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Synthesis of ethyl acetate from delactosed whey permeate by *Kluyveromyces marxianus*

A. Hoffmann, A. Franz, S. Nguyen, C. Löser, T. Walther

Institute of Natural Materials Technology, TU Dresden, Dresden, Germany

Background and Motivation

In 2013, around 180 million tons of whey were produced worldwide during milk processing [1]. The separation of proteins and lactose from whey results in the effluent stream of partially delactosed whey permeate (DWP). DWPs contain 150 − 200 g L^{-1} residual lactose, 40 − 80 g L^{-1} mineral salts and 20 − 60 g L^{-1} organic acids, which results in a BOD_5 of approximately 160 g L^{-1} [2]. DWP is used as animal food and fertilizer and, despite the high lactose content, often regarded as waste [2]. Therefore, microbial synthesis of value-added products from residual lactose offers an economic re-use of DWP. The Crabtree-negative yeast *Kluyveromyces marxianus* has turned out to be the most potent natural producer of ethyl acetate from lactose [3]. In the past years, ethyl acetate synthesis was successfully demonstrated with *K. marxianus* DSM 5422 cultivated in concentrated whey permeates (CWPs) as a substrate with yields over 50 % of the pathway maximum [4].

The challenge to utilize DWP, which is derived from CWP, for fermentation processes is the high mineral salt content which inhibits microbial growth and reduces the production performance [5, 6]. This inhibitory effect was reduced by appropriate dilution with water. Supplementation with a source of nitrogen and a mixture of trace elements resulted in an optimized DWP-based medium which enables satisfactory growth of *K. marxianus*. Synthesis of ethyl acetate by *K. marxianus* is induced at growth under iron-limited conditions, where highest synthesis rates are achieved under moderate iron limitation [3, 7]. DWP-borne organic acids, in particular citrate, can complex dissolved iron by chelate formation and thereby alter its bioavailability. Complexation of iron by citrate depends on the pH and the iron-citrate ratio [8]. Therefore, we investigated the impact of the pH and iron supplementation on the performance of synthesis of ethyl acetate by *K. marxianus* DSM 5422 during cultivation in DWP-based medium.

Experimental procedure

Ethyl acetate was synthesized with *K. marxianus* DSM 5422 in an aerated stirred bioreactor. DWP was provided by Sachsenmilch GmbH and contained about 170 g L^{-1} lactose, 50 g L^{-1} mineral salts, 20 g L^{-1} citrate and 400 µg L^{-1} iron. DWP was diluted in an 1:1 ratio with deionized water, autoclaved for 20 min at 121 °C, and supplemented with 7 g L^{-1} urea as a source for nitrogen and with trace-element solution (with or without iron). The 3,6-L stirred bioreactor (Infors HT) was filled with 1 L medium. After inoculation with a small amount of biomass (for minimizing the supply of biomass-bound iron), the cultivation was conducted at 40 °C and an aeration with 1 vvm air. The process began with a first cultivation phase at pH 5.1. At a CO_2 content of 0.1% in the exhaust gas, the pH was switched to different values from 5.1 to 5.9 in the second phase (Fig. 1). The gaseous concentrations of ethyl acetate and ethanol were analyzed in a high temporal resolution during the cultivation by gas chromatography. These gas-phase concentrations were used for balancing the microbial synthesis of the ester and ethanol (calculation of time-dependent liquid-phase concentrations, formed masses, and synthesis rates) by using a recently developed method [9].

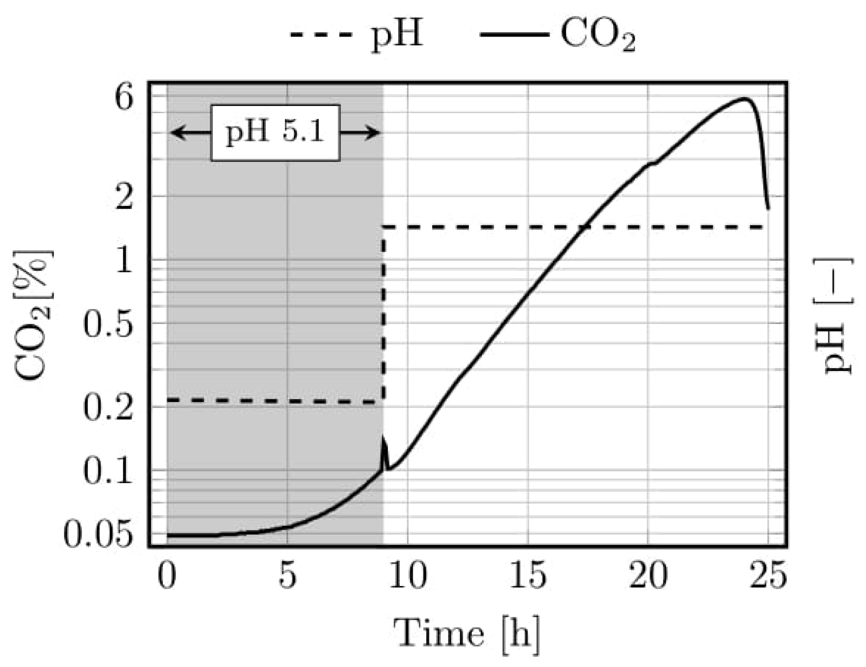

Fig. 1: Cultivations with pH shift. First cultivation phase at pH 5.1 and second cultivation phase at a desired pH.

Cultivations

In the yeast *K. marxianus*, synthesis of ethyl acetate is easiest induced by iron limitation [3]. Iron limitation reduces the capacity of the electron transport chain for NADH re-oxidation and diverts accumulating acetyl-CoA to ethyl acetate [10, 11]. In processes without iron limitation (when using iron-rich trace-element solution), *K. marxianus* converted lactose mainly into biomass and only a little ethyl acetate, without visible influence of the pH (Figs. 2 and 3). When iron was omitted from the medium (trace-element solution without iron), growth was limited by the DWP-borne iron, which resulted in a decreased biomass concentration and an increased formation of ethyl acetate (Fig. 2). The highest yield of ethyl acetate of 0.31 g g^{-1} (61 % of the theoretical maximum) was achieved at pH 5.1. Higher pH values during the second cultivation phase revealed decreasing ester yields and increasing ethanol yields (Fig. 3). Iron-limited chemostat cultivations of *K. marxianus* DSM 5422 have shown that moderate iron limitation favors synthesis of ethyl acetate while strong iron limitation intensifies ethanol synthesis ([7] and unpublished results from cultivations in mineral media). The results suggest that increasing pH values intensify the iron complexation by citrate [8] and thus reduce the bioavailability of iron which, in turn, leads to severe iron limitation and, as a consequence, to an increased ethanol formation. Ethanol formation is disadvantageous since *K. marxianus* DSM 5422 does not synthesize ethyl acetate from ethanol in significant amounts [3]. In this context, the pH-dependent synthesis behavior in the DWP-based medium offers a simple tool to regulate the production process which is aimed at maximizing the yield of ethyl acetate.

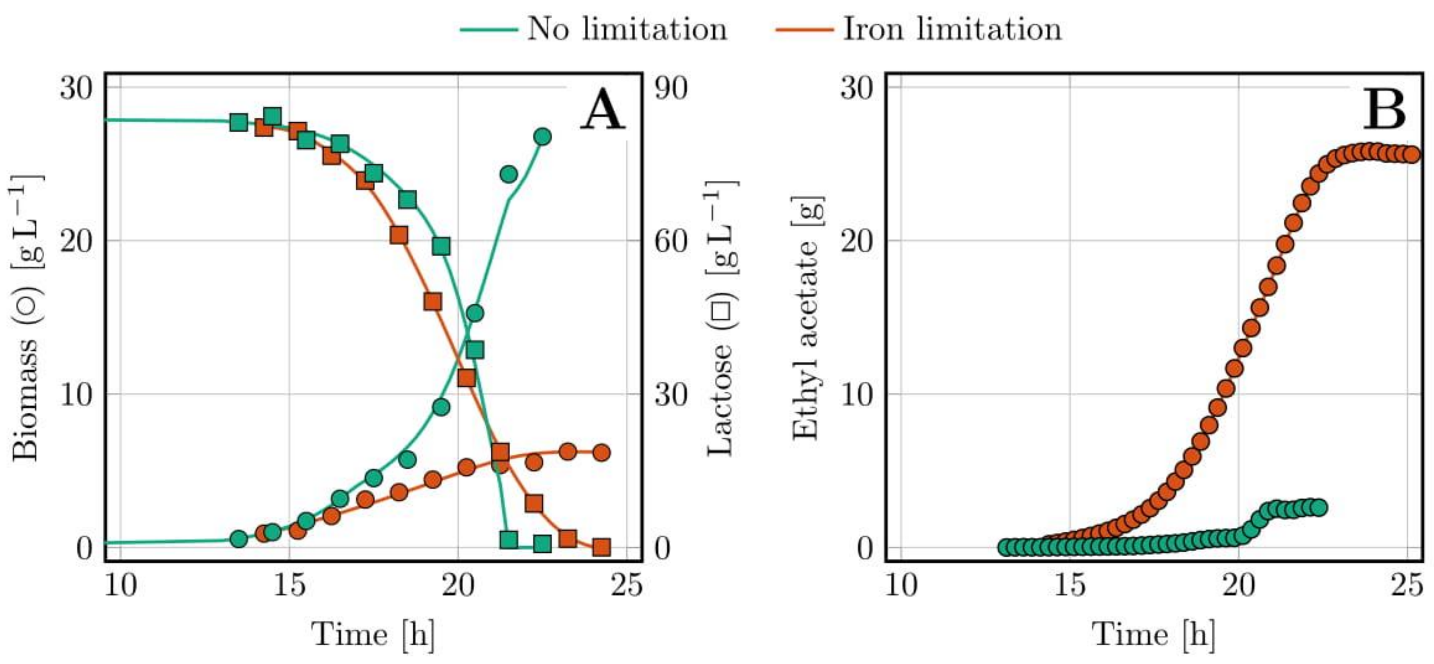

Fig. 2: Synthesis of ethyl acetate from DWP-borne medium by *K. marxianus* DSM 5422 under non-induced and iron-limited conditions at pH 5.1. (A) Conversion of lactose into biomass, **(B)** synthesized amount of ethyl acetate.

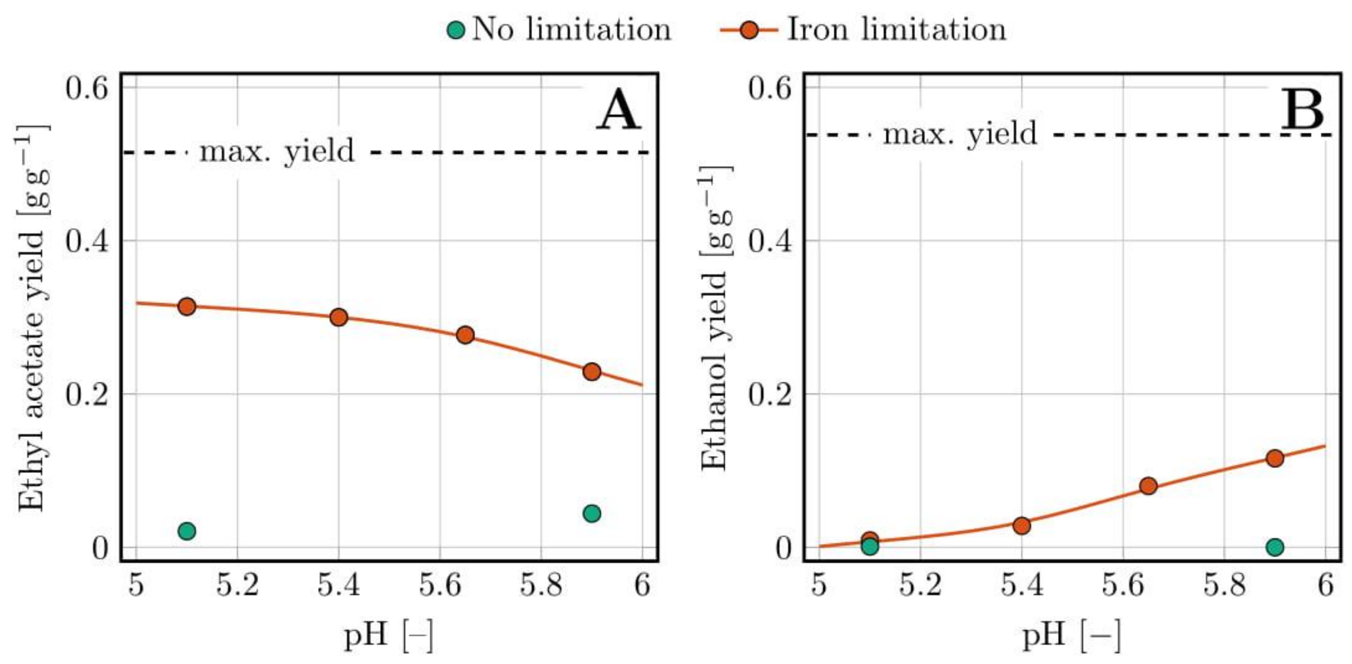

Fig. 3: Product yields of *K. marxianus* DSM 5422 under non-induced and iron-limited conditions at various pH values during the second cultivation phase. (A) Yield of ethyl acetate, **(B)** yield of ethanol; Iron-limited cultivations were performed as shown in Fig. 1.

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