

Butanol Tolerance in a Selection of Microorganisms

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Abstract Butanol tolerance is a critical factor affecting the ability of microorganisms to generate economically viable quantities of butanol. Current *Clostridium* strains are unable to tolerate greater than 2% 1-butanol thus membrane or gas stripping technologies to actively remove butanol during fermentation are advantageous. To evaluate the potential of alternative hosts for butanol production, we screened 24 different microorganisms for their tolerance to butanol. We found that in general, a barrier to growth exists between 1% and 2% butanol and few microorganisms can tolerate 2% butanol. Strains of *Escherichia coli*, *Zymomonas mobilis*, and non-*Saccharomyces* yeasts were unable to surmount the 2% butanol growth barrier. Several strains of *Saccharomyces cerevisiae* exhibit limited growth in 2% butanol, while two strains of *Lactobacillus* were able to tolerate and grow in up to 3% butanol.

Keywords Butanol · Tolerance · BioScreenC

Introduction

As an alternative liquid fuel, butanol offers distinct advantages because of its high energy content, miscibility with gasoline, octane rating, and low volatility [1]. With the increasing price of oil, there is renewed interest in producing butanol biologically [2, 3]. Butanol can be produced from anaerobic bacterial (*Clostridia*) fermentations in a process that also produces acetone and ethanol (“ABE” fermentation). These fermentations suffer from low yield, low productivity, and low titer. In batch fermentations, *Clostridia* are quite sensitive to butanol and are typically unable to produce [4, 5] or tolerate concentrations greater than 2% [6–8]. A couple of attempts have been made using mutagenesis or serial enrichment to increase butanol tolerance in *Clostridium acetobutylicum* ATCC824. In these experiments,

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an increase from tolerance to 0.5% butanol to 1.5% butanol was observed [6, 9, 10]. However, in one case, this mutagenic event resulting in increased tolerance did not also result in a higher butanol yield [6]. A compounding factor in *Clostridia* fermentation is the complex regulatory pathways involved in switching from acidogenesis to solventogenesis [11–14]. Thus, it has been difficult to make significant progress in engineering highly productive strains for ABE fermentations [15]. Many attempts have been made to develop economically viable methods of concurrent butanol removal and all have their disadvantages [15]. An alternative strategy would be to establish a butanol production pathway in an alternative host lacking these complex regulatory pathways. Recently, a non-fermentative pathway for production of butanol was described [16, 17]. An important consideration in selecting a host for butanol production is butanol tolerance. Reports of butanol tolerance in organisms other than *Clostridia* are few. In one report, *Lactobacillus hilgardii* was reported to be more tolerant to an ethanol challenge if pre-stressed by exposure to various solvents including butanol [18]. Two additional reports show that *Saccharomyces* and other yeast species can tolerate butanol at 1% up to near 2% [19, 20].

To evaluate the potential of alternative hosts for butanol production, we conducted a screening of a variety of microorganisms which are amenable to genetic engineering, are tolerant to hydrolysate inhibitors or ethanol, or are capable of fermentation at higher temperatures. The results of this initial screening will be useful for considering which organisms to engineer for butanol production.

Materials and Methods

Media and Strains

Strains used are listed in Table 1. Stock cultures of *Lactobacillus* were grown in MRS medium (Difco, 0881-17-5) at 30 or 37 °C without shaking. Stock cultures of *Escherichia coli* were grown in LB medium (Sigma, L-3022) at 30 or 37 °C with shaking at 220 rpm. Yeast stock cultures were grown in yeast-peptone-dextrose (YPD) medium (Sigma, Y-1375) at 30 °C with shaking at 220 rpm. Stock cultures of *Zymomonas mobilis* were grown in RMG medium, consisting of 10 g/L yeast extract, 2 g/L KH_2PO_4 , and 20 g/L glucose, at 30 °C with shaking at 100 rpm. Strains were acquired from the American Type Culture Collection (ATCC), the National Center for Agricultural Utilization Research (NRRL), and the Centraalbureau voor Schimmelcultures (CBS).

Growth Curves

Growth was monitored using the BioScreenC (GrowthCurvesUSA, NJ, USA). Strains were grown overnight and then inoculated into fresh media and incubated until early log phase with an optical density (OD_{600}) of 0.1–0.3 optical density units (ODU)/ml. The cells were harvested, washed with sterile H_2O , and inoculated into media containing various concentrations (v/v) of 1-butanol. YPD medium was used at 1/2 strength due its high background OD. Quadruplicates of each condition were aliquoted into wells in the honeycomb BioScreenC plate and wide-band OD (420–580 nm) was recorded. Growth temperatures were the same as those used to grow the cultures, 30 or 37 °C as appropriate. Shaking of the BioscreenC plate was performed for 5 s prior to each reading. Growth rates were calculated from the linear range of exponential growth. This typically occurred at an OD between 0.08 and 0.3 but varied for different species.

Results and Discussion

Tolerance to Butanol

Various strains were chosen to test the effect of 1-butanol on growth (Table 1). The growth rate was calculated from growth curves monitored using the BioScreenC. The results showed that 1-butanol is toxic at low levels to most of the selected microorganisms. With a few exceptions, in the presence of 1% butanol, the relative growth rates were about 60% as that in medium without butanol. Very few microorganisms, however, can tolerate and grow in 2% or higher butanol.

Of the non-*Saccharomyces* yeast species, only one, *Candida sonorensis*, was able to tolerate 2% butanol. However, *C. sonorensis* grew slowly and the initial OD did not double (Fig. 1). Both strains of *P. methanolica* were extremely sensitive to butanol and were not able to grow in 1% butanol. *Pachysolen tannophilus* and *P. guilliermondii* were also sensitive to 1% butanol and reached only 40% of their relative growth rates (Fig. 1). Strains of *Saccharomyces cerevisiae*, in general, were not able to tolerate and grow in 2% butanol and the haploid strain GY5196 could not tolerate 1% butanol (Fig. 2). Only three out of the ten strains grew in 2% butanol. The *S. cerevisiae* strains ATCC26602, ATCC20252, and Fali grew but were severely inhibited by 2% butanol reaching growth rates 10–20% of

Table 1 Strains screened for 1-butanol tolerance.

Genus	Species	Strain ^a	Reference ^b
<i>Candida</i>	<i>acidothermophilum</i>	ATCC20381	[35]
<i>Candida</i>	<i>sonorensis</i>	CBS6793	[36]
<i>Escherichia</i>	<i>coli</i>	DH5 α	
<i>Escherichia</i>	<i>coli</i>	K12	
<i>Escherichia</i>	<i>coli</i>	W3110	
<i>Lactobacillus</i>	<i>brevis</i>		
<i>Lactobacillus</i>	<i>delbrueckii</i>		
<i>Lactobacillus</i>		MONT4	[21]
<i>Pachysolen</i>	<i>tannophilus</i>	ATCC32691	[37]
<i>Pichia</i>	<i>guilliermondii</i>	NRRL Y-2075	[36]
<i>Pichia</i>	<i>methanolica</i>	CBS6515	[36]
<i>Pichia</i>	<i>methanolica</i>	ATCC46071	[36]
<i>Saccharomyces</i>	<i>cerevisiae</i>	D5A	[38]
<i>Saccharomyces</i>	<i>cerevisiae</i>	ATCC20252	[39]
<i>Saccharomyces</i>	<i>cerevisiae</i>	ATCC9763	[40]
<i>Saccharomyces</i>	<i>cerevisiae</i>	NRRL Y-1539	[41]
<i>Saccharomyces</i>	<i>cerevisiae</i>	JCM2216	[41]
<i>Saccharomyces</i>	<i>cerevisiae</i>	ATCC26602	[42]
<i>Saccharomyces</i>	<i>cerevisiae</i>	ATCC24859	[43]
<i>Saccharomyces</i>	<i>cerevisiae</i>	GY5196	
<i>Saccharomyces</i>	<i>cerevisiae</i>	ATCC4126	[44]
<i>Saccharomyces</i>	<i>cerevisiae</i>	Fali	Broin Inc.
<i>Zymomonas</i>	<i>mobilis</i>	ATCC31821	[45]
<i>Zymomonas</i>	<i>mobilis</i>	8b	[46]

^a Strain is only designated if known

^b Reference is only given if species or strain has been previously referenced with regards to relevant biomass to biofuels work. Unreferenced species and strains were already present in our laboratory from unknown previous sources

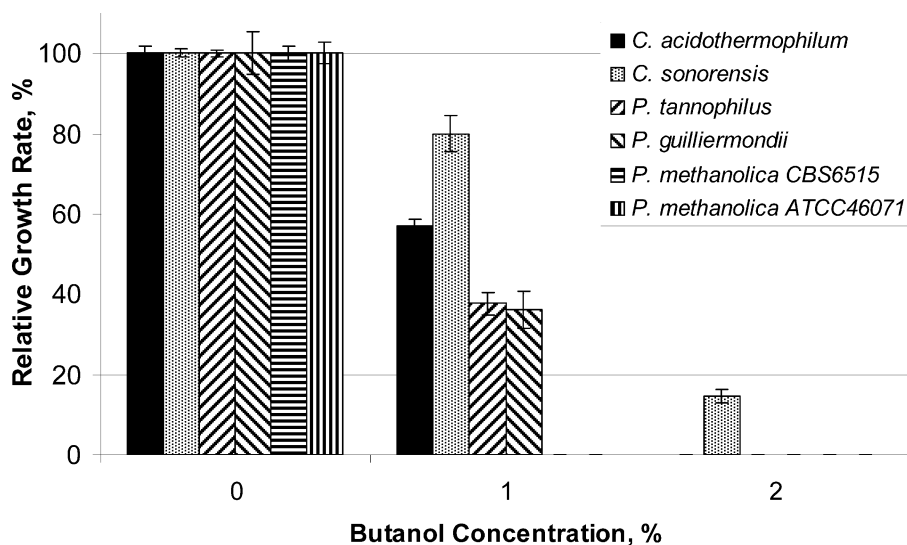


Fig. 1 Growth rates in butanol relative to medium without butanol of non-*Saccharomyces* yeast species

those in medium without butanol (Fig. 2). These strains reached cell densities of 2-, 3-, and 6-fold, respectively, of initial cell density in 24 h.

Yeast strains used for this study are able to ferment at increased temperatures and/or were tolerant to ethanol. *Candida acidotherophilum* is tolerant to a high concentration of ethanol (14%) at high temperature (40 °C). *C. sonorensis*, *P. tannophilus*, and *P. guilliermondii* have a fast growth rate based on our previous unpublished work and are tolerant to 40–42 °C. Strains of *P. methanolica* are tolerant to methanol and ethanol. The yeast strain *S. cerevisiae* D5A was tolerant to degradation products present in pre-treated hardwoods yet failed to grow in 2% butanol. The *S. cerevisiae* strain ATCC20252 was tolerant to 40 °C and 9.5% ethanol, whereas ATCC26602 was tolerant to 43 °C and 11%

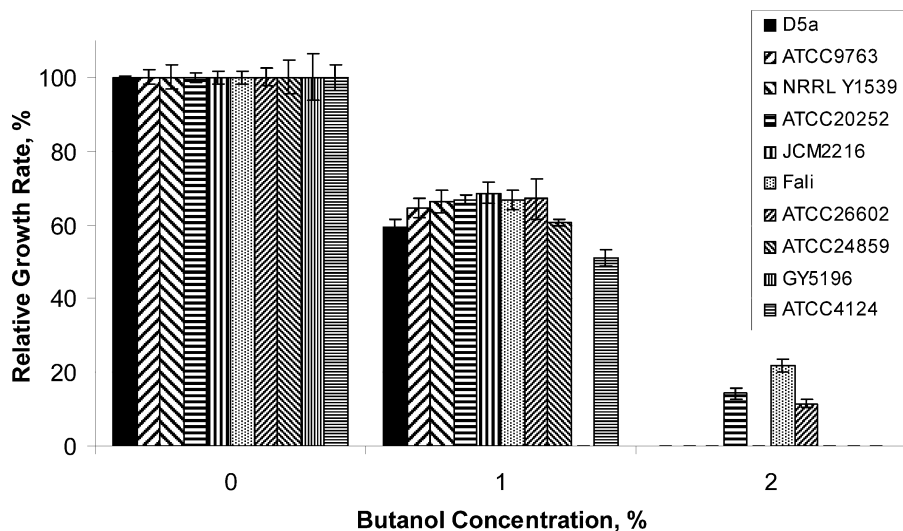


Fig. 2 Growth rates in butanol relative to medium without butanol of *Saccharomyces cerevisiae* strains

ethanol. Both strains grew somewhat in 2% butanol. Strain ATCC9763 was tolerant to 14% ethanol at 30 °C and 12% ethanol at 35 °C, and strain ATCC4126 was found to ferment optimally at 36 °C. The Fali yeast strain was used for commercial ethanol production. No correlation can be drawn about whether tolerance to hydrolysates, high temperature, or high ethanol concentrations in yeast provides any benefit to butanol tolerance.

Strains of *Lactobacillus* were more tolerant of butanol than yeast. Two strains, *L. delbrueckii* and *Lactobacillus brevis*, were able to grow in 2% butanol with relative growth rates of 55% and 58%, respectively (Fig. 3). These two strains were also able to grow in 2.5% butanol with relative growth rates of 30% and 44%, respectively (Fig. 3). *L. brevis* was able to grow in 3% butanol with a 30% growth rate relative to the no butanol control (Fig. 3). In 2% butanol, *L. delbrueckii* reached a cell density 90% of that of the cell density in the control culture grown without butanol and reached a cell density 10-fold of the initial cell density in 2.5% butanol in 24 h. Additionally, *L. delbrueckii* was grown at an increased temperature of 37 °C. Similarly, in 2% butanol, *L. brevis* reached a cell density 80% of that of the cell density in the control culture grown without butanol and reached a cell density 4-fold of the initial cell density in both 2.5% and 3% butanol in 24 h. The *Lactobacillus* strain MONT4 did not grow in 2% butanol.

The strains of *Lactobacillus* used were tolerant to the inhibitors present in hydrolysates [21] and are known to be acid- and bile-tolerant. Several reports in the literature show, however, that although tolerant to acid and bile, *Lactobacillus* strains are not tolerant to more than 1% (v/v) butanol [22–24].

Z. mobilis strains were sensitive to 2% butanol. *Z. mobilis* ATCC31821 grew slowly and the initial OD did not double (Fig. 4). The *Z. mobilis* 8b strain is relatively tolerant to acetic acid present in hydrolysates, while the parental ATCC31821 strain is tolerant to 13% ethanol.

Effects of Temperature

We investigated the effect of temperature on butanol tolerance in *E. coli*. The cultures grew faster at 37 °C; however, they were not as tolerant to 1% butanol as when grown at 30 °C.

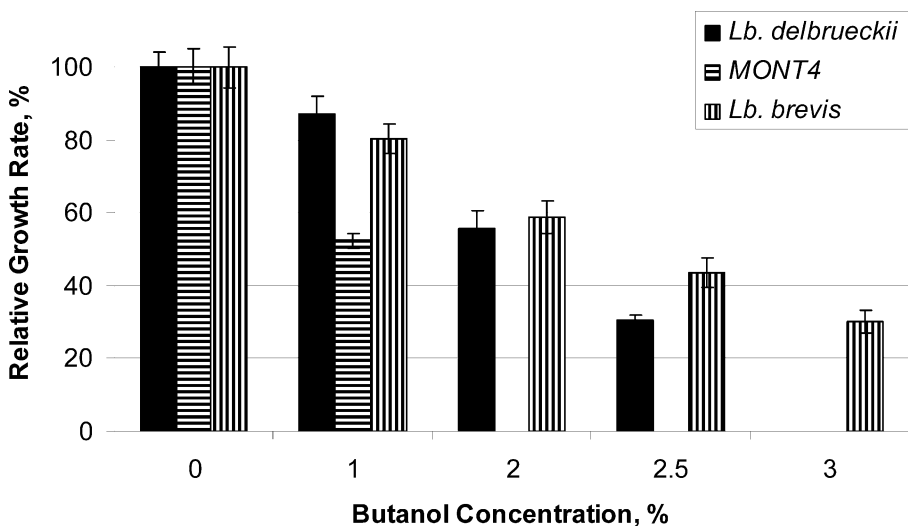


Fig. 3 Growth rates in butanol relative to medium without butanol of *Lactobacillus* strains

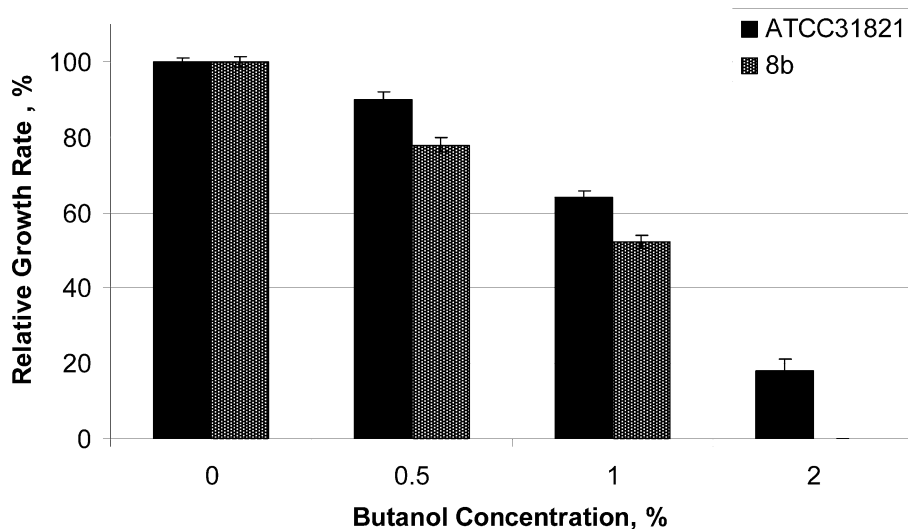


Fig. 4 Growth rates in butanol relative to medium without butanol of *Zymomonas mobilis* strains

Although a lower temperature improved growth in the presence of 1% butanol, it did not allow *E. coli* to grow at the higher concentration of 2% butanol (Fig. 5). A similar effect was observed for *C. acetobutylicum* ATCC824. The absolute growth rate was slower at the lower temperatures and growth was observed in 1.5% butanol, while at the higher temperature, the absolute growth rate was faster but the strain was not able to tolerate 1.5% butanol [6]. *E. coli* was included in the screening based on their increasingly being targeted for development as a butanologen [16, 17].

The results of current research show that microorganisms can be naturally tolerant to relatively high concentrations of butanol. These results suggest that tolerance to inhibitors

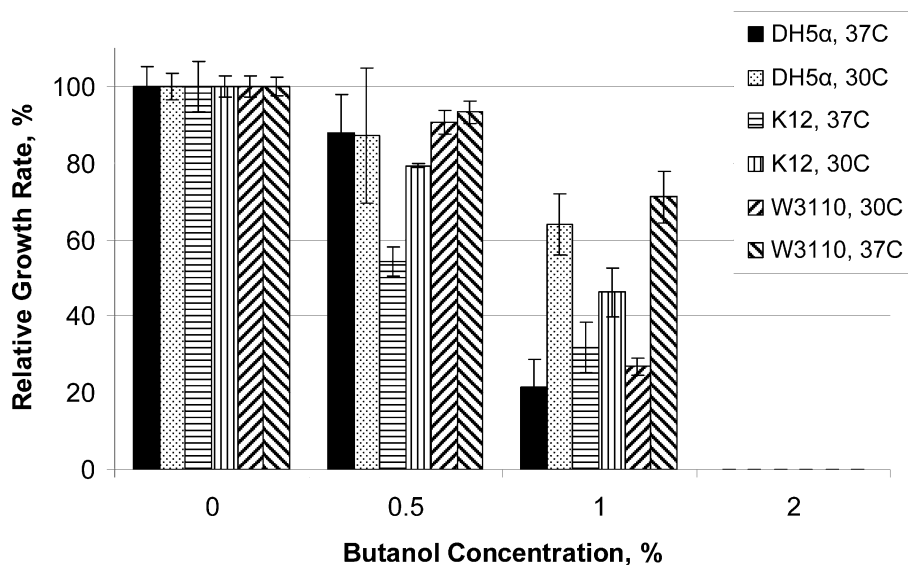


Fig. 5 Growth rates in butanol relative to medium without butanol for *Escherichia coli* strains at 30 and 37 °C

present in hydrolysates, high temperatures, or high ethanol concentrations may not be indicative of a strain's ability to tolerate butanol. It has been demonstrated, however, that solvent tolerance leads to tolerance of heavy metals and antibiotics [25].

Butanol is toxic due to inhibition of membrane transport systems and enzymes and membrane disruption [26–29]. Additionally, in *S. cerevisiae*, butanol has been shown to negatively impact initiation of translation [30]. Cellular responses to butanol and other toxic solvents vary by alteration of the cell membrane lipid composition, expulsion by efflux pumps, and adjustments in cellular membrane protein content [6, 8, 25, 31–34]. It appears that solvent tolerance is a complex mechanism and requires a robust general stress response.

Conclusions

In general, the majority of the 24 strains screened were not able to tolerate 2% butanol. The barrier to growth in the presence of butanol was between 1% and 2% butanol. These results are consistent with the reported growth barrier to strains of *Clostridium*. Typically, growth in the presence of 1% butanol resulted in a decrease of growth rate to about 60% of that in the absence of butanol. Two strains of *Lactobacillus* were able to grow in 2% butanol and reach a final OD 80–90% of that reached in the medium without butanol and may be a promising host for butanol production. Additionally, these strains were able to sustain limited growth in 2.5% and 3% butanol. Temperature affects the toxicity of butanol. A lower temperature allows better growth in limiting concentrations of butanol but does not allow growth in higher concentrations. Future experiments will broaden the range of microorganisms tested for butanol tolerance and further explore the detrimental effect of increased temperature on butanol tolerance.

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