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Transforming acid whey into a resource by selective removal of lactic acid and galactose using optimized food-grade microorganisms

Ge Zhao\textsuperscript{a}, Shuangqing Zhao\textsuperscript{a}, Line Hagner Nielsen\textsuperscript{b}, Fa Zhou\textsuperscript{a}, Liuyan Gu\textsuperscript{a}, Belay Tilahun Tadesse\textsuperscript{a}, Christian Solem\textsuperscript{a,*}

\textsuperscript{a} National Food Institute, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
\textsuperscript{b} DTU Health Tech, Department of Health Technology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

**HIGHLIGHTS**

- It is the first time to use \textit{C. glutamicum} to remove lactic acid from AW.
- \textit{C. glutamicum} mutants with enhanced lactate metabolic rate were isolated using ALE.
- Best adapted strain reduced time required for lactic acid removal from AW by 50%.
- The removal of lactic acid increased lactose yield of powder after spray drying.

**ABSTRACT**

The presence of lactic acid and galactose makes spray drying of acid whey (AW) a significant challenge for the dairy industry. In this study, a novel approach is explored to remove these compounds, utilizing food-grade microorganisms. For removing lactic acid, \textit{Corynebacterium glutamicum} was selected, which has an inherent ability to metabolize lactic acid but does so slowly. To accelerate lactic acid metabolism, a mutant strain G6006 was isolated through adaptive laboratory evolution, which metabolized all lactic acid from AW two times faster than its parent strain. To eliminate galactose, a lactose-negative mutant of \textit{Lactococcus lactis} that cannot produce lactate was generated. This strain was then co-cultured with G6006 to maximize the removal of both lactic acid and galactose. The microbially “filtered” AW could readily be spray dried into a stable lactose powder. This study highlights the potential of utilizing food-grade microorganisms to process AW, which currently constitutes a global challenge.
1. Introduction

Acid whey (AW) is a liquid leftover from acid-coagulated dairy products, including cream, ricotta and cottage cheese, and strained (Greek) yogurt (Rocha-Mendoza et al., 2021). This dairy side stream has a pH of 4.2–4.5 and a solid content of about 5–6.4 %, with lactose making up the majority (3.9–4.9%), followed by lactate/lactic acid (~0.8%) and smaller amounts of galactose and protein (Chandrapala et al., 2016). The projected global Greek yogurt market size is expected to reach $11.2 billion by 2027, driven by the increasing awareness of consumer health and dietary preferences (https://www.alliedmarketresearch.com/greek-yogurt-market-A06295). This growing demand for Greek yogurt will consequently lead to the generation of very large quantities of AW as a byproduct. Despite massive profits generated from Greek yogurt production, manufacturers have been struggling to find a more economical and environmentally sustainable way to dispose of the AW, driven by environmental policies that have become increasingly stringent (Chandrapala et al., 2016; Rocha-Mendoza et al., 2021). The biological oxygen demand (BOD) for degrading AW is high (BOD > 35,000 ppm) and costly treatment facilities are needed (Bolwig et al., 2019). In general, AW has been used as animal feed in raw form or for biogas production (Prazeres et al., 2012; Chen et al., 2016). However, these applications are not sufficiently attractive economically.

Processing AW to make lactose powder is an alternative and potential value-added application as most of the solid content of AW is lactose. One of the indispensable steps in this process is spray drying (Chen et al., 2016). However, spray drying AW is not an easy task since the high concentrations of lactic acid and low pH of AW interfere with spray drying, causing the powder to stick to the dryer walls, thereby reducing product quality and yield, and furthermore, there is a risk of damaging the spray dryer during the operation (Boonyai et al., 2004; Saffari et al., 2016). Moreover, it has been found that increased thermal stability and hygroscopicity caused by an elevated content of lactic acid promotes inter-particle adhesion, resulting in the generation of large particles during storage and a significant decrease in the shelf life of the powders (Chandrapala et al., 2016; Chandrapala & Vasiljevic, 2017).

Therefore, it is necessary to maximize the removal of this compound from AW in order to decrease operational problems in downstream spray drying and obtain higher-value powder from AW. To address these challenges, there have been attempts at using ultrafiltration, nanofiltration, and electrode dialysis or their combination to remove lactic acid (Chandrapala et al., 2016; Chen et al., 2016; Chandrapala et al., 2017; Dufton et al., 2019; Talebi et al., 2020). However, none of these strategies have proven to be sufficiently efficient. Moreover, the environmental impact of the above-mentioned processes is also tremendous (Dufton et al., 2019). Apart from lactic acid, the small amount of galactose present in AW could decrease the storage stability of spray-dried powders, as galactose has been reported to increase the tendency to caking (Rao et al., 2004; Thorakkkattu, 2020). Therefore, there is a need for developing new cost-efficient and environmentally friendly approaches for removing lactic acid and galactose from AW for its valorization.

Corynebacterium glutamicum, a Gram-positive bacterium, is widely used for the production of food and feed amino acids (Neuner and Heinzel, 2011). It has been granted “Generaly Recognized As Safe” (GRAS) status, has relatively few nutritional requirements and grows well in defined medium to high cell densities (Lee et al., 2016). Moreover, C. glutamicum is unable to metabolize lactose or galactose but grows well on lactate/lactic acid as the sole carbon and energy source (Sato et al., 2010). These characteristics make C. glutamicum a good candidate for removing lactic acid from AW while leaving its valuable constituents untouched.

Lactococcus lactis is a food-grade lactic acid bacterium (LAB), most commonly known for its involvement in cheesemaking (Ainsworth et al., 2014; Zhao et al., 2021). For this microorganism, it is well-known that lactose metabolism is plasmid-encoded (Siezen et al., 2005). The curing of this plasmid can be accomplished through protoplast formation or by using mutants such as ethidium bromide and acriflavine, and such strains are unable to metabolize lactose but still retain the ability to metabolize galactose (Gasson and Davies, 1980; Gasson, 1983). An L. lactis strain RD1M5, obtained through classical chemical mutagenesis, was previously isolated, which has almost completely lost its lactic acid-producing ability, and this strain grows quite well in dairy waste (Liu et al., 2020). A derivative of RD1M5 lacking its lactose plasmid has great potential to be used as a whole-cell catalyst to selectively remove galactose, especially since the main fermentation product of RD1M5 is acetoin, a volatile compound, that will be lost during spray drying.

The primary goal of the present study is to investigate whether C. glutamicum and L. lactis can be used as efficient whole-cell catalysts to selectively remove lactate and galactose in AW. First, the wild-type C. glutamicum ATCC 13032 strain is tested, revealing its potential for lactate removal, and afterwards, adaptive laboratory evolution (ALE) is used to obtain strains with improved performance. Candidates with improved performance are isolated and one of these is characterized in detail. Second, an effort is made to isolate an L. lactis strain deficient in lactate dehydrogenase, which is unable to metabolize lactose and this strain is subsequently tested for its galactose removing ability. Finally, spray-dried powders of microbially treated AW are prepared, and their quality compared to powders obtained from non-treated AW.

2. Materials & methods

2.1. Microorganisms and growth condition

C. glutamicum ATCC 13032 was cultivated in Brain Heart Infusion (BHI) broth or CGXII minimal medium containing 107 mM lactate (CGXIII, pH 6.5) at 30 °C and 200 rpm. RD1M5, a natural mutant of L. lactis subsp. lactis biovar diacetylactis SD96, has almost completely lost its lactic acid-producing ability (Liu et al., 2021), was grown aerobically in M17 medium with 1% lactose (LM17) or 1% glucose (GM17), or defined SAL medium (Solem et al., 2013) with 1% lactose (SALL), 1% glucose (SALG) or 1% galactose (SALGa).

2.2. Optimization of initial pH and initial OD600 of AW

AW with pH 4.2 was obtained from Arla Foods, and contained about 117 mM, 45 mM, and 107 mM of lactose, galactose, and lactate, respectively, which was sterilized at 95 °C for 30 min before use. An overnight BHI broth culture of wild-type C. glutamicum (WT) was centrifuged at 5000 × g for 5 min to collect cell pellets which were washed twice with sterile 0.85% NaCl solution and re-suspended in the same solution to a final OD600 of 5, 10, 20, 40, 80 and 160, respectively. The optimization experiments were conducted in 100 mL conical flasks, each containing 19 mL of AW. To compare the effects of pH on lactate metabolism rate in AW, the pH of AW was adjusted using ammonia solution to obtain different pH levels of 5, 5.5, 6, 6.5, 7, 7.5, and 8. Subsequently, 1 mL of cell suspension with OD600 of 20 was inoculated into AW at an initial OD600 of 1. To investigate the effects of initial OD600 on lactate metabolic rate, 1 mL of suspension with OD600 of 5, 10, 20, 40, 80, and 160 was transferred to 19 mL of AW with pH 6.5 to initial OD600 of 0.25, 0.5, 1, 2.4, and 8. Each experiment was performed in triplicates.

2.3. Adaptive evolution of C. glutamicum in CGXIII

Adaptation of C. glutamicum in CGXIII was carried out using a serial-transfer regime as illustrated in Fig. 2a. Cells from a frozen glycerol stock were streaked on the CGXIII plates with pH 6.5. Single colony was picked and inoculated in a glass tube containing 5 mL of BHI to obtain the preculture. Then an inoculum comprising 1% (v/v) of preculture was inoculated into 5 mL of CGXIII medium. After the culture had entered the growth phase, the inoculation was conducted again and this growth-and-dilution process was continued for approximately 3 months. For
every fifth passage, 800 µL of culture was taken and preserved through storage at –80 °C for future analysis. Frozen stocks from passages 10, 35, and 60 were streaked on CGXII medium agares plates with a pH of 6.5, and ten isolates forming larger colonies were picked and saved individually at –80 °C.

2.4. Initial performance comparison of WT and its adapted mutants

2.4.1. Growth in high-throughput microbio reactor

Single colonies of both the WT and its adapted mutants were inoculated into 24-well plates filled with 1 mL of CGXIII medium to prepare precultures, which were aerobically incubated at 30 °C. After 24 h of incubation, 50 µL of preculture was transferred into 48-well plates filled with 1 mL of CGXIII medium and then incubated at 30 °C using a Biolector (M2p-labs, Germany) with constant shaking at 800 rpm for 24 h. Subsequently, 500 µL of culture from each well was withdrawn to measure OD_{600}.

2.4.2. Lactate removal in AW

Lactate removal using the adapted derivatives was carried out in a 100 mL conical flask containing 20 mL of AW (pH 6.5) with an initial OD_{600} of 2. Samples were withdrawn at 0, 12, 18, and 24 h to determine lactate concentration. Each experiment was performed in triplicates.

2.4.3. Comparison of growth and lactate metabolism kinetics of WT and its adapted derivative G6006 in the flask

A single colony of both WT and G6006 strains was separately inoculated into a glass tube containing 10 mL of BHI broth. These cultures, along with their 10-fold serial dilutions, were incubated overnight at 30 °C in the presence of ethidium bromide (EB). RD1M5 was grown in GM17 broth overnight. Subsequently, 50 µL of preculture was transferred to 5 mL of fresh GM17 broth containing different concentrations of EB (0.5, 1, 2, 3, 4, and 5 µg/mL) and cultured for 12 h. After the curing treatment, serially diluted cultures were plated on 2,3,5-triphenyl tetrazolium (TTC) indicator agar and incubated for 48 h. TTC indicator agar is composed of (g/L): glucose (5), casein peptone (15), yeast extract (5), MgSO_{4} (0.5), ascorbic acid (0.5), agar (15), and TTC (0.1) (Liu et al., 2020). Then the dark red colonies were simultaneously inoculated into SALG, SALL, and SALGa medium. The isolates that only grew up in SALG and SALGa medium and produced almost no lactate were selected for experiments where their galactose-removing ability in AW was tested.

2.5. Whole genome sequencing

Whole genome sequencing of WT, G3507, and G6006 was conducted on the DNBseq platform by BGI China. The resulting raw paired-end 150-bp reads corresponding to these three strains were initially processed and then mapped to the reference genome (C. glutamicum ATCC 13032, Genbank Accession number: NZ_CP115148) using Geneious Prime 2023. Subsequently, single nucleotide variants (SNVs) were identified in both the parental strain and adapted strains using the same software. SNVs were deemed to be valid if they had a mutant frequency of >90% and a strand bias of <75%. Any SNVs that were found in both parental strains and adapted strains were excluded from the final analysis.

2.6. Transcriptomics analysis

Exponentially growing cells of both the WT and G6006 in CGXIII (pH 6.5) were harvested (at an OD_{600} around 1) and centrifuged at 4 °C at 7000 × g for 5 min. Then total RNA was extracted from the harvested cells using Qiagen RNeasy kit, and further purified using Qiagen RNase-Free DNase Set, respectively, according to the protocols of manufacturer. Each sample was analyzed in duplicates. RNA sequencing was performed by BGI-Tech (Hong Kong) using BGISEQ-500 sequencing platforms and about 2 GB of clean data with trimmed reads was obtained for each sample. The quality control of clean reads was performed using FastQC. Afterwards, the RNA sequence data were mapped to transcriptional region of the reference genome (ASM284740v1) using Geneious Prime 2023. DESeq2 algorithm was used to compare the gene-level expression of two groups. Differentially expressed genes (DEGs) were identified using cutoff thresholds for false discovery rate (FDR) < 0.05 and a log2 [fold change (FC)] > 1. To evaluate the global expression categories of G6006, Clusters of Orthologous Groups (COG) analysis was performed using eggNOG-mapper with an adjusted p-value (P_{adj}) < 0.05 as a threshold for statistical significance, excluding the non-descriptive category Function Unknown (category S).

2.7. Isolation of derivatives of RD1M5 cured of the lactose plasmid

Curing of the lactose plasmid was accomplished by growing the cells at 30 °C in the presence of ethidium bromide (EB). RD1M5 was grown in GM17 broth overnight. Subsequently, 50 µL of preculture was transferred to 5 mL of fresh GM17 broth containing different concentrations of EB (0.5, 1, 2, 3, 4, and 5 µg/mL) and cultivated for 12 h. After the curing treatment, serially diluted cultures were plated on 2,3,5-triphenyl tetrazolium (TTC) indicator agar and incubated for 48 h. TTC indicator agar is composed of (g/L): glucose (5), casein peptone (15), yeast extract (5), MgSO_{4} (0.5), ascorbic acid (0.5), agar (15), and TTC (0.1) (Liu et al., 2020). Then the dark red colonies were simultaneously inoculated into SALG, SALL, and SALGa medium. The isolates that only grew up in SALG and SALGa medium and produced almost no lactate were selected for experiments where their galactose-removing ability in AW was tested.

2.8. Co-cultivation of G6006 and LAC1

In order to simultaneously remove galactose and lactate from AW, an optimization experiment was carried out to determine the appropriate amount of LAC1 to be inoculated. In brief, cell suspensions of G6006 and LAC1 were simultaneously inoculated into 100 mL conical flasks containing 20 mL of AW (pH 6.5). The initial OD_{600} of G6006 was set at 2, while the initial OD_{600} values of LAC1 were set at 0, 0.25, 0.5, 1, 2, and 4, respectively. Samples were withdrawn at 0, 12, 18, and 36 h to determine lactate and galactose concentrations. Each experiment was performed in triplicates.

2.9. Spray drying of raw AW and processed AW

A Buchi-B290 laboratory mini spray dryer was employed to spray dry three distinct feed solutions: raw AW (RWL), lactate-removed AW, also known as de-lactate AW (AWL), and AW with both lactate and partial galactose removed (AWLG). Before the drying process, all feed solutions underwent pre-filtration using a sterile membrane (0.22 µm). During the experiment, the nozzle air flow rate and feed solution flow rate were maintained at 8 kg/h and 0.6 L/h, respectively, while the inlet and outlet temperatures were carefully controlled at 180 ± 5 °C and 75 ± 5 °C, respectively. After allowing sufficient time for the system to reach a steady-state condition (approximately 30 min), the spray dryer operated for 1 h. Following the drying process, the resulting powder was promptly collected, sealed, and stored in a desiccator containing silica gel beads until further analysis.

2.10. Lactose powder yield and powder properties

The effects of the presence of lactate and galactose on powder properties were assessed, focusing on lactose powder yield, hygroscopicity, and particle size distribution. Lactose powder yield was determined as the percentage weight fraction of the initial lactose content present in the AW that was successfully introduced into the spray dryer. Each experiment was performed in triplicates.

Hygroscopicity was assessed by measuring the final moisture content (g/100 g dry weight) after exposing the samples to an atmosphere with 40% and 80% relative humidity at 25 °C. The water content was then measured by weighing, following the method described by Thorakattu (2020). Each experiment was performed in triplicates.

The particle size distribution of powders was analyzed using
Mastersizer 2000 laser particle size analyzer (Malvern Instruments Ltd, Malvern, UK). Powders were dispersed using a dry sampling system, and the particle refractive index was set to 1.45. The median diameter $d_{(0.5)}$ and De Brouckere mean $D_{[4,3]}$ were calculated and selected to evaluate the particle size distribution.

2.11. Analytic methods

Optical density (OD$_{600}$) was measured with a UV-1600PC spectrophotometer (VWR, Denmark) at 600 nm, and pH was measured with a Lab 845 pH Meter (SI Analytics, Denmark). The lactate, lactose, and galactose were quantified on an HPLC system (Dionex, Sunnyvale, USA) equipped with a refractive index detector (Shodex RI-101; Showa Denko K.K., Tokyo, Japan) and an Aminex HPX-87H column (Bio-Rad, Hercules, USA) (Zhao et al., 2022).

3. Results and discussion

3.1. Effects of initial pH and initial OD$_{600}$ on lactate removal by WT

It has previously been shown that lactate can serve as the sole carbon and energy source to support the growth of C. glutamicum ATCC 13032 (Kato et al., 2010). Therefore, it was investigated if this strain could be used to remove lactate from raw AW with a pH of 4.2. The WT could hardly metabolize lactate in this low pH food waste (Fig. 1a), even with an initial OD$_{600}$ of 20 (data not shown), which may be due to an inability to maintain the proton motive force (PMF) and thus a high intracellular pH at the low pH (Follmann et al., 2009). To allow C. glutamicum to metabolize lactate in AW, the pH of the AW was adjusted to 5–8 with ammonia solution. As shown in Fig. 1a, when the initial pH of AW was adjusted to 5.5 and above, this enabled the WT to metabolize all lactate within 36 h and the final pH consistently increased to around 9.1, except when the initial pH was 5.5. However, the lactate metabolic rate began to decrease at a pH below 5.5. Among the different pH values tested, raw AW with a pH of 6.5 showed the lowest residual lactate concentration (9.98 mM) after 24 h of incubation. Therefore, this pH value was chosen as fixed pH when determining the optimal initial cell density.

In Fig. 1c lactate removal over time by the WT at different initial OD$_{600}$ can be seen. At the lowest initial OD$_{600}$ (OD$_{600}$ = 0.25), lactate was not completely metabolized in 36 h. Using a higher initial OD$_{600}$ accelerated the rate at which lactate disappeared, and the fastest lactate removal occurred at an initial OD$_{600}$ of 2, where the lactate was almost eliminated within 24 h. However, when the initial OD$_{600}$ was increased to 8, lactate disappeared more slowly. It is tempting to speculate that nutrient competition at an initial OD$_{600}$ of 8 between the bacteria could hamper their performance, eventually disrupting bacterial growth and lactate metabolism (Jeff Sumardee et al., 2020).

There have been no previous attempts at removing lactate from AW using C. glutamicum. The findings in this study indicate that C. glutamicum has great potential to do so, and by optimizing the initial pH and inoculum, almost complete elimination of the lactate could be accomplished in 24 h. It should be stressed that no other method or technology capable of eliminating lactate from AW, that also leaves galactose and lactose untouched, has been described in the past. It has been reported that C. glutamicum grows well in cheap defined media (Wang et al., 2018) and da Luz et al. (2017) achieved a maximum OD$_{600}$.
of 40.5 using their modified minimal medium, suggesting that there is a great potential for processing large volumes of AW using *C. glutamicum*.

### 3.2. Isolation of *C. glutamicum* mutants with improved ability to remove lactate from AW

Although lactate in AW can be almost completely removed within 24 h after optimization of extracellular process parameters such as pH and inoculum size, it is still relevant to improve the removal efficiency further as shorter processing time can decrease production costs. ALE has been widely investigated in recent years as a non-genetic engineering technique to improve substrate utilization rates in host organisms, which is particularly relevant due to strict regulations on genetically modified organisms (GMOs) in food production (Long and Antoniewicz, 2018). Therefore, to generate natural *C. glutamicum* strains with superior lactate metabolic rate, ALE was performed in a lactate minimal medium (CGXII). This strategy was founded on the premise that beneficial mutations might emerge over hundreds to thousands of generations, leading to the optimization of lactate utilization properties in *C. glutamicum*. It should be emphasized that the decision to directly

![Figure 2](image-url)

*Fig. 2.* Isolation of *C. glutamicum* mutants with higher lactate metabolic rates. (a) Outline of the ALE experiment. For the adaptation process, the WT strain was subcultured in CGXII medium (pH 6.5) at 30 °C and 200 rpm. After 10, 35, and 60 passages, cells were streaked on CGXII agar, and ten larger colonies from each plate were randomly isolated, resulting in three sets of ten isolates each. These sets were designated as G10, G35, and G60. (b) Growth monitoring of the WT and its mutants using a BioLector cultivation system, where growth curves for the WT and mutants are colored in cyan and purple, respectively. (c) Cell density of isolated colonies after 24 h cultivation in BioLector. (d) Lactate removal from AW (pH 6.5) by WT and strains of the G35 set at an initial OD$_{600}$ of 2. (e) Lactate removal from AW (pH 6.5) by WT and strains of the G60 set at an initial OD$_{600}$ of 2.
evolve *C. glutamicum* in the lactate minimal medium, rather than in the AW, was made due to the ease of monitoring strain growth in the former since the latter has a milky and cloudy appearance at pH above 5.0.

First, culture samples obtained after 10, 35 and 60 passages were plated on CGXIII, and ten randomly picked colonies from each passage, designated as the G10, G35, and G60 sets, were selected for characterization. From Fig. 2b and c, it is evident that the majority of the isolates grew faster and had a higher final OD_{600} than the WT, especially isolates from the 35 and 60 passages. Subsequently, the lactate metabolism ability of G35 and G60 sets was determined in AW. As shown in Fig. 2d and e, all the isolates from these two passages metabolize lactate in AW faster than the WT. Especially isolates from the 60 passage culture (G60 sets) stood out and a total of four strains named G6001, G6005, G6003, and G6006 could remove lactate within 18 h. However, only one isolate, named G3507, from the 35 passage culture (G35 sets) was also able to remove lactate within 18 h. The most efficient isolate/strain, G6006, was capable of almost eliminating lactate in AW after 12 h, nearly saving 12 h as compared to the WT. Additionally, this strain exhibited stable traits both during process use and storage.

### 3.3. The adapted strain G6006 grows better in defined CGXIII and metabolizes lactate faster

As mentioned, the growth of the evolved strains was monitored in CGXIII medium using a BioLector and then a robust strain G6006 was identified from the last passage culture, which could eliminate lactate in AW twice as fast as the WT. However, due to the relatively small scale of the Biolector experiments, it was challenging to perform a detailed characterization and comparison of the WT and the adapted G6006 strains, particularly regarding their maximum specific growth rate and lactate consumption rate. These properties are crucial for understanding the lactate removal ability of G6006 in AW. Characterization of the WT and G6006 using shake flask experiments (Fig. 3) confirmed that growth and lactate metabolism of G6006 was significantly faster than that of the WT at pH 6.5. It should be noted that both strains entered the stationary phase more slowly than they did when cultivated in a microtiter plate in the Biolector, which may be ascribed to more effective aeration in the Biolector microbioreactor and the reduced fermentation volume (Wang et al., 2018). Characterization is summarized in Table 1 and revealed that G6006 grew faster than the WT by 146%, and its lactate consumption rate (18.29 ± 0.14 mmol/h/g DW) correspondingly increased by 56%. In addition, the biomass yield (26.22 ± 0.04) of G6006 and final OD_{600} was increased by around 32%, which indicated that less lactate is used for the same biomass production in G6006. It has been reported that biomass of *C. glutamicum* can be used as a biosorption agent to remove and/or recover heavy metals from industrial wastewater (Choi and Yun, 2004), which provides a sustainable route for the reuse of G6006 biomass waste after it has been used to remove lactate from AW.

### 3.4. Scrutinizing the genomes of strains adapted to growth on lactate

To identify the mutations responsible for the faster growth phenotype of G6006, its genome as well as that of the parent strain were sequenced. The genome of G3507 from the G35 set of strains was also sequenced, as this strain required 25% less time to eliminate lactate in AW than the WT. In addition, the specific growth rate, lactate consumption rate, and biomass yield for this strain in lactate minimal medium were much greater than for the WT, however, the improvement was less than for G6006 (Table 1 and see supplementary material). As shown in Table 2, in total, 4 SNVs were identified in the genomes of G3507 and G6006, including 2 SNVs in intergenic regions and 2 SNVs in protein-coding regions. Interestingly, both G3507 and G6006 had an SNV between divergently oriented genes encoding an Acetyl-CoA carboxylase β-subunit (accD1) and a DUF485 domain-containing protein, respectively, which could potentially affect transcription of both genes by altering the promoter region. The remaining two mutations were in genes encoding regulators. One SNV detected in G3507 was a T to G change in sigM, encoding a sigma-70 family RNA polymerase sigma factor (SigM), leading to amino acid replacement of F by L. It has been reported that SigM is involved in the regulation of the heat and oxidative stress response in *C. glutamicum* (Nakunst et al., 2007). It is tempting to hypothesize that this mutation changed the target range/activity of SigM, thereby affecting the growth rate of G3507 under aerobic conditions. The other SNV detected in G6006 was an insertion of a G in the *whcA* gene, encoding a WhiB family transcriptional regulator, leading to a frameshift mutation. WhcA is known to play a negative role in the oxidative stress response (Bush, 2018). Park et al.,(2012) found that overexpressing *whcA* resulted in slow cell growth and increased

<table>
<thead>
<tr>
<th>Strains</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)</th>
<th>Lactate consumption rate (mmol/h/g DW)</th>
<th>Biomass yield (g DW/mol of lactate)</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.19 ± 0.01</td>
<td>11.70 ± 0.81</td>
<td>19.93 ± 0.34</td>
</tr>
<tr>
<td>G6006</td>
<td>0.46 ± 0.00</td>
<td>18.29 ± 0.14</td>
<td>26.22 ± 0.04</td>
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</table>

**Fig. 3.** Time course of OD_{600} (a) and lactate consumption (b) for WT and G6006 grown in CGXII medium with 0.67 % lactate (pH 6.5).
susceptibility to oxidants. Based on this available evidence, it can be inferred that the frameshift mutation in the whcA gene in G6006 is likely to decrease its activity, leading to decreased oxidative stress. This may explain why the G6006 has a faster growth rate and higher biomass yield.

3.5. RNA sequencing revealed strong changes in inorganic ion transport and metabolism in G6006

To reveal more about the underlying mechanism for the improved phenotypes, the transcriptomes of the WT and the adapted G6006 strain were compared. Principal-component analysis (PCA) confirmed the eligibility of data for analysis (see supplementary material). Total RNA from both *C. glutamicum* strains cultivated in CGXII during the exponential phase was prepared, processed, and analyzed. Differentially expressed genes (DEGs) revealed a total of 221 down-regulated genes and 192 up-regulated genes in the G6006 compared to WT (Fig. 4a). COG analysis revealed that most of the DEGs were in the category inorganic ion transport and metabolism (category P), where G6006 had 34 downregulated and 28 upregulated genes (Fig. 4b). Subsequently, amino acid metabolism and transport (category E) was strongly represented in G6006, with 21 downregulated genes and 13 upregulated genes, respectively. It was remarkable that the category involved energy production and conversion (category C) and carbohydrate transport and metabolism (category G) were also significantly enriched, with 20 and 10 downregulated and 11 and 15 upregulated genes, respectively. Changes in these two categories were expected since they contain most of the genes involved in cell-associated cell processes such as glycolysis, pentose phosphate pathway (PPP), and citrate cycle (TCA cycle). It has been reported that *pyc* (encoding pyruvate carboxylase) and *malE* (encoding malic enzyme) belonging to category C, are essential for *C. glutamicum* growing on lactate (Peters-wendisch et al., 1998; Gourdon et al., 2000). Transcript analysis found that *pyc* and *malE* were significantly upregulated in G6006, approximately 4.2-fold and 2.6-fold increases were observed (see supplementary material), which is consistent with previous studies showing that overexpression of *pyc* or *malE* enhanced the lactate metabolic rate (Peters-wendisch et al., 1998; Gourdon et al., 2000).

Previously, it was hypothesized that a mutation that lies in the promoter region of *accD4* and *cg0952* could affect their expression. However, transcriptomic analysis revealed that only the expression of *cg0952* was significantly changed (see supplementary material). *cg0952* is followed by and cotranscribed with *mctC* gene, encoding a monocarboxylate transporter that is involved in the uptake of pyruvate, acetate and propionate, but it has not been indicated that MctC is involved in lactate transport in *C. glutamicum* (Jolkver et al., 2009). These

### Table 2

<table>
<thead>
<tr>
<th>Strains</th>
<th>RP</th>
<th>Region</th>
<th>NC</th>
<th>AAC</th>
<th>product or (distance from CDS)</th>
<th>neared product</th>
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<tr>
<td>G3507</td>
<td>882,896</td>
<td>intergenic</td>
<td>(G6)</td>
<td>None</td>
<td>(344 bp) Acetyl-CoA carboxylase β-subunit</td>
<td>Cg0952</td>
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<tr>
<td>G6006</td>
<td>298,846</td>
<td>gene CDS</td>
<td>T &gt; G</td>
<td>F &gt; L</td>
<td>Sigma-70 family RNA polymerase sigma factor SigM</td>
<td></td>
</tr>
<tr>
<td>882,893</td>
<td>gene CDS</td>
<td>Frameshift</td>
<td>(G6)</td>
<td>None</td>
<td>(341 bp) Acetyl-CoA carboxylase β-subunit</td>
<td></td>
</tr>
</tbody>
</table>

**Footnotes:**

- RP: Reference position refers to the genome sequence of *C. glutamicum* ATCC 13032 (Accession number: NZ_CP115148).
- NC: Nucleotide change.
- AAC: Amino acid change.

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**Fig. 4.** Overall transcriptomic profile obtained in an analysis using DESeq2. (a) The volcano plot for DEGs between the strain WT and G6006. Gray, blue, and red dots represent genes with no significant differences, significantly down-regulated genes, and significantly upregulated genes, respectively; (b) COG analysis comparing expression between WT and G6006. Categories are as follows: C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism and transport; I, lipid metabolism and transport; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelope biogenesis; O, post-translational modification, protein turnover, and chaperone functions; P, inorganic ion transport and metabolism; Q, secondary structure; T, signal transduction; U, intracellular trafficking and secretion; V, defense.
findings lead to speculation that pyruvate derived from lactate could be more rapidly utilized intracellularly in G6006 as pyc was significantly upregulated, and thus less pyruvate excretion occurred, which resulted in significant downregulation of the cg0952-mctC operon in G6006 compared to WT as mctC expression is induced by pyruvate. However, this hypothesis needs to be experimentally verified. The other mutation caused a frameshift mutation in whcA, which might lead to enhanced tolerance to oxidative stress and thus positively affect the growth of cells (Choi et al., 2009; Park et al., 2012). Interestingly, a significant increase in the expression of whcA-regulated genes involved in the oxidative stress response was not detected in G6006 (Choi et al., 2009).

3.6. Isolation of lactose plasmid-cured derivatives of L. lactis RD1M5

In a previous study, RD1M5 was isolated after exposing RD01 to a chemical mutagen (Liu et al., 2020), a strain with an almost eliminated ability to produce lactic acid. RD1M5 can metabolize lactose and a lactose utilization gene cluster was found to be present on one plasmid (Dorau et al., 2020). To obtain a lactose-negative derivative of RD1M5, a plasmid curing experiment was carried out with ethidium bromide (EB). Since exposure of RD1M5 to 3 μg/ml EB resulted in a 99% reduction in viability, this concentration was used for isolating lactose-negative derivatives of RD1M5. After streaking a mutagenized RD1M5 culture on TTC agar plates, only 11 dark red colonies were found (dark red color = unable to produce lactic acid), indicating that RD1M5 readily reverts to producing lactic acid. Subsequently, the capacity of these 11 red colonies to metabolize lactose and galactose was investigated, and finally one isolate, LAC1, was identified, that had lost its ability to metabolize lactate, while retaining the ability to metabolize galactose (see supplementary material). Therefore, this strain was used for removing galactose in AW in combination with G6006 to remove lactate from AW.

3.7. Co-cultivation of strains G6006 and LAC1 to remove lactate and galactose from AW

In the initial experiment, it was observed that LAC1 exhibited a slow metabolism rate of galactose in pH-adjusted AW (data not shown), possibly due to the insufficient amounts of proteins/peptides to support the growth of LAC1. To accelerate the removal of galactose while removing lactate, LAC1 with varying initial OD600 was co-cultivated with G6006, which can produce amino acids, which may be utilized by LAC1 and therefore contribute to the growth of LAC1. As seen from Fig. 5a, when the initial OD600 of LAC1 is 1, 58.7% of the galactose can be consumed within 18 h. Further increasing the inoculum size of LAC1 slowed down the metabolism rate of galactose. However, when prolonging the fermentation time by 18 h, only an additional 14.9% of the galactose was removed, suggesting that the remaining nutrients from AW or the high pH caused by lactate removal were inadequate to sustain LAC1’s growth. It is noteworthy that the addition of LAC1 prolonged the time needed for depleting lactate, indicating that LAC1 negatively influences lactate removal by G6006. Taking into account the economic cost of time, an initial OD600 of 1 for LAC1 and an 18-hour metabolic period were selected for the complete elimination of lactate and partial removal of galactose. To completely remove galactose while removing lactate, it is necessary to screen for LAC1 mutants that rapidly metabolize galactose by non-genetic engineering methods, such as physical and chemical mutagenesis and adaptive evolution.

3.8. AW powder without lactate contains more lactose and is less hygroscopic

When lactate and galactose in AW are eliminated by microorganisms, other metabolites such as amino acid and acetoin are inevitably generated. To assess the effectiveness of the microbial treatment, a spray-dried powder was prepared using a laboratory mini spray dryer (Buchi-B290). According to Table 3, AWL powder was similar to AWLG powder in terms of lactose yield, hygroscopicity, and powder particle size distribution. In addition, AWLG powder did not have a greater degree of browning than AWL after two months’ storage (data not shown). These findings indicate that only lactate has an important influence on the yield of the process and properties of the AW powder. Thorakkattu (2020) observed that the addition of 0.3% galactose to deproteinized whey (DPW) and whey permeate could increase the hygroscopicity of the spray-dried powders obtained from these. In this study, galactose

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Unit</th>
<th>RAW</th>
<th>AWL</th>
<th>AWLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose yield (%)</td>
<td>%</td>
<td>31.64±16.20</td>
<td>49.56±18.45</td>
<td>50.45±3.17</td>
</tr>
<tr>
<td>Hygroscopicity at 40% relative</td>
<td>g/100g</td>
<td>5.88±1.22</td>
<td>3.32±0.42</td>
<td>3.17±0.23</td>
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<tr>
<td>Hygroscopicity at 80% relative</td>
<td>g/100g</td>
<td>28.15±5.68</td>
<td>18.45±2.81</td>
<td>16.20±1.22</td>
</tr>
<tr>
<td>Hygroscopicity at 90% relative</td>
<td>g/100g</td>
<td>243.0±49.56</td>
<td>112.0±50.45</td>
<td>95.3±3.12</td>
</tr>
<tr>
<td>Hygroscopicity at 100% relative</td>
<td>g/100g</td>
<td>95.3±49.56</td>
<td>55.8±50.45</td>
<td>47.8±3.12</td>
</tr>
</tbody>
</table>

Fig. 5. The effect of initial cell density (OD600) of LAC1 co-cultured with G6006 on (a) galactose removal and (b) lactate removal from AW.
was found to have a minor impact on the hygroscopic properties of the spray-dried powder, possibly because of the source of the AW, which could affect its composition and overall properties. As expected, microbial processing significantly increased the lactose yield after spray drying by up to about 49.56 ± 0.78% (p < 0.01), as compared to 31.64 ± 1.25% for the RAW. In addition, the results for hygroscopicity (Table 3) were also significantly different for the RAW and AWL powders, with values of 5.88 ± 0.02% (p < 0.001) and 3.32 ± 0.08, respectively, for relative humidity at 40%, and of 28.15 ± 0.18% (p < 0.01) and 18.45 ± 1.22% for relative humidity at 80%. These findings are consistent with results previously reported by Bédas et al., (2017), and can be explained by the fact that lactate is highly hygroscopic: the more lactate removed, the greater the reduction in hygroscopicity will be (Safiﬁari & Langrish, 2014). Murti et al., (2009) reported that as moisture content increases, the rate of stickiness development also increases. Therefore, hygroscopicity can be used to evaluate a powder’s tendency towards stickiness and caking. The lower hygroscopicity of AWL and AWLG powder thus indicates that it is less prone to lumping and caking during long-term storage. Also, the absence of lactate influenced the size distributions of powders, and resulted in finer particles with a size distribution with D(4,3) of 112.0 μm and (0.5) of 55.8 μm, compared to D(4,3) of 243.0 μm and (0.5) of 154.0 μm for the powder derived from the untreated AW.

4. Conclusions

For the first time, it was shown that C. glutamicum can eliminate lactate from AW. To reduce the time needed to remove lactate, G6006 derived from C. glutamicum ATCC 13032 through ALE was isolated. Co-cultivating G6006 with LAC1, a galactose-metabolizing L. lactis strain, enabled the removal of all lactate and part of the galactose. The approach was validated through spray drying of the microbiially treated AW, which produced a stable, low-hygroscopicity powder. These findings highlight C. glutamicum as an excellent catalyst for converting low-value AW into a valuable resource in a cost-effective and environmentally friendly manner.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christian Solem reports financial support was provided by Technical University of Denmark. Christian Solem reports a relationship with Technical University of Denmark that includes: employment.

Data availability

The data that has been used is confidential.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129594.


