Renewable Energy 98 (2016) 216–220

Contents lists available at ScienceDirect

Renewable Energy

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

Evaluation of oil palm front hydrolysate as a novel substrate for 2,3-butanediol production using a novel isolate *Enterobacter cloacae* SG1

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ARTICLE INFO

Article history:
Received 5 January 2016
Received in revised form
6 February 2016
Accepted 9 February 2016
Available online 28 February 2016

Keywords:
Batch fermentation
Pretreatment
Acid pretreated liquor
Pentose utilization
Biomass

ABSTRACT

The present work deals the production of 2,3-butanediol, an industrially important chemical, through biological route using a novel bacterial isolate. Batch fermentation trials for the production of 2,3-butanediol were carried out using the isolated strain *Enterobacter cloacae* SG-1. The study resulted 14.67 g/l of 2,3-butanediol with 48.9% yield using glucose as the carbon source. In order to replace the expensive glucose in the production media, non-detoxified oil palm frond hydrolysate was used as the carbon source and it resulted 2,3-butanediol yield of 7.67 g/l. Process parameters like pH, temperature and initial sugar concentration were optimized. The ability of strain *E. cloacae* SG-1 for utilization various pentoses and hexoses were evaluated and found that the strain can utilize both arabinose and glucose with a comparable 2,3-butanediol yield.

1. Introduction

2,3-Butanediol(BDO) is a valuable platform chemical that is commonly synthesized from petrochemical derivatives. In manufacture of aviation fuels, printing inks, perfumes, fumigants, explosives, plasticizers, food additives, moistening and softening agents [1]. It can be used as the substrate for the production of 1, 3-butadiene, diacetyl, methyl ethyl ketone and diurethane, that are the monomeric units for the synthesis of artificial rubber, food additives, high-quality aviation fuels and cosmetic products respectively. Occurrence of the stereoisomers in meso, D and L forms BDO increases its specific applications in the field of pharmaceutical as a carrier and anti-freezing agent [2].

Petroleum derivatives as raw materials for the production of monomers or value added chemicals are not entertaining, because of the increased price, declining resources and environmental issues [3]. Greener approach for 2,3-BDO production would be through microbial fermentation using cost effective raw materials like waste byproducts and lignocellulosic hydrolysate. In physiological metabolism of hexoses and pentoses via mixed acid pathway, bacteria can produce 2,3-BDO [4]. Several microorganisms are reported for 2,3-butanediol fermentation like *Bacillus licheniformis* [5], *Bacillus subtilis* [6], *Bacillus amyloliquefaciens* [7], *Paenibacillus polymyxa* [8], *Serratia marcescens* [9], *Klebsiella pneumonia* [10], *Klebsiella oxytoca* [11], *Enterobacter cloacae* [12] and *Enterobacter aerogenes* [13]. As 2,3-BDO occur in various stereo isomeric forms, the end product of each strain can be of any form based on the mode of cultivation and type of substrate. Either pentose or hexose converted to pyruvate by any metabolic pathway and it is then converts to ά-acetolactate, acetoin and 2,3-BDO. The key enzymes involved in 2,3-BDO production are α-acetolactate synthase, α-acetolactate decarboxylase, acetoin reductase (2,3-butanediol dehydrogenase) respectively [1]. Acetoin is the major intermediate produced during 2,3-BDO fermentation. The reaction between acetoin and 2,3-BDO is reversible so that there will be a high chance of converting the produced 2,3-BDO to acetoin. The fermentation conditions play a significant role in controlling the level of other byproducts of mixed acid pathway like ethanol, acetate, lactate, succinate, formate and also the intermediate acetoin in the fermentation broth [4].

Synthetic and purified carbon sources for fermentation increases the process cost, so readily available and inexpensive carbon source lignocellulosic materials can be utilized. Many

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http://dx.doi.org/10.1016/j.renene.2016.02.030
0960-1481/

lignocellulosic materials like agricultural wastes, wood hydrolysate [10], hexose rich non-grain plants such as Jerusalem artichokes [14] and food industry wastes like whey permeate [8], sugar beet molasses [15] had reported as the raw material for 2,3-BDO fermentation. Here we introduce oil palm frond (OPF) hydrolysate as a new substrate for 2,3-BDO production. Oil palm (*Elaeis guineensis*) is one of the major oil crop plant cultivated mainly in Malaysia and Indonesia. Palm oil is the highest consuming edible oil globally as well as in India. OPF is one of the major biomass during oil palm cultivation that contain 60% of the volume of total oil palm biomass residues. In palm cultivation OPF is treated as the byproduct that is usually burnt. Recently it was found that oil palm front contained large amount of fiber and less protein [16]. The high sugar content and daily availability make OPF as a best and unexploited source for 2,3-BDO production. These renewable sugars are better substrates as it is not disturbing any biological ecosystem, where few other substrates like corn starch, rice straw can be used as feed and fodder for cattle.

This study basically focuses on the efficiency of OPF hydrolysate as an alternative carbon source for the fermentation of 2,3-BDO using a newly isolated *E. cloacae* SG-1. Fermentation conditions and parameters were optimized in order to enhance the production of 2,3-BDO. Sugar utilization profile and growth pattern of the isolate during the fermentation process using both commercial glucose media and OPF derived sugar media were monitored.

2. Materials and methods

2.1. Raw material& dilute acid pre-treatment of OPF

Raw OPF were collected from a local plantation in Kerala, washed with tap water to remove the dirt, cut into small pieces of 3–4 cm in length and dried at 60 °C in hot air oven for 48 h. The dried OPF were milled using a knife mill and sieved so that the particle size were less than 0.5 mm. The biomass were stored at room temperature until used. The OPF was pretreated using 1.5% (v/v) concentrated H_2SO_4 at 121 °C for 45 min. The biomass loading during pretreatment was 10%. After pretreatment, the slurry was neutralized using 10 N NaOH and the filtered with muslin cloth. Quantification of total reducing sugar concentration in the acid pretreated liquor (APL) and biochemical compositional of raw and pretreated OPF were analyzed.

2.2. Media and microorganism

The bacterial strain was cultured in seed medium consist of peptone 10 (g/l), beef extract 10.0 (g/l), sodium chloride 5.0 (g/l). The 24hr old seed inoculum was transferred to fermentation medium consist of glucose 30.0 (g/l), yeast extracts 5.0 (g/l), K_2HPO_4 14.0 (g/l), KH_2PO_4 6.0 (g/l), $(NH_4)_2SO_4$ 2.0 (g/l), sodium citrate dehydrate 1.0 (g/l), $MgSO_4.7H_2O$ 0.2 (g/l) [13]. The initial pH of the media was maintained at 6.5. Fermentation was carried out at 30 °C, 200 rpm for 48 h. For optimizing inoculum age for 2,3-BDO fermentation, seed media of 6, 12, 18, 24, 30, 36 hold inoculums were taken and fermentation were carried out. Optimum inoculum size was found out by fermentation using 2–10% (v/v) inoculum, each 1 ml inoculum contained 7×10^9 CFU. Initial sugar concentration was optimized by varying glucose concentration from 10 to 60 g/l.

In the fermentation experiments with OPF derived sugars, neutralized APL was directly added to the media without detoxification. All media components were same except glucose and total sugar concentration was adjusted so that it equal to glucose concentration.

Different paddy field soil samples from Kollam district, Kerala, India were serially diluted using 1% saline and plated on 2% agar medium consists of glucose 50.0 (g/l), peptone 4.0 (g/l), yeast extract 4.0 (g/l) and NaCl 5.0 (g/l). The resultant colonies were selected and screened for 2,3-BDO fermentation and the product was evaluated by thin layer chromatography method as described by Saran et al. [17]. The isolate SG1, which produced the highest amount of 2,3-BDO, was selected for further studies. The isolate was maintained on nutrient agar slants at 4 °C throughout the study. 16srRNA gene sequencing of the strain shows 99% similarity with *E. cloacae* and named as *E. cloacae* SG1.

2.3. Analytical methods

Total reducing concentration in APL was estimated by DNS method [18]. Compositional analysis was done according to NREL protocol [19]. Growth pattern of organism was checked by measuring the optical density (OD) at 600 nm. The sugar concentration in the fermentation broth was estimated by high performance liquid chromatography (HPLC) (Shimadzu Prominence UFLC, Japan) with Rezex RPM-monosaccharide column (300 × 7.8 mm) with refractive index (RI) detector. The mobile phase was milliQ water with a flow rate of 0.6 ml/min. Column temperature was maintained at 80 °C. The concentration of 2,3-BDO in the fermentation broth was determined by HPLC equipped with a differential RI detector and organic acid (Aminex HPX-87H, 300 × 7.8 mm) column. The analysis was carried out at 65 °C using 0.01 N H_2SO_4 as the mobile phase. The flow rate of the mobile phase was 0.6 ml/min.

3. Results

3.1. Compositional analysis of OPF

The compositional analysis of raw and pretreated OPF showed that pre-treatment degraded a major portion of hemicellulose (Table 1). Comparatively cellulose content also increased after pretreatment because of the reduction of acid soluble compounds [20]. The total extractives include moisture, cellobiose, proteins etc. The sugar analysis of APL showed that it contain (g/l) glucose-28.45, xylose-30.97, galactose-3.97, mannose 6.05, the rest 30.61 consist of arabinose, fructose, cellobiose etc. (Data not shown).

3.2. Optimization of fermentation conditions

The optimum age of seed culture media was found to be 24 h (Fig. 1). The optimization of inoculums size revealed that 2% inoculum was best for the production of 2,3-BDO and the other inoculum load showed a decrease in 2,3-BDO production (Fig. 2).

The optimum fermentation time was found to be 24 h and beyond 24 h the concentration of 2,3-BDO was declining gradually and acetoin concentration tends to increase. pH was a major factor in the 2,3-butanediol fermentation. Because intracellular acidification is one of the key factor in production of 2,3-BDO, thus changing the metabolic flux towards neutral compounds [21]. A wide pH range from 5 to 8 was selected for optimization studies. At pH below 4.0 the organism cannot grow. Like many previous

Table 1
Compositional analysis of raw & pre-treated OPF.

Composition	Raw OPF	Pre-treated OPF
Cellulose (%)	38.04 ± 0.73	49.39 ± 3.13
Hemicellulose (%)	19.81 ± 0.17	8.16 ± 0.79
Lignin (%)	29.97 ± 1.14	18.79 ± 3.90
Ash (%)	0.46 ± 0.81	0.12 ± 0.02
Extractives (%)	11.72 ± 0.03	23.54 ± 0.01