SOCIEDAD LATINOAMERICANA DE BIOTECNOLOGÍA AMBIENTAL Y ALGAL

IV SOLABIAA
LATIN AMERICAN
CONGRESS

NOVEMBER 8 TO 13, 2015, FLORIANÓPOLIS, BRAZIL

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Abstract

Caproic acid (hexanoic acid), which is a six-carbon saturated fatty acid, currently is considered a valuable industrial commodity and has twice the value of ethanol per carbon atom. It can be produced by chain elongation from ethanol and acetic acid and it has been used in several industrial applications such as the food industry, medicine, and chemical industry. The chain elongation process with ethanol was used to evaluate the production of caproic acid in anaerobic granular and flocculent sludge, as well as goat ruminal liquid under different culturing conditions. Three different concentrations of substrate, namely 100/25, 200/50, and 400/100 mM of ethanol and mM acetic acid, respectively, were tested at two different pH levels of 5.5 and 7.0. A *n*-caproic acid concentration of ~ 3 g/L was obtained with the granular sludge as an inoculum at a pH of 5.5; however, the highest concentrations of ~ 4.5 g/L and 4.9 g/L was obtained with granular sludge and ruminal liquid at a pH 7.0, respectively. We, therefore, showed some inhibition of undissociated caproic acid at the lower pH value and an in-line extraction of caproic acid must be coupled to avoid such inhibition.

Keywords

Caproic acid; chain elongation; mixed culture.

INTRODUCTION

Anaerobic fermentation is an attractive approach to the biological production of chemicals or fuels from renewable resources. After post-processing these products can be alcohols, alkanes and fatty acids.

One example is to produce *n*-hexanoic acid or *n*-caproic acid (C6), because it has many industrial applications and it can be produced by chain elongation using ethanol as a source of reducing equivalents, carbon molecules, and energy to upgrade short-chain carboxylic acids (SCCAs), such as acetic acid (C2) and *n*-butyric acid (C4), into medium-chain carboxylic acids (MCCAs), such as caproic acid (C6) and caprylic acid (C8) (Steinbusch et al., 2011; Agler et al., 2012).

Recently, Steinbusch et al. (2011) reported *n*-caproic acid and *n*-caprylic acid production with a mixed culture from granular sludge withdrawn from a UASB reactor.In this study, we investigated the *n*-caproic acid production in mixed culture fermentation from wet organic waste with several different inocula, including granular and domestic sludge withdrawn

from UASB reactors and goat ruminal liquid.

MATERIAL AND METHODS

The experiment was conducted as a batch test with serum bottles in which MCCA production was evaluated by different culturing conditions. Three different sources of inoculum, namely rumen liquid from goats (RL) and sludge withdrawn from two upflow anaerobic sludge blanket (UASB) reactors treating municipal wastewater (DOM) and brewery effluent (GRAN), were tested for MCCA production during an experimental period of 14 days. Three different concentrations of substrates, namely 100 mM/25 mM; 200 mM/50 mM; and 400 mM/100 mM of ethanol and acetic acid, respectively, at pH range of 5.5 and 7.0 were tested to evaluate the best conditions for MCCA production. The bottles with a working volume of 100 mL contained 10.0 g of wet sludge plus CGM medium with ethanol and acetic acid as substrate and incubated at 37 ºC and shaken at 120 rpm. A chemical treatment was applied to inhibit methanogenic activity using 0.05% of chloroform. An anaerobic environment in the bottles was imposed by bubbling N_2 gas in the head space. MCCAs production was determined by HPLC. Statistically significant differences among treatments applied were evaluated by one-way analysis of variance (ANOVA). The significance levels are quoted at a 95% confidence level ($p \leq 0.05$).

RESULTS AND DISCUSSION

Table 2 shows the end products obtained after 14 days of batch tests at a pH range of 5.35-5.65. Carboxylic acids with a chain length of 5, 6, and 8 atoms of carbon were produced. The main products were *n*-butyric acid, *n*-caproic acid, and *n*-caprylic acid in all the different concentrations tested with both sludges. Mixed cultures were able to produce ~1.5-3 g/L of *n*-caproic acid in anaerobic fermentation after 14 days. The highest production rate ($p \leq 0.05$) was achieved at concentrations of 200 mM/50 mM of ethanol and acetic acid, respectively, compared with other concentrations (100/25 and 400/100 mM of ethanol and acetic acid, respectively) for both sludges at a pH of 5.5. Granular sludge, which was withdrawn from an UASB reactor treating brewery effluent, showed higher rates of *n*-caproic acid ($p \leq 0.05$) at all concentrations tested when compared with the domestic sludge, which was withdrawn from an upflow anaerobic sludge blanket (UASB) reactor treating domestic wastewater. The same trend was observed for the production of *n*-butyric acid for which higher rates of production were ~2 g/L in all concentrations tested.

Table 1. Carboxylic acids production after 14 days of anaerobic fermentation using domestic wastewater (DOM) and granular sludge (BE) as inocula at a pH range of 5.35-5.65.

Treatments	*n*-Butyric acid (g/L)	*n*-Caproic acid (g/L)	*n*-Caprylic acid (g/L
DOM (control)	0	0	0
DOM 100/25	0.8	1.3^{c}	0.3
DOM 200/50	0.5	1.5^{b}	0.5
DOM400/100	0.2	1.2^{c}	0.2
GRAN (control)	0	0	0
GRAN 100/25	1.8	2.2^{b}	0.6
GRAN 200/50	1.9	3.2^{a}	0.5
GRAN 400/100	1.9	1.7^{bc}	0.7

*values with different letters differ significantly ($p \leq 0.05$).

Table 2 shows the main products formed at a pH range of 6.8-7.2 after 14 days of batch tests using ethanol (200 mM) and acetic acid (50 mM) as a substrate. At this pH level, a higher production of *n*-caproic acid was obtained with GRAN and RL when compared with the pH level of 5.5. However, in the domestic sludge, only traces of *n*-caproic and *n*-caprylic acid were observed. Only traces of *n*-butyric acid were observed in both sludges and ruminal liquid.

Table 2. Carboxylic acids production obtained after 14 days of anaerobic fermentation using as seed inocula domestic wastewater (DOM), granular sludge (GRAN) and ruminal liquid (RL) at pH range of 6.8- 7.2.

	n-Butyric acid (g/L)	*n*-Caproic acid (g/L)	*n*-Caprylic acid (g/L)
DOM	0.2	0.2	0.2
GRAN	0.1	4.4	0.1
RL	0.3	4.9	0.2

The results demonstrate a promising production of MCCA, which consisted mainly of *n*-caproic acid, from acetate and ethanol by mixed culture fermentation in domestic, granular sludge, and ruminal liquid. Recently, Steinbusch et al. (2011) demonstrated that ethanol and hydrogen are suitable electron donors for caproate and caprylate formation during anaerobic fermentation of granular sludge. They reported high rates of *n*-caproic acid production (~8 g/L) in a batch reactor by chain elongation using ethanol and hydrogen as electron donors. Keanelly et al. (1995) reported a production of 4 g/L using ethanol and cellulose as substrate. In this study, the highest production of *n*-caproic acid was produced at a pH of 7.0 with granular sludge (4.4 g/L) and ruminal liquid (4.9 g/L) as inocula, respectively. At this level of pH, more ethanol is consumed than acetate. Similar results were obtained by Steinbusch et al. (2011), who reported higher production rates at pH 7.0 than at pH 5.5. In this study, we did not control the pH, however, we suppress methanogenic activity by adding 0.05% chloroform. Both MCCA producers and methanogenic bacteria compete for acetate as a substrate. Furthermore, the *n*-caproic acid formation from ethanol requires production of intermediates such as *n*-butyrate (Grootscholten et al., 2013; Vasudevan et al., 2014). It has been suggested that *Clostridum kluyveri* might be responsible for *n*-caproic acid production in mixed cultures (Ding et al., 2010), and Steinbusch et al. (2011) demonstrated that this organism dominated the microbial community during MCCA production. In addition, Agler et al. (2012) found a statistically significant correlation between *n*-caproic acid production and the abundance of this bacterium.During the ethanol–acetate metabolism of anaerobes, such as with clostridia, ethanol is converted to acetate while producing NADH and ATP *via* substrate level phosphorylation. Then, acetate is elongated in a cyclic pathway to *n*-butyrate. Finally, from *n*-butyrate and ethanol a chain elongation to caproate occurs in a similar cycle by coupling butyryl-CoA with acetyl-CoA (Seedorf, et al., 2008; Spirito et al., 2014). We observed that along the 14 days of fermentation, the concentration of *n*-butyric acid slightly decreased along with the ethanol concentration drop, confirming the consumption of *n*-butyrate for *n*-caproic acid formation. The same tendency was observed by (Grootscholten et al., 2013). They reported that 78% of consumed electrons from acetate and ethanol were converted to MCCA production, while 16% converted to *n*-butyrate.The highest concentration of undissociated caproic acid was ~3 g/L at a pH level of 5.5, which would have inhibit microbial activity and its own production in a long operating periods in systems with realistic influent streams. In such operating systems at this level of slightly acid pH, an in-line extraction of caproic acid must be coupled to avoid such toxicity as suggested by Vasudevan et al. (2014) and already implemented by Agler et al., (2012).

CONCLUSIONS

In this study, mixed culture fermentation was able to produce MCCAs from acetate and ethanol. The highest concentration of *n*-caproic acid was achieved at pH 7.0 for granular sludge and ruminal liquid. At a pH of 5.5, the highest concentration of *n*-caproic acid was obtained in the granular sludge.

ACKNOWLEDGEMENTS

This work was supported by the Brazilian National Council for Research and Technology (CNPq) and FUNCAP (Ceará Research Foundation).

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