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Butyric acid production by fermentation of waste hydrolysates

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Butyric acid is a valuable chemical with wide use in food and pharmaceutical industries. It is a precursor of butanol in acetone-butanol-ethanol fermentation (ABE). Usually, it is produced by different *Clostridia* strains from glucose as substrate. The aim of this study was to performed ABE fermentation by newly isolated *Clostridium beijerinckii* 4A1 strain, where glucose was replaced with hydrolysates obtained from several waste materials (distillers spent grains (DDGS), wood cellulose (WH) and spent coffee grounds (SCG)).The hydrolysates from spent grains and coffee grounds were prepared by acid hydrolysis followed by enzymatic hydrolysis. The wood cellulose hydrolysate was used as received from a local manufacturer. The direct use of waste biomass hydrolysates for butyric acid production was unsuccessful due to the toxicity of some products of the hydrolysis. For purification and detoxification, the hydrolysates were consecutively treated with Carrez solutions ($K_4[Fe(CN)_6]$, and $ZnSO_4$), $Ca(OH)_2$ and activated carbon. The waste biomass hydrolysates can be used after detoxification as substrates in ABE fermentation. Further optimization of the purification and detoxification as well as of the process parameter is necessary.

Keywords: Butyric acid, *Clostridium beijerinckii*, acetone-butanol-ethanol (abe) fermentation, waste biomass hydrolysates, hydrolysates' purification.

INTRODUCTION

Butyric acid (butanoic acid), a 4-carbon short-chain fatty acid, is widely used in chemical, food, and pharmaceutical industries.

Currently, butyric acid is predominantly produced from petrochemical feedstocks via chemical synthesis. Its production from renewable, low-cost biomass has attracted large attention in recent years.

In 1861, Louis Pasteur discovered that some rod-shaped microbes grew and produced butyric acid in the absence of air. The butyric-acid fermenting bacteria were divided into two groups: those producing mostly butyric acid as final product and those producing mostly butanol as final product. The latter process—called acetone–butanol–ethanol (ABE) fermentation—was one of the oldest known industrial fermentations. [1].

Many anaerobic microorganisms can produce butyric acid from sugars and other carbon sources. Generally, they pertain to the genera of *Clostridium, Butyrivibrio, and Butyribacterium* [2].

Usually, the optimal conditions for butyric acid fermentation are 35–37°C in the anerobic atmosphere (pure CO_2, N_2 or a 1:9 mixture of N_2 and CO_2 [3]) and a pH range of 4.5–7.0. The pH value depends on the objective of the bioprocess because the pH optima for acidogenesis and solventogenesis differ [4].

Glucose is the common carbon source for butyrate or butanol production with *Clostridium*q but *Clostridium* bacteria are able to utilize a wide range of sugars: hexoses, several pentoses and oligo- and polysaccharides. Lactose from whey [2, 5, 6], saccharose from molasses [7], starch [8], potato wastes [9], wheat flour [10], cellulose [11] are among the possible substrates. For industrial application, a non-pathogenic strain is preferred for environmental health and safety concerns.

Slow cell growth has a positive effect on butyrate productivity and selectivity. It may be evoked by carbon limitation in continuous or fed-batch processes. Higher butyrate concentrations may be obtained in fed-batch cultures than in continuous cultures. On the other hand, higher productivity may be achieved through the use of continuous cultures [12, 13]. Strains screen, substrates selection and innovation in fermentation techniques are in the research focus in order to make ABE fermentation engineering sustainable and economically feasible.

One of the ways to achieve cost-effective and industrial-scale fermentation of butyric acid is to use waste biomass as fermentation substrate. Lignocellulosic biomass is considered as the most abundant and inexpensive biomass on the earth [14] and it is a rich, sustainable carbon source which uses as a substrate helps in avoiding the global food crisis [15].

However, as a result of the pretreatment and hydrolysis of lignocellulose by diluted acids, several kinds of microbial inhibitors such as phenolic compounds, furan derivatives, some organic acids, etc. [6], which have been confirmed to seriously suppress cell growth and product synthesis [16].

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Although detoxification step has been proved to effectively reduce the toxicity of lignocellulosic hydrolysates, it may significantly increase the total costs of biofuels and bio-based chemicals due to the separation process step and sugar loss [17].

In this study, hydrolysates, obtained from different waste materials (distillers spent grains (DDGS), wood cellulose (WH) and spent coffee grounds (SCG)) were used as substrates for butyric acid production by newly isolated *Clostridium beijerinckii 4A1* strain.

MATERIALS AND METHODS

Raw materials

Spent coffee grounds were taken from a local cafeteria, DDGS a by-product of the ethanol production from grain was received from Almagest AG, Ihtiman, Bulgaria. The wood hydrolysate was obtained from local producer.

Strain and medium

In this work, a newly isolated *Clostridium beijerinckii* 4A1 strain from chickpea fermentation was used [18]. Reinforced Clostridial Medium (RCM) was used as a basic medium for strain maintenance. The inoculum was prepared by growing of 1 ml the culture in 10 ml sterile culture medium and incubated for 24 hours at 37 ºC at anaerobic conditions achieved by Anaerocult® A (Merck Millipore, Germany). The thus prepared inoculum was used in all subsequent experiments. The experiments were performed on hydrolysates, replacing glucose in RCM.

Hydrolysates preparation and purification

The hydrolysates from spent grains and coffee grounds were prepared as follows: 500 ml stock solution (10% w/v dry material) was hydrolyzed in an autoclave with 1% H_2SO_4 at 2 atm for 1 h. The resulting hydrolysate was treated with cellulase „Onozuka R-10" (Yakult Pharmaceutical Industries Co., Ltd. Japan) for 24h at pH=4.5 and 45 ºC (20 U/g substrate). Aliquots were taken for fermentation with appropriate reducing sugar concentration. The wood cellulose hydrolysate was used as received from a local manufacturer.

For purification and detoxification, the hydrolysates were treated with Carrez solutions ($K_4[Fe(CN)_6]$, and $ZnSO_4$) for protein precipitation. After filtration of the precipitated protein, $Ca(OH)_2$ was added to pH about 10 for partial removal of inhibitors and elimination of the excess of sulfate ions. The pH of the filtered solution was corrected with H_2SO_4 to 5.5 and Na_2SO_3 (0.1%) was added. The solution was boiled for 3 h. The initial volume of the solution was restored with distilled water, and activated carbon (10% w/v) was added for as complete as possible removal of furans, acids and other inhibitors. After shaking for 1 hour at room temperature the filtered hydrolysates were used for fermentation media preparation.

Analysis

The concentration of the target products (sugars, butyric acid, and butanol) was determined by HPLC. An Aminex HPX- 87H, 300x7,8 mm column and 0,01 N H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min were used. The biomass concentration was determined by optical density measurements at 620 nm with a spectrophotometer VWR UV-1600PC.

RESULTS AND DISCUSSION

The maximum theoretical yield of butyric acid determined from the following stoichiometric equations:

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2H_2 + 2CO_2 \quad (1)$$

$$6C_5H_{10}O_5 \rightarrow 5C_4H_8O_2 + 10H_2 + 10CO_2 \quad (2)$$

is 0.49 g/g from hexoses and 0.59 g/g from pentoses. In case of a cellulosic hydrolysate with undefined sugar ratio, the yield should be between these values. However, in a real fermentation, the yield would be lower due to production of acetic and lactic acids.

As it was mentioned above, during acid pretreatment of cellulosic substrates, together with individual sugars and oligosaccharides different organic compounds are also released - furan derivatives, phenolic compounds, and weak acids. These compounds are usually strong microbial inhibitors. For example, the furan derivatives in hydrolysates are derived from the degradation of monosaccharides. They are identified as notorious fermentation inhibitors, which have been confirmed to seriously suppress cell growth and product synthesis by disrupting cell membranes and nucleic acids, inhibiting the activity of key enzymes, and causing intracellular oxidative stress response [18]. Hence, when the lignocellulosic biomass was hydrolyzed by dilute acid, which is an efficient, economical and widely used method, the furan derivatives would become major inhibitors and severely inhibit cell growth.

Table 1 shows the concentration of reducing sugars in hydrolysates at a different stage of pretreatment. The purification led to a loss of sugars from 5 to 30%.

Usually, a typical ABE fermentation starts with about 60 g/l glucose or other substrate and continues from 32 to 72 h. Preliminary studies on the influence of glucose concentration showed that the optimal one for the strain *Clostridium beijerinckii* 4A1 is 20 g/l (Table 2).

Table 1. Reducing sugars concentrations of different substrates

Substrate	Reducing sugars, g/l		
	After acid hydrolysis	After enzyme hydrolysis	After purification
DDGS	19	24	23
SCG	21	26	18
WH	10	10	7

Table 2. Influence of the glucose concentration on the growth and production of *Clostridium beijerinckii* 4A1

Glucose g/l	Biomass g/l	Butyric acid g/l	C/N ratio
5	4.97	1.25	0.734
10	7.43	3.67	1.468
15	10,41	4.95	2.202
20	13,00	5.47	2.936
50	6,52	2.45	7.339

The results of a typical ABE fermentation by *Clostridium beijerinckii* 4A1 are presented in Figure 1.

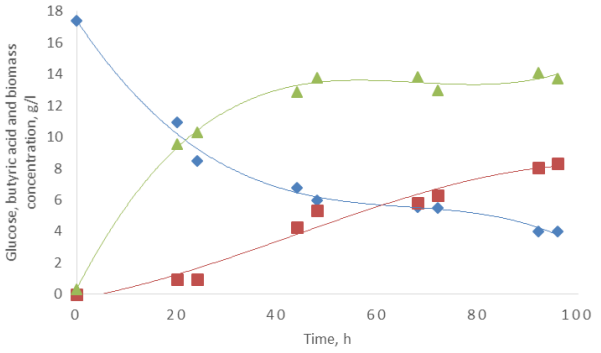

Fig. 1. Time course of glucose (◆), butyric acid (■) and biomass (▲). Initial glucose concentration ~20 g/l.

The first set of experiments for microbial butyric acid production were carried out with obtained hydrolysates (after acid and enzyme hydrolysis) added directly to the medium without purification. The initial reducing sugars concentration was 10 g/l.

The results of the experiments with non-treated hydrolysates are presented in Fig. 2.

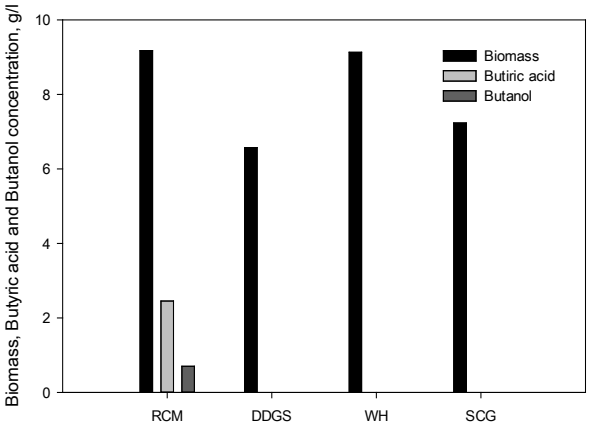

Fig. 2. Biomass growth and butyric acid and butanol production on untreated hydrolysates.

As can be seen from the figure, despite the good biomass growth neither butyric acid nor butanol were produced.

Baroi *et al.* [19] reported that *Clostridium tyrobutyricum* strain does not grow in pretreated by wet explosion and enzyme hydrolysis wheat straw hydrolysate.

Although detoxification step has been proved to effectively reduce the toxicity of lignocellulosic hydrolysate, it may significantly increase the total costs of biofuels and bio-based chemicals due to the separate process step and sugar loss [20].

After hydrolysates' purification and detoxification, according to the above described three steps scheme a new set of experiments was carried out. An RCM medium, containing about 7 g/l reducing sugars (this was the sugar's concentration in wood hydrolysate after detoxification treatment) was used as a control.

Purification of the hydrolysates improved the butyric acid production to 68-78% from the control. The results are shown in Fig. 3.

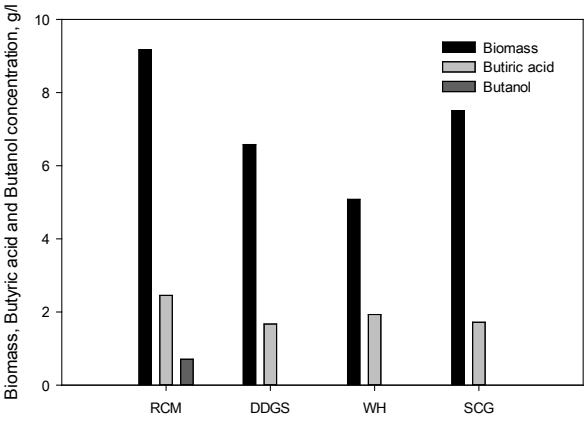

Fig. 3. Biomass growth and butyric acid and butanol production on treated hydrolysates.

Zhang *et al.* [21] reported differences in the degree of detoxification using different methods. While overliming removed more dialdehydes and diketones, treatment with active carbon (AC) removed more phenolic acids. The authors suggested that a combination of different detoxification methods was needed for ABE fermentation. The sequential overliming and AC treatment resulted in remarkable fermentability and high butanol yield (88% from the control with glucose as substrate).

CONCLUSIONS

The direct use of waste biomass hydrolysates for butyric acid production was unsuccessful due to the severe toxicity of some products of the hydrolysis. The waste biomass hydrolysates can be used, after detoxification, as substrates in ABE fermentation with good productivity (68-78% from the control).

Further optimization of the purification and detoxification, as well as, of the process parameter is necessary.

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