The yeast Saccharomyces cerevisiae is widely used in industrial biotechnology for the production of fuels, chemicals, food ingredients, food and beverages, and pharmaceuticals. To obtain high-performing strains for such bioprocesses, it is often necessary to test tens or even hundreds of metabolic engineering targets, preferably in combinations, to account for synergistic and antagonistic effects. Here, we present a method that allows simultaneous perturbation of multiple selected genetic targets by combining the advantage of CRISPR/Cas9, invivo recombination, USER assembly and RNA interference. CRISPR/Cas9 introduces a double-strand break in a specific genomic region, where multi-expression constructs combined with the knockdown constructs are simultaneously integrated by homologous recombination. We show the applicability of the method by improving cis,cis-muconic acid production in S. cerevisiae through simultaneous manipulation of several metabolic engineering targets. The method can accelerate metabolic engineering efforts for the construction of future cell factories.

The transcriptome and flux profiling of Crabtree-negative hydroxy acid producing strains of Saccharomyces cerevisiae reveals changes in the central carbon metabolism

Saccharomyces cerevisiae is a yeast cell factory of choice for the production of many bio-based chemicals. However, it is also a Crabtree-positive yeast and so it shuttles a large portion of carbon into ethanol, even under aerobic conditions. To minimise the carbon loss, ethanol formation can be eliminated by deleting pyruvate decarboxylase (PDC) activity. Deletion of PDC genes has a profound impact on S. cerevisiae physiology, and it is not yet well understood how PDC-negative yeasts are affected when engineered to produce other products than ethanol. In this study, we introduced pathways for the production of three hydroxy acids (lactic, malic, or 3-hydroxypropionic acid) into an evolved PDC-negative strain. We characterised these strains via transcriptome and flux profiling to elucidate the effects that the production of these hydroxy acids has on the host strain. The expression of lactic acid and malic acid biosynthesis pathways improved the maximum specific growth rate ($\mu_{max}$) of the strain by 64 and 20% respectively, presumably due to NAD+ regeneration. On the contrary, the 3HP pathways expression decreased the $\mu_{max}$. All strains showed a very high flux (>90% of glucose uptake) into the oxidative pentose phosphate pathway under batch fermentation conditions. The transcriptional profile was least affected by the production of lactic acid and more by malic or 3-hydroxypropionic acids. The study, for the first time, directly compares the flux and transcriptome profiles of several different hydroxy acid producing strains of an evolved PDC-negative S. cerevisiae and suggests directions for future metabolic engineering. This article is protected by copyright. All rights reserved.
Understanding metabolite transport gives an upper hand in strain development
Cell factories can be engineered for more efficient product secretion through modulation of membrane transporters.

Advances in synthetic biology of oleaginous yeast Yarrowia lipolytica for producing non-native chemicals
Oleaginous yeast Yarrowia lipolytica is an important industrial host for the production of enzymes, oils, fragrances, surfactants, cosmetics, and pharmaceuticals. More recently, improved synthetic biology tools have allowed more extensive engineering of this yeast species, which lead to the production of non-native metabolites. In this review, we summarize the recent advances of genome editing tools for Y. lipolytica, including the application of CRISPR/Cas9 system and discuss case studies, where Y. lipolytica was engineered to produce various non-native chemicals: short-chain fatty alcohols and alkanes as biofuels, polyunsaturated fatty acids for nutritional and pharmaceutical applications, polyhydroxyalkanoates and dicarboxylic acids as precursors for biodegradable plastics, carotenoid-type pigments for food and feed, and campesterol as a precursor for steroid drugs.
EasyCloneYALI: CRISPR/Cas9-based synthetic toolbox for engineering of the yeast Yarrowia lipolytica

The oleaginous yeast Yarrowia lipolytica is an emerging host for production of fatty acid-derived chemicals. To enable rapid iterative metabolic engineering of this yeast, there is a need for well-characterized genetic parts and convenient and reliable methods for their incorporation into yeast. Here, we present the EasyCloneYALI genetic toolbox, which allows streamlined strain construction with high genome editing efficiencies in Y. lipolytica via the CRISPR/Cas9 technology. The toolbox allows marker-free integration of gene expression vectors into characterized genome sites as well as marker-free deletion of genes with the help of CRISPR/Cas9. Genome editing efficiencies above 80% were achieved with transformation protocols using non-replicating DNA repair fragments (such as DNA oligos). Furthermore, the toolbox includes a set of integrative gene expression vectors with prototrophic markers conferring resistance to hygromycin and nourseothricin.
Energetic evolution of cellular Transportomes

Background: Transporter proteins mediate the translocation of substances across the membranes of living cells. Many transport processes are energetically expensive and the cells use 20 to 60% of their energy to power the transportomes. We hypothesized that there may be an evolutionary selection pressure for lower energy transporters.

Results: We performed a genome-wide analysis of the compositional reshaping of the transportomes across the kingdoms of bacteria, archaea, and eukarya. We found that the share of ABC transporters is much higher in bacteria and archaea (ca. 27% of the transportome) than in primitive eukaryotes (13%), algae and plants (10%) and in fungi and animals (5–6%). This decrease is compensated by an increased occurrence of secondary transporters and ion channels. The share of ion channels is particularly high in animals (ca. 30% of the transportome) and algae and plants with (ca. 13%), when compared to bacteria and archaea with only 6–7%. Therefore, our results show a move to a preference for the low-energy-demanding transporters (ion channels and carriers) over the more energy-costly transporter classes (ATP-dependent families, and ABCs in particular) as part of the transition from prokaryotes to eukaryotes. The transportome analysis also indicated seven bacterial species, including Neorickettsia risticii and Neorickettsia sennetsu, as likely origins of the mitochondrion in eukaryotes, based on the phylogenetically restricted presence therein of clear homologues of modern mitochondrial solute carriers.

Conclusions: The results indicate that the transportomes of eukaryotes evolved strongly towards a higher energetic efficiency, as ATP-dependent transporters diminished and secondary transporters and ion channels proliferated. These changes have likely been important in the development of tissues performing energetically costly cellular functions.
candidate promoters that strongly responded to glucose presence or absence. These promoters were characterized in small-scale batch and fed-batch cultivations using a quickly maturing rapidly degrading green fluorescent protein yEGFP3-Cln2PEST as a reporter. Expressing 3-hydroxypropionic acid (3HP) pathway from a set of selected regulated promoters allowed for suppression of 3HP production during glucose-excess phase of a batch cultivation with subsequent activation in glucose-limiting conditions. Regulating the 3HP pathway by the ICL1 promoter resulted in 70% improvement of 3HP titer in comparison to PGK1 promoter.

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, iLoop, Research Groups, Yeast Metabolic Engineering, Yeast Cell Factories, Department of Biotechnology and Biomedicine, Regulatory Genomics, Technical University of Denmark
Corresponding author: Workman, C. T.
Publication date: 2018
Peer-reviewed: Yes

Publication information
Journal: Frontiers in Bioengineering and Biotechnology
Volume: 6
Article number: 63
ISSN (Print): 2296-4185
Ratings:
Scopus rating (2018): CiteScore 4.04
Web of Science (2018): Impact factor 5.122
Web of Science (2018): Indexed yes
Original language: English
Keywords: 3-hydroxypropionic acid, Dynamic regulation, Inducible promoters, Strain engineering, Yeast Saccharomyces cerevisiae
Electronic versions:
fbioe_06_00063.pdf
DOIs:
10.3389/fbioe.2018.00063

Bibliographical note
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Source: Scopus
Source-ID: 85047881693
Research output: Contribution to journal › Journal article – Annual report year: 2018 › Research › peer-review

Metabolic Engineering and Molecular Tool Development in Yeast for Production of Bulk Chemicals
The brewers’ yeast Saccharomyces cerevisiae has long been a close friend of humanity. Yeast has been used in food and beverage production for thousands of years, and our close relationship with yeast has led to it becoming a model organism in research. Yeast was the first eukaryote to have its genome sequenced, and has helped in the study of human diseases, such as cancer. This extensive characterisation, along with its routine use in bioprocesses makes it an attractive target for metabolic engineering efforts.

The work presented in this thesis encompasses some of the work that has been undertaken as part of the Ph.D. programme. Herein, I present work towards the development of tools to improve the process of engineering S. cerevisiae, as well as to apply those and other existing metabolic engineering tools to gain insights into the metabolism of engineered yeast strains. This research focuses on the production of hydroxy acids, a class of carboxylic acid. In the first research study, we developed a new set of integrative yeast expression vectors based on the previously developed EasyClone system. We adapted this system for use with CRISPR-Cas9 to insert expression cassettes into the yeast genome without the need for also integrating markers. This increases the speed of the engineering cycle as no marker loop-out is required between transformations. It also negates the need for markers, both auxotrophic and dominant, which can alter native host physiology and potentially affect experimental results. We have made this vector toolkit available through the Addgene platform, and hope many will use it to facilitate their research in S. cerevisiae. Using our vector toolkit, we investigated different acetyl-CoA supply strategies and their impact on the production of 3-hydroxypropionic acid (3HP), a bulk chemical that is a common target for metabolic engineering. We discovered that expression of a bacterial pyruvate dehydrogenase complex was the most successful strategy, which increased 3HP titres by almost 100% when grown in a simulated fed-batch medium. In the second study, focus is shifted away from tool development and towards metabolic engineering. Here we engineered a Crabtree-negative strain of S. cerevisiae to produce three different hydroxy acids; lactate, malate, and 3HP. We characterised these strains, and sought to gain an understanding of how these heterologous
pathways influence the metabolism and physiology of the host strain. We performed 13C-based metabolic flux analysis to quantify the carbon flux distributions through the central carbon metabolism. Between the strains, we identified large differences in flux distribution, but each strain showed surprisingly high levels of flux through the pentose phosphate pathway, and only low levels of flux through the tricarboxylic acid (TCA) cycle. The third study attempts to use metabolic flux and transcriptomic analysis to identify key gene deletion strategies that can boost the production of 3HP in a host strain (ST938) capable of producing high levels of this hydroxy acid. Transcriptomic analysis revealed that much of central carbon metabolism was upregulated in ST938 compared to the WT strain, but metabolic flux analysis revealed lower proportional carbon flux through the TCA cycle. Metabolic flux analysis also revealed that only a small proportion of the available carbon flux was diverted into 3HP production. Computational approaches and insights from the transcriptomic analysis identified several gene deletion strategies that were then implemented in vivo. Of these strategies, the deletion of PRY1 was able to increase final 3HP titres by 27%.

General information
Publication status: Published
Organisations: Yeast Metabolic Engineering, Yeast Cell Factories, Novo Nordisk Foundation Center for Biosustainability
Contributors: Fabre, M. M. J.
Number of pages: 200
Publication date: 2018

Publication information
Place of publication: Kgs. Lyngby
Publisher: Technical University of Denmark (DTU)
Original language: English
Electronic versions:
MMJF_PhD_Thesis_Revision_for_Print.pdf

Method for production of moth pheromones in yeast
The present disclosure relates to methods for production of (Z)-11-hexadecen-1-ol in a yeast cell using desaturases and fatty acyl-CoA reductase. Also disclosed are methods for production of (Z)-11-hexadecenal in a yeast cell. Also disclosed are methods for production of (Z)-11-hexadecen-1-yl acetate in a yeast cell. The disclosure also provides for nucleic acid constructs and yeast cells useful for performing the present methods, as well as to pheromone compositions.

General information
Publication status: Published
Organisations: Yeast Metabolic Engineering, Research Groups, Novo Nordisk Foundation Center for Biosustainability
Contributors: Borodina, I., Holkenbrink, C., Dam, M. I., Löfstedt, C., Ding, B., Wang, H.
Publication date: 2018

Publication information
Country: United States
IPC: C07K14/435; C12N1/16; C12P7/04; C12P7/24; C12P7/64
Patent number: US2018162916
Filing date: 14/06/2018
Priority date: 26/06/2015
Priority number: EP20150174099
Original language: English
Electronic versions:
US2018162916A1.pdf

Bibliographical note
Also published as: BR112017027527 (A2) CN108138202 (A) EP3313997 (A1) WO2016207339 (A1)

Methods for producing fatty alcohols and derivatives thereof in yeast
The present invention relates to oleaginous yeast cells for the production of fatty alcohols and derivatives thereof, in particular desaturated fatty alcohols, saturated fatty acyl acetates and desaturated fatty aldehydes. Also provided are methods for obtaining such compounds, which are useful in pheromone compositions.

General information
Publication status: Published
Organisations: Yeast Metabolic Engineering, Research Groups, Novo Nordisk Foundation Center for Biosustainability
Contributors: Borodina, I., Holkenbrink, C., Dam, M. I., Löfstedt, C.
Publication date: 2018
Native chinese hamster ovary cell secretion signal peptides for production of recombinant polypeptides
The invention provides a genetically modified Chinese Hamster Ovary (CHO) cell for production of a recombinant polypeptide, comprising a transgene encoding a CHO-derived signal peptide fused to a recombinant polypeptide, wherein said signal peptide is capable of directing co-translational transport of said polypeptide in the cell resulting in enhanced levels of polypeptide secretion. The invention further provides a method for producing a recombinant polypeptide by using the genetically modified CHO cell. The invention further provides for the use of a CHO-derived signal peptide and a transgene encoding said signal peptide to enhance production of extracellular recombinant polypeptide by a genetically modified CHO cell.

Production of desaturated fatty alcohols and desaturated fatty acyl acetates in yeast
The present invention relates to the production of compounds comprised in pheromones, in particular moth pheromones, such as desaturated fatty alcohols and desaturated fatty acyl acetates and derivatives thereof, from a yeast cell.

Production of desaturated fatty alcohols and desaturated fatty acyl acetates in yeast
The present invention relates to the production of compounds comprised in pheromones, in particular moth pheromones, such as desaturated fatty alcohols and desaturated fatty acyl acetates and derivatives thereof, from a yeast cell.
Yeast cell factories for more sustainable pest control in agriculture/ Engineered Oleaginous Yeast Cell Factories for Fine Chemicals

General information
Publication status: Published
Organisations: Yeast Metabolic Engineering, Research Groups, Novo Nordisk Foundation Center for Biosustainability
Contributors: Borodina, I.
Pages: 13-13
Publication date: 2018

Host publication information
Title of host publication: The Danish Microbiological Society Annual Congress 2018 - programme & abstracts
Place of publication: Copenhagen, Denmark
Publisher: Danish Microbiological Society

BIOREFINE-2G — Result In Brief: Novel biopolymers from biorefinery waste-streams
Second generation biorefineries are all about creating value from waste, so it seems only right that the ideal plant should leave nothing behind. With this in mind, the BIOREFINE-2G project has developed novel processes to convert pentose-rich side-streams into biopolymers.

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering, Applied Metabolic Engineering
Contributors: Stovicek, V., Chen, X., Borodina, I., Förster, J.
Number of pages: 2
Publication date: 2017

Comparison of the metabolic response to over-production of p-coumaric acid in two yeast strains
The development of robust and efficient cell factories requires understanding of the metabolic changes triggered by the production of the targeted compound. Here we aimed to study how production of p-coumaric acid, a precursor of multiple secondary aromatic metabolites, influences the cellular metabolism of Saccharomyces cerevisiae. We evaluated the growth and p-coumaric acid production in batch and chemostat cultivations and analyzed the transcriptome and intracellular metabolome during steady state in low- and high-producers of p-coumaric acid in two strain backgrounds, S288c or CEN.PK. We found that the same genetic modifications resulted in higher production of p-coumaric acid in the CEN.PK background than in the S288c background. Moreover, the CEN.PK strain was less affected by the genetic engineering as was evident from fewer changes in the transcription profile and intracellular metabolite concentrations. Surprisingly, for both strains we found the largest transcriptional changes in genes involved in transport of amino acids and sugars, which were downregulated. Additionally, in S288c amino acid and protein biosynthesis processes were also affected. We systematically overexpressed or deleted genes with significant transcriptional changes in CEN.PK low and high-producing strains. The knockout of some of the downregulated transporters triggered a 20-50% improvement in the synthesis of p-CA in the CEN.PK high-producing strain. This study demonstrates the importance of transporters in the engineering of cell factories for production of small molecules.

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Yeast Cell Factories, iLoop, Research Groups, Yeast Metabolic Engineering, Chalmers University of Technology
Contributors: Rodríguez, A., Chen, Y., Khoomrung, S., Özdemir, E., Borodina, I., Nielsen, J.
CRISPR/Cas system for yeast genome engineering: advances and applications

The methods based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system have quickly gained popularity for genome editing and transcriptional regulation in many organisms, including yeast. This review aims to provide a comprehensive overview of CRISPR application for different yeast species: from basic principles and genetic design to applications.

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering
Contributors: Stovicek, V., Holkenbrink, C., Borodina, I.
Number of pages: 16
Publication date: 2017
Peer-reviewed: Yes

Bibliographical note
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
**Easyclone-markerfree: A vector toolkit for marker-less integration of genes into saccharomyces cerevisiae via CRISPR-Cas9**

**General information**
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Yeast Cell Factories, Yeast Metabolic Engineering, Synthetic Biology Tools for Yeast, Research Groups, Chalmers University of Technology, University of California at Berkeley
Number of pages: 2
Pages: 149-150
Publication date: 2017

**Host publication information**
Title of host publication: Synthetic Biology Conference, SEED 2017: Engineering, Evolution, and Design
Publisher: American Institute of Chemical Engineers
ISBN (Electronic): 9781510856738
Source: Scopus
Source-ID: 85048654053
Research output: Chapter in Book/Report/Conference proceeding – Article in proceedings – Annual report year: 2018 – Research – peer-review

**Engineering microbial fatty acid metabolism for biofuels and biochemicals**
Traditional oleochemical industry chemically processes animal fats and plant oils to produce detergents, lubricants, biodiesel, plastics, coatings, and other products. Biotechnology offers an alternative process, where the same oleochemicals can be produced from abundant biomass feedstocks using microbial catalysis. This review summarizes the recent advances in the engineering of microbial metabolism for production of fatty acid-derived products. We highlight the efforts in engineering the central carbon metabolism, redox metabolism, controlling the chain length of the products, and obtaining metabolites with different functionalities. The prospects of commercializing microbial oleochemicals are also discussed.

**General information**
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Yeast Metabolic Engineering, Research Groups, Chalmers University of Technology
Contributors: Marella, E. R., Holkenbrink, C., Siewers, V., Borodina, I.
Pages: 39-46
Publication date: 2017
Peer-reviewed: Yes

**Publication information**
Journal: Current Opinion in Biotechnology
Volume: 50
ISSN (Print): 0958-1669
Ratings:
BFI (2017): BFI-level 2
Scopus rating (2017): CiteScore 8.45 SJR 3.202 SNIP 2.205
Web of Science (2017): Impact factor 8.38
Web of Science (2017): Indexed yes
Original language: English
Electronic versions:
Engineering_microbial_fatty_acid_metabolism_for_biofuels_and_biochemicals.pdf
DOIs:

**Bibliographical note**
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Source: FindIt
Source-ID: 2392781800
Research output: Contribution to journal – Journal article – Annual report year: 2017 – Research – peer-review
Engineering of Yarrowia lipolytica for production of astaxanthin

Astaxanthin is a red-colored carotenoid, used as food and feed additive. Astaxanthin is mainly produced by chemical synthesis, however, the process is expensive and synthetic astaxanthin is not approved for human consumption. In this study, we engineered the oleaginous yeast Yarrowia lipolytica for de novo production of astaxanthin by fermentation.

First, we screened 12 different Y. lipolytica isolates for β-carotene production by introducing two genes for β-carotene biosynthesis: bi-functional phytoene synthase/lycopene cyclase (crtYB) and phytoene desaturase (crtI) from the red yeast Xanthophyllomyces dendrorhous. The best strain produced 31.1 ± 0.5 mg/L β-carotene. Next, we optimized the activities of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG1) and geranylgeranyl diphosphate synthase (GGS1/crtE) in the best producing strain and obtained 453.9 ± 20.2 mg/L β-carotene. Additional downregulation of the competing squalene synthase SQS1 increased the β-carotene titer to 797.1 ± 57.2 mg/L. Then we introduced β-carotene ketolase (crtW) from Paracoccus sp. N81106 and hydroxylase (crtZ) from Pantoea ananatis to convert β-carotene into astaxanthin. The constructed strain accumulated 10.4 ± 0.5 mg/L of astaxanthin but also accumulated astaxanthin biosynthesis intermediates, 5.7 ± 0.5 mg/L canthaxanthin, and 35.3 ± 1.8 mg/L echinenone. Finally, we optimized the copy numbers of crtZ and crtW to obtain 3.5 mg/g DCW (54.6 mg/L) of astaxanthin in a microtiter plate cultivation.

Our study for the first time reports engineering of Y. lipolytica for the production of astaxanthin. The high astaxanthin content and titer obtained even in a small-scale cultivation demonstrates a strong potential for Y. lipolytica-based fermentation process for astaxanthin production.

Metabolic engineering of yeast for fermentative production of flavonoids

Yeast Saccharomyces cerevisiae was engineered for de novo production of six different flavonoids (naringenin, liquiritigenin, kaempferol, resokaempferol, quercetin, and fisetin) directly from glucose, without supplementation of expensive intermediates. This required reconstruction of long biosynthetic pathways, comprising up to eight heterologous genes from plants. The obtained titers of kaempferol 26.57±2.66mgL−1 and quercetin 20.38±2.57mgL−1 exceed the previously reported titers in yeast. This is also the first report of de novo biosynthesis of resokaempferol and fisetin in yeast. The work demonstrates the potential of flavonoid-producing yeast cell factories.
Production of Steviol Glycosides in Recombinant Hosts

The invention relates to recombinant microorganisms and methods for producing steviol glycosides and steviol glycoside precursors.

General information
Publication status: Published
Organisations: Yeast Metabolic Engineering, Novo Nordisk Foundation Center for Biosustainability
Contributors: Douchin, V., Mikkelsen, M. D., Møller-Hansen, I.
Publication date: 2017

Publication information
Country: United States
IPC: C12N1/16; C12P19/44
Patent number: US2017218418
Filing date: 03/08/2017
Priority date: 07/08/2017
Priority number: US201515328365
Original language: English
Electronic versions:
US20170218418A1.pdf
Research output: Patent – Annual report year: 2018

Yeast Cell Factories for Production of Fuels and Chemicals

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering, CAS - Dalian Institute of Chemical Physics
Contributors: Borodina, I., Zhao, Z. K.
Number of pages: 1
Publication date: 2017
Peer-reviewed: Yes

Publication information
Journal: FEMS Yeast Research
Volume: 17
Issue number: 8
Article number: fox082
ISSN (Print): 1567-1356
Ratings:
BFI (2017): BFI-level 1
Microbial production of the flavonoids garbanzol, resokaempferol and fisetin
The invention provides a genetically modified micro-organism comprising one or more transgene for the production of one or more of the flavonoids garbanzol, resokaempferol and fisetin. The micro-organism may be a bacterial or yeast cell engineered to express a metabolic pathway for garbanzol, resokaempferol and/or fisetin biosynthesis. The invention further provides a method for producing garbanzol, resokaempferol and/or fisetin employing the genetically modified micro-organism of the invention. The genetically modified micro-organism may be used to convert a number of substrates and/or co-substrates into fisetin via a fisetin biosynthetic pathway.

A toolbox for accelerating strain engineering of oleaginous yeast Yarrowia lipolytica

Bibliographical note
Poster presentation
Source: PublicationPreSubmission
Source-ID: 127761289
Research output: Chapter in Book/Report/Conference proceeding › Conference abstract in proceedings – Annual report year: 2016 › Research › peer-review
EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR-Cas9

*Saccharomyces cerevisiae* is an established industrial host for production of recombinant proteins, fuels and chemicals. To enable stable integration of multiple marker-free overexpression cassettes in the genome of *S. cerevisiae*, we have developed a vector toolkit EasyClone-MarkerFree. The integration of linearized expression cassettes into defined genomic loci is facilitated by CRISPR/Cas9. Cas9 is recruited to the chromosomal location by specific guide RNAs (gRNAs) expressed from a set of gRNA helper vectors. Using our genome engineering vector suite, single and triple insertions are obtained with 90–100% and 60–70% targeting efficiency, respectively. We demonstrate application of the vector toolkit by constructing a haploid laboratory strain (CEN.PK113-7D) and a diploid industrial strain (Ethanol Red) for production of 3-hydroxypropionic acid, where we tested three different acetyl-CoA supply strategies, requiring overexpression of three to six genes each. Among the tested strategies was a bacterial cytosolic pyruvate dehydrogenase complex, which was integrated into the genome in a single transformation. The publicly available EasyClone-MarkerFree vector suite allows for facile and highly standardized genome engineering, and should be of particular interest to researchers working on yeast chassis with limited markers available.

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Yeast Metabolic Engineering, Synthetic Biology Tools for Yeast, Research Groups, Applied Metabolic Engineering, Chalmers University of Technology
Number of pages: 11
Pages: 1110-1117
Publication date: 2016
Peer-reviewed: Yes

Publication information
Journal: Biotechnology Journal
Volume: 11
Issue number: 8
ISSN (Print): 1860-6768
Ratings:
BFI (2016): BFI-level 1
Scopus rating (2016): CiteScore 3.2 SJR 1.29 SNIP 0.969
Web of Science (2016): Impact factor 3.649
Web of Science (2016): Indexed yes
Original language: English
Keywords: 3-hydroxypropionic acid, CRISPR-Cas9, Metabolic engineering, Saccharomyces cerevisiae
Electronic versions:
A_vector_toolkit_for_marker_less_integration_of_genes_into_Saccharomyces_cerevisiae_via_CRISPR_Cas9.pdf
DOIs: 10.1002/biot.201600147

Bibliographical note
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Research output: Contribution to journal › Journal article – Annual report year: 2016 › Research › peer-review

EasyCloneMulti: A Set of Vectors for Simultaneous and Multiple Genomic Integrations in *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* is widely used in the biotechnology industry for production of ethanol, recombinant proteins, food ingredients and other chemicals. In order to generate highly producing and stable strains, genome integration of genes encoding metabolic pathway enzymes is the preferred option. However, integration of pathway genes in single or few copies, especially those encoding rate-controlling steps, is often not sufficient to sustain high metabolic fluxes. By exploiting the sequence diversity in the long terminal repeats (LTR) of Ty retrotransposons, we developed a new set of integrative vectors, EasyCloneMulti, that enables multiple and simultaneous integration of genes in *S. cerevisiae*. By creating vector backbones that combine consensus sequences that aim at targeting subsets of Ty sequences and a quickly degrading selective marker, integrations at multiple genomic loci and a range of expression levels were obtained, as assessed with the green fluorescent protein (GFP) reporter system. The EasyCloneMulti vector set was applied to balance the expression of the rate-controlling step in the β-alanine pathway for biosynthesis of 3-hydroxypropionic acid (3HP). The best 3HP producing clone, with 5.45 g.L⁻¹ of 3HP, produced 11 times more 3HP than the lowest producing clone, which demonstrates the capability of EasyCloneMulti vectors to impact metabolic pathway enzyme activity.

General information
Engineering and systems level analysis of *Saccharomyces cerevisiae* for production of 3-hydroxypropionic acid via malonyl CoA reductase dependent pathway

In the future, oil- and gas-derived polymers may be replaced with bio-based polymers, produced from renewable feedstocks using engineered cell factories. Acrylic acid and acrylic esters with an estimated world annual production of approximately 6 million tons by 2017 can be derived from 3-hydroxypropionic acid (3HP), which can be produced by microbial fermentation. For an economically viable process 3HP must be produced at high titer, rate and yield and preferably at low pH to minimize downstream processing costs.
Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast

Whole-cell biocatalysts have proven a tractable path toward sustainable production of bulk and fine chemicals. Yet the screening of libraries of cellular designs to identify best-performing biocatalysts is most often a low-throughput endeavor. For this reason, the development of biosensors enabling real-time monitoring of production has attracted attention. Here we applied systematic engineering of multiple parameters to search for a general biosensor design in the budding yeast Saccharomyces cerevisiae based on small-molecule binding transcriptional activators from the prokaryote superfamily of LysR-type transcriptional regulators (LTTRs). We identified a design supporting LTTR-dependent activation of reporter gene expression in the presence of cognate small-molecule inducers. As proof of principle, we applied the biosensors for in vivo screening of cells producing naringenin or cis,cis-muconic acid at different levels, and found that reporter gene output correlated with production. The transplantation of prokaryotic transcriptional activators into the eukaryotic chassis illustrates the potential of a hitherto untapped biosensor resource useful for biotechnological applications.

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Synthetic Biology Tools for Yeast, Research Groups, Yeast Metabolic Engineering, iLoop, Bacterial Synthetic Biology, Technical University of Denmark, Evolva Biotech A/S, Evolva SA
Number of pages: 10
Pages: 951-958
Publication date: 2016
Peer-reviewed: Yes

Publication information
Journal: Nature Chemical Biology
Volume: 12
Issue number: 11
ISSN (Print): 1552-4450
Ratings:
BFI (2016): BFI-level 2
Scopus rating (2016): CiteScore 9.41 SJR 8.946 SNIP 3.061
Web of Science (2016): Impact factor 15.066
Web of Science (2016): Indexed yes
Original language: English
Keywords: Metabolic engineering, Model invertebrates, Synthetic biology, Transcription
DOIs:
10.1038/nchembio.2177

Engineering yeast for high-level production of stilbenoid antioxidants

Stilbenoids, including resveratrol and its methylated derivatives, are natural potent antioxidants, produced by some plants in trace amounts as defense compounds. Extraction of stilbenoids from natural sources is costly due to their low abundance and often limited availability of the plant. Here we engineered the yeast Saccharomyces cerevisiae for production of stilbenoids on a simple mineral medium typically used for industrial production. We applied a pull-push-block strain engineering strategy that included overexpression of the resveratrol biosynthesis pathway, optimization of the electron transfer to the cytochrome P450 monooxygenase, increase of the precursors supply, and decrease of the pathway intermediates degradation. Fed-batch fermentation of the final strain resulted in a final titer of 800 mg l(-1) resveratrol, which is by far the highest titer reported to date for production of resveratrol from glucose. We further integrated heterologous methyltransferases into the resveratrol platform strain and hereby demonstrated for the first time de novo biosynthesis of pinostilbene and pterostilbene, which have better stability and uptake in the human body, from
Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*

Melatonin is a natural mammalian hormone that plays an important role in regulating the circadian cycle in humans. It is a clinically effective drug exhibiting positive effects as a sleep aid and a powerful antioxidant used as a dietary supplement. Commercial melatonin production is predominantly performed by complex chemical synthesis. In this study, we demonstrate microbial production of melatonin and related compounds, such as serotonin and N-acetylserotonin. We generated *Saccharomyces cerevisiae* strains that comprise heterologous genes encoding one or more variants of an L-tryptophan hydroxylase, a 5-hydroxy-L-tryptophan decarboxylase, a serotonin acetyltransferase, an acetylserotonin O-methyltransferase, and means for providing the cofactor tetrahydrobiopterin via heterologous biosynthesis and recycling pathways. We thereby achieved de novo melatonin biosynthesis from glucose. We furthermore accomplished increased product titers by altering expression levels of selected pathway enzymes and boosting co-factor supply. The final yeast strain produced melatonin at a titer of 14.50 ± 0.57 mg L\(^{-1}\) in a 76h fermentation using simulated fed-batch medium with glucose as sole carbon source. Our study lays the basis for further developing a yeast cell factory for biological production of melatonin.

**General information**
Publication status: Published
Organisations: iLoop, Novo Nordisk Foundation Center for Biosustainability, Research Groups, Applied Metabolic Engineering
Number of pages: 8
Pages: 717-724
Publication date: 2016
Peer-reviewed: Yes

**Publication information**
Journal: Biotechnology Journal
Volume: 11
Production of steviol glycosides in recombinant hosts
The invention relates to recombinant microorganisms and methods for producing steviol glycosides and steviol glycoside precursors.

General information
Publication status: Published
Organisations: New Bioactive Compounds, Novo Nordisk Foundation Center for Biosustainability, Yeast Metabolic Engineering
Publication date: 2016

Publication information
IPC: A23L27/36; C07H15/256
Patent number: WO2016038095
Filing date: 17/03/2016
Priority date: 09/09/2014
Priority number: US201462048178P
Original language: English
Electronic versions: WO2016038095A2.pdf
Source: PublicationPreSubmission
Source-ID: 162259682
Research output: Patent › Patent – Annual report year: 2016 › Research

Production of steviol glycosides in recombinant hosts
The invention relates to recombinant microorganisms and methods for producing steviol glycosides and steviol glycoside precursors.

General information
Publication status: Published
Organisations: Yeast Metabolic Engineering, Novo Nordisk Foundation Center for Biosustainability
Contributors: Douchin, V., Mikkelsen, M. D., Møller-Hansen, I.
Publication date: 2016

Publication information
IPC: C07H1/08; C12N15/81
Patent number: WO2016023844
Filing date: 18/02/2016
Priority date: 07/08/2015
Rational and Evolutionary Engineering of Industrial Saccharomyces Cerevisiae Strains for Production of Chemicals from Xylose-Rich Feedstocks

**General information**
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering, Department of Chemical and Biochemical Engineering, CAPEC-PROCESS
Contributors: Stovicek, V., Lis, A. V., Borodina, I., Förster, J.
Number of pages: 1
Publication date: 2016

**Host publication information**
Title of host publication: Online Proceedings
Publisher: American Institute of Chemical Engineers
Electronic versions:
Rational_and_Evolutionary_Engineering.pdf

**Bibliographical note**
Poster and rapid fire presentation
Research output: Chapter in Book/Report/Conference proceeding › Conference abstract in proceedings – Annual report year: 2016 › Research › peer-review

Yeast cell factories for bio-based chemicals

**General information**
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering
Contributors: Borodina, I.
Number of pages: 1
Publication date: 2016
Peer-reviewed: Yes
URLs:
http://www.sustain.dtu.dk/

**Bibliographical note**
Sustain Abstract I-1
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 2016 › Research › peer-review

**EasyClone 2.0: expanded toolkit of integrative vectors for stable gene expression in industrial Saccharomyces cerevisiae strains**
Saccharomyces cerevisiae is one of the key cell factories for production of chemicals and active pharmaceuticals. For large-scale fermentations, particularly in biorefinery applications, it is desirable to use stress-tolerant industrial strains. However, such strains are less amenable for metabolic engineering than the standard laboratory strains. To enable easy delivery and overexpression of genes in a wide range of industrial S. cerevisiae strains, we constructed a set of integrative vectors with long homology arms and dominant selection markers. The vectors integrate into previously validated chromosomal locations via double cross-over and result in homogenous stable expression of the integrated genes, as shown for several unrelated industrial strains. Cre-mediated marker rescue is possible for removing markers positioned on different chromosomes. To demonstrate the applicability of the presented vector set for metabolic engineering of industrial yeast, we constructed xylose-utilizing strains overexpressing xylose isomerase, xylose transporter and five genes of the pentose phosphate pathway.

**General information**
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Applied Metabolic Engineering, Yeast Metabolic Engineering, Yeast Cell Factories
Microorganisms for efficient production of melatonin and related compounds.
Recombinant microbial cells and methods for producing 5HTP, melatonin and related compounds using such cells are described. More specifically, the recombinant microbial cell may comprise exogenous genes encoding one or more of an L-tryptophan hydroxylase, a 5-hydroxy-L-tryptophan decarboxylase, a serotonin acetyltransferase, an acetylserotonin O-methyltransferase; and means for providing tetrahydrobiopterin (THB), and can be further genetically modified to enrich one or more of tryptophan, S-adenosyl-L-methionine and acetyl coenzyme A. Related sequences and vectors for use in preparing such recombinant microbial cells are also described.

Application of synthetic biology for production of chemicals in yeast Saccharomyces cerevisiae
Synthetic biology and metabolic engineering enable generation of novel cell factories that efficiently convert renewable feedstocks into biofuels, bulk, and fine chemicals, thus creating the basis for biosustainable economy independent on fossil resources. While over a hundred proof-of-concept chemicals have been made in yeast, only a very small fraction of those has reached commercial-scale production so far. The limiting factor is the high research cost associated with the development of a robust cell factory that can produce the desired chemical at high titer, rate, and yield. Synthetic biology
has the potential to bring down this cost by improving our ability to predictably engineer biological systems. This review highlights synthetic biology applications for design, assembly, and optimization of non-native biochemical pathways in baker's yeast *Saccharomyces cerevisiae*. We describe computational tools for the prediction of biochemical pathways, molecular biology methods for assembly of DNA parts into pathways, and for introducing the pathways into the host, and finally approaches for optimizing performance of the introduced pathways.

**General information**
- Publication status: Published
- Organisations: Novo Nordisk Foundation Center for Biosustainability, Yeast Metabolic Engineering, Yeast Cell Factories
- Contributors: Borodina, I., Li, M.
- Number of pages: 12
- Publication date: 2015
- Peer-reviewed: Yes

**Publication information**
- Journal: *FEBS Yeast Research*
- Volume: 15
- Issue number: 1
- ISSN (Print): 1567-1356
- Ratings:
  - BFI (2015): BFI-level 1
  - Scopus rating (2015): CiteScore 2.56 SJR 1.213 SNIP 0.703
  - Web of Science (2015): Impact factor 2.479
- Web of Science (2015): Indexed yes
- Original language: English
- Keywords: Synthetic biology, Metabolic engineering, Yeast, Chemicals, S. cerevisiae
- DOIs: 10.1111/1567-1364.12213
- Source: PublicationPreSubmission
- Source-ID: 101501430
- Research output: Contribution to journal > Journal article – Annual report year: 2014 > Research > peer-review

**CasEMBLR: Cas9-Facilitated Multiloci Genomic Integration of In Vivo Assembled DNA Parts in *Saccharomyces cerevisiae***

Homologous recombination (HR) in *Saccharomyces cerevisiae* has been harnessed for both plasmid construction and chromosomal integration of foreign DNA. Still, native HR machinery is not efficient enough for complex and marker-free genome engineering required for modern metabolic engineering. Here, we present a method for marker-free multiloci integration of in vivo assembled DNA parts. By the use of CRISPR/Cas9-mediated one-step double-strand breaks at single, double and triple integration sites we report the successful in vivo assembly and chromosomal integration of DNA parts. We call our method CasEMBLR and validate its applicability for genome engineering and cell factory development in two ways: (i) introduction of the carotenoid pathway from 15 DNA parts into three targeted loci, and (ii) creation of a tyrosine production strain using ten parts into two loci, simultaneously knocking out two genes. This method complements and improves the current set of tools available for genome engineering in *S. cerevisiae*.

**General information**
- Publication status: Published
- Organisations: Novo Nordisk Foundation Center for Biosustainability, Synthetic Biology Tools for Yeast, Yeast Metabolic Engineering, Yeast Cell Factories, Bacterial Cell Factories, Research Groups, Bacterial Cell Factory Optimization
- Number of pages: 9
- Pages: 1226-1234
- Publication date: 2015
- Peer-reviewed: Yes

**Publication information**
- Journal: *ACS Synthetic Biology*
- Volume: 4
- Issue number: 11
- ISSN (Print): 2161-5063
- Ratings:
  - Scopus rating (2015): CiteScore 4.41 SJR 2.339 SNIP 0.964
  - Web of Science (2015): Indexed yes
CrEdit: CRISPR mediated multi-loci gene integration in Saccharomyces cerevisiae

Background: One of the bottlenecks in production of biochemicals and pharmaceuticals in Saccharomyces cerevisiae is stable and homogeneous expression of pathway genes. Integration of genes into the genome of the production organism is often a preferred option when compared to expression from episomal vectors. Existing approaches for achieving stable simultaneous genome integrations of multiple DNA fragments often result in relatively low integration efficiencies and furthermore rely on the use of selection markers. Results: Here, we have developed a novel method, CrEdit (CRISPR/Cas9 mediated genome Editing), which utilizes targeted double strand breaks caused by CRISPR/Cas9 to significantly increase the efficiency of homologous integration in order to edit and manipulate genomic DNA. Using CrEdit, the efficiency and locus specificity of targeted genome integrations reach close to 100% for single gene integration using short homology arms down to 60 base pairs both with and without selection. This enables direct and cost efficient inclusion of homology arms in PCR primers. As a proof of concept, a non-native beta-carotene pathway was reconstructed in S. cerevisiae by simultaneous integration of three pathway genes into individual intergenic genomic sites. Using longer homology arms, we demonstrate highly efficient and locus-specific genome integration even without selection with up to 84% correct clones for simultaneous integration of three gene expression cassettes. Conclusions: The CrEdit approach enables fast and cost effective genome integration for engineering of S. cerevisiae. Since the choice of the targeting sites is flexible, CrEdit is a powerful tool for diverse genome engineering applications.

General information
Publication status: Published
Organisations: Bacterial Cell Factories, Novo Nordisk Foundation Center for Biosustainability, Synthetic Biology Tools for Yeast, Research Groups, Yeast Metabolic Engineering, Bacterial Cell Factory Optimization
Number of pages: 11
Publication date: 2015
Peer-reviewed: Yes

Publication information
Journal: Microbial Cell Factories
Volume: 14
Issue number: 97
ISSN (Print): 1475-2859
Ratings:
BFI (2015): BFI-level 1
Scopus rating (2015): CiteScore 4.08 SJR 1.528 SNIP 1.221
Web of Science (2015): Indexed yes
Original language: English
Keywords: Metabolic engineering, CRISPR/Cas9, Genome editing, Saccharomyces cerevisiae, Carotenoid production, Genome integrations
Electronic versions:
CrEdit.pdf
DOIs:
10.1186/s12934-015-0288-3

Bibliographical note
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Source: FindIt
Source-ID: 275440697
Research output: Contribution to journal › Journal article – Annual report year: 2015 › Research › peer-review
CRISPR–Cas system enables fast and simple genome editing of industrial Saccharomyces cerevisiae strains

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.

De novo production of resveratrol from glucose or ethanol by engineered *Saccharomyces cerevisiae*

Resveratrol is a natural antioxidant compound, used as food supplement and cosmetic ingredient. Microbial production of resveratrol has until now been achieved by supplementation of expensive substrates, *p*-coumaric acid or aromatic amino acids. Here we engineered the yeast *Saccharomyces cerevisiae* to produce resveratrol directly from glucose or ethanol via tyrosine intermediate. First we introduced the biosynthetic pathway, consisting of tyrosine ammonia-lyase from *Herpetosiphon aurantiacus*, *p*_-coumaryl-CoA ligase from *Arabidopsis thaliana* and resveratrol synthase from *Vitis vinifera*, and obtained 2.73±0.05 mg L$^{-1}$ resveratrol from glucose. Then we over-expressed feedback-insensitive alleles of *ARO4* encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate and *ARO7* encoding chorismate mutase, resulting in production of 4.85±0.31 mg L$^{-1}$ resveratrol from glucose as the sole carbon source. Next we improved the supply of the precursor malonyl-CoA by over-expressing a post-translational de-regulated version of the acetyl-CoA carboxylase encoding gene *ACCF*; this strategy further increased resveratrol production to 6.39±0.03 mg L$^{-1}$. Subsequently, we improved the strain by performing multiple-integration of pathway genes resulting in resveratrol production of 235.57±7.00 mg L$^{-1}$. Finally, fed-batch fermentation of the final strain with glucose or ethanol as carbon source resulted in a resveratrol titer of 415.65 and 531.41 mg L$^{-1}$, respectively.

**General information**

Published: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Applied Metabolic Engineering, Yeast Metabolic Engineering
Contributors: Stovicek, V., Borodina, I., Förster, J.
Number of pages: 10
Pages: 13-22
Publication date: 2015
Peer-reviewed: Yes

**Publication information**

Journal: Metabolic Engineering Communications
Volume: 2
ISSN (Print): 2214-0301
Ratings:
Scopus rating (2015): CiteScore 2.75 SJR 1.215 SNIP 0.909
Original language: English
Keywords: CRISPR–Cas9, Genome editing, Industrial yeast, Biorefineries, Chemical production
Electronic versions:
Vratislav.pdf
DOIs:
10.1016/j.meteno.2015.03.001

**Bibliographical note**

This is an open access article under the CCBY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Source: PublicationPreSubmission
Source-ID: 110751585
Research output: Contribution to journal › Journal article – Annual report year: 2015 › Research › peer-review

De novo production of resveratrol from glucose or ethanol by engineered *Saccharomyces cerevisiae*

Resveratrol is a natural antioxidant compound, used as food supplement and cosmetic ingredient. Microbial production of resveratrol has until now been achieved by supplementation of expensive substrates, *p*-coumaric acid or aromatic amino acids. Here we engineered the yeast *Saccharomyces cerevisiae* to produce resveratrol directly from glucose or ethanol via tyrosine intermediate. First we introduced the biosynthetic pathway, consisting of tyrosine ammonia-lyase from *Herpetosiphon aurantiacus*, *p*-coumaryl-CoA ligase from *Arabidopsis thaliana* and resveratrol synthase from *Vitis vinifera*, and obtained 2.73±0.05 mg L$^{-1}$ resveratrol from glucose. Then we over-expressed feedback-insensitive alleles of *ARO4* encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate and *ARO7* encoding chorismate mutase, resulting in production of 4.85±0.31 mg L$^{-1}$ resveratrol from glucose as the sole carbon source. Next we improved the supply of the precursor malonyl-CoA by over-expressing a post-translational de-regulated version of the acetyl-CoA carboxylase encoding gene *ACCF*; this strategy further increased resveratrol production to 6.39±0.03 mg L$^{-1}$. Subsequently, we improved the strain by performing multiple-integration of pathway genes resulting in resveratrol production of 235.57±7.00 mg L$^{-1}$. Finally, fed-batch fermentation of the final strain with glucose or ethanol as carbon source resulted in a resveratrol titer of 415.65 and 531.41 mg L$^{-1}$, respectively.

**General information**

Published: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Applied Metabolic Engineering, Yeast Metabolic Engineering, Yeast Cell Factories, Chalmers University of Technology
Contributors: Li, M., Kildegaard, K. R., Chen, Y., Rodriguez Prado, E. A., Borodina, I., Nielsen, J.
Pages: 1-11
Publication date: 2015
Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via β-alanine

Microbial fermentation of renewable feedstocks into plastic monomers can decrease our fossil dependence and reduce global CO2 emissions. 3-Hydroxypropionic acid (3HP) is a potential chemical building block for sustainable production of superabsorbent polymers and acrylic plastics. With the objective of developing *Saccharomyces cerevisiae* as an efficient cell factory for high-level production of 3HP, we identified the β-alanine biosynthetic route as the most economically attractive according to the metabolic modeling. We engineered and optimized a synthetic pathway for *de novo* biosynthesis of β-alanine and its subsequent conversion into 3HP using a novel β-alanine-pyruvate aminotransferase discovered in *Bacillus cereus*. The final strain produced 3HP at a titer of 13.7±0.3 g・L⁻¹ with a 0.14±0.0 C-mol・C-mol⁻¹ yield on glucose in 80 hours in controlled fed-batch fermentation in mineral medium at pH 5, and this work therefore lays the basis for developing a process for biological 3HP production.
Establishment of a yeast platform strain for production of p-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis

Aromatic amino acids are precursors of numerous plant secondary metabolites with diverse biological functions. Many of these secondary metabolites are already being used as active pharmaceutical or nutraceutical ingredients, and there are numerous exploratory studies of other compounds with promising applications. p-Coumaric acid is derived from aromatic amino acids and, besides being a valuable chemical building block, it serves as precursor for biosynthesis of many secondary metabolites, such as polyphenols, flavonoids, and some polyketides. Here we developed a p-coumaric acid-overproducing Saccharomyces cerevisiae platform strain. First, we reduced by-product formation by knocking out phenylpyruvate decarboxylase ARO10 and pyruvate decarboxylase PDC5. Second, different versions of feedback-resistant DAHP synthase and chorismate mutase were overexpressed. Finally, we identified shikimate kinase as another important flux-controlling step in the aromatic amino acid pathway by overexpressing enzymes from Escherichia coli, homologous to the pentafunctional enzyme Aro1p and to the bifunctional chorismate synthase-flavin reductase Aro2p. The highest titer of p-coumaric acid of 1.93±0.26 g L⁻¹ was obtained, when overexpressing tyrosine ammonia-lyase TAL from Flavobacterium johnsonii, DAHP synthase ARO4K229L, chorismate mutase ARO7G141S and E. coli shikimate kinase II (aroL) in Δpdc5Δaro10 strain background. To our knowledge this is the highest reported titer of an aromatic compound produced by yeast. The developed S. cerevisiae strain represents an attractive platform host for production of p-coumaric-acid derived secondary metabolites, such as flavonoids, polyphenols, and polyketides.

Highly Active and Specific Tyrosine Ammonia-Lyases from Diverse Origins Enable Enhanced Production of Aromatic Compounds in Bacteria and Saccharomyces cerevisiae

Phenylalanine and tyrosine ammonia-lyases form cinnamic acid and p-coumaric acid, which are precursors of a wide range of aromatic compounds of biotechnological interest. Lack of highly active and specific tyrosine ammonia-lyases has previously been a limitation in metabolic engineering approaches. We therefore identified 22 sequences in silico using...
synteny information and aiming for sequence divergence. We performed a comparative in vivo study, expressing the genes intracellularly in bacteria and yeast. When produced heterologously, some enzymes resulted in significantly higher production of p-coumaric acid in several different industrially important production organisms. Three novel enzymes were found to have activity exclusively for phenylalanine, including an enzyme from the low-GC Gram-positive bacterium Brevibacillus laterosporus, a bacterial-type enzyme from the amoeba Dictyostelium discoideum, and a phenylalanine ammonia-lyase from the moss Physcomitrella patens (producing 230 μM cinnamic acid per unit of optical density at 600 nm [OD600]) in the medium using Escherichia coli as the heterologous host. Novel tyrosine ammonia-lyases having higher reported substrate specificity than previously characterized enzymes were also identified. Enzymes from Herpetosiphon aurantiacus and Flavobacterium johnsoniae resulted in high production of p-coumaric acid in Escherichia coli (producing 440 μM p-coumaric acid OD600 unit−1 in the medium) and in Lactococcus lactis. The enzymes were also efficient in Saccharomyces cerevisiae, where p-coumaric acid accumulation was improved 5-fold over that in strains expressing previously characterized tyrosine ammonia-lyases.

Production of 3-hydroxypropionic acid from glucose and xylose by metabolically engineered Saccharomyces cerevisiae
Biomass, the most abundant carbon source on the planet, may in the future become the primary feedstock for production of fuels and chemicals, replacing fossil feedstocks. This will, however, require development of cell factories that can convert both C6 and C5 sugars present in lignocellulosic biomass into the products of interest. We engineered Saccharomyces cerevisiae for production of 3-hydroxypropionic acid (3HP), a potential building block for acrylates, from glucose and xylose. We introduced the 3HP biosynthetic pathways via malonyl-CoA or β-alanine intermediates into a xylose-consuming yeast. Using controlled fed-batch cultivation, we obtained 7.37±0.17 g 3HP L−1 in 120 hours with an overall yield of 29±1% Cmol 3HP Cmol−1 xylose. This study is the first demonstration of the potential of using S. cerevisiae for production of 3HP from the biomass sugar xylose.
Development of new USER-based cloning vectors for multiple genes expression in Saccharomyces cerevisiae

Saccharomyces cerevisiae is one of the most widely used cell factory in industrial biotechnology and it is used for the production of fuels, chemicals, food ingredients, food and beverages, and pharmaceuticals. Such bioprocesses frequently require multiple rounds of metabolic engineering to obtain the production strain with the proper phenotype and product yield. However, the sequential number of metabolic engineering is time-consuming. Furthermore, the number of available selectable markers is also limiting the number of genetic modifications. To overcome these limitations, we have developed a new set of shuttle vectors for convenience of use for high-throughput cloning and selectable marker recycling. The new USER-based cloning vectors consist of a unique USER site and a CRE-loxP-mediated marker recycling system. The USER site allows insertion of genes of interest along with a bidirectional promoter of choice into the vector backbone with time- and cost-effective. The selectable marker cassette is flanked by loxP recognition sites for the CreA recombinase to allow reutilization of the same selectable marker. Furthermore, our USER vector set provides a choice of different selectable markers both auxotrophic and dominant markers for convenience of use. Our vector set also contains both integrating and multicopy vectors for stability of protein expression and high expression level. We will make the new vector system available to the yeast community and provide a comprehensive protocol for cloning in these vectors using USER cloning strategy.

USER-based vector set for high-throughput cloning and metabolic engineering in Saccharomyces cerevisiae

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering, CFB - Core Flow, iLoop, Applied Metabolic Engineering, Technical University of Denmark
Pages: 98
Publication date: 2013

Host publication information
Title of host publication: Proceedings of SB6.0 : The Sixth International Meeting on Synthetic Biology
Article number: PA-138
URLs:
Source: PublicationPreSubmission
Source-ID: 118364009
Research output: Chapter in Book/Report/Conference proceeding › Conference abstract in proceedings – Annual report year: 2013 › Research › peer-review

General information
Publication status: Published
Organisations: Novo Nordisk Foundation Center for Biosustainability, Research Groups, Yeast Metabolic Engineering, CFB - Core Flow, Applied Metabolic Engineering, Department of Systems Biology, Eucaryotic Molecular Cell Biology, iLoop