Nutritional Requirements of Solventogenic *Clostridium beijerinckii* SA-1 Growing Under Butanol Stress and Co-utilization of D-glucose/D-xylose

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Abstract

Recent advances in systems biology, omics, and computational studies allowed us to carry out data mining for improving biofuel production bioprocesses. Our interests address the need to find the requirements of microbial capabilities to biotransform both the hexose and the pentose fractions present in crop residues for the production of biobutanol. Using a meta-analysis approach of the transcriptional responses to butanol stress, we identified nutritional requirements of solvent tolerant strain *Clostridia beijerinckii* SA-1 (ATCC 35702). A systematic exploration and validation of media components lead to higher density cultures and more productive fermentation operations than are currently found.

Introduction

The well-known ability of solventogenic clostridia to utilize a large variety of carbon sources such as mono, oligo and polysaccharides containing pentose and hexose sugars present in plant biomass makes them ideal for biobutanol production. However, traditional fermentation processes and genetic modification of strains have been slow in overcoming the hurdles associated with operating continuous butanol production processes. For example, the final accumulation of solvent is limited by strain sporulation which is triggered by solvent toxicity (1) yielding the traditional transient production processes. New media formulations are essential for production and can make a considerable impact on selected or genetically engineered strains with desirable traits. However, optimization of media formulations for clostridia fermentation has been hindered by the inherent instability of butanol producing strains. For this reason, the solvent tolerant strain *Clostridia beijerinckii* SA-1 (ATCC 35702) (5) was re-evaluated and key ingredients that are often overlooked were identified using a meta-analysis approach.

Methods

Figure 1 Each strain was grown separately in serial individual flasks each containing increasing concentrations of butanol.
Figure 2 We analyzed publically available microarray data obtained from stress experiments of samples exposed to 50 mM butanol (3). The data were downloaded from the GEO database (1). The data was log_2 transformed and imported into JMP Genomics (SAS, Cary, NC). Afterwards, loess normalization was applied to preprocessed data. Following normalization, gene-specific effects were modeled in terms of the residuals. Two independent model-based approaches were applied to the normalized data: a **mixed model analysis** (2) and a **step-down quadratic regression model for pattern recognition** (3). The outcome of responsive genes from our analysis was then grouped into functional categories (Clusters of Orthologous Groups, For statistical significance the P-value ≤ 0.05 were considered significant http://www.ncbi.nlm.nih.gov/COG/grace/uni.html).
Figure 3 To assure the proliferation of *C. beijerinckii* and avoid nutrient limitation as a consequence of butanol and butyric acid toxicity, nutrients were independently added to the reactor (pulse) and when the observed response was an increase in biomass after pulsing a particular compound into the reactor, that compound, ("the limiting factor") was subsequently incorporated to the culture medium reservoir, which is referred to as the "shift". The pulse location for each component is indicated on the graph (↓). Compounds causing decrease or no change in biomass were not included in the culture medium. Final concentrations of each component incorporated in the working volume are shown in Table 1. Pulse shift experiments were conducted in the presence of excess sugars, D-glucose (30 g/L) and D-xylose (15 g/L).
Table 2 Two fermenters were connected in series and the harvest of the first fermenter became the feed stream to the second fermenter. Both fermenters were inoculated simultaneously and cultivated in batch conditions at 37°C with a pH of 6.5. The carbon sources were increased to 40 g/L for glucose and 20 g/L for D-xylose. When the cells in the first fermenter reached the log phase of growth, the medium feed was initiated. The volume in the first fermenter was maintained at 700 mL, while the volume of the second fermenter was modified according to the desired dilution rate. Three samples were taken once the steady state of the reactor was established with at least three retention times between each of the samples. Concurrently, the cell stability based on of the percentage of sporulation in the culture for both fermenters was monitored daily to confirm stationary consistency at the respective steady states.

Objectives

1. **Use meta-analysis to define possible nutritional limitations for *C. beijerinckii* SA-1 (ATCC 35702) by examining published transcriptome data of *C. acetobutylicum* growing under butanol stress.**
2. **Validate the nutritional component limitations for *C. beijerinckii* SA-1 (ATCC 35702) using pulse-shift technique to formulate a new medium.**
3. **Characterize the responses of *C. beijerinckii* SA-1 (ATCC 35702) in the new medium to environmental fluctuations in pH, temperature, growth, solvent production rates and glucose/xylose consumption.**

Results

Comparative analysis of the specific growth rates of *C. beijerinckii* strains (NRRL B-527 (wild-type) and SA-1/ATCC 35702) growing in the presence of increasing butanol concentrations.

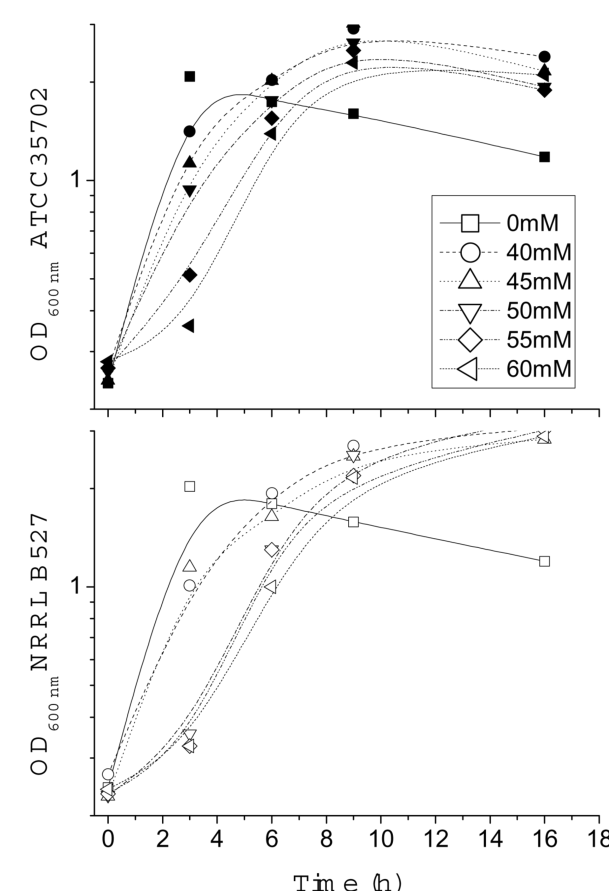

Figure 1. Batch growth kinetics of *C. beijerinckii* NRRL B-527 (wild-type) and SA-1/ATCC 35702 strains grown at 37°C in RCM medium containing increasing concentrations of butanol (0, 40, 45, 50, 55 and 60 mM). *C. beijerinckii* (SA-1/ATCC 35702) is represented by solid symbols connected with solid lines, while the *C. beijerinckii* (NRRL B-527) is represented by open symbols connected with solid lines.

❖The butanol tolerant strain SA-1/ATCC 35702 maintained higher specific growth rates compared to the growth rate of NRRL B-527 in the presence of increasing butanol concentrations.
Higher resistance to butanol toxicity should theoretically allow the strain to produce greater quantities of butanol.

Identification of possible nutrient limitations performing meta-analysis on transcriptome data from *C. acetobutylicum* ATCC 824 response to butanol stress.

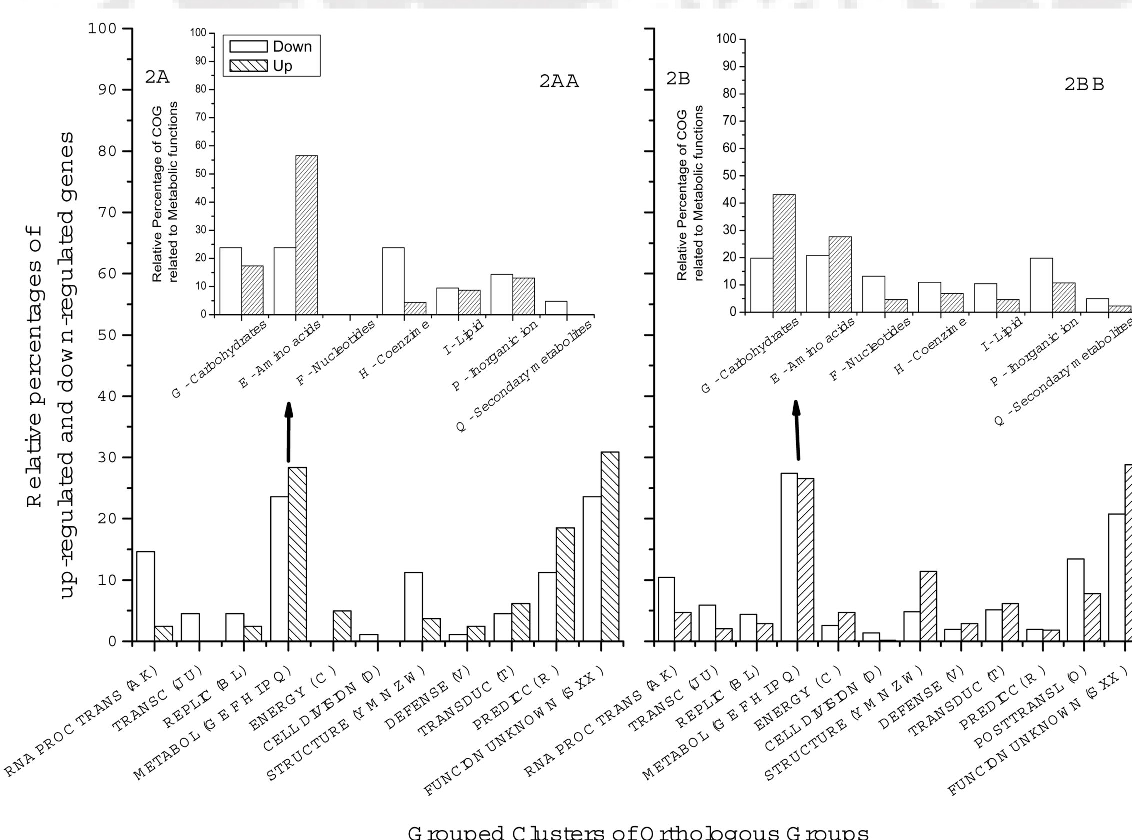

Figure 2. Results of the analysis and grouping of the responsive genes when cells from *C. acetobutylicum* ATCC 824 were exposed to 50 mM butanol compared to non-exposed controls. The analysis includes short time-course microarray experiments of culture samples exposed to or in the absence of butanol for 10, 30, 45, 60, 120, 360, 720, and 1440 min. Figure 2A shows frequencies of genes demonstrating a kinetic linear pattern of up or down-regulation when compared to genes showing a flat pattern or no regulation. Figure 2B shows the frequencies of significantly (P=0.05) up- and down-regulated genes exposed to 50 mM butanol compared to non-exposed controls [e.g. differential of Time*Treatment = (360 min Butanol)-(360 min Control)].

Validation of the consolidated medium by the chemostat pulse and shift technique.

Compound	Concentration
KH_2PO_4	3 g/L
NH_4Cl	3 g/L
$(NH_4)SO_4$	0.5 g/L
NaCl	0.01 g/L
EDTA	0.1 m/M
Yeast Extract	2 g/L
Tween 80	1 ml/L
Trace compounds prepared by filtration and aseptically added to the medium.	
$MgSO_4 \cdot 7 H_2O$	0.1 g/L
$CaCl_2$	22 mg/L
$MnCl_2 \cdot 4 H_2O$	20 mg/L
$FeSO_4 \cdot 2 H_2O$	20 mg/L
$CuSO_4 \cdot 5 H_2O$	6.4 mg/L
$CoCl_2 \cdot 6 H_2O$	6.4 mg/L
$ZnSO_4$	8.8 mg/L
$(NH_4)_6Mo_7O_{24} \cdot 4 H_2O$	4.4 mg/L
Biotin	1 mg/L
p-amino benzoic acid	2 mg/L
Thiamine – HCl	2 mg/L
Cysteine	0.1 g/L
Asparagine	0.1 g/L
Tryptophan	0.1 g/L
Histidine	0.1 g/L
Glutamine	0.1 g/L
Riboflavin	0.001 g/L
Vitamin B_{12}	0.001 g/L

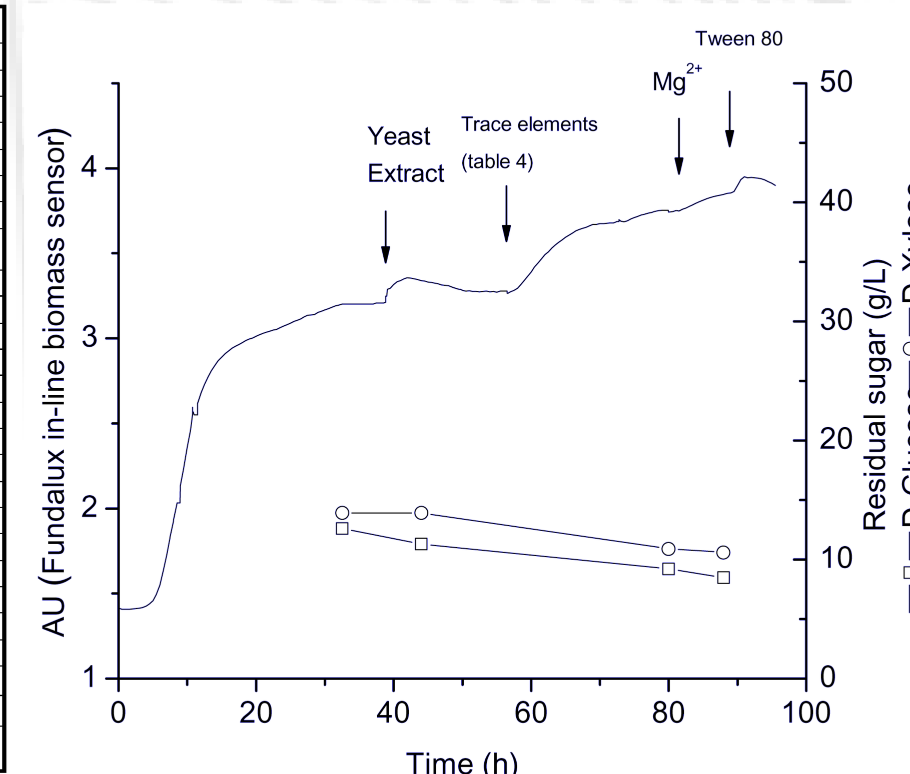

Table 1. Composition of the validated medium formulated in this study to cultivate *C. beijerinkii* SA-1. Validated medium composition was obtained by applying the pulse and shift optimization technique (Figure 3). Carbon source(s) are not included in the formula.

Figure 3. To assure the proliferation of *C. beijerinckii* and avoid nutrient limitation as a consequence of butanol and butyric acid toxicity, nutrients were independently added to the reactor (pulse) and when the observed response was an increase in biomass after pulsing a particular compound into the reactor, that compound, ("the limiting factor") was subsequently incorporated to the culture medium reservoir, which is referred to as the "shift". The pulse location for each component is indicated on the graph (↓). Compounds causing decrease or no change in biomass were not included in the culture medium. Final concentrations of each component incorporated in the working volume are shown in Table 1. Pulse shift experiments were conducted in the presence of excess sugars, D-glucose (30 g/L) and D-xylose (15 g/L).

Evaluation of SA-1 performance during the solventogenic phase at different physiological states employing a two-stage continuous process.

Table 2. *C. beijerinckii* SA-1/ATCC 35702 cultivated in a two-stage, one feed stream chemostat culture containing D-glucose (40 g/L) and D-xylose (20 g/L) in the newly validated medium at 37°C and pH 6.5. The dilution rate (D) and pH were evaluated in the second reactor.

Reactor Number	Overall D (h^{-1})	pH	Acetate	Butyrate	Acetone	Butanol	D-glucose	D-xylose	Butanol Yield	Vegetative cells	Spores
			Fermentation Products (g/L)				Consumed Sugar (%)		(mol/mol substrate)	CFU/mL	Spores/mL
First Reactor	0.102	6.5	3.3 ± 0.6	6.3 ± 0.5	0.2 ± 0.1	0.5 ± 0.3	69.2	31.0			
	0.04	6.5	1.6 ± 0.8	6.4 ± 3.0	0.2 ± 0.1	0.4 ± 0.2	93.3	59.1	0.018	7.3 E7	2.3 E8
	0.05	6.5	1.2 ± 1.0	4.0 ± 0.8	0.5 ± 0.2	1.8 ± 0.8	96.9	67.6	0.078	1.4 E8	1.5 E8
Second Reactor	0.06	6.5	2.0 ± 1.0	1.6 ± 1.0	5.3 ± 0.6	7.2 ± 0.2	83.2	52.9	0.380	1.8 E8	1.3 E8
	0.04	Free	2.7 ± 0.2	3.2 ± 1.2	1.1 ± 0.1	3.7 ± 0.4	95.7	47.5	0.183		
	0.05	Free	2.0 ± 0.4	3.5 ± 0.2	0.5 ± 0.1	2.0 ± 0.1	96.6	32.7	0.105		
	0.06	Free	2.0 ± 0.1	3.2 ± 0.9	0.4 ± 0.1	2.1 ± 0.5	93.5	57.6	0.100		

❖The core of our work addressed the biphasic nature of butanol production by *C. beijerinkii* SA-1 by designing our process in two stages: The first stage evaluated the process parameters and variables that influence product formation, cell density, and culture stability. The first stage was employed to determine the conditions that would support the highest possible butyric acid concentration, which would subsequently trigger and allow for solventogenesis. In the second stage, the goal was to obtain the highest butanol concentration possible.

The butanol yield obtained for *C. beijerinckii* SA-1/ATCC 35702 varied from 0.018 to 0.38 mol (butanol produced per mol of substrate consumed) and was dependent on the working conditions of the second reactor.
The highest butanol concentration was obtained when the dilution rate of the second reactor was 0.06 h^{-1} and had a pH of 6.5.
The optimal pH results confirmed the sensitivity of *C. beijerinckii* SA-1/ATCC 35702 to butyric acid.

Resolving the optimum environmental conditions of reactor operation improved our understanding of organic acid metabolism for the initiation of solvent production by *C. beijerinckii* SA-1/ATCC 35702.

Conclusions

•The results obtained after examining the batch growth kinetics of the wild-type and SA-1 strains in the presence of butanol or butyric acid (data not shown) emphasizes the complexity of solvent and acid toxicity in *Clostridia*, reinforcing the need for a flexible genomic approach for discovering genes involved in solvent tolerance.

•The nutritional demands of the strain *C. beijerinckii* SA-1/ATCC 35702 under stress were met by including optimal concentrations of metals and/or vitamins in the medium.

•The two stage fermentation process makes it possible to continually co-fermenting a mixture of D-glucose and D-xylose and generate butanol at a concentration of 7.2 g/L comparable to previous studies (6).

•Surprisingly, we identified an increased sensitivity to butyric acid by SA-1/ATCC 35702. This discovery was unexpected since cells require acid accumulation in the fermentation broth as a pre-requisite for triggering butanol formation and sporulation (data not shown).

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