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Research paper

New contributions for industrial n-butanol fermentation: An optimized *Clostridium* strain and the use of xylooligosaccharides as a fermentation additive

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ABSTRACT

n-Butanol is a chemical widely used in industry, with an approximate annual output of 4 million tons and a market valued at USD 8.6 billion. It was first produced by ABE fermentation (acetone - n-butanol - ethanol) at the beginning of the twentieth century using molasses as a substrate, an expensive raw material that made the process economically unfeasible. In this work, we developed a second-generation fermentation process using cellulosic sugar as the substrate. A strain of *Clostridium saccharoperbutylacetonicum* has been evolved to n-butanol resistance, and we have found that the addition of xylooligosaccharides to the culture medium leads to a significant increase in the n-butanol yield and productivity. As a result, an n-butanol titer of 12.5 g/L and a productivity of 0.43 g/L.h were obtained, a respective gain of 17% and 58% relative to the initial process. These advances indicate that second-generation technologies can make viable the production of renewable n-butanol.

1. Introduction

n-Butanol is a four-carbon alcohol widely used as a chemical feedstock for the manufacturing of butyl glycol ether, butyl acetate, plasticizer and esters used in coating, enamels, and lacquers. Energetically, n-butanol is also a promising chemical to be used as a biofuel or a biofuel additive, since it could be blended with gasoline or even used as a pure biofuel without any modification in vehicle engines [1,2]. The high demand for n-butanol is reflected in its global market, which was valued at US$ 7.86 billion in 2014 and is expected to reach US$ 9.9 billion by 2020 [3]. The ABE (acetone - n-butanol - ethanol) fermentation using *Clostridium* bacteria, a well-established fermentation process, was largely used in the beginning of 20th century for the production of solvents like acetone and n-butanol. However, due to the high price of fermentation substrates and low solvent yields, most of the marketed n-butanol is now produced through petrochemical routes [1]. Recently, the growth of the n-butanol market combined with environmental concerns related to the use of petrochemicals has renewed the interest in the production of n-butanol through fermentation processes.

Nevertheless, several obstacles must be overcome prior to implementation of the ABE fermentation at industrial scale. The main shortcomings of this process are the low yield and productivity of solvents due to the toxicity of high n-butanol concentrations, and the high cost of substrates used as raw material for solvent production [1,4]. Therefore, three industrial inputs are, currently, required to enable the commercial production of n-butanol through ABE fermentation: a low-cost substrate, an optimized fermentation process and an effective microorganism, capable of producing high titers of n-butanol.

Recent developments in the production of cheaper sugars (pentoses and hexoses) from lignocellulosic biomass (second-generation technology) generate a favorable scenario for ABE fermentation [5]. Consequently, various research groups are attempting to develop robust strains able to produce considerable amounts of n-butanol [6–9]. Several studies also aim to optimize the fermentation process with lignocellulosic biomass by exploring different types of pretreatment, improving the enzymatic hydrolysis, cultures conditions and culture media [5,6,10–12]. To be used as a substrate, the lignocellulosic biomass has to be pretreated under high temperatures so that the complex structures formed by cellulose and hemicellulose chains can be broken, into fermentable sugars, like glucose and xylose [13,14]. The

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lignocellulosic hydrolysate resultant from the hydrolysis of lignocellulosic biomass contains high sugar concentration, however, it often also has a wide range of toxic compounds, including weak acids, furans and phenolic compounds that can inhibit the microbial metabolism, decreasing the performance of the fermentative process [2,15].

Several pretreatment technologies are available to be used on an industrial scale such as steam explosion, acid pretreatment, alkaline pretreatment, ammonia fiber explosion, organosolv and others [14]. For the ABE fermentation, the organosolv technology based on SEW (SO_2-ethanol-water) for producing cellulose pulp has shown several advantages over other methods, especially considering that this technology decreases the concentration of fermentation inhibitors by eliminating the long heat-up time required for other processes [16]. Moreover, the cellulose pulp can be partially hydrolyzed before fermentation and fully hydrolyzed during the fermentation in the context of simultaneous saccharification and fermentation (SSF), with the advantage of reducing the amount of enzyme cocktail required in the hydrolysis process. During the SSF process, it is possible to increase the saccharification rates since the glucose is immediately consumed by bacteria, decreasing its inhibitory effect on enzymes [17].

Although second-generation technology is the most promising strategy to enable the implementation of n-butanol fermentation at an industrial scale, it is still necessary to optimize the fermentation process and to develop a *Clostridium* strain that shows an excellent fermentative performance and is capable of reaching high yields and productivity of n-butanol from lignocellulosic hydrolysates containing toxic compounds. In this context, with the aim of improving the strain tolerance and minimizing the adverse impact of the process constraints, many strategies using metabolic engineering are being developed [18–21]. However, the tolerance for n-butanol and hydrolysate inhibitors are characteristics of a complex phenotype involving multiple loci, which makes it difficult to engineer the strains. Therefore, several studies have applied adaptive evolution as the primary method to enhance the butanol production, and the fermentative performance of these evolved strains seem promising [22–25].

Notwithstanding the promising results reported in literature about fermentation technologies and strains for n-butanol production, there is no process being employed at industrial scale. Considering the current status of n-butanol technology and aiming to solve the main bottlenecks of ABE fermentation using a low-cost technology, we propose a fermentative process by combining of three factors: (1) a less toxic lignocellulosic hydrolysate produced by SEW technology; (2) a SSF process using cellulose pulp produced from SEW technology; and (3) an hyper-butanol producing *Clostridium* strain obtained through adaptive evolution.

2. Materials and methods

2.1. Bacterial strain

All the experiments were performed using the strain *Clostridium saccharoperbutylacetonicum* DSM 14923 obtained from Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures.

2.2. Culture maintenance and inoculum preparation

The strain *C. saccharoperbutylacetonicum* DSM 14923 is routinely maintained as a vegetative cell in a 30% glycerol stock solution at −80 °C. In order to propagate the cells and prepare the inoculum for batch fermentation, 1 mL of frozen culture containing around 3×10^8 cells/mL was inoculated in 50 mL of sterilized and anaerobic Clostridial Nutrient Medium - CNM (e.g. Sigma – Aldrich, that comprises 0.5 g/L of L-cysteine hydrochloride, 5 g/L of D(+)-glucose, 10 g/L of beef extract, 5 g/L of peptone, 3 g/L of sodium acetate, 5 g/L of sodium chloride, 1 g/L of starch, 3 g/L of yeast extract, 0.5 g/L of agar) and incubated in an anaerobic chamber (Don Whitley/DG-250 with an atmosphere of 80% N_2, 10% CO_2 and 10% of H_2) at 32 °C for 20 h. After the incubation period, the culture was centrifuged and the recovered cells used as inoculum for batch fermentation.

2.3. Adaptive evolution assay

To obtain a tolerant and hyper-butanol producer strain, *C. saccharoperbutylacetonicum* was repeatedly cultivated in Clostridium Nutrient Medium – CNM containing n-butanol at several concentrations. Using preliminary tests, we identified the minimum growth inhibitory concentration of n-butanol as 5 g/L. Thus, the adaptive evolution assays were started utilizing this concentration. At regular intervals, of three days, an aliquot of the *C. saccharoperbutylacetonicum* was transferred to fresh medium containing the same concentration of n-butanol and always keeping the same initial number of cells (OD_{600} ~ 1). When the culture presented an improvement in growth rate, a culture aliquot was transferred to a medium containing an additional 1 g/L of n-butanol. The maximum n-butanol concentration used was 9 g/L. After a total of 22 transfers (approximately 47 generations), an aliquot of the final culture was streaked on CNM plates containing 9 g/L of n-butanol. The two largest colonies were selected for tests of tolerance in liquid culture medium containing 7 g/L and 9 g/L of n-butanol, for analysis of fermentation performance and finally for genome sequencing.

2.4. Batch fermentation

The batch fermentation assays were performed in 100 mL Schott Glass bottles with a working volume of 50 mL. Hydrolysate Medium or Clostridium Synthetic Medium were used as culture medium. The Hydrolysate Medium was prepared using around 50 g/L of total sugars from sugarcane straw hydrolysate provided by American Process Inc. (Table 1), 4 g/L of yeast extract, 2 g/L of ammonium sulfate and 50 ppm of antifoam. The Clostridium Synthetic Medium was prepared in the same way excepted by using synthetic sugars (glucose and xylose) in the same proportion found in the sugarcane straw hydrolysate. The pH of the medium was adjusted to 6.5 using 3 M of NaOH, and the medium was sterilized by autoclaving at 121 °C and 1 atm for 20 min before bacteria inoculation. The loosely capped media bottle was incubated in the anaerobic chamber at 32 °C for 20 h to reach the anaerobiosis before bacteria inoculation. The batch fermentation assays using Hydrolysate Medium were performed in four different conditions: (1) only Hydrolysate Medium without any supplementation; (2) Hydrolysate Medium supplemented with 20 g/L of dry matter of sugarcane straw cellulose pulp or hardwood cellulose pulp (Aspen pulp); (3) Hydrolysate Medium supplemented with 3% (w/w – grams of enzyme cocktail/g cellulose pulp at dry matter) of enzyme cocktail Cellic® CTec3 (Novozymes); and (4) Hydrolysate Medium supplemented with 20 g/L of dry matter of sugarcane straw cellulose pulp or hardwood cellulose pulp and 3% (w/w) of enzyme cocktail Cellic® CTec3 (Novozymes).

Moreover, to verify the effect of cellulose pulp concentration in fermentation profile, batch fermentation assays containing different amounts of sugarcane straw cellulose pulp (0 g/L, 1 g/L, 0.5 g/L, 2 g/L and 4 g/L) were performed. All the experiments were also

Table 1
Content of sugars and organic acid in Hydrolysate Medium.

	Compound (g/L)
Glucose	44.80
Xylose	3.25
Galactose	0.40
Arabinose	0.06
Mannose	0.26
Acetic acid	1.63