract. Preliminary experiments indicated that the organism cannot grow in the medium when yeast extract is omitted but can ferment cellobiose (data not shown). The final cell concentration was 1.6 mg of protein (dry weight)/mL. Because *C. thermocellum* is sensitive to oxygen, cell manipulations were performed in sealed bottles that had been purged with nitrogen.

Pressurized Incubations. Hyperbaric fermentations were carried out in a 100 mL high-pressure reactor (Parr Minireactor), using a syringe pump (Isco model 500D) to control the system pressure. Prior to all pressurized experiments, the reactor was heated to 60 °C and the air was purged from the reactor at low pressure using the selected gas. Cellobiose (29 mM) was added to the resting cell preparation in a serum bottle, and a portion (75 mL) of this mixture was immediately loaded into the reactor using a syringe. The remaining cell mixture (approximately 75 mL) was incubated in a serum bottle under atmospheric nitrogen at 60 °C, and this served as a control. The reactor and the control bottle were both mixed at a constant rate for all experiments. The reactor was pressurized at a constant rate to the selected pressure over 30 min. The procedure for liquid sampling from the pressurized reactor consisted of removing 2 mL of liquid to account for the sample tube volume and removing an additional 2 mL of sample for further analysis. The piston pump was operated in constant-pressure mode to maintain pressure in the reactor. A concurrent sample was obtained from the control using a syringe. Samples from both the reactor and atmospheric control were immediately frozen (−4 °C). All experiments were run in duplicate.

Liquid Solvent Incubations. Incubations in the presence of liquid solvents were performed using 75 mL of resting cell preparations at atmospheric pressure. Prior to the incubations, the liquid solvents were thoroughly washed using deionized water to remove any water-soluble impurities and then sparged with oxygen-free nitrogen. Resting cells from identical preparations were simultaneously incubated under two conditions: a medium in the absence of liquid solvent (control experiment) and a medium in contact with 10 mL of excess liquid solvent (two-phase experiments). All experiments were run in duplicate and vigorously mixed to create a good dispersion. Cellobiose was added to each bottle so the final concentration in the aqueous phase was 29 mM. Samples were removed at regular intervals from all bottles using a syringe.

Analyses. Cellobiose, glucose, and lactate in cell-free culture supernatant were measured by an enzymatic method as previously described.[13] Ethanol and acetate concentrations were determined in acidified samples by gas chromatography using a column (1.83 m × 4 mm) packed with Supelco SP-1000 (1% H_3PO_4, 100−120 mesh). Nitrogen was used as a carrier gas, and the inlet and detector temperatures were 185 and 190 °C, respectively. The oven temperatures increased from 125 to 135 °C at a rate of 9 °C/min after an initial isothermic period of 0.5 min.

Data Analysis. Pressurized treatments were randomized and conducted sequentially in time. Microorganisms exhibit slightly different characteristics each time a new culture is grown up from the seed culture. Therefore, raw data include differences due to the bacterial culture and differences due to treatment effects. Because the study was only concerned with the impact of pressurized solvent compared to conventional fermentation, the pressurized treatment was normalized against the control treatment to remove the effects related to differences in the bacterial cultures.

The production of each of the products (ethanol, acetate, and lactate) was compared between the pressurized reactor and the control by normalization to the final concentration of the product in the control. The final concentration in the control was taken as 100%, and the progress made toward this production for the control was calculated as follows:

$$P_{Ct} = \left(\frac{[P]_{Ct} - [P]_{Ci}}{[P]_{Cf} - [P]_{Ci}}\right) \times 100 \qquad (1)$$

where P_{Ct} is the percent of product produced in the control at time t, t is the sampling time being evaluated, [P] is the product concentration, i is the initial time, f is the final time, and the C subscript stands for the control treatment.

The production of each product in a pressurized reactor was normalized against the final concentration in the control. The final concentration in the control was taken as 100%, and the progress made toward this production for the pressurized reactor was calculated as follows:

$$P_{Pt} = \left(\frac{[P]_{Pt} - [P]_{Pi}}{[P]_{Cf} - [P]_{Ci} + [P]_{Pi} - [P]_{Ci}}\right) \times 100 \qquad (2)$$

where the subscript P stands for the pressurized reactor and P_P is the percent of the product produced in pressurized treatment compared to the final control concentration at time t. The percent of product produced has been adjusted for differences in starting concentrations between the control and the pressurized reactor.

Prediction of log *P*. The *n*-octanol/water partition coefficient P of a substance is defined as the ratio of the molarity of the substance at infinite dilution in the two saturated phases. log P values are generally reported at 298 K and 0.1 MPa. The GCA-EOS (the group contribution associating equation of state)[14−16] was used to predict the *n*-octanol/water partition coefficient for compressed and supercritical solvents[8] at elevated pressures and temperatures. GCA-EOS has been used

extensively to describe the extraction of oxychemicals from aqueous solutions using supercritical and near-critical solvents.[14−16] Because GCA-EOS cannot accurately predict the densities of the water and octanol phases, it cannot reliably determine the molarity of the solvent in each phase. To avoid this problem, we define the P' based on mole fractions as follows:

$$\log P' = \log(X_i^{O}/X_i^{W}) \tag{3}$$

where X_i is the mole fraction of the solvent i in the octanol-rich (O) or water-rich (W) phase. Equation 3 can be related to the more traditional molarity-based log P at 298 K and 0.1 MPa by

$$\log P' = \log(M_i^{O}/M_i^{W}) + 0.82 \tag{4}$$

where M_i is the molarity of solvent i. log P values for the liquid hydrocarbon solvents were taken from Vermuë et al.[17] and converted to density-independent units using eq 4.

Results

We have previously shown[7] that the incubation of *C. thermocellum* at high pressures with nitrogen, a non-extractive compressed solvent, has little effect on the overall metabolic activity of resting cells. This work also demonstrated that carbon dioxide caused an almost complete inhibition of metabolism and so is not an appropriate extracting solvent for this system. Further work has demonstrated that metabolism of resting cells of *C. thermocellum* is possible in compressed ethane and propane.[7,8] Because the presence of organic and supercritical solvents has been shown to affect product selectivities and enantioselectivities in other systems,[1,10,11] the effect of compressed hydrocarbon solvents on the selectivity of product formation by *C. thermocellum* incubated in a biphasic system was investigated.

The presence of different compressed solvents had a variety of effects on the fermentation of cellobiose by *C. thermocellum*. Incubation with 7.0 MPa nitrogen had little effect on the production of ethanol relative to the nonpressurized control (Figure 2). However, incubation in the presence of ethane and propane significantly impacted the amount of ethanol produced, especially at times greater than 3 h. At 10 h, incubation with ethane produced 51% as much ethanol as was produced in the control and incubation with propane produced 45% of the ethanol in the control. All compressed solvents reduced the rate and extent of acetate production (Figure 3). Incubation with nitrogen, ethane, and propane produced 61%, 30%, and 11% of the acetate in the control, respectively, after 10 h. An increased selectivity of metabolism to ethanol relative to acetate production was also apparent in pressurized incubations (Figure 4). The ethanol-to-acetate ratio increased from 0.7 in the control to 1.1 while incubated in the presence of nitrogen, an unexpected result because nitrogen incubation is primarily an indicator of pressure effects. Incubation with compressed propane gave a substantial increase in selectivity of ethanol, resulting in an ethanol-to-acetate ratio of 3.2 at 10 h. Previous work has shown that the ratio of ethanol to acetate produced by the microorganism is influenced by hydrogen accumulation in the medium and subsequent effects on end-product pathways.[18] The effects of pressure and the compressed solvents may be due to a rerouting of

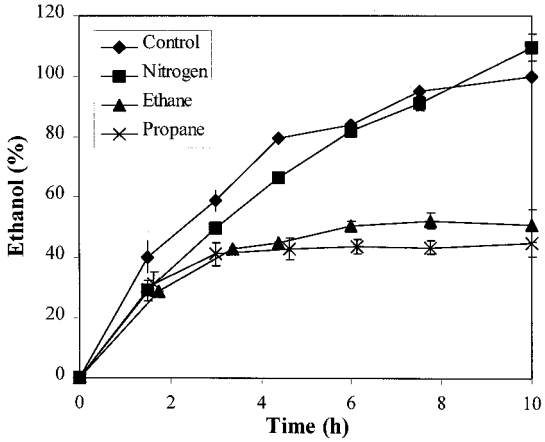

Figure 2. Effect of nitrogen, ethane, and propane at 7.0 MPa and 333 K on the production of ethanol by *C. thermocellum*. Error bars represent the standard error of duplicate incubations. Non-visible error bars are less than the size of their respective symbols. Ethanol (%) represents the percentage of ethanol formed in pressurized treatments as compared to the concentration in nonpressurized control bottles. Ethanol production in the control bottle at 10 h is defined as 100%. An average concentration of 9.9 mM ethanol was present in the control bottle after 10 h of incubation.

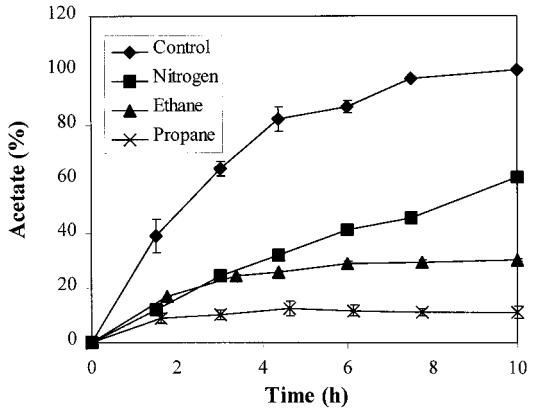

Figure 3. Effect of nitrogen, ethane, and propane at 7.0 MPa and 333 K on the production of acetate by *C. thermocellum*. Error bars represent the standard error of duplicate incubations. Non-visible error bars are less than the size of their respective symbols. Acetate (%) represents the percentage of acetate formed in pressurized treatments as compared to the concentration in nonpressurized control bottles. Acetate production in the control bottle at 10 h is defined as 100%. An average concentration of 15.8 mM of acetate was present in the control bottle after 10 h of incubation.

reducing equivalents, an as yet unexplained effect on hydrogen formation, or a decrease in the activity of a specific enzyme(s). Further work is needed to determine the exact nature of this phenomenon.

Incubation in the presence of compressed solvents also had a dramatic impact on lactate production by *C. thermocellum* (Figure 5). Lactate formation in the presence of nitrogen was nearly the same as that in control incubations, but ethane and propane caused a dramatic decrease in lactate production. After 10 h, incubation with compressed ethane produced 32% of the lactate in the control and compressed propane produced 7% of the lactate in the control. To more completely evaluate the effect of solvent chemical structure (or aliphatic chain length) on metabolism, lactate production was also measured in the presence of a range of liquid solvents (Figure 6). There was a direct positive relationship between the carbon chain length of the

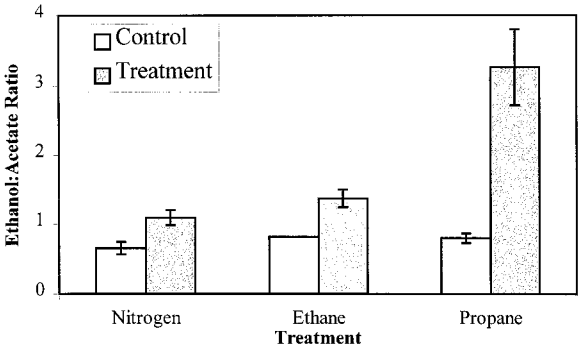

Figure 4. Effect of nitrogen, ethane, and propane at 7.0 MPa and 333 K on the ratio of ethanol to acetate produced by *C. thermocellum*. Error bars represent the standard error of duplicate incubations.

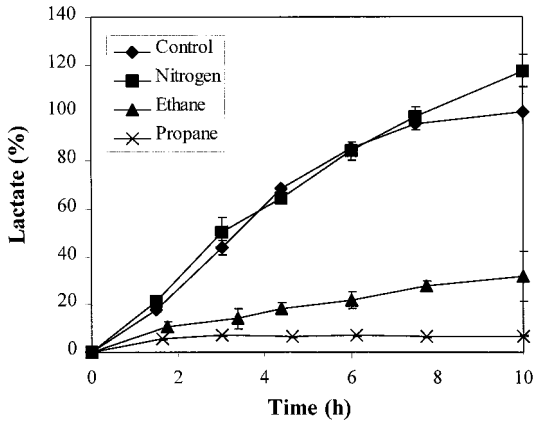

Figure 5. Effect of nitrogen, ethane, and propane at 7.0 MPa and 333 K on the production of lactate by *C. thermocellum*. Error bars represent the standard error of duplicate incubations. Nonvisible error bars are less than the size of their respective symbols. Lactate (%) represents the percentage of lactate produced in the pressurized treatment as compared to the concentration in nonpressurized control bottles. Lactate production in the control bottle at 10 h is defined as 100%. An average concentration of 18.7 mM of lactate was produced in the control bottle after 10 h of incubation.

hydrocarbon solvents in biphasic incubations and lactate formation. Incubation with pentane, hexane, and heptane produced less than 1% of the lactate in the control, while incubation with decane and dodecane produced 12% and 97% of the lactate in the control, respectively, after 24 h.

The activity of the extracellular hydrolase of *C. thermocellum*, which can convert cellobiose (a glucose dimer) to glucose, does not appear to be inhibited by solvents and pressure in the same manner as the metabolic pathways operating within the cells. Incubation with compressed solvents showed an increase in the amount of glucose accumulating in the fermentation media (Table 1). Very little glucose accumulated in the fermentation medium when *C. thermocellum* was in contact with nitrogen at 7.0 MPa (less than twice the glucose in the control); however, glucose accumulation was very high in the presence of ethane (13 times as much glucose as in the control) and propane (12 times as much as in the control) at 10 h.

Discussion

The incubation of *C. thermocellum* with liquid and compressed hydrocarbon solvents clearly causes significant shifts in metabolism. However, the exact mechanism of solvent effects on whole cells is poorly understood.[1] The toxicity of solvents dissolved in the aqueous phase has been attributed to solvent incorporation within membrane lipids, causing changes in membrane fluidity, disrupting membrane functions, and inactivating or denaturing membrane-associated proteins.[1,19] Such effects presumably negatively impact the ability to transport essential nutrients across the cell membrane. Furthermore, high solvent concentrations may lead to cell lysis.[1] These inhibitions are referred to as "molecular toxicity".[1,19,20] When excess solvent is present, allowing the formation of two distinct phases, phase toxicity can develop because of direct cell−solvent contact, the extraction of nutrients from the aqueous phase, extraction of essential cellular components, diffusional constraints due to the adherence of the cells to interfaces or entrapment in emulsion, or a combination of these effects.[19,20]

Selection of appropriate liquid and compressed extracting solvents for biocatalysis is constrained by solvent biocompatibility. Attempts have been made to develop solvent selection criteria based on physical properties such as Hildebrand solubility parameters, solvent density, and log *P*.[1] Generally, solvents with log *P* values of less than 2 (or log $P' < 3$) are not suitable for biocatalysis, while solvents with log *P* values of greater than 4 (or log $P' > 5$) are biocompatible.[21] We have previously predicted log P' values for these compressed solvents (1.9 for nitrogen, 2.4 for ethane, and 3.4 for propane) using GCA-EOS.[8] The log P' values

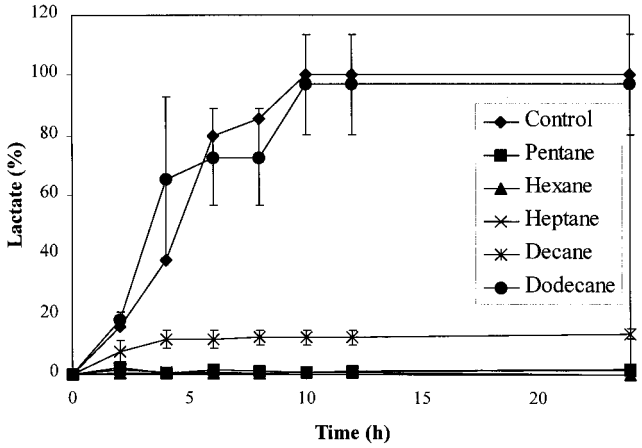

Figure 6. Effect of excess liquid solvent at 0.1 MPa and 333 K on the production of lactate by *C. thermocellum*. Error bars represent the standard error of duplicate incubations. Nonvisible error bars are less than the size of their respective symbols. Lactate (%) represents the percentage of ethanol produced in the control and percentage of lactate formed in the pressurized treatment as compared to the concentration in nonpressurized control bottles. Ethanol production in the control bottle at 24 h is defined as 100%. Incubation with pentane, hexane, and heptane produced less than 1% of the lactate in the control.

Table 1. Effect of Compressed Solvents at 7.0 MPa and 333 K on Glucose Accumulation in the Fermentation Media[a]

	control (mM)	treatment (mM)
nitrogen	1.31 (0.14)	1.97 (0.08)
ethane	0.94 (0.18)	12.20 (1.01)
propane	1.16 (0.27)	14.27 (0.03)

[a] Control incubations were performed at 0.1 MPa, and treatment incubations were performed at 7.0 MPa. Values represent glucose accumulated in a medium after 10 h of incubation. Values in parentheses represent the standard error of duplicate incubations.

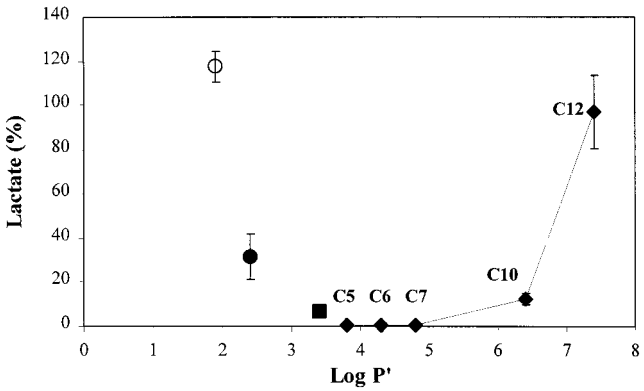

Figure 7. Relationship between the percentage of lactate produced (compared to a control) by *C. thermocellum* and the log *P′* of the compressed and liquid solvents used in this study. The log *P′* of the compressed solvents was calculated using GCA-EOS. (○) Nitrogen, 7.0 MPa. (●) Ethane, 7.0 MPa. (■) Propane, 7.0 MPa. (◆) Atmospheric liquid solvents in increasing order of log *P′* are pentane (C5), hexane (C6), heptane (C7), decane (C10), and dodecane (C12). Error bars represent the standard error of duplicate incubations. Nonvisible error bars are less than the size of their respective symbols.

correlated well with the total metabolic activity (the ratio of the total products formed in the treatment to the total products formed in the control) in liquid hydrocarbon solvents; however, log *P′* failed to describe the increased biocompatibility seen with compressed hydrocarbon solvents.[8] The correlation of lactate formation by *C. thermocellum* with log *P′* (Figure 7) follows trends similar to those seen for the total activity with compressed and liquid hydrocarbon solvents.[8] This indicates that the formation of lactate is a good indicator of solvent biocompatibility.

The positive relationship between lactate formation and solvent biocompatibility might be explained by a change in cellobiose flux through the metabolic pathways. It has recently been demonstrated in our laboratory (data not shown) and by other groups[22] that lactate formation in clostridial bacteria is triggered when cellobiose consumption rates are high. Hence, more biocompatible solvents which permit high rates of cellobiose consumption lead to increased rates of lactate formation.

A hypothesized decrease in the cellobiose uptake caused by less biocompatible solvents is further supported by the buildup of glucose outside the cell with solvents such as ethane and propane (Table 1). If the cellobiose uptake rate is low (as seen with less biocompatible solvents), large amounts of glucose will accumulate outside the cell because of extracellular hydrolase activity. On the basis of this result, it appears that, unlike the cellular metabolic steps, this extracellular enzymatic activity is relatively unaffected by the compressed solvents. This hydrolase activity is apparently very robust given the fact that glucose accumulation occurs even when incubated with supercritical carbon dioxide (data not shown).

The increased rate of cellobiose conversion to fermentation products by cells with increasing solvent biocompatibility indicates that solvents affect activity via membrane inhibitions. For the less biocompatible solvents (ethane and propane), there was a decreased rate of cellobiose uptake by the microorganism and hence less lactate was formed (less than 40% when compared to the control). The decreased rate of consumption is consistent with the partial or total inactivation of the membrane transport proteins. Alternatively, more biocompatible solvents (nitrogen and dodecane) have higher rates of cellobiose uptake and thus form more lactate (more than 80% when compared to the control).

Numerous research groups have attributed the toxicity to whole cells by organic media to the inactivation of the membrane and the membrane transport protein function.[1,19,23] Our work shows that incubation of whole cells in a biphasic, compressed solvent system may also lead to disruption of the membrane transport function. Furthermore, the presence of compressed solvents does not inhibit all cellular function. Thus, the potential exists to manipulate cellular metabolic activity and product selectivity by careful selection of solvent properties.

Conclusions

The application of supercritical fluids to bioprocessing with whole cells currently lags the technology in liquid organic solvents. This work shows potential for the use of compressed solvents for extractive bioconversions using whole cells. The presence of compressed solvents offers the opportunity to tune product selectivity with solvent choice by causing significant shifts in the fermentation pathways of *C. thermocellum*. Compressed solvents lead to an increase in the ratio of ethanol to acetate produced by the organism, which may be due to a possible rerouting of metabolic reducing equivalents. Furthermore, lactate formation was decreased in the presence of compressed and liquid solvents, and this is apparently related to a reduction in the rate of cellobiose uptake. These results are consistent with the claim that incompatible solvents may affect the membrane and membrane transport systems of the microorganism. In addition to the commercial significance of combining supercritical fluid technology and whole cell synthesis, the use of compressed solvents affords a unique opportunity to examine metabolic processes in a single pressure-tunable solvent.

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