 doi:10.20944/preprints201704.0085.v1

Article

Isoprenoids Production from Lipid-Extracted Microalgal Biomass Residues Using the Engineered *E. coli*

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Abstract: Microalgae are recognized as a third generation feedstock for biofuel production due to its rapid growth rate and lignin-free characteristic. In this study, the lipid extracted microalgal biomass residues was used as the material to produce isoprene, α-pinene and β-pinene with the engineered *E. coli* strain. We adopted an optimal sulfuric acid hydrolysis method (1:7 ratio of solid to acid solution, 32 % (w/v) concentration of sulfuric acid solution at 90℃ for 90 min) to convert holocellulose into glucose efficiently (6.37 g/L) and explored a novel detoxification strategy (phosphoric acid/calcium hydroxide) to remove inhibitors notably. 55.32 % acetic acid, 99.19 % furfural and 98.22 % 5-HMF (5-hydroxymethylfurfural) were cut down with the phosphoric acid/calcium hydroxide method, and the fermentation concentration of isoprene (223.23 mg/L), α-pinene (382.21 μg/L) and β-pinene (17.4 mg/L) using the detoxified hydrolysate as the carbon source account for approximately 86.02 %, 90.16 % and 88.32 % of those produced by the engineered *E. coli* strain fermented on pure glucose, respectively.

Keywords: lipid extracted microalgae, isoprenoids, detoxification

1. Introduction

As the simplest member of isoprenoids, isoprene is an important platform chemical which could be used for medicines, pesticides fragrances, especially rubber synthetic [1-3]. Its derivatives, α-pinene and β-pinene, therefore, have the potential to be used for aviation fuel production owing to the compact structure and reactive olefin functionality properties [4, 5]. Due to the bottleneck of feedstock, biotechnology can be applied to isoprenoids production by using more economical resources.

At first, starch (potato, wheat etc.) was used, the first generation feedstock, as the material to produce bioethanol and other biobased materials. However, large amount of crop was consumed during the fermentation, which could lead to severe food shortage, especially in the developing countries [6]. And later, in the past two decades, second generation feedstock, lignocellulosic materials (straw, wood and grass), were explored in biofuel production. Yet, these second generation materials were not applied to commercialization due to their low yield, high cost resulting from hydrolysis process owing to inherent lignin in particular, but lignocellulosic materials are cheap, renewable and don't have competitiveness with food supply [6, 7]. Therefore, it requires a third generation feedstock to both satisfy the demand for biofuel production on a large scale and maintain ecological balance at the same time. Currently, researchers proposed algae as an ideal alternative for biofuel production for its rapid growth, a unicellular or simple multicellular structure, lignin-free property and easy availability on earth [8].

Hence, in this paper, we utilized the lipid extracted microalgal biomass residues (LMBRs) as the feedstock to biosynthesize isoprene and its derivatives (α-pinene, β-pinene) using the engineered *E. coli*. It began with conversion of LMBRs into fermentable sugar, and then the microbial fermentation was performed to generate bio-based isoprenoids. However, as it is rather time- and money-

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consuming, the LMBRs were hydrolyzed with sulfuric acid rather than enzyme during the hydrolysis process. Finally, 6.37 g/L of glucose was achieved based on the following hydrolysis condition: ratio of solid to acid (1:7), acid concentration (32 %), hydrolysis temperature (90 ℃), hydrolysis time (90 min). Since inhibitors (weak acid, furfural, 5-HMF) were formed during acid hydrolysis process [9], we adopted five different detoxification strategies with recombination to remove the inhibition consequence on the fermentation[9-13]. Among five methods, phosphoric acid/calcium hydroxide detoxification combination, the best detoxification way, was applied to remove acetic acid, furfural and 5-HMF about 55.32 %, 99.19 % and 98.22 % respectively. Finally, 223.23 mg/L of isoprene, 382.21 μg/L of α-pinene and 17.4 mg/L of β-pinene were obtained with the engineered *E. coli* strain fermented on the detoxified hydrolysate of LMBRs, accounting for about 86.02 %, 90.16 % and 88.32 % of isoprene, α-pinene and β-pinene production by *E. coli* strain using pure glucose, respectively.

2. Methods and materials

2.1. Materials

LMBRs used in this study were kindly provided by Prof. Tianzhong Liu (Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, China) which were the residual *Chlorella* biomass derived from oil extraction processes[14]. Briefly, algae was mixed with ethanol at 1.5 MPa, 120 ℃ for 50 min, after cooled to room temperature, the residual algae and the oil solution was separated with centrifugation. Finally, the residual algae was collected to be used in this study. LMBRs were oven dried at 60 ℃ and milled to 60 mesh size. Sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$), phosphoric acid (H_3PO_4) were bought from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ion exchange resin (D310) was purchased from Tianjin Nankai university resin company (Tianjin, China). All of chemical reagents were analytical reagent grade.

2.2. Compositions analysis

The compositions of LMBRs were determined with previous methods. Cellulose and hemicellulose were analyzed according to the method of NREL Laboratory Analytical Procedure (LAP) [15]. This method is used to detect the monomeric sugars (glucose, xylose, arabinose, galactose and mannose) by HPLC to calculate the cellulose and hemicellulose content[15]. Total protein and lipid were measured based on the previous studies[16, 17]. Protein content was calculated according to the total nitrogen content of LMBRs and Nitrogen Factor. Lipid was firstly transfed into fatty acid methyl esters, and then wasdetermined according to the fatty acid methyl esters content. Ash analysis of LMBRs was measured according to the NREL Laboratory Analytical Procedure (LAP)[18]. Ash was detected according to residue weight after dry oxidation at 550 to 600 ℃. And organic solvent extractives (O.S.E) were analyzed with TAPPI Standard Methods[19]. O.S.E of LMBRs was extracted by Soxhlet extraction apparatus, then the extractive was dried at 105 ±3 ℃ to detect the O.S.E content according the weight of extractive and none extracted sample. Water component was measured with gravimetric method after dried completely.

2.3. Optimization of Acid hydrolysis process

Acid hydrolysis was optimized with four kinds of factors including time, temperature, concentration of sulfuric acid and the ratio of solid to acid. After lipid-extracting, the recovering microalgae residues was collected with filtering apparatus. Then, the residues were washed with distilled water till the pH reached neutral and dried at 60 ℃. The hydrolysis condition of hydrolysis condition was explored according to *Table 1*. And the glucose concentration was detected and calculated by the standard using HPLC. All of experiments were repeated three times.

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Table 1. Hydrolysis condition was explored at four kinds of factors of LMBRs

Optimizing factor	Scope of optimizing factor	Immovable factors
Ratio of solid to acid	1:3, 1:5, 1:7, 1:9	20 % (w/v) concentration of sulfuric acid solution, at 80 ℃ for 30 min
Concentration of sulfuric acid	28 % (w/v), 30 % (w/v), 32 % (w/v), 34 % (w/v)	1:7 ratio of solid to acid solution, at 80 ℃ for 30 min
temperature	80 ℃, 90 ℃, 100 ℃, 110 ℃	1:7 ratio of solid to acid solution and 32 % (w/v) concentration of sulfuric acid solution for 30 min
Time	30 min, 60 min, 90 min, 120 min	1:7 ratio of solid to acid solution and 32 % (w/v) concentration of sulfuric acid solution, at 90 ℃

2.4. Inhibitor and sugar analysis of hydrolysate

Inhibitors and glucose were analyzed using HPLC equipped with a refractive index (RID) detector, and the concentration of inhibitors and glucose were calculated by converting peak area to gram via their calibration curves. The HPX-87 Bio-Rad Ion Exclusive Column (300×7.8 mm, USA) was used for glucose detection, 0.005 M sulfuric acid was supplied at the mobile phase with a flow rate of 0.6 ml/min, and the column temperature was 55 ℃. The concentrations of furfural and 5-HMF were determined with C-18 column (Nucleosil 100-5 C18, Merck, Darmstadt, Germany) with a gradient of 5-100% (v/v) methanol and 0.025% (v/v) of trifluoroacetic acid with a flow rate of 0.8 ml/min, formic acid and acetic acid were determined with AS11HC column which eluted with 80% (v/v) water and 20% (v/v) of a mixture consisting of 0.4 m M Na OH and methanol (50% v/v) at a flow rate of 1.4 m L/min [9].

2.5. Detoxification

To produce isoprenoids with engineered *E. coli*, inhibitors should be removed first because them would be generated when hydrolyzing the microalgae biomass with sulfuric acid. As is shown in the following, five strategies have been developed to remove the inhibitors in the acidolysis hydrolysate based on the previous studies with some modifications[9-13]:

A: The hydrolysate was adjusted to pH 10 with sodium hydroxide, and then the solution was readjusted by sulfuric acid to pH 5. Anhydrous sodium sulphite was added into the solution with the loading concentration 1 g/L and heated to 100 ℃ for 15 min. Then, 1 % (w/v) activated carbon was mixed in the solution and incubated at 40 ℃ with shaking at 200 rpm for 1 h.

B: The hydrolysate was neutralized with calcium hydroxide. After that, 1 % (w/v) activated carbon was added into the solution and incubated at 40 ℃ with shaking at 200 rpm for 1 h.

C: Sodium hydroxide was used to regulate the pH of the hydrolysate to 5.0, and then 1 % (w/v) activated carbon was added into the solution and incubated 40 ℃ with shaking at 200 rpm for 1 h.

D: Anion exchange resin (D301, China) was put into the hydrolysate with the loading concentration of 20 % (w/v) until the pH to 5.5. The mixed solution was kept at 24 ℃, with shaking 200 rpm for 1 h.

E: The pH of the hydrolysate was initially adjusted to 7.0 with calcium hydroxide, after that, the pH was readjusted to 5.5 with phosphoric acid.

All of the hydrolysate was filtered with decompress filter to obtain the supernate after the inhibitors being removed with different detoxification methods.

2.6. Biosynthesis and analysis of isoprenoids using the engineered E. coli strains

Engineered *E. coli* strain, YJM25 was used during isoprene fermentation [20], YJM29 was utilized in α-pinene biosynthesis[21] and FHR-2 (*E. coli* BL21 (DE3) (pACYDuet-1-*mvaE-mvaS-GPPS2-QH6*, pTrcHis2B-*ERG8-ERG12-ERG19-IDI1*) was applied for β-pinene production[22]. The fermentation procedure was carried out as reported in the previous study[23] with some modifications. Shake-flask experiments were performed in triplicate using a series of 600 ml sealed

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shake flasks containing 100 ml fermentation medium including glucose 2 g/L or suitable concentration of acid hydrolysates. Optical density (OD) of the bacteria was measured with a spectrophotometer (Cary-50, Varian Inc. USA) at a wavelength of 600 nm. The isoprene, α-pinene and β-pinene production were analyzed as described earlier[20-22] by a gas chromatograph (GC) equipped with a flame ionization detector (FID) and a HP-1 column (30 m×0.25 mm×0.25 μm, Agilent). The concentration of target production was calculated with peak area on the bases of a standard curve.

3. Results and discussion

3.1. Chemical composition of LMBRs

As is shown in Fig 1, LMBRs mainly consists of hollocellulose (18.03 %), protein (48.12 %), ash (24.35 %), lipid (0.28 %) and water (9.20 %) leaving only a tiny amount of lipid (0.28 %). It suggested that lipid has almost been extracted completely. What is more delighting is that lignin was not found anymore as it created the most unfavorable threat to lignocellulose hydrolysis rejection. Therefore, it must be removed prior to hydrolysis[24]. As we all know, to get rid of lignin during the process of pretreatment is rather costly and time-consuming as well. Hence, compared with lignocellulose (rice straw, switchgrass etc.), LMBRs (with absence of lignin) was considered the ideal alternative as the biofuel-producing material [25].

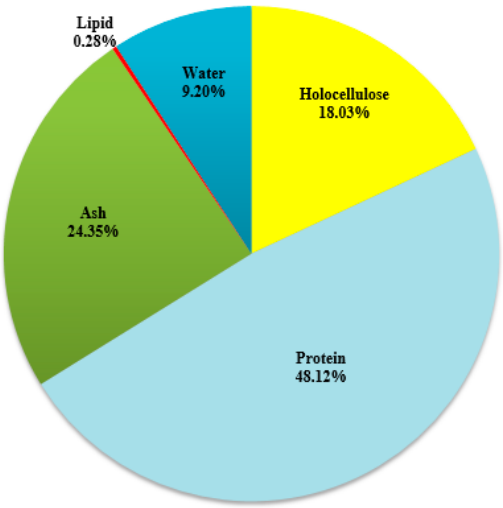

Fig 1. Chemical composition of lipid extracted microalgae biomass.

3.2. Optimization of acid hydrolysis condition

In this study, the ''one-factor at-a-time'' optimization strategy was applied to augment hydrolytic efficiency by optimizing time, temperature, acid concentration and ratio of solid to acid solution respectively[26, 27]. Fig 2 has shown that the maximal glucose concentration (6.37 g/L) was obtained under the hydrolysis condition of 1:7 ratio of solid to acid solution, 32 % (w/v) concentration of sulfuric acid solution at 90℃ for 90 min whose combined optimization effect could contribute to an approximately 142-fold increase in glucose concentration.

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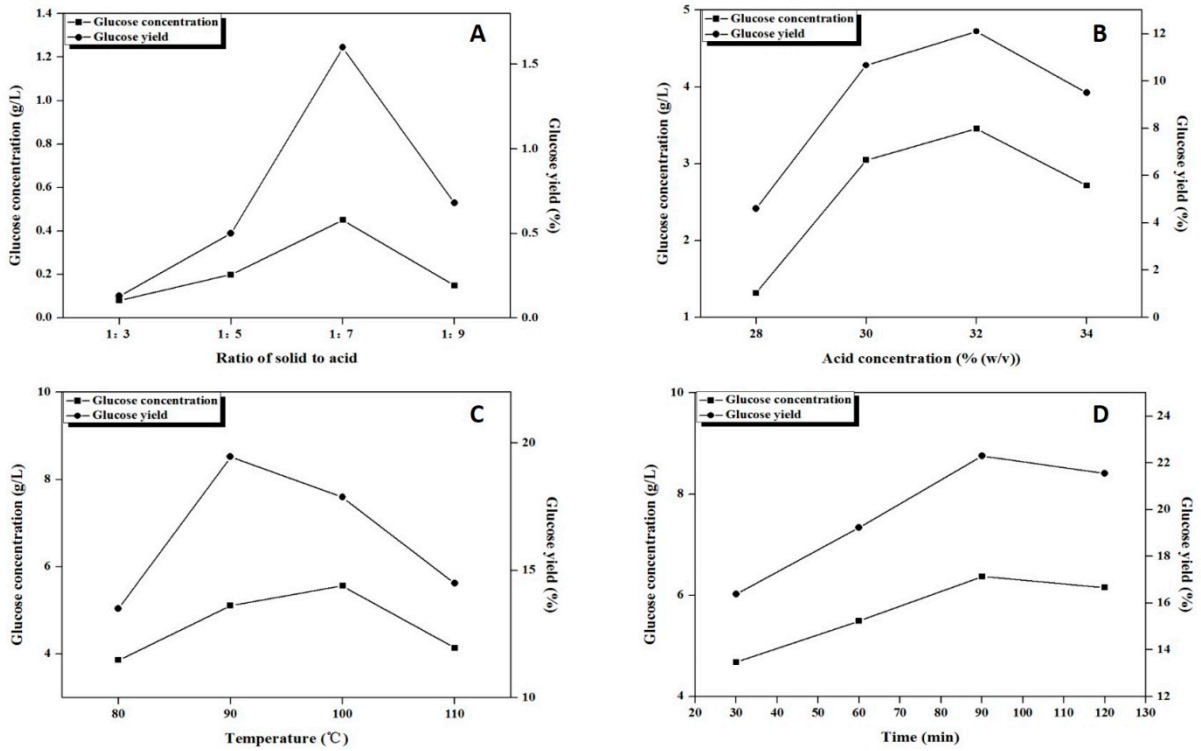

Fig 2. Effects of acid hydrolysis condition on the glucose concentration and yield. A: Effect of ratio of solid to acid on acid hydrolysis efficiency; B: Effect of concentration of sulfuric acid on acid hydrolysis efficiency; C: Effect of temperature on acid hydrolysis efficiency; D: Effect of reaction time on acid hydrolysis efficiency.

3.3. Inhibitor changes with different detoxification methods

Although acidolysis reaction could convert the carbohydrate into fermentable monomeric sugar, by-product compounds (weak acid, furfural and 5-HMF, etc.) were formed simultaneously, which could inhibit the microorganism from producing high value-added products[28]. Those toxic compounds could undoubtedly affect the target product yield. Due to inhibitor only derived from holocellulose and lignin, however, there was no lignin exist in LMBRs, therefore, inhibitors come from holocellulose degradation. Compared with other inhibitors, acetic acid is formed primarily by hydrolysis of acetic groups of hemicellulose, furfural and 5-HMF are derived from pentoses and hexoses, respectively[29]. Considering acetic acid, furfural and 5-HMF as the major inhibitors existed in the hydrolysate solution, Thus, we adopted five different kinds of detoxification strategies to explore the optimal detoxification method and analyzed the changes in the concentration of three types of inhibitors (acetic acid, furfural and 5-HMF). As is shown in Fig 3, compared with the raw hydrolysate, the concentration of all three types of inhibitors was reduced more or less in five different detoxification hydrolyzates. Interestingly, in the detoxification hydrolysate using E method, the acetic acid, furfural and 5-HMF were reduced about 55.32 %, 99.19 % and 98.22 %, respectively. It showed that E detoxification method has the most efficient on the removal of three above-metioned inhibitors.

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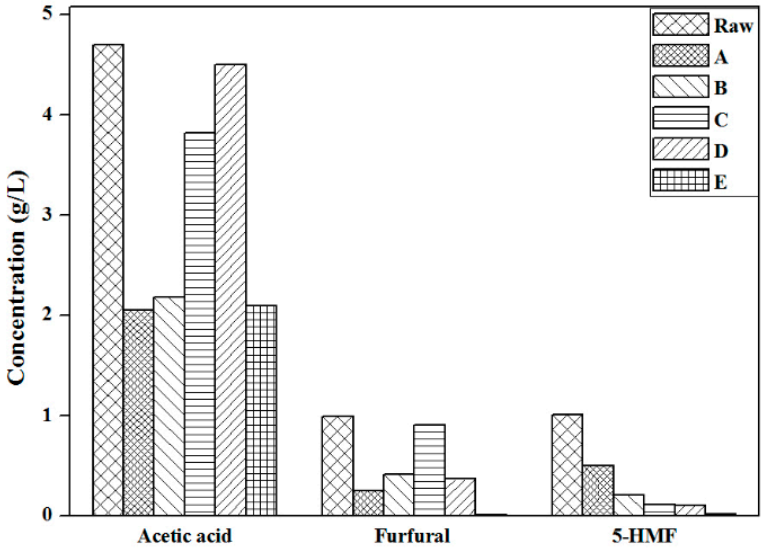

Fig 3. Inhibitors concentration of raw and detoxification hydrolysates with A-E five different detoxification strategies. A-E five detoxification methods are corresponding to the A-E five methods described at "methods and materials" list respectively.

To date, many of investigations have shown that neutralization, overliming, activated charcoal, ion exchange resin and reducing agents have the ability to remove the inhibitors of the acidolysis hydrolysate [9-13]. In the present study, we employed five different above-mentioned detoxification methods. And finally, we observed that each of them has potentially removed acetic acid, furfural and 5-HMF inhibitors in some degree. The most significance of the result is that among these five detoxification strategies, calcium hydroxide and phosphoric acid combination could eliminate the three inhibitors mostly. Compared with other detoxification methods, calcium hydroxide/phosphoric acid combination strategy assumes a series of advantages such as lower cost, easier operation and better capacity to remove inhibitory compounds. Though methods A and B could decrease more than half of the acetic acid volume, the concentration of the furfural and 5-HMF inhibitors still remains too high. The hydrolysates after detoxified by methods C and D have a lower 5-HMF concentration, however, the acetic acid of the two hydrolysates was removed rarely. As a result, when cost, removed efficiency and difficulty of detoxification process are all taken into consideration, method E has the highest potential to be used in the future industrialization field.

3.4. Effect of detoxification on isoprenoids production

Fig 4 has presented the isoprene produced by the engineeried *E. coli* YJM25 with seven different carbon sources including pure glucose, raw hydrolysate, hydrolysates detoxified by A-E methods. As can be shown, compared with the raw hydrolysate fermentation (160.26 mg/L), isoprene yield produced by using group E hydrolysate (223.23 mg/L) was increased by up to about 40 % after the E detoxification, representing 86.02 % of that produced by using pure glucose as carbon source, which is much higher than those using other five groups of hydrolysate (A-D and raw) and lower than that using the pure glucose fermentation (259.52 mg/L). Therefore, calcium hydroxide/phosphoric acid detoxification method was proved to be better than other four kinds of detoxification methods for isoprene production with *E. coli*. As is expected, Fig 5 and Fig 6 have also demonstrated that, in comparision to raw hydrolysate fermentation, the yield of α-pinene and β-pinene were increased about 35 % and 52 % when using hydrolysate detoxified by E method respectively. And α-pinene and β-pinene production accounted for about 90.16 % and 88.32 % of the yield biosynthesized on pure glucose. These results revealed that the *E. coli* fermentability was improved greatly after using calcium hydroxide/phosphoric acid detoxification strategy.

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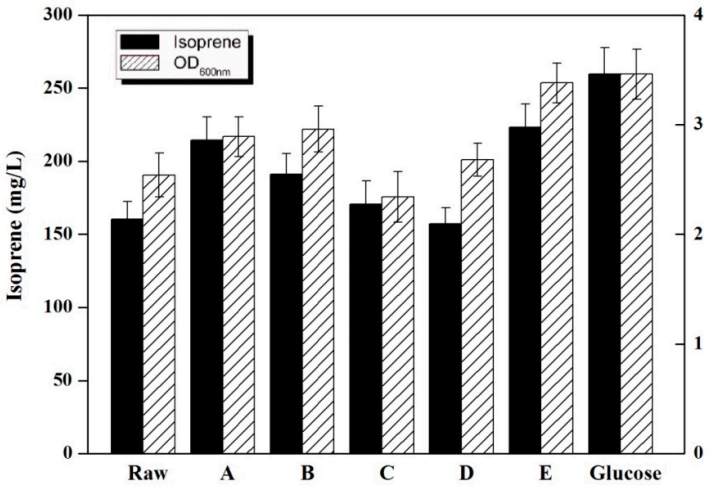

Fig 4. Isoprene production by the YJM25 using different carbon sources containing pure glucose, raw and detoxification hydrolysates. When OD_{600} reaches ~0.6, cultures was induced at 30 ℃ for 24 h using 0.5 mM IPTG. The experiment was performed in triplicate.

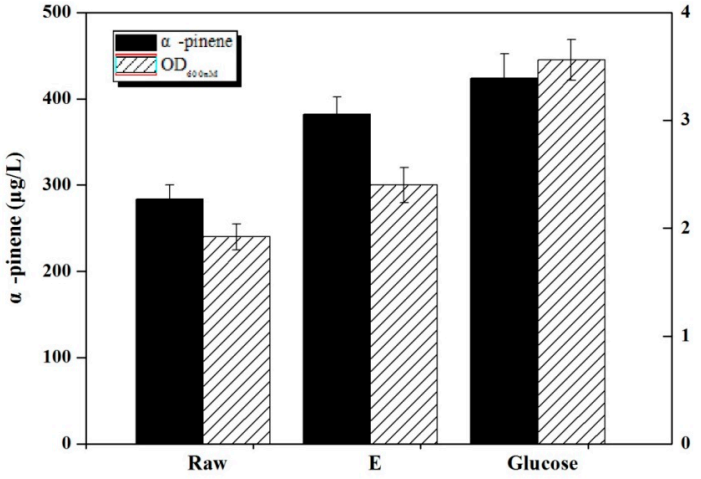

Fig 5. α-pinene production by the YJM29 using different carbon sources containing pure glucose, raw and detoxification hydrolysate with E method. When OD_{600} reaches ~0.6, cultures was induced at 30 ℃ for 24 h using 0.5 mM IPTG. The experiment was performed in triplicate.

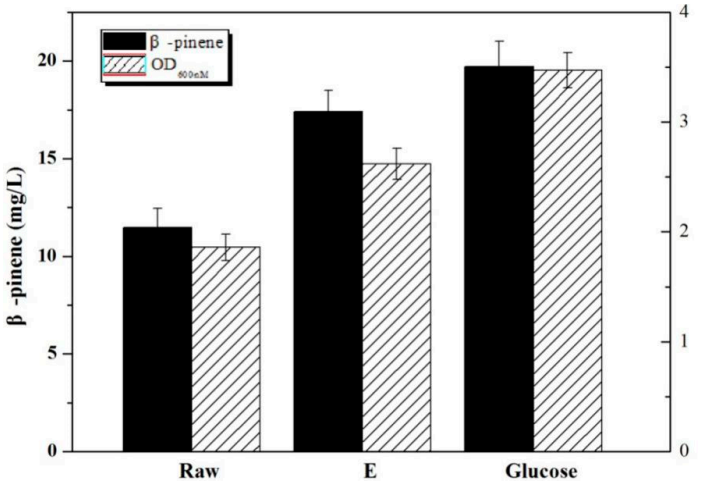

Fig 6. β-pinene production by FHR-2 using different carbon sources containing pure glucose, raw and detoxification hydrolysate with E method. When OD_{600} reaches ~0.6, cultures was induced at 30 ℃ for 24 h using 0.5 mM IPTG. The experiment was performed in triplicate.

Microorganism growth could be restricted by toxic inhibitive compounds such as furfurals, 5-HMF and organic acid[30]. Among those inhibitors, furfural was found to be able to inactivate the cell replication by breaking down the single-strand[31-34]. Organic acid (acetic acid) derived from hemicellulose could cross the cell membrane, which resulted in the lower cell pH than normal and consequently inhibited cell activity [33, 35]. Ultimately, the cell activity of *E. coli* was inhibited and directly reduced its fermentation ability. As shown in Fig 3 and Fig 4, compared to other different carbon sources, the hydrolysate with E detoxification method had the least inhibitors concentration and consequently achieved the highest isoprene yield.

Chandel AK *et al* has reported that although the toxicity of acetic acid on microorganisms is lower than furans (furfural and 5-HMF), the synergistic toxicity is possibly more severe when furans are in conjunction with acetic acid[30]. In this study, we have achieved the similar result: as shown in Fig 3 and Fig 4, the amount of acetic acid in both hydrolysates detoxified of A and E was similar while the furans was higher in A hydrolysate than in E hydrolysate, which resulted in the lower isoprene production from A hydrolysate than in E hydrolysate. It suggested that the reason for lower isoprene production from A hydrolysate might be the synergistic toxicity of acetic acid and furans.

4. Conclusions

LMBRs were potential material for biofuel production due to the absence of lignin and fermentable sugar existing in microalgae residual biomass. 6.37 g/L glucose was achieved after hydrolyzing the lipid extracted microalgae with 1:7 ratio of solid to acid solution, 32 % (w/v) concentration of sulfuric acid at 90 °C for 90 min. In order to increase the production of isoprene, α-pinene and β-pinene, inhibitors including acetic acid, furfural and 5-HMF of acid hydrolysate were removed about 55.32 %, 99.19 % and 98.22 % respectively by the new method (phosphoric acid/calcium hydroxide). Finally, 223.23 mg/L isoprene, 382.21 μg/L α-pinene and 17.4 mg/L β-pinene were produced, which accounted for about 86.02 %, 90.16 % and 88.32 % of pure glucose fermentation, respectively. Therefore, lipid extracted microalgae was regarded as a promising material for isoprenoids and other bio-based chemicals production after the acid hydrolysate was detoxified by phosphoric acid/calcium hydroxide mixture.

Acknowledgements: The authors appreciate Professor Tianzhong Liu for providing the LMBRs. This work was financially supported by the Natural Science Foundation of Shandong Province, China (Grant no. ZR2015BM021), the National Natural Science Foundation of China (Grant no. 21572242), the Project of Science and Technology for People's Livelihood of Qingdao (no. 15-9-2-94-nsh), the special project of science and technology development for construction (Grant no. JK2015-22), the Talents of High Level Scientific Research Foundation (Grant no. 6631113326) of Qingdao Agricultural University, the National Natural Science Foundation of China (Grant no. 31300599), the Talents of High Level Scientific Research Foundation (Grant no. 6631113318) of Qingdao Agricultural University, and theNationalNatural Science Foundation ofChina (Grant no. 31172012/c1506).

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