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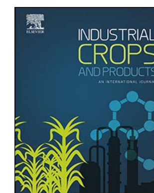

Research paper

Phenolic compounds removal from sweet sorghum grain for efficient biobutanol production without nutrient supplementation

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ABSTRACT

Industrial scale production of biobutanol has been hampered by substrate cost and availability. Sweet sorghum grain is an inexpensive substrate for acetone-butanol-ethanol (ABE) production by *Clostridium acetobutylicum*. Amylolytic activity of *C. acetobutylicum* eliminates the need for the hydrolysis of starchy grain prior to fermentation. However, untreated grain contains phenolic compounds, i.e. tannins, which exhibit inhibitory effects against amylolytic activity and ABE fermentation. Less than 3 g/L ABE was obtained from untreated sweet sorghum grain at different substrate concentrations. Concentration of 0.2 mM gallic acid equivalent (GAE) of sorghum tannins was detected as the critical concentration which inhibits severely ABE fermentation. Applying a multi-stage hot water treatment resulted in tannins removal and significant enhancement in total ABE production up to 18 g/L. For efficient butanol production from 40, 60, and 80 g/L sorghum grain, hot water treatment with two, five, and six stages were found to be essential for efficient butanol production, respectively. Moreover, the amylolytic activity of *C. acetobutylicum* was inhibited by sorghum grain tannins, more than twice as high as the effects on the ABE fermentation pathway. Furthermore, unlike most substrates, sweet sorghum grain could provide all nutrients required for ABE fermentation, eliminating the need for supplementing expensive additional nutrients.

1. Introduction

The global demand for energy is exceeding the sustainable capacity of fossil resources available on the earth and this causes serious environmental and energy security issues. Developing a platform for supplying alternative liquid transportation fuels is of significant importance for sustainability revolution from non-renewable resources to renewable resources (Qureshi and Blaschek, 2001). In recent years, biologically produced butanol, i.e., biobutanol, has been suggested as a "drop-in" liquid biofuel, which can be utilized in the current transportation fuel infrastructures (Bergthorson and Thomson, 2015; Da Silva Trindade and dos Santos, 2017). High energy content, low volatility and corrosiveness, high octane number, and the possibility to be blended with gasoline at the desired ratio are among its advantages (Amiri et al., 2014; Heidari et al., 2016; Kheyrandish et al., 2015; Sasaki et al., 2014; Sirisantimethakom et al., 2016). Besides, butanol is an important industrial chemical, widely used in food, cosmetics, plastic, and chemical industries (Heidari et al., 2016).

Bacteria of the genus *Clostridium*, e.g., *Clostridium acetobutylicum*, are able to produce butanol anaerobically, in the so-called acetone–butanol–ethanol (ABE) fermentation process (Jones and Woods, 1986). Although ABE process was one of the largest industrial scale fermentation process in the first half of the 20th century, most of the biobutanol production plants throughout the world were closed after World War II, due to rapid development in the petroleum industry and production of acetone and butanol using cheaper petroleum-based raw materials (Ezeji et al., 2004). However, rise in the oil price along with climate changes associated with the steadily increasing demand for fossil fuels and recent advances in the genetic engineering techniques, omics technologies and systems biology tools provide an opportunity to re-establish the ABE fermentation as an economically competitive process (Heidari et al., 2016; Lee et al., 2008).

Since substrate cost has a significant influence on the final price of butanol produced via ABE process (up to 60% of the total price), finding a cost effective substrate is essential to improve the economic viability of the ABE process (Maiti et al., 2016; Shao and Chen, 2015). Therefore, in recent years, considerable efforts have been directed toward the use of lower cost substrates, agricultural residues and industrial wastes for ABE production (Amiri and Karimi, 2015; Heidari et al., 2016; Jafari et al., 2016; Mechmech et al., 2016; Sasaki et al., 2014).

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Sorghum is a crop with the ability to grow on saline soils (comprising 5–10% of the world's arable lands) and drought-prone areas (Whitfield et al., 2012). This important ability along with a number of interesting characteristics such as little need of fertilizers, easy sowing, high carbohydrate content comparable with corn, and high biomass content make sorghum a unique candidate to be used as an industrial crop (Almodares et al., 2011; Udachan et al., 2012). Among all the varieties, sweet sorghum or *Sorghum bicolor* (L.) Moench is of great interest because of its rapid growth rate, high sugar and biomass content, and adaptability (Almodares et al., 2011). Sweet sorghum grain resembles corn in many features specially starch content (Udachan et al., 2012); however, due to its dark color, high fiber content, pronounced flavor, and very low gluten content, some varieties of sorghum grain, e.g., red sorghum, do not have the least acceptable quality for using as a food, e.g., in the form of flour. For instance, the gritty flour obtained from sweet sorghum grain cannot be cooked into bread and pastries (Mehboob et al., 2015). Furthermore, the recovery of starch from the grain is not plausible due to the relatively high amounts of cross-linked proteins in the grain structure (Mehboob et al., 2015). Thus, sweet sorghum grain can be considered as an industrial crop with important advantages especially for biobutanol production (Whitfield et al., 2012).

The solvent producing *Clostridia* with amylolytic characteristics can directly utilize starchy raw materials. Therefore, despite ethanol production processes, in which grain should be hydrolyzed by commercial enzymes prior to the fermentation by yeast strains, butanol production through ABE fermentation using *Clostridia* does not need any separate hydrolysis process (Jesse et al., 2002). However, some varieties of sweet sorghum grain have noticeable amounts of tannins in their structure, i.e., tannin-containing varieties, which is a serious inhibitor of *C. acetobutylicum* (Heidari et al., 2016).

In this study, sweet sorghum grain was evaluated for biobutanol production. It was shown that production of butanol, acetone, and ethanol from sweet sorghum grain can be significantly improved up to the theoretical yields after removing tannin compounds using multi-stage hot water treatment. Furthermore, the treated sweet sorghum grain can be used as the sole nutritional source in the fermentation by *C. acetobutylicum,* leading to efficient butanol production without supplementing the culture medium with additional nutrients. The threshold amount of tannins in sweet sorghum grain for efficient butanol production was determined.

2. Materials and methods

2.1. Microorganism and inoculum preparation

C. acetobutylicum NRRL B-591 was provided by Persian Type Culture Collection (PTCC), cultivated in cooked meat medium (60 g/L cooked meat and 4 g/L glucose), and preserved in glycerol. For seed culture preparation, cryo-preserved *C. acetobutylicum* cells were firstly cultivated in cooked meat medium for 18 h. The inoculum was then prepared from the resulting seed culture by a second cultivation in a medium containing 20 g/L glucose, 3 g/L peptone, and 1 g/L yeast extract. P2-stock solution, i.e., vitamins, minerals, and buffers, was also added to the medium with a ratio of 1% (v/v) (Soni et al., 1987). P2 solution contained (g/L): KH_2PO_4, 50; K_2HPO_4, 50; ammonium acetate, 220; Para-amino-benzoic acid, 0.1; thiamin, 0.1; biotin, 0.001; $MgSO_4{\cdot}7H_2O$, 20; $MnSO_4{\cdot}H_2O$, 1; $FeSO_4{\cdot}7H_2O$, 1; NaCl, 1 (Kheyrandish et al., 2015). Serum-bottles filled with 50 mL medium were sealed with a butyl rubber stopper, fastened with an aluminum crimp and purged by oxygen-free nitrogen for 3 min to maintain anaerobic conditions. After autoclaving at 121 °C for 20 min, 0.5 mL filter-sterilized P2 stock solution was added to each bottle. The medium was inoculated by 3 mL seed culture and cultivated for 18 h at 37 °C and 160 rpm.

2.2. Raw materials

Sweet sorghum grains, *S. bicolor* (L.) Moench, were kindly provided by Pakan Bazr Co. (Isfahan, Iran). Sweet sorghum grains were washed twice by tap water to eliminate any dust and trash and then dried at 50 °C for 48 h. Dried grains were milled, sieved to obtain a homogenous powder within 80 and 20 mesh size, and used for ABE production.

2.3. Multi-stage hot water treatment

After primary dust and trash removal, the grains were treated by multi-stage hot water treatment. In the first stage, the grains were mixed with distilled water at 75 °C and 100 rpm with 10% (w/v) solid to water ratio for 5 min. It was found that prolonging the treatment had insignificant effect on the inhibitory effect of sweet sorghum grain (unpublished data). The treated grains were filtered through a 20 sized sieve. After filtration, the treated grains obtained from the first stage, i.e., "TG_1", were mixed again with fresh water for 5 min at 100 rpm and 75 °C in the second stage of the treatment, denoted to the two-stage treated grain, i.e., "TG_2". Likewise, TG_3, TG_4, TG_5, TG_6, and TG_7 denote grains treated by 3, 4, 5, 6, and 7 consecutive stages as described, respectively. The treated grains were dried at 50 °C for 48 h and prepared for milling as described in Section 2.2.

2.4. ABE fermentation

Serum-bottles (118 mL) with 50 mL working volume were used in all fermentations. The bottles were filled with glucose, pure starch, or sorghum grain powder (20, 40, 60 and 80 g/L) as carbon sources. All media, unless stated else, were provided with PY (peptone–yeast extract) and P2 medium as supplementary nutrients at concentrations of (g/L): peptone, 3; yeast extract, 1, and P2 stock solutions.

The bottles were capped and purged with pure nitrogen for 3 min to provide anaerobic conditions. After that, the bottles were autoclaved for 20 min at 120 °C. Then they were inoculated with 3 mL (6%) of an early prepared culture grown in PGY + P2 medium and incubated at 37 °C and 160 rpm (Kheyrandish et al., 2015).

2.5. Enzymatic hydrolysis

For enzymatic hydrolysis, 50 mL of 60 g/L solutions of untreated sorghum grain and TG_6 were prepared in 118 mL serum-bottles, in duplicate. The enzymatic hydrolysis was conducted based on the method reported by Barcelos et al. (2011). The bottles were capped and autoclaved for 20 min at 120 °C for sterilization and gelatinization of the starchy powder. After cooling to 85 °C, the bottles were opened under sterile conditions and α-amylase (Liquozyme, Novozymes, Denmark) was added to these media, at the ratio of 0.01 (w/w) enzyme to starchy grains powder. These solutions were then shaken for 2 h at 100 rpm and 85 °C. After that, the bottles were cooled to 65 °C and glucoamylase (Spirizyme, Novozymes, Denmark) was added at the ratio of 0.04% (w/w) enzyme to starchy grains powder. The bottles were then capped and purged with sterilized nitrogen for 3 min and placed in a shaker incubator for 24 h at 80 rpm and 65 °C. After 24 h, the enzyme hydrolysis is accomplished and 1 mL aliquots of each medium was centrifuged and analyzed for glucose content using a glucose kit. Then the bottles were inoculated with 3 mL of an early prepared culture grown in PGY + P2 medium and incubated at 37 °C and 160 rpm for 72 h. Finally, duplicate samples were taken from these bottles to be analyzed by gas chromatography (GC) for solvents production.

2.6. Analytical procedures

Starch contents of the treated and untreated sorghum grains were measured according to the method described by Li et al. (2015) and stated on dry basis. The starch recovery yields were then determined

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