Fuel 134 (2014) 477–484

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

The effects of single and combined cellulosic agrowaste substrates on bioethanol production

Esra Imamoglu *, Fazilet Vardar Sukan

Department of Bioengineering, Faculty of Engineering, University of Ege, 35100 Bornova, Izmir, Turkey

HIGHLIGHTS

- Bioethanol production was carried out by *Escherichia coli* KO11.
- Importance of the combination of cellulosic substrates.
- The growth rate increased with increasing substrate C/N ratio.

ARTICLE INFO

Article history:
Received 6 July 2013
Received in revised form 14 February 2014
Accepted 28 May 2014
Available online 18 June 2014

Keywords:
Bioethanol
Escherichia coli KO11
Cotton stalks
Rice hulls
Combination of cellulosic substrates

ABSTRACT

The aim of this study was to investigate the effect of the single and combined substrates, namely rice hulls and/or cotton stalks hydrolysates media prepared by dilute acid hydrolysis on the bioethanol production using *Escherichia coli* KO11 for the economic feasibility of efficient production system. The cells were incubated on an orbital shaker at a shaking frequency of 160 rpm under semi-anaerobic conditions at the temperature of 30 °C during 96 h of the fermentation period. The ethanol concentration reached the maximum level of 20.69 g/L with the maximum yield of 0.44 (g ethanol/g reducing sugar) when the biomass concentration was 2.32 g/L corresponding to a growth rate of 0.023 h^{-1} at the highest C/N ratio (27.13) in the combination of 70% rice hulls and 30% cotton stalk hydrolysate medium at the end of the fermentation period of 96 h. The experimental results indicated that the combination of cellulosic substrates proves to have positive effects on the bioethanol production.

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1. Introduction

The EU target for 2020 is for biofuels to contribute 10% of the energy used by the transport sector. Currently, bioethanol is mainly produced from wheat, corn, rye and sugar beet, crops which are also used for human consumption [1]. As a result of the use of these agricultural crops for the bioethanol productions, the world food balance could be adversely affected and the price of feed and food could rise. The practice of planting crops solely for the production of energy could also result in exploitation of forests and arable lands. Furthermore, the increasing dependency on oil imports and the growing emissions of greenhouse gases are the two main concerns which justify the introduction of public policy incentives in Europe for developing lignocellulosic (second generation) ethanol [2]. On the other hand, the integration of second generation ethanol (derived from lignocellulosic materials) with the first generation ethanol production (conventional; derived from sugar and starch crops) may require a lower investment, since some operations (e.g. concentration, fermentation, distillation, storage and cogeneration) may be shared between both plants [3].

Lignocellulosic biomass obtained as agricultural byproducts and industrial residues is an abundant, inexpensive, and renewable source of sugars, and is a desirable feedstock for the sustainable production of liquid fuels and chemical products through the biorefinery processes [4,5]. Agricultural residues are easier than wood to use as feedstocks for biofuels due to their lower lignin and higher hemicellulose contents [6]. Moreover, the residues (mainly lignin) may also be used as fuel for the production of steam and electricity [7,8]. In this respect, the sustainable production of bioethanol from lignocellulosic biomass is expected to become one of the most credible alternatives within a few years [2]. One of the major difficulties that would be faced by bioethanol technology developers will be the geographical region of feedstock, logistics and annual crop yield [9].

The price of U.S. produced bioethanol is approximately $ 2.39 per gallon in 2013, as reported by NEO (Nebraska Energy Office)

* Corresponding author. Tel./fax: +90 2323884955.
E-mail address: esraimamoglu@yahoo.com (E. Imamoglu).

http://dx.doi.org/10.1016/j.fuel.2014.05.087
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[10]. As reported by TMENR (Turkish Republic Ministry of Energy and Natural Resources) [11], the total consumption of fuel-oil was 22 million tons, 3 million tons and 160 thousand tons of which were benzene and bioethanol, respectively. According to TUIK (Turkish Statistical Institute) [12], 900 thousand tons of paddy was harvested from 99 thousand ha of planting areas and 180 thousand tons of rice hulls removed. On the other hand, 2.6 million tons of cotton was harvested from 481 thousand ha of planting areas and 15.5 million tons of cotton stalks obtained in Turkey. As a result, the ratios of waste to product (W/P) were 1/5 and 6/1 for rice hulls and cotton stalks, respectively in Turkey. Rice hulls and cotton stalks consist of 28.6% and 47.1% cellulose, 28.6% and 24.1% hemicelluloses, 24.4% and 22% lignin and 18.4% and 6.3% extractive matter, respectively [13,14].

Escherichia coli KO11 was genetically engineered to produce ethanol from pentose and hexose sugars by inserting genes encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) from the bacterium *Zymomonas mobilis*. *E. coli* KO11 can efficiently metabolize complex mixtures of sugars derived from the acid hydrolysis of lignocellulosic biomass [15]. It is more resistant to toxic compounds such as organic acids and furans generated from lignocellulose hydrolysis than the ethanol-producing bacterium *Z. mobilis*. *E. coli* needs minimal growth requirements and has a higher growth rate than *Saccharomyces cerevisiae* [16]. The KO11 strain was tested successfully on various hydrolysates under semi-industrial conditions in fermentations up to 10,000-L capacity [17,18]. In an interlaboratory comparison of the performance of various bacterial and fungal ethanologens on a xylose-rich corn cob hydrolysate, *E. coli* KO11 showed the highest ethanol yield and was considered to be the most promising ethanol producer [18,19]. On the other hand, the most commonly used microorganism for industrial ethanol production is *S. cerevisiae*. The recombinant xylose fermenting strain *S. cerevisiae* TMB3400 [20] have been used in simultaneous saccharification and fermentation (SSF) of sugar cane bagasse [21] showing that the co-fermentation of xylose and glucose can be achieved in SSF [22].

The aim of this study was to compare the effects of the single and combined hydrolysates media on the bioethanol production by *E. coli* KO11 and to evaluate the effects of various ratios of the combinations of rice hulls and cotton stalks economically.

2. Materials and methods

2.1. The preparations and dilute acid pre-treatments of cellulosic agrowaste substrates

The rice hulls (RH) were obtained from Sah Gida San. Tic. Ltd. Sti., Ipsala, Edirne, Turkey, and the cotton stalks (CS) were obtained from the University of Ege, Faculty of Agriculture, Izmir, Turkey. They were milled in a hammer mill (Brook Crompton Series 2000, UK) to pass through a 1 mm screen in order to obtain a homogeneous particle size and increase the yield of acidic hydrolysis. The milled cellulosic substrates were dried in an oven (Memmert GmbH, Germany) at 70 °C for one night.

Each of these milled cellulosic substrates at a solid loading of 30% (w/w) was mixed with 0.4 M H_2SO_4 and pretreated in an autoclave at 121 °C under the pressure of 0.10 MPa for 60 min. The detoxification process (overliming method) was carried out using 340 mM $Ca(OH)_2$ at 60 °C for 30 min with rapid mixing of 400 rpm. Each hydrolysate was adjusted to initial pH 6 with 6 M KOH and then separated (10,000 rpm, 30 min) using centrifuge separator (Westfalia Separator Mineral oil Systems GmbH D-59302, Germany) to remove any precipitate formed before using it as substrate [23].

If necessary, they were thoroughly mixed to form the hydrolysate combinations of 50% (v) RH and 50% (v) CS, 60% (v) RH and 40% (v) CS, 70% (v) RH and 30% (v) after obtaining liquid phase as a hydrolysate from each solid substrate. The dry Luria–Bertani (LB) medium ingredients except glucose were added to the single and combined hydrolysates media and were not sterilized for fermentations.

2.2. Strain maintenance and preparation of Inocula

E. coli KO11 was provided by courtesy of Professor L.O. Ingram (University of Florida, U.S.A.). The recombinant *E. coli* KO11 is the derivative of *E. coli* B and contains the chloramphenicol acyl transferase gene (*cat*) and the *Z. mobilis* genes encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) for ethanol production. Stock cultures were stored in 40% glycerol at −80 °C.

The cells from a single well-isolated colony were inoculated into 250 mL cotton-plugged-conical flasks containing 50 mL of modified Luria–Bertani medium [24] with 50 g/L glucose. The cultures were incubated under static conditions for 16 h at 30 °C in the absence of antibiotic. Then, the cells were harvested by centrifugation (6000*g*, 5 min and 5 °C) and used as inocula for the experiments at the initial dry weight of 0.267 g cell/L.

2.3. Bioethanol production conditions

The cells were incubated on an orbital shaker (IKA® KS 4000ic Thermoshake, Werke GmbH & Co. KG, Germany) with a 20 mm shaking diameter at a shaking frequency of 160 rpm in 250 mL glass bottles containing 150 mL of media (the single or combined hydrolysates with dry LB ingredients) at 30 °C under semi-anaerobic conditions during 96 h of the fermentation period. Bottles were equipped with a gas outlet, and a sampling port through appropriately drilled polypropylene screw caps. The gas outlet port was fitted with a sterile 0.22 μm filter (Sartorius, Germany) to allow the escape of CO_2 formed during fermentation. No pH adjustments were made to shaken cells in the bottles throughout the fermentation period.

2.4. Experimental analysis

Optical density (OD) was measured at 550 nm in a spectrophotometer (Unicam–Helios Alpha, Cambridge, UK). OD_{550} was converted to dry cellular weight using a standard curve developed (1 OD_{550} = 0.32 g/L of dry cellular weight).

The total carbohydrate was determined using the phenol–sulphuric acid method at the absorbance value of 490 nm [25]. Reducing sugar concentration was analyzed using the dinitrosalicylic acid (DNS) method where the absorbance was measured at 540 nm [26] by UV/VIS spectrophotometer (GE Healthcare Ultrospec 1100 pro, UK).

Samples collected from the bottles were centrifuged at 7379*g* for 15 min via a micro-centrifuge (Sigma, USA 1-14 (10014)). The supernatant was then filtered through 0.22 μm cellulose acetate filters. Ethanol (EtOH), volatile fatty acids (VFAs: butyrate (HBut), isobutyrate (IsoBut), caprionate (HCap), isocaprionate (IsoCap), heptanoic acid (Hepta), propionate (HPr), valate (HVal) and isovalate (IsoVal)) and formic acid (HFor) concentrations were measured using a Gas Chromatograph (6890 N Agilent Technologies Network GC System) equipped with a flame ionization detector and a DB-FFAP 30 m × 0.32 mm × 0.25 mm capillary column (J&W Scientific, USA) [27]. The acetic acid (HAc), lactic acid (HLac), succinic acid (HSuc), 2,3-butanediol (2,3-BD), glucose and xylose were determined and quantified by HPLC (Thermo Scientific, USA) with a Phenomenex Rezex RHM Monosaccharide (H+) 300 mm × 7.8 mm ion exchange column, using a Thermo Refractive Index Detector (Thermo Scientific, USA) as reported by Gungormusler et al. [28].

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