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Published in:
Metabolic Engineering Communications

Link to article, DOI:
10.1016/j.meteno.2015.09.001

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Strucko, T., Magdenoska, O., & Mortensen, U. H. (2015). Benchmarking two commonly used Saccharomyces cerevisiae strains for heterologous vanillin--glucoside production. Metabolic Engineering Communications, 2, 99-108. DOI: 10.1016/j.meteno.2015.09.001
Benchmarking two commonly used *Saccharomyces cerevisiae* strains for heterologous vanillin-β-glucoside production

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**Abstract**

The yeast *Saccharomyces cerevisiae* is a widely used eukaryotic model organism and a key cell factory for production of biofuels and wide range of chemicals. From the broad palette of available yeast strains, the most popular are those derived from laboratory strain S288c and the industrially relevant CEN.PK strain series. Importantly, in recent years these two strains have been subjected to comparative “-omics” analyzes pointing out significant genotypic and phenotypic differences. It is therefore possible that the two strains differ significantly with respect to their potential as cell factories for production of specific compounds. To examine this possibility, we have reconstructed a *de novo* vanillin-β-glucoside pathway in an identical manner in S288c and CEN.PK strains. Characterization of the two resulting strains in two standard conditions revealed that the S288c background strain produced up to 10-fold higher amounts of vanillin-β-glucoside compared to CEN.PK. This study demonstrates that yeast strain background may play a major role in the outcome of newly developed cell factories for production of a given product.

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1. Introduction

The recent achievements in the field of systems biology and metabolic engineering combined with a wide array of molecular biology tools has established the yeast *Saccharomyces cerevisiae* as a key cell factory for heterologous production of scientifically and industrially relevant products. The latter comprise a large variety of products ranging from low-value bulk chemicals and biofuels (e.g., ethanol) to food additives (e.g., flavors and colorants) and high value pharmaceuticals (e.g., recombinant proteins) (Kim et al., 2012; Hong and Nielsen, 2012). Today, a range of different *S. cerevisiae* strain backgrounds are available for the yeast community of which BY (S288c), W303 and CEN.PK are the most frequently used (van Dijken et al., 2000). The variety of strains has been developed by different laboratories to suit a range of diverse research goals within different disciplines such as genetics, physiology and biochemistry. For example, CEN.PK strain (Entian and Kötter, 2007), is a popular platform for physiological as well as metabolic engineering studies whereas S288c, the first eukaryote to be sequenced (Goffeau et al., 1996), was mainly used for genetic studies, but has in recent years been increasingly used as an alternative platform for metabolic engineering experiments (Kim et al., 2012; Hong and Nielsen, 2012). Specifically, recent surveys show that over the past ten years the two strains were used in more than 50% of the analyzes with CEN.PK series being the most popular (approx. 37%-CEN.PK vs. 24%-S288c and its derivatives) (Kim et al., 2012; Hong and Nielsen, 2012). The importance of the two strains as cell factories is further substantiated by an extensive multi-laboratory efforts which were made for systematic comparisons of S288c and CEN.PK (Rogowska-Wrzesinska et al., 2001; Canelas et al., 2010). Genetic differences of these strains were revealed first by microarray studies (Daran-Lapujade et al., 2003) and more recently genotype to phenotype relation was investigated after whole-genome sequencing of the CEN.PK113-7D strain (Otero et al., 2010; Nijkamp et al., 2012). For example, the sequencing comparison studies revealed that 83 genes were absent in CEN.PK relative to S288c (Nijkamp et al., 2012) and that more than 22,000 single nucleotide polymorphisms (SNPs) exist between the two strains. 13,000 of the SNPs are distributed in 1843 open reading frames (ORFs) and the activity of a large number of proteins may therefore differ in the two strains; especially since 35% of these SNPs result in amino-acid residue substitutions. The remaining 9000 SNPs, which are mainly distributed in the intergenic regions, may potentially impact gene expression and thereby protein levels in the two strains. The fact that the highest enrichment of SNPs was detected in genes involved in carboxylic acid, organic acid, and carbohydrate metabolism, as well as, in nitrogen, amino acid, lipid and aromatic compound metabolism suggests that the basic metabolism in the two strain background may be quite different (Otero et al., 2010). Importantly, based on the combined “-omics” analyzes, several phenotypic differences between the two strains were assigned to

**Keywords:** Yeast, Cell factory, Strain choice, Heterologous production, Vanillin-glucoside, Shikimate pathway

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http://dx.doi.org/10.1016/j.meteno.2015.09.001

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these mutations, e.g., differences in galactose uptake and ergosterol biosynthesis, etc. in two strains (Otero et al., 2010).

The fact that the two main S. cerevisiae backgrounds for construction of cell factories are genetically/phenotypically quite different raises the possibility that heterologous production of a given compound in CEN.PK and in S288c may result in significantly different production modes revealed major differences in the VG production.

Characterizations of both VG cell factories in two different cultivation scenarios where the optimal genetic background for cell factory construction can be successfully selected based on a systems biology model for yeast cell factories.

2. Materials and methods

2.1. DNA cloning procedures

The DNA fragments used for vector construction were amplified by PCR with PfuX7 polymerase developed by Nørholm (2010) using the primers listed in the Table 1. Molecular coning was done by uracil-specific excision reagent (USER™) as previously described in (Geu-Flores et al., 2007; Nour-Eldin et al., 2006). Final constructs were validated by sequencing (StarSEQ™, Germany). The genes constituting the de novo VG pathway (1-3DSD, 2-ACAR, 3-EntD, 4-HsOMT, and 5-UGT) and a set of bidirectional promoter (pPGK1/pTEF1) were amplified by PCR from the appropriate vector templates (see Table 2). The pathway genes and promoters were assembled into vectors designed to integrate on chromosome XII (Mikkelsen et al., 2012). A total of three plasmids were produced: pXII1-23 (pPGK1::ACAR; pTEF1::EntD), pXII2-54 (pPGK1::UGT; pTEF1::HsOMT) and pXII5-01 (pTEF1::3DSD), see Fig. S1. For a full list of plasmids used or constructed in this work see Table 2.

2.2. Strain construction

The genotype and source of the strains used in this study is given in the Table 3. Two different background strains CEN.PK113-11C and X2180-1A (isogenic to S288c (Mortimer and Johnston, 1986)) were used as hosts for reconstruction of the de novo vanillin-β-glucoside (VG) pathway. All yeast constructs were generated by high efficiency transformation method (lithium acetate/polyethylene glycol/single carrier DNA) previously described by Getz et al. (1992).

To construct two yeast strains containing the VG pathway, vectors pXII1-23, pXII2-54 and pXII5-01 harboring the following genes (ACAR and EntD), (UGT and HsOMT) and (3DSD), respectively, were digested with NotI restriction enzyme (Fermentas-Thermo Fisher Scientific) and gel-purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Lifesciences). The individual gene

Fig. 1. De novo vanillin-β-glucoside pathway in S. cerevisiae assembled by Hansen et al. (2009). Colored boxes represent metabolites of the pathway: PAC – protocatechuic acid, PAL – protocatechuic aldehyde, VAC – vanillic acid, VAN – vanillin, VG – vanillin-glucoside. Black thick arrows show enzymatic reactions by heterologous enzymes: 3DSD – 3-dehydroshikimate dehydratase (Podospora pauciseta), ACAR – aromatic carbonylic acid reductase (Neurospora sp.), EntD – phosphopantetheine transferase (Escherichia coli), HsOMT – O-methyltransferase (Homo sapiens) and UGT – UDP-glucosyltransferase (Arabidopsis thaliana). Gray arrows and boxes (left side of the picture) represent simplified shikimate biosynthetic pathway and native parts of yeast metabolism: PPP – pentose phosphate pathway, DAHP – 3-deoxy-o-arabinoheptulosonate 7-phosphate, 3-DHS – 3-dehydroshikimic acid, Trp – tryptophan, Phe – phenylalanine, Tyr – tyrosine. Key enzymes of the shikimate pathway: AR03 and AR04 – DAHP synthase isoenzymes and AR01 – pentafunctional enzyme catalyzing DAHP conversion to chorismate.
targeting substrates were transformed iteratively into both yeast strain backgrounds in three consecutive transformations. To prevent undesired production of toxic intermediates especially protocatechuic aldehyde (PAL), the 3DSD gene was cloned at the latest step. After each round of transformation the URA3 marker was eliminated by direct repeat recombination and counter-selection step. After each round of transformation the URA3 marker was recycled.

Complete gene deletions of BGL1 and ADH6 were achieved using method described by Güldener et al. (1996). PCR fragment carryingloxP-KanMX-loxP flanked by 40nt long segment homologous to sequences of Up- and Down-stream of the appropriate open reading frame (ORF) to be deleted were amplified form plasmid pLG6. The KanMX marker was excised by expressing Cre recombinase from the vector pSH47 (Güldener et al., 1996). All gene targeting events were validated by diagnostic PCR using specifically designed primer pairs (see Table 1).

To eliminate any auxotrophies, two engineered strains S-VG-aux and C-VG-aux were sexually crossed to S288c and CEN.PK110-based. Genetic cross and selection procedures were performed as described in (Treco and Winston, 2008). Schematic flowchart representing the strain construction is depicted in Fig. S2.

### 2.3 Media

For cloning purposes lysogeny broth (LB) (Bertani, 1951) supplemented with 100 mg/L of ampicillin (Sigma-Aldrich) was used for growing of Escherichia coli DH5α. All media used for genetic manipulations of yeast were prepared as previously described by Sherman et al. (1986), with minor modifications of synthetic medium where leucine concentration was doubled to 60 mg/L. All yeast transformants with gene integrations were selected on synthetic complete media lacking uracil (SC-ura). For subsequent round of transformations.

### Table 1

List of the primers used in this study. All sequences are presented in 5′–3′ direction, standard capital letter are gene specific sequences, bold letters represent USER specific tails, regular underline letters represent targeting sequences for appropriate gene deletions. Italic letters represent translational enhancer sequence (Nakagawa et al., 2008; Cavener and Ray, 1991).

| Name                  | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| Primers for cloning purposes                                         |                                                                          |
| PGR_ R                | ACCCGTTGAUGCCGCTTTGTTTATTTTGGTG                                           |
| TEF_F+                | CGTGGAGUCCCGCACACAACTACTACGCTC                                           |
| TEF_R+                | ACCTAGTCGCCCTAGTCTATTGCGATCTTGTG                                        |
| DSD_F+                | AGGCGATACGIAAAATAATGGCTACATAAAGGCAA                                       |
| DSD_R+                | CACCGGAUTTTAGCGACATTCGCAAGCC                                             |
| ACAR_F                | ATCAACGCGGAAAATGGCTGATAGTAAAAATACACG                                    |
| ACAR_R               | CACCGGAATTTAACATGCAATGCAATGCAAGCC                                         |
| hOMT_F                | ATCAACGCGGAAAAATCATCGACAGCAACACAG                                       |
| hOMT_R+              | CGTGCGAATACAGGCACACCAGCACAGAC                                            |
| PPT1_F+               | ATTTTTGGTTTACTACCTTTCTCTGTTTCTATTACCAAACATCTTACGCGTGAGGTC               |
| PPT1_R+               | CATTAGAAATTTAGCTTAATAGGGGACTGACAGGCGTTAGTCAGTACATATACCTCAA              |
| UGT1_F                | GAGGAAAAATTTAGCAAACACACACAAAGGCAAAGAACATCTTACGCGTGAGGTC                 |
| UGT1_R               | GTTAAAAGAGAGGAGCGTACTATTATCAGAGCGTCTAGTATATACCTCAA                       |
| Deletion primers       |                                                                          |
| BGL1_del_F            | CTGGCAAGAAGAACCACAAAT                                                     |
| BGL1_del_R            | CGAAGAAGCGCTGACATTC                                                      |
| ADH6_del_F            | CCGCGAGATGTTATGCTGTTTAT                                                  |
| ADH6_del_R            | CTGAGTAAACTCTCTTCTCTGACAGT                                               |
| Verification primers   |                                                                          |
| XII-1-up-out-sq        | TCATCTCTCTCTTTCTTGTGTTAC                                                 |
| XII-2-up-out-sq        | AGTTGGAAGAACAGGATAGGTT                                                   |
| XII-3-up-out-sq        | GTTGTGGTTTFFFTTCTGGG                                                     |
| C1_TADH1_F            | GCGATATCAGCACTAAXXACG                                                   |
| BGL1_VF               | CGGTCCTTCAATCTTACGCAITTC                                                 |
| BGL1_VR               | CAAAGGGGTTGTCTTATGTTAT                                                  |
| ADH6_VF               | CATGTCCTTGCCACAGACATGCA                                                  |
| ADH6_VR               | AAAATCTGTCGAAGGCTAGGCT                                                    |
| Primers for sequencing  |                                                                          |
| S1_TEF_F              | TTGGGTGATAGCTCTTCTGTTG                                                    |
| S1_PGR_R              | GAGGAAAAATTTAGCAAACACACACAAAGGCAAAGAACATCTTACGCGTGAGGTC                 |
| S2_ACAR_F             | TAGCAGTATTAGCTGCTGACGT                                                   |
| S2_ACAR_R            | GCGATATCAGCACTAAXXACG                                                   |
| YF19                  | GCCTACAGAAAGAAGATCGAATCCAGATACGCA                                       |
| YF21                  | GACCTACAGAAAGAAGATCGAATCCAGATACGCA                                       |

### Table 2

List of the plasmids used and constructed in this study.

| Name     | Genetic element | Reference |
|----------|-----------------|-----------|
| pXII1-23 | pPGK1::ACAR, pTEF1::EntD | This study |
| pXII1-54 | pPGK1::UGT, pTEF1::HsOMT | This study |
| pXII5-01 | pTEF1::3DSD       | This study |
| pXII1-23 |             |           |
| pXII1-54 |             |           |
| pXII5-01 |             |           |
| pS5-2G  | pPGK1::pTEF1     | Partow et al. (2010) |
| pH500   | 3DSD            | Hansen et al. (2009) |
| pH674   | ACAR            | Hansen et al. (2009) |
| pH589   | EntD            | Hansen et al. (2009) |
| pH543   | HsOMT           | Hansen et al. (2009) |
| pH665   | UGT             | Hansen et al. (2009) |
| pUG6    | KanMX           | Güldener et al. (1996) |
| pXII6    | Cre recombinase  | Güldener et al. (1996) |
Table 3
List of the yeast strains used in this study.

| Name                  | Genotype                                                                 | Reference                                                                 |
|-----------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|
| X2180-1A (ura3)       | MATa SUC2 gal2 mal mel flo1 fl08-1 hap1 ho his1 bio6                      | Public domain (Martiner and Johnston, 1986)                                |
| S288c                 | MATa SUC2 gal2 mal mel fl01 f108-1 hap1 ho bio1 bio6                      | ATCC 204508                                                               |
| CEN.PK113-11C         | MATa MAL2-8C SUC2 ura3-2 his3a                                           | Peter Kötter                                                               |
| CEN.PK110-16D         | MATa MAL2-8C SUC2 tryp-1-39                                              | This study                                                                 |
| C-VG-aux              | MATa MAL2-8C SUC2 ura3-2 his3a                                           | This study                                                                 |
| S-VG-aux              | MATa MAL2-8C SUC2 ura3-2 his3a                                           | This study                                                                 |
| S-VG                  | MATa MAL2-8C SUC2 ura3-2 his3a                                           | This study                                                                 |

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URA3 marker was recycled through direct repeat recombinant and selected on synthetic complete media containing 30 mg/L uracil and 740 mg/L 5-fluoroorotic acid (5-FOA) (Sigma-Aldrich).

Yeast transformants with necessary gene deletions were selected on Yeast Extract Peptone Dextrose (YPD) plates supplemented with 200 mg/L of G418 (Sigma-Aldrich) (Guldener et al., 1996). The medium composition is as follows: 10 g/L of yeast extract, 20 g/L of peptone and 20 g/L of glucose and 20 g/L of agar.

A defined minimal medium previously described by Verduyn et al. (1992) with glucose as a carbon source was used for S. cerevisiae cultivations in batch and chemostat cultures. For batch cultivations the medium was supplemented with 30 g/L of glucose (Canelas et al., 2010), whereas for feed media for continuous cultivations contained 7.5 g/L of glucose. The medium was composed of: 7.5 g/L (NH₄)₂SO₄, 3 g/L KH₂PO₄, 0.75 g/L MgSO₄·7H₂O, 1.5 mL trace metal solution, 1.5 mL/L vitamins solution, 0.05 mL/L anti-foam 204 (Sigma-Aldrich). Trace metal solution contains 3 g/L FeSO₄·7H₂O, 4.5 g/L ZnSO₄·7H₂O, 4.5 g/L CaCl₂·6H₂O, 0.84 g/L MnCl₂·2H₂O, 0.3 g/L CoCl₂·6H₂O, 0.3 g/L CuSO₄·5H₂O, 0.4 g/L NaNO₃·2H₂O, 1 g/L H₃BO₃, 0.1 g/L KI and 15 g/L Na₃EDTA·2H₂O. Vitamins solution includes 50 mg/L b-vitamin, 200 mg/L para-amino benzoic acid, 1.0 g/L nicotinic acid, 1.0 g/L Ca-pantothenate, 1.0 g/L pyridoxine HCl, 1.0 g/L thiamine HCl and 25 mg/L pinitol. Glucose was autoclaved separately and vitamins solutions were sterile filtered (pore size 0.2 μm Ministart®-Plus, Sartorius AG, Germany) and added after autoclaving.

2.4. Batch and chemostat cultivations

For each biological replica separate colonies of engineered yeast strains from YPD plate were inoculated to the 0.5 L shake flask with 50 mL of the previously described minimal medium (pH 6.5). Pre-cultures were incubated in an orbital shaker set to 150 rpm at 30 °C until mid-exponential phase OD₆₀₀ = 5 and directly used for inoculation. In this experiment, batch cultivations were performed in duplicates and continuous cultures – in triplicates.

Batch cultivations were performed under aerobic conditions in 1 L fermenters equipped with continuous data acquisition (Sartorius, B. Braun Biotech International, GmbH, Melsungen, Germany) with a working volume of 1 L. Fermenters were inoculated with initial OD₆₀₀ = 0.05. To ensure aeration a stirrer speed was 600 rpm and airflow rate to 1.0 v.v.m. (60 L/h). The temperature was maintained at 30 °C during the cultivation and pH = 5.0 level was controlled by automatic addition of 2 M NaOH or 2 M H₂SO₄. The batch cultures were sampled in regular intervals through both glucose and ethanol growth phases. Glucose-limited chemostat cultures were grown in the same conditions as previously described for batch cultivations. Chemostats were initiated as batch cultivations with starting glucose concentration of 15 g/L and OD₆₀₀ = 0.05 and switched to continuous mode in early exponential phase. Minimal medium was fed at a constant dilution rate of 0.1 h⁻¹ (Canelas et al., 2010). The working volume of 1 L was kept constant by a level based outlet. Samples were taken after at least five residence times (50 h) of constant biomass and carbon dioxide concentration readings.

In both cultivation experiments exhaust gas composition was constantly monitored by off gas analyzer (1311 Fast response triple gas, Innova) combined with Mass Spectrometer (Prima Pro Process MS, Thermo Fisher Scientific, Winsford UK). No elevated ethanol concentration in the exhaust was detected.

The biomass concentration was monitored by measuring both optical density at 600 nm wavelength (OD₆₀₀) and cell dry weight (DW) in the cultivation broth. DW was estimated using a UV mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan), biomass samples were diluted with distilled water to achieve OD₆₀₀ reading within 0.1–0.4 range. DW measurements were performed using polyethersulfone (PES) filters with a pore size of 0.45 μm Montamīl™ (Membrane Solutions, LLC). The filters were pre-dried in a microwave oven at 150 W and weighed. A known volume of cultivation broth (5 mL) was filtered and then washed with approx. 15 mL of distilled water. Finally, the filters with biomass were dried in the microwave oven at 150 W and cell DW was determined (Olsson and Nielsen, 1997).

2.5. Extracellular metabolite measurements

Extracellular metabolites were determined by high performance liquid chromatography (HPLC) analysis. Two distinct HPLC methods were applied for analysis of different groups of extracellular metabolites.

The submerged cultivation samples for yeast primary metabolites were centrifuged at 12,000 g for 2 min, supernatant was transferred to a new tube and stored at –20 °C until further analysis. Glucose, ethanol, glycerol, pyruvate, succinate and acetate were determined by high performance liquid chromatography (HPLC) analysis using an Aminex HPX-87H ion-exclusion column (Bio-Rad Laboratories, Hercules, CA). The column temperature was kept at 60 °C and the elution was performed using 5 mM H₂SO₄ with constant flow rate of 0.6 mL min⁻¹. Metabolite detection was performed by a RI-101 differential refractometer detector (Shodex) and an UVD340U absorbance detector (Dionex) set at 210 nm.

Samples for quantification of vanillin-β-glucoside and its pathway metabolites were prepared as follows: 1 mL of cultivation broth and 1 mL of 96% ETOH was carefully mixed by vortex and centrifuged at 12,000 g for 2 min, supernatant was transferred to a
new tube and stored at −20 °C until further analysis. Extracellular vanillin-β-glucoside (VG), vanillin (VAN), protocatechuic acid (PAC), protocatechuic aldehyde (PAL) and vanillic acid (VAC) were quantified using Agilent 1100 series equipment with a Synergi Polar-RP 150 × 2 mm 4 μm column (Phenomenex). A gradient of acetonitrile (ACN) with 1% tetra-fluoroacetic acid (TFA) and water with 1% TFA at a constant flow rate of 0.5 mL/min was used as mobile phase. The elution profile was as follows: 5% ACN – 1 min, 5% ACN to 30% ACN – 8 min, 30% ACN to 100% ACN – 1 min, 100% ACN – 1 min, 100% ACN to 5% ACN – 3 min. The column was kept at 40 °C and metabolite detection was performed using a UV diode-array detector set to 230 and 280 nm.

2.6. Intracellular metabolite measurements

Samples for intracellular co-factor metabolites were taken during steady state conditions at the end of continuous cultivation. The samples were quenched and extracted as previously described by Villas-Bôas et al. (2005); 5 mL of culture broth was sprayed into pre-cooled (−40 °C) falcon tube containing 20 mL of 60% methanol, spun down for 2 min at 5000 g in precooled centrifuge (−10 °C) and extracted using boiling ethanol method (Villas-Bôas et al., 2005) followed by evaporation under nitrogen. The samples were re-dissolved in 300 μL eluent A (10 mM tributylamine and 10 mM acetic acid). The analysis was carried out on an Agilent 1290 binary UHPLC system coupled with an Agilent 6460 triple quadrupole mass spectrometer (Torrance, CA, USA). The MS was operated in negative ion and multiple reactions monitoring mode. Separation of 0.5 μL samples was performed by ion-pair chromatography, as described in detail in Magdenoska et al. (2013) using 10 mM tributylamine as ion pair reagent. The gradient used was: 0–12 min 0–50% B, 12–12.5 min 50–100% B, 12.5–14 min 100% B, 14–14.5 min 100–0% B, 14.5–19.5 min 0% B. External standard calibration method was used for quantification. The calibration curves were constructed by preparing calibration solutions ranging from 1 to 100 μg/mL for ATP and 0.3–25 μg/mL for NADPH and UDP-glucose. Both the quenched extracts and the calibration solutions were spiked with 60 μL mixture containing 150 μg/mL [U-13C] ATP. The quantification was carried out using Mass Hunter Quantitative analysis software (version B.06.00).

3. Results

3.1. De novo pathway reconstruction in CEN.PK and S288C backgrounds

To compare CEN.PK and S288C for their ability to produce VG in a fair manner, it was necessary to insert all genes identically in both strain backgrounds. In the original VG producing strain, the individual genes of the pathway were inserted by repeated integrations into the TP11 promoter region in an S288C based strain (Hansen et al., 2009). Consequently, the gene order is ill-defined and not easy to reconstruct in CEN.PK. We therefore introduced the VG pathway in the two strain backgrounds, S288C (isogenic isolate X2180-1A (Mortimer and Johnstone, 1986)) and CEN.PK113-11C (Entian and Kötter, 2007) in a defined manner. Specifically, the five genes used by Hansen et al. (2009) for VG production were integrated into three sites located on chromosome XII (see Fig. 2), which are part of a defined gene expression platform we have previously established (Mikkelsen et al., 2012). Importantly, prior to integration, we compared the up- and downstream sequences at these integration sites in CEN.PK and S288C and found that they differ by only a single SNP, a C in CEN.PK and G in S288C, which is present in the upstream targeting sequence of the XII-5 site. The five genes are therefore inserted into a genetic context, which is essentially identical. Additionally, two genes ADH6 and BGL1 were deleted in both strains to minimize unwanted side reactions with VG pathway metabolites. Finally, for both genetic backgrounds, auxotrophic markers were eliminated by sexual back crossing to wild-type variants of the two strain backgrounds to produce two prototrophic VG producing strains, C-VG (CEN.PK based) and S-VG (S288C based), which we used for further analysis.

3.2. Physiological characterization of vanillin-β-glucoside producing strains during batch and continuous fermentations

Before evaluating the VG production ability of the two strain backgrounds, we first assessed whether VG production affected the overall physiology of the C-VG and S-VG strains. Hence, they were grown in batch and as continuous cultures in well-controlled bioreactors under standard laboratory cultivation conditions (Canelas et al., 2010). Like for wild-type strains, the overall growth profiles exhibited by the S-VG and C-VG strains in batch reactors were composed by two growth phases. One initial growth phase where all glucose was fermented (GF phase); followed, after the diauxic shift, by a second growth phase where all accumulated ethanol was respiro-fermented (ER phase). In contrast, C-VG and S-VG grew slower than the corresponding wild-type strains and both strains displayed identical growth rates on glucose (Table 4) higher (14%) in C-VG as compared to S-VG, but in our experiment this difference was not significant (p > 0.33). A similar difference has previously been observed for the parental S288C and CEN.PK strains grown at the same conditions (van Dijken et al., 2000; Canelas et al., 2010). It has previously been shown that CEN.PK grows faster than S288C at these conditions, μmax ≈ 0.4 h⁻¹ vs. μmax ≈ 0.3 h⁻¹, respectively (Canelas et al., 2010; Otero et al., 2010). In contrast, C-VG and S-VG grew slower than the corresponding wild-type strains and both strains displayed identical growth rates on glucose (μmax ≈ 0.2 h⁻¹). Further analysis of the cultivation broth obtained at different time points during growth, showed that the production profiles of five primary metabolites (ethanol, pyruvate, succinate, glyceral, and acetate) in the central carbon metabolism of S-VG and C-VG were similar to what has previously been observed with the corresponding wild-type strains (Fig S3). Among the remaining parameters – rCyc, rFta, and rCOX, only the latter varied between the two strains as it was approx. three-fold higher in the S-VG (p < 0.002). Finally, we also note that S-VG displayed a growth efficiency during the ER phase which was nearly twice as long with S-VG (23 h) than with C-VG (12 h) partly due to a much longer delay from the diauxic shift to exponential growth is resumed. This deficiency has also been previously reported for wild-type S288C (Otero et al., 2010).

In chemostats, steady-state conditions with constant production of biomass as well as stable readings of carbon dioxide and oxygen by the off-gas analyzer where obtained for both strain
ethanol respiratory growth phase. In the original VG producing strain constructed by Hansen et al. (2009), various levels of intermediates were observed due to the production of VG and its intermediates of the two strains (Fig. 5C). As a result, TCV was 45% higher with S-VG as compared to C-VG. Yields and productivities on glucose during exponential growth and during steady state condition are presented in the Table S1). The VG yields were almost identical during the different growth phases throughout the entire batch cultivation (Fig. 5A and B). The VG yields were almost identical with the two strains during the GF phase (Fig. 5C). The total carbon ending up in VG pathway metabolites (TCV) is 14% lower in C-VG as compared to S-VG. This effect is mainly due to less accumulated PAC in C-VG (37% less in C-VG, p < 0.05). In contrast, with S-VG, a two-fold higher (p < 0.05) VG yield was generated during the ER phase than with C-VG despite that the amounts of intermediates accumulating at the end of the phase were similar in both strains (see Fig. 5C). As a result, TCV was 45% higher with S-VG as compared to C-VG. Yields and productivities on glucose during exponential growth and during steady state condition are represented in the Table S1).

To further understand why VG yields were higher with S-VG than with C-VG, we determined whether production of VG and

| Cultivation mode | Batch | Chemostat |
|------------------|-------|-----------|
| Strain           | S-VG  | C-VG      |
| Glucose µ, h⁻¹   | 0.209 ± 0.002 | 0.199 ± 0.002 |
| Ethanol µ, h⁻¹   | 0.05 ± 0.01   | 0.08 ± 0.01   |
| Yₓ (C-mol/C-mol) | 0.133 ± 0.014 | 0.151 ± 0.001 |
| rGlc             | 60.7 ± 8.0    | 49.9 ± 0.60   |
| rEth             | 25.9 ± 3.1    | 20.9 ± 1.00   |
| rGly             | 4.05 ± 0.05   | 1.40 ± 0.13   |

* The dilution rate used in this study.

The dilution rate used in this study.

 backgrounds. For the C-VG strain this was achieved in less than 5 residence times (50 h) after feeding was initiated, whereas for the S-VG strain it took more than 8 residence times (80 h), see Fig S4. Importantly, at a dilution rate of 0.1 h⁻¹, both strains propagated exclusively by glucose respiratory metabolism (Van Hoek, 1998) as no production of ethanol, glycerol and organic acids was observed (see Table 4). The C-VG strain produced significantly more (seven percent, p < 0.01) biomass as compared to S-VG, on the contrary, the specific glucose uptake rate was approx. seven % higher (p < 0.01) in S-VG strain.

3.3. High VG yields with S288c are generated during its prolonged ethanol respiratory growth phase

VG production in the S-VG and C-VG strains was initially compared at 45 h after both cultures had reached stationary phase. In the original VG producing strain constructed by Hansen et al. (2009), various levels of intermediates were observed due to an unbalanced heterologous VG pathway (Hansen et al., 2009), (Brochado et al., 2010). In agreement with this, analyzes of the extracellular metabolite levels in the cultivation broth showed that PAC, PAL, VAC, and VAN accumulated in both S-VG and C-VG in batch cultivation. The yields of each of the intermediates were similar in the two strains (Fig. 4) with PAC being far the most prominent metabolite. In fact, PAC accounted for ~70% and 75% of the carbon ending up as intermediates in C-VG and S-VG strains, respectively. However, when the two strains were analyzed for VG production, we surprisingly observed that twice as much VG was produced with the S-VG strain than with the C-VG strain (p < 0.05). This finding prompted us to determine and compare the production of VG and its intermediates of the two strains during the different growth phases throughout the entire batch cultivation (Fig. 5A and B). The VG yields were almost identical with the two strains during the GF phase (Fig. 5C). The total carbon ending up in VG pathway metabolites (TCV) is 14% lower in C-VG as compared to S-VG. This effect is mainly due to less accumulated PAC in C-VG (37% less in C-VG, p < 0.05). In contrast, with S-VG, a two-fold higher (p < 0.05) VG yield was generated during the ER phase than with C-VG despite that the amounts of intermediates accumulating at the end of the phase were similar in both strains (see Fig. 5C). As a result, TCV was 45% higher with S-VG as compared to C-VG. Yields and productivities on glucose during exponential growth and during steady state condition are represented in the Table S1).
VG-intermediates was proportional to biomass during all time-points. Based on this analysis, we observed for both strains that the efficiencies of TCV, VAC and VG production were higher in the ER phase as compared to the GF phase. In contrast, for both strains PAC was produced with equal efficiency in the two phases Fig. 6. After the diauxic shift, C-VG quickly entered a new state where these metabolites were produced in amounts proportional to biomass. With S-VG such states are also achieved for TCV, PAC and VAC although, for TCV and VAC, more time was required for these states to be reached in this strain as compared to C-VG. In contrast, with S-VG a state where VG was produced proportionally to biomass was never achieved as the VG production efficiency increased during the entire phase, see Fig. 6D.

3.4. Steady-state VG production at glucose limitation is higher in S288c than in CEN.PK

Next, we investigate VG production in continuous cultures where we could obtain strictly glucose respiratory conditions for both strains, see above. Dramatically, this analysis revealed that the amounts of TCV, VAC and VG production were significantly increased to levels four- and ten-fold higher with S-VG than with the C-VG strain \( (p<9E-9, p<2E-10, \text{respectively}) \), see Fig. 7. When the levels of intermediates were inspected, we observed that with S-VG, the majority (68%) of the TCV ended up in VG, whereas the rest of the carbon ended up in PAC (18%) and in VAC (13%). In contrast, VG constituted only 20% of the TCV in the C-VG strain. The remaining carbon was mainly ending up in VAC (approx. 70%) and only little (less than 5%) in PAC.

Four co-factors, ATP, NADPH, SAM and UDP-Glc, are used for formation of VG. In the paper by Canelas et al. (2010) where “-omics” data for the two background strains were compared, the levels of three of these cofactors (ATP, NADPH and UDP-Glc) were measured. We were able to quantify ATP and therefore determined the concentrations of this metabolite in C-VG and S-VG to investigate whether ATP levels were altered due to the presence of the VG pathway. Analysis of samples obtained at steady state showed that concentrations of ATP were 10.6 \( \mu \text{mol/g DW} \) and 8.5 \( \mu \text{mol/g DW} \) for C-VG and S-VG, respectively. These numbers are somewhat higher than the corresponding numbers reported by Canelas et al. and this may be due to lab to lab differences. Importantly, the relative ATP levels of the two strains (C-VG to S-VG) is 1.2 in our experiment as well as in theirs (Canelas et al., 2010). This indicates that the presence of the VG pathway does not change ATP levels in the two strains despite that 10-fold more VG is formed in S-VG as compared in to C-VG. Due to the absence of internal standards for NADPH and UDP-Glc, we were not able to compare our data with those in the literature. However, UDP-Glc
levels were 0.5 μmol/g DW for both strains. These results were also confirmed by a high resolution mass spectrometer, where similar chemical profiles were obtained for both strains. The NADPH levels measured in C-VG and S-VG in our study were close to the limit of detection preventing a comparison between the two strains.

4. Discussion

The importance of *Saccharomyces cerevisiae* in the development of novel cell factories is demonstrated by the large number of industrially relevant substances that can now be produced in this organism (Kim et al., 2012; Hong and Nielsen, 2012; Nevoigt, 2008). Several laboratory yeast strains have been used for this purpose with S288c and CEN.PK being the most popular (Hong and Nielsen, 2012; Canelas et al., 2010). However, despite that the two strains are genetically and physiologically very different (van Dijken et al., 2000; Canelas et al., 2010; Otero et al., 2010; Nijkamp et al., 2012); these differences are rarely used to determine, which strain background should be chosen as a cell factory for de novo production of a given compound. In this study, we therefore investigated whether choice of yeast strain background for production of vanillin-β-glucoside (VG) is an issue that can be advantageously considered. Our finding that heterologous production of VG was dramatically more efficient in an S288c based strain compared to CEN.PK in both batch and continuous cultivations demonstrates that this is indeed the case.

Our physiological characterizations of the C-VG and S-VG strains show that the S-VG strain produces significantly more VG in both batch and continuous cultivations. One explanation could be a higher flux of carbon into the VG pathway in the S-VG strain. In agreement with this, both the first intermediate in the VG pathway (PAC) and TCV accumulate to significantly higher levels with S-VG as compared to with C-VG during both continuous and batch cultivations, see Figs. 4 and 7. For batch cultivation, we note this is true at all time points examined, see Fig. 6.

Carbon for VG production is recruited from the shikimate pathway, which is a part of the aromatic amino acid biosynthesis and, which is well characterized in yeast (Teshiba et al., 1986; Braus, 1991). The pathway is tightly regulated via two 3-deoxy-D-arabinohexulosonate 7-phosphate (DAHP) synthase isoenzymes Aro3 and Aro4, which are feedback inhibited by phenylalanine and
In this context, we note that in the case of CEN.PK derived strains, the flux through the shikimate pathway is increased more than four-fold if feedback inhibition is eliminated (Luttik et al., 2008); and that this feature has been successfully exploited in a metabolic strategy to increase the yield of CEN.PK strains producing the plant flavonoid naringenin, which is derived from phenylalanine and tyrosine (Koopman et al., 2012). Interestingly, two of the genes in the shikimate pathway, ARO1 and ARO3, contain ten SNPs that result in amino acid substitutions. Moreover, a large number of SNPs are distributed in the up- and downstream regions of the genes in the shikimate pathways. It is therefore likely that the level and/or activities of enzymes of the shikimate pathway are different in the two strain backgrounds. In agreement with this, the available "omics" datasets presented by Canesas et al. (2010) show that the intracellular concentration of shikimic acid (the direct metabolite of 3-DHS) is significantly higher in S288c than in CEN.PK strains at similar conditions in batch and continuous cultivations. Moreover, they also reported that under chemostat conditions CEN.PK strains are able to maintain higher intracellular amino-acid pools, which was suggested, and later shown, to be a result of increased protein turnover rate (Hong et al., 2012).

One feature that could influence the VG production is the capacity of the two strains to adapt to toxic intermediates. This is derived from phenylalanine and tyrosine (Koopman et al., 2012). In this context, we note that in the two strain backgrounds, the ability of the two strains to adapt to toxic intermediates, especially PAL and VAN (Hansen et al., 2009), may also vary and influence yields. Even if they do not, accumulation of these intermediates varies slightly in the two strain back grounds and may therefore differentially influence VG yields. To this end, we note that the growth rates for C-VG and S-VG are reduced as compared to the corresponding strains that do not contain the VG pathway.

Since, the VG yield for S-VG is approximately 20-fold below the maximum theoretical yield; the effects described above are unlikely due to carbon being channeled into the VG pathway at the expense of other destinations and functions. An interesting question that still remains to be answered is therefore how the two strain backgrounds develop as VG producers as yields are improved by e.g. metabolic engineering. Similarly, it would be interesting to address whether some of the obvious genetic differences pointed out above can be transferred from S-VG to C-VG to improve the VG yields in the latter.

## 5. Concluding remarks

We have shown that heterologous production of VG differs dramatically in two different strain backgrounds. Our analyzes suggest that for strains like CEN.PK and S288c where high quality "omics" datasets are available it may be possible to predict, which of the two strain backgrounds would be the better producer. As additional data constantly accumulate we expect that such qualified guesses can be made in an increasingly safe manner. However, many other less characterized strain backgrounds exist, and it is likely that one of these strains may be a superior producer of VG. Rapid construction of strains with genetically comparable setups for VG production would be desirable. To this end, we have recently shown that multiple genes can be simultaneously integrated into three different sites in our expression platform in one step by using three different selectable markers (Jensen and Strucko, 2013). Similarly, simultaneous integration of multiple genes have also been achieved via CRISPR-Cas9 technology (Mans et al., 2015; Jakobčinas et al., 2015). Importantly, since similar strain differences are likely to apply for the production of other metabolites, we strongly recommend to test more than one genetic background during construction of a novel cell factory.

### Acknowledgments

We thank Jens Nielsen and Morten Kielland-Brandt for comments on this manuscript, Kristian Fog Nielsen for supporting

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**Table 5**

| Genes | SNPs | Amino acid substitution |
|-------|------|-------------------------|
| **Total** | **Non-synonymous** |
| ARO1 | 15 | T2251I, P337S, S517P, N844T, M1141K, V1386I, G1576A |
| ARO3 | 23 | K141R, E214D, S349T |
| ARO4 | – | – |
| ARO7 | – | – |
metabolite analyzes; and Anna-Lena Heins and Ted Johansen for facilitating fermentation experiments. This work was supported by Grant 6063-00323B from the Danish Council for Strategic Research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.meteno.2015.09.001.

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