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Impact of butyric acid on butanol formation by *Clostridium pasteurianum*

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HIGHLIGHTS

- Butanol yield from glycerol can be increased through butyric acid.
- Butyric acid conversion requires primary carbon source.
- Butyric acid uptake only at low pH.
- Online process data shows metabolic switches.

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ABSTRACT

The butanol yield of the classic fermentative acetone–butanol–ethanol (ABE) process has been enhanced in the past decades through the development of better strains and advanced process design. Nevertheless, by-product formation and the incomplete conversion of intermediates still decrease the butanol yield. This study demonstrates the potential of increasing the butanol yield from glycerol though the addition of small amounts of butyric acid. The impact of butyric acid was investigated in a 7 L stirred tank reactor. The results of this study show the positive impact of butyric acid on butanol yield under pH controlled conditions and the metabolic stages were monitored via online measurement of carbon dioxide formation, pH value and redox potential. Butyric acid could significantly increase the butanol yield at low pH values if sufficient quantities of primary carbon source (glycerol) were present.

1. Introduction

Production of butanol by *Clostridia* spp. is a historical process of increasing relevance in the current socio-economic environment (Jang et al., 2012; Lee et al., 2008). Progress has been made towards reducing product inhibition through process engineering solutions such as insitu butanol recovery via gas stripping, adsorption and pervaporation as well as by several biological/genetic engineering methods to reduce toxicity and decrease byproduct formation (Ezeji et al., 2007; Ezeji et al., 2003; Karcher et al., 2005; Maddox et al., 1995). Multiple low-cost feedstocks such as lignocellulosic residues (Gao and Rehmann, 2014b; Morone and Pandey, 2014; Wen et al., 2014), dedicated energy crops (Gao et al., 2014a; Sarchami and Rehmann, 2014; Zhang and Ezeji, 2014), food residues (Raganati et al., 2015), etc. have been evaluated. A promising feedstock is crude glycerol from biodiesel production due to constant availability at industrial scale and possible co-generation of butanol at existing biodiesel facilities (Jensen et al., 2012a; Khanna et al., 2013b).

Clostridium pasteurianum can produce butanol from glycerol through the acetone–butanol–ethanol process (ABE process). Genetic modifications as well as suitable process conditions ensure the predominant formation of the desired product (e.g. butanol) (Ezeji et al., 2007). Nevertheless, by-products such as 1,3-propanediol and butyric acid often remain in the fermentation broth lowering the butanol yield of the process (Malaviya et al., 2012; Zheng et al., 2009). In the case of butyric acid, there exists a metabolic pathway allowing for re-uptake and further conversion to butanol, hence there is no apparent necessity for butyric acid accumulation. The pathway from butyric acid to butanol is relatively short, not only from a metabolic point of view, but also with respect to the energy content (Fig. 1). The standard Gibbs free energy of formation (at 25 °C and 101.3 kPa) of butyric acid ($\Delta_f G° = -352.63$ kJ mol^{-1}) is very close to butanol ($\Delta_f G° = -171.84$ kJ mol^{-1}), especially in comparison to the substrate glycerol ($\Delta_f G° = -488.52$ kJ mol^{-1}) (Thauer et al., 1977). However, successful uptake and further conversion of butyric acid

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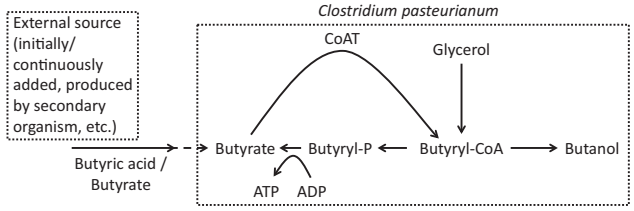

Fig. 1. Metabolic pathway for the formation and uptake of butyrate/butyric acid. Butyrate/ butyric acid can be produced by *C. pasteurianum* itself, or added artificially.

will directly increase butanol yield (Gallardo et al., 2014) and hence is a desired metabolic pathway to activate.

Butyric acid typically accumulates as an intermediate during batch fermentation and can later on be taken up again by *C. pasteurianum*. Additionally, it can be added from an external source, either as a simple co-substrate or produced by a secondary organism during co-culture fermentation. For instance, *Eubacterium limosum* converts carbon monoxide into butyric acid (Genthner and Bryant, 1982). A CO enriched gas stream (possible derived through biomass gasification) could be used for in situ product recovery and as a carbon source for such a secondary microorganism. The reported growth conditions of *C. pasteurianum* and *E. limosum* indicate that such a process might be feasible, which will be investigated by this group in the future. However, a detailed techno-economical discussion of such a process is beyond the scope of this study and requires additional data.

Due to the chemical properties of butyric acid the compound reversibly dissociates into hydrogen protons and non-protonated butyrate depending on the pH value. This dependency is illustrated in Fig. 2 and shows that above the butyric acid's specific pK_a-value of pH 4.82, less than 50% of the compound is protonated. However, most organisms are only able to assimilate the uncharged and, therefore, completely protonated form of a given substrate (Ahn et al., 2011; Casal et al., 1996; Netik et al., 1997). Consequently, to enable butyric acid uptake by *C. pasteurianum* low pH values are necessary. Although pH values lower than 4.82 would be optimal for butyric acid uptake, there has to be a compromise between optimal growth conditions of *C. pasteurianum*, formation of butanol and uptake of butyric acid. Cultivation of *C. pasteurianum* has been reported in a wide pH range between 5 and 7.5, often in a buffered, yet not controlled pH environment (Jensen et al., 2012b; Khanna et al., 2013a; Moon et al., 2011). The degree of butyric acid accumulation is variable in the literature and not fully understood.

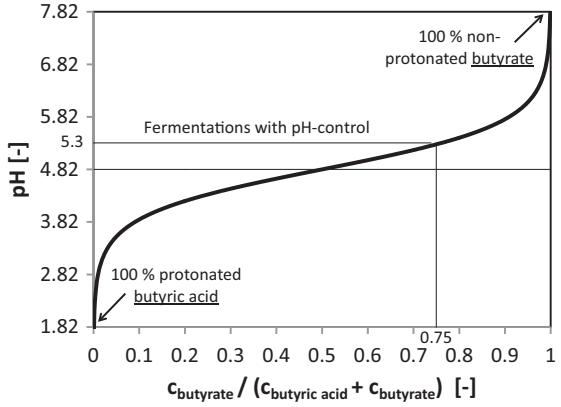

Fig. 2. Dissociation of butyric acid (pK_a = 4.82). Under pH-controlled conditions at set point 5.3, the compound is present as 25% butyric acid and 75% butyrate (Calculations based on Henderson–Hasselbalch equation).

Therefore, the aim of this study is to characterize process conditions for butyric acid uptake by *C. pasteurianum* under non pH-controlled as well as under pH-controlled conditions in a stirred tank reactor. Additionally the ability to increase the overall butanol yield through an external butyric acid source was investigated.

2. Methods

2.1. Strain and media

The anaerobic strain *C. pasteurianum* DSM 525 (DSMZ, Germany) was used in this study. All *C. pasteurianum* pre-cultures were conducted in complex medium which contained: 15 g L^{-1} glycerol, 10 g L^{-1} peptone, 10 g L^{-1} beef extract, 3 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 1 g L^{-1} soluble starch, 3 g L^{-1} sodium acetate. Experiments in a stirred tank reactor were performed in Biebl-medium according to Biebl (2001) which contained: glycerol 30–45 g L^{-1}, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 5 g L^{-1} $(NH_4)_2SO_4$, 0.2 g Lg L^{-1} $MgSO_4{\cdot}7H_2O$, 0.02 g L^{-1} $CaCl_2{\cdot}2H_2O$, 0.05 g L^{-1} $FeSO_4$, 1 g L^{-1} yeast extract, 2 mL L^{-1} trace element solution. The trace element solution contained: 10 mL L^{-1} HCl, 1.5 g L^{-1} $FeCl_2{\cdot}4H_2O$, 0.19 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.1 g L^{-1} $MnCl_2{\cdot}4H_2O$, 0.07 g L^{-1} $ZnCl_2$, 0.063 g L^{-1} H_3BO_3, 0.036 g L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.024 g L^{-1} $NiCl_2{\cdot}6H_2O$, 0.017 g L^{-1} $CuCl_2{\cdot}2H_2O$. All chemicals used were of analytical grade.

2.2. Cultivation conditions

Pre-cultures were conducted in three steps in complex medium (cultivation time given in parentheses). The first step was cultivated in 100 mL Erlenmeyer flasks filled with 10 mL (17 h), scaled up to 250 mL Erlenmeyer flasks filled with 50 mL (8 h) and finally to 500 mL Erlenmeyer flasks filled with 250 mL (12 h). Cultures were incubated at 35 °C on a shaker set to 200 rpm.

Main cultures were grown in a 7 L stirred tank reactor Labfors 4 (Infors, Bottningen, Switzerland) with a liquid volume of 5 L. All cultures were performed in Biebl-medium at 35 °C. Depending on the experimental setup, the pH was either buffered with 2 g L^{-1} $CaCO_3$ or adjusted to pH 5.3 with 3 M KOH and 3 M H_2SO_4. Stirring and gas flow rate were adjusted to 400 rpm and 0.6 L min^{-1}, respectively. To prevent foam formation, Antifoam 204 (Sigma, St. Louis, MO) was used as necessary.

2.3. Analytics

Concentrations of glycerol, butanol, butyric acid, ethanol, acetic acid and 1,3-propanediol were measured via HPLC (Agilent 1260 infinity, Agilent, Santa Clara, USA) using an Agilent Hi-Plex H (7.7 × 300 mm) column operating at 40 °C with a refractive index detector (RID). 5 mmol L^{-1} H_2SO_4 was used as mobile phase with a flow rate of 0.5 cm^3 min^{-1}. Carbon dioxide was measured by an exhaust gas analyzer (Gas analyzer CO_2/O_2, Infors HT, Bottningen, Switzerland), pH via pH probe (Hamilton, Reno, USA) and redox potential by a redox probe (Mettler-Toledo, Delaware, USA).

3. Results and discussion

3.1. Comparison of butyric acid uptake in buffered and pH controlled system

The aim of this experimental setup was to demonstrate butyric acid uptake under pH-controlled conditions in comparison with the commonly used buffered fermentation system. The buffered system had an initial pH of 6.3 and a buffer concentration of