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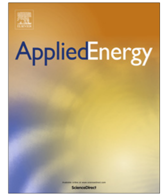

# Feasibility of acetone–butanol–ethanol fermentation from eucalyptus hydrolysate without nutrients supplementation

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## HIGHLIGHTS

- This is the first study on the use of eucalyptus for ABE production.
- ABE could be produced without nutrients supplementation.
- Cellulase SS supplied nitrogen source for strain's growth.
- Production of 13.1 g/L ABE using diluted eucalyptus hydrolysate.

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## ABSTRACT

The economic feasibility of acetone–butanol–ethanol (ABE) fermentation is greatly affected by the type of raw material used. The easy availability of eucalyptus from marginal environments is an alternative feedstock for use as raw material to reduce the production cost. In this study, hydrolyzed eucalyptus was used for ABE production without any nutrients supplementation. Increasing the solid concentration in the eucalyptus slurry from 6.7% (*w*-dry matter/*v*) to 25% led to an increase in the initial glucose concentration from 33.7 g/L to 86.7 g/L after enzymatic hydrolysis. Dosed cellulases not only hydrolyzed cellulose but also supplied nitrogen source for ABE producing strain. However, ABE production from the obtained hydrolysate decreased when the solid concentration was increased to more than 10%. The maximum ABE of 12.3 g/L was obtained at 10% solid concentration, with an initial glucose concentration of approximately 40 g/L. In addition, the fermentation capability of eucalyptus hydrolysate was found to be improved by diluting the hydrolysate, which prevented inhibition by substrate and fermentation inhibitors. Finally, ABE concentration was improved to 13.1 g/L by diluting the hydrolysate from the initial solid concentration of 25% to an initial glucose concentration of 45 g/L, which resulted in ABE productivity of 0.109 g/L/h and ABE yield of 0.413 g/g. Thus, the high ABE production from eucalyptus makes it a potential feedstock for biofuel production.

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## 1. Introduction

Lignocellulosic materials are the most abundant, sustainable, and cost-effective biomass. Among the diverse types of available lignocelluloses, eucalyptus, a woody plant, is a good feedstock option owing to its higher cellulose content and compositional uniformity [1]. Moreover, eucalyptus has been successfully planted in many parts of the world, particularly in several marginal environments that are otherwise not suitable for food crop cultivation [2].

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The fast-growing eucalyptus can also serve as a potential source of carbohydrates in several biotechnological processes. Eucalyptus hydrolysate has been used as a fermentation substrate after pretreatment and hydrolysis for the production of various value-added products such as xylitol [3], ethanol [4], lactic acid [5], fumaric acid [6], and single-cell protein [7]. Thus, eucalyptus is an attractive substrate candidate for the production of various bioconversion products.

The rapid exhaustion of fossil fuels in addition to the inflation and fluctuation in the prices of crude oil has stimulated considerable interest in fuel production from alternative sources such as lignocellulosic materials via biological processes [8]. Butanol is an alternative fuel that has earned much attention for its irreplaceable advantages. It is not only less volatile, explosive, corrosive, and hygroscopic than ethanol but also has energy content similar to that of gasoline and is compatible with gasoline in any proportion [9]. However, although more favorable than fossil fuels for several reasons, the use of ABE is economically less viable due to the high cost of food-based substrate in ABE fermentation. Therefore, the abundant availability of lignocellulosic materials and their non-edible characteristics make them an attractive substrate [10]. To date, several lignocellulosic materials such as switchgrass [11], rice straw [12], corncob [13], barley straw [14], and wheat straw [15] have been successfully used in ABE fermentation. However, ABE production from eucalyptus has not been reported.

In general, in addition to the pretreatment, hydrolysis, or detoxification of lignocellulosic material, supplementation of several nutrients to the fermentation medium is a crucial step in the fermentation of the lignocellulosic hydrolysate by microorganisms. Nutrients including nitrogen sources, buffer reagents, metal ions, and vitamins are supplemented to the medium to compensate for the nutritional deficiencies of lignocellulosic hydrolysates. Several researchers have supplemented modified P2 solution (as a semisynthetic medium containing buffer, minerals, and vitamins) or yeast extract to several lignocellulosic hydrolysates so as to enhance ABE production [16–18]. Although supplementation of several nutrients is considered to enhance ABE production from lignocellulosic hydrolysate, it would be preferable to avoid nutrient supplementation to reduce the overall production cost and to simplify the medium composition.

In this study, we used eucalyptus pretreated by steam explosion (cellulose as the major component) as a substrate for ABE production. The objective of the study was to evaluate the feasibility of using eucalyptus hydrolysate for ABE fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 and to investigate the critical issues therein.

## 2. Materials and methods

### 2.1. Steam explosion pretreatment of eucalyptus

Eucalyptus was pretreated by steam explosion using a steam gun kindly provided by the Japan Gasoline Corporation (JGC), Japan. Briefly, eucalyptus chips were air-dried, milled to a size of <10 mm, and then loaded directly into the steam gun for pretreatment. In the steam explosion treatment, the loaded eucalyptus chips were subjected to a temperature of 230 °C under a pressure of 3 MPa with a residence time of 3 min; the steam explosion-treated eucalyptus was then released from the steam gun by rapid depressurization of the vessel, causing the material to expand into a stainless steel cyclone. The eucalyptus sample was then water-washed to recover the water-insoluble solid fraction for further use, that is, for enzymatic hydrolysis and fermentation. The glucan content of the water-insoluble solid fraction was estimated to be approximately 0.48 g/g-dry eucalyptus.

### 2.2. Enzymatic hydrolysis of steam-exploded eucalyptus

First, the moisture content of the eucalyptus water-insoluble solid fraction was determined and then the fraction was used for the preparation of eucalyptus slurry with varying solid concentrations (*w*-dry matter/*v*) of 6.7%, 8.3%, 10%, 12.5%, 16.7%, and 25%. Cellulase SS of 211 filter paper units (FPU)/mL (Nagase Chemtex Co.; Osaka, Japan) was used in the enzymatic hydrolysis after setting it to a cellulase dosage of 175 FPU/g-dry matter. Slurries of steam-exploded eucalyptus with different solid concentrations were hydrolyzed using cellulase at 50 °C for 48 h with an agitation speed of 120 rpm after the initial pH was adjusted to 5.0 with 3 M KOH. After the enzymatic hydrolysis, the eucalyptus slurry was centrifuged at 2300*g* for 30 min to separate the supernatant from the solid residues. Then, the supernatant was filtrated through a 0.45-μm cellulose-acetate filter (ADVANTEC; Tokyo, Japan) to remove the suspended particles; the obtained eucalyptus hydrolysate was stored at 4 °C until use.

### 2.3. Microorganism and medium

The spore stock of *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was prepared in potato glucose (PG) medium and stored at 4 °C [19]. For pre-culturing, tryptone–yeast extract (TY) medium with the following composition was used: in deionized water (per liter), 20 g glucose, 2 g yeast extract (Difco™; Becton Dickinson; Franklin Lakes, NJ, USA), 6 g tryptone (Difco™), 2.6 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $KH_2PO_4$, and 10 mg $FeSO_4 \cdot 7H_2O$. The initial pH was adjusted to 6.5 with 3 M KOH, and the medium was sterilized at 115 °C for 15 min [20,21].

### 2.4. ABE fermentation

Seed culturing was performed by transferring 1 mL of the spore suspension to 9 mL of the PG medium, followed by providing heat shock for 1 min in boiling water, and then incubation in the AnaeroBox (Hirasawa Co. Ltd.; Tokyo, Japan) chamber at 30 °C for 24 h [22]. Pre-culture was initiated by transferring 10% (*v*/*v*) of the seed culture into the TY medium and further incubating in the AnaeroBox chamber at 30 °C for 15 h.

ABE fermentations were performed using a working volume of 50 mL eucalyptus hydrolysate at different solid concentrations in serum bottles without any nutrient supplementation. The initial pH of the eucalyptus hydrolysate was adjusted to 6.5 with 3 M KOH. After sparging the media with oxygen-free nitrogen to ensure anaerobic conditions, the serum bottles were sealed with butyl rubber and an aluminum crimp cap and sterilized by autoclaving at 115 °C for 15 min. The batch fermentation was initiated by inoculating the pre-culture broth at an inoculum size of 10% (*v*/*v*) [20]. Each experiment was performed a minimum of two times to ensure reproducibility.

### 2.5. Analysis

The theoretical glucan content of the steam-exploded eucalyptus was determined based on monomer content measured after a two-step acid hydrolysis procedure according to the Laboratory Analysis Protocol (LAP) developed by the National Renewable Energy Laboratory (Golden, CO, USA) [23]. Growth of *C. saccharoperbutylacetonicum* N1-4 was determined by measuring the optical density at 562 nm ($OD_{562nm}$) on a spectrophotometer (V-530; JASCO; Tokyo, Japan) [20,21]. The collected samples were centrifuged at 13,000*g* for 10 min; the supernatant was filtered through a 0.45-μm filter and diluted for the quantitative determination of the solvents and sugars. The sugar concentration was determined by high-performance liquid chromatography (HPLC) (US HPLC-1210;

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