Metabolic Engineering Communications 11 (2020) e00134

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

Metabolically engineered recombinant *Saccharomyces cerevisiae* for the production of 2-Deoxy-*scyllo*-inosose (2-DOI)

Ahmed J. Al-Fahad [a], Mohamed B. Al-Fageeh [a], Najeh M. Kharbatia [b], Salim Sioud [b], Radhakrishnan Mahadevan [c,*]

[a] National Center of Biotechnology, King Abdulaziz City for Science and Technology (KACST), Riyadh, 11442, Saudi Arabia
[b] Analytical Chemistry Core Laboratory, King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia
[c] Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON, M5S 3E5, Canada

ARTICLE INFO

Keywords:
2-Deoxy-*scyllo*-inosose
2-Deoxy-*scyllo*-inosose synthase
Scyllo-quercitol
(−)-*Vibo*-quercitol

ABSTRACT

Saccharomyces cerevisiae is a versatile industrial host for chemical production and has been engineered to produce efficiently many valuable compounds. 2-Deoxy-*scyllo*-inosose (2-DOI) is an important precursor for the biosynthesis of 2-deoxystreptamine-containing aminoglycosides antibiotics and benzenoid metabolites. Bacterial and cyanobacterial strains have been metabolically engineered to generate 2-DOI; nevertheless, the production of 2-DOI using a yeast host has not been reported. Here, we have metabolically engineered a series of CEN.PK yeast strains to produce 2-DOI using a synthetically yeast codon-optimized *btrC* gene from *Bacillus circulans*. The expression of the 2-Deoxy-*scyllo*-inosose synthase (2-DOIS) gene was successfully achieved *via* an expression vector and through chromosomal integration at a high-expression locus. In addition, the production of 2-DOI was further investigated for the CEN.PK knockout strains of phosphoglucose isomerase *(Δpgi1)*, D-glucose-6-phosphate dehydrogenase *(Δzwf1)* and a double mutant *(Δpgi1, Δzwf1)* in a medium consisting of 2% fructose and 0.05% glucose as a carbon source. We have found that all the recombinant strains are capable of producing 2-DOI and reducing it into *scyllo*-quercitol and (−)-*vibo*-quercitol. Comparatively, the high production of 2-DOI and its analogs was observed for the recombinant CEN.PK-btrC carrying the multicopy *btrC*-expression vector. GC/MS analysis of culture filtrates of this strain showed 11 times higher response in EIC for the *m/z* 479 (methyloxime-tetra-TMS derivative of 2-DOI) than the YP-btrC recombinant that has only a single copy of *btrC* expression cassette integrated into the genomic DNA of the CEN.PK strain. The knockout strains namely Δpgi1-btrC *and* Δpgi1Δzwf1-btrC, that are transformed with the *btrC*-expression plasmids, have inactive Pgi1 and produced only traces of the compounds. In contrast, Δzwf1-btrC recombinant which has intact *pgi1* yielded relatively higher amount of the carbocyclic compounds. Additionally, ^{1}H-NMR analysis of samples showed slow consumption of fructose and no accumulation of 2-DOI and the quercitols in the culture broth of the recombinant CEN.PK-btrC suggesting that *S. cerevisiae* is capable of assimilating 2-DOI.

1. Introduction

Kanamycin and other related antibiotics have become a drug of choice for several diseases including the multi-drug resistant Tuberculosis. An important intermediate for the production of 2-deoxystreptamine containing aminoglycosides antibiotics such as Kanamycin, Hygromycin and Butirosin is 2-DOI(Kudo et al., 1999a). Hence, the production of 2-DOI is of significant interest. The six-membered carbocyclic compound is a valuable starting material, since it be easily converted into a variety of benzoid and aromatic derivatives(Hansen and Frost, 2002; Kakinuma et al., 2000). However, currently, chemical routes have very low yield of 2-DOI and require multistep reactions, hazardous chemicals and expensive reagents(Yamauchi and Kakinuma, 1992; Yu and Spencer, 2001). In contrast, synthetic biology and metabolic engineering approaches have several advantages for the production of 2-DOI including selectivity and the ability to obtain high conversion yields.

The formation of 2-DOI from D-glucose-6-phosphate(G6P) is the first step in the biosynthesis of aminoglycosides and this pathway to kanamycin and other derivatives has been recently mapped(Kharel et al., 2004). This important step is catalyzed by a 2-Deoxy-*scyllo*-inosose

* Corresponding author.
E-mail address: krishna.mahadevan@utoronto.ca (R. Mahadevan).

https://doi.org/10.1016/j.mec.2020.e00134
Received 14 October 2019; Received in revised form 22 May 2020; Accepted 23 May 2020

synthase (2-DOIS) which accelerates an intramolecular C–C bond formation and cyclization of the precursor G6P. (Furumai et al., 1979; Kakinuma et al., 1989; Kudo et al., 1997; Rinehart Jr and Stroshane, 1976; Yamauchi and Kakinuma, 1992, 1995bib_Yamauchi_and_Kakinuma_1992bib_Yamauchi_and_Kakinuma_1995). In particular, the 2-DOIS (*btrC*) from the butirosin producer *Bacillus circulans* SANK 72073 was firstly overexpressed in *Escherichia coli* and structurally characterized(Kudo et al., 1999b). The stability of BtrC has encouraged researchers to exploit this enzyme for heterologous expression and 2-DOI high production. Consequently, recombinant microorganisms such as *E. coli* and the cyanobacterium *Synechococcus elongatus PCC 7942* have been able to produce high amount of 2-DOI *via* the cloning and overexpression of *btrC*. Interestingly, 99% biotransformation of D-glucose to 2-DOI was achieved by a *btrC*-recombinant *E. coli* strain carrying the knockout of *pgi*, *zwf*, and *pgm* genes which encode phosphoglucose isomerase, G6P dehydrogenase, and phosphoglucomutase respectively and using a medium supplemented with mannitol and D-glucose as carbon source(Kogure et al., 2007). More recently, *btrC* was successfully expressed in the cyanobacterium *Synechococcus elongatus* PCC 7942. Without using a carbon source, a recombinant of *S. elongatus* was able to produce 400 mg/L of 2-DOI directly from CO_2(Watanabe et al., 2018). Another example for a high titer of 2-DOI production has been reported after the disruption of the genes encoding the isomerase (*pgi*) and the phosphoglucomutase *(pgcA)* in *Bacillus subtilis* and using the knockout strains for heterologous expressions of 2-DOIS genes. For example, the natural *btrC* gene and a codon optimized *tobC* gene from *Streptomyces tenebrarius* were separately introduced into the double knockout strain of *B. subtilis* to produce 2.3 and 37.2 g/L of 2-DOI, respectively. The production of 2-DOI was further improved by adding a dual carbon source glycerol to yield 38.0 g/L(Lim et al., 2018)

Although 2-DOI has been successfully generated by metabolically engineered bacterial and cyanobacterial strains, no attempt has been reported for the production of 2-DOI using recombinant yeast strains. *S. cerevisiae* is a safe microorganism and shows fast adaptation to pH changes and inhibitors (Borodina and Nielsen, 2014). In addition, *S. cerevisiae* is an aminoglycoside-resistant and has been used as a model system to study genes corresponding to aminoglycosides interaction(Prezant et al., 1996). In this study, we constructed recombinants CEN.PK and knockout strains of *S. cerevisiae* to produce 2-DOI using a codon optimized and synthesized *btrC*. The production of 2-DOI was achieved and evaluated for all the recombinants strains on a medium containing mainly fructose as a carbon source. Multicopy *btrC* expression plasmid in CEN.PK (CEN.PK-btrC) yielded higher amount of 2-DOI (11 fold increase) than the mutant YP-btrC which has one single copy of the *btrC* expression cassette chromosomally integrated. We found that the CEN.PK strain has a 2-Deoxy-*scyllo*-inosose reductase (2-DOIR) that converts 2-DOI into *scyllo*-quercitol and (−)-*vibo*-quercitol. The knockout strains, especially the recombinants Δpgi1-btrC and Δpgi1Δzwf1-btrC which have the disruption of *pgi1* produced traces of the 2-DOI and its analogs. Among the knockout strains, the recombinant Δzwf1-btrC produced the highest amounts of the compounds. This is the first report for the heterologous expression of *btrC* and the production of 2-DOI and its reduced derivatives in a yeast host. These intermediates are industrially important precursors for antibiotics, cosmetic agents and other useful chemicals. Our work would provide an alternative route than the shikimate pathway and pave the way for further metabolic engineering of these aminoglycoside intermediates in yeast.

2. Material and methods

2.1. Strains and culture conditions

The strains used in this study were DH5α *E. coli* (New England Biolabs) which was used for cloning and plasmid amplification. The bacterial cells were grown at 37 °C in Luria–Bertani (LB) broth medium supplemented with 100 μg/mL of carbenicillin. *S. cerevisiae* strain CEN.PK 116A (ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; Mata) was used as a host. For yeast competent cells preparation, yeast strains were routinely cultivated in 2X Yeast extract Peptone Dextrose (2XYPD) medium or Yeast extract Peptone Fructose (2XYFD) medium and at 30 °C. In addition, yeast synthetic dropout medium supplements without the appropriate auxotrophic markers, 2% fructose and 0.05% glucose were used for selection and 2-DOI production.

2.2. Gene knockout

The knockout of the targeted genes was preformed using bipartite gene-targeting strategy(Catlett et al., 2003). A plasmid consist of *ura3* auxotrophic marker (pRSII426 was a gift from Steven Haase, Addgene plasmid # 35470)(Chee and Haase, 2012) was purchased from Addgene and used as a template for PCR to generate two DNA fragments. High-Fidelity KOD Xtreme™ Hot Start DNA Polymerase (Merck Millipore) was used for PCR amplification of the construction of the knockout cassettes and the expression plasmid. Primers were ordered from (Eurofins Genomics).

Firstly, the selectable marker was split into two parts using PCR oligonucleotides which were intended to generate the two parts of DNA with an overlapping nucleotide sequence (494 bp) from the *ura3* gene. The first part was called (5'ura) which sized 887 bp and contains the *ura3* promoter and around three quarters upstream of the *ura3* gene, whereas the second part (3'ura) had a size of 800 bp including truncated *ura3* gene and its terminator. The two amplicons were flanked by *loxP* sites using designed primers F-loxP-5'ura3 and R-3'ura3-loxP (Table 1).(Güldener et al., 1996) To generate the disruption cassette for *pgi1* and *zwf1* around 850 bp of the homologous regions of the targeted gene were linked to the two inactive marker parts *via* fusion PCR.

Before yeast transformation, 1.5 μg of each part of the disruption cassette was generated and cleaned with Monarch PCR & DNA Cleanup Kit. LiAc/SS carrier DNA/PEG transformation procedure was performed as the protocol described by Gietz and his colleagues(Gietz et al., 1995). All the knockout transformants were then selected on uracil yeast synthetic drop-out media containing 2% fructose 0.05% glucose(Aguilera, 1987). A number of mutants from each knockouts were subjected to diagnostic PCR to confirm the right disruptions of the targeted genes. The ura3 marker was then removed by growing the knockout strains on 2XYFD liquid medium for an overnight. Cells of 100 μl cultures were collected by centrifugation and washed then streaked on plates containing 5-fluoroorotic acid for counterselection(Güldener et al., 1996). The double knockouts *Δpgi1Δzwf1* was preformed following the same procedure of disrupting *pgi1* on the *Δzwf1* mutant that had been its selection marker eliminated.

2.3. The expression of btrC

The 2-DOIS (*btrC*) gene was codon-optimized for *S. cerevisiae* expression and synthesized by GenScript (Piscataway, USA). To generate a promoter and a terminator for *btrc* expression, genomic DNA of *S. cerevisiae* was extracted following the procedure as described in (Lõoke et al., 2011). The Glyceraldehyde-3-phosphate dehydrogenase promoter (GAPp) and the alcohol dehydrogenase terminator (ADH1t) were PCR-amplified using primers listed in (Table 1). The primers of *btrC* amplification were designed to include tails from the promoter and the terminator nucleotide sequences. Then, fusion PCR technique was applied to join the three fragments. The expression construct was used for a direct chromosomal integration and the formation of the plasmid expression.

2.4. Chromosomal integration of btrC

To create a stable recombinant with a high chromosomal *btrc*-expression, one integration site (YPRCΔ15) of the *S. cerevisiae* genomic DNA was chosen. The YPRCΔ15 locus was reported to have a high

Table 1
Oligonucleotides used in this study.

Primer name	5' → 3' sequence
F-loxP-5'ura3	ATAACTTCGTATAATGTATGCTATACGAAGTTATcggcatcagagcagattgta
R-5'ura3	ccttgtcatctaaacccacac
F-3'ura3	ggagttagttgaagcattaggtc
R-3'ura3-loxP	ATAACTTCGTATAGCATACATTATACGAAGTTATcctgatgcggtattttctcc
F-5hom-pgi1	atgtccaataactcattcactaacttc
R-5hom-pgi1	ATACATTATACGAAGTTATgtaacgaccaccgaccc
F-3hom-pgi1	GCTATACGAAGTTATctgtctggtcggctattg
R-3hom-pgi1	gttcttaggtatatatttaagagcg
5check-pgi1	ggtcctttcttcataatcaatgcttt
3check-pgi1	cttggacgctgttcaataatgta
F-5hom-zwf1	cgaaaaaaataccgtcatatctgtctt
R-5hom-zwf1	ATACATTATACGAAGTTATgtctctgattatgcctatagagtcgaaa
F-3hom-zwf1	GCTATACGAAGTTATgtgatgcagaaccatctgttacaaa
R-3hom-zwf1	ccttcgtatcttctggcttagtcaCCTTCGTATCTTCTGGCTTAGTCA
3check-zwf1	gacttagccgataaatgaatgtgctt
F-GAP-P	tcagttcgagtttatcattatcaatac
R-GAP-P	tttgtttgtttatgtgtgtttattcg
F-ADH1-T	gcgaatttcttatgatttatg
R-ADH1-T	gaaatggggagcgatttgc
F-btrC	cgaataaacacacataaacaaacaaaATGACTACAAAGCAAATCTG
R-btrC	cataaatcataagaaattcgcTTATAAACCTTCTCTAATAACTTC
F-GAP-*Not*I	AATATAATTTAAgcggccgctcagttcgagtttatcattatcaatac
R-ADH1-*Xho*I	ATTATTAATATAcctcgaggaaatggggagcgatttgc
R-5YPRC15-UNS1	cgagacagcctgagaatggatgcgagtaatgGGTAAACATTTCAACACACCGTT
F-UNS8-loxP	cctcgtctcaaccaaagcaatcaacccatcATAACTTCGTATAATGTAT
UNS1-UNS8	cattactcgcatccattctcaggctgtctcgcctcgtctcaaccaaagcaatcaacccatc
F-GAPp-UNS10	gttccttatcatctggcgaatcggacccactcagttcgagtttatcattatcaatac
R-loxP-UNS7	agtagcggaaatgtcagagccagcgtcttgATAACTTCGTATAGC
UNS7-UNS10	caagacgctggctctgacatttccgctactgttccttatcatctggcgaatcggacccac
R-ADH1t-3'YPCR15	TGAGTTGTTAGAGCTGTTACAAGTTACgaaatggggagcgatttgc
F-3'YPRC15-ADH1t	gcaaatcgctcccccatttcGTAACTTGTAACAGCTCTAACAACTCA
R-3'YPRC15	GACATCTATGAAACACCCATAAAGCA

transcription level(Bai Flagfeldt et al., 2009). For target integration, homologous recombination of three constructs namely 5'YPRC15-loxP-5'ura, 3'ura3-loxP-GAPp-btrC-ADH1t and ADHt-3'YPRC15 was needed (Fig. 1). A homologous region (5'YPRC15) from the YPRCΔ15 sequence was produced by PCR using primers of which the reverse primer had a tail of (UNS8) a unique nucleotide sequences (UNS). Another UNS1 was introduced to the loxP-5'ura part by PCR using a forward primer(Torella et al., 2014). The two amplicons were linked together using UNS bridges and Ligase Cycling Reaction (LCR) technique(de Kok et al., 2014). The second part was constructed using the same LCR technique. The reaction mixtures were used as templets for PCR amplification. The third fragment was easily linked using fusion PCR. 1μMole of each construct was generated and transformed into CEN.PK strain competent cells. A number of colonies were picked and PCR-diagnosed for the right integration. The ura3 marker was looped-out after growing on rich medium.

2.5. Plasmid construction

An expression Yeast/E.coli shuttle vector (pRSII425) was a gift from Steven Haase (Addgene plasmid # 35468)(Chee and Haase, 2012). The *btrc* expression cassette was PCR amplified for subcloning using primers that were designed to introduce *Not*I site at the 5′ end of the GAPp and the *Xho*I at the 3' end of the ADH1t. The expression cassette was inserted into the multicloning site of the desired plasmid following the standard protocol (Fig. 1). All DNA fragments and the pRSII425 vector were cleaned by Monarch PCR & DNA Cleanup Kit (NEB) prior digestion with high-fidelity restriction endonucleases and ligation. Restriction enzymes, T4 DNA ligase were supplied from (New England Biolabs). In some cases, gel purifications were performed using Monarch DNA Gel Extraction Kit

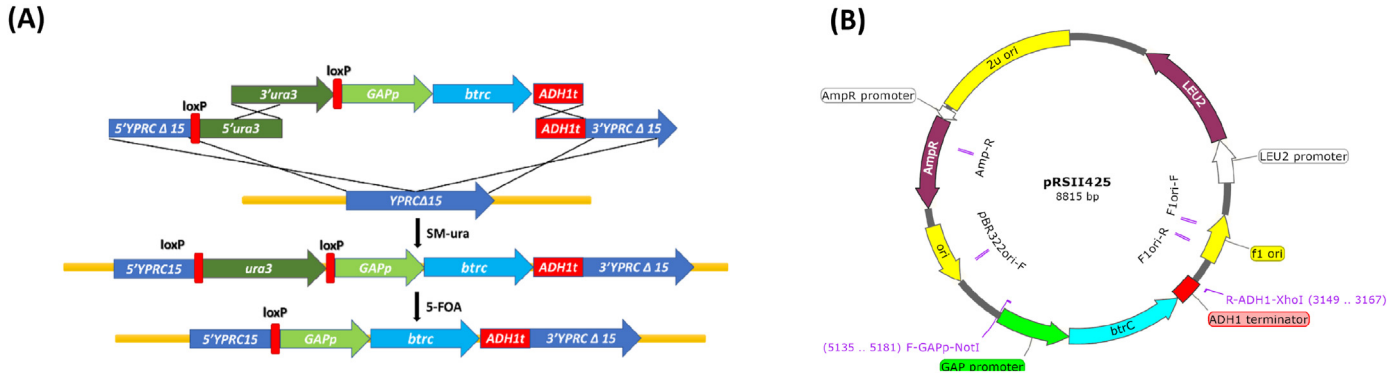

Fig. 1. An illustration for the expression of *btrC* codon-optimized **A)** The chromosmal integration of the *btrC* expression casstte into the YPRCΔ15 site. Three PCR products were generated for locus targeting and two crossover were needed for full integration of the expression cassette. The ura3 marker was looped-out and counterselection was done by growing on 5-fluoroorotic acid **B)** The cloning of *btrc* recombinant DNA into the *Not*I and *Xho*I sites of the expression Yeast/E.coli shuttle vector (pRSII425).

(NEB). The ligation reaction mixture was used for *E. coli* transformation to propagate the constructed plasmid. Ampicillin-resistant colonies were picked for diagnostic PCR and plasmid isolation was using the GeneJET Plasmid Miniprep Kit from (Thermo Fisher Scientific). Confirmation of insertion was done by DNA sequencing using the primers of the GAP promoter and the ADH1 terminator.

2.6. GC/MS analysis

2.6.1. Sample derivatization

5 ml of yeast culture were centrifuged at 30000 xg for 5 min at room temperature. The supernatant was then collected and filtered through a (Millex-GP, 0.22 μm) syringe filter. Accurately, 1 mL aliquot from each sample were transferred to 2 mL Eppendorf vials and evaporated in CentriVap concentrator (Labconco). 50 μL of 2% (wt/v) Methoxyamine HCl in pyridine (Pierce, USA) were added and samples were incubated in an oven at 60 °C for 1 h. All samples were left to cool down at room temperature and 100 μL of (N,O-Bis(trimethylsilyl)trifl uoroacetamide) with 1% (vol/vol) trimethylchlorosilane solution (Thermo Scientific, USA) BSTFA, derivatization agent, were added to each sample vial. The vials again were incubated at 60C for 30 min and centrifuge for 5 min at 10K rpm. Clear solution of each sample was transferred to Gas Chromatography (GC) vial deactivated inserts and injected into Gas chromatography–mass spectrometry GC/MS.

2.6.2. GC/MS instrument method

One microliter of the derivatized solution was analyzed using single quadrupole GC-MS system (Agilent 7890 GC/5975C MSD) equipped with EI source at ionization energy of 70 eV. The temperature of the ion source and mass analyzer was set to 230 °C and 150 °C, respectively, and solvent delay of 8.0 min. The mass analyzer was auto tuned according to the manufacturer's manual and the scan was set from 35 to 700 with scan speed 2 scans/s. Chromatography separation was performed using DB-5MS fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent J&W scientific, Folsom, CA), chemically bonded with 5% phenyl 95% methylpolysiloxane cross-linked stationary phase. Helium was used as the carrier gas with constant flow rate of 1.0 mL min^{-1}. The initial oven temperature was held at 80 °C for 4 min, then ramped to 300 °C at a rate of 6.0°Cmin^{-1}, and held at 300 °C for 10 min. The temperature of the GC inlet port and the transfer line to the MS source was kept at 200 °C and 320 °C, respectively. One microliter of the derivatized solution of the sample was injected into split/splitless inlet using an auto sampler equipped with 10 μL syringe. The GC inlet was operated under splitless mode.

2.6.3. Data analysis

Metabolites in all samples processed using Agilent MSD Chemstation software and were identified using NIST 14 database. The expected TMS-derivatized compounds were searched for manually in every chromatogram throughout the samples.

2.7. HPLC/HR-MS analysis

The analysis was performed using a Thermo LTQ Velos Orbitrap high resolution mass spectrometer (Thermo Scientific, Pittsburgh, PA, USA) equipped with a heated ESI ion source. The mass scan range was set to 100–2000 m/z, with a resolving power of 100 000. The m/z calibration of the LTQ-Orbitrap analyzer was performed in the positive ESI mode using a solution containing caffeine, MRFA (met-arg-phe-ala) peptide and Ultramark 1621 according to the manufacturer's guidelines. The ESI was performed with a heated ion source equipped with a metal needle and operated at 4 kV. The source vaporizer temperature was adjusted to 400 °C, the capillary temperature was set at 270 °C, and the sheath and auxiliary gases were optimized and set to 40 and 20 arbitrary units, respectively.

The separation of the biological extracts was performed using an Eclipse XDB C18 150 × 4.6 mm column packed with 5 μm particles size. The separation was achieved using a gradient composed of water/methanol. The mobile phase solvents were composed of A: 100% water + (0.1% formic acid) and B: 100% methanol + (0.1% formic acid). The gradient elution program is summarized in Table 1. The injection volume was 10 μL and the flow rate was set to 400 μL/min. XcaliburTM software (Thermo Scientific) was used for method development and data treatment.

2.8. Analysis of time-course ^{1}H-NMR

Erlenmeyer flasks 250 ml containing 50 ml of yeast synthetic dropout medium without leucine, 2% fructose 0.05% glucose were inoculated with the recombinant CEN.PK-btrC and a control strain CEN.PK (carrying the empty plasmid pRSII425). The non-baffled flasks were incubated at 30 °C and shacked at 250 rpm for 6 days (aerobic growth). After every 24 h, 1 ml was withdrawn from the two flasks, centrifuged, filtered and dried using nitrogen blow-down evaporator. All samples were then redissolved in 500 μl D2O and submitted for 1H-NMR experiment on a 600 MHz NMR spectrometer (JEOL).

3. Result & discussion

To achieve high production of 2-DOI, *btrC* gene was first codon-optimized for expression in *S. cerevisiae* in order to attain high and efficient protein expression. A strong and constitutive promoter GAP was selected to drive high expression level of 2-DOIS. The 2-DOI expression cassette was integrated into a high expression chromosomal site (YPRCΔ15)(Bai Flagfeldt et al., 2009). The resulting mutant was named YP-btrC. In addition, the construct was subcloned into a high-copy number 2 micro yeast vector (pRSII425) and introduced to CEN.PK *S. cerevisiae* and the knockout strains *Δpgi1*, Δzwf1 and the double knockout *Δpgi1Δzwf1*. Consequently, the recombinant strains were called CEN.PK-btrC, Δpgi1-btrC, Δzwf1-btrC and Δpgi1Δzwf1-btrC respectively.

After plasmid transformation and incubation for 3–4 days colonies appeared on fructose synthetic leucine dropout agar plates. The recombinant colonies had a distinct phenotype compared to the control colonies of CEN.PK strain transformed with the empty-plasmid (pRSII425). Specifically, the CEN.PK-btrC recombinant colonies had irregular shapes (Fig. 2.). All the recombinants were then cultured in 50 ml minimal yeast synthetic drop-out medium supplements without leucine and containing 2% fructose and 0.05% glucose. We decided to use this medium for a couple of reasons. The leucine drop-out would retain the expression vector and enhance the plasmid high copy number. The sugar content is optimal for the knockout strains carrying Δ*pgi1* as they cannot grow on glucose as a main carbon source(Aguilera, 1986). Unlike *E. coli*, the disruption of *pgi1* makes glucose toxic for *S. cerevisiae* since the mutant cannot efficiently reoxidize the accumulated NADPH and meet the need for $NADP^{+}$ in oxidative metabolism(Boles et al., 1993). Hence, the utilization of fructose as a main carbon source is necessary for the production of 2-DOI. The precursor D-glucose-6-phosphate (G6P) is a point branch for many of metabolic pathways. In case of growth on fructose, the main supply of G6P is through the conversion of D-fructose-6-phosphate (F6P) into G6P by the isomerase (Pgi1). Predictably, the recombinant knockout strains namely, Δpgi1-btrC and Δpgi1Δzwf1-btrC which have the isomerase inactive produce lower amounts of 2-DOI (Fig. 3.).

The knockout strains are slow-growing compare to the wild type and therefore, all strains were incubated at 30C° for 6 days along with the control strain carrying the empty plasmid without the btrc-expression cassette. The cultures filtrates were subjected to GC/MS analysis and metabolites were scanned and identified using NIST 14 database. A peak corresponding to 2-deoxy-*scyllo*-inosose methyloxime-tetrakis-O-trimethylsilyl (2DOI-MeOX-4TMS) derivative was detected at a retention time (*Rt*) of 25.4 min. The mass fingerprint matches exactly the reported EI spectra of 2-DOI in the literature (figSS1Supplementary Figs. S1

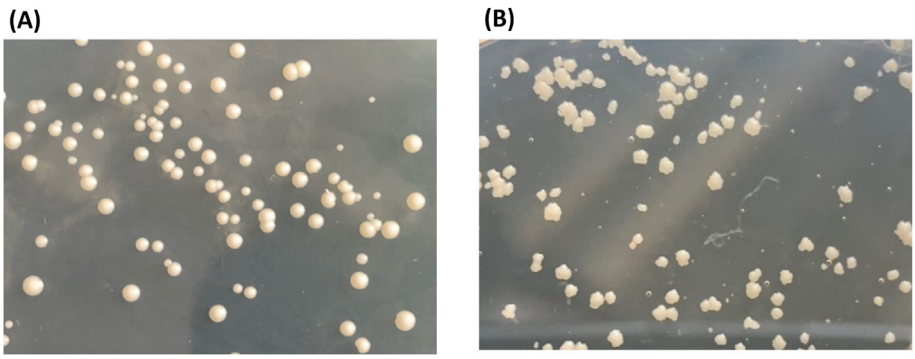

Fig. 2. The change in the phenotype of yeast colonies growing on fructose synthetic leucine dropout agar plates **A)** CEN.PK strain transformed with the empty-plasmid pRSII425, **B)** CEN.PK-*btrC* recombinant strain carrying *btrC* plasmid expression shows colonies with irregular shapes.

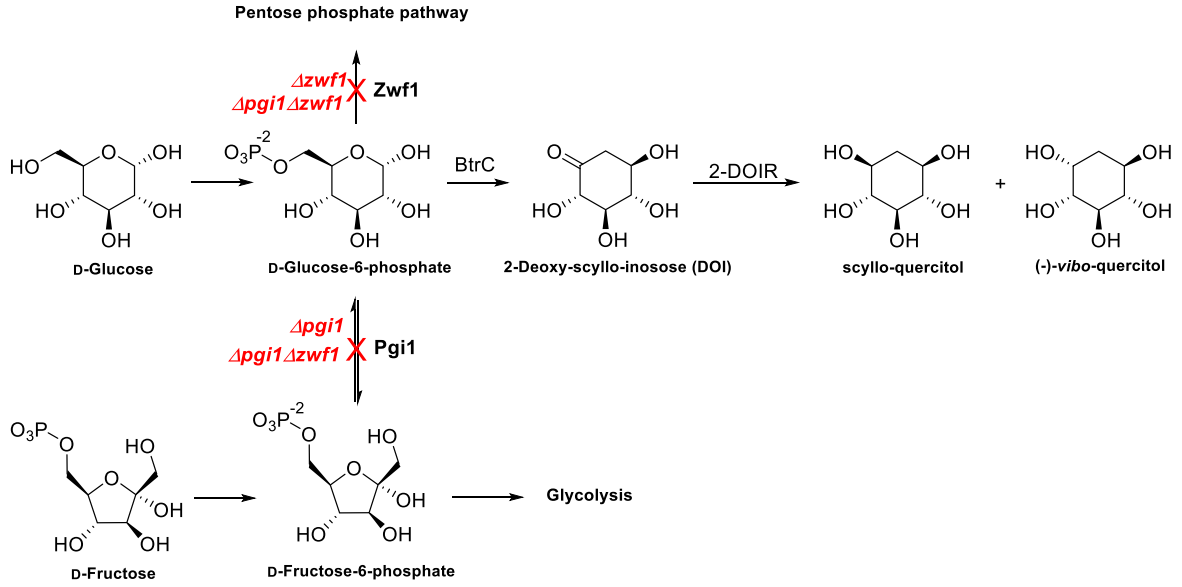

Fig. 3. Schematic presentation of the metabolic pathway of 2-DOI and its analogs and the effect of the genes knockout of *pgi1* and *zwf1*. Inactivation of Pgi1 reduce dramatically the production of 2-DOI from fructose.

and S2).(Ara et al., 2018) A comparative analysis between the control and all recombinant strains showed the absence of the peak in the control and Δpgi1-btrC metabolic profiles. Also, other peaks at retention times 27.3 and 28.8 min were present in the samples but not in the control (figSS3Supplementary Figs. S3 and S4). A close inspection into their EI spectra revealed similarity to the spectra corresponding to *(−)-vibo*--quercitol-pentakis-O-trimethylsilyl (VQ-5TMS) and *scyllo*-quercitol (SQ-5TMS) (Fig. 4)..(Ara et al., 2018)

Noticeably, the production of *scyllo*-quercitol was proportional to the 2-DOI production. On the other hand, *(−)-vibo*-quercitol could be only detected in the culture filtrates of the recombinants CEN.PK-btrC and Δzwf1-btrC. Since we did not have pure standards of the three compounds, the product evaluation for the recombinant strains was based on comparative analysis of the extracted ion chromatograms (EIC) of the peaks area count for the corresponding ions *m/z* 479 (2-DOI derivative) and 524 (*vibo* and *scyllo*-quercitol silylation derivatives). CEN.PK-btrC yielded the highest amount of the three compounds and around 11 times higher than YP-btrC which has only a single copy of the *btrC* expression construct. Additionally, Δzwf1-btrC had a considerable amount of 2-DOI with (7.5 fold more) among the knockout strains, whereas the mutant Δpgi1Δzwf1-btrC showed only a trace (Fig. 5.)

Further confirmation was obtained from the HPLC/HR-MS comparative analysis. The HPLC/HR-MS analysis came in agreement with the GC/MS results. The culture filtrates of all the recombinants and the control were analyzed using a high resolution LTQ-Orbitrap analyzer in the positive ESI mode. Extracted ion chromatogram of the sodiated *scyllo*-quercitol and *(−)-vibo*-quercitol ions $[M+Na]^+$ at *m/z* 187.05824 for all the recombinant strains samples against the control of CEN.PK strain carrying pRSII425 empty plasmid showed that the compounds eluted at *Rt* 3.7 min. The peak of the compounds was present at different levels in all the recombinant strain samples but absent in the control (Fig. 6.)

Our result showed the successful overexpression of the yeast codon-optimized 2-DOIS gene (*btrC*) and the production of 2-DOI by metabolically engineered *S. cerevisiae* strains. For all producing recombinant strains, *S. cerevisiae* was able to convert 2-DOI into its reduced analogs *scyllo*-quercitol and *(−)-vibo*-quercitol. Similarly, 116 yeast strains were found to assimilate 2-DOI and one of which was reported to reduce 2-DOI into *scyllo*-quercitol and *(−)-vibo*-quercitol.(Ara et al., 2018). The strong production was observed by CEN.PK-btrC carrying the multicopy expression vector compare to the recombinant YP-btrC which has only one copy of the *btrC* expression cassette. The recombinant knockouts namely Δpgi1-btrC and Δpgi1Δzwf1-btrC produced only traces since the precursor G6P was limited by the in activation of Pgi1. Δzwf1-btrC, on the other hand, produces noticeable amounts of the compounds but not as high as the CEN.PK-btrC. This may be attributed to the fact that Zwf1 plays the main source for the cellular NADPH and the disruption of ZWF1

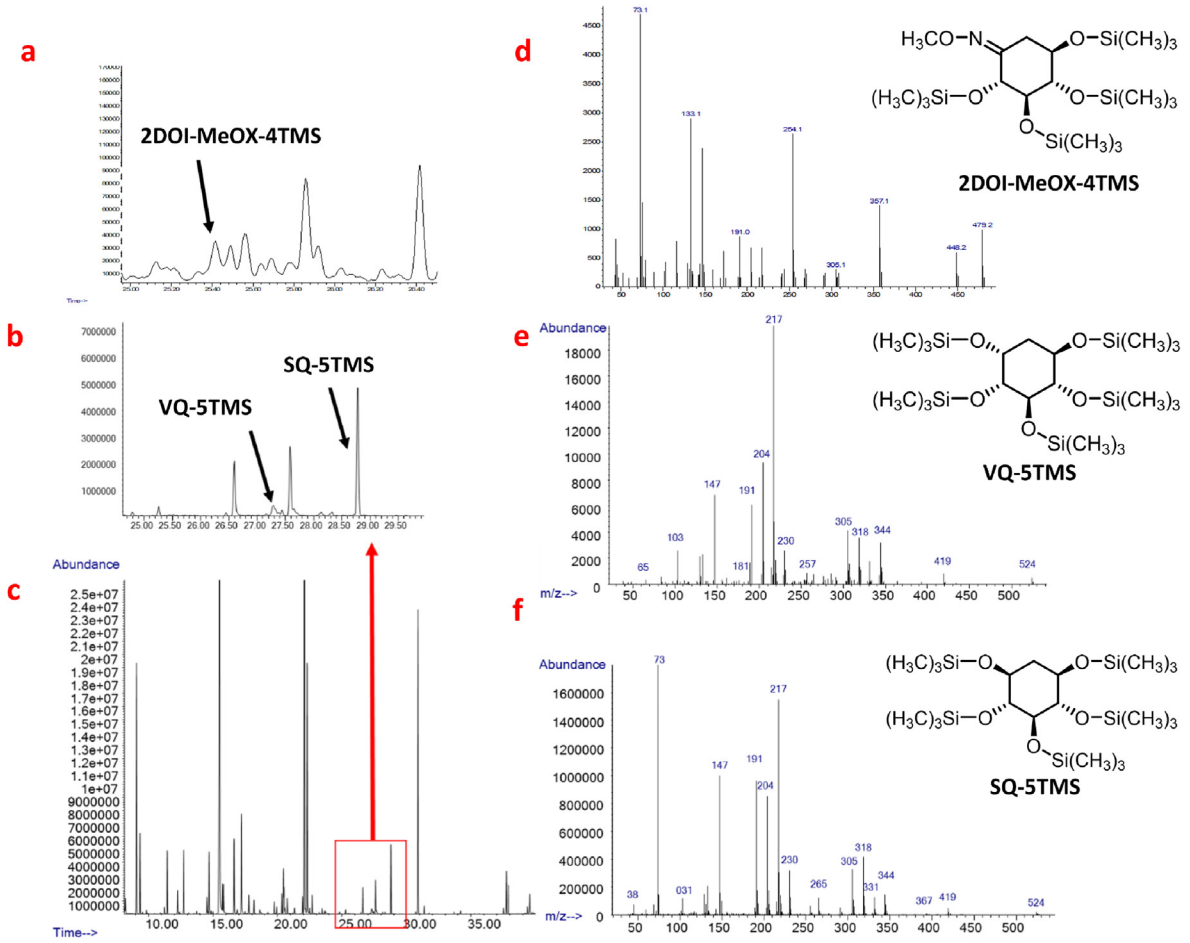

Fig. 4. The figure shows a GC/MS chromatogram of CEN.PK-btrC chemical profile **(c)** demonstrating the retention times for the three compounds and their EI mass spectra. The identification of these metabolites was confirmed using NIST 14 database and by matching the MS spectrum with literature. **a)** The methyloxime-tetra-TMS derivative of 2-DOI was detected at *Rt* 25.4 min. **b)** The silylation derivatives of *vibo* and *scyllo*-quercitol were eluted at *Rt* 27.3 min and 28.8 min respectively. **d)** The EI spectra of 2DOI-MeOX-4TMS *m/z* 479 $M^{+\bullet}$. **e)** The EI spectra of VQ-5TMS *m/z* 524 $M^{+\bullet}$. **F)** The EI spectra of SQ-5TMS *m/z* 524 $M^{+\bullet}$.

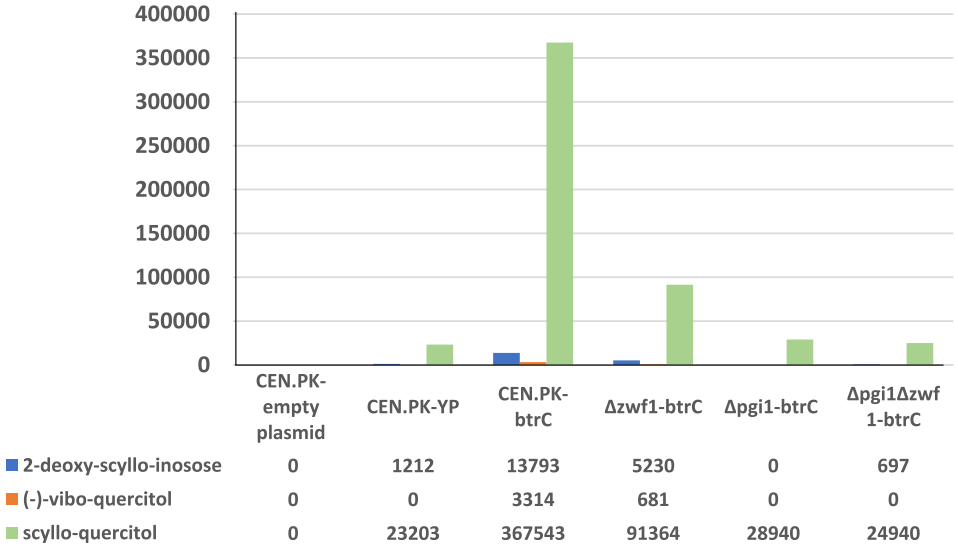

Fig. 5. The extracted ion chromatograms (EIC) of the peaks area count comparative analysis of the corresponding ions m/z 479 (methyloxime-tetra-TMS derivative of 2-DOI) and m/z 524 (*vibo* and *scyllo*-quercitol the silylation derivatives). CEN.PK-btrC yielded the highest amount of the three compounds and around 11 times higher than YP-btrC. The strain Δzwf1-btrC had a considerable amount of 2-DOI with (7.5 fold more) among the knockout strains, whereas the mutant Δpgi1Δzwf1-btrC showed only a trace.

perhaps causes NADPH starvation. (Castegna et al., 2010; Minard and McAlister-Henn, 2005). As a result any discrepancy in $NADP^+$/NADPH ratio will impact metabolic network and profile(Celton et al., 2012). The double mutant *Δzwf1, Δpgi1* was not able to grow on glucose and grows very slowly on fructose compared to the other single knockout mutants. In addition, the introduction of *btrc* plasmid expression significantly

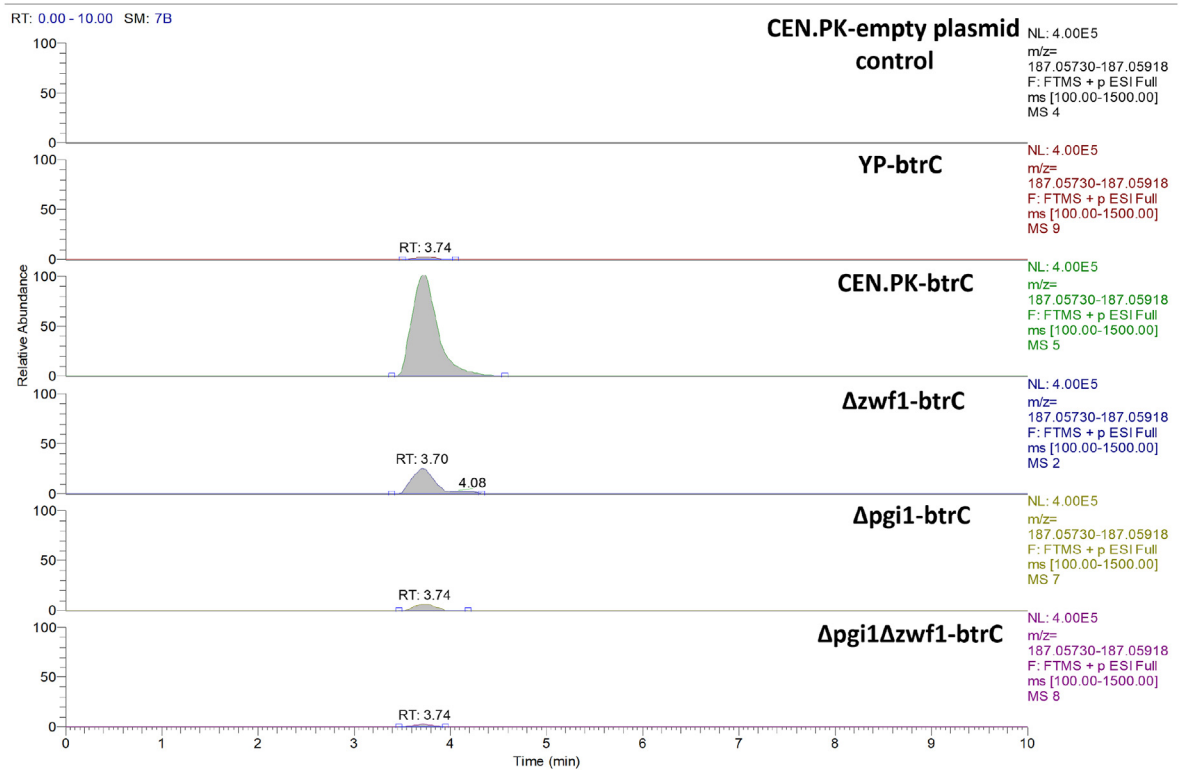

Fig. 6. HPLC/HRMS comparative analysis of extracted ion chromatograms of the sodiated *scyllo*-quercitol and (−)-*vibo*-quercitol ions $[M+Na]^+$ at *m/z* 187.05824 for all the recombinant strains samples against a control of CEN.PK strain carrying pRSII425 empty plasmid. The compounds eluted at RT 3.7 min and present variably in all the recombinant strain samples but absent in the control.

affected the fitness of the recombinant Δpgi1Δzwf1-btrC and leading to lower amounts of 2-DOI. ^{1}H-NMR comparative analysis of samples from the time-course of the recombinant CEN.PK-btrC showed slow metabolism of fructose over four days and no accumulation of 2-DOI and its reduced analogs whereas, complete consumption of fructose was observed after 48h for the control (figSS5Supplementary Figs. S5 and S6). Like many yeast strains, it is highly plausible that *S. cerevisiae* is capable of assimilating 2-DOI.

4. Conclusions

In this study, we have integrated the first step in the production of aminoglycosides, namely, the 2-DOIS gene from *Bacillus circulans* into *S. cerevisiae* CEN.PK strains and have demonstrated the production of 2-DOI along with its reduced analogs namely, *scyllo*-quercitol and (−)-*vibo*-quercitol. In particular, additional deletions to enhance the flux of glucose into this pathway resulted in lower yields of 2-DOI production due to changes in the redox metabolism and to the centrality of D-glucose-6-phosphate (G6P) intermediate for cellular growth. Although the knockout of the phosphoglucose isomerase and D-glucose-6-phosphate dehydrogenase genes did not improve the production yield of 2-DOI and quercitols, the deletion of the responsible genes for 2-DOI reduction would improve the production of 2-DOI. This result strongly suggests the assimilation of the cyclitols by *S. cerevisiae*, since 2-DOI and its quercitol products were not accumulated by the recombinant CEN.PK-btrC, which also demonstrated slow consumption of fructose, Nevertheless, this study provides an avenue for the further understanding of 2-DOI metabolism and optimization of this pathway through the use of computational strain design tools (Maia et al., 2016) and possibly the use of dynamic control strategies (Gupta et al., 2017; Venayak et al., 2015, 2018bib_Venayak_et_al_2015bib_Venayak_et_al_2018) to manage the transition between growth and production of this compound.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the Infectious Diseases Program, National Center for Biotechnology, King Abdulaziz City for Science and Technology (KACST) for the financial and intellectual support and Mohammed Alarawi (KAUST). Prof. Radhakrishnan Mahadevan would like to acknowledge funding from the Grand Challenges Canada program for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00134.

References

Aguilera, A., 1986. Deletion of the phosphoglucose isomerase structural gene makes growth and sporulation glucose dependent in Saccharomyces cerevisiae. Mol. Gen. Genet. 204, 310–316. https://doi.org/10.1007/bf00425515.

Aguilera, A., 1987. Mutations suppressing the effects of a deletion of the phosphoglucose isomerase gene PGI1 in Saccharomyces cerevisiae. Curr. Genet. 11, 429–434. https://doi.org/10.1007/BF00384603.

Ara, S., Yamazaki, H., Takaku, H., 2018. Isolation of 2-deoxy-scyllo-inosose (DOI)-assimilating yeasts and cloning and characterization of the DOI reductase gene of Cryptococcus podzolicus ND1. J. Biosci. Bioeng. 125, 397–406. https://doi.org/10.1016/j.jbiosc.2017.10.019.

Bai Flagfeldt, D., Siewers, V., Huang, L., Nielsen, J., 2009. Characterization of chromosomal integration sites for heterologous gene expression in Saccharomyces cerevisiae. Yeast 26, 545–551. https://doi.org/10.1002/yea.1705.

Boles, E., Lehnert, W., Zimmermann, F.K., 1993. The role of the NAD-dependent glutamate dehydrogenase in restoring growth on glucose of a Saccharomyces

cerevisiae phosphoglucose isomerase mutant. Eur. J. Biochem. 217, 469–477. https://doi.org/10.1111/j.1432-1033.1993.tb18266.x.

Borodina, I., Nielsen, J., 2014. Advances in metabolic engineering of yeast Saccharomyces cerevisiae for production of chemicals. Biotechnol. J. 9, 609–620. https://doi.org/10.1002/biot.201300445.

Castegna, A., Scarcia, P., Agrimi, G., Palmieri, L., Rottensteiner, H., Spera, I., Germinario, L., Palmieri, F., 2010. Identification and functional characterization of a novel mitochondrial carrier for citrate and oxoglutarate in Saccharomyces cerevisiae. J. Biol. Chem. 285, 17359–17370. https://doi.org/10.1074/jbc.M109.097188.

Catlett, N.L., Lee, B.-N., Yoder, O., Turgeon, B.G., 2003. Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet. Rep. 50, 9–11. https://doi.org/10.4148/1941-4765.1150.

Celton, M., Goelzer, A., Camarasa, C., Fromion, V., Dequin, S., 2012. A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in Saccharomyces cerevisiae. Metab. Eng. 14, 366–379. https://doi.org/10.1016/j.ymben.2012.03.008.

Chee, M.K., Haase, S.B., 2012. New and redesigned pRS plasmid shuttle vectors for genetic manipulation of Saccharomyces cerevisiae. G3 Genes Genom. Genet. 2, 515–526. https://doi.org/10.1534/g3.111.001917.

de Kok, S., Stanton, L.H., Slaby, T., Durot, M., Holmes, V.F., Patel, K.G., Platt, D., Shapland, E.B., Serber, Z., Dean, J., Newman, J.D., Chandran, S.S., 2014. Rapid and reliable DNA assembly via ligase cycling reaction. ACS Synth. Biol. 3, 97–106. https://doi.org/10.1021/sb4001992.

Furumai, T., Takeda, K., Kinumaki, A., Ito, Y., Okuda, T., 1979. Biosynthesis of butirosins. II. Biosynthetic pathway of butirosins elucidated from cosynthesis and feeding experiments. J. Antibiot. (Tokyo) 32, 891–899. https://doi.org/10.7164/antibiotics.32.891.

Gietz, R.D., Schiestl, R.H., Willems, A.R., Woods, R.A., 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11, 355–360. https://doi.org/10.1002/yea.320110408.

Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., Hegemann, J.H., 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. 24, 2519–2524. https://doi.org/10.1093/nar/24.13.2519.

Gupta, A., Reizman, I.M., Reisch, C.R., Prather, K.L., 2017. Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nat. Biotechnol. 35, 273–279. https://doi.org/10.1038/nbt.3796.

Hansen, C.A., Frost, J.W., 2002. Deoxygenation of polyhydroxybenzenes: an alternative strategy for the benzene-free synthesis of aromatic chemicals. J. Am. Chem. Soc. 124, 5926–5927. https://doi.org/10.1021/ja0176346.

Kakinuma, K., Nango, E., Kudo, F., Matsushima, Y., Eguchi, T., 2000. An expeditious chemo-enzymatic route from glucose to catechol by the use of 2-deoxy-scyllo-inosose synthase. Tetrahedron Lett. 41, 1935–1938. https://doi.org/10.1016/S0040-4039(00)00064-2.

Kakinuma, K., Ogawa, Y., Sasaki, T., Seto, H., Otake, N., 1989. Mechanism and stereochemistry of the biosynthesis of 2-deoxystreptamine and neosamine C. J. Antibiot. (Tokyo) 42, 926–933. https://doi.org/10.7164/antibiotics.42.926.

Kharel, M.K., Subba, B., Basnet, D.B., Woo, J.S., Lee, H.C., Liou, K., Sohng, J.K., 2004. A gene cluster for biosynthesis of kanamycin from Streptomyces kanamyceticus: comparison with gentamicin biosynthetic gene cluster. Arch. Biochem. Biophys. 429, 204–214. https://doi.org/10.1016/j.abb.2004.06.009.

Kogure, T., Wakisaka, N., Takaku, H., Takagi, M., 2007. Efficient production of 2-deoxy-scyllo-inosose from d-glucose by metabolically engineered recombinant Escherichia coli. J. Biotechnol. 129, 502–509. https://doi.org/10.1016/j.jbiotec.2007.01.016.

Kudo, F., Hosomi, Y., Tamegai, H., Kakinuma, K., 1999a. Purification and characterization of 2-deoxy-scyllo-inosose synthase derived from Bacillus circulans. A crucial carbocyclization enzyme in the biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics. J. Antibiot. (Tokyo) 52, 81–88. https://doi.org/10.7164/antibiotics.52.81.

Kudo, F., Tamegai, H., Fujiwara, T., Tagami, U., Hirayama, K., Kakinuma, K., 1999b. Molecular cloning of the gene for the key carbocycle-forming enzyme in the biosynthesis of 2-deoxystreptamine-containing aminocyclitol antibiotics and its comparison with dehydroquinate synthase. J. Antibiot. (Tokyo) 52, 559–571. https://doi.org/10.7164/antibiotics.52.559.

Kudo, F., Yamauchi, N., Suzuki, R., Kakinuma, K., 1997. Kinetic isotope effect and reaction mechanism of 2-deoxy-scyllo-inososse synthase derived from butirosin-producing Bacillus circulans. J. Antibiot. (Tokyo) 50, 424–428. https://doi.org/10.7164/antibiotics.50.424.

Lim, J.H., Hwang, H.H., Lee, N.J., Lee, J.W., Seo, E.G., Son, H.B., Kim, H.J., Yoon, Y.J., Park, J.W., 2018. Enhanced biosynthesis of 2-Deoxy-scyllo-inososse in metabolically engineered Bacillus subtilis recombinants. Front. Microbiol. 9, 2333. https://doi.org/10.3389/fmicb.2018.02333.

Lõoke, M., Kristjuhan, K., Kristjuhan, A., 2011. Extraction of genomic DNA from yeasts for PCR-based applications. Biotechniques 50, 325–328. https://doi.org/10.2144/000113672.

Maia, P., Rocha, M., Rocha, I., 2016. In silico constraint-based strain optimization methods: the quest for optimal cell factories. Microbiol. Mol. Biol. Rev. : MMBR (Microbiol. Mol. Biol. Rev.) 80, 45–67. https://doi.org/10.1128/MMBR.00014-15.

Minard, K.I., McAlister-Henn, L., 2005. Sources of NADPH in yeast vary with carbon source. J. Biol. Chem. 280, 39890–39896. https://doi.org/10.1074/jbc.M509461200.

Prezant, T.R., Chaltraw Jr., W.E., Fischel-Ghodsian, N., 1996. Identification of an overexpressed yeast gene which prevents aminoglycoside toxicity. Microbiology (Reading, England) 142 (Pt 12), 3407–3414. https://doi.org/10.1099/13500872-142-12-3407.

Rinehart Jr., K., Stroshane, R.M., 1976. Biosynthesis of aminocyclitol antibiotics. J. Antibiot. (Tokyo) 29, 319–353. https://doi.org/10.7164/antibiotics.29.319.

Torella, J.P., Lienert, F., Boehm, C.R., Chen, J.-H., Way, J.C., Silver, P.A., 2014. Unique nucleotide sequence–guided assembly of repetitive DNA parts for synthetic biology applications. Nat. Protoc. 9, 2075. https://doi.org/10.1038/nprot.2014.145.

Venayak, N., Anesiadis, N., Cluett, W.R., Mahadevan, R., 2015. Engineering metabolism through dynamic control. Curr. Opin. Biotechnol. 34, 142–152. https://doi.org/10.1016/j.copbio.2014.12.022.

Venayak, N., Raj, K., Jaydeep, R., Mahadevan, R., 2018. An optimized bistable metabolic switch to decouple phenotypic states during anaerobic fermentation. ACS Synth. Biol. 7, 2854–2866. https://doi.org/10.1021/acssynbio.8b00284.

Watanabe, S., Ozawa, H., Kato, H., Nimura-Matsune, K., Hirayama, T., Kudo, F., Eguchi, T., Kakinuma, K., Yoshikawa, H., 2018. Carbon-free production of 2-deoxy-scyllo-inosose (DOI) in cyanobacterium Synechococcus elongatus PCC 7942. Biosci. Biotechnol. Biochem. 82, 161–165. https://doi.org/10.1080/09168451.2017.1411777.

Yamauchi, N., Kakinuma, K., 1992. Biochemical studies on 2-Deoxy-scyllo-inosose, an early intermediate in the biosynthesis of 2-deoxystreptamine. J. Antibiot. (Tokyo) 45, 756–766. https://doi.org/10.7164/antibiotics.46.1916.

Yamauchi, N., Kakinuma, K., 1995. Enzymic carbocycle formation in microbial secondary metabolism. The mechanism of the 2-deoxy-scyllo-inosose synthase reaction as a crucial step in the 2-deoxystreptamine biosynthesis in Streptomyces fradiae. J. Org. Chem. 60, 5614–5619. https://doi.org/10.1021/jo00122a049.

Yu, J., Spencer, J.B., 2001. Convenient synthesis of 2-deoxy-scyllo-inosose and 2-deoxy-scyllo-inosamine: two key intermediates on the biosynthetic pathway to aminoglycoside antibiotics. Tetrahedron Lett. 42, 4219–4221. https://doi.org/10.1016/S0040-4039(01)00612-8.