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Simultaneously down-regulation of multiplex branch pathways using CRISPRi and fermentation optimization for enhancing β-amyrin production in *Saccharomyces cerevisiae*

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ABSTRACT

The production of β-amyrin in *Saccharomyces cerevisiae* is still low due to the inability of effectively regulating the endogenous metabolic pathway for competitive synthesis of β-amyrin precursors. In this study, we focused on two branches of β-amyrin synthetics pathway that consume β-amyrin precursors (2,3-oxidosqualene and cytosolic acetyl-CoA) and regulated related genes (*ADH1, ADH4, ADH5, ADH6, CIT2, MLS2* and *ERG7*). We developed a CRISPRi method by constructing a multi-gRNA plasmid to down-regulate the seven genes simultaneously, which is reported for the first time in *S. cerevisiae*. The average transcription inhibition efficiency of the seven genes reached as high as 75.5%. Furthermore, by optimizing the fermentation condition (including pH, inoculum size, initial glucose concentration and feed of glucose or ethanol) and increasing extracellular transportation via supplying methyl-β-cyclodextrin, β-amyrin concentration of engineered strain SGibSdCg increased by 44.3% compared with the parent strain SGib, achieving 156.7 mg/L which was the highest concentration of β-amyrin reported in yeast. The one-step down-regulation of multiple genes using CRISPRi showed high efficiency and promising future in improving the yields of natural products.

1. Introduction

β-amyrin is a pentacyclic triterpenoid compound and a key precursor of many other triterpenoids such as glycyrrhetinic acid and soybean saponin. β-amyrin has many physiological functions such as anti-inflammatory, anti-hyperglycemic and hypolipidemic functions [1]. The industrial production of β-amyrin depends on plant extraction or chemical synthesis [2–6]. However, the low extraction efficiency and the low synthetic yield lead to the high economic costs.

In our previous work, β-amyrin has been successfully produced in *S. cerevisiae* [7]. Through introducing a β-amyrin synthase (βAS) from *Glycyrrhiza glabra* converting endogenous 2,3-oxidosqualene in mevalonic acid (MVA) pathway to β-amyrin, strengthening precursor by overexpressing *ERG20* and *ERG9* and introducing analogous genes *IDI1* and *ERG1*, the biosynthesis of β-amyrin in *S. cerevisiae* was realized [7]. However, the supplementary of precursor (acetyl-CoA and 2,3-oxidosqualene) is still insufficient to further improve β-amyrin production, which is a substantial bottleneck limiting its industrial application. The regulation of competitive pathways of β-amyrin production will further increase the titer of β-amyrin.

Down-regulating or deleting the competing pathways have been shown as effective approaches to increase the production of target metabolite. For example, the titer of mevalonate increased by 41-fold compared with the wild-type strain by deleting and repressing some genes in MVA branch pathways [8]. In another study presented the bifunctional dynamic control that can provide simultaneous upregulation and downregulation of cellular metabolism for engineered biosynthesis, and this allows muconic acid biosynthesis to reach 1.8 g/L [9]. Other study used CRISPRi to down-regulate essential genes sucA and sucB in tricarboxylic acid (TCA) cycle and the final strain could

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produce 0.42 g/L glutarate, which was increased by 40% compared with the parental strain [10]. Presently, several approaches for gene knock-out or knock-down have been developed for balancing exogenous and endogenous pathways, such as Cre/LoxP, ZFN, TALENs and RNAi [11–14]. Besides, CRISPR/Cas9 is currently widely used in genome editing due to its high efficiency, specificity and multi-gene editability. It enables to trigger multi-gene mutations in one step, which is much more precise and efficient compared with traditional methods [15–17]. Nuclease inactivated dead Cas9 protein (dCas9) can inhibit the extension of RNA polymer and thus downregulate the transcription of single or multiple genes, which is well-known as CRISPRi [18,19]. For example, Lian et al. [20] used CRISPRi to down-regulate the transcriptional level of *ERG7* to increase β-carotene production and down-regulate the transcriptional level of *MNN9* to increase the production of *Trichoderma reesei* endoglucanase in *S. cerevisiae*. Therefore, CRISPRi may be a powerful tool to down-regulate the branch pathways in β-amyrin biosynthesis.

In this study, CRISPR/dCas9 system was designed and constructed in the β-amyrin production strain, named as *S. cerevisiae* SGibSd. An efficient multi-gene one-step transcriptional inhibition method was succeeded to increase β-amyrin production. Furthermore, we optimized the fermentation conditions of the engineered yeast and the highest yield of β-amyrin to our knowledge was obtained. This method gives a reference for rapid and multiple manipulations of desired metabolic pathways.

2. Materials and methods

2.1. Strains, plasmids and media

Strains and plasmids are listed in Table 1. Strains were grown in YPD media with 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose. Engineered yeasts carrying pRS42K plasmids (EUROSCARF, Frankfurt, Germany) were maintained in YPD media with 200 mg/L G418 (Genview, Florida, USA).

2.2. Strain construction for expressing dCas9

The *S. cerevisiae* codon-optimized dCas9 fused with two C-terminal SV40 nuclear localization signal sequences was PCR amplified from plasmid PUC57-dCas9. The dCas9 expression cassette containing *TEF1* promoter and *CYC1* terminator was then generated through overlap extension PCR (OE-PCR). Co-transforming 500 ng of dCas9 expression cassette and URA3 auxotrophic selection marker into δ1 site in genome of strain SGibS by standard electroporation produced the strain expressing dCas9. This strain was named SGibSd and used for all further manipulations.

2.3. Design of gRNA and plasmids construction

To design specific gRNAs for targeted genes *ERG7, CIT2, MLS1, ADH1, ADH4, ADH5* and *ADH6*, all potential gRNA targets (sRNA) in these genes along with promoter *SNR52p* and terminator *SUP4t* (*SNR52p*-gRNA-*SUP4t*) were predicted through RNAfold web server at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi. From these predictions, only gRNAs with two structural traits including free single strand sRNA exposed to gRNA and complete hairpin handle for dCas9 binding were left as target gRNAs.

To form the complete gRNA module SNR52p-gRNA-SUP4t, SNR52p and SUP4t were amplified with primers containing target sRNA from plasmids pUC57-SNR52p and pUC57-SUP4t respectively, and then were assembled by OE-PCR. Single gRNA expression vector was constructed by co-transforming gRNA (500 ng) with a 39 bp flanking overlap homology sequences that can direct assembly into a pRS42K plasmid (300 ng) digested with *Sal*I and *Bam*HI into the strain SGibSd.The gene *LacZ* encoding β-galactosidase was for verifying the confidence of the gRNA design and to determine effect of CRISPR/dCas9 in yeast. The *LacZ* expression cassette *FBA1p-LacZ-CYC1t* formed by OE-PCR was co-transformed with corresponding gRNA module and linearized pRS42K, resulting in the different validation strains.

By searching for 20-bp target-specific complementary region in the *ERG7* and predicting the correct folding of gRNA with this sequence, we identified a target sequence beginning at the 601st base at downstream of ATG of the ERG7. The single gRNA expression vectors were then constructed by co-transforming gRNA and linearized pRS42K plasmid digested with *Sal*I and *Bam*HI into dCas9-expressing yeast.

Multiple gRNA expressing plasmid was efficiently constructed by one step assembling method. Firstly, each complete gRNA was produced through OE-PCR as stated for constructing single gRNA expression vector. In order to assembly multiple gRNA modules in sequence without interference each other, additional 20 bp of random complementary sequences were presented at 5′ upstream regions of forward primer for amplifying *SNR52p* in one gRNA module and reverse primer for amplifying *SUP4t* in another adjacent gRNA module. Then, multiple gRNA expressing plasmid was produced via assembling multiple gRNA modules with flanking overlap homology sequences into linearized pRS42K *in vivo* after co-transformation.

2.4. Quantitative PCR

Fresh cells were harvested by centrifuging at 5000 × *g* for 5 min, and the total RNA was isolated using Yeast RNA Kit (OMEGA, Doraville, GA) following the manufacturer's instructions. Five hundred nanogram of RNA was converted to cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, USA) under standard conditions. Quantitative PCR reactions were performed with LightCycler SYBR Green I Master Kit according to the manufacturer's instructions. Reactions were run on a LightCycler® 96 system (Roche) using the housekeeping gene *ACT1* as a reference gene. Primers used for *ERG7, CIT2, MLS1, ADH1, ADH4, ADH5* and *ADH6* are shown in Table S2. Relative ratio represents the gene expression relative to housekeeping gene *ACT1*. Repression ratio (%) is the ratio of decreased mRNA when repressed by CRISPRi system relative to mRNA without repression.

2.5. β-galactosidase assay

Cells grown to midlog phase were used to determine β-galactosidase activity. β-galactosidase was assayed by measuring hydrolysis of the

Table 1
Strains used in this study.

Strain	Description	Source
SGibS	*S.cerevisiae*INVSc1::NTS::$*P_{ADH1}$-IDI-T_{ADH1}-$*P_{ALA1}$-ERG20-T_{ALA1}-$*P_{GPM1}$-ERG9-T_{GPM1}-$*P_{TYS1}$-ERG1-T_{TYS1}-$*P_{FBA1}$-bAS-T_{FBA1}- P_{TEF1}-hphNT1-T_{CYC1}	Zhang et al., 2015
SGibSd	SGibS:: δ1:: P_{TEF1}- *dCas9-2NLS* -T_{CYC1}-*URA3*	This study
SGibSdL	SGibSd:: pRS42K:: FBA1p-LacZ -CYC1t	This study
SGibSdLn	SGibSd:: pRS42K:: $gRNA_{(Ln)}$- P_{FBA1}-*LacZ* -C_{CYC1}, n stand for different gRNA of LacZ (n = 1–5)	This study
SGibSdE	SGibSd:: pRS42K:: $gRNA_{(E)}$	This study
SGibSdC	SGibSd::δ2::P_{TYS1}-*PDC1*-P_{GPM1}-*ALD6*-P_{FBA1}-*ACS1*$_{SE}$-T_{TYS1}-P_{FBA1}-*ADH2*-*LEU2*	This study
SGibSdCg	SGibSdC:: pRS42K:: $gRNA_{(E)}$- $gRNA_{(C)}$ - $gRNA_{(M)}$ - $gRNA_{(A1)}$ - $gRNA_{(A4)}$ - $gRNA_{(A5)}$ - $gRNA_{(A6)}$	This study

chromogenic substrate, O-nitrophenyl-β-Dgalactoside (ONPG). The amount of O-nitrophenol formed can be measured by determining the absorbance at 420 nm. Since whole cells present have light scattering, the absorbance at 420 nm is the sum of the absorbance due to O-nitrophenol and the cells. The contribution of light scattering was thus determined by measuring the absorbance at 550 nm where O-nitrophenol doesn't absorb. The light scattering at 420 nm is 1.75× the light scattering at 550 nm, so the absorbance of O-nitrophenol is determined by subtracting 1.75 × OD550 nm. The corrected absorbance is then used to calculate the activity of β-galactosidase.

2.6. Preparation of seed liquid and fermentation

The *S. cerevisiae* project deposited in a low temperature refrigerator at −80 °C was streaked onto YPD agar medium, 30 °C for 36 h. A single colony was picked in a 100 mL shake flask with 30 mL YPD at 30 °C, 200 rpm for 36 h.

For flask fermentations, the prepared seed liquid was inoculated into a 250 mL shake flask with 40 mL YPD at 30 °C, 200 rpm for 120 h. The initial OD_{600} was 0.1.

For fermenters, the prepared seed liquid was inoculated into a 2.5 L-fermentor containing 1.5 L YPD medium at a certain inoculation amount, and the fermentation temperature was controlled at 30 °C and the stirring speed was 250 rpm. Control the pH of the system using a 0.05 mM phosphate buffer system during initial pH optimization.

2.7. Metabolites assay

β-amyrin production during the fermentation course was determined as described previously [21]. β-amyrin was extracted using hexane from cell lysate. For GC/MS analysis, the extracts were trimethylsilylated with 50 μL of N-methyl-N (trimethyl sylil) trifluoro acetamide (Sigma-Aldrich) for 30 min at 80 °C [22]. GC/MS was performed using a GCMS-QP2010 Plus (Shimadzu) with a DB-5MS column (Agilent). For quantification of metabolites including β-amyrin, squalene and ergosterol, samples were run in same column condition using a GC2010 (Shimadzu) with a TG-5MS column (Thermo).

3. Results and discussion

3.1. Strengthening of cytosolic acetyl-CoA synthesis

The initial substrate of the synthesis of β-amyrin is cytosolic acetyl-CoA, but the strict regionalization of yeast cells results in a low cytosolic acetyl-CoA flow [23,24]. Cytosolic acetyl-CoA can mainly be synthesized by the pyruvate dehydrogenase branch, which involves the decarboxylation of pyruvate to form acetaldehyde, the dehydrogenation of acetaldehyde to acetic acid and the activation of acetic acid to acetyl-CoA. In this pathway, ethanol can be reversed to acetaldehyde by alcohol dehydrogenase *ADH2p* in the case of glucose limitation (Fig. 1a). Shiba et al. [25]found that the modification of the pyruvate dehydrogenase pathway promoted the supply of acetyl-CoA and increased the production of sesquiterpene. Overexpression of *ADH2* also contributes to the synthesis of acetyl-CoA [26]. To enhance cytosolic acetyl-CoA, the strain SGibSdC was obtained by overexpressing pyruvate dehydrogenase *PDC1p*, acetaldehyde dehydrogenase ALD6p, acetyl-CoA synthase ACS1p and ADH2p on the basis of SGibSd (Fig. 1a). By overexpressing these genes, the concentration of β-amyrin increased by only 9.5%, but the yield of β-amyrin did not increase significantly (Fig. 1b), indicating that the balance of metabolic flux within the cell may require more synergistic regulation.

3.2. Branch-suppression strategies for enhancing β-amyrin synthesis

There are at least two metabolic branches competing for the synthetic precursors of β-amyrin. One is the ergosterol synthesis pathway,

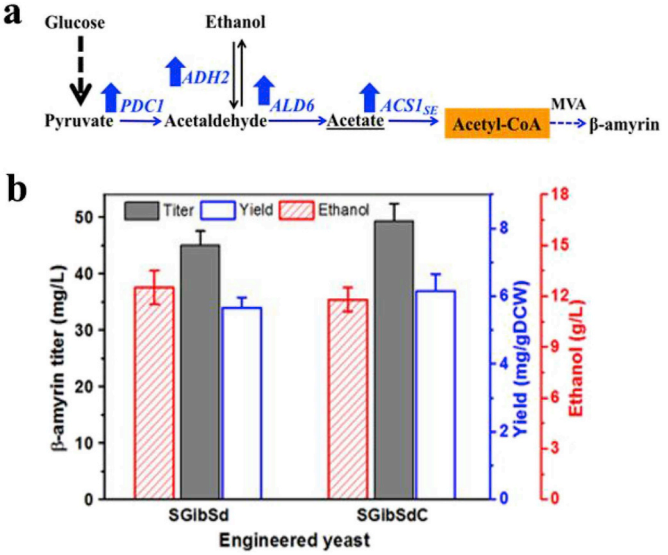

Fig. 1. Metabolic strategy to improving β-amyrin production by strengthening pyruvate dehydrogenase pathway. (a) Pyruvate dehydrogenase pathway and the overexpressed genes (in blue); **(b)** β-amyrin concentration and yield and ethanol concentration at 24 h fermentation, $n = 3$.

which is essential to the normal growth of cells but competes for the direct precursor of β-amyrin synthesis, 2,3-oxidosqualene (Fig. 2). Another is the metabolic pathways of cytosolic acetyl-CoA (Fig. 2). β-amyrin is synthesized from cytosolic acetyl-CoA as the initial substrate, but acetyl-CoA in yeast cells is strictly localized and separated into mitochondria, peroxisomes, nucleus and cytoplasm [27]. Acetyl-CoA cannot be output to the cytoplasm due to the lack of translocator. A large proportion of acetyl-CoA can re-enter into the mitochondria by glyoxylate cycle [28], reducing the cytosolic acetyl-CoA content. Moreover, cytosolic acetyl-CoA is generated via pyruvate dehydrogenase branch, in which acetaldehyde and acetate are the intermediate metabolites. In *S. cerevisiae*, acetaldehyde is mainly converted to ethanol, while acetate can transfer into peroxisome to form peroxisomal acetyl-CoA. These transformations further decrease the cytosolic acetyl-CoA for β-amyrin synthesis. Hence, enhancing the transformation from pyruvate to acetyl-CoA would improve the production of β-amyrin. In this study, we attempted to downregulate the genes in these competing pathways for β-amyrin production, including *ERG7* in ergosterol synthesis, *ADH1*, *ADH4, ADH5* and *ADH6* in ethanol production, and *CIT2* and *MLS1* in consumption of peroxisomal acetyl-CoA (Fig. 2). Malate synthase MLS1p catalyzes glyoxylate to malate and depletes cytosolic acetyl-CoA. Peroxisomal acetyl-CoA converts to citrate by citrate synthase CIT2p, which promotes cytosolic acetate entering into peroxisome and reduces amount cytosolic acetate. ADH1p, ADH4p and ADH5p locate in the cytoplasm, while ADH6p localization is not yet clear, which are all mainly responsible for the production of ethanol [29–31].

To redirect flux for enhancing β-amyrin production, we attempted to modify flux through regulating the sterol biosynthetic pathway. It has been indicated that downregulation of lanosterol synthase (ERG7) could provide more 2,3-oxidosqualene substrate for β-amyrin production (Supplementary Material Fig. S1). It showed that the transcriptional level of *ERG7* decreased by 78.5% via CRISPRi, leading to an increase of only 7.9% of β-amyrin concentration (Table 2). Repression of *ERG7* resulted in the accumulation of squalene by 5 folds although it caused no significant change in FPP, IPP and ergosterol yields (Table 2), which agrees with a previous report [32]. The dramatic increase in squalene upon downregulation of *ERG7* probably concerns with feedback regulation of *ERG1*, as the reduced *ERG7* transcriptional level caused the accumulation of 2,3-oxidosqualene and thus the suppression

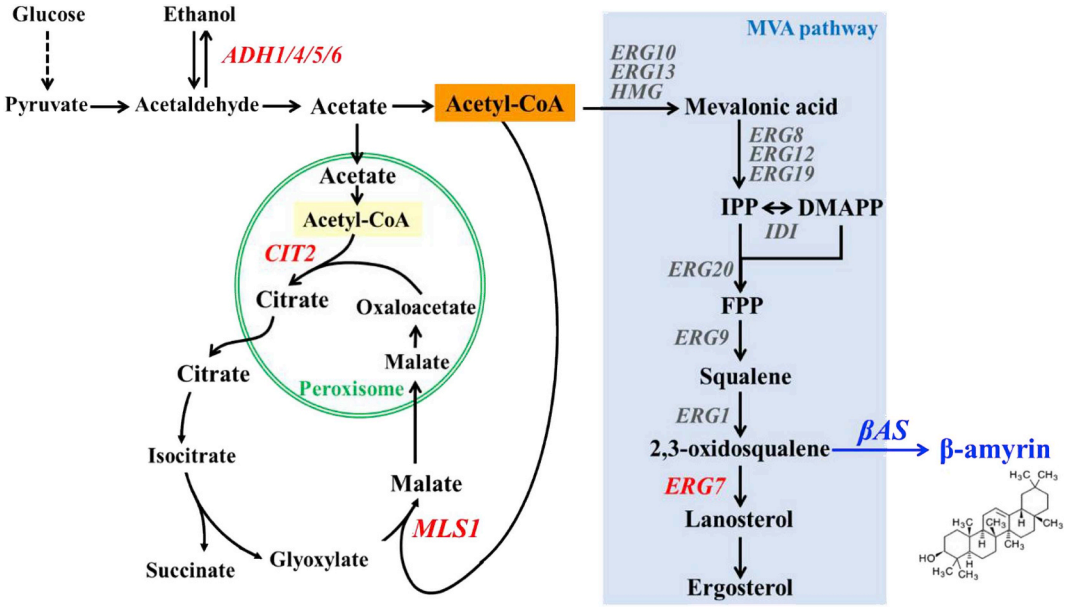

Fig. 2. Overview of the biosynthesis pathway of β-amyrin in *S. cerevisiae*. Genes in red were supposed to be down-regulated to improve β-amyrin production.

of *ERG1* [33]. Similar levels of ergosterol implied that ERG7 is not the flux-limiting enzyme or other regulation of the pathway was initiated in response to the reduced *ERG7* transcription to maintain ergosterol production for normal cell growth [32,34]. As a result, the down-regulation of *ERG7* has limited effect on improving β-amyrin production. It is regarded that multiplex target regulation is necessary in the complex metabolism network. For example, researchers obtained higher mevalonic acid levels by simultaneously regulating five genes (*ERG9, BTS1, ROX1, YJL064W* and *YPL062W*) comparing to one or two genes [8]. Herein, CRISPRi is used to down-regulate genes to improve β-amyrin production.

3.3. Developed CRISPRi system for down-regulating multiple gene expression

To construct CRISPR/dCas9 system for repressing target genes in *S. cerevisiae*, dCas9 gene was integrated into chromosome, and gRNA expression cassettes were constructed in a plasmid which is convenient for gRNA tuning. For dCas9 expression, a yeast codon optimized D10A/H840A double mutant version from *S. pyogenes* Cas9 fused to two 54 copies of a nuclear localization sequence was expressed from a yeast constitutive promoter *TEF1p* (Fig. 3a) [35]. gRNA expression was controlled through the RNA polymerase III small nucleolar RNA (snoRNA) *SNR52* promoter and the yeast tRNA gene SUP4 terminator on plasmid pRS42K [18]. The resulted gRNA molecule consists of a nucleotide (nt) target-specific complementary region, a dCas9-binding hairpin (dCas9 handle) and a *SUP4t* terminator (Fig. 3b) [18].

To determine whether designed gRNAs led to efficient repression of gene expression, the gRNA expression plasmid with a β-galactosidase reporter system (*LacZ* cassette) was constructed (Fig. 3c). Five gRNAs complementary to different regions of the LacZ coding sequence were designed (Fig. 3d and f). The result indicated that gRNAs targeting the *LacZ* DNA strand could repress the gene expression. The transcription repression efficiency ranged from 8.8% to 82.5% and correspondent LacZ activity decreased by 5%–87.5% (Fig. 3d). This also indicated that the structure of gRNA was crucial importance to achieve best transcription repression via CRISPR/dCas9 (Fig. 3e). It was proposed that the most efficient gRNA has two characterization: (1) target-specific complementary region was single-stranded and exposed outside gRNA molecule; (2) gRNA had the entire handle structure to bind dCas9 protein. Furthermore, this repression effect was highly stable during the fermentation process (Supplementary Material Fig. S2), which demonstrates that CRISPR/dCas9 could yield efficient repression of gene expression in yeast. Based on the advantages of CRISPR/dCas9 system in transcriptional regulation, the multi-gene transcriptional down-regulation was designed. Since the different gRNA expression modules have the same promoter and terminator sequences, these repeat sequences make them difficult to directly connect to multiple gRNA expression cassettes. Thus, in order to construct a multiple gRNA expression plasmid, 20 bp random sequences were added to each gRNA module by PCR and then assembled using the DNA assembler yeast homologous recombination (Fig. 4d). The gRNA sequences were designed with the gene sequences of *ADH1, ADH4, ADH5, ADH6, CIT2* and *MLS1* as templates. Meanwhile, the above six gRNAs together with *ERG7* gRNA (Supplementary Material Table S1) were co-transformed into strain SGibSd. Ten colonies were randomly selected to verify the positive clones. The proportion of positive clones showed that the assembly efficiency was 90% when transforming one gRNA, but the assembly efficiency decreased with the increase of the number of transformed gRNAs. The efficiency of transforming seven gRNAs was 40% (strain SGibSdCg) (Supplementary Material Fig. S3). Thus, the assembly method in this study is efficient for constructing multiple gRNAs in one vector. Fig. 4a shows the transcriptional inhibitory effect of the seven genes of the engineering strain SGibSdCg during the 72 h fermentation process. gRNA-mediated dCas9 proteins have significant inhibitory effects on the transcriptional level of seven genes. The average inhibition rate of seven genes is 75.5% (Fig. 4a and c). The concentration of β-amyrin produced by engineered strain SGibSdCg was increased to

Table 2
Fermentation performances of SGibSd and SGibSdE.

Strain	Biomass (g/L)	β-amyrin (mg/L)	β-amyrin per unit cell (mg/g DCW)	Farnesol (mg/L)	Isopentenyl alcohol (mg/L)	Squalene (mg/L)	Ergosterol (mg/L)
SGibSd	7.95 ± 0.87	44.98 ± 2.50	5.66 ± 0.38	0.10 ± 0.02	0.27 ± 0.03	0.51 ± 0.09	10.80 ± 1.06
SGibSdE	8.05 ± 1.06	48.55 ± 3.84	6.03 ± 0.47	0.15 ± 0.02	0.28 ± 0.04	2.58 ± 0.22	10.22 ± 1.13

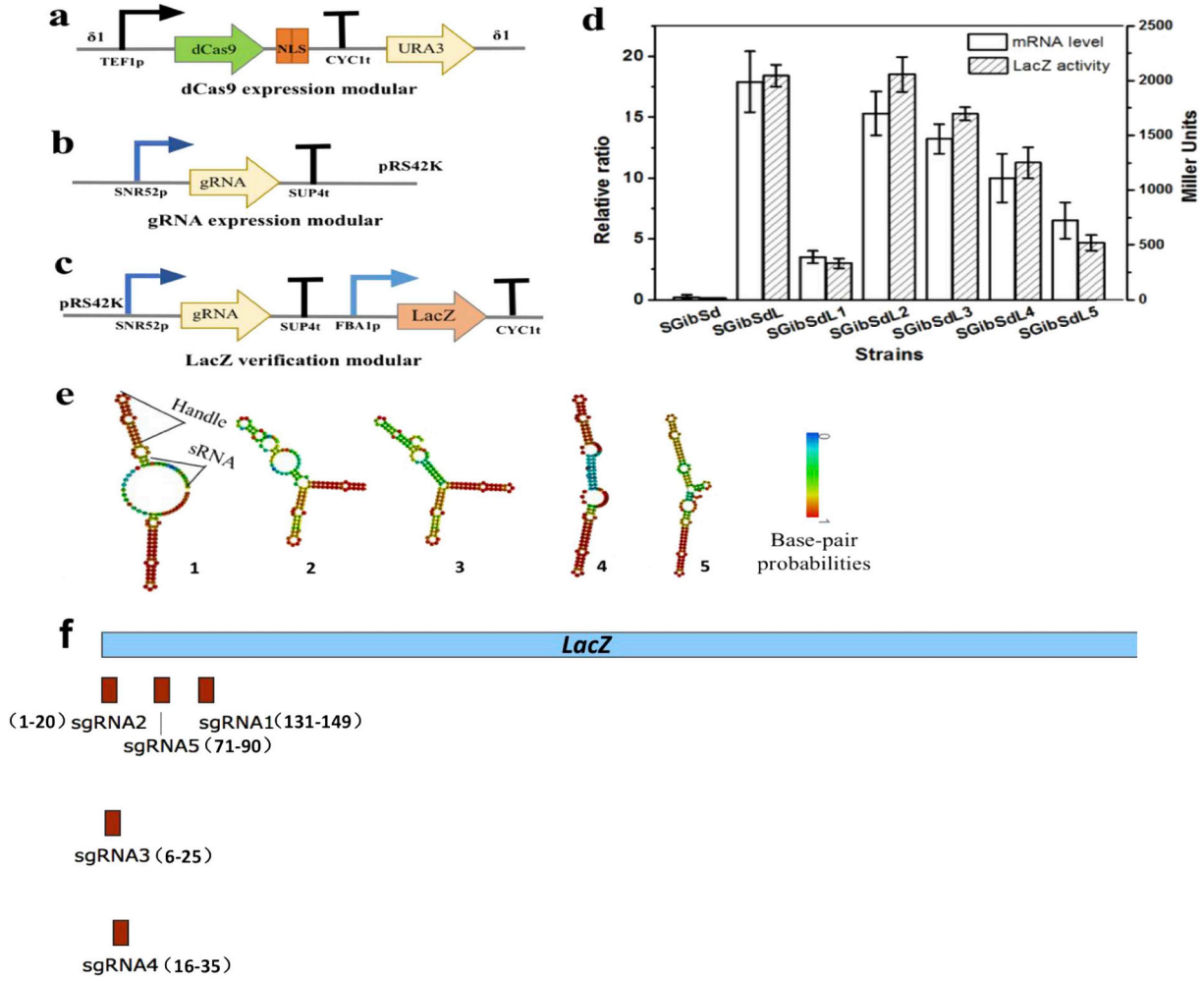

Fig. 3. Construction of CRISPRi system and the verification of repression efficiency of different gRNA backbones. (a) Modular construction of dCas9 expression cassette; **(b)** gRNA expression cassette; **(c)** gRNA expression cassette with a *lacZ* reporter; **(d)** repression efficiency of different gRNA backbone structures reflected by mRNA level and LacZ activity, $n = 3$; **(e)** topological structures of the LacZ gene gRNAs. **(f)** the positions of the gRNAs targeting LacZ.

59.68 ± 7.12 mg/L (increased by 42.2%) (Fig. 4b). The yield of β-amyrin reached 6.28 mg/g DCW, which increased by 11.0% compared with strain SGibSdC. This result demonstrated the potential of the CRISPRi system in yeast pathway engineering applications.

3.4. Optimization of β-amyrin fermentation

As acetyl-CoA production is closely related to cell growth, the production of β-amyrin is coupled with biomass. Studies have shown that the growth and metabolism of *S. cerevisiae* was affected by the culture conditions, such as the concentration of glucose, metabolic products, dissolved oxygen and biomass [36], indicating that growth environment will affect the distribution of acetyl-CoA and the production of β-amyrin ultimately. Therefore, we optimized the basal culture conditions of *S. cerevisiae* SGibSdC and analyzed the factors that affected the microbial metabolic rate, including pH, inoculum size and initial glucose concentration. The results showed that the most favorable condition for the formation of β-amyrin was pH = 5.0, initial OD = 0.3 and initial glucose concentration 35 g/L (Supplementary Material Fig. S4). Fed-batch fermentations with glucose or ethanol were designed and carried out to increase β-amyrin production. Glucose or ethanol was fed every 12 h during fermentation. With the increasing concentration of feeding glucose, the yield of β-amyrin gradually increased and reach highest (76.9 mg/L) when feeding 5 g/L glucose (Supplementary Material Fig. S5a). Feed of ethanol can also improve the yield of β-amyrin. The yield of β-amyrin reached maximum (108.7 mg/L) when ethanol concentration increased to 7 g/L. This yield increased by 90.2% compared with the batch fermentation (Supplementary Material Fig. S6a).

Several studies have confirmed that the accumulation of intracellular heterogenous products will feedback to inhibit the pathways of key enzymes or affect cell growth [37,38], β-amyrin can not be transported to extracellular due to the lack of the transporter in yeast. Therefore, we envisioned some compounds that can transport β-amyrin to extracellular. Methyl-β-cyclodextrin (MβCD) is a polysaccharide with a circular topology, which has been widely used to dissolve and stabilize nonpolar drugs because of its structural characteristics of hydrophobic cores and hydrophilic surfaces. Researchers found that MβCD can help terpenoids to be transported outside the cell [39]. Therefore, the effect of MβCD in medium on the extracellular transport of β-amyrin was studied (Fig. 5a). We found that as the addition of MβCD increased, more β-amyrin was transported extracellular. When the amount of MβCD added reached 40 mM, it did not further promote the transport of β-amyrin, which seems to be saturated. We found that the transport of 1 mg β-amyrin required about 1 mM MβCD. Besides, total concentrations of β-amyrin in the cell and medium increased as the increasing of MβCD, indicating the extracellular transport alleviate the cell burden from β-amyrin and increased the production of β-amyrin. Through feeding ethanol and MβCD (Fig. 5b), we found that the extracellular β-amyrin of strain SGib and SGibSdCg accounted for about 20% of the total productions. The yield of

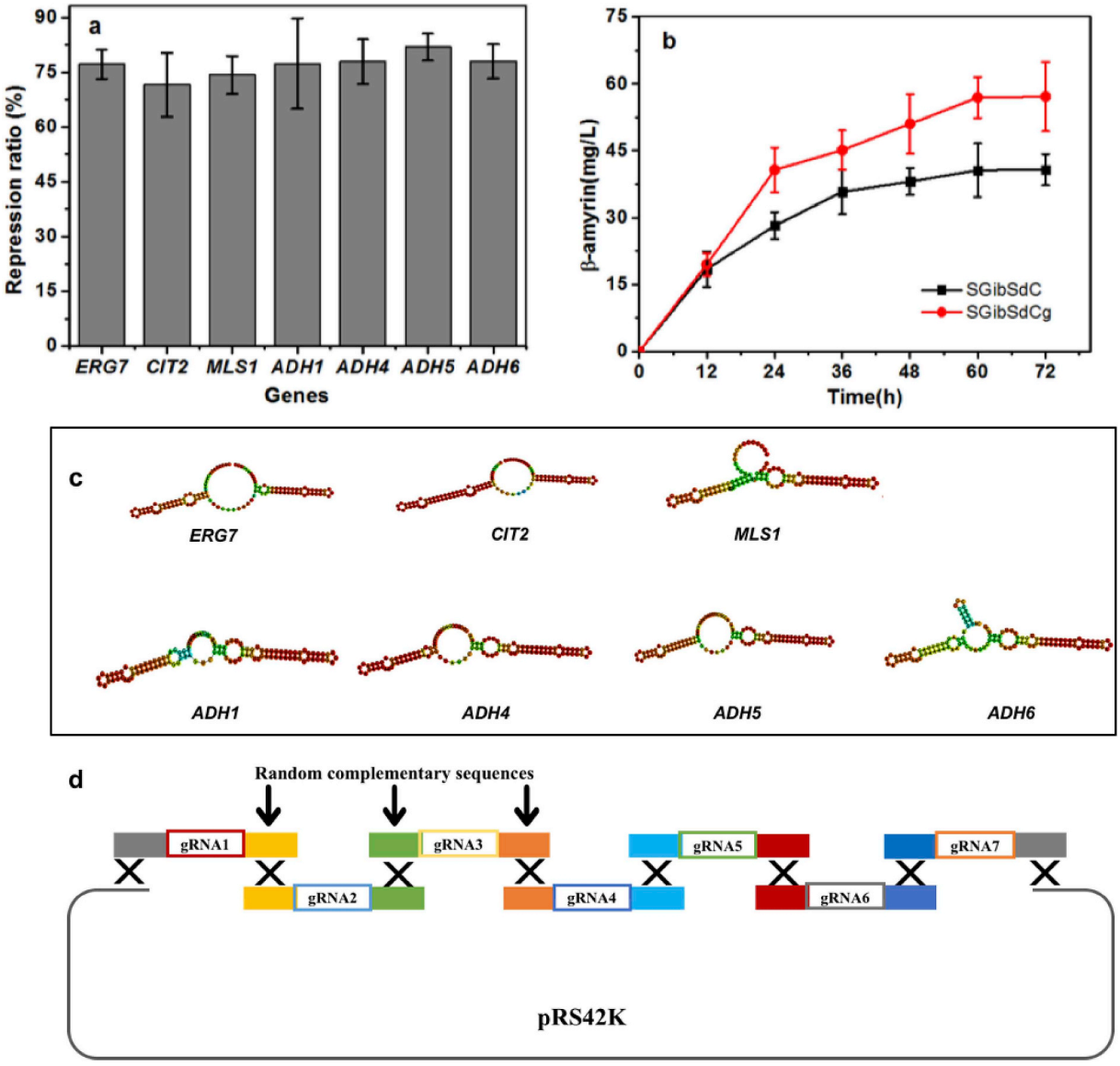

Fig. 4. One step down-regulation of competitive pathway for β-amyrin synthesis using CRISPRi. (a) repression ratio of the seven genes by CRISPRi; **(b)** β-amyrin concentrations during the fermentation before (SGibSdC) and after (SGibSdCg) the transcriptional regulation using CRISPRi. $n = 3$; **(c)** the RNAfold structure prediction for ERG7, CIT2, MLS1, ADH1, ADH4, ADH5, and ADH6 genes;**(d)**construction of multiplex gRNA expression plasmid.

β-amyrin of SGibSdCg was up to 156.7 ± 8.62 mg/L, which was 30.1% higher than that of no MβCD and was 44.3% higher than SGib.

Due to the high cost of MβCD, its application to large scale fermentation is limited. It is possible to consider replacing MβCD with other compounds that have lower cost or producing endogeneous MβCD by yeast. In the future, we also can find β-amyrin transporter or modify existing transporters by direct evolution to solve the transport problem of β-amyrin.

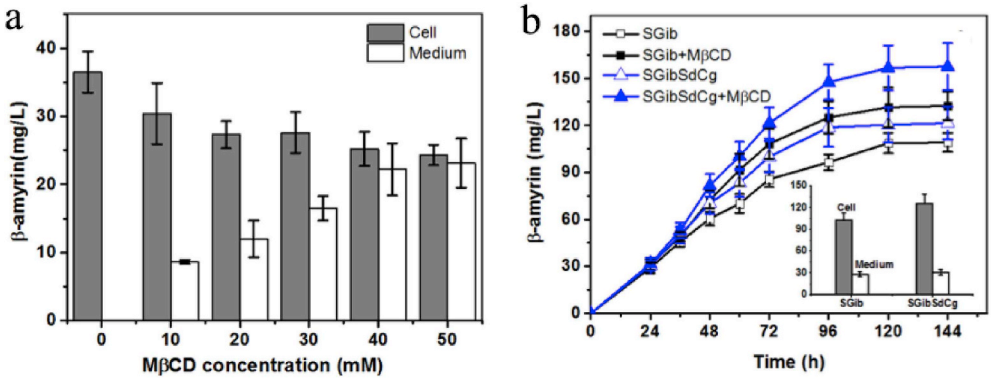

Fig. 5. Effect of MβCD on β-amyrin production in 2.5 L fermentors. (a) Effects of MβCD dosage on intracellular and extracellular β-amyrin concentrations of strain SGib in flask fermentation. Samples were withdrawn at 120 h; **(b)** Effects of MβCD supplementation on β-amyrin production of strain SGib and SGibSdCg in ethanol fed-batch fermentation. 20 mM/L MβCD was added every 12 h. Samples were withdrawn every 12 h before 96 h, samples were withdrawn every 24 h before 144 h, $n = 3$.

4. Conclusion

In this study, CRISPRi system was constructed in β-amyrin producing strain *S. cerevisiae* SGib to repress the competitive pathway for β-amyrin production. *LacZ* gene was employed in CRISPRi system to determine the effect of gRNA structure on the efficiency of transcription inhibition. By repressing *ERG7*, β-amyrin concentration increased by only 7.9%. To further improve β-amyrin production, seven genes in the competitive pathways including *ADH1, ADH4, ADH5, ADH6, CIT2, MLS2* and *ERG7* were one-step knocked-down using CRISPRi. The average degree of transcription inhibition of these genes reached 75.5%. β-amyrin concentration of the engineered strain reached 59.68 ± 7.12 mg/L. Furthermore, fermentation conditions and the dosage of MβCD were optimized. As a result, β-amyrin concentration reached 156.7 ± 8.62 mg/L, which was 44.3% higher than the original strain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2019.02.002.

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