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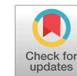
Research paper

Production of medium-chain carboxylic acids by anaerobic fermentation of glycerol using a bioaugmented open culture



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ABSTRACT

Biological production of carboxylic acids during glycerol fermentation *via* the chain elongation process was investigated. Glycerol is a by-product of the biodiesel production process and a convenient feedstock alternative for the carboxylate platform. In batch experiments, glycerol was used as substrate, whereas sludge (granular and flocculent) and ruminal liquid served as inocula. To improve the production of carboxylates, bioaugmentation with *Clostridium acetobutylicum* ATCC 824 was performed. Maximal production of *n*-caproic and *n*-caprylic acids was achieved with the use of the granular sludge. Bioaugmentation with *C. acetobutylicum* enhanced production of *n*-caproic acid in the presence of 100 mM ethanol. However, increasing ethanol concentration to 200 mM had no further enhancing effect on *n*-caproic production with or without bioaugmentation. The enhancement of *n*-caproic acid production was probably due to the increased production of *n*-butyric acid by *C. acetobutylicum*, because *n*-butyric acid is an important intermediate in the chain elongation process when ethanol is used as an electron donor.

1. Introduction

Glycerol is a by-product of the biodiesel production process and is derived from the transesterification reaction of oils from oilseeds and animal fats [1]. Despite residual glycerol is already used by the chemical industry, the increased demand for biodiesel may lead to an overproduction of this by-product. Biodiesel industry generates about 1 kg of glycerol per 10 kg of produced biodiesel [2]. According to the Organization for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization (FAO) of the United Nations [3], approximately 3.2×10^6 tons of glycerol have been generated from biodiesel production in 2015 worldwide [3]. Furthermore, an increase to about 3.8×10^6 tons is predicted for 2024, so alternative uses of this by-product must be developed.

Pure glycerol can be used by the food, pharmaceutical, chemical, and cosmetics industries [4]. Non-purified (residual) glycerol seems to be a promising feedstock to biological processes, because it contains a high organic matter fraction (chemical oxygen demand [COD] of $\sim 1260\,g\,L^{-1}$) [5], and is highly available on the market at low cost. Alternative uses of residual glycerol have been investigated for producing renewable energy *via* synthesis of hydrogen [1], methane [6], ethanol [7], butanol [8], as well as a variety of chemicals, such as 1,2-propanediol [2], 1,3-propanediol (1,3-PD) [9], 2,3-butanediol [10], and organic acids [11]. However, to the best of our knowledge, this is the first report regarding the production of medium-chain carboxylic acids (MCCAs), which are organic acids with 6–12 carbons, using residual glycerol as substrate.

MCCAs are used in the production of fragrances, pharmaceuticals, feed additives, antimicrobials, lubricants, rubbers, and dyes [12]. Among MCCAs, *n*-caproic acid, with a chain of 6 carbons, is a bio-based precursor of biofuel [13], and can be used as flavorants [14], and supplements in swine and poultry feed for control of enteric diseases [15]. Traditionally, *n*-caproic acid is produced *via* a petrochemical platform or extracted from oil seeds. However, the biological synthesis can be a more environmentally friendly alternative [14]. Recently, production of *n*-caproic and *n*-caprylic acids by an open culture from

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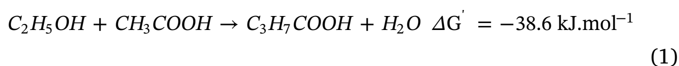
the granular sludge withdrawn from an upflow anaerobic sludge blanket (UASB) reactor was reported [16]. Agler and collaborators [17] achieved a high rate of *n*-caproic acid production that exceeded $2\ g\ L^{-1}\ d^{-1}$ using open microbial cultures. According to Spirito and co-authors [18], the use of mixed culture was chosen due its several advantages, i.e. non-sterile environment; easily adaptable microorganism consortia to various substrates; ability to operate in a (semi)-continuous mode.

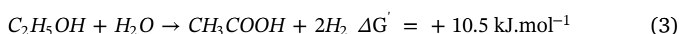
The biological production of *n*-caproic acid can occur by the carboxylic chain elongation process, in which short-chain carboxylic acids are converted to MCCAs with six or eight carbon atoms [19] *via* the reverse ß-oxidation metabolic pathway [20]. Equations (1) and (2) show the conversion of acetic acid and ethanol to *n*-butyric acid and then, to *n*-caproic acid [19]. Although it is thermodynamically possible, this reaction is not energetically self-sufficient because not enough adenosine triphosphate (ATP) is formed. Coupled with the biochemical pathway described in Equation (3), one mol of ATP has to be obtained from the oxidation of ethanol [18]. The latter reaction generates sufficient energy for producing *n*-butyric and *n*-caproic acids (Equations (1) and (2)). The whole process produces free Gibbs energy (ΔG°) of −366.0 kJ, and 5 mol of *n*-caproic acid (Equation (4)), resulting in $73.2\ kJ\ mol^{-1}$ of produced *n*-caproic acid.

If other substrates, such as glycerol, are the only carbon source and energy, it would be necessary to include preliminary fermentation for producing the chain elongation precursors (acetic acid and/or *n*-butyric acid and ethanol), which is shown in Equation (5) [21]. Several studies have reported high yields of acetic and *n*-butyric acids, and ethanol from crude glycerol [22,23]. Coupled with the biochemical pathway described in Equation (5), six mols ATP and four mols of $NADPH_2$ are generated, which can be used for providing energy for the reactions of Equations (1) and (2) without oxidation of ethanol. In this case, more ethanol, which serves as the electron donor for the chain elongation process, is available.

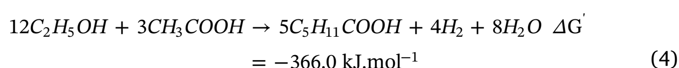


$$C_2H_5OH + CH_3COOH \rightarrow C_3H_7COOH + H_2O \quad \Delta G^{'} = -38.6\ kJ.mol^{-1} \tag{1}$$

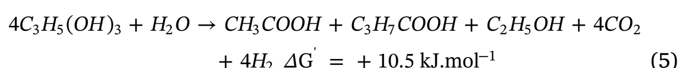
$$C_2H_5OH + C_3H_7COOH \rightarrow C_5H_{11}COOH + H_2O \quad \Delta G^{'} = -38.8\ kJ.mol^{-1} \tag{2}$$



$$C_2H_5OH + H_2O \rightarrow CH_3COOH + 2H_2 \quad \Delta G^{'} = +10.5\ kJ.mol^{-1} \tag{3}$$



$$12C_2H_5OH + 3CH_3COOH \rightarrow 5C_5H_{11}COOH + 4H_2 + 8H_2O \quad \Delta G^{'} = -366.0\ kJ.mol^{-1} \tag{4}$$



$$4C_3H_5(OH)_3 + H_2O \rightarrow CH_3COOH + C_3H_7COOH + C_2H_5OH + 4CO_2 + 4H_2 \quad \Delta G^{'} = +10.5\ kJ.mol^{-1} \tag{5}$$

Very few currently isolated microorganisms can synthetize *n*-caproic acid, and among them are *Eubacterium pyruvativorans* [24], *Megasphaera elsdeni* [25], *Clostridium* sp. *BS-1* [26], and *Clostridium kluyveri* [27]. Despite production of *n*-caproic acid by other microorganism, the most well-known producer is *C. kluyveri* [14,28]. When complex substrates are used as carbon and energy sources, other species in the open culture are needed to produce the precursors (acetic and *n*-butyric acids) for chain elongation. Therefore, one way to improve the carboxylic chain elongation process could be by the addition of a culture of a specific microorganism in a process referred to as bioaugmentation. This procedure has been used for increasing methane and hydrogen production with archaeal, facultative, and acidogenic microorganisms [29,30].

This work aimed to improve medium-chain carboxylic acids production (with emphasis in *n*-caproic acid) in batch mode process by biological route deviation using a bioaugmented mixed culture and residual glycerol as feedstock. The process was evaluated by using different sources of inoculum, bioaugmentation with *Clostridium acetobutylicum* ATCC 824, and utilizing different concentrations of ethanol used as the electron donor. Finally, the microbial community present in the inocula was assessed using denaturing gradient gel electrophoresis (DGGE) of the PCR products.

2. Material and methods

2.1. Glycerol source

Residual glycerol as substrate was kindly provided by Petrobras from the Quixadá Biodiesel Production Plant, State of Ceará, Quixadá, Brazil. There, glycerol was generated from transesterification of soybean oil (56%) and beef tallow (44%). The resulting product had the following chemical composition: glycerol 78.4%, methanol 3.0%, non-glycerol organic matter 1.2%, ashes 4.8%, and moisture 12.6%. The concentration of sodium chloride was 4.8%, which is an indication that ashes was due to NaCl. The chemical oxygen demand (COD) concentration was $1374\ g\ L^{-1}$.

2.2. Inocula and microorganism for bioaugmentation

Three different sources of inoculum were used: 1) flocculent sludge (FS) withdrawn from a UASB reactor treating municipal wastewater containing total volatile solids (TVS) of $55\ g\ L^{-1}$; 2) granular sludge (GS) withdrawn from a UASB reactor treating brewery effluent containing TVS of $26\ g\ L^{-1}$; and 3) goat ruminal liquid obtained by stomach tubing (RL) containing TVS of $34\ g\ L^{-1}$.

C. acetobutylicum ATCC 824 was purchased from the American Type Culture Collection (ATCC). Stock cultures were maintained as suspensions in 30% glycerol and stored at −80 °C. The stock culture was transferred to the activating Reinforced Clostridial Medium with the following composition ($g.L^{-1}$): glucose (5), beef extract (10), peptone (10), sodium chloride (5), yeast extract (3), sodium acetate (3), soluble starch (1), L-cysteine hydrochloride (0.5), and agar (0.5). Activation was carried out for 72 h at pH 7.0, and temperature of 37 °C. After activation, 10% (v/v) of this culture was transferred to the inoculum Clostridium Growth Medium (CGM), containing residual glycerol ($5\ g\ L^{-1}$) as the main sources of carbon and energy, and the following constituents ($g.L^{-1}$): $(NH_4)_2SO_4$ (2.0), K_2HPO_4 (2.4), KH_2PO_4 (1.8), Na_2HPO_4 (0.6), $MgSO_4$ (0.1), and $1\ mL\ L^{-1}$ of trace elements solution diluted in 5 M HCl ($g.L^{-1}$): $FeSO_4{\cdot}7H_2O$, (10), $CaCl{\cdot}2H_2O$ (2.0), $ZnSO_4{\cdot}5H_2O$ (2.0), $MnSO_4{\cdot}4H_2O$ (0.5), $CuSO_4{\cdot}5H_2O$ (1.0), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.1), and $Na_2B_4O_7{\cdot}10H_2O$ (0.02). Cells were grown in anaerobic conditions created by saturation of the medium and headspace by N_2 gas for 1 min, harvested during the exponential growth phase (based on the optical density at 600 nm), and used to inoculate the bioreactors.

2.3. Experimental set-up

Serum 250-mL flasks with the working volume of 100 mL equipped with rubber stoppers and aluminum caps were used as bioreactors. All flasks were filled with CGM as the cultivation medium at an initial pH of 6.5, and 10 g of the mixed culture. Cellular suspension of $1\ g\ L^{-1}$ of *C. acetobutylicum* ATCC 824 (10% v/v) was used for the bioaugmentation assays. Ethanol (100 or 200 mM) was added as the electron donor to allow *n*-butyric and *n*-caproic acid synthesis [16]. To inhibit methanogenic activity, a chemical treatment with a 0.05% chloroform solution was used [31]. Anaerobic environment in the serum flasks was created by saturating the headspace with N_2 gas. All experiments were performed in triplicate.

Biogas volume was measured in the flasks by liquid replacement [32] at days 2, 7, and 14 (end of the experiment). This was performed by a gas collection system with an inverted Mariotte bottle filled with saline solution (NaCl of $25\ g\ L^{-1}$), which was acidified to pH 2.0 using H_2SO_4. The displaced liquid volume corresponded to the volume of produced biogas. Biogas was extracted from the headspace for

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