

## RESEARCH ARTICLE

# High-level recombinant protein production with *Corynebacterium glutamicum* using acetate as carbon source

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## Abstract

In recent years, biotechnological conversion of the alternative carbon source acetate has attracted much attention. So far, acetate has been mainly used for microbial production of bioproducts with bulk applications. In this study, we aimed to investigate the potential of acetate as carbon source for heterologous protein production using the acetate-utilizing platform organism *Corynebacterium glutamicum*. For this purpose, expression of model protein eYFP with the promoter systems T7lac and tac was characterized during growth of *C. glutamicum* on acetate as sole carbon source. The results indicated a 3.3-fold higher fluorescence level for acetate-based eYFP production with T7 expression strain MB001(DE3) pMKEx2-eyfp compared to MB001 pEKEx2-eyfp. Interestingly, comparative eyfp expression studies on acetate or glucose revealed an up to 83% higher biomass-specific production for T7 RNAP-dependent eYFP production using acetate as carbon source. Furthermore, high-level protein accumulation on acetate was demonstrated for the first time in a high cell density cultivation process with pH-coupled on-line feeding control, resulting in a final protein titer of 2.7 g/L and product yield of 4 g per 100g cell dry weight. This study presents a first proof of concept for efficient microbial upgrading of potentially low-cost acetate into high-value bioproducts, such as recombinant proteins.

## INTRODUCTION

Beyond its traditional use for fermentative amino acid production, the industrial platform organism *Corynebacterium glutamicum* has gained considerable importance as alternative microbial cell factory for heterologous protein production (Freudl, 2017; Lee & Kim, 2018; Liu et al., 2015). Especially with respect to

secretory protein production, it offers beneficial characteristics such as (i) low amounts of endogenous extracellular proteins, (ii) low proteolytical activity in the culture supernatant and (iii) the ability to directly transport proteins across the cytoplasmic membrane with high efficiency. Accordingly, large libraries of signal peptides (Hemmerich et al., 2016) and genome-reduced strains (Hemmerich et al., 2020), as well as

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biosensor-based monitoring tools (Bakkes et al., 2021), have already been established for screening and optimization of protein secretion performance in *C. glutamicum*. In addition, the Gram-positive bacterium represents an endotoxin-free cell factory, thus eliminating the need for endotoxin removal and simplifying downstream processing in case of biopharmaceutical proteins. So far, a first commercial expression system known as CORYNEX® has already been developed by the Company Ajinomoto Co., Inc. As a well-established and GRAS-classified workhorse of white biotechnology, *C. glutamicum* also exhibits easy genetic accessibility (Linder et al., 2021), fast growth up to cell densities higher than 80 g/L cell dry weight (Kiefer, Merkel, Lilge, Hausmann et al., 2021; Knoll et al., 2007), and robustness towards substrate gradients typically found in industrial-scale bioreactors (Buchholz et al., 2014; Limberg et al., 2017). Beyond that, the relatively high tolerance to various lignocellulose-derived inhibitors has enabled this bacterium to produce several value-added compounds from alternative biorefinery feedstocks (Becker et al., 2018; Lange et al., 2017, 2018; Mao et al., 2018; Sasaki et al., 2019).

With regard to a future bio-based industry, the C2 compound acetate represents a potential next-generation platform substrate generated by lignocellulose depolymerization and multiple other alternative routes (Kiefer, Merkel, Lilge, Henkel et al., 2021). In fact, the microbial production of value-added products from pure acetate or acetate-containing biorefinery streams has truly gained increasing interest over the past decade. Starting from compounds directly derived from microbial acetate metabolism (e.g. succinate or microbial lipids), strain and tolerance engineering of acetate-utilizing microorganisms has largely expanded the portfolio of acetate-based bioproducts (Kim et al., 2021; Kutscha & Pflügl, 2020). However, the potential of bioprocess strategies for efficient biotechnological conversion of acetate as potentially inhibitory microbial substrate is still rather underexploited. In a previous study, we demonstrated a novel bioprocess for efficient acetate conversion using *C. glutamicum* as biocatalyst (Kiefer, Merkel, Lilge, Hausmann et al., 2021). By applying an automated pH-coupled online feeding control, *C. glutamicum* wild-type ATCC 13032 was cultivated up to high cell densities using lignocellulosic acetic acid derived from beech wood depolymerization. In addition to the presented biomass accumulation on acetate, this bioprocess was recently adapted for biomass-decoupled production of the promising C5 platform chemical itaconic acid using engineered *C. glutamicum* strain ICD<sup>R453C</sup> (Merkel et al., 2022).

In the present work, we aimed to evaluate the potential of acetate as carbon source for production of recombinant proteins. Since the commonly used prokaryotic protein production hosts *Escherichia coli* and *Bacillus subtilis* naturally exhibit rather poor or even no growth in

presence of acetate (Arnold et al., 2019), this study focused on *C. glutamicum* as acetate-utilizing, alternative protein cell factory. For exemplary intracellular protein expression, the fluorescence model protein eYFP derived from *Aequorea victoria* GFP (Ormö et al., 1996) was chosen as a target protein. In the first part of this work, eYFP expression during growth of *C. glutamicum* on acetate as carbon source was characterized with the expression vectors pMKEx2 (Kortmann et al., 2015) and pEKEx2 (Eikmanns et al., 1991) containing the IPTG-inducible promoter systems T7lac and tac, respectively. In addition, the T7 RNA polymerase (T7 RNAP)-dependent production of eYFP on acetate was quantitatively compared with that on glucose as commonly used carbon source for fermentation. Lastly, the heterologous protein accumulation with *C. glutamicum* T7 expression system was studied under high cell density conditions in a fed-batch bioreactor process using pure lignocellulosic acetic acid as combined carbon source and pH titrant. The results of this study demonstrate the capability of platform organism *C. glutamicum* for efficient acetate-based protein production and further supports the outstanding potential of acetate as next-generation carbon source in a future biotechnology.

## EXPERIMENTAL PROCEDURES

### Bacterial strains, plasmids and culture media

All bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strain maintenance was carried out at  $-80^{\circ}\text{C}$  using 30% (v/v) glycerol stocks. If not stated otherwise, the used chemicals were of analytical grade and obtained from Carl Roth GmbH. Cloning host strain *E. coli* BL21-Gold(DE3) was routinely grown at  $37^{\circ}\text{C}$  in LB complex medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl). First precultures of *C. glutamicum* strains were cultivated at  $30^{\circ}\text{C}$  in 2x yeast extract tryptone (YT) complex medium (16 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) supplemented with 5 g/L of Na-acetate or glucose. Second precultures and main cultivations in shake flasks were carried out using modified CGXII<sub>a</sub> minimal medium (pH 7.0) described by Kiefer, Merkel, Lilge, Hausmann et al. (2021), containing either 13.7 g/L Na-acetate or 10 g/L glucose as carbon source. For high cell density cultivations in bioreactors, modified CGXII<sub>b</sub> high cell density minimal medium (pH 6.8) from Kiefer, Merkel, Lilge, Hausmann et al. (2021) was used as batch medium. For the pH-coupled fed-batch phase, the first feed solution contained pure lignocellulosic acetic acid ( $\geq 99.9\%$  purity) from Lenzing AG, a biorefinery product of pulp production from beech wood (Lenzing™ Acetic Acid Biobased). The second feed medium referred to as CGXII feed solution was comprised of 105 g/L urea,

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic or genotype	References
Strain		
<i>E. coli</i> BL21-Gold(DE3)	F <sup>-</sup> <i>ompT hsdS</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>dcm</i> <sup>+</sup> Tet <sup>r</sup> <i>gal</i> λ(DE3) <i>endA Hte</i>	Agilent Technologies
<i>C. glutamicum</i> ATCC 13032	Biotin-auxotrophic wild-type strain	Abe et al. (1967)
<i>C. glutamicum</i> MB001	Genome-reduced wild-type strain ATCC 13032 with in-frame deletion of chromosomal prophage regions CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752) and CGP3 (cg1890-cg2071)	Baumgart et al. (2013)
<i>C. glutamicum</i> MB001(DE3)	MB001 derivative with chromosomally encoded T7 RNAP gene 1 (cg1122-P <sub>lacI</sub> - <i>lacI</i> -P <sub>lacUV5</sub> - <i>lacZ</i> α-T7 gene 1-cg1121)	Kortmann et al. (2015)
Plasmid		
pEKEx2	Kan <sup>R</sup> ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression under control of the tac promoter (P <sub>tac</sub> , <i>lacI</i> <sup>q</sup> , pBL1 <i>oriV</i> <sub>Cg</sub> , pUC18 <i>oriV</i> <sub>Ec</sub> )	Eikmanns et al. (1991)
pEKEx2- <i>eyfp</i>	Kan <sup>R</sup> ; pEKEx2 vector containing <i>eyfp</i> gene	Hentschel et al. (2012)
pMKEx2	Kan <sup>R</sup> ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression under control of the T7 promoter (P <sub>lacI</sub> , <i>lacI</i> , P <sub>T7</sub> , <i>lacO</i> <sub>1</sub> , N-term. Strep-tag II, MCS, C-term. His-tag, pHM1519 <i>ori</i> <sub>Cg</sub> ; pACYC177 <i>ori</i> <sub>Ec</sub> )	Kortmann et al. (2015)
pMKEx2- <i>eyfp</i>	Kan <sup>R</sup> ; pMKEx2 vector containing <i>eyfp</i> gene	Kortmann et al. (2015)
pMKEx2-His(6)- <i>eyfp</i>	Kan <sup>R</sup> ; pMKEx2 vector containing His(6)- <i>eyfp</i> gene	This study

10 g/L KH<sub>2</sub>PO<sub>4</sub>, 10 g/L K<sub>2</sub>HPO<sub>4</sub> and 2 mg/L D-biotin. In case of plasmid-containing strains, batch cultivation media for shake flask and bioreactor experiments were supplemented with the appropriate kanamycin concentrations of 50 µg/ml (*E. coli*) or 25 µg/ml (*C. glutamicum*), respectively.

## Cloning procedure and plasmid construction

DNA manipulation and cloning was performed using standard laboratory protocols (Sambrook & Russell, 2001). Restriction enzymes, T4 DNA ligase and DNA polymerases were purchased from New England Biolabs (NEB) GmbH. Oligonucleotides were synthesized from Eurofins Genomics Germany GmbH. Genes were amplified by PCR (peqSTAR 96X; VWR GmbH) using Q5® High-Fidelity DNA Polymerase from NEB. Plasmid DNA derived from *E. coli* was isolated using innuPREP Plasmid Mini Kit (Analytik Jena AG). Purification of PCR products or DNA fragments was carried out with QIAquick PCR & Gel Cleanup Kit (QIAGEN GmbH). Transformation of ligation mixtures into CaCl<sub>2</sub>-competent cells of *E. coli* BL21-Gold(DE3) was done via heat-shock procedure. Preparation of competent *C. glutamicum* cells and transformation of plasmid DNA by electroporation (0.1 mm gap, 1250V) was performed according to Tauch et al. (2002) using the Eporator® from Eppendorf AG. Recombinant clones were screened by colony PCR using *Taq* 5x Master Mix from NEB and new expression vectors were confirmed by Sanger sequencing at Eurofins.

For the construction of expression vector pMKEx2-His(6)-*eyfp*, a 779bp fragment was amplified from original vector pMKEx2-*eyfp* using oligonucleotides His(6)-*eyfp\_fw* (5' AAAACATGCCATGGGACATCATCATCATCACGG CAGCGGTGTGAGCAAGGGCGAGGAG3') and His(6)-*eyfp\_rv* (5'AAAACGCGGATCCTTATCTAGACTTGTACAG CTCG 3') to synthetically add the hexahistidine sequence upstream of the *eyfp* gene. A flexible linker sequence (Gly-Ser-Gly) was added in between to ensure that the histidine affinity tag remains flexible and accessible for binding to the chromatography resin. After restriction digestion with NcoI-HF and BamHI-HF, the amplified PCR product was ligated into the multiple cloning site of the double-digested vector backbone of pMKEx2-*eyfp* to obtain the modified expression vector referred to as pMKEx2-His(6)-*eyfp*.

## Batch cultivations in shake flasks

Shake flask cultivations were routinely performed at 30°C and 130rpm in an incubator shaker (New Brunswick™/Innova® 44; Eppendorf AG) using baffled flasks with a filling volume of 10% (v/v). Inoculum preparation was comprised of two pre-cultivation steps starting from 100 µl of the respective glycerol stock. First precultures of the *C. glutamicum* strains were grown in a total volume of 25 ml 2xYT complex medium. Depending on the used carbon source of the main culture, either 5 g/L Na-acetate or 5 g/L glucose were supplemented. After incubation for about 18h, 2 ml of the first preculture (=2% v/v) was used to inoculate a second preculture containing 100 ml of the respective modified CGXII<sub>a</sub> minimal medium

composition. Subsequently, second precultures were incubated for about 22 h. Main cultivation experiments were inoculated to an initial  $OD_{600nm}$  of 1 ( $=0.23 \text{ g}_{CDW}/\text{L}$ ) using an appropriate volume of the second precultures. Shake flask cultivation experiments were carried out as biological duplicates.

## Fed-batch bioreactor cultivations

Fed-batch cultivations on acetate were conducted in a 42 L stirred-tank bioreactor system from ZETA GmbH equipped with three 6-bladed Rushton turbine impellers, four baffle plates and mechanical foam centrifuge as described elsewhere (Hoffmann et al., 2020). For high cell density cultivation of *C. glutamicum* strain MB001(DE3) harbouring expression vector pMKEx2-*eyfp* or pMKEx2-His(6)-*eyfp*, our recently developed pH-coupled feeding strategy was used (Kiefer, Merkel, Lilge, Hausmann et al., 2021). Briefly, microbial consumption of acetate ( $\text{Ac}^-$ ) from the culture medium leads to an increasing pH value due to equimolar removal of protons ( $\text{H}^+$ ). Therefore, acetate feeding can be coupled to the online pH control by using its pure, non-dissociated acetic acid form (HAc) as pH titrant. The experimental bioreactor set-up and cultivation conditions were the same as described in our previous study (Kiefer, Merkel, Lilge, Hausmann et al., 2021). Differing from this, the culture pH during the fed-batch phase was regulated to a setpoint of  $7.3 \pm 0.1$ . For supply of nitrogen (urea) and replenishment of other essential nutrients (phosphate and D-biotin), addition of CGXII feed solution was coupled to acetate feeding via an automated feeding programme (C/N feeding ratio = 10 C-mole/N-mole). For preparation of the inoculum, second precultures with a total volume of 700 ml modified CGXII<sub>h</sub> high cell density minimal medium were grown for about 22 h in shake flasks. Main cultivations in the bioreactor with a starting volume of 10 L were then inoculated to an initial  $OD_{600nm}$  of about 1 ( $=0.23 \text{ g}_{CDW}/\text{L}$ ). The cultivation temperature was kept constant at 30°C and dissolved oxygen (DO) concentration was maintained above 30% by a proportional–integral–derivative (PID) controller regulating the stirred speed (200 to 1000 rpm) and gas aeration rate (10–21 lpm). If necessary, oxygen-enriched gas was applied to ensure sufficient oxygen transfer at high cell densities. Bioreactor cultivation processes were carried out as biological duplicates.

## Cultivation and protein analytics

During cultivation, samples of the culture broth were taken in regular intervals. For analysis of bacterial growth, the optical density at 600 nm ( $OD_{600nm}$ ) was

measured with a spectrophotometer (Biochrom WPA CO8000; Biochrom Ltd.). Biomass concentrations ( $c_{\text{biomass}}$ ) were calculated from  $OD_{600nm}$  values using a correlation factor ( $\alpha = 4.3$ ) previously determined (Kiefer, Merkel, Lilge, Hausmann et al., 2021). For quantification of glucose, acetate and ammonium concentrations in the culture supernatants, enzymatic assay kits from R-Biopharm AG were used. Prior to analysis, samples of 2 ml were centrifuged for 10 min at 4°C and 14,000 rpm (5430 R, FA-45-30-11 rotor; Eppendorf AG) and cell-free supernatants were stored at  $-20^\circ\text{C}$ .

For analysis of *eyfp* expression by fluorescence measurement, samples of 1 ml culture broth were harvested as described before (14,000 rpm, 10 min and 4°C) and cell pellets were routinely stored at  $-20^\circ\text{C}$ . For measurements, pellets were resuspended in 1 ml of saline and rediluted to an  $OD_{600nm}$  of 0.1. The diluted cell suspensions were transferred into 96-well microtiter plates (200  $\mu\text{l}$ /well) and *eyfp* fluorescence (ex/em 512 nm/527 nm) was measured in a multimode plate reader (Enspire®; Perkin Elmer Inc.) with saline as blank.

To analyse recombinant eYFP production by SDS-PAGE analysis, an appropriate volume of culture broth was centrifuged for 10 min at 4°C and 4700 rpm (Heraeus X3R, TX-750 rotor; Thermo Fisher Scientific GmbH) to obtain at least 0.1 g of wet biomass and pellets were stored at  $-20^\circ\text{C}$ . For cell extract preparation, thawed pellets were resuspended (20% m/v) in ice-cold lysis buffer (20 mM Na-phosphate, 500 mM NaCl, pH 7.4) supplemented with Halt™ Protease Inhibitor Cocktail from Thermo Fisher Scientific GmbH. Next, 500  $\mu\text{l}$  of cell suspension were mixed with 500  $\mu\text{l}$  ( $=1.25 \text{ g}$ ) of glass beads ( $\varnothing$  0.1 mm) and cell disruption was performed on a Vortex-Genie™ 2 mixer equipped with TurboMix™ attachment (Scientific Industries Inc.). To avoid overheating, five cycles of 2 min shaking at maximum speed (3200 rpm) were conducted with 1 min intervals on ice in between. The resulting crude cell extract was centrifuged for 30 min at 4°C and 14,000 rpm (5430 R, FA-45-30-11 rotor; Eppendorf AG) to remove insoluble cell debris and the protein concentration was measured according to Bradford (1976) using Roti® Quant (Carl Roth GmbH). The soluble protein fraction was then used to analyse the *eyfp* expression by Laemmli SDS-PAGE (Laemmli, 1970) using 4%–20% Mini-PROTEAN® TGX™ Precast Protein Gels and Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, USA). After Coomassie staining with Roti®Blue quick, protein gels were scanned with imaging system Quantum ST5 (Wilber Lourmat Deutschland GmbH). PageRuler™ Plus (10–250 kDa) from Thermo Fisher Scientific GmbH was used as protein ladder. As a reference protein, an in-house expressed and purified His(6)-eYFP standard was used. Densitometric quantification of eYFP protein bands was performed with GelAnalyzer 19.1 software ([www.gelanalyzer.com](http://www.gelanalyzer.com)) by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc.



## Isolation and IMAC purification of His(6)-eYFP

For exemplary purification of recombinantly produced His(6)-eYFP, culture broth samples of 40 ml from high cell density cultivation of *C. glutamicum* MB001(DE3) pMKEx2-His(6)-*eyfp* were harvested for 10 min at 4°C and 4700 rpm (Heraeus X3R, TX-750 rotor; Thermo Fisher Scientific GmbH), and biomass pellets were stored at -20°C. For intracellular target protein isolation, one thawed cell pellet (~7 g) was resuspended in 100 ml of ice-cold binding buffer (20 mM Na-phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. Cell disruption was performed in a high-pressure homogenizer (APV 2000, SPX Flow Technology Germany GmbH, Norderstedt, Germany) using five cycles at a pressure of 1400 bar. For separation of insoluble cell debris, the crude cell extract was centrifuged for 30 min at 4°C and 11,348 rpm (Heraeus X3R, Fiberlite™ F15-8 ×50cy rotor, Thermo Fisher Scientific GmbH), and the soluble protein fraction was used for subsequent protein purification.

Purification of His(6)-eYFP from the soluble protein fraction was performed via immobilized metal affinity chromatography (IMAC) using an automated chromatography system (ÄKTA™ start; Cytiva Europe GmbH). For capturing the recombinant protein, a prepacked 5 ml HisTrap™ FF column precharged with Ni Sepharose™ 6 Fast Flow was used. The column was equilibrated with five column volumes (CV) of binding buffer. Unbound proteins were washed out with about 10 CV of binding buffer. The bound His(6)-eYFP fraction was eluted with a one-step gradient (100%) of elution buffer (20 mM Na-phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Pooled fractions were processed to remove imidazole with a buffer exchange using PD-10 desalting columns from Cytiva. The recovery procedure for isolation and purification of His(6)-eYFP was performed in technical duplicates.

## Data analysis

Specific growth rates  $\mu$  [ $\text{h}^{-1}$ ] and substrate-specific biomass yields  $Y_{\text{XIS}}$  [ $\text{g}_{\text{CDW}}/\text{g}_{\text{acetate}}$ ] were determined as previously described by Kiefer, Merkel, Lilge, Hausmann et al. (2021). Biomass-specific fluorescence values [ $\text{AU}/\text{g}_{\text{CDW}}\cdot\text{L}$ ] were given by the ratio of fluorescence [AU] and respective biomass concentration [ $\text{g}_{\text{CDW}}/\text{L}$ ]. Relative target protein contents of eYFP [%] were expressed as the percentage proportion of total soluble protein. Biomass-specific product yields  $Y_{\text{PIX}}$  [ $\text{mg}_{\text{eYFP}}/\text{g}_{\text{CDW}}$ ] were calculated from the produced eYFP level and respective biomass concentration. Volumetric productivities of eYFP production [ $\text{g}/\text{L}\cdot\text{h}$ ] were determined using the produced amount of eYFP, corresponding bioreactor volume [L] and process time [h]. Graphs were

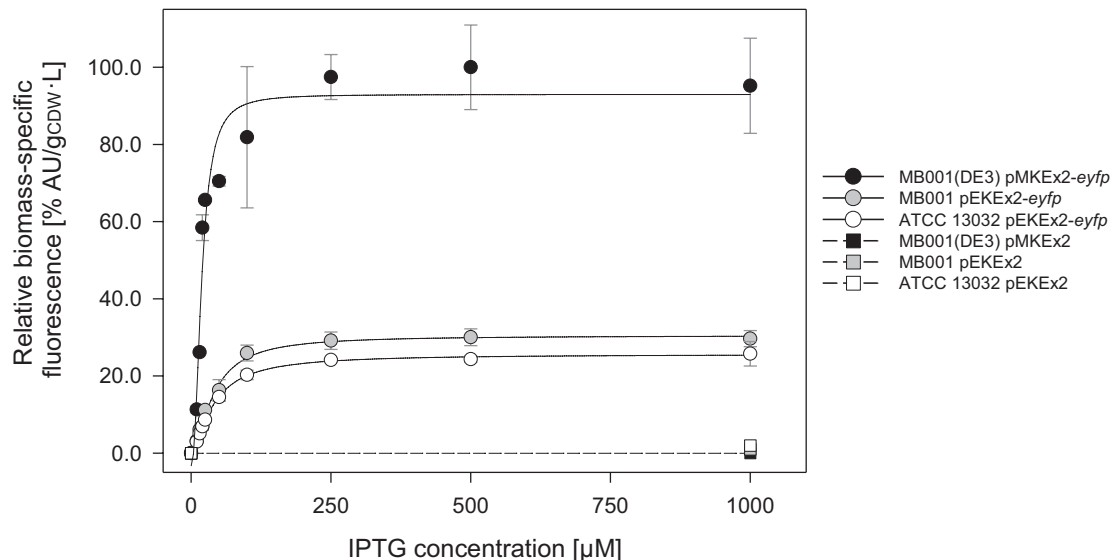
generated using the scientific graphing and data analysis software SigmaPlot 14.0 (Systat Software Inc.). For fitting the experimental data of biomass-specific fluorescence, a 4-parameter sigmoidal Hill fit was used.

## RESULTS AND DISCUSSION

### Characterization of IPTG-dependent eYFP production on acetate using T7lac and tac promoter system

To investigate the feasibility of acetate-based protein production, expression of heterologous model protein eYFP in *C. glutamicum* was first studied using two different promoter systems. For this purpose, the expression vector pMKEx2 (Kortmann et al., 2015) based on the strong IPTG-inducible T7lac promoter was chosen. Combined with the prophage-free *C. glutamicum* strain MB001(DE3) carrying a chromosomally integrated T7 RNA polymerase gene under control of the *lacUV5* promoter, this expression system has been already shown to be powerful for glucose-based protein expression in *C. glutamicum* (Kortmann et al., 2015). As a comparison, the well-established expression vector pEKEx2 (Eikmanns et al., 1991) containing the IPTG-inducible *tac* promoter with weaker promoter strength was tested, which is recognized by the corynebacterial RNA polymerase itself. To analyse the respective eYFP production on acetate, *C. glutamicum* strains MB001(DE3) pMKEx2-*eyfp*, MB001 pEKEx2-*eyfp* and wild-type strain ATCC 13022 pEKEx2-*eyfp* (see Table 1) were grown in shake flasks with modified CGXII<sub>a</sub> minimal medium containing 10 g/L acetate as sole carbon source. The IPTG-dependent expression of the target protein was induced at an  $\text{OD}_{600\text{nm}}$  of 2 ( $=0.47 \text{ g}_{\text{CDW}}/\text{L}$ ) using varying inductor concentrations of 0, 10, 15, 20, 25, 50, 100, 250, 500 and 1000  $\mu\text{M}$  IPTG. For a quantitative comparison of the protein production performances, the eYFP-specific fluorescence of the bacterial cultures was measured in the late-exponential growth phase ( $=6 \text{ h}$  of post-induction growth). Fluorescence signals were correlated to the respective biomass concentrations to consider the slightly differing final cell densities of the different strains. A diagrammatic comparison showing the biomass-specific eYFP fluorescence of the different *C. glutamicum* strains is provided in Figure 1.

For both promoter systems tested, a characteristic IPTG dose-dependent saturation curve as previously shown for eYFP production during growth of *C. glutamicum* on glucose (Kortmann et al., 2015) was also observed for acetate as carbon source. When cultivated in the presence of IPTG, maximum biomass-specific eYFP fluorescence signals could be detected at inductor concentrations above 250  $\mu\text{M}$  for all three tested strains. However, higher IPTG concentrations did not result in significantly enhanced fluorescence values, obviously



**FIGURE 1** Effect of IPTG concentration on heterologous eYFP production in *C. glutamicum* with different promoter systems using acetate as sole carbon source. *C. glutamicum* strains MB001(DE3) pMKEx2-*eyfp* (black circles), MB001 pEKEx2-*eyfp* (grey circles) and ATCC 13032 pEKEx2-*eyfp* (white circles) were grown at 30°C and 130 rpm in baffled shake flasks with 50 ml modified CGXII<sub>a</sub> minimal medium containing 10 g/L acetate. Heterologous expression of target gene *eyfp* was induced at an optical density of 2 (=0.47 g<sub>CDW</sub>/L) using varying IPTG concentrations ranging from 0 to 1000 µM. After 6 h of post-induction growth, the biomass-specific eYFP fluorescence levels of the bacterial cell suspensions were measured in the late-exponential growth phase. Production performance is shown relative to that of MB001(DE3) pMKEx2-*eyfp*, which was set to 100%. Strains MB001(DE3) (black squares), MB001 (grey squares) and ATCC 13032 (white squares) harbouring the respective empty vectors without *eyfp* gene were grown as negative control strains. Values and error bars represent means ± standard deviations from biological duplicate cultivations.

indicating IPTG-saturated production levels. Compared with strain MB001 pEKEx2-*eyfp* (30.0 ± 2.2% AU/g<sub>CDW</sub>·L at 500 µM IPTG), a 3.3-fold higher maximum biomass-specific eYFP fluorescence was found for T7 expression strain MB001(DE3) pMKEx2-*eyfp* (100 ± 11% AU/g<sub>CDW</sub>·L at 500 µM IPTG). This is in accordance with the results from Kortmann et al. (2015), where a 3.5-fold higher specific fluorescence in glucose-containing CGXII minimal medium has been described for eYFP production under control of the T7lac promoter. For wild-type strain ATCC 13032 pEKEx2-*eyfp*, a 3.9-fold lower maximum biomass-specific fluorescence of 25.8 ± 3.2% AU/g<sub>CDW</sub>·L (1000 µM IPTG) was observed. When comparing the eYFP production levels between the pEKEx2-*eyfp* harbouring strains, prophage-free strain MB001 showed a clearly higher fluorescence level (up to 22.4%). This is in agreement with a previous study, where the same was reported for eYFP production on glucose due to presumably higher plasmid copy number in the genome-reduced strain (Baumgart et al., 2013). Accordingly, this also holds true for eYFP production during growth of MB001 on acetate as carbon source. For the negative control strains harbouring the empty vectors pMKEx2 and pEKEx2 without *eyfp* gene, no background fluorescence could be detected. Interestingly, bacterial growth performances of the different strains were quite unaffected by the respective inducer concentration added and minor growth differences could be rather explained due to biological variations (Figure S1). It can thus be concluded that the phenomenon known as ‘metabolic

burden’ (Bentley et al., 1990) typically described for heterologous protein production is obviously not observed for overexpression of eYFP as target protein. In fact, the same has been also reported for T7 RNAP-dependent production of fluorescence model protein GFP with *E. coli* in a previous study, where almost no negative effect on cell growth could be observed (Li & Rinas, 2020).

To summarize, heterologous production of model protein eYFP in *C. glutamicum* based on acetate as carbon source was demonstrated with two different, commonly used IPTG-inducible promoter systems. In this context, no inhibitory effect of acetate on production performances of the *C. glutamicum* strains could be obviously found, although acetate was still present in concentrations above 8 g/L at the time point of induction. Moreover, the T7 RNAP-driven expression system was also identified to be powerful for acetate-based protein production. For this reason, further experiments in this study were only focused on *C. glutamicum* strain MB001(DE3) pMKEx2-*eyfp*.

## Comparison of T7 RNAP -dependent eYFP production using acetate and glucose as carbon sources

Since carbohydrate-based substrates such as glucose are still the commonly used biotechnological carbon sources, the next aim was to quantitatively compare the T7 RNAP-dependent eYFP protein production on

acetate to that on glucose. For this purpose, *C. glutamicum* strain MB001(DE3) pMKEx2-*eyfp* was cultivated in shake flasks with modified CGXII<sub>a</sub> minimal medium containing either 10 g/L acetate or 10 g/L glucose as sole carbon source. Expression of *eyfp* gene was induced at an OD<sub>600nm</sub> of 2 (=0.47 g<sub>CDW</sub>/L) using an IPTG concentration of 1 mM to guarantee maximum induction. To quantitatively compare the heterologous protein production on the different substrates, the eYFP fluorescence of the bacterial cultures was analysed until reaching the stationary growth phase (=10 h of post-induction growth). The corresponding graphs showing biomass growth, substrate consumption and eYFP production for the different carbon sources are illustrated in Figure 2.

As shown in Figure 2A, biomass concentrations of up to 6.0 ± 0.1 g<sub>CDW</sub>/L were achieved for growth of MB001(DE3) pMKEx2-*eyfp* on 10 g/L glucose as carbon source ( $Y_{XIS} = 0.54 \pm 0.01$  g<sub>CDW</sub>/g<sub>glucose</sub>). In contrast to this, maximum cell densities reached for 10 g/L acetate were shown to be 1.6-fold lower (3.7 ± 0.1 g<sub>CDW</sub>/L) due to the lowered biomass yield of 0.36 ± 0.00 g<sub>CDW</sub>/g<sub>acetate</sub>. However, maximum growth rates on acetate ( $\mu_{\max}^{\text{acetate}} = 0.44 \pm 0.01$  h<sup>-1</sup>) were found to be competitive to that on glucose as carbon source ( $\mu_{\max}^{\text{glucose}} = 0.43 \pm 0.01$  h<sup>-1</sup>). This is in accordance with our previous study, where  $\mu_{\max}$  values of 0.45 h<sup>-1</sup> were found for batch culture studies of wild-type strain ATCC 13032 on 10 g/L acetate (Kiefer, Merkel, Lilge, Hausmann et al., 2021).

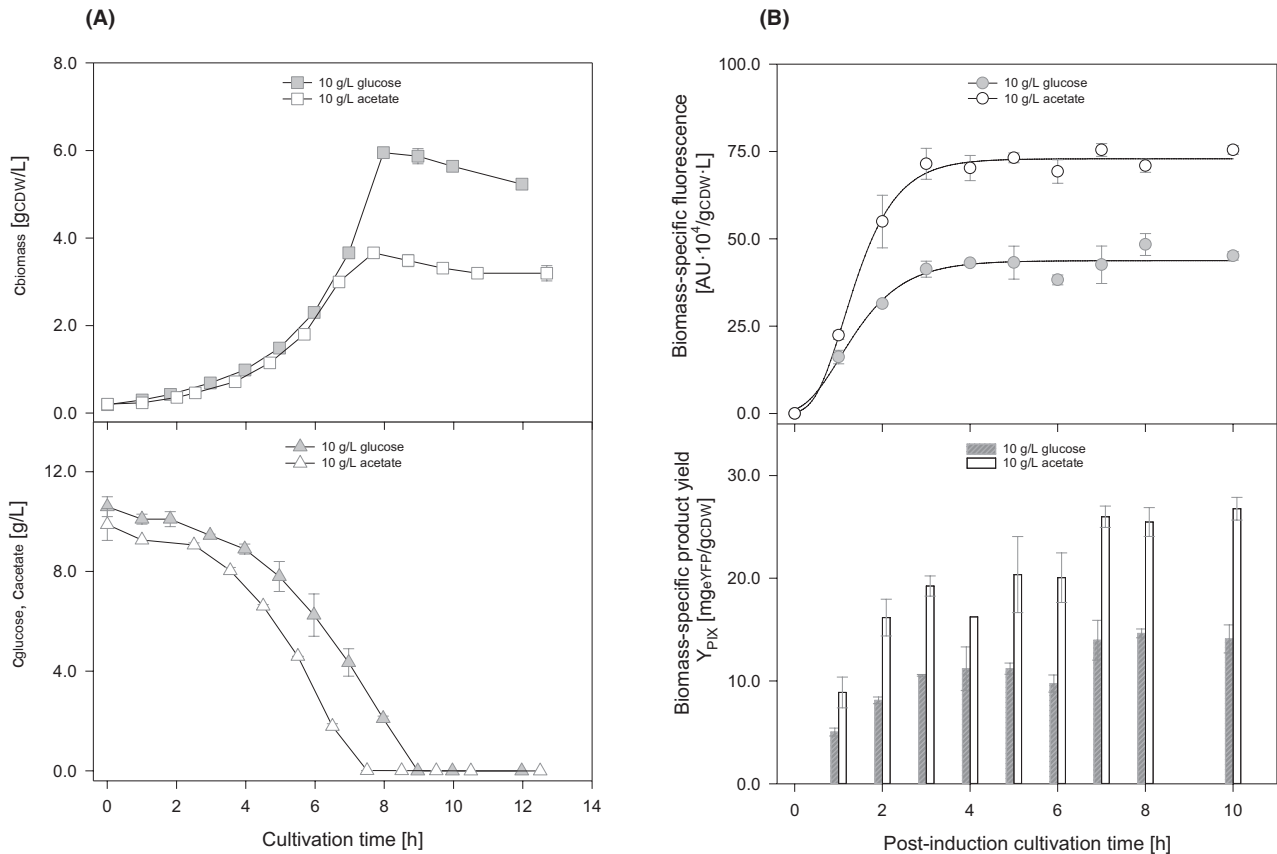
With respect to heterologous protein production, comparable maximum eYFP fluorescence values could be measured for growth on glucose (207 ± 33 AU·10<sup>4</sup>) and acetate (225 ± 13 AU·10<sup>4</sup>), respectively (see Table S1). However, when considering the much lower cell densities achieved on acetate, a drastically higher biomass-specific fluorescence level was surprisingly found for acetate-based eYFP production (Figure 2B). While the specific fluorescence for growth on 10 g/L glucose reached a maximum of 48.4 ± 3.1 AU·10<sup>4</sup>/g<sub>CDW</sub>·L (t = 8 h induction), a 1.6-fold higher value of 75.5 ± 1.3 AU·10<sup>4</sup>/g<sub>CDW</sub>·L (t = 10 h induction) could be achieved for 10 g/L acetate as substrate. In order to compare the eYFP production not only on fluorescence activity but also on a level of protein amount, the intracellular target protein contents were additionally determined. As can be seen in the pattern of the respective biomass-specific product yields ( $Y_{PIX}$ ), the same was also true for eYFP production on protein level. In fact, a 1.8-fold higher  $Y_{PIX}$  (26.8 ± 1.1 mg<sub>eYFP</sub>/g<sub>CDW</sub>) was reached for growth and eYFP production of MB001(DE3) pMKEx2-*eyfp* on acetate compared with that on glucose (14.6 ± 0.4 mg<sub>eYFP</sub>/g<sub>CDW</sub>). Interestingly, the results thus indicate that T7 RNAP-dependent protein production in *C. glutamicum* was more efficient for acetate as carbon source. This finding is rather unexpected, and the reason thereof remains unclear,

as common fermentations of *C. glutamicum* rely on carbohydrate-based carbon sources with glucose being the most commonly used carbon and energy source of this bacterium. One may speculate that microbial growth on acetate via the glyoxylate cycle as anaplerotic pathway is associated with a higher precursor supply for eYFP biosynthesis or even a higher plasmid copy number of pMKEx2-*eyfp*. Apart from this, the presence of glucose for a long time period after IPTG addition might have affected the induction of the chromosomally encoded T7 RNAP gene itself by high levels of cAMP. Even though the targeted point mutations in the chromosomal *lacUV5* promoter are known to reduce its sensitivity towards catabolite repression, it has been reported that it is not fully eliminated, and the highest target protein expression can be observed in absence of glucose (Novy & Morris, 2001). Therefore, a stronger expression and activity of T7 RNAP resulting in higher target protein expression at low cAMP levels might be another possible explanation for improved eYFP production in acetate-containing CGXII minimal medium. However, further investigations such as plasmid copy number determination or intracellular cAMP level measurement might be necessary to clarify the reasons for improved eYFP production during growth on acetate as carbon source.

Despite the fact that final cell densities of MB001(DE3) pMKEx2-*eyfp* for growth on glucose were 62% higher, the higher biomass-specific production performance for growth on acetate was found to yield a quite similar eYFP titer of approximately 86 mg per L of culture (Table S1). With regard to a high cell density cultivation process with integrated biomass-coupled product formation, acetate might thus potentially present a beneficial substrate for target protein accumulation due to the 83% higher biomass-specific product yield  $Y_{PIX}$ . In theory, acetate-based production of model protein eYFP might thus enable higher product titers at the same biomass concentration compared with glucose as carbon source.

### High cell density cultivation of *C. glutamicum* T7 expression system on acetate for high-level eYFP accumulation

In general, high cell density fed-batch cultures are commonly the method of choice for accumulation of heterologous target proteins (Choi et al., 2006). Accordingly, the next goal of this study was to establish acetate-based protein production with *C. glutamicum* T7 expression system under high cell density conditions in a 42L stirred-tank bioreactor. For this purpose, our recently described fed-batch process strategy based on a pH-coupled online feeding control for acetate supply (Kiefer, Merkel, Lilge, Hausmann et al., 2021) was used for growth and eYFP production of *C.*



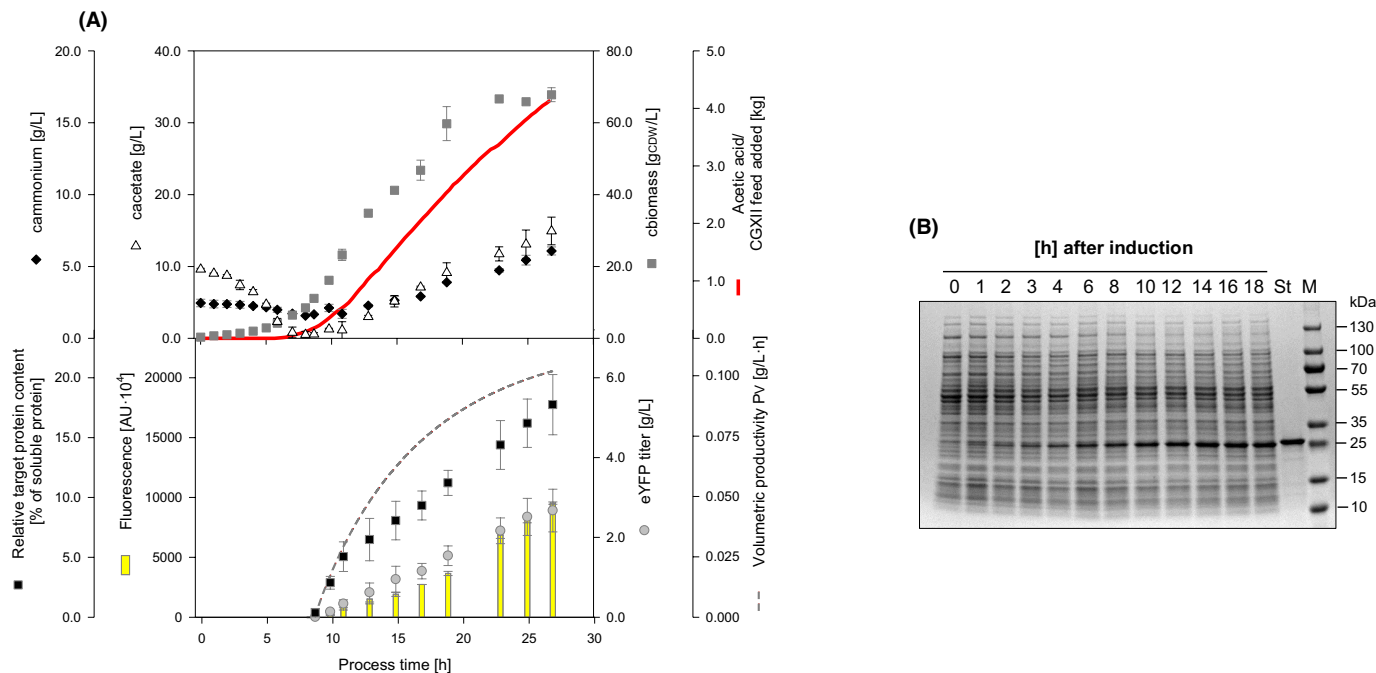
**FIGURE 2** Comparison of T7 RNAP-dependent eYFP production in *C. glutamicum* MB001(DE3) using acetate or glucose as carbon source. *C. glutamicum* strain MB001(DE3) pMKEx2-*eyfp* was grown at 30°C and 130 rpm in baffled shake flasks with 200 ml modified CGXII<sub>a</sub> minimal medium containing either 10 g/L acetate (white symbols) or 10 g/L glucose (grey symbols) as sole carbon source. Heterologous expression of target gene *eyfp* was induced at an optical density of 2 (=0.47 g<sub>CDW</sub>/L) using an IPTG concentration of 1 mM. Values and error bars represent means ± standard deviations from biological duplicate cultivations. (A) Time-courses of biomass growth (squares) and substrate consumption (triangles) during cultivation on the respective carbon sources. (B) Time-courses of biomass-specific fluorescence (circles) and biomass-specific product yield Y<sub>PiX</sub> (bars) during the post-induction growth on the respective carbon sources.

*glutamicum* MB001(DE3) pMKEx2-*eyfp*. After an initial batch phase with a starting concentration of 10 g/L acetate, a fed-batch phase with pH-coupled supply of pure acetic acid (HAc) was followed. For compensating the pH-decreasing effect by consumption of ammonia as nitrogen source, the latter was fed in form of urea present in CGXII feed solution (C/N feeding ratio = 10 C-mole/N-mole). The production phase for heterologous eYFP accumulation was initiated by addition of 1 mM IPTG upon reaching a cell density of OD<sub>600nm</sub> 50 (=11.6 g<sub>CDW</sub>/L). Graphs showing the results of the bioreactor fed-batch cultivation are presented in Figure 3. Obtained cultivation data and calculated parameters are further summarized in Table 2.

Within the initial batch phase of the bioreactor cultivation, a cell density of 4.3 ± 0.5 g/L cell dry weight was reached after about 6 h (Figure 3A). In the end of this process phase, residual acetate and ammonium concentrations of 2.3 ± 0.4 and 2.0 ± 0.2 g/L were detected in the culture supernatant. In the subsequent pH-coupled fed-batch phase, a total amount of 4188 ± 113 g acetate was fed by the auto-regulated pH control resulting in a

final biomass concentration of up to 67.8 ± 1.9 g<sub>CDW</sub>/L after 27 h. With respect to the growth performance of *C. glutamicum* MB001(DE3) pMKEx2-*eyfp* under bioreactor conditions, μ<sub>max</sub> values of 0.42 ± 0.00 h<sup>-1</sup> were determined. However, the specific growth rate was found to decline after a process time of about 10 h, although acetate as carbon source was sufficiently present in the culture medium (1.3 ± 0.1 g/L). Consequently, the μ<sub>overall</sub> for the feeding phase clearly decreased to a value of 0.08 ± 0.00 h<sup>-1</sup>. It should be noted that this finding is consistent with our previous study, where the same trend was observed for fed-batch cultivations of *C. glutamicum* ATCC 13032 without product formation (Kiefer, Merkel, Lilge, Hausmann et al., 2021). More strikingly, this is also true for the slight accumulations of acetate and ammonium over time, reaching final concentrations of 14.9 ± 1.9 and 6.1 ± 0.3 g/L in the end. The reason for this can be explained by the shifting bacterial C/N consumption ratio, as already described for the previous wild-type cultivations. When reaching a process time of about 13 h, the C/N consumption ratio started to clearly exceed the constantly applied





**FIGURE 3** Fed-batch cultivation of *C. glutamicum* MB001(DE3) for T7 RNAP-dependent eYFP production under high cell density conditions using acetate as carbon source. *C. glutamicum* strain MB001(DE3) pMKEx2-*eyfp* was grown at 30°C in a stirred-tank bioreactor with an initial volume of 10 L modified CGXII<sub>b</sub> high cell density minimal medium. Feeding of pure acetic acid (HAc) was coupled to the pH control of the bioreactor system. Heterologous expression of target gene *eyfp* was induced at an optical density of 50 (=11.6 g<sub>CDW</sub>/L) using an IPTG concentration of 1 mM. Values and error bars represent means ± standard deviations from biological duplicate cultivations. (A) Time-courses of biomass growth (dark grey squares), fed amounts of acetic acid and CGXII feed solution (red line), concentrations of acetate (white triangles) and ammonium (black diamonds), eYFP titer (grey circles), volumetric productivity (grey dashed line), relative target protein content (black squares) and fluorescence level (yellow bars) during the cultivation process. (B) SDS-PAGE analysis showing the soluble protein fractions at different post-induction cultivation time points. Equivalent amounts of total protein (6 μg) were loaded onto the gel and protein bands were visualized by Coomassie brilliant blue staining. St, purified His(6)-eYFP protein standard; M, protein marker with molecular weights indicated in kDa.

C/N feeding ratio of 10 C-mole/N-mole (Figure S2). As cultivation continued, nitrogen oversupply from CGXII feed solution consequently caused an overfeeding of acetate due to the pH-increasing effect of excess ammonia acting as a base.

With regard to acetate-based protein production, a high eYFP production level was found under high cell density conditions in the bioreactor. In fact, the fluorescence intensity of the bacterial culture steadily increased after IPTG induction ( $t = 9$  h), reaching a maximum of  $9537 \pm 98 \text{ AU} \cdot 10^4$  after 27 h of cultivation. A highly correlating trend could be also observed for the intracellular eYFP titer, which increased up to a maximum of  $2.7 \pm 0.3$  g/L until the end of cultivation. In comparison to production performance under batch conditions in shake flasks, maximum values for fluorescence level and eYFP titre could be enhanced by factors of 42 and 35, respectively. Beyond that, the relative target protein content was found to be 2.2-fold higher compared to shake flask cultivations ( $7.9 \pm 0.1\%$ ) and steadily increased up to a maximum of  $17.7 \pm 2.5\%$  of soluble protein. This is reflected by the representative expression pattern shown in SDS-PAGE analysis (Figure 3B), where an eYFP protein band with continuously increasing intensity could be observed at a size slightly above 25 kDa (theoretical

mass: 27 kDa). The extended post-induction cultivation time of 18 h realized under fed-batch conditions was also associated with a 1.5-fold higher biomass-specific product yield  $Y_{\text{PIX}}$  compared to batch cultivations with 10 h of post-induction growth ( $40.0 \pm 1.0 \text{ mg}_{\text{eYFP}}/\text{g}_{\text{CDW}}$  vs.  $26.8 \pm 1.1 \text{ mg}_{\text{eYFP}}/\text{g}_{\text{CDW}}$ ). Interestingly, the overall productivity  $P_V$  was still found to be maximal ( $0.10 \pm 0.02 \text{ g/L} \cdot \text{h}$ ) until the end of cultivation. This gives reason to assume that an extended production phase could also lead to even higher eYFP protein titers. However, due to the steadily increasing substrate concentrations which might have caused growth (and product) inhibition as previously shown for wild-type ATCC 13032 (Kiefer, Merkel, Lilge, Hausmann et al., 2021), fed-batch cultivation experiments were certainly stopped after a total process time of 27 h. For this reason, the presented acetate-based production process may thus be further optimized to first avoid substrate accumulation over time and second to extend the production phase for reaching even higher product accumulation. This might be either realized by a manual increase of the C/N feeding ratio in the later process phase as already demonstrated (Kiefer, Merkel, Lilge, Hausmann et al., 2021) or by applying a model-based feeding control which continuously adapts the applied C/N feeding ratio to the actual C/N

**TABLE 2** Cultivation data and calculated parameters for fed-batch high cell density cultivation of *C. glutamicum* MB001(DE3) pMKEx2-*eyfp* using acetate as carbon source. Values and error bars represent means  $\pm$  standard deviations from biological duplicate cultivations.

Cultivation parameter	Values
Process time [h]	27
$c_{\text{biomass}}^{\text{max}}$ [g <sub>CDW</sub> /L]	67.8 $\pm$ 1.9
Reactor volume [L]	15.9 $\pm$ 1.3
$m_{\text{biomass}}^{\text{max}}$ produced [g <sub>CDW</sub> ]	1074 $\pm$ 115
Acetate added/consumed [g]	4188 $\pm$ 113/ 3966 $\pm$ 131
Ammonia added/consumed [g]	240 $\pm$ 4/ 165 $\pm$ 7
$Y_{\text{XIS}}$ [g <sub>CDW</sub> /g <sub>acetate</sub> ]	0.27 $\pm$ 0.02
$\mu_{\text{max}}$ [h <sup>-1</sup> ]	0.42 $\pm$ 0.00
$\mu_{\text{overall}}$ feeding phase [h <sup>-1</sup> ]	0.08 $\pm$ 0.00
Fluorescence [AU·10 <sup>4</sup> ]	9537 $\pm$ 98
Biomass-specific fluorescence [AU·10 <sup>4</sup> /g <sub>CDW</sub> ·L]	208 $\pm$ 1
Relative target protein content [% of soluble protein]	17.7 $\pm$ 2.5
eYFP titer [g/L]	2.7 $\pm$ 0.3
$Y_{\text{PIX}}$ [mg <sub>YFP</sub> /g <sub>CDW</sub> ]	40.0 $\pm$ 1.0
$P_{\text{V}}$ overall [g/L·h]	0.10 $\pm$ 0.02

consumption ratio of the bacterial culture. Additionally, an optimization of the induction time point for initiating the production phase might affect the final product titer. However, the presented bioprocess was rather intended to be a first proof of concept demonstrating the feasibility of acetate-based protein production in platform organism *C. glutamicum* under industry-relevant fed-batch cultivation conditions.

To the best of our knowledge, there is only one study in literature so far dealing with recombinant protein production using acetate as carbon source (Leone et al., 2015). This is most likely due to the fact that acetate is generally reported to inhibit recombinant protein production, which is why most studies have only focused on minimizing or fully eliminating acetate formation during heterologous protein production (De Anda et al., 2006; Jensen & Carlsen, 1990; Lozano Terol et al., 2019; Waegeman et al., 2013; Wong et al., 2008). In the only study from Leone and colleagues, a sweet protein (MNEI) was recombinantly produced on acetate with average concentrations of about 180 mg/L using *E. coli* T7 expression system. However, the described production experiments were only restricted to batch cultures and did not include any process strategies for fed-batch cultivations. Compared to general research works which study heterologous protein production in *C. glutamicum* fed-batch cultivations using common carbohydrate carbon sources, the protein titer and overall productivity achieved in our presented acetate-based bioprocess can be regarded as highly competitive (Table 3). For instance, the recombinant enzymes  $\alpha$ -amylase, endoxylanase and

**TABLE 3** Summary of existing literature dealing with heterologous protein production in fed-batch cultivations of *C. glutamicum* strains (2015–2022)

Target protein	Cellular localization	<i>C. glutamicum</i> host strain	Carbon source (type of medium)	Product titer [g/L]	Process time [h]	Productivity <sup>a</sup> [g/L·h]	References
eYFP	Intracellular	MB001(DE3)	Acetate (defined)	2.7	27	0.1	This study
Cutinase-GFP11	Extracellular	ATCC 13032 K9	Glucose (defined)	1.5	25	0.06	Bakkes et al. (2021)
PINP	Extracellular	CGMCC1:15647	Glucose (semi-defined)	1.2	44	0.03	Sun et al. (2020)
Endoxylanase	Extracellular	CGMCC1:15647 $\Delta$ cspB2 $\Delta$ c/pSlnX	Maltose (semi-defined)	1.8	44	0.04	Zhang et al. (2019)
SUMO-NT-proBNP	Intracellular	CGMCC1:15647 $\Delta$ c/pC $\Delta$ porB $\Delta$ mepA	Glucose (semi-defined)	0.3 <sup>b</sup>	28	0.01	Peng et al. (2019)
$\alpha$ -Amylase	Extracellular	ATCC 13032	Glucose (semi-defined)	0.8	21	0.04	Yim et al. (2015)
Endoxylanase	Extracellular	ATCC 13032	Glucose (semi-defined)	1.1	32	0.03	Yim et al. (2015)
Camelid antibody fragment (VHH)	Extracellular	ATCC 13032	Glucose (semi-defined)	1.6	30	0.05	Yim et al. (2015)
Phospholipase C	Extracellular	ATCC 13869	Glucose (semi-defined)	5.5	55	0.1	Ravasi et al. (2015)

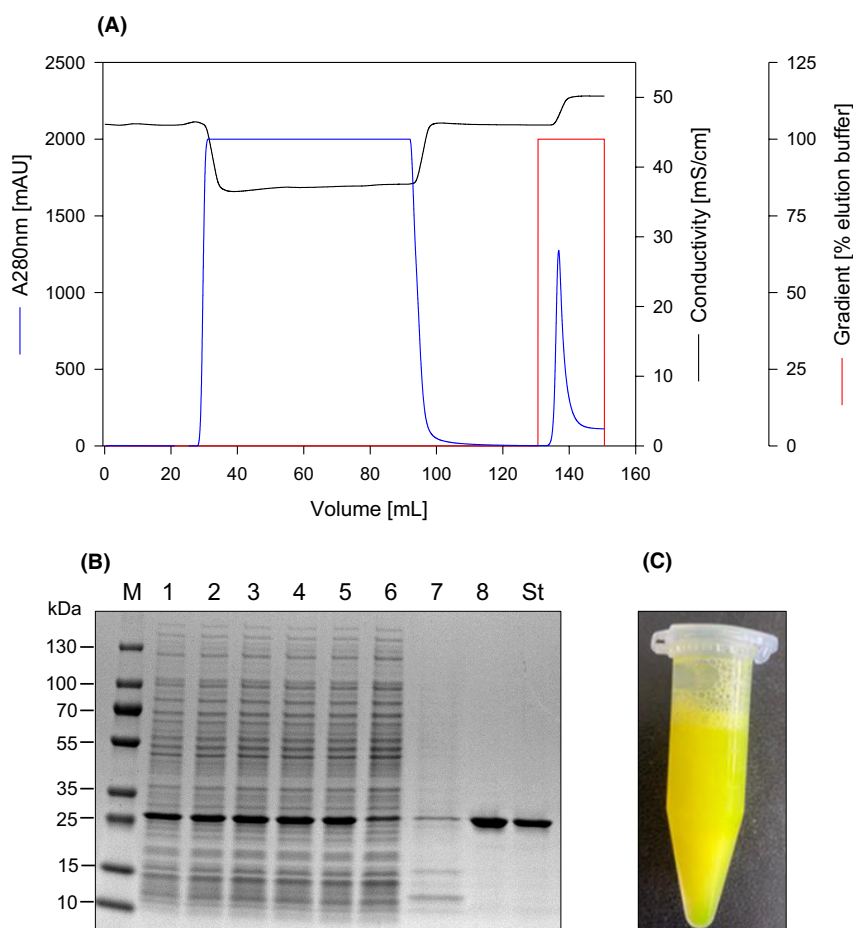
<sup>a</sup>Calculated from the given maximum titer and respective process time.

<sup>b</sup>Calculated from the given yield of 29.96 mg/gDCW.

cutinase have been produced in highest titers of 0.8 g/L (Yim et al., 2015), 1.8 g/L (Zhang et al., 2019) and 1.5 g/L (Bakkes et al., 2021), respectively. In addition, product titers of 1.6, 0.3 and 1.8 g/L have been achieved for production of the biopharmaceutical relevant proteins VHH (Yim et al., 2015), SUMO-NT-proBNP (Peng et al., 2019) and PINP (Sun et al., 2020). When comparing the product titers, it must be considered that many target proteins of other studies have been produced in an extracellular manner in contrast to intracellular protein accumulation in this study. So far, only the study from Ravasi et al. (2015) reported a higher titer of 5.5 g/L for heterologous production of Phospholipase C in a fed-batch cultivation of *C. glutamicum* ATCC 13869. In conclusion, the results of this study indicate that acetate might be also a highly promising and competitive alternative platform substrate for production of heterologous proteins in industry-relevant titers (g/L).

## Recombinant production and purification of a His(6)-tagged eYFP variant

To demonstrate the feasibility of the established acetate-based production process for subsequent product recovery up to pure protein, the bioprocess was finally applied to an affinity-tagged eYFP variant. For this purpose, we synthetically added a hexahistidine sequence to the 5' end of the *eyfp* gene construct from Kortmann et al. (2015) in order to allow a simple one-step protein purification from the cell lysate. The resulting expression vector pMKEx2-His(6)-*eyfp* was transformed into *C. glutamicum* strain MB001(DE3) and the newly constructed expression strain was applied for acetate-based His(6)-eYFP production under high cell density conditions as described before. To isolate the expressed target protein, biomass samples from 40 ml



**FIGURE 4** Exemplary isolation and purification of His(6)-tagged eYFP produced in high cell density cultivation of *C. glutamicum* MB001(DE3) on acetate as carbon source. Biomass pellets (~7 g) from 40 ml of culture broth were disrupted with a high pressure homogenizer. The soluble protein fraction was subsequently used for His(6)-eYFP purification by IMAC using an automated chromatography system. (A) Chromatogram of a representative purification run showing the courses of UV signal (blue line), conductivity (black line) and used gradient concentration (red line). (B) SDS-PAGE analysis showing the soluble protein fractions at different disruption cycles (lanes 1–5) as well as flow-through, wash and elution fractions from protein purification (lanes 6–8). Equivalent amounts of total protein (6  $\mu$ g for lanes 1–6, 0.6  $\mu$ g for lane 7, 2  $\mu$ g for lane 8) were loaded onto the gel and protein bands were visualized by Coomassie brilliant blue staining. St, purified His(6)-eYFP protein standard; M, protein marker with molecular weights indicated in kDa. (C) Picture taken from glowing, purified His(6)-eYFP elution fraction.

culture broth were disrupted using a high-pressure homogenizer. Thereafter, the soluble protein fractions were used for His(6)-eYFP purification by immobilized metal affinity chromatography (IMAC).

As shown in **Figure 4A**, a sharp peak of the UV signal in the chromatogram (blue line) visible after elution with a one-step gradient indicated that the recombinantly produced His(6)-eYFP was successfully isolated and captured. A dominant protein band with a size of ~27 kDa in SDS-PAGE analysis (**Figure 4B**) confirmed the presence of the overexpressed target protein in the different disruption cycles (lanes 1–5). As can be seen in the eluted peak fraction (lane 8), a highly purified target protein band with a purity of approximately  $88 \pm 2\%$  was obtained. In total, an amount of  $42 \pm 2$  mg purified His(6)-eYFP could be recovered from 40 ml culture broth by using this simple capturing purification step. Taken together, the exemplary high-level recombinant production based on acetate as carbon source followed by subsequent target protein recovery was also successfully demonstrated for the newly constructed His(6)-tagged eYFP variant.

## CONCLUDING REMARKS

Until now, most research efforts have rather focused on microbial conversion of acetate into bio-based products with bulk applications, such as platform chemicals (succinic acid, itaconic acid) or bioplastics (polyhydroxyalkanoates). In this work, the potential of acetate for biotechnological production of recombinant proteins as high-value products was demonstrated. Here, the Gram-positive platform bacterium *C. glutamicum* has been proven to be a powerful platform for acetate-based protein production at g/L scale. Compared with previous research works, the finding of our study is quite surprising and unique due to the fact that the presence of acetate is generally reported to strongly inhibit protein production performance in the Gram-negative cell factory *E. coli*. To conclude, this study is the first in which a recombinant protein has been produced in a fed-batch cultivation using (lignocellulosic) acetate as carbon source. Beyond that, the high efficiency of the T7 RNAP-dependent protein production system in *C. glutamicum* has been proven for the first time under high cell density conditions in a bioreactor. The novelty of this study presents a proof of concept for efficient microbial conversion of potentially low-cost acetate into recombinant proteins by acetate-tolerant platform organisms, such as *C. glutamicum*. The presented acetate-based bioprocess could thus be also used to produce (extracellular) target proteins with industrial relevance (e.g. food proteins, industrial enzymes and biopharmaceutical proteins) in order to meet the needs of a future bio-based industry.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests or personal relationships that influenced the work reported in this paper.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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