Valorization of by-products from soybean-based biodiesel production plant for 1,3-propanediol production

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Abstract

Purpose: The increase of biodiesel production has resulted in the production of significant quantities of by-products. Soybean-based biodiesel production generates soybean meal and crude glycerol as by-products. These by-product streams could be valorized in bioprocessing concepts to generate value-added products, including 1,3-propanediol (PDO).

Methods: Soybean meal and crude glycerol were utilized for the formulation of nutrient-complete fermentation media for the production of PDO. The nutrient-rich hydrolysate derived from soybean meal supplemented with crude glycerol was evaluated as the sole fermentation media for PDO production by two bacterial strains. Fed-batch fermentations using crude glycerol and commercial nutrients and/or soybean meal hydrolysate were carried out. The inhibitory effect of metabolite products along with salts present in crude glycerol was investigated in microplate assays.

Results: Both *Citrobacter freundii* strains could efficiently utilize crude glycerol to synthesize PDO. Maximum PDO production employing crude glycerol reached 33.6 and 35 g/L using *C. freundii* VK-19 and FMCC-8 respectively. The valorization of soybean meal hydrolysate as nutrient rich supplement resulted in increased yield and productivity for both strains. An inhibition from lactic acid, formic acid and acetic acid in concentrations higher than 20 g/L was observed for both strains. Significant inhibition by sodium salt was observed in both strains.

Conclusion: Soybean meal hydrolysate and crude glycerol were successfully metabolized by both bacterial strains to generate PDO. The by-products produced during the fed-batch fermentation have negative effect on PDO production and hence process optimization could be further undertaken aiming to reduce the inhibitory metabolites.

Keywords

1,3-propanediol (PDO), crude glycerol, soybean meal, *Citrobacter freundii*, bioprocess

Introduction

The inevitable exhaustion of fossilized resources comprises a key driving factor to undertake significant steps for the transition of the currently fossil-dependent economy towards a bio-based economy. New biorefining processes have been established to sustainably convert renewable natural resources into bio-based chemicals and biofuels. Ongoing development and implementation of biorefinery processes focus on biofuels production such as bioethanol, biodiesel, biogas, biomethanol, synthetic biofuels and biohydrogen [1]. Inauguration of mandates and policies on biofuels utilization has induced an increase in first generation biodiesel production. Biodiesel is produced by transesterification or alcoholysis process of vegetable oils or animal fats with methanol. Soybean, sunflower seed, cottonseed, rapeseed and palm oils are mainly used for biodiesel production. The growing demand for biodiesel production from oilseeds coincides with the generation of vast amounts of by-products, namely oilseed meals and crude glycerol. Currently, the predominant market outlet for oilseed meals is as animal feed supplements. Composition of oilseed meals may significantly differ; still the predominant components are protein, carbohydrates, mineral and phenolic compounds, hence providing a flourishing resource for the development of consolidated bioprocesses to be further integrated in biorefinery concepts. Oilseed meals could be fractionated to generate protein concentrates or protein isolates antioxidants, hulls and fibre as value-added products [2, 3, 4]. Nonetheless, in the viewpoint of restructuring industrial biodiesel facilities, the development of a two-stage bioprocess based on rapeseed and sunflower meal, including solid state fermentation (SSF) and enzymatic hydrolysis, exhibits an alternative to formulate fermentation rich feedstocks aiming to substitute the conventional and expensive nutrient supplements [5]. On the other hand, glycerol is used in cosmetic and food manufacturing industry. The purity of crude glycerol exiting biodiesel producing plants ranges from 66-90 % [6]. The main contaminants found in crude glycerol streams are water, sodium or potassium salts depending on the catalyst and unreacted triglycerides. Although purification methods have been proposed, their implementation is hindered in small scale facilities. Recently, crude glycerol has been widely evaluated in the fermentative production of specialty chemicals including 1,3-propanediol (PDO), 2,3-butanediol, acrolein, succinic acid and propionic acid [7].

PDO is one of the bulk chemicals used as an intermediate in the manufacturing of polymers, cosmetics and lubricants. PDO is mainly produced by chemical synthesis through hydration of acrolein or hydroformylation of ethylene oxide which requires high temperature, high pressure and expensive catalysts [8]. Alternatively, PDO can be synthesized by fermentative bioconversion processes attained mainly by bacteria belonging to the genera *Clostridium*, *Enterobacter*, *Lactobacillus*, *Citrobacter* and *Klebsiella*. Market demand for PDO is rapidly flourishing whilst projected to reach 150 kt by 2019 [9].

Process development for enhanced bioconversion yields along with the cost of fermentation substrate entail the prevailing bottlenecks in the industrialization of bio-based PDO.

Therefore, many microbial bioconversion processes have been proposed and evaluated for the fermentative production of PDO from renewable resources including extensive research on glycerol. In this study, the effect of crude glycerol and the potential use of soybean meal hydrolysate as nutrient source for PDO production by two different *Citrobacter freundii* strains was investigated during batch and fed-batch experiments. Varying initial nitrogen concentrations in the form of amino acids and peptides using soybean meal hydrolysates were also assessed during bioreactor trials. Moreover, the inhibitory effect on microbial growth of *C. freundii* strains by the main products of the fermentation as along with the salts usually presented in crude glycerol was undertaken.

Materials and methods

Microorganisms

An industrial strain of *Aspergillus oryzae* isolated from soy sauce starter at the company Amoy Food LTD (Hong Kong) was used in solid state fermentations (SSF) for the production of crude enzyme consortia, essentials for the hydrolysis of soybean meal. Two bacterial strains *C. freundii* FMCC-8 and *C. freundii* VK-19 previously isolated and identified by the Department of Food Science and Human Nutrition, AUA, were used for 1,3-propanediol (PDO) fermentation.

Raw materials

Crude glycerol and soybean meal (SBM) were obtained from the biodiesel plant Petroleo Brasileiro (Petrobras) and originated from soybean oil transesterification.

Solid state fermentation (SSF)

Solid state fermentations were carried out at 250 mL Erlenmeyer flasks containing 5 g of SBM sterilized at 121 °C for 20 min. Prior to each SSF, the fungal strain *A. oryzae* was sporulated according to the method reported by Kachrimanidou et al. [10]. The moisture content was adjusted at 65 % (w/w, db) by means of inoculation with a fungal spore suspension and subsequently, the flasks were incubated at 30 °C for 48h.

Production of soybean meal hydrolysate

After 48 h, the fermented solids were suspended in sterilized distilled water and macerated using a kitchen blender followed by vacuum filtration in order to obtain the enzyme consortia extract. The enzymatic rich extract was further applied in SBM hydrolysis with an initial proteolytic activity of 5.32 U/mL. The hydrolysis of SBM was conducted in 1-L Duran bottles containing an initial SBM solid concentration of 50 g/L. The Duran bottles were placed in a water bath at 45°C while agitation was achieved with magnetic stirrers.

Bacterial fermentations

Batch and fed-batch fermentations were performed in a 1-L bioreactor (New Brunswick Scientific, USA) with a working volume of 0.8 L. Fermentation cultures were inoculated with 10% (v/v) inoculum in exponential growth phase. The incubation temperature was 30˚C, agitation rate was 180 rpm and pH value was regulated at 7.0 using 5 M NaOH and 5M HCL solution. In the batch bioreactor trials evaluating the effect of initial glycerol concentration, crude glycerol used as the sole carbon source in varying concentrations supplemented with the nutrient medium reported by Metsoviti et al. [11] with few modifications. In the batch bioreactor trials where SBM hydrolysate was valorized as nitrogen and minerals supplement, SBM hydrolysate was diluted in order to reach different initial free amino nitrogen concentration (FAN), while crude glycerol was used as carbon source under the optimized initial concentration. During the fed-batch experiments pure and crude glycerol were used as carbon source under the optimized initial concentration with the above modified medium. Also, fed-batch experiments were conducted using crude glycerol and SBM hydrolysate under the optimized initial concentrations. During fed-batch fermentations, when glycerol concentration was lower than 10 g/L, a concentrated glycerol solution (500 g/L) was added into the medium in order to maintain the glycerol concentration in range of 10-15 g/L.

Microplate inhibition assay

The inhibition of various organic acids along with PDO, K^+ and Na^+ salts on *C. freundii* VK-19 and FMCC-8 growth was examined using a 96-well microtitre plate system (Infinite M200-PRO, TECAN). For the inhibition trials different organic acids, namely acetic acid, succinic acid, formic acid, lactic acid, as well as PDO and salts in the form of K_2SO_4 and NaCl were used at an initial concentration ranging from 1 to 90 g/L.

Results

Effect of initial glycerol concentration on PDO production

The composition of crude glycerol is mainly affected by the origin of oil feedstock, the conditions of the transesterification reaction as well as the post-treatments involved in biodiesel production. Crude glycerol generated from biodiesel plants primarily consists of residual methanol, unreacted fatty acids, methyl esters of fatty acids, glycerides and water. Moreover, significant quantities of inorganic salts and micro-nutrients such as calcium, magnesium, phosphorous could be encountered in crude glycerol streams [7]. In this study the feasibility of crude glycerol derived from soybean-based biodiesel plant was evaluated for PDO production by *C. freundii* bacterial strains. As demonstrated in Table 1, PDO production increased with increasing glycerol concentrations for the bacterial strain *C. freundii* VK-19, reaching a maximum PDO production of 40.2 g/L when an initial glycerol concentration of 136 g/L was employed. Nonetheless, the increment in glycerol concentration entailed a gradual increase in by-product formation thus reducing the glycerol conversion yield. On the other hand, for the experiments performed with the

strain *C. freundii* FMCC-8, the maximum PDO production (18.6 g/L) was achieved when an initial glycerol concentration of 41.6 g/L was used. Notwithstanding, maximum PDO yield and productivity of 0.51 g/g and 0.63 g/L/h, respectively were achieved when the initial glycerol concentration employed was 20 g/L glycerol. In addition, it was also observed that increasing substrate concentration resulted in a progressive decrease in cell mass production demonstrating substrate inhibition towards *C. freundii* FMCC-8.

Table 1 Effect of initial glycerol concentration on growth and PDO production by *C. freundii* VK19 and FMCC-8 strains on batch fermentations.

Time (h)	Initial glycerol (g/L)	μmax (h^{-1})	DCW (g/L)	PDO (g/L)	Yield (g/g)	Productivity (g/L/h)	AA (g/L)	SA (g/L)	FA (g/L)	LA (g/L)	EtOH (g/L)
Citrobacter freundii VK-19											
18	31.6	0.36	1.7	14.7	0.47	0.82	5.6	2.6	1.4	4.3	1.5
26	53.0	0.27	2.9	26.3	0.50	1.01	8.0	3.4	3.1	3.8	3.5
29	71.8	0.24	3.3	31.3	0.45	1.08	8.4	1.9	2.3	6.8	0.5
56	90.5	0.23	4.3	37.2	0.43	0.66	8.1	3.0	1.6	15.8	2.2
85	136.2	0.12	4.5	40.2	0.37	0.47	6.6	7.3	0	21.7	2.8
Citrobacter freundii FMCC-8											
17	20.9	0.22	3.5	10.7	0.51	0.63	4.2	1.7	0.8	1.6	0.8
25	31.3	0.35	3.0	15.0	0.49	0.60	4.0	1.7	1.1	2.8	0.0
31	41.6	0.24	3.0	18.6	0.45	0.60	5.0	2.2	1.0	4.2	1.3
45	52.0	0.20	2.8	13.9	0.45	0.31	3.3	1.6	0.0	3.3	0.0

Effect of initial FAN concentration of soybean meal hydrolysate on PDO production

Soybean meal was employed for the production of a nutrient rich hydrolysate that was subsequently evaluated as fermentation feedstock for the production of PDO by two *C. freundii* strains. The produced hydrolysate was then diluted to achieve different initial FAN concentrations and was further evaluated as fermentation supplement to elucidate the effect on microbial proliferation and PDO synthesis. Crude glycerol was implemented at an initial concentration of 50 g/L and 20 g/L for *C. freundii* VK-19 and FMCC-8 respectively. The results from batch fermentation using different initial FAN concentrations for *C. freundii* strains are demonstrated in Figure 1. An initial FAN concentration of 400 mg/L proved to be optimum for cultivation of *C. freundii* VK-19 resulting in 27.9 g/L PDO production corresponding to a bioconversion yield of 0.53 g/g and a productivity of 1.07 g/L/h. Likewise, concerning the experiments conducted with *C. freundii* FMCC-8 and soybean meal hydrolysate, PDO production proved to be similar within the range of the different initial FAN concentrations implying that SBM hydrolysate can provide the essential nutrients to sustain microbial growth and PDO production. Compared to the experiments conducted with the commercial nutrients the results obtained using SBM hydrolysate were enhanced for

both *C. freundii* strains demonstrating that SBM hydrolysate could serve as the sole nutrient source for to support microbial growth and PDO production.

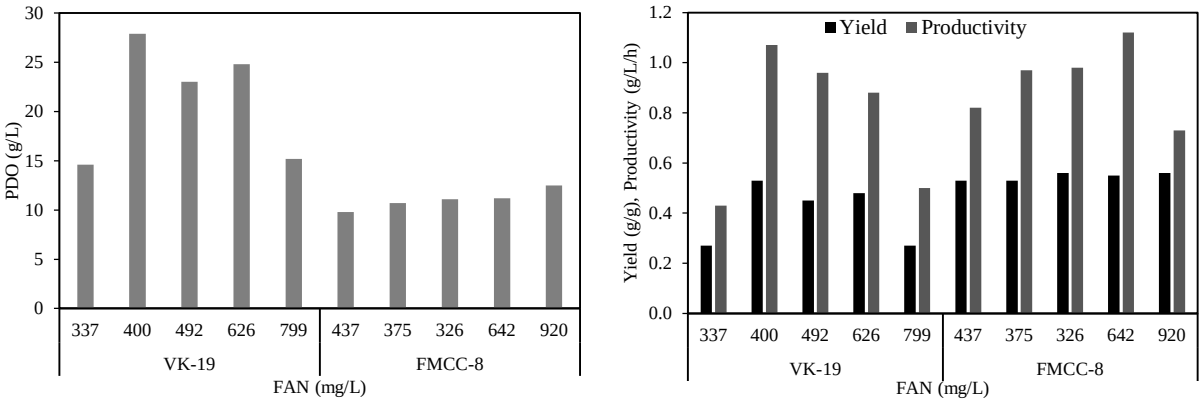

Figure 1 Effect of FAN concentration on PDO production, yield and productivity

Fed batch fermentations

Tthe results of the fed-batch cultivations for both bacterial strains performed with different type of glycerol are presented in Table 2.

Table 2 Results of fed-batch fermentations using two *C. freundii* bacterial strains

Glycerol	Nitrogen & nutrients	Time (h)	PDO (g/L)	Yield (g/g)	Productivity (g/L/h)	Acetic acid (g/L)	Lactic acid (g/L)	Succinic acid (g/L)	Formic acid (g/L)	EtOH (g/L)
					C. freundii FMCC-8					
pure	Commercial	43	43.6	0.28	1.01	14.9	3.6	8.2	16.3	7.1
crude	Commercial	74	39.5	0.54	0.53	9.8	7.0	3.1	0.0	1.5
crude	SBM	54.5	37.7	0.50	0.69	9.8	7.5	3.8	1.6	1.4
					C. freundii VK-19					
pure	commercial	71	34.2	0.33	0.61	7.6	9.7	2.8	6.8	3.1
crude	commercial	67	34.0	0.37	0.51	7.4	20.4	2.2	2.7	1.5
crude	SBM	60	36.8	0.43	0.61	9.0	14.3	3.6	4.5	1.7

In the case of *C. freundii* VK-19 maximum PDO production of 34.2 g/L and 34.0 g/L was achieved using pure and crude glycerol respectively. The higher conversion yield was obtained when crude glycerol was implemented as the carbon source, although the fermentation time was prolonged resulting in lower productivity (0.52 g/L/h). The valorization of crude glycerol and SBM hydrolysate results in increased PDO yield and productivity (0.43 g/g and 0.61 g/L/h respectively).

Regarding fed-batch experiments with *C. freundii* FMCC-8, when pure glycerol was evaluated maximum PDO synthesis reached 43.6 g/L that decreased to 35 g/L using crude glycerol as carbon source. The

presence of impurities seemed to impose a negative effect on biomass production entailing a proximate reduction of 67% in the latter culture. In fed-batch experiments with SBM hydrolysate and crude glycerol, PDO production reached 37.7 g/L with conversion yield of 0.55 g/g. In all cases the by-products formation, mainly lactic acid and acetic acid, accumulated in high concentrations and may impose inhibitory effects on PDO production.

Inhibitor effect of metabolites and impurities of crude glycerol on growth of C. freundii FMCC-8 and VK19 strains

During the fermentation of glycerol, microorganisms generate various metabolites including succinic acid (SA), formic acid (FA), acetic acid (AA), lactic acid (LA) and ethanol (EtOH) in order to obtain energy in the form of ATP. The composition of by-products differs depending on the microbial strain and the fermentation conditions. Formation of acetic acid and ethanol is necessary in order to supply energy for growth and maintenance. However, the formation of pyruvate-derived by-products may cause an inhibitory effect on growth and likewise on PDO production. In this study, the effect of organic acids and PDO on microbial proliferation of *C. freundii* VK-19 and FMCC-8 was evaluated in microtiter plate experiments. Figure 2 illustrates the normalized specific growth rate (μ) achieved with each component in various concentrations for *C. freundii* VK-19 and FMCC-8 respectively. Both strains demonstrate similar inhibition behavior on growth concerning LA, AA and FA whereas the specific growth rates decreased exponentially as the concentration of these acids was increased. The effect of SA and PDO on the proliferation of *C. freundii* strains was less pronounced even at higher concentrations.

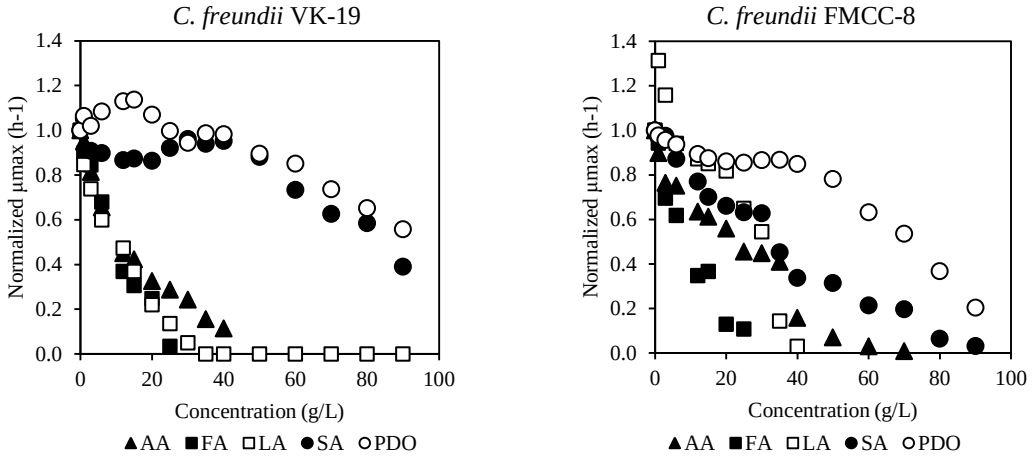

Figure 2 Inhibitory effect of metabolites

The effect of Na^+ and K^+ salts was also evaluated in microtiter experiments for *C. freundii* strains. Figure 3 demonstrates the specific growth rate obtained after microtiter fermentations in the presence of Na^+ and

K^+ salts added in the form of NaCl and K_2SO_4 in various concentrations for *C. freundii* VK-19 and FMCC-8 respectively. The addition of K_2SO_4 did not affect the bacterial growth for any of the strains but in fact, the presence of K_2SO_4 enhanced the cell growth leading to increased specific growth rates. On the other hand, a linear reduction of μ_{max} was observed with increasing concentrations of Na^+.

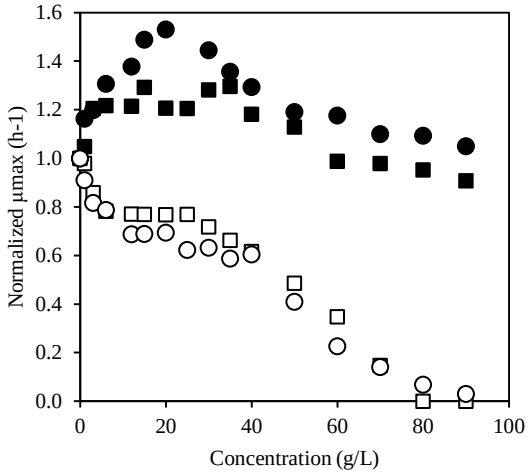

Figure 3 Inhibitory effect of Na^+ (unfilled symbols) and K^+ (filled symbols) salts for *C. freundii* VK-19 (cycles) and FMCC-8 (squares).

Conclusions

In the present study the by-products generated from soybean-based biodiesel plant were evaluated for PDO production. Crude glycerol and SBM hydrolysates were implemented in fed-batch cultures using *C. freundii* VK-19 and FMCC-8. The effect of inhibitors was also investigated, indicating an inhibition from LA, FA and AA in concentrations higher than 20 g/L for both strains. Apparently, *C. freundii* strains were more tolerant to SA and PDO even in high concentrations. The effect of crude glycerol impurities was also undertaken, demonstrating enhanced bacterial growth from K+ salts, whereas Na+ salts exhibited a restraining factor for both strains.

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