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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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.

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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 α-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
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 during oil palm 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 stalk, 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 H2SO4 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), KH2PO4 14.0 (g/l), KH2PO4 6.0 (g/l), (NH4)2SO4 2.0 (g/l), sodium citrate dehydrate 1.0 (g/l), MgSO4.7H2O 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 × 108 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 Prominance UPLC, 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 H2SO4 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.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Raw OPF</th>
<th>Pre-treated OPF</th>
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<tbody>
<tr>
<td>Cellulose (%)</td>
<td>38.04 ± 0.73</td>
<td>49.39 ± 3.13</td>
</tr>
<tr>
<td>Hemicellulose (%)</td>
<td>19.81 ± 0.17</td>
<td>8.16 ± 0.79</td>
</tr>
<tr>
<td>Lignin (%)</td>
<td>29.97 ± 1.14</td>
<td>18.79 ± 3.90</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>0.46 ± 0.81</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Extractives (%)</td>
<td>11.72 ± 0.03</td>
<td>23.54 ± 0.01</td>
</tr>
</tbody>
</table>
reports on 2,3-BDO fermentation, E. cloacae SG1 gives maximum diol yield at pH 6.5 [4]. The optimized temperature for fermentation was 37 °C (Data not given). When the organism was cultivated at other temperature the product yield was comparatively less (Fig. 3).

3.3. Fermentation using different sugars

Initial sugar concentration for 2,3-BDO fermentation was selected as 30 g/l because, at this concentration highest BDO concentration with maximum yield obtained. While increasing sugar concentration beyond 60 g/l, a problem of dark coloration in the media or charring of sugars during sterilization encountered. So, further increase in sugar concentration was not opted for the studies. The organism was able to utilize both hexoses and pentoses for 2,3-BDO production (Fig. 5). When arabinose was given as a sole carbon source, production of 13.06 g/l 2,3-BDO was monitored, which is comparable to glucose (14.67 g/l), while the yield was 43.53% and 48.9% respectively. With xylose as a carbon source, the 2,3-BDO production was very low (4.65 g/l) and the yield was 15.5%. Earlier reports shows that many potent BDO producers efficiently utilize xylose [22] (Figs. 4 and 6).

3.4. Growth pattern in glucose v/s OPF media

In OPF media the bacteria take a longer lag phase than glucose...
media. This may be because of the inhibitors present in the APL like furfural and hydroxymethyl furfural which normally inhibit the bacterial growth [23]. After 24 h of fermentation 7.24 g/l of BDO was obtained using OPF media while glucose media gives 11.48 g/l. Complete utilization of sugar was seen using glucose media, while using OPF media there are some residual sugars (less than 5%) mainly xylose and glucose. This may be because of the presence of inhibitors, the additional compounds in the APL may reduce the availability to glucose.

4. Discussion

Pre-treatment of biomass using dilute acid, alkali, steam explosion, liquid hot water and wet oxidation along with enzymatic digestion [24] was already best studied for various biomass like sugar cane bagasse, rice straw, wheat straw etc. [25]. There are very few reports on pre-treatment of OPF using dilute acid. Dilute acid could be a cost effective, efficient pre-treatment technique for hemicellulose removal. The residual cellulose and lignin fraction could be utilized for enzymatic digestion for ethanol or 2,3-BDO production. Bioethanol production from oil palm biomass using various fungi were previously reported [26]. The compositional analysis of raw and pretreated OPF revealed that the chemical composition of OPF contain a major portion of cellulose and lignin. OPF contain high amount of fiber and very low protein [16]. There will be slight changes in the chemical composition according to plant variety, geographical conditions etc. Hemicellulose content in selected biomass was comparatively low, but it can also provide a significant amount of reducing sugars in a single operation of dilute acid pretreatment at 121 °C for 45 min that was optimized in laboratory conditions (Data not given).

2,3-BDO production from various agro-industrial lignocellulosic biomass was successfully done in the past few decades itself. Raw materials like rice straw, sugar cane bagasse [31], sugar cane molasses [27], corn cob molasses [28], de-protienated whey, hydrolyzed whey permeate [8] were already well studied for 2,3-BDO production using different bacterial strains. Fermentation methods were either batch or fed-batch, accordingly yields might varied. But in general, the 2,3-BDO yield from lignocellulosic was comparatively low [13,31]. So the requirement of cost effective, an alternative substrate which could produce a higher amount of diol using with minimal media came into the study. Report [26] shows that OPF is a cost effective substrate with simple pre-treatment conditions and minimal addition of salts could produce ethanol. Wong et al. [31] used enzymatic hydrolysate of sugar cane bagasse and rice straw for 2,3-BDO production and a concentration of 8.26 g/l and 24.6 g/l could achieve with a yield of 15 and 62% respectively and the study was using a Klebsiella strain. But in this study using non-detoxified OPF hydrolysate 7.67 g/l 2,3-BDO with 25.56% yield could be obtained. While comparing the enzyme cost and additional operational cost in the previous studies [31], dilute acid pre-treatment OPF could be more efficient for economical large scale production. There are a few reports on E. cloacae for 2,3-BDO production. Saha and Bothast [12] reported a simultaneous saccharification and fermentation procedure for corn fiber hydrolysate using an E. cloacae which produced 12 g/l of 2,3-BDO. Dai et al. [27] recently reported an E. cloacae which gives 90.8 g/l 2,3-BDO from sugar cane molasses. Perego et al. [13] had also reported an E. aerogenes for 2,3-BDO fermentation from various food industry wastes, but the BDO concentration was lesser than the present study. When compared to the earlier reports of microbial 2,3-BDO production [1] the maximum yield were in a range of 45–50% with various Klebsiella, Serratia, Enterobacter strain. In this study also we could attain a similar yield compared to the past reports [1,31]. While considering the opportunistic pathogenicity compared to Klebsiella and Serratia, E. cloacae would be a better answer for the large scale production of BDO using lignocellulosic biomass like OPF.

Fermentation using different sugars clearly showed that E. cloacae SG1 can utilize both glucose and arabinose very well and almost complete utilization of all sugars in batch culture was noted. The strain SG-1 is a native strain of paddy field soil and with sophisticated fermentation methodology it can be utilized for larger scale production of 2,3-BDO. Process parameters like pH, temperature, initial sugar concentration, inoculum size [28] and inoculum age are major factors influencing the 2,3BDO yield, hence these parameters were optimized with commercial glucose media. pH play a major role in controlling the byproducts concentration [29]. During fermentation pH changes were noted in the fermentation broth from 6.5 to 5. This was because of the acid byproducts of mixed acid pathway. Alkaline pH of 8.0 showed a total reduction of both acetoin and BDO concentration.

The study observed that incubation temperature of 37 °C was found to be best for E. cloacae SG-1, while some previous studies suggests that 30 °C was optimum for some strains of E. cloacae [30]. Initial sugar concentration for batch fermentation was fixed to 30 g/l with 49.8% of BDO yield. Increasing sugar concentration had a positive effect on diol concentration, but gradually the yield was decreasing. Isolate was equally utilizing both glucose and arabinose in its growth. Even though xylose concentration in APL was very low, and xylose fermentation alone giving only 15.5% yield, more than 95% xylose in APL was completely utilized by the organism within 24 h.

5. Conclusions

The newly isolated E. cloacae SG1 was found to be a good producer of 2,3-BDO, which can utilize both glucose and arabinose. OPF was found to be a promising renewable carbon source for the production 2,3-BDO. Non-detoxified acid pretreated OPF hydrolysate media could give 7.67 g/l of BDO by batch culture. Media engineering and detoxification of OPF hydrolysate could increase the diol yield.

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References


