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CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains

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ABSTRACT

There is a demand to develop 3rd generation biorefineries that integrate energy production with the production of higher value chemicals from renewable feedstocks. Here, robust and stress-tolerant industrial strains of *Saccharomyces cerevisiae* will be suitable production organisms. However, their genetic manipulation is challenging, as they are usually diploid or polyploid. Therefore, there is a need to develop more efficient genetic engineering tools. We applied a CRISPR–Cas9 system for genome editing of different industrial strains, and show simultaneous disruption of two alleles of a gene in several unrelated strains with the efficiency ranging between 65% and 78%. We also achieved simultaneous disruption and knock-in of a reporter gene, and demonstrate the applicability of the method by designing lactic acid-producing strains in a single transformation event, where insertion of a heterologous gene and disruption of two endogenous genes occurred simultaneously. Our study provides a foundation for efficient engineering of industrial yeast cell factories.

1. Introduction

In addition to its traditional use in baking, brewing and wine-making, *Saccharomyces cerevisiae* is an important host in industrial biotechnology. This yeast is the host of choice for the first and second generation biorefineries for ethanol fermentation, which respectively use food biomass (corn starch, sugarcane) or ligno-cellulosic feedstocks (straw, corn stover, wood). *S. cerevisiae* is also applied for production of several enzymes and pharmaceutical proteins (e.g., insulin, hepatitis and human papillomavirus vaccines) and it is being developed for the production of advanced biofuels, such as farnesene and isobutanol, and fine chemicals, such as resveratrol or nootkatone (Borodina and Nielsen, 2014). The production of fuels and chemicals in biorefineries requires robust strains, tolerant to the common stresses in the industrial setting, such as low pH, high ethanol concentrations, fluctuating temperatures and the presence of various inhibitors (Demeke et al., 2013).

While there are many of such industrial strains, often adapted to the specific environments over long-time evolution, these strains are genetically more complex and hence less amenable to genetic manipulation than well-studied haploid laboratory strains (Le Borgne, 2012). Strain improvement requires several rounds of genetic interventions, such as introduction of heterologous genes and whole metabolic pathways, and complete or partial removal of the activity of endogenous genes to guide the metabolic flux towards the products of interest (Li and Borodina, 2015). In haploid laboratory strains, single genes can be disrupted and the resulting mutants easily selected and studied. This is difficult to achieve in diploid and polyploid industrial strains exhibiting often also aneuploidy, since multiple alleles of a gene are present and all the copies have to be inactivated. The classical PCR-based gene deletion strategy, based on replacement of the targeted allele with a selection marker cassette, relies on several laborious selection and screening processes and on the availability of an appropriate selection marker (Wach et al., 1994). For industrial strains this procedure is very time consuming and sometimes even infeasible.

In the past several years, new genome editing approaches based on the use of specific endonucleases, performing double strand breaks (DSB) and stimulating cell repair mechanisms, have emerged and caused a breakthrough in the field of synthetic biology. The transcription activator-like effector nucleases (TALENs) contain DNA-binding domain, which can be customized to recognize any sequence of choice, and FokI endonuclease cleavage domain (Li et al., 2011). Zinc finger nucleases (ZFNs) are

generated by fusion of a cleavage domain and a DNA-binding zinc finger domain. Such chimeric nucleases cleave the chromosomal DNA in the target area and the subsequent repair via homologous recombination (HR) or non-homologous end joining (NHEJ) results in gene disruption or another desired change in the affected genome (Urnov et al., 2010).

More recently, an alternative genome editing approach clusters of regularly interspaced palindromic repeats–CRISPR-associated nuclease 9 (CRISPR–Cas9), based on RNA-guided nuclease activity, emerged (Cong et al., 2013; Mali et al., 2013). *Streptococcus pyogenes* Cas9 endonuclease is originally a part of the bacterial immune system, where it forms ribonucleoprotein complex with two small RNA molecules and performs sequence-specific cleavage of the invading DNA (Horvath and Barrangou, 2010). So called CRISPR (cr) RNA is transcribed from the genome-encoded clusters of foreign DNA, processed and annealed to trans-activating (tracr) RNA molecule. Such complex then guides Cas9 to the target sequence (Gasiunas et al., 2012). It has been shown that target recognition by Cas9 requires a 20-bp sequence within crRNA, which base pairs with the target genomic sequence and a NGG trinucleotide, called protospacer adjacent motif (PAM), immediately downstream the target sequence. The parts of tracrRNA and crRNA, essential for Cas9 activity, can be combined into a single chimeric guide molecule (gRNA) with a target sequence on its 5′ end. By redesigning the 5′ end of the gRNA molecule the system can be programmed to target any desired sequence (Jinek et al., 2012). This represents a great advantage compared to TALENs and ZFNs, which are generally very time-consuming and costly to design. Several studies demonstrating the use of CRISPR–Cas9-mediated genome editing, such as single and multiplex gene disruptions and targeted insertions have been reported in bacteria (Bikard et al., 2013), human cells (Mali et al., 2013), mice (Cong et al., 2013), flies (Gratz et al., 2013) or worms (Dickinson et al., 2013). DiCarlo et al. (2013) have demonstrated efficient single gene disruptions in haploid *S. cerevisiae* cells, engineered with CRISPR–Cas9, when the donor template for homology directed repair (HDR) of the double strand break was co-transformed. Recently, simultaneous multiple gene disruptions in yeast have been reported (Bao et al., 2014), even in polyploid strain (Ryan et al., 2014).

In this study, we address the questions whether CRISPR–Cas9 method is suitable for engineering of strains isolated from various industrial settings and if there are potential limitations based on lower genetic accessibility of such strains. We also aim at showing whether these can be easily engineered for production of valuable chemicals. We demonstrate that a CRISPR–Cas9 genome editing tool can be used for efficient disruption of multiple alleles of the genes and for DNA insertion without specific adjustments, in several unrelated industrial strains. Firstly, we develop and test the method using the *ADE2* gene as the disruption target and green fluorescent protein (GFP) as insertion fragment for simultaneous disruption and knock-in. Secondly, we demonstrate the applicability of the CRISPR–Cas9 method by single-step construction of lactate-producing yeast, where both alleles of *PDC1* and *PDC5* genes are disrupted and heterologous lactate dehydrogenase genes are inserted. Furthermore, we show that it is important to consider potential off-target effects of the system, despite their relatively low frequency.

2. Materials and methods

2.1. Strains and cultivation conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cells were grown at 30 °C in standard yeast peptone dextrose

(YPD) medium supplemented with 20 g/l agar for preparation of solid medium. For cultivation of *pdc1Δpdc5Δ* strains, the glucose was replaced by 20 g/l ethanol or defined mineral medium (Jensen et al., 2014) containing 10 g/l glucose and 1 g/l sodium acetate was used. For selection, the media were supplemented with 200 mg/l G418 sulfate, 200 mg/l hygromycin B, or 100 mg/l nourseothricin. For selection on acetamide, conditions described in Solis-Escalante et al. (2013) were adopted. To examine sporulation capability, the strains were grown overnight on pre-sporulation plates (20 g/l yeast extract, 30 g/l nutrient broth, 50 g/l glucose, 20 g/l agar), subsequently inoculated on sporulation plates (10 g/l potassium acetate, 50 mg/l zinc acetate, 10 mg/l adenine, 20 g/l agar) and incubated for 4 days at room temperature. *Escherichia coli* strain DH5α was used as a host for cloning procedures and plasmid propagation. *E. coli* cells were grown at 37 °C in Lucia–Bertani (LB) medium containing 100 mg/l ampicillin.

2.2. Spore and GFP fluorescence evaluation

Spores were observed under Zeiss Primo Star transmitted-light bright-field microscope. After 1-h digestion of spore wall in 2.5 mg/ml solution of Glucanex (Sigma-Aldrich), the tetrads were dropped on YPD plates. Tetrad dissection was performed under Zeiss Axio Scope.A1 dissection microscope and the spores were grown for 2 days. Screening of fluorescent protein-expressing colonies was performed by visual scoring using the Safe Imager benchtop blue light transilluminator (Invitrogen). Measurement of GFP fluorescence was carried out in Corning® black flat-bottom 96-well microtiter plates using Biotek Synergy MX multi-mode plate reader in 485/512 nm emission/excitation wavelength.

2.3. Cloning and strain construction

The plasmids with dominant selection markers were constructed using USER fusion (Nour-Eldin et al., 2010) (Supplementary Table 1). The biobricks were amplified by PCR with PfuX7 polymerase (Nørholm, 2010) under the following conditions: 98 °C for 2 min, 30 cycles of 98 °C for 10 s, 54 °C for 10 s, 72 °C for 30 s/1 kb, 72 °C for 10 min. The used primers and templates are listed in Supplementary Tables 1 and 2. The dominant marker gene cassettes were synthesized by GeneArt (Life Technologies). DNA fragments were gel purified and incubated in HF buffer (New England Biolabs) together with USER enzyme (New England Biolabs) for 25 min at 37 °C followed by incubation at 25 °C for 25 min. Completed reactions were transformed into chemically competent *E. coli* cells. Synthetized GFP gene was provided by Morten Nørholm (Technical University of Denmark) (Toddo et al., 2012). Synthetized ʟ-lactate dehydrogenase gene (*ldhL*) from *Lactobacillus plantarum* (Supplementary File 1) was firstly digested with *Kpn*I and *Sal*I restriction endonucleases and ligated into plasmid pE1 (Borodina et al., 2015), digested with the same enzyme pair. GFP gene, *ldhL* gene and *Streptococcus pyogenes* Cas9 gene (DiCarlo et al., 2013) were PCR amplified and inserted along with *TEF1* promoter into integrative plasmids by USER cloning (Jensen et al., 2014). Parental vectors (Supplementary Table 1) were digested with AsiSI endonuclease and subsequently nicked with Nb.BsmI and assembled with desired biobricks as described above. The integrative vectors were linearized with *Not*I restriction endonuclease before yeast transformation. To construct the plasmids with specific gRNA cassettes, we PCR-amplified the whole plasmid with phosphorylated primers, containing the desired target sequences on their 5′ end. For this reaction we used Phusion Hot Start II DNA polymerase (Thermo Scientific). The PCR product was subsequently self-circularized using T4 DNA ligase (Thermo Scientific) according to manufacturer's protocol and treated with *Dpn*I endonuclease.

Table 1
List of strains used in the study.

Strain	Ploidy	Description	Source
CEN.PK113-7D (MATa)	Haploid	Laboratory strain	Peter Kötter, Johann Wolfgang Goethe University Frankfurt
CBS7960	Diploid[a]	Brasilian bioethanol producer from cane-sugar syrup	Silas Villas-Bôas, University of Auckland
CLIB382	Diploid[a]	Irish brewer's strain	Silas Villas-Bôas, University of Auckland
Ethanol Red (MATa/α)	Diploid	Industrial bioethanol producer	Fermentis (A Lesaffre division)

[a] Determined in this study using flow cytometry analysis of the DNA content.

For homologous recombination-mediated gene replacement, the disruption cassettes were prepared by PCR (Reid et al., 2002). One cassette consisted of the upstream *ADE2* region and the first 2/3 part of kanMX marker, and the other cassette contained the last 2/3 part of kanMX marker and the downstream sequence of *ADE2*. Both fragments were made by fusion PCR (Yon and Fried, 1989) and then simultaneously transformed to yeast cells. 90-bp dsDNA oligos (Supplementary Table 2) used as template for DSB repair were synthesized by Integrated DNA Technologies. Gene-carrying fragments, such as GFP or *ldhL* expression cassettes, used alternatively as DSB repair templates, were prepared with primers containing 40-bp overhangs to particular genomic sequence bordering potential Cas9 cutting site and integrative plasmids (Supplementary Table 1) as templates.

2.4. Yeast transformation

Yeast cells were transformed by PEG/LiAc method according to Gietz and Woods (2006). The heat shock time was prolonged to 90 min for strain CBS7960 in order to get higher transformation efficiency.

2.5. Propidium iodide staining and flow cytometer analysis

Yeast cells were grown in cell culture tubes in 1 ml YPD for 2 days with shaking (200 rpm). The cells were collected by centrifugation, washed in 1xSSC buffer (150 mM sodium chloride, 15 mM sodium citrate; pH=7), resuspended in $1\times$ SSC/70% ethanol solution and stored at $-20\,°C$ overnight. The fixed cells were centrifuged, resuspended in $1\times$ SSC buffer with 0.25 mg/ml RNase A and treated overnight at $37\,°C$. The samples were then treated with 1 mg/ml proteinase K for 1 h at $37\,°C$. The cells were collected and stained in $1\times$ SSC+10 µg/ml propidium iodide (PI) for 1 h at room temperature in the dark. The stained cells were stored at $4\,°C$ until analysis. The samples were briefly sonicated and analyzed with BD LSRFortessa cell analyzer equipped with blue 488-nm laser (50 mW power) and 695/40-nm bandpass filter. Histograms were acquired in linear mode. The data was analyzed with BD FACSDiva software.

2.6. HPLC analysis

Concentrations of lactic acid, glucose, pyruvic acid, ethanol, acetate, succinate and glycerol in culture supernatants were determined by HPLC (UltiMate 3000, Dionex). The samples were analyzed for 30 min using Aminex HPX-87H ion exclusion column with a 5 mM H_2SO_4 flow of 0.6 ml/min. The temperature of the column was $60\,°C$. The refractive index at $45\,°C$ and the UV absorption at 210 nm were measured. Glucose, lactic acid, glycerol and ethanol were detected using RI-101 Refractive Index Detector (Dionex). Pyruvate, succinate and acetate were detected with DAD-3000 Diode Array Detector at 210 nm (Dionex). The data was acquired and analyzed with Chromeleon software.

3. Results and discussion

3.1. Assessment of ploidy of the industrial strains CBS7960 and CLIB382

Industrial strains Ethanol Red, which is a commercial strain commonly used in many first generation ethanol plants, CBS7960 Brazilian ethanol producer from sugar-cane syrup and an Irish brewer's strain CLIB382, isolated from super-attenuated beer, were included in this study (Table 1). As a representative of an easily accessible and well-defined laboratory strain, CEN.PK113-7D was chosen. This strain is widely used for metabolic engineering and as a reference strain in systems biology and functional genomics studies (Daran-Lapujade et al., 2003; Canelas et al., 2010). To estimate the ploidy of the industrial strains, we determined the relative DNA content of the cells stained with propidium iodide by flow cytometry. Based on this analysis, all the chosen industrial strains were assessed as diploids when compared to the known haploid DNA content of CEN.PK strain (Fig. 1).

3.2. Construction of tools for Cas9-mediated genetic manipulation in industrial yeast

To perform Cas9-mediated genome editing in industrial yeast strains an approach, developed by DiCarlo et al. (2013), was adapted. The system uses *S. pyogenes* Cas9 nuclease-encoding gene, codon-optimized for yeast, and a single chimeric gRNA, which carries the 20-bp target sequence, and the structural module essential for Cas9 activity (Jinek et al., 2012). The gRNA molecule is transcribed from the expression cassette containing regulatory elements of RNA polymerase III (DiCarlo et al., 2013). For use in prototrophic yeast strains, a replicative (CEN/ARS element containing) vector carrying Cas9 gene with dominant kanMX selection marker and 2µ-replicative vector carrying gRNA molecule with dominant natMX selection marker were prepared (Fig. 2A). Specific sequence on the 5′ end of the gRNA molecule, targeting a gene of interest (GOI), was introduced on the primer used for amplification of the entire plasmid, allowing easy and cost-effective generation of gRNA targeting vector by PCR and ligation, followed by *E. coli* transformation (Fig. 2A). The suitable target sequence was selected using CRISPy online Internet tool (Ronda et al., 2014), which was adapted to the CEN.PK genome sequence (Jakočiūnas et al., 2015). The criteria for the selection were: minimal off-target effects elsewhere in the genome, proximity to the N-terminus of the coding region of the gene, and conserved sequence in order to target the gene in various unrelated strains. To achieve the maximal efficiency of disruption, a 90-bp dsDNA donor oligo was designed. The oligo would introduce a STOP codon into the PAM sequence behind the target site of a gene of interest upon a homologous recombination (Fig. 2B). Some other single nucleotide changes in the "seed" sequence of gRNA target sequence (Jinek et al., 2012) and frameshift mutation behind the introduced STOP codon were also included.
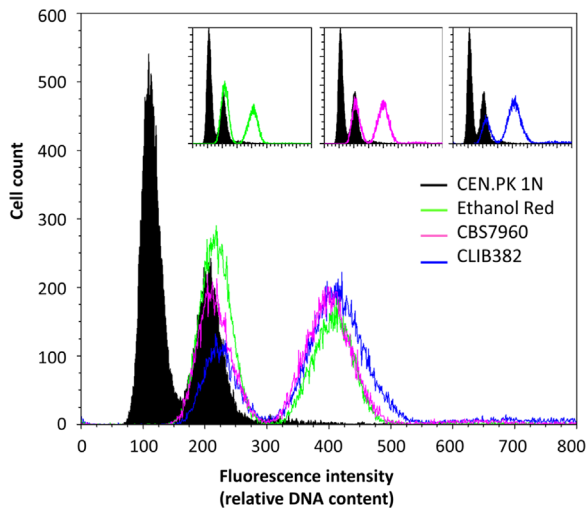
Fig. 1. Determination of strain ploidy. Uniparametric histogram shows distribution of PI stained cells according to their relative DNA content as examined by flow cytometry. CEN.PK113-7D strain of known ploidy was included as a reference. Individual histograms overlaid with 1N (haploid) CEN.PK histogram for comparison are included at the upper part of the picture. First peak represents population of G1-phase cells, second peak population of G2-phase cells.

3.3. Disruption of ADE2 in industrial strains

To analyze the suitability and efficiency of CRISPR–Cas9 as a tool for gene disruption in various industrial strains, a proof-of-concept in the form of Cas9-mediated *ADE2* gene disruption was selected (Fig. 3A). *ADE2* encodes phosphoribosylaminoimidazole carboxylase, which catalyzes a step in the *de novo* purine nucleotide biosynthetic pathway. The *ade2Δ* mutant phenotype can be recognized by red color of the colonies, since the mutant cells, deprived of adenine, accumulate red purine precursors in the vacuole (Ugolini and Bruschi, 1996). As a control experiment, single-allele replacement by PCR-generated marker cassette was performed in haploid CEN.PK and in diploid Ethanol Red strain. As expected, the red mutant colonies were observed only in the case of haploid CEN.PK, as in the Ethanol Red only one of the two *ADE2* alleles was disrupted (Fig. 3B). As expected, the phenotype of Ethanol Red *ade2Δ*: kanMX/*ADE2* strain did not differ from the phenotype of parental Ethanol Red strain revealing that elimination of both alleles must be performed to achieve mutant phenotype. Using the CRISPR–Cas9 system, the cells, expressing Cas9 gene either from a replicative plasmid or from a genomic location, were transformed with a 2μ-replicative vector, carrying the gRNA molecule along with templates for DSB repair and selected for presence of both markers. High efficiency of *ADE2* disruption was observed in all strains reaching the values of 95%, 65%, 78% and 66% in CEN.PK, Ethanol Red, CBS7960 and CLIB382, respectively (Fig. 3C and D). This shows that using the two plasmid system, which (probably due to insufficient expression of a guiding RNA component) resulted in the method inefficiency in previous study (Bao et al., 2014), high disruption efficiency can be achieved. Furthermore, the presented system allows for high disruption efficiency in several unrelated industrial strains, when no further adjustments in terms of gRNA expression, which have been reported recently (Ryan et al., 2014), are needed. The simplicity of a gRNA plasmid generation allows for fast evaluation of the disruption efficiency of any gene of interest without synthetic and cloning procedures.

The gRNA plasmid transformation efficiency was lower in the case of strains expressing Cas9, when compared to the wild type cells. This negative effect was more pronounced in those industrial strains that have generally lower transformation efficiency (Supplementary Fig. 1) and also when Cas9 was expressed from genomic location rather than from an episomal vector (not shown). The decreased transformation efficiency was also observed in laboratory strains expressing Cas9 and could be explained by the toxicity caused by off-target effects of the CRISPR–Cas9 system (DiCarlo et al., 2013). It has been shown that high level of Cas9 expression itself from a 2μ-based vector decreases the fitness of the cells and needs to be balanced (Ryan et al., 2014). However, the growth of the strains was not in any way affected by Cas9 carried alone on a low copy number vector (Fig. 2A), even under control of the strong *TEF1* promoter (not shown). It therefore appears reasonable to maintain Cas9 expression relatively low and stable while keeping the expression of gRNA high (e.g., by using a 2μ vector; Fig. 2A) to ensure high editing efficiency. Yet, the low transformation efficiency of Cas9-expressing strains could be a limiting factor, when some hardly accessible strains are considered to be engineered. We addressed this limitation by further decreasing Cas9 expression by using a weaker promoter (*ADH1*) (Fig. 2A). With this setup, the yield of transformants was significantly higher, and even in case of CBS7960 strain, exhibiting generally low transformation efficiency, we obtained five times more transformants (Supplementary Fig. 1). At the same time, the gene disruption efficiency was similar as before (Fig. 3D). When the dsDNA oligo was omitted, surprisingly high frequency of up to 16% of *ade2Δ* mutants was observed in CEN.PK strain. The disruption efficiency was much lower, but still significant in Ethanol Red and CLIB382 strains (around 3%) and not evaluated in strain and CBS7960 strain due to low number of positive transformants (Fig. 3D). This is in contrast with the previous findings, where the frequency of mutation in the absence of donor DNA was only around 0.1% (DiCarlo et al., 2013). Omission of the donor dsDNA also had further negative effect on the transformation efficiency as shown also previously (DiCarlo et al., 2013) (Supplementary Fig. 1). The negative controls, when the gRNA vector was omitted and only dsDNA was introduced, did not yield any mutant colonies among several thousand clones observed.

The affected parts of the *ADE2* gene were sequenced in a few randomly selected clones of CEN.PK and Ethanol Red strains. The majority of the red colonies, resulting from transformation with gRNA and donor dsDNA, contained the desired premature STOP codon (Supplementary Fig. 2). A few clones of CEN.PK also contained indel-caused frame-shift mutations. This signals that the DSB was repaired in other way than the homologous recombination with the donor dsDNA oligo. Mainly in case of Ethanol Red, where the proportion of non-mutant colonies is higher, some of the white colonies contained substitute mutations introduced with dsDNA donor in the expected cleavage region, but without the premature stop codon. Here, we did not observe any other random sequence changes, such as insertions or deletions, evidencing repair by NHEJ. This reveals that the DSB had occurred, the repair template had been delivered into the cells, but not entire donor sequence was inserted. The mutant clones, obtained by transformation with gRNA plasmid only, contained exclusively frame-shift mutations (Supplementary Fig. 2).

3.4. Simultaneous disruption of ADE2 and knock-in of GFP in industrial strains

To evaluate the possibility of simultaneous gene disruption and knock-in, we generated a DNA knock-in cassette, containing GFP-coding gene under control of *TEF1* promoter. The cassette had 40-bp flanking sequences, homologous to the genome targeting region (Fig. 2B). The cassette was co-transformed along with the *ADE2*-targeting gRNA plasmid into Cas9-expressing cells of CEN.PK and Ethanol Red, CBS7960 and CLIB382 strains instead of the
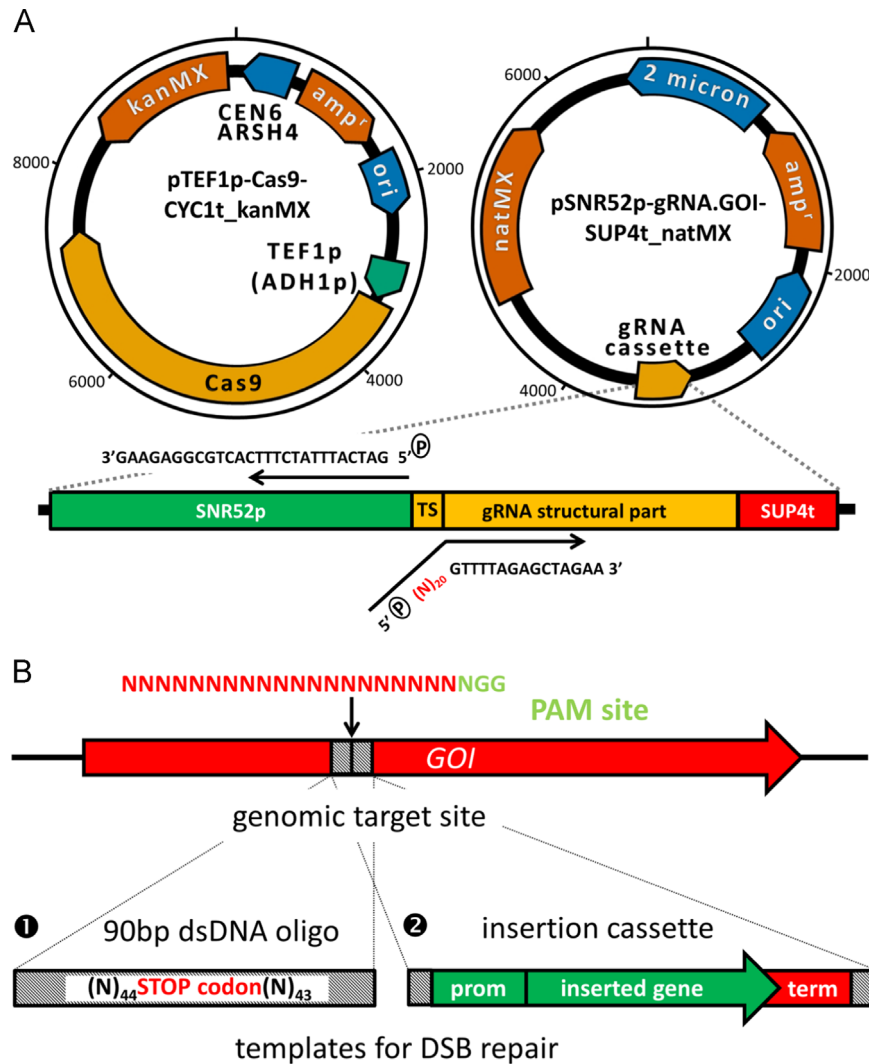
Fig. 2. Tools for Cas9-mediated genome editing in industrial strains. (A) Schematic illustration of replicative (CEN/ARS containing) plasmid carrying Cas9 gene, controlled by *TEF1* (or *ADH1*) promoter, and kanMX marker for selection. Alternatively, insertion of P_{TEF1}-Cas9 into the genome using integrative plasmid (not displayed) was performed. The vector with *TEF1* promoter controlling Cas9 expression was used unless otherwise stated. The two-micron-based replicative plasmid contains the gRNA expression cassette and natMX dominant marker for selection. The detailed illustration of gRNA cassette is displayed below the plasmid maps, including design of phosphorylated (P) primers used for PCR amplification of the plasmid with forward primer containing specific 20-bp target sequence (TS). (B) Illustration of target site of a gene of interest (GOI) in the genome, Cas9 target site being part of gRNA sequence and followed by PAM motif is highlighted. Templates, 90-bp dsDNA oligo introducing STOP codon into the coding sequence (1) and a gene expression cassette disrupting the coding sequence (2), used for DSB repair are displayed below.

donor dsDNA oligo (Fig. 3A). The efficiency of disruption (occurrence of red colonies) was comparable to the efficiency obtained with donor dsDNA oligo, when 10 μg of the knock-in cassette were used and significantly increased (reaching values of 97%, 82%, 92% and 81% in CEN.PK, Ethanol Red, CBS7960 and CLIB382, respectively) when increased amounts of gRNA plasmid (3 μg) and the repair cassette (15 μg) were used (Fig. 3D). This strongly suggests that when a gRNA vector and the appropriate repair DNA template are delivered into the cells, the presented system does not appear to have any other significant limitations. Bao et al. (2014) reported a way to facilitate the delivery of the donor DNA template by harboring it in the spacer of the crRNA array on plasmid. However, this system has limitations in the length of the sequence, which can be cloned as a HDR template, and apparently cannot be used for insertions of larger (heterologous) gene cassettes (Bao et al., 2014).

To uncover the number of GFP copies inserted in the genome of haploid and diploid strains, we performed measurement of GFP fluorescence of cell populations of randomly selected GFP-positive *ade2Δ* clones CEN.PK (1 N) and Ethanol Red (2 N) strains,

cultivated in mineral medium supplemented with adenine. Additionally, we evaluated haploid populations, derived from the four spores of ten red fluorescent Ethanol Red clones. All the haploid spores showed *ade2Δ* phenotype and GFP fluorescence, which was comparable to the level of fluorescence of haploid CEN.PK clones and approximately twice lower than GFP fluorescence of parental diploid cells (Supplementary Fig. 3). This reveals that all the tested diploid cells contained homozygous insertions of the reporter gene. Furthermore, the number of clones, exhibiting GFP fluorescence, was evaluated. Between 45% and 52% of the mutant red colonies of all strains showed GFP fluorescence (Fig. 3D). Successful Cas9-mediated insertion of heterologous marker gene cassette was achieved in laboratory strain and one polyploid strain, but only when different (tRNA-based) promoters driving expression of gRNA molecule were used (Ryan et al., 2014). PCR and sequence analysis of the *ADE2* gene of several non-fluorescent red CEN.PK and Ethanol Red clones showed that in most cases the expression cassette was not entirely inserted. Instead, different parts of the template DNA were used for DSB repair resulting in disruption of *ADE2* but not in expression of the reporter gene. This is consistent
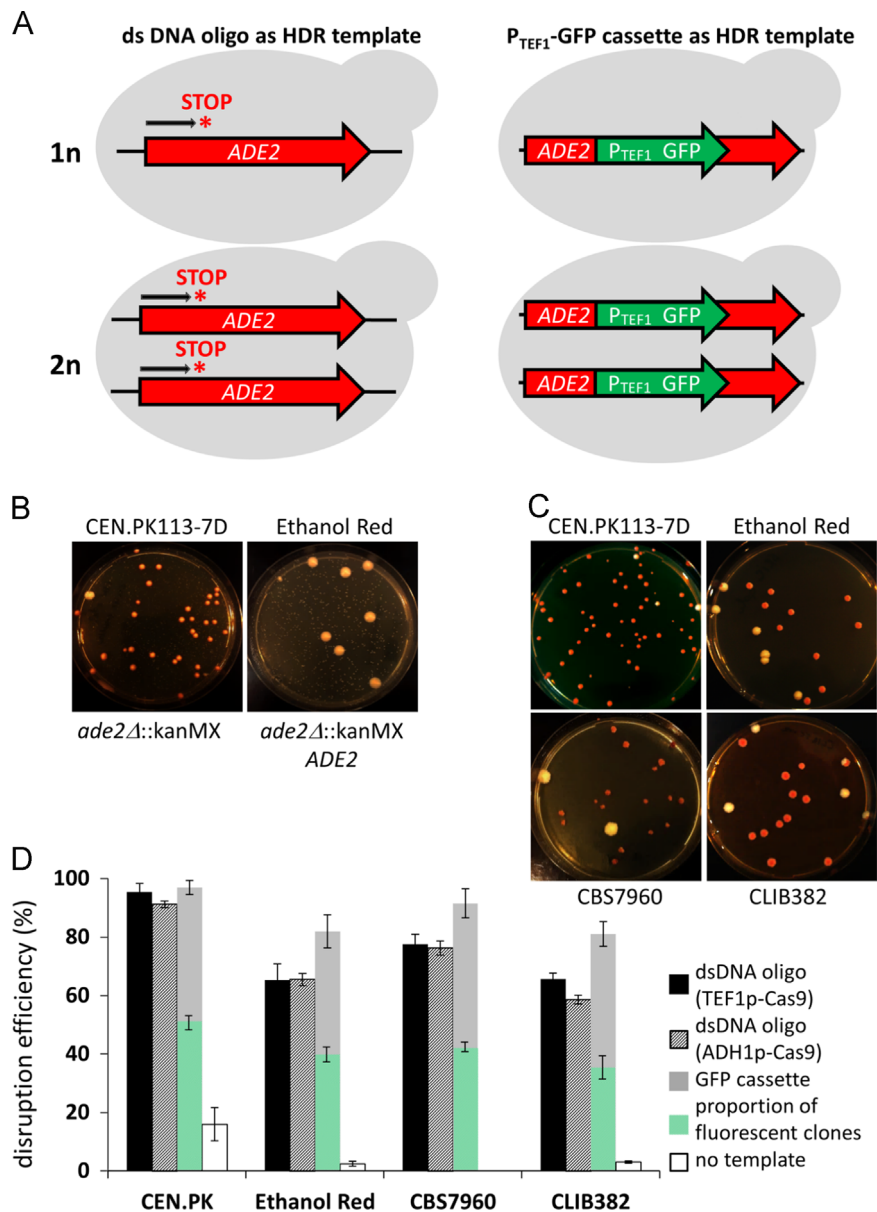
Fig. 3. Disruption of *ADE2* gene – proof-of concept. (A) Illustration of Cas9-mediated genomic changes in *ADE2* coding sequence. Introduction of STOP codon into coding sequence of *ADE2* gene in haploid – 1*n* and diploid – 2*n* cells, left; disruption of *ADE2* sequence with P_{TEF1}-GFP expression cassette, right. (B) Colonies of the strains with single-allele *ADE2* disruption performed with PCR-generated marker cassette disruption. Red color of the colony represents *ade2Δ* phenotype. (C) Colonies of the Cas9-expressing strains selected for presence of gRNA plasmid, being transformed along with 1 nmol/μl 90-bp dsDNA donor. (D) Disruption frequency of *ADE2* gene (number of red colonies per total number of transformants selected for presence of both Cas9-carrying and gRNA-carrying vectors) in analyzed strains. Black columns show the mutation frequency when dsDNA oligo donor is used as a repair template and Cas9 expression is driven by *TEF1* promoter, dashed columns represent the situation when Cas9 expression is driven by *ADH1* promoter, gray columns show the mutation frequency when 15 μg of P_{TEF1}-GFP cassette is used instead of dsDNA oligo donor and white columns represent the case when any repair template is omitted. Green pattern columns show percentage of fluorescent clones among *ade2Δ* mutants. Error bars represent standard deviation (SD, $N=3$ or $N=2$ in case of ADH1p-Cas9 experiment).

with previous experiments when 90-bp dsDNA donor was used as repair template. There, a desired stop codon was introduced but not the other single nucleotide changes (SNPs) the template contained. In some Ethanol Red clones, even the introduction of premature stop codon sometimes did not occur meanwhile the other SNPs appeared (Supplementary Fig. 2). However, some CEN.PK red and non-fluorescent clones contained indel mutations. This reveals that such cells probably did not gain the donor DNA template and the double strand break was repaired by NHEJ or other kind of error-prone mechanisms (Daley et al., 2005). Surprisingly, a few of the white colonies expressed GFP. Some of them contained the P_{TEF1}-GFP cassette in the appropriate location disrupting *ADE2,* suggesting, at least in case of haploid CEN.PK

strain, reversion to Ade$^+$ phenotype by the suppression of the *ade2Δ* mutation (Achilli et al., 2004). On the other hand, few clones contained the GFP gene integrated somewhere else, leaving the *ADE2* gene intact. As random insertion of the cassette not coupled with any selection is highly improbable and could not be detected in the control experiments, the wrong integration may be caused by off-target effects of CRISPR–Cas9 system, which have also been reported in other organisms (Hsu et al., 2013; Fu et al., 2013). Relatively low off-target change occurrence reveals that such effects can hardly be noticed, when only few clones are sequenced as in Ryan et al. (2014). Thus, the possibility of the off-target effect should always be taken into account when designing the gRNA molecule (Cho et al., 2013).

The disruption frequency is apparently dependent on the strain background due to different HDR-mediated repair activity of the particular strain and correct insertion of DNA donor. The latter explains the different disruption efficiencies obtained in experiments using either dsDNA oligo or a gene expression cassette as repair templates. When larger DNA cassette as a repair template is introduced, it always leads to the disruption of a gene, even if the cassette is not entirely inserted. In case of the donor dsDNA oligo, the premature STOP codon is not incorporated in some cases, resulting in lower efficiency. However, the efficiency of the presented system appears to be high enough to execute simultaneous disruption and knock-in of a heterologous gene expression cassette in three genetically complex strains. The presented two plasmid setup keeps low and stable expression of Cas9 to avoid any potential undesired sequence changes caused by its imbalanced expression, and high (*SNR52* promoter driven) expression of a gRNA molecule. In future, feasibility of targeting multiple genes in different industrial strains by simultaneous expression of multiple gRNAs at the same time by the approaches reported recently (Bao et al., 2014; Ryan et al., 2014) will be an attractive task.

### 3.5. Efficient removal of CRISPR–Cas9 tools results in stable marker-free genome alterations

An advantage of the CRISPR–Cas9 editing approach is that no selection marker is supposed to be left in the genome of the engineered strains since the plasmids, used for execution of the desired changes, can be cured out of the cells. To demonstrate the stability of introduced changes and marker-free nature of the method, we streaked few GFP expressing *ade2Δ* clones of both CEN.PK and Ethanol Red strains and grew them overnight on non-selective YPD plates. Several hundred cells were then plated on YPD plates. All the colonies maintained their original phenotype, i.e. they were red and showed comparable level of GFP fluorescence. The colonies were replica-plated on fresh YPD plates supplemented with either G418 or nourseothricin, and around 50% and 60% of CEN.PK and Ethanol Red cells, respectively, lost CEN/ARS Cas9-carrying plasmid. The gRNA 2μ-based plasmid was lost in the majority of cells resulting in stable marker-free phenotype (Supplementary Fig. 4). Keeping the selection, the Cas9-carrying vector can be maintained in the cells without any impact on cell fitness (Section 3.3), while the 2μ vector can be easily replaced by another gRNA vector targeting another gene of interest. This represents a remarkable advantage when compared to laborious approaches requiring marker insertion and subsequent particular marker recycling strategy (Guldener et al., 1996).

3.6. Disruption of pyruvate decarboxylase genes PDC1 and PDC5 in an industrial strain

To demonstrate the use of the CRISPR–Cas9 genome editing tool for metabolic engineering, we decided to engineer CEN.PK and industrial Ethanol Red strain for production of the industrially important chemical ʟ-lactic acid (Borodina and Nielsen, 2014). Several studies describing strategies for engineering of yeast strains for lactate production have been reported (Ishida et al., 2006; Van Maris et al., 2004a). These involve attenuation or elimination of pyruvate decarboxylase activity, transforming pyruvate to acetaldehyde, which is subsequently transformed to ethanol (Pronk et al., 1996), and expression of heterologous lactate dehydrogenase for conversion of pyruvic acid to lactic acid (Porro et al., 1995). *S. cerevisiae* contains three pyruvate decarboxylase isoenzymes, where the major isoforms are Pdc1p and Pdc5p (Flikweert et al., 1996). Disruption of *PDC1* and *PDC5* has previously been shown to result in inability of the strains to grow on glucose as the sole carbon source (Pronk et al., 1996), most likely

due to redox co-factor imbalance, and dependence on C2-carbon sources for generation of cytoplasmic acetyl-CoA, needed for lipids biosynthesis. To balance NADH overproduction in the *pdc1Δpdc5Δ* strains, we first introduced *L. plantarum* ʟ-lactate dehydrogenase (*ldhL*) gene into the genomes of Cas9-expressing CEN.PK and Ethanol Red strains. The resulting strains did not produce lactic acid under aerobic conditions (data not shown). Subsequently, the Pdc1$^-$/Pdc5$^-$ phenotype was introduced (Fig. 4A). As the homology of *PDC1* and *PDC5* genes is 95%, we designed a single gRNA for targeting both genes. The donor dsDNA oligo was designed to introduce a premature stop codon into *PDC1* and *PDC5* sequences (Fig. 4A). The *ldhL*- and Cas9-containing yeast strains were co-transformed with *PDC*-targeting gRNA and the donor dsDNA oligo. The resulting transformants were analyzed by PCR and sequencing of the targeted *PDC1* and *PDC5* loci (Supplementary Fig. 5). Seven out of the ten analyzed CEN.PK clones contained disruption mutations in all *PDC1* and *PDC5* alleles, with one of them containing frame-shift indel mutation and the rest STOP codons. For Ethanol Red strain, nine of the twenty five tested clones contained the desired mutations in both *PDC1* and *PDC5* genes. Several of the clones contained substitute mutations along the potential cleavage site, revealing improper integration of the dsDNA donor (Supplementary Fig. 5). All the clones verified for the presence of *pdc1Δpdc5Δ* mutations were grown aerobically on mineral medium with 10 g/l of glucose supplemented with 1 g/l of acetate and the concentration of lactate in the fermentation broth was analyzed by HPLC. While all seven CEN.PK derived strains produced certain amount of lactic acid, two out of nine of the Ethanol Red derived clones did not produce any lactic acid suggesting heterozygosity in one or both *PDC* alleles not uncovered by sequencing or suppression of their mutations. Selected CEN.PK and Ethanol Red lactate-producers were inoculated to initial OD=0.1 from overnight culture and cultivated aerobically in mineral medium in deep-well plates for 5 days. Production of metabolites, as analyzed by HPLC, and growth properties were determined in a time-course experiment (Fig. 4B). Both strains exhibited much slower growth and glucose consumption when compared to the parent strains, which depleted the carbon sources in the cultivation medium in 24 h (including produced ethanol) in case of CEN.PK or even faster in case of Ethanol Red (data not shown). Lactate-producer, derived from CEN.PK strain, grew even slower when compared to its Ethanol Red counterpart. Expectedly, the glucose consumption was more efficient in case of the engineered Ethanol Red. After 100 h of cultivation, the residual glucose concentration was 1.25 ± 0.6 g/l and 4.08 ± 0.08 g/l of glucose in Ethanol Red and CEN.PK, respectively. However, the final titer of lactic acid was 2.74 ± 0.11 g/l in CEN.PK and 2.52 ± 0.03 g/l in Ethanol Red resulting in yield of 0.61 ± 0.05 g and 0.35 ± 0.03 g lactic acid per 1 g consumed glucose, respectively. This confirms that lactic acid yields are also strongly dependent on the strain background (Branduardi et al., 2006). Expectedly, the achieved lactic acid titers were low due to the low glucose consumption by Pdc1$^-$/Pdc5$^-$ cells. The strain performance could be improved by adaptive laboratory evolution, as has been shown in the previous studies (Van Maris et al., 2004a; Van Maris et al., 2004b), but this was not the scope of this paper. Production of pyruvate was slightly higher in Ethanol Red derivative and there was no (Ethanol Red) or very low (CEN.PK) production of ethanol (Fig. 4B). Acetate concentration remained more or less at the initial concentration when CEN.PK lactate-producer was examined, revealing no significant production or consumption during the time-course, whereas significant drop in acetate concentration occurred in Ethanol Red strain (Fig. 4B). As for the other metabolites, glycerol and succinate levels were very low not exceeding concentrations of 0.08 g/l in CEN.PK strain and 0.16 g/l in Ethanol Red.
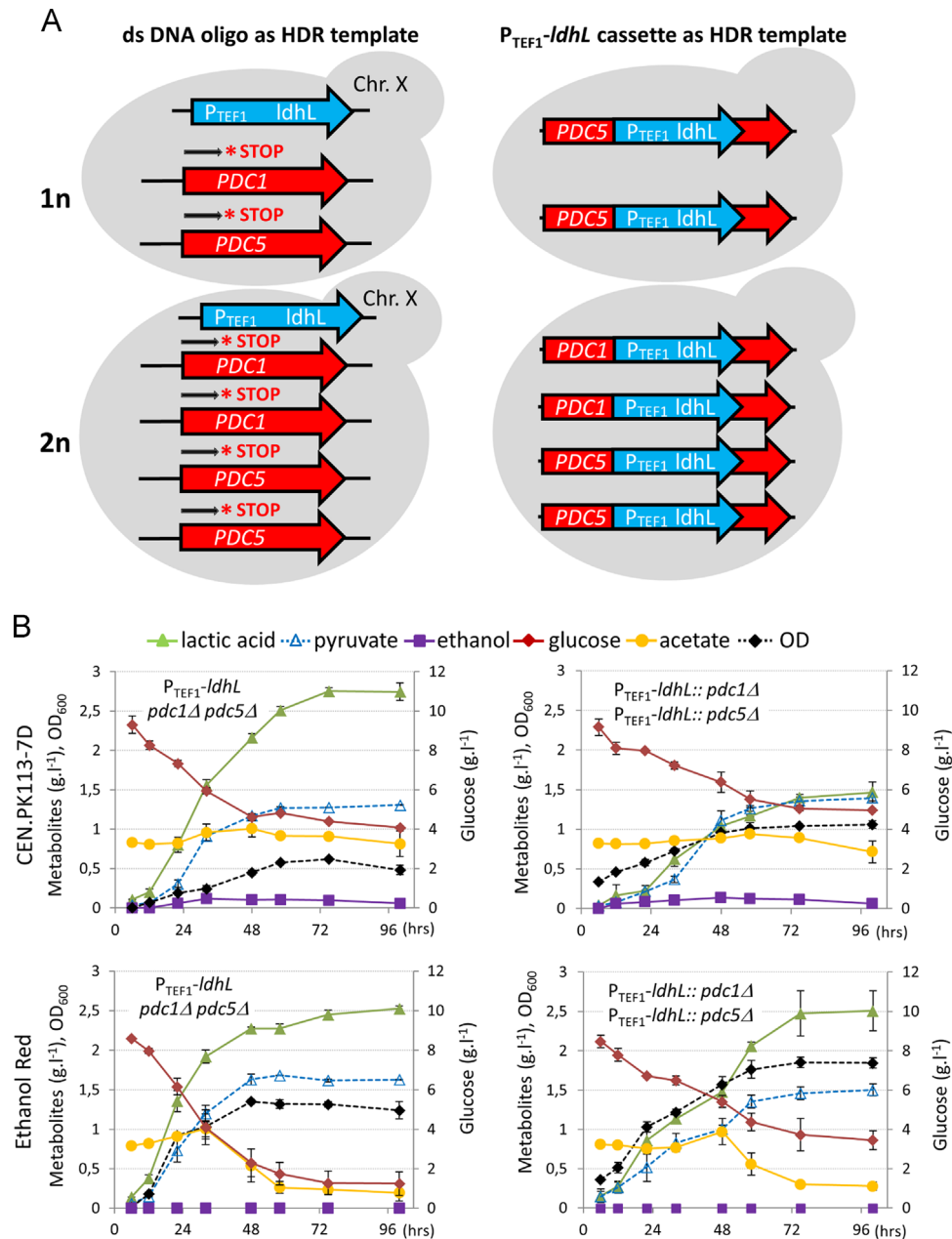
Fig. 4. Construction of lactic acid producing yeast strains. (A) Illustration of one-step Cas9-mediated *PDC1* and *PDC5* gene disruption in strains containing *ldhL* gene inserted in chromosome X, left; and *PDC1* and *PDC5* disruption by P_{TEF1}-*ldhL* expression cassette, right. Haploid strain – 1*n*, diploid strain – 2*n*. (B) Time-course metabolite profile (lactic acid – closed triangles, glucose – closed diamonds, pyruvate – dashed line with open triangles, ethanol – closed squares, acetate – closed circles) and growth properties (OD – dashed line with closed diamonds) of strains engineered for production of lactic acid. Glucose concentration is plotted on the secondary *y* axis. Upper charts represent CEN.PK derived strains. Lower charts represent Ethanol Red derived strains. Genotypes of the strains are in the left corner of each chart, P_{TEF1}-*ldhL pdc1Δ pdc5Δ* represents strains containing *ldhL* gene inserted in chromosome X and non-sense mutations in *PDC1* and *PDC5* genes, P_{TEF1}-*ldhL: : pdc1Δ* P_{TEF1}-*ldhL: : pdc5Δ* represents strains with disruption of *PDC1* and *PDC5* by P_{TEF1}-*ldhL* expression cassette. Samples from three biological replicates were taken at marked time points. Error bars represent standard deviation (SD, *N*=3).

3.7. Simultaneous disruption of pyruvate decarboxylase genes PDC1 and PDC5 and knock-in of lactate dehydrogenase in an industrial strain

To demonstrate one-step generation of lactic acid producing strains we constructed *ldhL* expression cassette flanked with 40-bp homologous region designed to disrupt *PDC1* and *PDC5* genes and transformed the cassette together with gRNA plasmid to Cas9-expressing cells (Fig. 4A). Mutant strains verified for presence of *ldhL* gene inside *PDC1* and *PDC5* coding sequence, were cultivated as described before and supernatant analyzed by HPLC. Since the growth of both CEN.PK and Ethanol Red lactate-producing

derivatives was affected even more when compared to their two-step procedure created counterparts (Section 3.6), cells were inoculated to higher initial OD=0.5. The growth defect might be caused by lower expression level of *ldhL* and NADH accumulation. The expression level might have been influenced by insertion of the *ldhL* gene inside the coding region of *PDC* genes, instead of insertion to a verified insertion site performed before (Section 3.6), providing high level of expression of a heterologous gene (Jensen et al., 2014). The slow growth was accompanied with slow glucose consumption and also lower lactic acid production in CEN.PK strain resulting in titer of 1.47 ± 0.13 g/l when only 3.6 ± 0.19 g of glucose was consumed. In case of Ethanol Red, the growth and

glucose consumption was also slower. In 100 h 5.1 ± 0.65 g/l of glucose was consumed, while 2.5 ± 0.3 g/l lactate was secreted (i.e. yield of 0.49 ± 0.03 g lactic acid per 1 g glucose) (Fig. 4B).

4. Conclusions

In this study, we demonstrated the suitability of CRISPR–Cas9 for genetic manipulation of unrelated prototrophic polyploid industrial yeast strains. CRISPR–Cas9 allowed highly efficient single-step gene disruption in diploid industrial strains. We also illustrated that Cas9-mediated gene targeting can be combined with simultaneous gene knock-in into the target site in all tested strains. Decreased expression of Cas9 and increasing the amount of a gRNA vector and a DNA repair template is beneficial towards increasing disruption efficiency and overall yield of positive transformants. Moreover, we showed the simultaneous inactivation of two conditionally essential genes (*PDC1* and *PDC5*), each present in two alleles, in the industrial Ethanol Red strain. When Cas9 and gRNA are introduced on episomal vectors, the latter can be cured from the yeast, effectively resulting in marker-free permanent modification. The described CRISPR–Cas9 method is easily applicable and universal tool, which can be quickly adapted and tested for any genome editing change in a *S. cerevisiae* strain of choice and greatly facilitates the metabolic engineering of industrial strains.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.meteno.2015.03.001.

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