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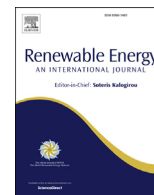

Acetone-butanol-ethanol (ABE) fermentation using the root hydrolysate after extraction of forskolin from *Coleus forskohlii*

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ABSTRACT

The biomass obtained after the extraction of forskolin from the roots of *Coleus forskohlii* was evaluated as a substrate for the production of acetone-butanol-ethanol (ABE). The spent biomass constituting more than 90% of the raw material showed 50–70% carbohydrates with starch and cellulose being the major constituents. This study was undertaken to optimize enzymatic hydrolysis of *C. forskohlii* roots for maximum release of fermentable sugars and subsequent fermentation to ABE. The root biomass was hydrolyzed using the Stargen® 002 and Accellerase® 1500. Cocktail of both enzymes (16U Stargen® 002 and 60 FPU Accellerase® 1500) could produce 41.2 g/l of total reducing sugars (glucose equivalent to 32.33 g/l). The production of ABE was optimized in a batch fermentation using *Clostridium acetobutylicum* NCIM 2877. The maximum ABE production using the root hydrolysates was 0.55 g/l. Pretreatment with lime and Amberlite XAD-4 increased the production of total solvent to 5.33 g/l.

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1. Introduction

Coleus forskohlii Briq, a herbal plant belonging to the *Lamiaceae* family, is native to India and is reported in Ayurvedic *Materia Medica* under the Sanskrit name Makandi and Mayini [1]. Presently, about 40,000 acres of land is under cultivation of *C. forskohlii* in India, Africa and South East Asia for its tuberous roots [2]. The cultivation may provide an average yield of 800–1000 kg/ha of dry tubers which can be improved to 2000–2200 kg/ha of dry tubers by applying proper practices. The cultivation of *C. forskohlii* has been on an increase due to its commercial utilization [3]. The roots of the *C. forskohlii* plant are a unique source of forskolin (FSK), a labdane diterpene compound. FSK has been shown to be useful in the treatment of asthma, glaucoma, cardiovascular diseases and certain types of cancer [4].

The spent biomass obtained after extraction of forskolin from the root has no commercial value. It constitutes more than 90% of carbohydrate-rich raw material which could be used as a substrate for the production of value added chemicals and fuels such as ABE solvents. Currently, tonnes of *C. forskohlii* root biomass is either dumped or burnt which are environmentally hazardous. There is an increased interest among the researchers to develop various strategies to utilize waste biomass for useful and value-added purpose.

Increase in petroleum prices and depletion of fossil fuels are the key reasons for ongoing search of energy alternatives worldwide [5]. Bioconversion of waste biomass to alcoholic fuels such as bioethanol, biobutanol, and biodiesel is rapidly emerging as an area of interest among researchers [6]. Currently, countries like USA and Brazil contribute 20–30% of biobutanol production in fuel market, while in Asia the bioethanol production is at a very early stage of development [7,8]. Biobutanol has attracted the attention of researchers and investors due to its various advantages over other biofuels such as like high heating value, low freezing point, high hydrophobicity, and low heat of vaporization that are closer to gasoline [9–11]. The current production of n-butanol is about 5–6 million tons per year with a worldwide market sale of US$7–8.4 billion [12]. The market demand is anticipated to increase dramatically, if n-butanol can be produced cost-effectively [13].

Butanol can be obtained from renewable biomass by ABE fermentation [14]. *Clostridium acetobutylicum* utilizes a range of carbon sources to produce butyric acid and acetic acid in the first phase (acidogenesis), and acetone, butanol and ethanol in the second phase (solventogenesis). The process of biobutanol production

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consists of several unit operations such as pretreatment of the biomass, *clostridial* ABE fermentation, and recovery of the desired product. The pretreatment of the biomass varies with its chemical makeup (starch rich, sugar rich or lignocellulosic materials).

The cost of the fermentation substrate conclusively decides the economics of biobutanol production [15]. The use of inexpensive, abundantly available, sustainable and renewable feedstock such as lignocellulosic materials (agricultural waste, paper waste, wood chips, etc.) has the potential to reduce the cost of biobutanol production. These agricultural biomass sources cost much less (US$24–75/ton) than the traditional substrates [16]. The use of lignocellulosic biomass such as wheat straw [17], wheat bran [18], barley straw [19], corn stover and switch grass [20], corn fiber [21] and wood [22] for solvent production has been reported earlier.

The lignocellulosic biomass is pretreated to fractionate the lignin, hemicelluloses and cellulose prior to hydrolysis and subsequent ABE fermentation. The types of pretreatments and their merits and demerits have been reviewed by Alvira et al. [23] and Sun and Cheng [24]. The hydrolysis of lignocelluloses results in the formation of some inhibitory components such as formic acid, acetic acid, levulinic acid, furfural and hydroxymethyl furfural [25] which limits the ABE fermentation. Several detoxification methods have been previously investigated for the removal of these inhibitors. These include lime treatment, evaporation, and adsorption using ion exchange resin or activated charcoal to biological treatment including laccase and peroxide enzymes [26–29]. Sun and Liu [30] investigated the application of membrane filtered sugar maple wood extract hydrolysates for ABE production and found that overliming treatment can significantly improve the ultimate butanol concentration from 0.8 g/l to 7 g/l.

The objective of this study was to investigate ABE production from *C. forskohlii* root hydrolysate using *C. acetobutylicum*. The root biomass of *C. forskohlii* was characterized to determine the carbohydrate content which was further hydrolyzed enzymatically to fermentable sugars. The phenolic compounds present in the root hydrolysates were removed by pretreatment using $Ca(OH)_2$ and resin i.e. Amberlite XAD-4. The pretreated root hydrolysate was subsequently used for ABE fermentation.

2. Materials and methods

2.1. Materials

C. forskohlii roots were procured from Salem, Tamilnadu, India. Dried roots were ground in a mill fitted with 18 mesh to a particle size <1 mm and stored in air tight containers for further studies. Peptone, meat extract, yeast extract, glucose, starch, NaCl, sodium acetate, L-cysteine, ammonium acetate, K_2HPO_4, KH_2PO_4, p-amino benzoic acid, thiamine HCl, biotin, $FeSO_4$, $MnSO_4$, $MgSO_4$, $Ca(OH)_2$, NaOH and HCl was purchased from Hi Media Laboratories, Mumbai, India. Amberlite XAD-4 resin was purchased from Sigma Aldrich, Mumbai, India. Glucose oxidase-peroxidase (GOD-POD) kit was purchased from Accurex Biomedical Pvt. Ltd. Mumbai, India. Enzymes (Stargen® 002 and Accellerase® 1500) were gifted by Gencor International, Mumbai, India. All the chemicals used were of analytical grade.

2.2. Proximate analysis and carbohydrate profiling of C. forskohlii roots

Moisture, ash, fat and nitrogen content were determined by standard AOAC Official methods [31]. Total carbohydrate content of sample was calculated by difference [32], rather than by direct analysis. Dried and powdered *C. forskohlii roots* samples were used for carbohydrate profiling as per the earlier reports [33,34]. Carbohydrate components were fractionated into free sugars, oligosaccharides, starch, pectin, hemicellulose, cellulose and lignin.

2.3. Optimization of parameters for enzymatic saccharification of C. forskohlii roots

The principal components of *C. forskohlii roots* are starch and cellulose. Hence Stargen® 002 and Accellerase® 1500 were used individually and/or in combination in order to determine the optimum conditions for complete saccharification of starch and cellulose.

Slurry of *C. forskohlii* roots (10% w/v) was prepared in sodium citrate buffer by gentle stirring using a magnetic stirrer. Starch breakdown was carried out by using Stargen® 002 which contains *Aspergillus kawachi* α-amylase expressed in *Trichoderma reesei* and a glucoamylase from *Trichoderma reesei* that work synergistically to hydrolyze granular starch substrate to glucose. The pH of slurry (3.5–5.5), hydrolysis temperature (30 °C–70 °C) and substrate concentration (5–25% w/v), Stargen® 002 (4–16 U/g dry weight of *C. forskohlii roots*) and incubation time (6–30 h) were optimized for maximum release of reducing sugars.

Cellulose breakdown was carried out by using Accellerase® 1500 which is a mixture of cellulase and glucosidase that works synergistically to hydrolyze cellulosic substrate to glucose. The pH of slurry (3.5–5.5), hydrolysis temperature (30 °C–60 °C), incubation time (8–40 h), Accellerase® 1500 (30–120 FPU/g dry weight of *C. forskohlii roots*) and substrate concentration (5–25% w/v) were optimized for maximum release of reducing sugars. Individual and cocktail effect of enzymes on starch and cellulose hydrolysis was performed under the optimum parameters of saccharification. Reducing sugars released was analyzed by 3,5-dinitrosalicylic acid (DNS) method and expressed as % w/w of *C. forskohlii* root powder. Residual glucose was detected by glucose oxidase-peroxidase (GOD-POD) kit [35].

2.4. Detoxification of C. forskohlii root hydrolysates

Two pretreatments *viz*. overliming and passing through hydrophobic polymeric resin, were evaluated for detoxification of *C. forskohlii* root hydrolysate.

In the pretreatment by overliming, the hydrolysate was placed on a magnetic stirrer and heated to 50 °C. $Ca(OH)_2$ was added gradually and mixed by using magnetic stirrer until the pH reached 9 to 10. The hydrolysate was then maintained at 50 °C for 30 min [36]. The hydrolysate was vacuum filtered to remove the recovered solids ($CaSO_4$). The pH of the overlimed filtrate was adjusted to 6.5 with 0.1 N HCl and used for ABE fermentation.

Amberlite XAD-4 resin was obtained from Sigma Aldrich (Mumbai, India). Amberlite XAD resins are nonionic macroreticular cross linked polymers of styrene divinyl benzene with a mean pore diameter of approximately 140 Å. Amberlite XAD-4 (1.5:1) was added to the hydrolysate and allowed to equilibrate at room temperature (28 ± 2 °C) for 3 h. The resin-hydrolysate slurry was then filtered through Whatman #1 filter paper. The filtered hydrolysate was then adjusted to pH 6.5 with 0.1 N HCl and used for ABE fermentation [37].

A combined detoxification wherein the root hydrolysate was first treated with lime and then followed by Amberlite XAD-4 resin was also carried out.

2.5. Determination of total phenolic content of C. forskohlii root hydrolysate

The amount of total phenolics before and after the pretreatment of *C. forskohlii* root hydrolysates was determined with the Folin-

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