Monitoring of Extracellular TCA Cycle Intermediates in Mammalian Cell Culture

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Abstract: Some intracellular intermediate metabolites can also be found in the medium supernatant at micromolar concentrations. In this work, we are investigating extracellular concentrations of five organic acids (succinic, malic, fumaric, citric and isocitric acid) during cell growth and after viral infection of MDCK cells.

Key words: influenza, vaccine, mammalian, MDCK cells, adherent, TCA cycle, organic acids, metabolism, extracellular, intracellular, batch cultivation, monitoring, T-flask, spinner, bioreactor, chromatography, anion-exchange

1. INTRODUCTION

One research focus of our group is the optimization of an influenza vaccine production process with the adherent cell line MDCK (Madin-Darby canine kidney) on microcarriers [1]. In addition to monitoring basic metabolites the measurement of extracellular amino acid levels was already established [1, 2]. Further information about metabolism could be achieved by analyzing intracellular intermediates. But some of these metabolites can also be found in the medium supernatant in cell culture [3]. Since no active transport for organic acids is reported for our cell line, they might be released by disruption of dead cells, leakage by protonated diffusion through membranes or transporters for e.g. monocarboxylates [4, 5] or amino acids [6]. Our goal is to gain additional information about metabolism by the investigation of the accumulation of intermediates from central carbon metabolism in the culture broth.

R. Smith (ed.), Cell Technology for Cell Products, 603–605.
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2. MATERIALS AND METHODS

Cell culture, virus infection and standard analytics was done according to Genzel *et al*. [1]. Cells (start concentration 0.4-1.3 x 10^5 cells/mL) were grown in T-flasks (175 mL, 125 mL wv) (Greiner), spinner bottles (200 mL wv) or 5L-stirred tank bioreactor (B. Braun Biotech) on Cytodex 1 microcarriers (2-3 g/L, GE Healthcare).

Cultivation supernatant was mixed with methanol/formic acid solution in a ratio 3:8, followed by drying. Samples were dissolved in Milli-Q H_2O prior to analysis. Chromatography for malate, succinate, fumarate, citrate, isocitrate was performed with a method adapted from Koswig *et al*. [7] on a DX-320 system (Dionex) using an AS-11 (2 x 250 mm) column with conductivity and UV-detection. A KOH gradient was generated automatically by an eluent generation system, no $NaHCO_3$ was added. Injection volume was 20 µL; flow rate was set to 0.25 mL min^{-1}.

3. RESULTS AND DISCUSSION

Peaks were identified by standard addition, UV-signal and retention times. For quantification, standards with seven different concentrations were randomly inserted between samples for each sequence. Clear differences could be seen in typical chromatograms of samples at the beginning and the end of cultivation. Identified peaks were quantified (see Fig. 1), but also several not identified peaks showed major changes.

For the three different cultivation modes, cell as well as glucose, lactate, glutamine, glutamate and ammonia concentrations followed typical profiles of batch cultivations. For the bioreactor experiment, metabolite concentrations were changed to starting concentrations by medium exchange before virus infection. After virus infection, a clear decline of viable cells could be seen accompanied by an increase of dead cells. Figure 1 shows the concentrations of the investigated organic acids during the cultivations. At t = 0 h, all samples already contained organic acids coming from serum and peptone. The level of all organic acids was constant during the first 48 h of cultivation, followed by an increase for approximately the next 48 h. Other experiments from our group have shown clear changes in amino acid metabolism [1] and cell cycle distribution in this time interval. Both the experiment in the T-flask and in the spinner-flask showed for malate, fumarate and citrate a continuous accumulation until the end of cultivation, whereas succinate and isocitrate seemed to reach a constant level between 96 and 120 h. There is no indication that the concentration changes of the organic acids were correlated to dead cells, because succinate and isocitrate

stopped increasing during the end of cultivation, when dead cells accumulated. Additionally, calculations showed that intracellular concentrations of metabolite pools (values from literature) do not suffice to result in the change of concentration after lysis of dead cells. Furthermore, in chromatograms from samples after virus infection additional peaks appeared, which were probably substances released by dead cells. With regard to these results, we assume that depending on the intracellular concentrations of organic acids, a corresponding unspecific leakage occurred. The exact mechanism however cannot be explained so far.

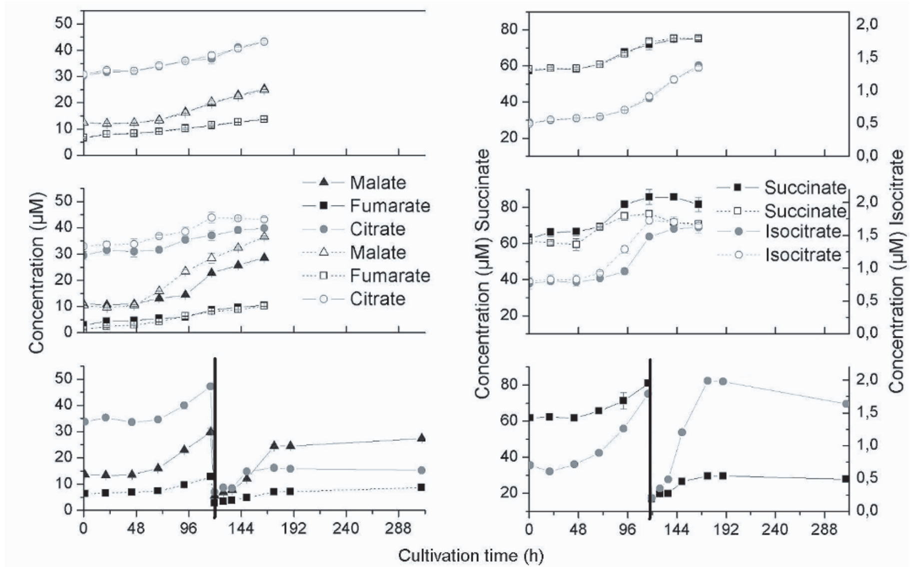

Figure 1. Concentrations of organic acids during cultivation: first row: T-flask, second row: spinner flask, third row: bioreactor (virus infection at t = 120 h, vertical line).

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