Enhancing acetic acid and 5-hydroxymethyl furfural tolerance of *Clostridium saccharoperbutylacetonicum* through adaptive laboratory evolution

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**A R T I C L E  I N F O**

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- Adaptive laboratory evolution
- Hemicellulosic hydrolysate inhibitors
- Tolerance
- *Clostridium saccharoperbutylacetonicum*
- Genomic analysis

**A B S T R A C T**

In this study, adaptive laboratory evolution (ALE) was applied to isolate four strains of *Clostridium saccharoperbutylacetonicum* able to grow in the presence of hemicellulosic hydrolysate inhibitors unsupported by the parental strain. Among them, isolate RAC-25 presented the best fermentative performance, producing 22.1 g/L of ABE and 16.7 g/L of butanol. Genome sequencing revealed a deletion in the arabinoose transcriptional repressor gene (*araR*) and a mutation in the anti-sigma factor I that promoted a downregulation of *sigI*. Gene expression analysis indicated high expression of genes related to H⁺-pumps (ATP synthases), proline biosynthesis (gamma phosphate reductase) and chaperonins (*GroL*), suggesting an integrated mechanism that is probably coordinated by the repression of *sigI*. Therefore, in addition to highlighting the power of ALE for selecting robust strains, our results suggest that *sigI* and *araR* may be interesting gene targets for increased tolerance toward inhibitor compounds relevant for lignocellulosic biofuels production.

**1. Introduction**

Butanol has emerged as a “superior biofuel” when compared to ethanol; offering advantages such as higher energy density, a less corrosive nature, higher octane number and higher hydrophobicity [1]. Additionally, butanol and its derivatives may be used in other important applications such as surface coating, plasticizing agent and as diluents; verifying the versatility and market interest of this compound [2]. Traditional butanol production has been based in the petrochemical industry; though recently, biobutanol production has received renewed interest due to its contribution to reducing the exhaustion of natural resources, environmental pollution and global warming [3]. However, biobutanol production is not economically competitive with the petrochemical-based butanol, due to the high cost of feedstock (usually molasses) and low butanol yield and productivity [3,4]. Thus, the opportunity for using low cost and abundant agro-industrial waste, which is mainly composed of lignocellulosic biomass as feedstock, opens a new chapter in the biobutanol development process [5-8].

Lignocellulosic biomass is the most abundant renewable carbon source on Earth, consisting of a composite material, primarily formed by two types of polysaccharides (cellulose and hemicellulose), and the complex aromatic compound lignin [8]. Given the recalcitrant nature of lignocellulosic biomasses, the access to whole sugars present in the plant cell walls can be carried out by two sequential hydrolysis steps; a thermochemical pretreatment to obtain the hemicellulosic hydrolysate, and an enzymatic hydrolysis of the cellulose fraction to generate the hexose-rich stream [9]. Hemicellulosic hydrolysate is a pentose rich liquor composed primarily of xylose [10] and also of inhibitory compounds formed during the pretreatment step, which can negatively affect the microbial cells and the fermentation process [11]. The presence of these toxic compounds affects cells in several ways: inhibition of cell growth by affecting glycolytic and fermentative enzymes,
degradation of DNA, disruption of cell membrane and disturbance of ATP generation due to dissipation of the proton motive force [12,13]. In terms of inhibitory effects on living cells, it is well known that weak acids present in lignocellulosic-derived streams act by uncoupling energetic metabolism due to the effect of weak acids [11]. Phenolic compounds have been reported to be toxic even at low concentrations, reducing cell growth and ABE (acetone, butanol, and ethanol) production [14].

Detoxification methods such as the use of lime, peroxidases, activated charcoal, surfactant and ion-exchange resin adsorption [15-18] have been proposed to reduce the hydrolysates toxicity. Despite its effectiveness, the detoxification process involves a series of separation and purification steps that can sharply increase the overall cost of the process and limit its economic feasibility [19].

Strategies based on adaptive laboratory evolution (ALE) have been proposed as a valuable tool to enrich favorable genetic changes to obtain robust microbial cells that can withstand different inhibitor compounds. The concept of ALE or evolutionary engineering involves two approaches: repeated batch cultivation or prolonged chemostat with the presence of selective pressures to produce desired genetic variants [20]. Guo et al. obtained through continuous culture cultivation a high inhibitor tolerant mutant of C. beijerinckii. able to produce 12.9 g/L of ABE using non-detoxified hydrolyzate from corn fiber [21]. Wang et al., applying a long term adaptive evolution strategy in non-detoxified corn stover hydrolysate, obtained a robust Corynebacterium glutamicum mutant with a high tolerance to various lignocellulose-derived inhibitors [22]. The evolved strain increased the conversion rate of typical lignocellulose derived inhibitors (furfural, 5-hydroxymethylfurfural, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, and acetic acid) into less toxic compounds, better glucose consumption and an increase of 68.4 % in glutamic acid production compared to the parental strain [22].

In this present work, we subjected C. saccharoperbutylactonicum (14923) isolate to adaptive laboratory evolution, to increase its tolerance to the main lignocellulosic derived inhibitors present in hemiecellulosic hydrolysate. The evolved strains were characterized at the genomic level and compared to the wild-type hydrolysate.

2. Material and methods

2.1. Bacterial strains and maintenance

The C. saccharoperbutylactonicum (14923) isolate was acquired from the German Collection of Microorganisms and Cell Culture (DSMZ). The strain was activated and propagated following the supplier’s recommendations. Cultures were routinely maintained as a 2 mL suspension in glycerol (20 % w/v) and stored at -80 °C until experimentation.

2.2. Culture media preparation

Culture medium was prepared according to Zetty et al. [23]. Strain reactivation (pre-culture) was carried out in Reinforced Clostridia Medium (RCM, Fluka, Sigma-Aldrich, Spain), at 30 °C and inside an anaerobic chamber. For all fermentation experiments, strains were cultured in mineral medium (MM) that contained, in g/L: xylose, 55; yeast extract, 5; KH_{2}PO_{4}, 0.75; K_{2}HPO_{4}, 0.75; NaCl, 1; MgSO_{4}.7H_{2}O, 0.4; MnSO_{4}.H_{2}O, 0.4; FeSO_{4}.7H_{2}O, 0.01; ammonium acetate, 4.3 and supplemented with l-asparagine, 2; para-aminobenzoic acid, 0.1; and biotin, 0.001. For adaptive laboratory evolution (ALE) experiments, cultivation was performed in MM (as described above) containing around 55 g/L of xylose, supplemented with hemiecellulosic hydrolysate (HH) at different percentages (v/v), according to each step of the evolution protocol.

2.3. Hemicellulosic hydrolysate production

Hemicellulosic hydrolysate production was obtained throughout the hydrothermal pretreatment, and carried out at the pilot plant facility of the Brazilian Biorenewables National Laboratory (LNBR/CNPENM, Campinas, Brazil) following the procedure described in detail from our previous work [24]. The liquor was concentrated 5-times in a pilot evaporator at the following operating conditions: pressure: 475 mbar; distillate: 80 °C; temperature: 110 °C-115 °C. Thereafter, the concentrated liquor was centrifuged at 9000 rpm at 10 °C for 20 min. The pH was set to 6.5 using NH_{4}OH 25 % (w/v). Finally, the HH was centrifuged at 8000 rpm for 30 min, filter-sterilized (0.22-µm polyethersulfone top filter; Nalgene, Rochester, NY, USA) for sterilization and removal of insoluble materials that would make it difficult to measure cell growth by absorbance. The filtered hydrolysate was stored in sterile glass bottles at -4 °C until use. Two batches of HH were produced and inhibitor compounds and sugars characterized and used in ALE experiments (Table 1). The same previously described protocol was used [24].

2.4. Adaptive laboratory evolution (ALE)

An adaptive laboratory evolution (ALE) strategy was used to obtain robust C. saccharoperbutylactonicum cells able to grow in media containing inhibitors derived from HH. For this purpose, a wild-type strain of C. saccharoperbutylactonicum was submitted to serial batch cultivation in MM, supplemented with increasing concentrations of HH (from 20 to 40 %, v/v). The initial concentration of 20 % of HH (HH-20) was based on preliminary data of wild type strain growth on medium containing different concentrations of HH (20 %, 50 % and 100 %, v/v) (Fig. 1S, Supplementary Material). All the fermentations were carried out in anaerobic chambers at 30 °C with an initial pH of 6.5. Firstly, the cells were cultivated in 20 mL of RCM the exponential phase was achieved in 600 mµ poly
calculated according to the following equation:
\[ DT = \frac{\text{Duration} \times \text{Culture}*\log(2)}{\log(\text{Final Concentration})} \]
\[ = \log(\text{Initial concentration}) \]

To isolate individual colonies from the EP-40 (40 % HH), a 2 mL aliquot was cultivated in 15 mL of RCM until the exponential phase, and subsequently plated onto solid media (RCM). The largest colonies were selected, cultivated in RCM, and stored in 20 % glycerol at -80 °C.

2.5. Evaluation of mutants for tolerance to acetic acid and HMF

The nine largest EP-40 colonies isolated from a solid plate (RCM) were evaluated for tolerance to acetic acid and 5-hydroxymethylfurfural (HMF). For this we carried out batch fermentations in 50 mL of MM containing xylose (60 g/L), acetic acid (5 g/L), and HMF (0.04 g/L) to compare the growth profile and fermentative performance of the mutants and the wild type strain (WT). All fermentations were carried out in duplicate in an anaerobic chamber at 30 °C. The initial pH was set to 4.95 and monitored off-line during fermentation using a pH meter (Metrohm). Cell growth was determined by measuring OD_{600nm} during cultivation. Samples were collected at 0, 24, 48, 72, 96, 120 and 144 h, and centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was transferred into 2 mL microtubes and stored at -4 °C until further analysis. The concentrations of the solvents (acetone, n-butanol, and ethanol), sugars (glucose and xylose), and acids (acetic and butyric) were determined using high-performance liquid chromatography (HPLC) with a refractive index (RI) detector coupled to an Aminex HPX-87H column (BioRad). The mobile phase was 5 mM sulfuric acid with a flow rate of 0.8 mL/min. All column conditions were as follows: the mobile phase was acetonitrile in water (1:8) with 1 % acetic acid and a flow rate of 0.8 mL/min. All samples were previously filtered using a 0.22 μm Millipore HX PVDF membrane filter. The culture growth was determined by measuring the optical density at 600 nm (OD_{600nm}) using a UV–vis spectrophotometer (Thermo Scientific - Evolution 60S, Ann Arbor, Michigan, USA) [23].

2.6. Genomics

The total genomic DNA (gDNA) of four mutants selected from the 9 evaluated mutants were extracted using the Wizard Genomic DNA purification kit (Promega). The extracted gDNA was purified using PowerClean® DNA Clean-Up Kits (Mo Bio Laboratories) to ensure the sample quality. The DNA library was built by Nextera DNA sample preparation kits (Illumina Inc., San Diego, CA, USA) and the fragmented sample was analyzed utilizing a Bioanalyzer (2100) with a 12,000 DNA assay kit (Agilent). The libraries were pooled in equimolar ratios and subsequently submitted to paired-end sequencing on MiSeq instrument with one 150 × 150nt paired-end mode (Illumina platform); according to standard procedures of the Brazilian Biorenewables National Laboratory (LNBR/CNPEM, Campinas, Brazil), which resulted in about 300x average coverage of each sample.

2.7. Next generation sequencing (NGS) data analysis

The NGS pipeline consisted of the following steps: Fastq files→; FastQC→; Trimmomatic→; BWA-MEM/Bowtie2→; Mpileup→; Varscan→; SnpEff [26–33]. For mutation analysis, the default setting in Bowtie2 was used for alignment and mapping [34]. The representative genome of Clostridium saccharoperbutylicum N1-4 (HMT) with taxonomy (ID) of 931276 (N1-4 (HMT) – ASM34088v1) was used as a reference genome for alignment. The results from the mapping were used to identify single nucleotide polymorphisms (SNPs), and insertions and deletions (indels) between the mutants and wild type. The results were further validated with the automatic Prokaryotic variant calling software Snippy. Genome annotation was done using Prokka and the aligned genomes and the SNP-indels were evaluated through viewing in IGV, Integrated Genome Browser [32]. Also, structural variants of the mutations were searched using the Delly software [35]. The mutations were also validated with different bioinformatics web platforms like Galaxy Melbourne and Patric. The types of mutations were classified using the SnpEff variant effect prediction software [30]. Further, the adverse of the mutations on protein sequences was predicted using Provean.

2.8. RNA isolation

Cell cultivation was carried out in MM containing 55 g/L of xylose, 5 g/L of acetic acid and 0.04 g/L of HMF, with an initial pH of 6.5. The pH used in this experiment was defined to allow the wild type strain to grow. For RNA isolation, 2 mL of culture was harvested and 4 mL of RNAprotect bacteria reagent (Qiagen, US) (1:2) was added immediately to stabilize and protect RNA from degradation. The material was mixed and incubated for 10 min at room temperature, centrifuged to obtain cell pellets, and stored at -80 °C for the following steps. For cell wall lysis; 200 μL of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8) containing lysozyme (15 mg/mL) (ThermoFischer, USA) and 20 μL of protease K (20 mg/mL) were added, and cells carefully re-suspended. The material was then incubated at room temperature for 10 min. In continuation, we added 700 μL of RLT buffer (with beta-mercaptoethanol) and mixed vigorously, followed by the addition of 500 μL of ethanol. The RNA was purified using an RNAeasy mini kit (Qiagen Inc, CA) according to the manufacturer’s instructions. After the extraction, RNA was treated with Turbo DNase free kit (Invitrogen) following the manufacturer’s protocol. RNA quality was analyzed using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US) and the concentration was determined using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA).

2.9. RT-qPCR analysis of selected genes

Total RNA samples were used to synthesize the cDNA using the reagent Superscript II transcriptase reverse kit (Invitrogen, USA) according to the manufacturer’s protocol. The first round of end-point PCR was performed and the products were separated by agarose gel electrophoresis and purified with GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, US). The amplicons were subjected to a 10-fold serial dilution (from 10^{-1} to 10^{-5}) and used to construct a standard curve. RT-qPCR reactions were performed according to Borin et al. along with the five best points of the standard curve and the cDNA samples from the experiments (see above), to keep the same conditions for standards and experimental samples (relative standard curve method) [35]. Primer sequences and genes analyzed are provided in Table 2. All RT-qPCR reactions were carried out in Viia 7 Real-Time PCR system (Applied Biosystems, Life Technologies) using the following amplification conditions: activation for 10 min at 95 °C followed by 40 cycles of denaturation (15 s at 95 °C), annealing and extension (1 min at 60 °C). Data normalization was performed using quantification obtained from the housekeeping genes 1 and 2 (Table 2), and all reactions were conducted in triplicate. Statistical significance of the results was determined using analysis of variance ANOVA (Tukey’s test), with a significance level of 95 % (p < 0.05). Analyses were performed using the GraphPrism 7.0 (GraphPad Software, San Diego, CA, USA).

2.10. Scanning electron microscopy

Morphology of bacterial cells (mutants and wild type) were analyzed using scanning electron microscopy (SEM). All strains were cultivated in individual batches on RCM in 15-mL shake flasks without agitation. All fermentations were carried out at 30 °C inside an anaerobic chamber.
Table 2: Primers used for RT-qPCR analyses of gene expression in wild type and mutants (RAC-21 and RAC-25).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Locus tag</th>
<th>Function</th>
<th>Primers 5'-3' (forward, reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rplD</td>
<td>CSPA_RS00900</td>
<td>Interacts with and stabilizes bases of the 16S rRNA that are involved in rRNA selection in the A site with the mRNA backbone. 30S ribosomal protein S12. With S4 and S5 plays an important role in translational accuracy</td>
<td>GAGTGTCTGAGAGGATTAAATGCG</td>
</tr>
<tr>
<td></td>
<td>Housekeeping 1</td>
<td></td>
<td>GATCTACCTTTGGCCCTTGGA</td>
</tr>
<tr>
<td>rpsL</td>
<td>CSPA_RS06500</td>
<td>Ribosomal protein S2 belongs to the universal ribosomal protein uS2 family.</td>
<td>GAGAGGAGTTACATTTCCGGA</td>
</tr>
<tr>
<td>sigD</td>
<td>CSPA_RS16265</td>
<td>Sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released.</td>
<td>TACCTTCATACCTACTTGC</td>
</tr>
<tr>
<td>pnuA</td>
<td>CSPA_RS01990</td>
<td>Catalyzes the NADPH-dependent reduction of L-glutamate 5-phosphate into L-glutamate 5-semialdehyde and phosphate.</td>
<td>CTGAGATCGGTGTCTTTGGT</td>
</tr>
<tr>
<td>apdB</td>
<td>CSPA_RS03060</td>
<td>Produces ATP from ADP in the presence of a proton gradient across the membrane.</td>
<td>GCTACTATCCTGTGTAATGAGCACTTC</td>
</tr>
<tr>
<td>prol</td>
<td>CSPA_RS02180</td>
<td>Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.</td>
<td>GAGTTGTGGAGAGGATTAAATGCG</td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1. Adaptive Laboratory Evolution of C. saccharoperbutylacetonicum in the presence of hemicellulosic hydrolyase (HH)

ALE was carried out in batch cultivations with an initial concentration of 20% HH (HH-20) diluted in MM. This initial concentration was based on preliminary growth profile of the wild type strain on medium containing different concentrations of HH (20%, 50% and 100%, v/v) (Fig. 1S, Supplementary Material). The subsequent cultivations at increased concentrations of HH were applied when a reduction or a stabilization of the doubling time (DT) was observed along with the cultivation rounds. We then progressively increased the HH fraction in the MM. The progression of ALE for C. saccharoperbutylacetonicum under increasing concentrations of HH (25%, 33% and 40%, v/v) is depicted in Fig. 1.

At HH-20 cultivations, five repetitive batch cultivations (rounds) were performed, encompassing 13 generations. In this first step, cells were able to grow at a fairly constant DT over the five rounds, suggesting that at 20% concentration (HH-20) the inhibitor titers did not severely impact microbial cells. The evolution experiment was continued by changing to a medium containing 25% HH (HH-25), starting with the evolved population (EP-20). Under this condition, eight rounds were performed for a total of 27 generations. The DT progressively decreased during the cultivations, with a reduction of 30% of DT in the last four rounds, in comparison to the first four rounds. Subsequently, we continued the evolution protocol in the presence of HH-33, starting with EP previously obtained (EP-25). After nine rounds and 22 generations in this condition, we were able to obtain an evolved population (EP-33) with a reduction of 48% in DT in the last five rounds compared to the first four rounds. In the last step of ALE, we challenged the EP-33 with HH-40. Cells were submitted to 17 rounds of cultivation, comprising of 66 generations under this condition. The results indicate that until round nine, the DT was practically unchanged. However from round 10 to 14, this parameter increased consistently. This fact can be explained by fact that the HH used in these experiments (batch 2) presented higher concentration of inhibitors compared to the first batch, and consequently appeared to be more toxic to the cells at the same concentration (HH-40) (Table 1). To facilitate data analyses, we can consider cultivations from round one to nine performed with the first batch of HH as separate; and from round ten to seventeen as another that utilized the higher inhibitor concentration compared to the first batch, and consequently that the HH used in these experiments (batch 2) presented higher concentration of inhibitors (Table 1). To facilitate data analyses, we can consider cultivations from round one to nine performed with the first batch of HH as separate; and from round ten to seventeen as another that utilized the higher inhibitor HH batch. In this case, we can divide the ALE with HH-40 into two parts. In the first, cells were evolved over 24 generations and a significant improvement in DT or final OD was not observed. In the second, we observed an increase of DT in the initial rounds due to the higher inhibitor concentration of the new HH batch, followed by a substantial decrease in DT. After approximately 130 generations, the adopted ALE strategy resulted in an evolved population (EP-40) with an improved fitness in HH supplemented media; with a 26% reduction in DT, in comparison to the cultivations with HH-20 and HH-40 (last three rounds). Finally, in order to obtain isolates from this EP-40, cells were plated onto solid RCM medium, and large colonies were selected and stored (Fig. 2).

3.2. Evaluation of evolved isolates towards acetic acid and HMF

The isolation of single EP-40 colonies from a solid plate (RCM) resulted in 9 colonies (mutants); to be evaluated for tolerance to acetic acid and HMF, previously identified (Table 1S, Table 2S and Fig. 2S, Supplementary Material) as the inhibitors that most negatively impact C. saccharoperbutylacetonicum growth. The isolates from the ALE experiment (EP-40 isolates) were evaluated in MM containing acetic acid and HMF. The concentration of inhibitors (5 g/L of acetic acid and 0.04 g/L of HMF) used in this work were higher than those present in the medium with HH-40. Cultivation under the presence of acetic acid and HMF showed that not all isolated mutants were able to grow under such conditions (Fig. 3).

Only four isolates (named RAC-2, RAC-8, RAC-21, and RAC-25) were...
able to reach an OD higher than 1.0. On the other hand, the wild type strain was not able to grow at all, confirming that ALE under HH promoted improved resistance toward acetic acid and HMF. Moreover, RAC-25 was able to achieve a cell density (OD<sub>600nm</sub>) of 9.14 in media without inhibitors (data not shown). Regarding butanol titer, mutant RAC-25 was able to produce 16.6 g/L of butanol (Y<sub>BUT/S</sub>=0.32 g/g); which is, to the best of our knowledge, the highest titer reported for batch cultures in a medium with a high concentration of acetic acid.

It is well known that solventogenic Clostridia spp. have a typically biphasic metabolism, where during the first phase (acidogenic) acids are produced (acetic acid and butyric acid) concomitantly with microbial growth. Consequently, due to the low pH promoted by acid production, cells switch their metabolism to the next phase (solventogenic); in which the acids (acetic and butyric) are re-assimilated into solvents (acetone, butanol, and ethanol). Butanol production via this route is known as the "hot channel" [37]. Another alternative pathway is when butanol is directly produced from acetyl-coenzyme A (CoA) through butyryl-CoA, and is known as the "cold channel" [37]. In literature, it is reported that 15.1 g/L is the maximum titer of butanol produced by <i>C. saccharoperbutylacetonicum</i> under normal conditions without inhibitors (Fule, 2017). The direct butanol forming hot channel has been described as playing a pivotal role in enhanced butanol production in comparison to cold channel [37]. Shinto et al. developed a model that showed <i>C. saccharoperbutylacetonicum</i> N1-4 has a robust metabolic network in the acid and solvent producing pathways [38].

The results obtained in our work suggest that the mutants (RAC-2, RAC-8, RAC-21, and RAC-25) with high tolerance to inhibitors (acetic acid and HMF) tend to preferentially produce butanol from the “hot channel” instead of the “cold channel”, since small amount of acids (acetic and butyric) were produced during fermentation (Fig. 4). Our results are consistent with results obtained by Jin et al., where they observed a down regulation of the metabolic flux towards the acid formation branch ("cold channel"), and an up-regulation of the metabolic flux toward the ABE formation branches ("hot channel"); and consequently improved <i>C. acetobutylicum</i> fermentation of a non-detoxified wheat straw hydrolysate supplemented with sodium sulfite [39].

Mutant RAC-25 displayed a remarkable fermentative performance in the presence of inhibitors, consuming 84% of the sugars and producing 22.1 g/L of ABE (Y<sub>ABE/S</sub> =0.42 g/g) (Table 3S, supplementary material). Moreover, RAC-25 was able to achieve a cell density (OD<sub>600nm</sub>) of 7.0 similar to that observed for the wild type strain (OD<sub>600nm</sub> of 9.14) in media without inhibitors (data not shown). Regarding butanol titer, mutant RAC-25 was able to produce 16.6 g/L of butanol (Y<sub>BUT/S</sub>=0.32 g/g); which is, to the best of our knowledge, the highest titer reported for batch cultures in a medium with a high concentration of acetic acid. In literature, 15.1 g/L is reported as the maximum titer of butanol produced by <i>C. saccharoperbutylacetonicum</i> under normal condition without inhibitors [14].

The remaining mutants (RAC-2, RAC-8, and RAC-21) were able to consume around 50% of the sugars and produce similar titers of butanol and ABE solvents. Nevertheless, mutant RAC-2 achieved the highest butanol (Y<sub>BUT/S</sub>=0.34 g/g) and ABE yield (Y<sub>ABE/S</sub> =0.50 g/g) despite consuming less sugar (46.7%) than the other mutants. Normally, both acetic and butyric acids are produced together with ATP generation in the acidogenic phase. These acids are then taken up for the production of butanol and ethanol, during the solventogenic phase, thus enabling an electron sink [37]. Another explanation for the high conversion yield observed in acetate containing media is the possible increased conversion of acetate to butanol to reduce its toxicity. Thus, a significant fraction of the solvents produced by the mutant RAC-2 can be formed from acetate. It is important to mention that an in-depth investigation, using metabolic modelling, for example, could be conducted to test this hypothesis, although no metabolic model for <i>C. saccharoperbutylacetonicum</i> is available until now.

In summary, our results revealed that the amount of butanol secreted by the mutant RAC-25 (16.6 g/L) in a batch fermentation exceeds previously reported limits for butanol tolerance for this bacteria [23]; which leads us to conclude that the adaptive evolution brought genetic
mutations that not only promoted tolerance to acetic acid and HMF but also increased the ABE production.

3.3. Genomic analysis of the evolved isolates

The results presented in the previous sections strongly support that the ALE strategy has generated mutants with higher growth capabilities as well as the best solvent production in the presence of inhibitors (acetic acid and HMF) compared to the parental strain. To provide more information about the different phenotype obtained through ALE, we sequenced the genome of mutants (RAC-2, RAC-8, RAC-21, and RAC-25) and compared them to the wild type. Mutations were identified by whole-genome re-sequencing and each genome was compared with the parental strain (ID129676) in Genbank (NCBI). The results of the alignment process for each strain generated a mean mapping ratio of about 99.98% with high genome coverage (a least mean value of 123.7×) for each strain, which implied excellent quality for variant calling (Table 4S, Supplementary Material). The obtained mutations,

![Graphs showing profile of acid and solvent production of mutants RAC-2 (A), RAC-8 (B), RAC-21 (C) and RAC-25 (D) during fermentation in mineral media containing acetic acid (5 g/L) and HMF (0.04 g/L).]

Table 3
Summary of mutations found in evolved strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation Type</th>
<th>Position</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAC-2</td>
<td>Stop gained</td>
<td>Glu428*</td>
<td>CSPA_RS22950</td>
<td>Catalyzes the phosphorylation on incoming sugar substrates</td>
</tr>
<tr>
<td></td>
<td>Deletion</td>
<td>F171fs</td>
<td>CSPA_RS16265</td>
<td>Promote the attachment of RNA polymerase to specific initiation sites</td>
</tr>
<tr>
<td></td>
<td>Missense</td>
<td>Gly30Ser</td>
<td>CSPA_RS00360</td>
<td>Protein involved in the pathway lipoprotein biosynthesis</td>
</tr>
<tr>
<td></td>
<td>Deletion</td>
<td>F171fs</td>
<td>CSPA_RS1415</td>
<td>Probably involved in glucitol uptake (carbohydrate transport)</td>
</tr>
<tr>
<td>RAC-8</td>
<td>Missense</td>
<td>Gly191Gly</td>
<td>CSPA_RS14135</td>
<td>Probably involved in glucitol uptake (carbohydrate transport)</td>
</tr>
<tr>
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<td>Missense</td>
<td>Gly30Ser</td>
<td>CSPA_RS00360</td>
<td>Protein involved in the pathway lipoprotein biosynthesis</td>
</tr>
<tr>
<td></td>
<td>Stop gained</td>
<td>Glu428*</td>
<td>CSPA_RS22950</td>
<td>Catalyzes the phosphorylation on incoming sugar substrates</td>
</tr>
<tr>
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<td>Deletion</td>
<td>F171fs</td>
<td>CSPA_RS14135</td>
<td>Promote the attachment of RNA polymerase to specific initiation sites</td>
</tr>
<tr>
<td>RAC-21</td>
<td>Missense</td>
<td>Leu33Ser</td>
<td>CSPA_RS14135</td>
<td>Probably involved in glucitol uptake (carbohydrate transport)</td>
</tr>
<tr>
<td></td>
<td>Missense</td>
<td>Gly191Gly</td>
<td>CSPA_RS14135</td>
<td>Probably involved in glucitol uptake (carbohydrate transport)</td>
</tr>
<tr>
<td></td>
<td>Stop gained</td>
<td>Ser25*</td>
<td>CSPA_RS22950</td>
<td>Involved in the regulation of arabinose metabolism (repressor)</td>
</tr>
<tr>
<td>RAC-25</td>
<td>Missense</td>
<td>Leu233Trp</td>
<td>CSPA_RS14135</td>
<td>Uncharacterized protein</td>
</tr>
<tr>
<td></td>
<td>Missense</td>
<td>Lys271fs</td>
<td>CSPA_RS14135</td>
<td>Anti-sigma factor for Sigl regulation through direct interaction</td>
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</tbody>
</table>
related genes, and functional information are summarized in Table 3. Results indicate that some of the mutations were shared among the isolated mutants, while others were exclusively present in one of the mutants. To facilitate data analysis, we arranged the mutants into two groups: Mutants RAC-2, RAC-8 and RAC-21 who shared mutations in similar genes (CSPA_RS22950, CSPA_RS16450, CSPA_RS16265), while mutant RAC-25 presented mutations in different genes (CSPA_RS22795, CSPA_RS19575 and CSPA_RS16260).

The ability of biological systems to respond to various environmental or nutritional changes is directly correlated to biochemical and genetic networks [40]. In this sense, several genes are necessary for this complex process. Among them, we can mention the recognition by RNA polymerase associated with alternative sigma factors. We noted that two of the mutations found were present in genes related to sigma factors. Mutants RAC-2, RAC-8 and RAC-21 showed a deletion in gene CSPA_RS16265, which produces the RNA polymerase sigma factor I (σI). Sigma factors are normally responsible for producing a multi-domain subunit of bacterial RNA polymerase, and therefore it plays an important role in transcriptional initiation [41]. Beyond that, this gene (σI) is also involved in the regulation of cell wall metabolism in response to heat stress in Bacillus [42]. So far, this is the first work revealing a possible role of this specific sigma factor (σI) in solventogenic Clostridium spp., since most of them have been reported in Bacillus spp. [43–45]. On the other hand, mutant RAC-25 showed a mutation (missense type) in the CSPA_RS16260 gene which produces the anti-sigma factor responsible for the down-regulation of sigma factor I (σI). Many works have described the involvement of transcriptional factors in stressful conditions, as well as strategies to enhance tolerance to many inhibitor compounds by manipulating these transcriptional factors [46–49]. Considering the mutations found in all the mutants, it was expected that RAC-2/RAC-8/RAC-21 showed a down regulation of σI, since they presented a deletion in the gene responsible for σI expression. On the other hand, regarding the mutant RAC-25, we expected a high expression of σI due to a mutation in the anti-σI gene, responsible for the σI gene regulation.

Furthermore, we have also identified mutations in genes involved in membrane transport and the transcriptional regulators of carboxydrates. The mutant RAC-25 presented a mutation (stop gained) in the CSPA_RS22795 gene that belongs to the GntR transcriptional regulator family; which is a large group of proteins present in diverse bacteria and regulates various biological processes. This gene (CSPA_RS22795), named araR, is responsible for the repression of genes related to arabinose metabolism and the pentose phosphate pathway in Clostridium spp [50]. In Gram positive organisms the arabinose operon is negatively regulated by araR, binding to operator regions of the arabinose operon in the absence of arabinose. On the other hand, in the presence of arabinose the sugars bind to araR promoting conformational changes and preventing its binding to DNA [51]. It has been reported that concomitant downregulation of XylR and/or araR may improve mixed-sugar utilization in solventogenic Clostridium species [52]. In a study conducted by Zhang et al. (2012), the researchers used a comparative genomic approach to identify AraR-binding DNA motifs and reconstruct AraR regulons in nine different Clostridium spp. The results obtained indicated that the expression of genes related to the pentose phosphate pathway, like nik (CAC1348), tal (CAC1347) and ptk (CAC1343), were up-regulated in the absence of arabinose in the mutant strain (araR inactivation) in comparison to wild type [50]. Their study corroborates with our results obtained from mutant RAC-25, which indicate that the mutation in gene araR could de-repress genes involved in xylose metabolism and improve sugar uptake (Fig. 3, Table 3 and Fig. 3S, Supplementary Material).

It has been shown that the inactivation of the XylR transcriptional repressor has been associated with increased utilization of xylose as the main substrate in C. beijerinckii and C. acetobutylicum [53,54]. A study conducted by Xiao et al. (2017) evaluated a point mutation in DNA dependent RNA polymerase (rpoB) regarding osmotolerance and succinic acid production in E. coli. The authors showed that the mutation rendered E. coli resistant to osmotic stress, probably due to improved cell growth and viability via enhanced sugar uptake under stress conditions, and activated a potential “pre-defense” mechanism under non-stressed conditions [55]. Another mutation (stop gained) shared by mutants RAC-2, RAC-8 and RAC-21 is present in gene CSPA_RS22950 (gldB), which encodes the glucose specific EIICBA protein component of the PTS (phosphotransferase system) system. The PTS system carries out both catalytic and regulatory functions in microbial cells. It plays an important role in transport mechanism of carbohydrate substrate, catalyzing both the accumulation and chemical conversion (phosphorylation) [56]. Since it has an important role in sugar uptake, we expected the mutation in CSPA_RS22950 (stop gained) would impact negatively the microbial growth and butanol production. Indeed this mutation showed a negative effect on microbial cells, impacting the substrate uptake and energy metabolism in MM with (Table 3) and without inhibitors (Fig. 3S, Supplementary Material). As mentioned before, this could be a strategy of cells, similar to catabolic repression, to consume the acetate present in media to avoid the deleterious effect caused by this acid at high concentrations. However, it is important to point out that there is a lack of knowledge describing a possible strategy to overcome hostile acidic conditions. Therefore, additional studies are required to deeply investigate this hypothesis of carbon catabolite repression (CCR) to promote acetate consumption.

3.4. RT-qPCR analysis of selected genes

Based on the results presented above, we hypothesized that in the first group (RAC-2, RAC-8 and RAC-21) sigma factor expression should be decreased, whereas in the second group (RAC-25) its expression should be increased when compared to the parental strain. To verify our hypotheses, we evaluated the expression level of sigma factor I and other genes related to stress conditions in two mutants from each group (RAC-21 and RAC-25), in comparison to the wild type strain. The genes investigated were: σI (CSPA_RS16265), proA (CSPA_RS00190), groL (CSPA_RS02180) and aptP (CSPA_RS3060) (Fig. 5).

The results indicated that the expression of σI was significantly different (p < 0.005) in the mutants studied (RAC-21 and RAC-25) compared to the wild type (WT) in all tested cultivation times (15, 24 and 48 h) (Fig. 5). Moreover, the mutant RAC-21 did not express the σI, as expected, due to the deletion of this gene, confirming the results obtained in the genome sequencing. On the other hand, the mutant RAC-25 surprisingly revealed a lower expression of the σI gene in comparison to the wild type (WT). Down-regulation of σI might be explained by the fact that the mutation in the anti-sigma factor can affect the mechanism responsible for “switching-off” the sig I protein; promoting a phenotype similar to the other mutants (RAC-2, RAC-8 and RAC-21). In the work performed by Minty et al. (2010), experimental evolution was applied to obtain E. coli mutants tolerant to exogenous isobutanol. Their results showed that many isobutanol tolerant strains presented a reduced activity in RpoS (sigma factor), probably related to a mutation in hfq or acrAB. They concluded that the mechanism for adaptation to isobutanol was based on cell envelope remodeling and stress response attenuation [57]. In another work, Riordan et al. showed that the inactivation of alternative sigma factor S4 (rhoN) affected the expression of stress resistance genes, most notably the gad genes required for GDAR (glutamate-dependent acid resistance); promoting an increase in acid resistance in the mutant strain [58]. Our results with σI suggest that the low expression of σI can promote an improvement in tolerance of C. saccharoperbutylicum towards acetic acid and HMF. However, it is important to note that until now, no other work has described which genes are regulated by σI (CSPA_RS16265) in solventogenic Clostridium spp.

Beyond σI, we also evaluated the expression of other genes involved in stressful conditions (proA, atpD and groL). The expression of the
gamma-glutamyl phosphate reductase (proA) gene, that is involved in L-proline biosynthesis [59], was also evaluated (Fig. 5). The results indicated a higher expression level of proA throughout cultivation in both mutants (RAC-21 and RAC-25) in comparison to the wild type. However, statistical analysis showed only differences between RAC-25 and WT at 15 h of cultivation. The results suggest that high expression of proA could be related to the improved tolerance to lignocellulosic inhibitors, in this case to acetic acid and HMF. Our data corroborates with results obtained by Liao et al. (2018), who showed that overexpression of some genes in (proA, proB, and proC) C. acetobutylicum to enhance proline biosynthesis promoted an excellent ability to withstand inhibitors (formic acid, ferulic acid, p-coumaric acid and syringaldehyde); and efficiently fermented undetoxified hydrolysates from different raw materials (soybean straw, rice straw, and corn straw) [60].

The groL gene which produces the 60 kDa chaperonin was also evaluated. This gene is responsible for preventing misfolding and promoting the refolding; and proper assembly of unfolded polypeptides generated under stress conditions. Our results indicate higher expression of groL in mutants (RAC-21 and RAC-25) at 15 and 24 h of cultivation (Fig. 5). Statistical analysis only showed significant differences between mutants and the wild type at 15 h, and between mutant RAC-25 and WT at 24 and 48 h. In a study conducted by Tomas et al. (2003), it was observed that the overexpression of the groELS gene in C. acetobutylicum promoted an increase of butanol tolerance and solvent production [61]. In another study the researchers constructed a recombinant strain of C. beijerinckii NCIB 8052 to overexpress groES and groEL and observed a higher solvent production, even under ferulic acid stressed conditions; providing a good candidate strain for biomass hydrolysate fermentation [62].

Finally, to investigate the acid tolerance of mutants, we evaluated the expression of H′ATPase (ATP synthase); since the response to organic acids, cells have demonstrated an increase in membrane H′ATPase activity through dissipation of plasma membrane potential induced by the weak acids [63]. It is known that uncharged weak-acids can diffuse freely across plasmatic membrane. Due to a more neutral intracellular pH, charged anions and protons are retained within cell, and cytoplasmic protons are expelled by membrane bound H′ATPase [63, 64]. Beyond disrupting internal pH homeostasis, weak acids can also affect lipid organization and function of cellular membranes [65]. The data obtained regarding ATP synthase subunit beta expression showed a higher expression in mutants RAC-25 and RAC-21 at 15 and 24 h of cultivations in comparison to the wild strain; presenting statistically significant differences only at 15 h between RAC-25 and WT (Fig. 5). At the end of cultivation (48 h), all the strains (mutants and WT) showed a decreased expression of this gene. In recent work, Mamata et al. applied adaptive laboratory evolution to improve Lactobacillus delbrueckii FMI performance at low pH (4.5), and showed a 1.80-fold increase in lactic acid production compared to the parental strain. Moreover, the evolved strain exhibited a higher H′ATPase activity, as well as a higher H′ATPase gene expression compared to the parent strain [65]. Guan et al. performed comparative genomics and transcriptomics analysis in an acid-tolerant strain of Propionibacterium acidipropionic to understand the microbial response of cells to acid stress during fermentation. The results showed that genes involved in ATP synthesis were found to differ in copy numbers between the two strains (evolved and parental strain). Thus, they concluded that several transporters, membrane proteins, and the ATP synthase delta chain contributed to phenotype differences between the wild type strain and an acid-resistant mutant [66]. The result confirms our data, supporting that an up-regulation of both ATP synthases (beta and delta subunits) may contribute to the enhanced acid tolerance displayed by RAC-21 and RAC-25 mutants.

3.5. Scanning electron microscopy (SEM)

In the adaptive laboratory evolution strategy, we observed some cellular morphological changes during cultivations under routine light microscopy observation (data not shown). Therefore, we decided to investigate these changes in morphology using SEM. Images of three mutants (RAC-2, RAC-21, and RAC-25) and the wild type in the mid-exponential phase of cultivation (15 h) were obtained by SEM (Fig. 6). The images revealed the differences between wild type (Fig. 6A) and evolved strains (Fig. 6B-D). It is shown that the mutants were much more elongated (almost 2 twofold) in length in comparison to the wild
type (control experiment). We believe that these changes can be related to the mutation found in \textit{sig I} and \textit{anti-sig I} which directly affect \textit{sigI} expression, as previously observed in Fig. 5. Alterations in cell morphology have been described as a visible indicator of bacterial strategies to tackle different environmental stress conditions [67]. In recent work, Zhang et al. performed a comparative transcriptome analysis of a \textit{C. beijerinckii} degenerated strain and the wild type 8052 strain. They found that morphological and physiological changes in the degenerated strain DG-8052 were related to disturbed expression of sigma factors; affecting aspects of sugar transport and metabolism, sporulation, chemotaxis and solventogenic pathways [68].

4. Conclusions

In this work, four robust strains of \textit{C. saccharoperbutylacetonicum} able to withstand a high concentration of acetic acid and HMF were successfully obtained through ALE. The genome analysis indicated that a down-regulation of \textit{sigI} can be directly involved in the improved tolerance of those strains. Moreover, the genes involved in membrane transport and metabolism of carbohydrates seem to be linked to a cellular strategy for adaptation to the challenging environment promoted by inhibitors. Our results bring important information about genes directly related to tolerance mechanism of cells, suggesting interesting targets for future metabolic engineering to obtain robust strains of \textit{C. saccharoperbutylacetonicum}.

CRediT authorship contribution statement

Rafael F. Alves: Conceptualization, Investigation, Data curation, Methodology, Writing - original draft. Ana M. Zetty-Arenas: Investigation, Writing - review & editing. Huseyin Demirci: Investigation, Writing - review & editing. Oscar Dias: Investigation, Writing - review & editing. Isabel Rocha: Supervision, Writing - review & editing. Thiago O. Basso: Conceptualization, Supervision, Writing - review & editing. Sindelia Freitas: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2020.11.013.

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