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Research paper

# Methanol as an alternative electron donor in chain elongation for butyrate and caproate formation

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ABSTRACT

Chain elongation is an emerging mixed culture biotechnology converting acetate into valuable biochemicals by using ethanol as an external electron donor. In this study we proposed to test another potential electron donor, methanol, in chain elongation. Methanol can be produced through the thermochemical conversion of lignocellulosic biowaste. Use of methanol in chain elongation integrates the lignocellulosic feedstocks and the thermochemical platform technologies into chain elongation. After such integration, the feedstocks for chain elongation are solely from 2nd generation biomass resources. A proof-of-principle study of chain elongation using methanol and acetate was performed in both a batch and a continuous experiment. In the batch experiment, butyrate (191 mMC) and caproate (3 mMC) production from methanol and acetate was observed. A mixed culture microbiome taken from a previous chain elongation reactor fed with ethanol was responsible for the observed organic acid production. The continuous experiment was performed in an upflow anaerobic bioreactor (UAB). The hydraulic retention time (HRT) was 36 h and the operational period lasted for 45 days. In the continuous experiment, butyrate production (Rate > 30 mMC/day) was observed; the caproate concentration was below the detection limit during the entire continuous operational period. In both experiments, methanol and acetate were both substrates contributing to the butyrate production. To the authors' current knowledge, this study is the first attempt at a mixed culture fermentation utilising methanol and acetate for biochemical production. Further research should focus on elevating the butyrate production rate and concentration in the continuous operation of methanol chain elongation, which may stimulate caproate formation.

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## 1. Introduction

Pollution caused by combustion of fossil fuels has triggered a shift towards using cleaner and more renewable alternative feedstocks for chemical and fuel production. Organic waste is a potential carbon resource for chemical and fuel production. Organic waste is non-fossil based, abundantly available and does not compete with food production. Utilising organic waste to produce biochemicals and biofuels can offer a win-win solution. The carbon in organic waste is recovered while a contribution to the demand for renewable chemicals and fuels can be met.

Chain elongation is a novel mixed culture biotechnology which converts organic waste into precursors of biofuels and biochemicals [1,2]. Chain elongation employs a reactor microbiome that converts intermediary fermentation products derived from organic waste (e.g. acetate, $CO_2$ and ethanol) into valuable biochemicals, the so-called “medium chain fatty acids” (MCFAs, saturated fatty acids containing 6–12 carbons; e.g. caproate, heptanoate and caprylate) [1–3]. Compared with pure culture biotechnologies, advantages of mixed culture biotechnologies include: no sterilization requirement, an adaptive capacity to changing conditions owing to microbial diversity and the capacity to use mixed substrates [4]. The products of chain elongation, MCFAs, can be used as commodity chemicals [5,6] or serve as precursors of various biofuels and biochemicals [7–9]. The current production of MCFAs relies on coconut and palm kernel oils [10], which are produced on environmentally undesired plantations. The potential environmental consequences [11] include Greenhouse Gas (GHG) emission [12], biodiversity loss [13] and competition for arable land with food production [14,15]. Chain elongation offers a process that can

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produce MCFAs with a reduced land footprint depending on its feedstocks. Acetate and $CO_2$ are two essential substrates used in chain elongation. Both acetate and $CO_2$ can be abundantly produced from various organic waste feedstocks through a biochemical conversion process, i.e. acidification, with minimal land requirement [7].

Ethanol is also an essential substrate for chain elongation. To carry out chain elongation either an ethanol-containing waste stream (e.g. corn fermentation beer) is used as the feedstock [1], or an addition of ethanol during the fermentation is required [16]. Ethanol contributes to at least two-thirds of carbon in the end product of chain elongation, as for example in caproate [7,17]. Currently ethanol is produced mainly from crops like sugarcane and corn [18]. These crops require arable land for their production and, in most cases, are more costly compared with an organic waste feedstock. Reducing or replacing the use of crop-based ethanol in chain elongation is of importance in order to further improve the environmental sustainability and cost-effectiveness of chain elongation.

Lignocellulosic biowaste is one of the potential organic waste streams that can be used to reduce or replace the crop-based ethanol in chain elongation. For example, lignocellulosic bioethanol can be produced by employing enzymatic hydrolysis and fermentation. This process is currently under development and it may be commercially available to replace the sugarcane- and corn-based bioethanol in the near future [19]. Vasudevan et al. demonstrated another possible use of lignocellulosic biowaste in chain elongation. Synthesis gas produced through thermochemical processing of lignocellulosic biowaste was converted into bioethanol through a pure culture fermentation process. The bioethanol produced from synthetic gas was then used as the feedstock for chain elongation [20].

In this study we investigated another strategy for using lignocellulosic biowaste in chain elongation: the use of methanol as an alternative electron donor in chain elongation. Methanol can be produced from synthesis gas or several other waste streams through chemical processes [21]. Moreover, the production process of lignocellulosic methanol has been commercialised and implemented [19]. The use of methanol in chain elongation can expand the feedstock range of chain elongation and increase the resource security for the production of MCFAs.

The usage of methanol for the biological formation of MCFAs was attempted in four previous studies with monocultures. Keneally and Waselefsky blended methanol into the growth medium for a pure culture *Clostridium kluyveri*, a known bacterium elongating short chain fatty acids (SCFAs; saturated fatty acid containing less than 6 carbons) and ethanol into MCFAs. It was reported, however, that methanol was not metabolised by *C. kluyveri*. The microorganism *Eubacterium limosum*, on the other hand, was reported to produce small amounts of caproate from methanol and SCFAs in a pure culture incubation [22–24]. Genthner et al. (1981) showed the production of butyrate (35.68 mMC; mMC = millimolar carbon) and caproate (0.78 mMC) from a pure culture *E. limosum* growing on methanol (50 mMC) and acetate (60 mMC) [22]. Lindley et al. (1987) also reported the caproate production with a pure culture *E. limosum* growing on methanol (100 mMC), $CO_2$ and butyrate (400–1600 mMC) with a yeast extract supplement (0.5 g/L) [23]. Tarasov et al. (2011) lately reported caproate production from methanol and $CO_2$ by a pure culture *E. limosum* but the actual data were not given [24]. So far a mixed culture fermentation converting methanol and SCFAs into MCFAs has not been reported. Nevertheless, the existence of these pure culture studies implies the potential feasibility of such fermentation process.

This study investigated the feasibility of using methanol and acetate as the substrates for chain elongation (Hereinafter referred to as "methanol chain elongation") to produce butyrate and caproate with a mixed culture. These are typical chemicals produced by chain elongation using ethanol and acetate as the substrates (Hereinafter referred to as "ethanol chain elongation"). Both batch and continuous methanol chain elongation experiments were performed. The batch tests aimed at demonstrating the proof-of-principle of methanol chain elongation. Several combinations of substrates and inoculum were examined in the batch experiments. Following the batch experiments, an upflow anaerobic bioreactor (UAB) was set up to demonstrate the feasibility of continuous methanol chain elongation.

## 2. Material and method

### 2.1. Batch experiment

Two batch experiments were carried out in this study, the 1st batch experiment and the 2nd batch experiment. The codes and experimental conditions of both the 1st and 2nd batch experiments can be found in Table 1. The 1st batch experiment was done to test a set of various combinations of the substrates and inoculum. Two types of inocula were used in the 1st batch experiment, i.e. the mixed culture inoculum with (+E) or without pure culture *Eubacterium limosum* (ATCC 8486) addition. The mixed culture inoculum was taken from the fermentation broth of a UAB that was used for performing ethanol chain elongation [2,25]. The added pure culture *E. limosum* was incubated in a batch prior to the inoculation to ensure its activity, as further described in the Supplementary Material (SM). The effect of the addition of methanol (Me) and acetate (Ac) were also examined in the 1st batch experiment. Two blanks (B and B + E) without methanol and acetate addition were used to quantify the contribution of the yeast extract to the organic acid formed during the batch experiments. Yeast extract is a commonly used substance in chain elongation for supporting the microbial growth [2,3,23,26–29].

The 2nd batch experiment was carried out to enrich the biomass for later use, i.e. as inoculum for the continuous methanol chain elongation experiment. For the 2nd batch experiment, the mixed culture fermentation broth from the MeAc + E was used as the sole inoculum. MeAc + E had the highest butyrate production from methanol and acetate in the 1st batch; therefore it was used as the inoculum for further research. The procedure for preparing the batch test and the inoculum are documented in the SM. All batch experiments in this study were done in triplicate.

The composition of the growth medium was adapted from the previous ethanol chain elongation studies [3,30]. The medium contained $NH_4H_2PO_4$ − 3.6 g/L, $MgCL_2.6H_2O$ − 0.33 g/L, $MgSO_4.7H_2O$ − 0.2 g/L, $CaCl_2.2H_2O$ − 0.2 g/L, KCl − 0.15 g/L, yeast extract − 1 g/L, Vitamin B solution 1 ml/L and trace element solution 1 ml/L [28]. Varying amounts of methanol and acetate were added in different batches as shown in Table 1. In the 1st batch experiment, the effects of methanol (100 mM) and acetate (50 mM, in the form of sodium acetate) supplements on the mixed culture fermentation were studied. In the 2nd batch experiment, methanol (100 mM), acetate (50 mM) and $CO_2$ were all used as substrates. Moreover, the methanol concentration was later (at Day7) elevated to 200 mM to prevent the substrate depletion.

A gas exchanger was used to flush the headspace of all the batches. The headspace was first vacuumed and subsequently filled with pure nitrogen gas up to 1.5 bar. This procedure was repeated for 5 times, then the headspace was vacuumed again and filled with the desired headspace composition (a gas mixture containing 80% $N_2$ and 20% $CO_2$ up to 1.5 bar). The gas exchanger may have trace amounts of impurities; moreover, not all the batch bottles were

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