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Use of Cranberry Bush (*Viburnum opulus* L.) Fruit Pomace as a Renewable Substrate for Biobutanol Production by *Clostridium beijerinckii* in the Presence of Sodium Dithionite

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Abstract
The present study aims to evaluate the use of cranberry bush fruit pomace (CBFP) (*Viburnum opulus* L.), which has recently become popular raw material, as a substrate in the presence of a reducing agent to increase biobutanol production by *Clostridium beijerinckii* DSMZ 6422. For this purpose, some factors were optimized, including the pretreatment, initial concentration of CBFP (5–20%), different types of reducing agents (ascorbic acid, L-cysteine, sodium dithionite and sodium sulfite), different concentrations of sodium dithionite (2.5–15 mM), inoculum concentration (5%, 10%, and 20%), and fermentation time (24–96 h). The maximum biobutanol, total ABE, biobutanol yield, and biobutanol productivity were 9.45 g/L, 12.08 g/L, 0.21 g/g, and 0.13 g/L/h in the medium containing enzymatically hydrolyzed 10% CBFP, 10 mM sodium dithionite, and 20% inoculum at the end of 72 h, respectively. These findings demonstrate that CBFP can be considered as a sustainable, economical, and viable substrate on biobutanol production for the first time in the literature.

Keywords Industrial food waste · ABE fermentation · Reducing agent · Anaerobic microorganism

Introduction

Biobutanol, with its renewable and sustainable properties, attracts attention to remove adverse effects such as greenhouse emmision due to fossil fuels. Biobutanol is a 4-carbon and long-chain alcohol. It has been used in a wide variety of fields to produce materials such as antibiotics, hormones, paints, and plastics for many years [1]. Biobutanol as a biofuel has superior properties compared to bioethanol and biodiesel. The features such as high energy content, easy mixing with gasoline and diesel, low vapor pressure, low solubility in water, and low corrosivity make butanol safer, more effective, and more applicable than the other biofuels [1]. Therefore, butanol has a growing market value. In present, over 1.2 billion gallons of butanol are needed globally each year, with a market value of over $6 billion [2].

Biobutanol is microbially produced by solventogenic *Clostridium* species under anaerobic conditions by ABE fermentation, which consists of two phases as solventogenesis and acidogenesis [2]. Biobutanol is produced mostly with a low yield on a large scale in anaerobic conditions [3]. Therefore, reducing agents have recently attracted attention for removing oxygen from the medium, increasing cofactors such as ATP and NADH and some gene and enzyme activities of microorganism on the biobutanol production process [4]. Different reducing agents, such as L-cysteine, sodium dithionite, and sodium sulfite, are used in the literature to increase yield and concentration in hydrolysis and fermentation processes in biofuel production [4, 5]. In this context, in the present study, the effects of different reducing agents on biobutanol and ABE concentrations were investigated due to their positive effects.

With their wide variety of enzymes, *Clostridium* species have a considerable potential to ferment both hemicellulose-originated pentoses and cellulose-originated hexoses from different feedstocks. In the biobutanol production process, it has been shown in the literature that the use of nutrient-rich substrates increases both microbial growth and solvent production, unlike costly synthetic media. In the literature, the first (agricultural products), second (lignocellulosic wastes), and third generation (microalgae) raw materials appear as cost-effective renewable

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substrates. Thus, they provide a significant advantage in the sustainability of production pathways for effective biobutanol production. Among these raw materials, second generation lignocellulosic raw materials, which are inedible, abundant, and rich in nutrients, are the most investigated sources on biobutanol production [6]. Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin [7]. In addition, they significantly affect microbial growth with their lipids, proteins, minerals, vitamins, carotenoids, and phenolics [6].

Lignocellulosic biomass, including agricultural, industrial, and municipal waste, is generated 200 billion tons annually worldwide [8]. In Turkey, the waste materials released in domestic, agricultural, and industrial areas are quite high. In particular, the production of fruits, beverages and spice crops reached 26.8 tons in 2022, according to Turkish Statistical Institute data [9]. This also indicates the production of a high proportion of industrial food waste. Recently, the cranberry bush (CB) plant (*Viburnum opulus* L.), which can also be evaluated as a lignocellulosic raw material, is widely consumed in the beverage industry in Turkey with its beneficial properties. Furthermore, the CB plant has been frequently used in areas such as medicine, cosmetics, and food. It has natural antioxidant properties with different bioactive compounds such as dietary fiber, saponins, essential oils, iridoids, triterpenes, carotenoids, vitamin C, and phenolic compounds. It is used to treat various diseases such as cancer, diabetes, cough, high blood pressure, digestion problems, and bleeding [10]. Therefore, there is a high level of processing and production for obtaining its juice and extract. CB with its beneficial biocompounds can support microbial growth and metabolic activities at a high rate compared with the other lignocellulosic materials [11, 12]. At the end of the processing of CB, a large amount of fruit pomace is released, which is inedible, worthless, and cost-free. Moreover, releasing these wastes into the environment may cause various problems. In this context, CB fruit pomace (CBFP) is an effective feedstock in terms of sustainability, cost-effectiveness, and viability for biobutanol production. Therefore, the use of CB fruit pomace (CBFP) as a carbon source in biobutanol production will be investigated for the first time in the literature in the current study.

This study aimed to increase biobutanol production from CBFP by *C. beijerinckii* DSMZ 6422 and the use of reducing agents to increase solvent concentration. For this purpose, parameters such as pretreatment, initial CBFP concentration, different types and concentrations of reducing agents, inoculum concentrations, and fermentation time were optimized. To the best of our knowledge, there is no report on the usage of CBFP on biobutanol production.

Materials and Methods

Microorganism, Raw Material, and Reducing Agents

C. beijerinckii DSMZ 6422 was supplied from the culture collection of Ankara University. The pre-culture of *C. beijerinckii* was grown in anaerobic Hungate tubes containing the standard P2 medium with a working volume of 15 mL for 24 h at 37 °C [13]. The standard P2 medium contains (g/L) the following: glucose 60 and yeast extract 1 for carbon and nitrogen source; K_2HPO_4 0.5, ammonium acetate 2.2, and KH_2PO_4 0.5 for acetate buffer; para-amino-benzoic acid 0.001, thiamine 0.001, and biotin 0.00001 for vitamins; and $MgSO_4{\cdot}7H_2O$ 0.2, $MnSO_4{\cdot}7H_2O$ 0.01, $FeSO_4{\cdot}7H_2O$ 0.01, and NaCl 0.01 for minerals [14]. Furthermore, resazurin was added in the standard P2 medium to indicate anaerobic conditions. For the standard P2 medium, a solution containing glucose, yeast extract, and resazurin was prepared and purged by 99.9% nitrogen gas for anaerobic conditions. Then, the medium was sterilized 121 °C for 15 min and cooled. Stock solutions of buffer, mineral, and vitamin were sterilized by a 0.2-µm membrane filter and added to the cooled medium through a disposable sterile injector. A total of 10% microorganism culture was anaerobically inoculated in the standard P2 medium.

CBFP was obtained from Nahita Natural Products Inc., a local company in Niğde/Turkey. CBFP was dried in an oven at 70 °C overnight. The completely dried CBFP was ground to 0.2 cm in an industrial mill (Miprolab/Turkey).

Ascorbic acid (Merck), L-cysteine (Merck), sodium sulfite (Merck), and sodium dithionite (SD) (Fluka) as reducing agent were obtained from the chemical inventory of the Biotechnology Research Laboratory, Ankara University.

Pretreatment and Hydrolysis of Raw Material

For the pretreatment, the desired concentrations of CBFP were pretreated with 1% H_2SO_4 and autoclaved at 121 °C for 15 min [15]. The hydrolyzates obtained after physicochemical pretreatment were filtered with Whatman No. 1 filter paper and stored at + 4 °C. Before experiments, the pH of the CBFP solutions after acidic physicochemical pretreatment was adjusted at 6.8 ± 0.2 by adding 0.2 M NaOH.

For the enzymatic hydrolysis, the pH of the pretreated whole sample was adjusted to 4.8 using 50 mM citrate buffer. Then, 15 FPU/g cellulase enzyme (CelliCTec2) was added to the solution and incubated in a shaking water bath at 50 °C for 72 h [16]. The hydrolyzate was kept in a 90 °C water bath for 10 min to stop the enzymatic activity at the end of 72 h. The hydrolyzate was filtered with Whatman No. 1 filter paper and used in the experiments.

Fermentation Experiments

The Effect of Reducing Agents on Biobutanol Production

The effect of ascorbic acid, L-cysteine, sodium sulfite, and SD as a reducing agent on the biobutanol fermentation was investigated in the standard P2 medium [3]. For this purpose, a stock solution for each reducing agent was prepared and sterilized with a 0.2-µm membrane filter. Using these stock solutions, reducing agents were added at 10 mM concentration in the sterile standard P2 media containing carbon and nitrogen source and P2 solutions (acetate, mineral, and vitamin). Fermentation was monitored after inoculating 10% (v/v) *C. beijerinckii* DSMZ 6422 pre-culture. In addition, the effect of resazurin, a redox indicator, was determined in the presence of reducing agents in the standard P2 medium.

The Effect of Initial CBFP Concentration on Biobutanol Production

The effect of three different initial concentrations (5%, 10%, and 20%) of dried and grounded CBFP on biobutanol production was examined before (CBFP-E, physicochemical pretreatment) and after (CBFP + E, physicochemical + biological pretreatment) enzymatic hydrolysis. The CBFP hydrolyzates were prepared according to the "Pretreatment and Hydrolysis of Raw Material" section. For fermentation, the Hungate tubes were prepared with P2 media containing CBFP hydrolyzate with a working volume of 15 mL. No synthetic glucose was added to these tubes, which only contained fermentable sugars from CBFP. The fermentation media were treated with 99.9% nitrogen gas to create anaerobic conditions. The purged anaerobic tubes were autoclaved. P2 solutions (acetate, mineral, and vitamin) and the 24-h *C. beijerinckii* DSMZ 6422 pre-culture (%10 (v/v)) were inoculated into the prepared fermentation media containing CBFP and incubated at 37 °C for 96 h [17]. In addition, the resazurin as an indicator was added to the medium to ensure the anaerobic conditions. Acetone, butanol, and ethanol concentrations were determined by gas chromatography (GC) with taking samples at specified intervals. In addition, sugar concentrations in the medium were analyzed during the fermentation period.

The Effect of Different Concentrations of SD in the Presence of Optimum CBFP Concentration

To determine the effect of SD concentration on biobutanol production, the fermentation media containing 10% CBFP and resazurin were supplemented with increasing (from 2.5 to 15 mM) SD concentrations [18]. For this purpose, using the prepared stock solution, SD was added to the sterile and purged fermentation media from 2.5 to 15 mM concentrations through a sterile injector. After 10% bacterial inoculation, the solvent production was monitored for 96 h.

The Effect of Inoculum Concentration on Biobutanol Production

After fixing the 10% CBFP and SD concentration, different inoculation rates (5%, 10%, and 20%) were tested to determine the effect of microorganism concentration on biobutanol production. The 24-h *C. beijerinckii* pre-culture was inoculated at the specified rates into media containing 10% CBFP, SD, and P2 solutions and incubated for 96 h at 37 °C. The fermentation samples taken at specified intervals were analyzed for sugar and total solvent (ABE) concentrations.

Analytical Methods

GC (GC 2010 Shimadzu-Japan) with a flame ionization detector (FID) and a RESTEK RTX-Wax capillary column (60-m long with 0.25-mm inner diameter) was used to measure the concentration of acetone, butanol, and ethanol in the fermentation medium [19]. A total of 1 mL of the sample was centrifuged at 9.500 rpm for 10 min before analysis. Then, the supernatant was filtered using a 0.22-µm membrane filter. A total of 1 µL of the sample was inserted from the injection port in split mode (split ratio 100). In the analysis process with GC, after being initially set at 50 °C, the temperature of the column was raised to 150 °C. The FID and injection port temperatures were maintained at 150 °C and 160 °C, respectively. A total of 1.85 mL/min and 190.4 mL/min were column and total flow rate, respectively. The flame ionization and carrier gas were hydrogen and nitrogen, respectively.

The total reducing sugar concentrations were analyzed by the dinitrosalicylic acid method (DNS) [20]. Absorbance measurements for reducing sugar were performed at 540 nm using a Shimadzu UV 2001 model spectrophotometer.

The cellulose content of CBFP was determined according to standard ISO protocol 5498–1981 [21], and cellulose measurement was detected by an external laboratory (Düzen Norwest/Ankara). Hemicellulose and lignin contents of CBFP were determined according to Li et al. [22].

The protein content of CBFP was tested using the Lowry Method [23] and spectrophotometrically measured at 660 nm using a Shimadzu UV 2001 model spectrophotometer. A serial dilution of BSA (bovine serum albumin) was used to create the standard curve. The protein content of CBFP was calculated using the slope value obtained from the standard curve.

According to Nielsen [24], the moisture content of CBFP was detected. For this, 3 g of CBFP in a petri dish was kept in an oven for 1 h at 130 °C.

The water-holding capacity was measured according to Ibrahim et al. [25]. Centrifugation was performed using 3 g of sample ($M_{initial}$), and the solution was centrifuged at 3000 rpm for 25 min after being dissolved in 25 mL of distilled water. The pellet was weighed as M_{final} after being dried in an oven at 50 °C for 30 min.

The following equations were used to compute kinetic parameters such as biobutanol yield, productivity, and sugar consumption rate [26, 27]:

$$\text{Biobutanol Yield(g/g)} = \text{Biobutanol concentration(g/L)/Sugar consumed(g/L)} \quad (1)$$

$$\text{Biobutanol Productivity(g/L/h)} = \text{Biobutanol concentration(g/L)/Fermentation time(h)} \quad (2)$$

$$\text{Sugar Consumption Rate(\%)} = [(\text{Initial sugar(g/L)} - \text{Residual sugar(g/L)})/\text{Initial sugar(g/L)}] \text{ x } 100 \quad (3)$$

homogeneity test was performed to evaluate the equality of variances within groups for a calculated variable. Fermentation media were specified as a factor, and each kinetic parameter was defined as the Y dependent variable in the ANOVA analysis model.

Results and Discussion

Physical and Structural Properties of CBFP

The content of the raw material used on biofuel production can affect microbial activity. Structural features such as the amount of cellulose, protein, and inhibitors in the raw material affect solvent production, while factors such as moisture and water-holding capacity are effective in obtaining fermentative sugar from the raw material. The physical and structural properties of CBFP for biobutanol production in the current study are shown in Table 1.

According to the data in Table 1, the cellulose, hemicellulose, lignin, protein, water-holding capacity, and moisture of CBFP were determined as 16.5%, 38.98%, 18.96% 4.78%, 44.47%, and 8.43%, respectively. Similar to the results of this study, Ozrenk et al. [12] found the cellulose and protein content of different genotypes of CB fruits as 16.6–18.7% and 5.67 to 6.71%, respectively. Akbulut et al. [11] determined 18.07 ± 0.68% cellulose and 6.48 ± 0.58% protein in CB fruits. In addition, the hemicellulose and lignin content of CBFP are compatible with similar fruits. King and Bolling [28] determined as 32.08% hemicellulose and 22.7% lignin in aronia berry pomace. In this context, CBFP is an alternative potential material for biobutanol production with its cellulose, hemicellulose, and lignin content.

Statistical Analysis

Statistical analysis for the biobutanol concentration and kinetic parameters such as biobutanol yield, biobutanol productivity, and sugar consumption rate was determined using variance analysis (ANOVA) test by commercial IBM SPSS version 25. The $p \leq 0.05$ was the significance level. An analysis of multiple comparisons of the fermentation media was performed using Tukey's post hoc test. In addition, a

Moisture content is an important factor in the pretreatment of lignocellulosic raw materials. More than 10% moisture content reduces the pretreatment efficiency. Accordingly, the moisture content of the CBFP used in the present study is low. Alifakı et al. [29] similarly determined 7.69% moisture in CB fruit powder.

The low water-holding capacity of lignocellulosic material offers a higher homogenization and saccharification possibility in the pretreatment stage. In this study, water-holding capacity was determined as 44.47% in CBFP dry matter. For other fruit wastes in the literature, Gouw et al. [30] found that the water-holding capacities of apple, blueberry, red raspberry, and cranberry pomace (dry matters) were 9.27 g water/g DW, 8.29 g water/g DW, 7.71 g water/g DW, and 8.70 g water/g DW, respectively. In addition, the water-holding capacity indicates the amount of cellulose in the lignocellulosic biomass. When the cellulose between the fiber molecules increases, the penetration of water decreases. Thus, the water-holding capacity decreases [25]. According to this, the cellulose content of CBFP matches its water-holding capacity value. Similarly, in the study of Ibrahim et al. [25], while the cellulose content of corn husk was 15.3%, its water-holding capacity was 37.4%.

Table 1 Physical and structural properties of dried CBFP

Properties (%)	Content
Cellulose	16.5 ± 0.00
Hemicellulose	38.98 ± 2.56
Lignin	18.96 ± 0.35
Protein	4.78 ± 0.04
Water-holding capacity	44.47 ± 0.13
Moisture	8.43 ± 0.08

The current study shows that CBFP is a promising raw material in terms of applicability, economy, and efficiency on biobutanol production with its physical and structural properties.

Biobutanol Fermentation Experiments

The Effect of Reducing Agents on Biobutanol Production

In the literature, the positive effects of reducing agents on biofuel production have been shown for the saccharification of the raw material and the fermentation process. For example, the addition of 15 mM reducing agents (sodium sulfite, sodium dithionite, or dithiothreitol) to the pretreatment liquid from Norway spruce resulted in a 31–54% higher glucose. The researchers emphasized that reducing agents have the potential to react with inhibitor compounds in the pretreatment liquid. Thus, inhibitors are easily removed from the medium. They also indicated that sodium dithionite was effective in wide enzyme ranges [31]. In another study, spruce wood and sugarcane bagasse were investigated using sodium dithionite and sodium sulfite as reducing agents in different fermentation strategies to increase fermentation efficiency. With separate hydrolysis and fermentation (SHF), the ethanol productivities of spruce wood and sugarcane bagasse increased from 0.2 to 2.5 g/L/h and from 0.9 to 3.9 g/L/h using sodium dithionite, respectively. These values were higher than the use of control fermentation medium containing glucose and mannose without inhibitors [18]. In addition, reducing agents such as ascorbic acid and L-cysteine were used in the pretreatment and fermentation process in the literature. Therefore, in the current study, the effect of ascorbic acid, L-cysteine, sodium sulfite, and SD as a reducing agent was examined initially in the standard P2 medium to improve the biobutanol fermentation.

The data in Fig. 1 shows the highest ABE concentrations in 10 mM of different reducing agents in the presence or absence of resazurin. Among reducing agents, the SD with 7.35 g/L biobutanol was selected as the most effective agent in the presence of resazurin (Fig. A). In addition, in the absence of resazurin, the ethanol and acetone concentrations with 10 mM SD were higher than the values in the other reducing agents (Fig. B).

The presence of other reducing agents resulted in similar biobutanol values. Moreover, the presence or absence of resazurin in the standard P2 media did not affect the biobutanol concentrations for ascorbic acid and L-cysteine. However, resazurin increased solvent concentration in the P2 medium containing SD, while the absence of resazurin positively affected the fermentation with sodium sulfite. The sodium sulfite can cause slightly higher biobutanol production in the absence of resazurin, depending on the pH change in the medium and its association with resazurin molecules [32, 33]. Resazurin is usually used as an indicator for anaerobic conditions in the standard P2 medium. Furthermore, its use as a reducing agent and its combinations with other reducing agents have been shown in the literature to provide anaerobic conditions in biobutanol production [34, 35]. According to Fukushima et al. [35], the photocatalytic interaction of cysteine and resazurin accelerated oxygen uptake by using cysteine as a reducing agent for anaerobic conditions. Therefore, the effectiveness of resazurin was also investigated in the current study. In addition, the biobutanol concentration was determined as 3.80 g/L in the standard P2 medium containing resazurin. Accordingly, the addition of 10 mM SD to the standard P2 media with and without resazurin improved 1.93 and 1.53 times the biobutanol concentration compared to in the standard P2 medium (3.80 g/L), respectively.

The highest biobutanol results shown in Fig. 1 were also statistically analyzed. According to the results, by statistically multiple comparisons of sodium dithionite with other reducing agent groups and standard P2 medium, the *p*-value was determined as 0.000, and a significant difference was observed. In addition, *p*-values in multiple comparisons of ascorbic acid-L-cysteine and standard P2 medium-sodium sulfite were 1.000 and 0.096, respectively. This shows that there is no difference in these groups.

The time course of ABE concentration and sugar content in the standard P2 media containing 10 mM SD in the presence or absence of resazurin is shown in Fig. 2. The initial sugar concentration of 63.65 g/L decreased to 26.97 g/L and 32.69 g/L in Res + and Res − media containing 10 mM SD at the end of the 96 h, respectively. In addition, biobutanol concentrations were enhanced to 7.35 g/L and 5.85 g/L in Res + and Res- media containing 10 mM SD. When the fermentation process was examined, biobuanol production was significantly accelerated after 48 h. Similarly, the same tendency is seen in sugar consumption. Moreover, no the acetone and ethanol concentrations were significantly changed after 24 h. As a result of the study, the most effective reducing agent was determined as SD, and further experiments were performed with SD and resazurin.

The Effect of Initial CBFP Concentration on Biobutanol Production

Different initial CBFP concentrations, which are a crucial factor in the fermentation of biobutanol, were examined in the present study. High sugar concentrations inhibit the fermentation process and microbial growth early. In addition, the low sugar concentrations are insufficient for the solventogenesis phase [36]. Therefore, pretreated 5%, 10%, and 20% CBFP concentrations with or without enzymatic hydrolysis were evaluated in the present study. Figure 3 depicts the acetone,

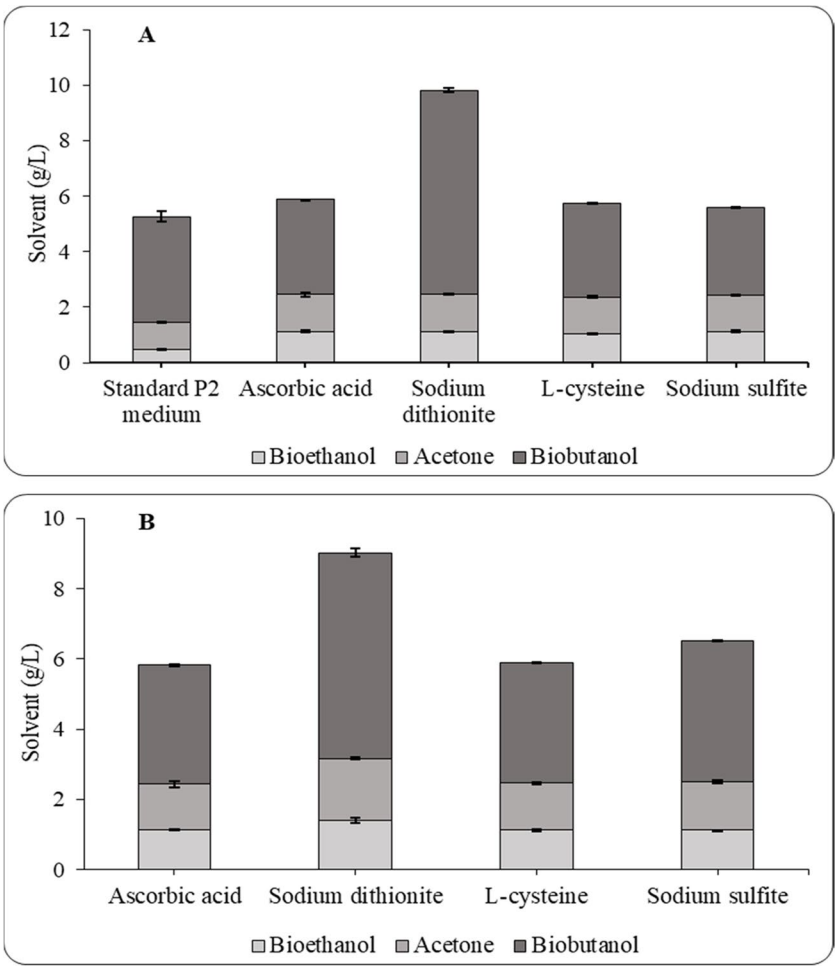


Fig. 1 The effect of different reducing agents on ABE production in the presence or absence of resazurin in the standard P2 medium (10% inoculum, pH 6.8, 37 °C, 96 h, P2 medium solutions + 60 g/L glucose, 10 mM reducing agent). **A** ABE production in the presence of resazurin. **B** ABE production in the absence of resazurin

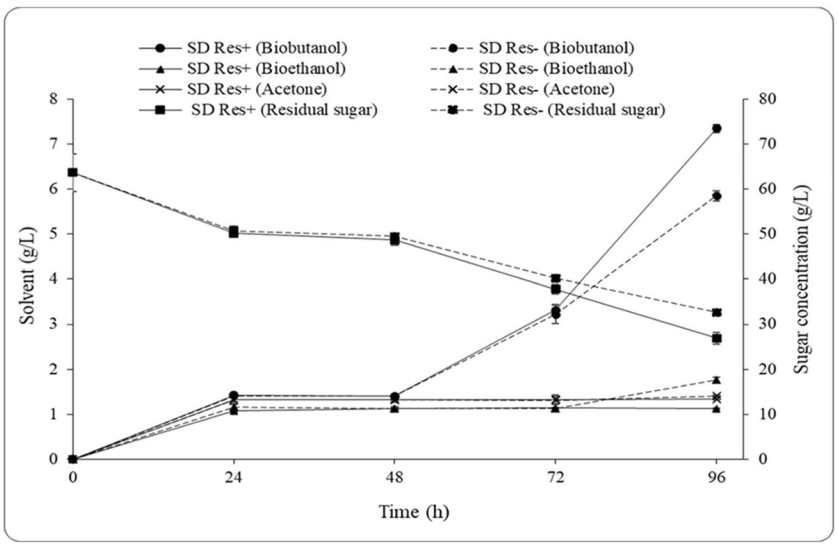


Fig. 2 Time course of ABE concentration and sugar content in P2 medium containing 10 mM SD in the presence or absence of resazurin (10% inoculum, pH 6.8, 37 °C, 96 h, P2 medium solutions + 60 g/L glucose). Res +, medium with resazurin; Res −, medium without resazurin

biobutanol, ethanol, and the initial sugar concentrations at different CBFP loadings. The initial sugar concentration gradually increased from 21.26 to 113.73 g/L with enzymatic hydrolysis and increasing CBFP concentration.

The same trend was not observed for biobutanol production. Increasing the CBFP concentration from 5 to 10% gradually raised the biobutanol concentration. However, when the CBFP concentration was 20%, the biobutanol

values for CBFP − E and CBFP + E sharply decreased. It has been stated in the literature that high substrate concentrations inhibit cell growth and limit the fermentation [37]. In addition, it has been shown in the literature that some biologically active substances in CB fruits have antimicrobial effects on some microorganisms [38]. This indicates that cell growth may be limited at high CBFP concentrations in the current study. Moreover, according to Pratto et al. [37], substrate concentrations above 15% (> 60 g/L sugar) are unfavorable, which may inhibit solvent production. Additionally, they indicated that substrate concentrations lower than 10% w/v (> 40 g/L sugar) may not provide the required sugar concentrations for optimum cell growth and solvent production.

In Fig. 3, the highest biobutanol concentration was obtained as 5.69 g/L with 57.39 g/L of initial sugar in the presence of 10% CBFP + E. Moreover, the biobutanol concentrations of 5% CBFP − E, 5% CBFP + E, 10% CBFP − E, 20% CBFP − E, and 20% CBFP + E were 3.42 g/L, 4.14 g/L, 4.65 g/L, 2.48 g/L, and 2.62 g/L, respectively. There are studies in the literature that show a similar trend compared to the other lignocellulosic materials. For example, using the hydrolysates of cauliflower waste after detoxification, Khedkar et al. [39] obtained 2.99 ± 0.10 g/L biobutanol in the presence 59.7 ± 0.2 g/L initial sugar. However, in the current study, the higher biobutanol concentration was obtained without exposing the CBFP hydrolyzate to any detoxification process. Qin et al. [40], who used food wastes for biobutanol production, investigated various concentrations of waste ranging from 30 up to 150 g/L. The biobutanol was fixedly increased when food waste was enhanced from 30 to 120 g/L. There was no noticeable solvent production when the initial food waste was raised over 120 g/L. In the other study, 8.8 ± 0.3 g/L biobutanol was obtained using 10% pretreated sugarcane straw biomass without detoxification. Moreover, 15% pretreated sugarcane straw biomass without detoxification resulted in the production of 2.1 ± 0.1 g/L biobutanol [37]. Luo et al. [41] also obtained 13.9 g/L butanol concentration from mixed substrates (25%:75%) of corn stover, a lignocellulosic material, and cassava at 10% solid loading by simultaneous saccharification and fermentation.

The ethanol and acetone concentrations in 5% and 10% CBFP loadings without enzymatic hydrolysis were higher compared to the values in 5% and 10% CBFP + E loadings (Fig. 3). On the other hand, similar concentrations for acetone and ethanol were observed in the medium containing 20% CBFP with and without enzymatic hydrolysis. In addition, the fermentation time that obtained the highest biobutanol concentration was 96 h for 5% CBFP − E, 5% CBFP + E, 10% CBFP − E, and 10% CBFP + E and 72 h for 20% CBFP − E and 20% CBFP + E.

Biobutanol concentrations from 5%, 10%, and 20% CBFP with and without enzyme hydrolysis were statistically analyzed. As a result of the analysis, *p*-values for 5%, 10%, and 20% CBFP were 0.001, 0.036, and 0.836 on the biobutanol concentrations, respectively. According to this, while the use of enzymatic hydrolysis created a significant difference on butanol production from 5 and 10% CBFP, it was ineffective at 20% CBFP.

According to Fig. 3, the use of 15 FPU/g cellulase was highly effective at increasing substrate concentrations in terms of sugar conversion. However, higher CBFP concentration was not investigated due to low biobutanol concentration, although there was no homogenization problem due to low water-holding capacity. In addition, enzyme activity and sugar conversion decrease at high biomass concentrations. In the study of Rahnama et al. [42], sugar conversion increased from 1 to 5% at increasing biomass concentration in the presence of the optimum enzyme. However, similar

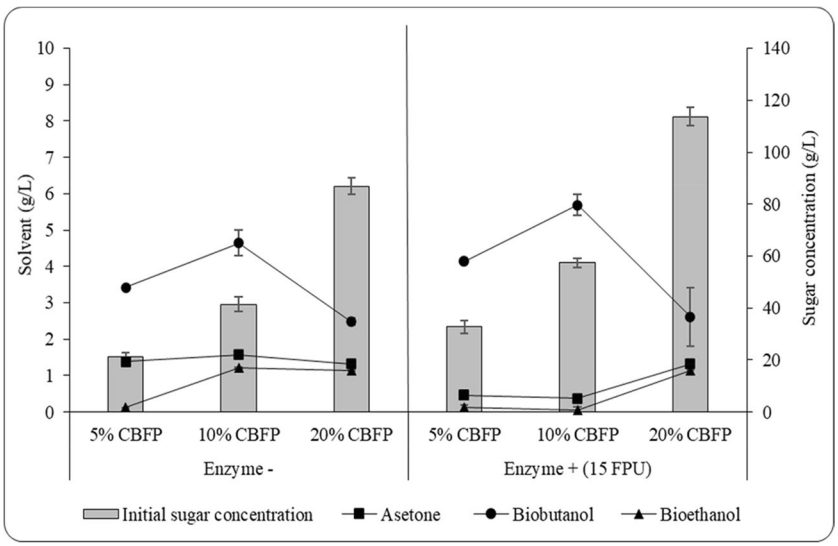


Fig. 3 The effect of pretreatment and initial CBFP concentrations on ABE production in P2 media without glucose (pretreatment: 1% H_2SO_4, 121 °C, 15 min; hydrolysis: 15 FPU/g substrate cellulase enzyme, pH 4.8, 72 h, 50 °C; fermentation: 10% inoculum, pH 6.8, 37 °C, 96 h, P2 media solutions + CBFP hydrolyzate)

sugar concentrations were obtained at 5% and 7% biomass concentrations.

According to the results obtained from the study, an effective sugar conversion was performed by enzymatic hydrolysis. Furthermore, effective butanol production was achieved with the favorable properties of CBFP.

In the industrial scale production of biobutanol, main drawbacks such as substrate cost, anaerobic conditions, low butanol yield, and butanol toxicity significantly increase the whole cost of the production process. In this context, the motivation of this study is to select cost-effective substrates, support anaerobic conditions, and increase butanol concentration in the large-scale production of biobutanol. In the study, CBFP was evaluated because of its promising features for a raw material (such as being inedible, its abundance in high amounts and rich content for microbial growth). The results show that CBFP increases the biobutanol concentration by 49.73% compared to the standard P2 medium. In this sense, the abundance and easy availability of lignocellulosic raw materials and the fact that they support butanol production can lead to cost-effective production in industry. This issue is also discussed in the literature. Kumar et al. [43] determined that cellulosic materials (switchgrass, corn stover, wheat straw, barley straw, and bagasse) and sugarcane are economically very suitable materials for biobutanol production and determined the production cost between 0.59$ and 0.75$. Ashani et al. [44] conducted a techno-economic analysis on biobutanol production from municipal solid waste with three different scenarios. As a result of the study, the scenario, including feed handling, pretreatment, detoxification, hydrolyze and fermentation and ABE separation, resulted in a production cost of 0.70$ is quite profitable in large-scale production. According to these studies, utilizing a very low-cost carbon source such as CBFP for biobutanol production makes the process economically feasible. Furthermore, the use of CBFP on biobutanol production contributes to environmental waste management. In this context, the abundance of CBFP and its favorable physical and structural properties show that it is a feasible and economical raw material in the large-scale production of biobutanol. In this context, the results obtained from this study suggest an important alternative raw material approach for the literature on the use of lignocellulosic raw materials.

To determine the efficiency of SD in the presence of CBFP, its different concentrations in the range 2.5–15 mM were studied. As seen in Fig. 4, the highest biobutanol concentration was determined as 7.82 g/L at the end of 72 h in the presence of 10 mM SD and 10% CBFP + E. In addition, the acetone and ethanol concentrations were 1.34 g/L and 1.13 g/L in this medium, respectively. When the SD concentration increased from 2.5 to 10 mM, biobutanol increased from 3.45 to 7.82 g/L at the end of 72 h. In addition, 2.5–10 mM concentration range of SD resulted in a higher concentration than the butanol concentration obtained in the presence of 10% CBFP + E and 0 mM SD. This increase can be explained by the strong reactions between sodium dithionite and oxygen molecules [45]. Thus, the anaerobic conditions were supported and the microorganism produced a higher biobutanol concentration. In addition, SD is used in the detoxification of lignocellulosic materials for biofuel production [5]. According to this, preventing adverse effects of inhibitory compounds derived from CBFP by SD enabled the improvement of the fermentation process.

A sharp decrease in butanol concentration was observed when the SD concentration was 12.5 and 15 mM in this medium. This decrease may be due to high concentrations of SD inhibiting cell growth and solvent production [3]. In addition, fermentation accelerated in the presence of 10 mM SD compared to the 0 mM SD and the highest concentration was obtained at 72 h. When the results obtained from all

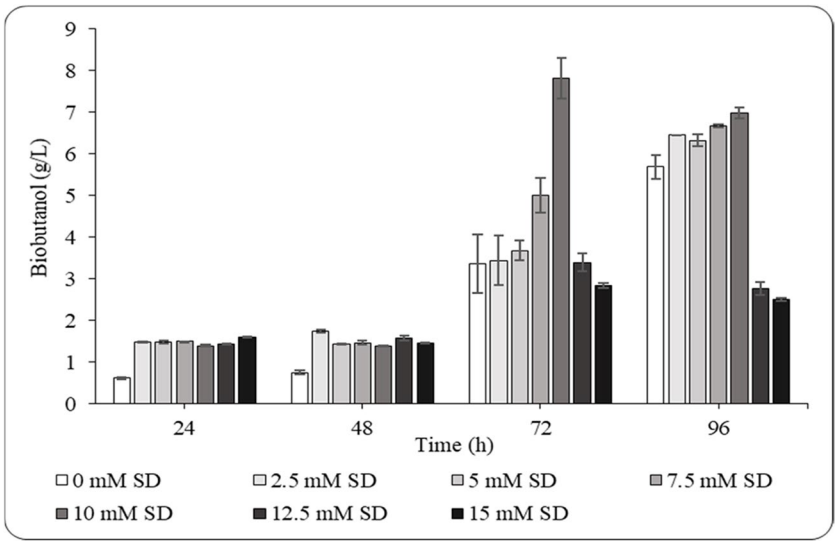


Fig. 4 The effect of different SD concentrations on biobutanol production in the medium containing enzymatically hydrolyzed 10% CBFP and the time course of biobutanol fermentation (pretreatment: 10% CBFP, 1% H_2SO_4, 121 °C, 15 min; hydrolysis: 15 FPU/g substrate cellulase enzyme, pH 4.8, 72 h, 50 °C; fermentation: 10% inoculum, pH 6.8, 37 °C, 96 h, P2 media solutions + 10% CBFP + E + SD)

groups at the end of 72 h were evaluated, the *p*-value was determined as 0.001 by variance analysis.

There are very few studies in the literature that examine how SD affects biobutanol production. However, Daengbussadee et al. [3] investigated the SD range of 0.125–1.0 mM to create anaerobic conditions on biobutanol production from sweet sorghum juice. The highest butanol concentration were obtained as 8.51 g/L using 0.25 mM SDTN. In addition, there are studies on the use of SD in the production of other bioalcohols. Alriksson et al. [18] performed simultaneous saccharification and fermentation (SSF) of sugarcane bagasse using 10 mM SD or 10 mM hydrogen sulfite. As a result of this study, ethanol concentration increased 7.5 times and 5.0 times compared to the control group, respectively. In the study of Cavka et al. [46], the supplementation of 10 mM SD to medium, including 2.5 mM coniferyl aldehyde caused a 3 times increase in the ethanol yield and 9 times increase in the glucose consumption rate.

Accordingly, the results obtained in the present study are comparable with the literature. In this study, the addition of SD was very effective in keeping the inhibitors released after pretreatment and enzymatic hydrolysis, and in supporting the anaerobic conditions during the fermentation stage. This approach resulted in 37.43% an increase in fermentation medium containing only 10% CBFP + E.

The Effect of Inoculum Concentration on Biobutanol Production

Inoculum concentration with factors such as pH, temperature, and substrate type significantly affects the biobutanol production. The optimal inoculum concentration changes depending on the substrate and microorganism utilized [47]. In the current study, the increasing inoculum concentration from 5 to 20% increased the biobutanol concentration in the fermentation medium prepared with 10% CBFP + E and 10 mM SD. Figure 5 shows the change in biobutanol during the fermentation at all inoculum concentrations. The maximum biobutanol concentration was obtained as 9.45 g/L using 20% inoculum of *C. beijerinckii* DMSZ 6422. According to this, 20% inoculum rate in P2 medium containing 10% CBFP and 10 mM SD caused an 20.84% increase in the biobutanol concentration compared with 10% inoculum rate. Similarly, a rise in inoculum size from 5 to 20% resulted in an increase from 0.28 ± 0.03 to 0.76 ± 0.01 g/L in the study of Al-Shorgani et al. [48]. Shukor et al. [47] showed that the increasing concentrations of inoculum significantly increased the production of butanol in the ABE process. In addition, the authors indicated that this effect may be caused by a decrease in the lag phase of growth. Because the lag phase of growth is decreased by an increase in the inoculation of cells. Thus, this situation promotes microbial cell growth and increases the solventogenesis phase and butanol synthesis. In addition, the maximum fermentation time was determined as 72 h. At the end of 96 h, biobutanol concentration slightly decreased.

When 20% inoculum is compared statistically with other concentrations, *p*-values are 0.002 for 5% inoculum, 0.038 for 10% inoculum, and 0.017 for 10% inoculum in standard P2 medium.

As a result of the production of biobutanol by ABE fermentation, acetone and ethanol are also produced in the fermentation process. In this context, as seen in Table 2, the highest total ABE with 1.21 g/L ethanol and 1.42 g/L acetone was produced by *C. beijerinckii* DSMZ 6422 as 12.08 g/L in a medium containing 10% CBFP + E and 10 mM SD with 20% inoculum. This total ABE concentration is noticeably higher than the standard P2 medium containing 10 mM SD, which produced 9.82 g/L of total ABE. Furthermore, biobutanol yield, productivity, and sugar

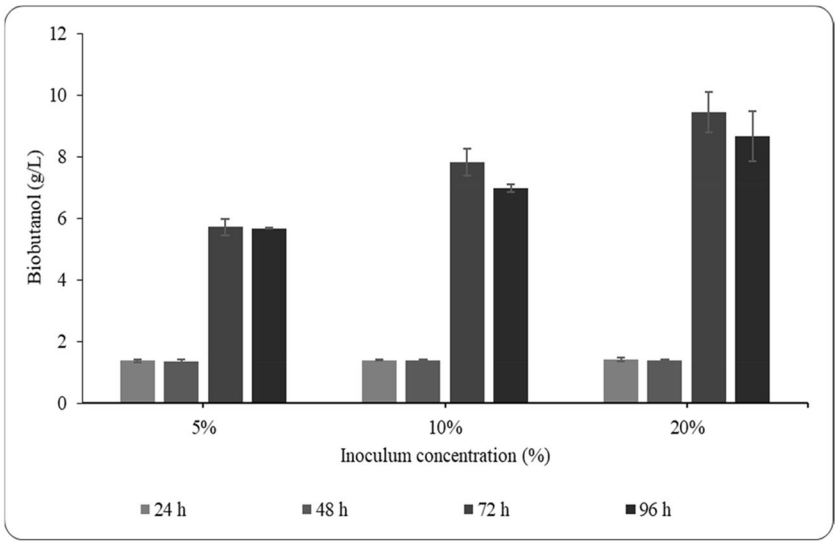


Fig. 5 The effect of inoculum concentration on biobutanol production in the presence of 10 mM SD and CBFP (pretreatment: 10% CBFP, 1% H_2SO_4, 121 °C, 15 min; hydrolysis: 15 FPU/g substrate cellulase enzyme, pH 4.8, 72 h, 50 °C; fermentation: pH 6.8, 37 °C, 96 h, P2 media solutions + 10% CBFP + E + 10 mM SD)

Table 2 Kinetic parameters at the end of 72 h for different inoculum concentrations (10% CBFP + E + 10 mM SD + P2 media solutions, pH 6.8, 37 °C)

Parameters	The medium containing 10% CBFP and 10 mM SD			P2 medium containing 10 mM SD *
	5% inoculum	10% inoculum	20% inoculum	%10 inoculum
Biobutanol (g/L)	5.72 ± 0.26^{a}	7.82 ± 0.43^{b}	9.45 ± 0.65^{c}	7.35 ± 0.09^{b}
Total ABE (g/L)	8.17 ± 0.27^{d}	10.29 ± 0.44^{e}	12.08 ± 0.63^{f}	9.82 ± 0.10^{e}
Biobutanol yield (g/g)	0.17 ± 0.02^{g}	$0.20 \pm 0.05^{g,h}$	0.21 ± 0.04^{h}	0.20 ± 0.00^{h}
Biobutanol productivity (g/L/h)	0.08 ± 0.00^{i}	0.11 ± 0.02^{j}	0.13 ± 0.02^{k}	0.08 ± 0.00^{i}
Sugar consumption rate (%)	57.92 ± 3.28^{l}	69.93 ± 4.24^{m}	80.59 ± 0.31^{n}	57.62 ± 0.10^{l}

The major variations in parameters are shown by different letters in superscripts within the same row. Statistically significant ($p \leq 0.05$)

*Values determined at the end of 96 h of fermentation

consumption rate were increased from 0.17 to 0.21 g/g, from 0.08 to 0.13 g/L/h, and from 57.92 to 80.59%, respectively, when the inoculum concentration raised from 5 to 20% in the presence of 10% CBFP + E + 10 mM SD. A similar change was found by Al-Shorgani et al. [48], who investigated the effect of inoculum size on ABE production from POME. The study showed an increase in butanol productivity from 0.002 and 0.006 g/L/h, increasing the inoculum size from 5 to 20%. Razak et al. [49] obtained a biobutanol yield of 0.11 g/g from oil palm decanter cake hydrolyzate at the highest inoculum concentration of 16.20% as an optimum size using CCD analysis. Chen et al. [50] determined the highest biobutanol yield of 0.22 g/g with high cell concentration from non-pretreated rice straw hydrolyzate.

Compared to previous studies, the present study suggests an effective and comparable approach by using 10% CBFP + E and 10 mM SD on the high biobutanol productivity and yield. Thus, the using both CBFP and SD in fermentation can improve the production process of biobutanol in terms of economy and efficiency.

Statistical Analysis for Optimized Conditions

According to the statistical analysis of results in Table 2, significant differences were observed for biobutanol concentrations in four different fermentation media. The *p*-value is 0.004, and the *F*-value is 26.704. Moreover, when the homogeneity distributions are compared, it shows that similar values are obtained in the P2 medium containing CBFP sugars or standard P2 medium containing synthetic glucose in the presence of 10 mM SD and 10% inoculum. The *p*-value for these two groups is 0.326. This shows that CBFP can be used as an alternative medium to the standard P2 medium.

Furthermore, when the biobutanol results obtained from the optimized conditions were statistically analyzed, the *p*-value and *F*-value were determined as 0.000 and 57.635, respectively. The values given in Table 3 show the relationship between the standard P2 medium, standard P2 medium containing 10 mM SD and resazurin, P2 medium containing 10% CBFP + E, P2 medium containing 10% CBFP + E and 10 mM SD, and P2 medium containing 10% CBFP + E, 10 mM SD, and 20% inoculum. These results show that there is a statistically significant difference between the standard P2 medium and the medium prepared with CBFP which is a promising feedstock for biobutanol production.

Table 3 ANOVA of biobutanol concentrations obtained from the different fermentation media

Sum of squares ($\Sigma(xi - x)^2$)	Degree of freedom (*df*)	Mean square (X^2)	*F*	*p*-value
37.138	4	9.284	57.635	0.000

Conclusion

The present study demonstrates the successful fermentation of CBFP as a cost-effective lignocellulosic substrate for biobutanol production. It is seen that CBFP significantly increases microbial activity for fermentation with its sugar and nutrient content. Moreover, according to the results obtained from the study, the use of SD as a reducing agent significantly improved the fermentation process by supporting anaerobic conditions and preventing the negative effects of inhibitor compounds in the medium prepared with CBFP. This study reports for the first time in the literature that CBFP is a promising raw material for biobutanol production.

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Author Contribution Nazlıhan Tekin: investigation, writing article draft; Mariye Nur Çelik: ınvestigation; Sevgi Ertuğrul Karatay: conceptualization, methodology, data analysis, writing—review and editing; Gönül Dönmez: resources.

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Data Availability No data was used for the research described in the article.

Declarations

Competing Interests The authors declare no competing interests.

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