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Microbial Production of Ethyl Acetate from Non-Sterile Delactosed Whey Permeate by *Kluyveromyces marxianus*

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ABSTRACT

This work investigates the feasibility of utilizing non-sterile delactosed whey permeate (DWP) for microbial production of ethyl acetate by *Kluyveromyces marxianus* DSM 5422. The results indicate efficient carbon source utilization within 12 hours, with a maximum biomass-specific ethyl acetate synthesis rate of 0.92 g g^{-1} h^{-1}. Moreover, *K. marxianus* demonstrates robust growth and suppresses the proliferation of autochthonous bacteria present in non-sterile DWP. These findings underscore the potential for sustainable and energy-efficient microbial ethyl acetate production processes using non-sterile dairy by-products.

Keywords: Ethyl acetate. Delactosed whey permeate. Non-sterile Process. *Kluyveromyces marxianus*.

1 INTRODUCTION

In 2020, around 4 million tons of ethyl acetate were produced worldwide[1]. The current production relies exclusively on energy-intensive petrochemical processes, although microbial synthesis of this ester from sugar-rich resources has high economic potential[2]. In the past, we have shown that high amounts of ethyl acetate can be produced by the Crabtree-negative yeast *Kluyveromyces marxianus* from concentrated whey permeate[3–5] and even from the more problematic delactosed whey permeate (DWP)[6], both of which are currently underutilized by-products of the dairy industry. Whey permeates are typically burdened with autochthonous bacteria, necessitating sterilization via autoclaving—a process that is energy-intensive and impractical for large-scale industrial applications. To address these challenges, our current study explores the feasibility of utilizing non-sterile DWP as a substrate for ethyl acetate production by *K. marxianus*. This approach aims to reduce energy costs associated with sterilization and capitalize on the potential of DWP as a low-cost feedstock, thereby laying the groundwork for a sustainable and economically viable microbial production process.

2 MATERIAL & METHODS

Ethyl acetate was synthesized using the yeast *Kluyveromyces marxianus* DSM 5422. The cultivation was conducted in a 3.6 L stirred bioreactor (Infors HT, Switzerland) with a working volume of 1 L. The bioreactor was maintained at a temperature of 40°C, with pH control set to either 5.1 or 5.9. Aeration was provided at a rate of 1 vvm. Delactosed whey permeate (DWP) was obtained from Sachsenmilch GmbH (Germany). One liter DWP contained approximately 170 g lactose, 50 g mineral salts, 20 g citrate, and 400 µg iron. To prepare the fermentation medium, DWP was diluted in a 1:1 ratio with deionized water. The diluted DWP was supplemented with 7 g L^{-1} urea as a nitrogen source and with a trace element solution lacking[3] iron to induce ethyl acetate formation.

The preculture was prepared in shaking flasks containing DWP medium without iron. It was inoculated from a YGC agar plate and incubated for 12 hours at 40 °C and 220 rpm. Cells from the preculture were centrifuged and resuspended in 4 mL DWP medium before inoculation into the bioreactor. During the cultivation process, the gaseous concentrations of air components, ethyl acetate, and ethanol were quasi-continuously monitored using process mass spectrometry (MS)[6]. In addition, ethyl acetate, ethanol, and acetaldehyde were detected and quantified in both the gas and liquid phases using gas chromatography coupled with a flame ionization detector (GC-FID)[3]. The concentrations of sugars (lactose and galactose) and organic acids (including citric acid and acetic acid) were measured using high-performance liquid chromatography[6].

The gas phase concentrations of ethyl acetate and ethanol were used to calculate their corresponding concentrations in the liquid phase. These data were then utilized to determine the total masses produced and the synthesis rates of these compounds. These calculations were performed using a recently developed method[7].

3 RESULTS & DISCUSSION

In *K marxianus*, the synthesis of ethyl acetate is primarily induced by iron limitation[8]. When iron availability is restricted, the capacity of the electron transport chain for NADH re-oxidation is reduced, leading to an accumulation of acetyl-CoA, which is then diverted towards ethyl acetate production[9,10]. In processes without iron limitation, *K. marxianus* predominantly converts lactose into biomass with minimal ethyl acetate production, regardless of the pH (Fig. 2). In these iron-sufficient conditions (using trace element solution with iron), the production of ethyl acetate remains low and consistent across different pH levels. Conversely, when iron was omitted from the medium (trace element solution without iron), the yeast's growth was constrained by the naturally low iron content of the DWP, resulting in lower biomass concentrations and a significant increase in ethyl acetate formation (Fig. 2).

Under these iron-limited conditions, the process's efficiency was markedly influenced by the pH, achieving a maximum ethyl acetate yield of 67.4 % of the theoretical maximum at pH 5.1 using sterilized DWP medium[6].

To reduce energy costs, we investigated the use of non-sterilized DWP medium. During aerobic batch cultivation at pH 5.1, *K. marxianus* DSM 5422, with an initial inoculum of 0.3 g L^{-1}, completely utilized the lactose, galactose, and lactate present in the DWP mediumn within 12 hours (Fig. 1). Throughout the initial 5 hours, biomass formation was predominant, as indicated by a respiration coefficient close to 1 (Fig. 1A). This phase was followed by a rapid increase in biomass-specific ethyl acetate synthesis (Fig. 1E), reaching a peak rate of 0.92 g g^{-1} h^{-1} due to the onset of iron limitation. The synthesis rate gradually declined as the lactose was depleted. These observations align closely with the results obtained from iron-limited processes on sterilized DWP medium.

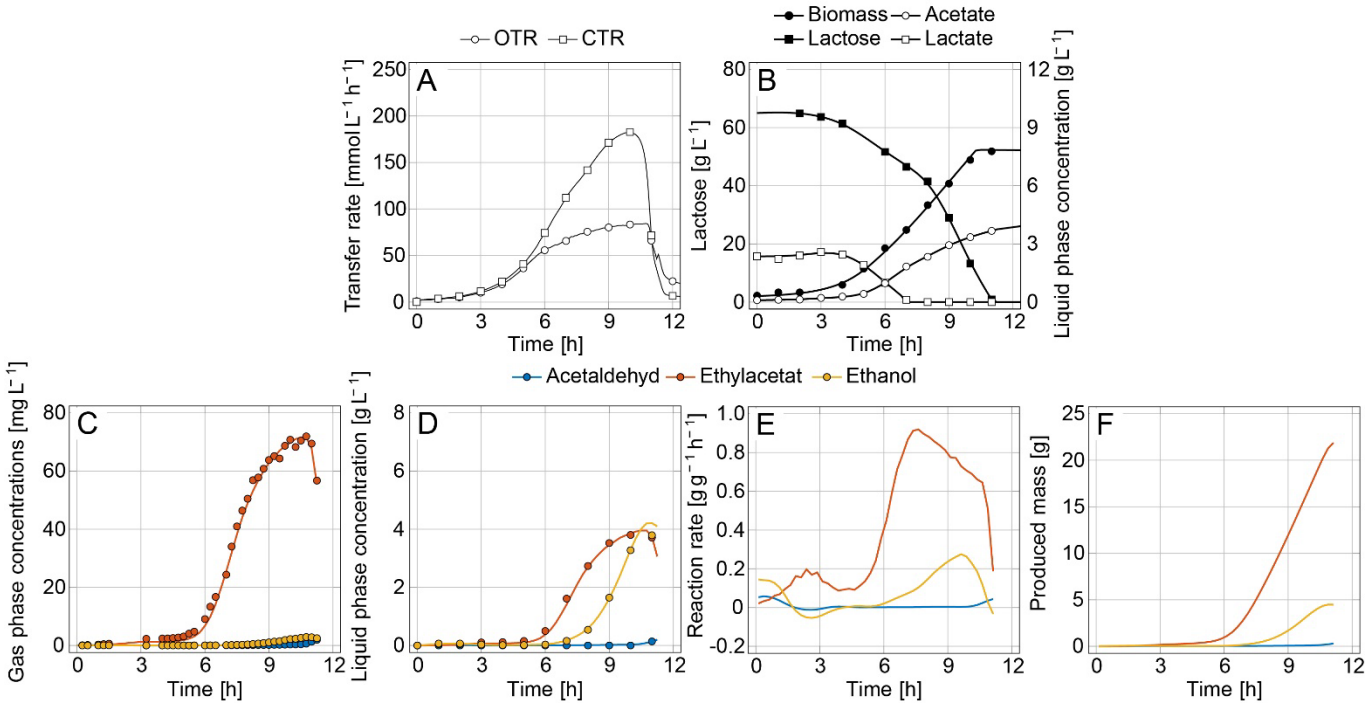

Figure 1 Kinetics of VOC synthesis by *K. marxianus* DSM 5422 during the aerobic batch cultivation on non-sterile DWP medium at pH 5.1. (A) CTR and OTR; (B) Lactose, biomass, acetate and lactate concentration; (C) Exhaust gas phase concentrations measured by GC-FID and MS; (D) Liquid phase concentrations; (E) Biomass-specific reaction rates; (F) masses of formed VOCs.

The pH dependence observed in the iron-limited processes with non-sterilized DWP is less pronounced compared to the sterilized medium (Fig. 2). The sterilization process, involving autoclaving at 121 °C for 20 minutes, likely leads to the formation of potential toxic Maillard reaction products, which could affect the pH dependence additionally to the pH-dependent iron-citrate complex formation[11]. This nuanced impact highlights the complexity of medium composition changes due to sterilization.

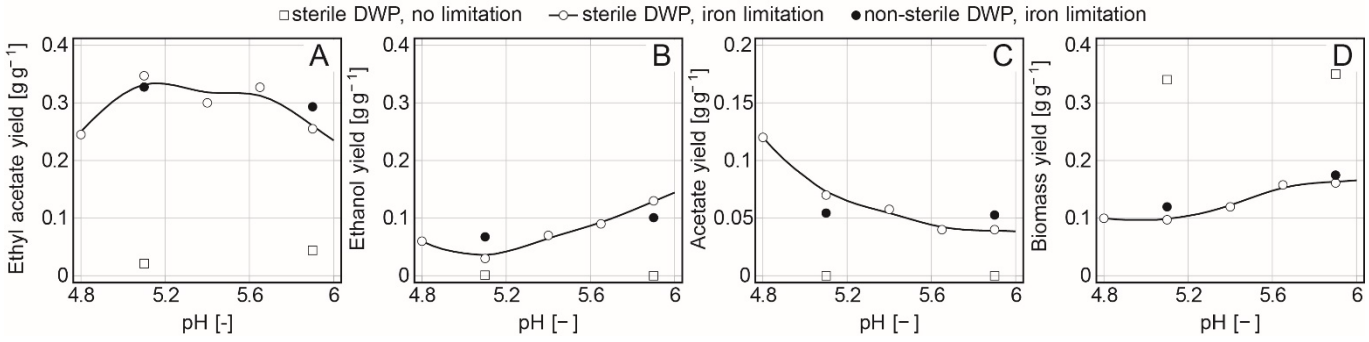

Figure 2 Product yields of *K. marxianus* DSM 5422 under non-limited and iron-limited conditions with sterile[6] and non-sterile DWP medium at various pH values. (A) Ethyl acetate, (B) Ethanol, (C) Acetate, and Biomass yield.

Despite the presence of autochthonous bacteria in the non-sterile DWP, their growth was effectively suppressed by *K. marxianus*. Initial observations indicated that bacterial growth could occur after 10 hours in the absence of *K. marxianus*. However, under the applied conditions, the robust growth *K. marxianus*, rapid consumption of lactose, and ethyl acetate formation inhibited bacterial proliferation. By the time of lactose depletion, no bacterial presence was detectable via microscopy.

4 CONCLUSION

This work demonstrates the effectiveness of using non-sterile delactosed whey permeate (DWP) as a substrate for the microbial production of ethyl acetate by *Kluyveromyces marxianus* DSM 5422. Within 12 hours, the yeast efficiently utilized all available carbon sources, achieving high biomass-specific rates and yields of ethyl acetate. Additionally, *K. marxianus* effectively

suppressed the growth of autochthonous bacteria, maintaining process stability and product purity. These findings highlight the potential for extending batch to repeated-batch processes under non-sterile conditions to further enhance process efficiency.

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