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Engineering *Saccharomyces cerevisiae* for production of simvastatin

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Abstract

Simvastatin is a semisynthetic cholesterol-lowering medication and one of the top-selling statins in the world. Currently, industrial production of simvastatin acid (SVA) is a multistep process starting from the natural product lovastatin. For this reason, there is significant interest in direct production of simvastatin from a microbial host. In this study, six heterologous biosynthetic genes were introduced into *Saccharomyces cerevisiae* and the acyl-donor dimethylbutyryl-*S*-methyl mercaptopropionate (DMB-SMMP) was added, resulting in initial production of 0.5 mg/L SVA. Switching the yeast strain from JHY686 to BJ5464-NpgA increased total polyketide production to over 60 mg/L and conversion from dihydromonacolin L acid to monacolin J acid (MJA) was increased from 60 to 90% by tuning the copy number of the P450 *lovA*. Increasing the media pH to 8.7 led to a further 10-fold increase in SVA production. Optimized chemical lysis of the cell walls *in situ* after maximum MJA production led to 55 mg/L SVA titer, representing nearly complete conversion from MJA and a 110-fold increase in titer from the initial SVA production strain. The yeast strains developed in this work can be used as an alternative production method for SVA, and the strategies employed can be broadly applied for heterologous production of other fungal polyketides and semisynthetic compounds in yeast.

Graphical abstract

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Keywords

Simvastatin; natural products; polyketides; *Saccharomyces cerevisiae*

1. Introduction

Small molecule secondary metabolites produced by plants, bacteria, and fungi have been an abundant source of pharmaceuticals; nearly half of the drugs approved by the U.S. Food and Drug Administration (FDA) from 1994–2008 were derived from natural products. Polyketides are one of the major classes of natural products, and fungi in particular have been important sources of polyketide pharmaceuticals. The diverse biological activities of polyketides originate from their structural complexity, but this same complexity poses challenges for developing cost-effective methods of chemical synthesis. For this reason, fermentation of the native producing strains, followed by semi-synthesis when necessary, is used to produce nearly all commercial polyketide-derived pharmaceuticals. The natural product lovastatin is a polyketide that was first isolated from the filamentous fungus *Aspergillus terreus* in 1979 and has since become a major drug used to treat hypercholesterolemia. Simvastatin is a semisynthetic compound derived from lovastatin, with a single methyl group addition on the side chain, and is one of the most highly prescribed cholesterol lowering medications, with annual sales topping \$5 billion before generics were permitted in 2007, and it is listed as an essential drug by the World Health Organization.

The lovastatin pathway has been well-studied through genetic and biochemical characterizations (Fig. 1). LovB, a highly reducing polyketide synthase (HR-PKS), and LovC, an enoyl reductase, together synthesize dihydromonacolin L acid (DMLA) from one acetyl-CoA and eight malonyl-CoA units, along with a methyl donor, *S*-adenosyl methionine (SAM), and the cofactor, nicotinamide adenine dinucleotide phosphate (NADPH). DMLA is then released from LovB by the thioesterase LovG and converted to monacolin L acid (MLA) and then monacolin J acid (MJA) by the cytochrome P450 monooxygenase (P450) LovA. During the multistep oxidation reactions, LovA receives reducing equivalents from its redox partner, the cytochrome P450 reductase (CPR). The diketide synthase LovF synthesizes the α -*S*-methylbutyryl-ACP product, and the acyl unit is transferred to MJA by the acyltransferase LovD, completing the synthesis of lovastatin acid (LVA).

The semisynthetic analog simvastatin, with an α,α -dimethylbutyryl side chain, is a more effective cholesterol lowering drug and is the active pharmaceutical ingredient of the blockbuster drug Zocor®. The semisynthesis of simvastatin from lovastatin is a multistep chemical processes that include protection and deprotection steps, requiring multiple purifications steps and is of poor atom economy. Our lab developed a biocatalytic process to shorten the chemical semisynthetic steps. We identified that LovD is a promiscuous acyltransferase and can directly transfer a small molecule acyl donor, α -dimethylbutyryl-*S*-methyl-mercaptopyruvate (DMB-SMMP), to MJA to yield SVA. Further protein engineering efforts led to a LovD variant, LovD9, that has an ~ 1000-fold increase in activity towards DMB-SMMP in the synthesis of SVA from MJA. Although this biocatalytic

process is used industrially to produce SVA, it still requires separate fermentation of *A. terreus* to produce lovastatin, followed by chemical hydrolysis to yield MJA and the use of LovD9 produced from *Escherichia coli*. Therefore, there remains significant interests in developing a one-step fermentation process for producing simvastatin from a single organism.

The native lovastatin host, *A. terreus*, has proven resistant to engineering attempts to produce simvastatin directly in our lab and in others⁶. *E. coli*, while successfully used to express LovD9, cannot express functional forms of the polyketide synthase LovB. A coculture of MJA-producing *A. terreus* and LovD9-expressing *E. coli* would require non-trivial engineering to ensure stable growth and maintenance of both organisms. Though cell-free approaches avoid the challenges of balancing organism growth and uptake of exogenously added substrates, current methods of preparing fungal extracts for cell-free synthesis are expensive and often limited by low levels of protein expression (especially with large PKS enzymes and endosome-bound P450s)⁷. Refactoring the pathway to MJA in model yeast organisms is therefore an attractive approach. Recently, lovastatin was produced in *Pichia pastoris* coculture, which required separate preparations of the two *P. pastoris* strains expressing the upstream DMLA synthesis pathway or the downstream pathway to convert DMLA to LVA. In addition to requiring balancing the growth of these two strains, the limited ability of MJA and DMB-SMMP to cross the *P. pastoris* cell membrane could prove challenging for SVA production. Therefore, we focused on producing simvastatin in *Saccharomyces cerevisiae*. From our previous work in identifying the function of LovG, *S. cerevisiae* was used as a heterologous host to produce 20 mg/L MJA via expression of LovG, LovC, LovB, *A. terreus* CPR, and LovA from three separate 2 μ plasmids¹¹. Here, we aimed to improve MJA production in *S. cerevisiae* through refactoring of the native *A. terreus* MJA pathway and to develop a one-pot process in which SVA can be produced (Fig. 1).

2. Materials and Methods

2.1 Strains and medium

Saccharomyces cerevisiae strains descended from BJ5464- NpgA and BY4741 were compared as biosynthetic hosts. Strains and modifications are summarized in Table 1. Engineered yeast strains were grown either in SD medium lacking leucine, tryptophan, histidine, and/or uracil where applicable, or in YPD medium (2% dextrose), supplemented with 0.2 g/L hygromycin or G418 when applicable. *E. coli* strains were used for cloning and followed standard recombinant DNA techniques.

2.2 Plasmid and strain construction

DNA restriction enzymes were acquired from New England Biolabs, NEB. Q5® High-Fidelity DNA Polymerase (NEB) and AccuPrime™ Pfx DNA Polymerase (Invitrogen™) were used for PCR.

Gene knockouts and integrations were accomplished via CRISPR-Cas9, following the protocol outlined in mode iv from Horwitz *et al.* pCRCT was a gift from Huimin Zhao

(Addgene plasmid # 60,621) bearing iCas9. The plasmids bearing gRNAs encoded antibiotic markers KanMX and HygR. For integrations and knockouts in the BJ5464-descended strains, the plasmid was transformed as three linear pieces: One piece with the iCas9 cassette and two with the plasmid backbone and gRNA cassette that used the gRNA target sequence as a homology arm. The gRNA target sequences were designed using E-CRISP and CRISPRdirect. Genome sequences were retrieved from the *Saccharomyces* Genome Database (SGD). The gRNA cassettes were ordered as gBlocks from IDT. Oligonucleotides were also ordered from IDT. Plasmids assembled via yeast homologous recombination (YHR) in the course of the first knockout at some loci were purified and sequenced. These assembled plasmids were used for subsequent modifications at the same loci in other strains and are listed in Table S5.

Metabolite production plasmids are listed in Table 2. Plasmids were assembled via yeast homologous recombination, restriction-digestion cloning, or SLIC. Primers are listed in Table S1. Gene sequences and sources are listed in Table S2. Gblock sequences are listed in Table S3. Integration loci and gRNA target sequences are listed in Table S4. Additional plasmids used during construction are listed in Table S5.

2.3 Yeast cultivation and lysis

Transformation of *S. cerevisiae* strains was accomplished by the standard lithium acetate method. Transformants were selected on plates of YPD with 0.2 g/L hygromycin or G418, or on uracil, leucine, histidine and/or tryptophan dropout SD medium. Colonies of JHY686 and descendants were visible 48 hours after plating while colonies of YRC01 and descendants required 72 hours. Single colony transformants were restreaked onto YPEG or onto SD dropout media if plasmid-bearing. Starter cultures were prepared by inoculating 2 – 3 mL of selective media with a portion of the yeast restreak. This starter culture was shaken at 28 °C and 250 rpm for 24 –48 h. Next, the appropriate volume of starter culture was spun down and resuspended in 9 mL of YPD to reach OD₆₀₀ = 0.1. This new culture was aliquoted to 3 culture tubes, 3 mL in each, for each condition to be tested. The cultures were shaken at 28 °C and 250 rpm for 72 hours until lysis, unless otherwise noted.

For glass bead mechanical lysis, 1.5 mL of 0.5 mm diameter glass beads from Biospec Products were added to the three mL culture, along with 250 µL of 1 M Tris-HCl buffer pH=8.7. The culture was then vortexed for 30 seconds three times, resting on ice for 1–5 min between vortexing rounds. For chemical lysis, 250 µL of Y-PER™ Yeast Protein Extraction Reagent from ThermoFisher Scientific or the described amounts of 3-(N,N-dimethylmyristylammonio)propanesulfonate (SB3–14) from Sigma-Aldrich were added to the culture tube, along with 250 µL of 1 M Tris-HCl buffer pH=8.7. After lysis, 4 or 16 µL 0.2 g/mL DMB-SMMP in DMSO was added to each culture. DMB-SMMP was synthesized as previously described. Finally, cultures were shaken at 28 °C and 250 rpm. Directly before lysis and 24 hours post-lysis 100 uL samples were taken, unless otherwise noted. In the case of YCB09, 1 µL of 0.2 g/mL DMB-SMMP in DMSO was added at 24, 36, 48, and 60 hours post inoculation into YPD and samples were taken 72 hours post inoculation.

2.4 Culture extraction and quantification

In a 1.5 mL microcentrifuge tube, 100 μ L culture and ~40 μ L 0.5 mm glass beads were combined. This was extracted twice with an organic phase of 200 μ L 2% trifluoroacetic acid (TFA) in ethyl acetate (EtOAc). Samples were vortexed for 30 seconds and centrifuged for 5 minutes before the organic phase was removed. After extraction, the organic phase was dried in a refrigerated CentriVap Concentrator from LabConco for 1 hour. Samples were resuspended in four times the original sample volume of 0.1 M NaOH in methanol (MeOH). For separate cell and media extraction, the cell pellet was spun down and the supernatant aspirated and stored in a separate tube. The two fractions were separately extracted following the above protocol. Chromatography analyses were performed on a Shimadzu 2020 EVLC-MS (Phenomenex kinetex, 1.7 μ m, 2.0 $\times$ 100 mm, C18 column) using positive and negative mode electrospray ionization with a linear gradient of 5–95% acetonitrile (MeCN)–H₂O supplemented with 0.1% (v/v) formic acid in 15 min followed by 95% MeCN for 5 min with a flow rate of 0.3 mL/min. Standard curves were produced using DMLA, MJA, and SVA. The DMLA standard curve was used to quantify MLA by assuming an equivalent mass response factor. All measurements were taken in biological triplicate unless otherwise noted.

2.5 Growth rate studies

A portion of cells from a single-colony restreak of the appropriate culture was inoculated into 2–3 mL starting culture YPD. This starter culture was shaken at 28°C and 250 rpm for 24 h. Next, 100 μ L of YPD was inoculated to OD₆₀₀ = 0.01 in a Corning96fc UV transparent plate with the applicable conditions. The plate was covered with Microseal® ‘B’ PCR Plate Sealing Film, adhesive, optical #msb1001 from BioRad. Growth curves were measured on a Tecan Infinite M200 Pro plate reader. The program temperature was set for 29.0°C, with 28.5°C minimum and 30.0°C maximum. The program was run for 99 cycles of 120 seconds of linear 5 mm shaking (330.5 rpm), absorbance was measured at 600 nm, then 1000 seconds of orbital 3 mm shaking (218.3 rpm). Conditions were tested in biological triplicate, and the means were reported.

2.6 Plasmid copy number measurements

Colony forming units (CFU) assays were performed by removing 5 μ L samples of culture, diluting the cultures 20,000-fold in sterile water, and plating 5 μ L on 60 mm diameter plates containing either YPAD or selective minimal media with uracil, leucine, or tryptophan dropouts. YPAD plates were done in technical duplicate and dropout plates were done in technical singlet. Each time point was done in biological triplicate.

Total DNA extraction was performed using the method from Lööke *et al.* The volume of culture sample taken was equivalent to one OD₆₀₀ unit. An OD₆₀₀ unit of 1 is the number of cells dissolved in 1 mL that gives OD₆₀₀ = 1. Samples were spun down for 30 seconds at 15,000 rpm and supernatant was aspirated. The resulting pellet was frozen at –20°C. Later the pellet was resuspended in 100 μ L water and 10 μ L of the resuspension was used for the extraction. The concentration of the resulting extracted DNA was measured and 2 ng was used per qPCR reaction, performed using Luna® Universal qPCR Master Mix from NEB on a Bio-Rad CFX96™ Real-Time System and C1000™ Thermal Cycler. Results were

quantified using the standard curve method. The reference gene used was *alg9*. The targeted genes representing the three plasmids were *lovB*, *lovG*, and *lovD9*. The primers used for qPCR are in Table S1.

3. Results and Discussions

3.1 MJA pathway construction and production of SVA in *S. cerevisiae* BY4741 series

To construct an SVA production strain, we split our proposed pathway into three modules as shown in Fig. 1. Module 1 involves the synthesis of the first dedicated precursor, DMLA, and requires the expression of *LovB*, *LovG*, and *LovC*. Module 2 involves the conversion of DMLA to MLA and MJA and requires the expression of *LovA* and *CPR*. The final module requires expression of *LovD9* and exogenous addition of DMB-SMMP to afford SVA. The *ADH2* promoter and terminator were chosen for all the gene cassettes. *ADH2p* is a strong promoter that is repressed in the presence of glucose and induced in the presence of ethanol. Reports from different groups have described that use of *ADH2p* for episomal expression leads to greater and more sustained expression, and can even allow successful production from plasmid-based genes through fermentation in complex media³⁴. The initial *S. cerevisiae* strain we tested was JHY686, which is derived from the BY4714 series, and has repaired petite tendency, increased respiration growth rate, required protease knockouts for PKS expression, and integration of the phosphopantetheinyl transferase gene *npgA* to enable posttranslational modification of the expressed PKSs. To accommodate rapid genome edits in this strain, we integrated a single copy of *iCas9* in the *YPRCTy1–2* locus using *leu2*-marker mediated integration, forming YCB03. Subsequent integrations and knockouts were then accomplished via transformation of the gRNA cassette and the plasmid backbone as two separate pieces, as discussed in mode iv of Horwitz *et al.* For sequential genome modifications, we alternated the marker on the gRNA backbone between one conferring resistance to hygromycin and another conferring resistance to G418. This allowed for rapid sequential genome edits without curing plasmids in between steps.

LovB catalyzes over thirty separate reactions in the synthesis of DMLA, so synthesis of this first dedicated precursor was anticipated to be the limiting step. Therefore, we decided to express Module 1 on high copy plasmids. In order to optimize DMLA production, we compared *lovC* with a related homologue. The mevastatin (also called compactin) cluster of *Penicillium citrinum* has been found to contain many genes with significant homology and identical putative functions to those in the lovastatin pathway³⁵. The enoyl reductase *MlcG*, homologue of *LovC* in *P. citrinum*, has been shown to be functionally equivalent to *LovC* in the lovastatin pathway when combined with *LovB* *in vitro*. DMLA production in *S. cerevisiae* carrying plasmids encoding *lovB*, *lovG*, and *mlcG* instead of the *A. terreus* native *lovC* led to 50% increase in DMLA titer (Fig. S1), therefore we chose to express *mlcG* in Module 1. YCB03 was transformed with the Module 1 plasmids pLovB (*lovB*) and pCB27 (*mlcG*, *lovG*), leading to an initial DMLA titer of 10 mg/L (Fig. 2A).

The next step was to incorporate Module 2, *lovA* and *CPR*, into the DMLA-producing strain to afford conversion to MLA and MJA. As is common amongst P450s, *LovA* is targeted to the endoplasmic reticulum (ER)³⁶. Recently, Trenchard *et al* showed that expression of an ER-targeted P450 from a high copy plasmid can cause ER membrane proliferation stress and

lower production of the desired heterologous product. To avoid this, Module 2 was integrated into the genome of the production strains. The *A. terreus* *CPR* was integrated at the *ura3* locus in YCB03, forming YCB05. Knocking out *pyc2* (involved in reverse glycolysis) or *nte1* (involved in lipid biosynthesis) has been shown to increase the acetyl-CoA pool in yeast. As acetyl-CoA is a precursor to malonyl-CoA, these mutations could increase production of DMLA as well. Therefore, *nte1* and *pyc2* in YCB05 were knocked out sequentially and replaced by the *lovA* expression cassette, forming YCB06 and YCB07 respectively. After transformation with the Module 1 plasmids (pLovB and pCB27), total polyketide titer in YCB06 and YCB07 increased to 20 mg/L and 25 mg/L, respectively.

Despite doubling the total polyketide titer, conversion from DMLA to MJA was only 5–10% (Fig. 2A). For the next step, we hypothesized that further converting MJA to SVA would increase the flux of DMLA to MJA. To test this, we integrated a copy of *lovD9*, the evolved version of the acyltransferase *lovD*, in the *HO* locus of YCB07, forming YCB09. YCB09 transformed with pLovB and pCB27 was cultured in YPD and DMB-SMMP was added exogenously at 24, 36, 48, and 60 hours post inoculation to a final concentration of 1.22 mM. While expressing LovD9 increased the percent conversion of DMLA to MJA to 44%, the final SVA titer was only 0.5 mg/L, indicating that conversion from MJA to SVA was the limiting step, and the total polyketide production decreased to 20 mg/L (Fig. 2A, B). Based on these initial results, we aimed to increase the total amount of polyketide produced, followed by improving conversion through the pathway to MJA and SVA.

3.2 Optimization of Total Polyketide Production and Conversion to MJA

Due to the low initial production of polyketide in YCB03, production of DMLA was explored in YRC01, a descendent of BJ5464-NpgA with the *A. terreus* *CPR* integrated in the genome at the *ura3* locus. Comparison of these strains transformed with pLovB and pCB21 showed that YRC01 produced twice as much polyketide as YCB03, justifying a shift in base strain (Fig. S2). JHY686 has a doubling time of 1.6 hours compared to the 2.4-hour doubling time of YRC01, so the lower titer in polyketide production could be due to JHY686-descended strains having higher carbon flux towards growth than polyketide production. CRISPR-Cas9 was used for gene knockouts and integrations in YRC01. In order to avoid any marker-mediated integrations and to reduce any effects genomic expression of iCas9 had on polyketide production, we expressed iCas9 and the gRNA cassette together on the same plasmid. In addition to the donor DNA with appropriate homology for the targeted locus, strains targeted for genome edits were transformed with three linear pieces of DNA that were assembled by homologous recombination *in vivo* into a single plasmid expressing the iCas9 cassette and gRNA cassette for targeting the desired locus.

Using this system, copies of *lovA* were integrated into either the *X-2* or the *pyc2* locus to yield YCB11 and YCB19, respectively. CRISPR-Cas9 mediated integrations in YRC01 proved to be less efficient than in JHY686, potentially due to sequence differences between the genome of YRC01 and the reference yeast genome from SGD. After transforming pLovB and pCB21, the polyketide titers from YRC01, YCB11 and YCB19 were measured to be 65 mg/L, 30 mg/L and 42 mg/L, respectively (Fig. 3A). Together, these results indicate that the genome expression of *lovA* decreases production but the *pyc2* knockout can improve

total polyketide production. With only one integrated copy of *lovA*, the percent conversion from DMLA to MJA was 60 – 65%, indicating that more copies of *lovA* may be necessary for sufficient production of MJA.

To increase the amount of LovA expressed in the different hosts, we transformed a third plasmid, pCB19 (*lovD9* and *lovA*), into YRC01, YCB11, and YCB19 along with the Module 1 plasmids. This significantly improved conversion of DMLA to MJA as 85%–90% conversion was observed in all three strains (Fig. 3A). The total polyketide titer, however, varied between the strains. For YRC01, both genome (as in YCB11) and episomal expression (with pCB19) of LovA led to decreases in total titer from 66 mg/L to 31 and 30 mg/L, respectively. This indicates the decrease in yield is a result of introducing the P450 into the yeast host, regardless of copy number of the gene. However, addition of pCB19 to YCB11 and YCB19, which both already expressed single integrated copies of *lovA*, did not further decrease the total polyketide titer. Notably, YCB19 transformed with all three plasmids had the highest polyketide production with 90% conversion of DMLA to MJA at 72 hours post inoculation. Measurements of the polyketides produced by this strain over time indicated that MJA titer peaked at 75 mg/L, 72 hours post inoculation (Fig. 3B). Contrasting the productivity of YCB11 and YCB19 further underscores the importance of the *pyc2* knockout for improving polyketide production. Our studies also showed that the gene dosage for P450 enzymes from biosynthetic pathways needs to be systematically tuned in *S. cerevisiae* for each pathway.

3.3 Characterizing plasmid stability and copy numbers

YCB19 transformed with pLovB, pCB21 and pCB19 represents the best strain to date in the production of MJA, the immediate precursor to simvastatin. The stability of the three 2 μ plasmids in YCB19 was measured by determining the percentage of cells maintaining each plasmid over the 72-hours culture period. The copy numbers of the plasmids provide an initial estimate for how many integrated copies of each pathway gene would be needed to reach a similar production level.

Copy numbers of each of the plasmids in YCB19 were measured at 24, 48, and 72 hours after inoculation (Fig. 3B, Table 3). The copy number of each plasmid was similar at 24 and 72 hours after inoculation, indicating that the plasmid copy number was stable for this period of culturing. Notably, each plasmid was only carried by ~40–50% of the cells at any time point, resulting in a significantly lower copy number of each plasmid when averaged over all cells. This indicates that similar levels of production and conversion to MJA could potentially be achieved by integrating less than ten copies of *lovA*, rather than the twenty or more copies of pCB19 maintained by the cells. The percentage of cells carrying all three plasmids simultaneously could be even lower than 40%, which would indicate that even lower integrated copy numbers of the needed genes would be sufficient to reach similar levels of production.

3.4 Development of an *in situ* lysis process to overcome substrate and enzyme localization differences

With higher conversion to MJA achieved, the final step was to improve conversion of MJA to SVA. In the initial SVA production strain (YCB09 transformed with pLovB and pCB27), DMB-SMMP was added throughout the 72-hour culturing period and less than 1 mg/L SVA was produced. Several possible reasons were examined for this low conversion. First, growth curves of *S. cerevisiae* in the presence of DMB-SMMP indicated that it is toxic to yeast growth, which could contribute to the lower total polyketide production (Fig. S3). Second, LovD9 has optimal activity at pH 8.0 to 9.5, while the pH of *S. cerevisiae* grown in YPD is significantly lower. The intracellular pH inside of *S. cerevisiae* is 6–7 while the extracellular pH is 5–6. Third, different localizations of MJA, DMB-SMMP, and LovD9 might prevent high conversion of MJA to SVA. Finally, simvastatin and lovastatin have both been shown to greatly inhibit yeast growth at 1.3 mM exogenous concentrations of these statins.

To circumvent DMB-SMMP toxicity, DMB-SMMP was added to YCB19 transformed with the three plasmids only at 72 hours after inoculation, when the concentration of MJA was the highest, and samples were taken 24 hours later. However, this led to only 0.5 mg/L SVA (Fig. 4B). Next, Tris-HCl buffer at pH 8.7 was added with DMB-SMMP to the cultures at 72 hours post-inoculation to improve the activity of LovD9. Increasing the culture pH led to a five-fold increase in SVA production to 5.9 mg/L (Fig. 4B), however, the percent conversion from MJA to SVA was still less than 15%.

To further increase conversion, we investigated the localization of the produced MJA. Separate metabolite analysis of the cell pellet and the media indicated that 98% of the produced MJA has been secreted to the extracellular space (Fig. S4). As DMB-SMMP was added exogenously, a significant portion of this substrate would also be in the media. With both substrates extracellular and LovD9 expressed intracellularly, conversion to SVA was therefore expectedly low. One possible solution is to remove the cell wall barrier at the time of MJA to SVA conversion. Cell-free reactions have been used to improve production of natural products, but these systems have required spinning down cultures and resuspending in a lysis buffer or otherwise concentrating the extract, which introduces significant process steps and scale-up barriers.

For this reason, we decided to lyse the cells *in situ* at 72 hours post inoculation when MJA titer was highest (Fig. 3B, Fig. 4A). Several lysis methods were tested, all performed with the increased media pH and addition of 1.22 mM DMB-SMMP. Initially, the cells were lysed *in situ* using glass beads, however decreased conversion relative to the whole cells was observed (Fig 4B). We then tested lysis efficiency using Y-PER and the cost-effective detergent 3-(N,N-Dimethylmyristylammonio)propanesulfonate (SB3–14) as chemical lysis agents. Y-PER and SB3–14 were both found to lead to improved SVA production, with 5 g/L SB3–14 giving the highest titer of SVA at 32 mg/L (Fig. 4B). We further determined that 2 g/L SB3–14 was the minimum concentration required to lyse the cells completely (Fig. S5).

Using 2 g/L of SB3–14 as the lysis agent, we monitored the conversion of MJA to SVA over time. The highest concentration of SVA occurred 16 hours after lysis (Fig. 4C,D). At longer

time, the titer of SVA decreased, while that of MJA increased. This is expected as LovD can catalyze the reverse reaction which is the hydrolysis of SVA to MJA. Analysis of the breakdown of polyketide products formed at the time points taken indicated that the concentrations of DMLA and MLA remained low and constant throughout. Adding an increased amount of DMB-SMMP led to production of 55 mg/L SVA and over 95% conversion from MJA (Fig. 4C,D). This represents the highest level of SVA produced in yeast using fermentation approaches and an 110-fold increase from the initial SVA production reached.

4. Conclusion

In this study, we refactored the LVA biosynthetic pathway from *A. terreus* into *S. cerevisiae* and found that higher polyketide production was reached in the BJ5464-descended strains than in the faster growing BY4741-descended strains. We found that incorporation of the P450 *lovA*, whether integrated or episomally expressed, decreased total polyketide production. However, total polyketide production can be increased by knockouts of *pyc2* and *nte1*, and could compensate for the drop in titer upon *lovA* overexpression. High copy expression of *lovA* was necessary for the near complete conversion of DMLA to MJA. The initial SVA production from the top MJA-producing strain was 0.5 mg/L, which was increased 5-fold via pH optimization and 110-fold via optimized *in situ* lysis, reaching 55 mg/L SVA and nearly 100% conversion from MJA. This work marks the first time SVA has been produced using a single heterologous host, *S. cerevisiae*, and the first time *in situ* lysis has been used for production of a semisynthetic natural product. This provides a novel strategy that can be used by future researchers for production of semisynthetic polyketides in yeast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Produced MJA and SVA heterologously in *S. cerevisiae*.
- Increased conversion to MJA through high copy expression of a fungal P450.
- Developed an *in situ* lysis process that increased MJA to SVA conversion 110-fold.
- Produced 55 mg/L SVA in *S. cerevisiae*, reaching nearly complete conversion from MJA.

Figure 1:

Lovastatin and simvastatin biosynthesis. In Module 1, LovB (polyketide synthase) and LovC (enoyl reductase) use one acetyl-CoA and eight malonyl-CoA units to form the polyketide backbone which is released from LovB by LovG (thioesterase), forming DMLA (dihydromonacolin L acid). In Module 2, LovA (cytochrome P450 monooxygenase) catalyzes the conversion of DMLA to MLA (monacolin L acid) and then to MJA (monacolin J acid). LovA is reduced by its partner CPR (cytochrome P450 reductase). In Module 3, the semisynthetic acyl donor DMB-SMMP (α,α -dimethylbutyryl-S-methyl-

mercaptopropionate) is transferred to MJA by an evolved version of LovD (acyl transferase) to form SVA (simvastatin acid). In the native pathway, LovF (polyketide synthase) provides the acyl side chain which is transferred by the wild type LovD to form LVA (lovastatin acid).

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Figure 2:

Polyketide production in the BY4741-descended strains. All strains contain Module 1 plasmids pLovB and pCB27 (A) Total polyketide production in green. The percent conversion of DMLA to MJA is in orange. Each point represents measurement from a different sample. Black horizontal lines indicate median values. (B) Breakdown of polyketide production in YCB09. Results are from triplicate runs. Black horizontal lines represent the median.

Figure 3: Polyketide production in the BJ5464-descended strains. All strains contained the Module 1 plasmids, pLovB and pCB21. (A) Polyketide production from YRC01, YCB11, and YCB19 at 72 hours post inoculation. The final row in the table, 2 μ lovA plasmid, refers to the presence or absence of pCB19. Total polyketide production in green. Percent conversion from DMLA to MJA is in orange. Each point is a different sample. Black horizontal lines indicate median values. (B) Breakdown of polyketide production in highest MJA producing

strain, YCB19 transformed with pLovB, PCB21 and pCB19 at 24, 48, and 72 hours post inoculation into YPD. Results in triplicate. Black horizontal lines represent median values.

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Figure 4: SVA production from the highest MJA producing strain. (A) Schematic showing the production protocol. In step i, the yeast was picked into selective media. After 24 hours, triplicate cultures of YPD were inoculated from the seed in step ii. After 72 hours of culturing, the pH was adjusted, DMB-SMMP was added, and then the appropriate lysis method was used, as shown in step iii. Cultures were shaken for up to 24 hours more while samples were taken. (B) SVA concentration 24 hours after lysis and DMB-SMMP feeding using different lysis methods. The pH of all the cultures was adjusted except for the one labeled none. Each point is a different sample. The horizontal black line represents the median. (C) Time course of percent conversion of MJA to SVA after addition of 2 g/L of SB3-14 (3-(N,N-Dimethylmyristylammonio)propanesulfonate), a chemical lysis reagent, and varied concentrations of DMB-SMMP. Results in triplicate. The horizontal lines represent the median values. (D) Breakdown of polyketide production of the highest SVA producing conditions from the time course in (C).

Table 1:*S. cerevisiae* strains used in this work.

Strain	Parent	Genotype modifications to parent	Reference
BY4741	S288C	<i>MATa his3⁻1 leu2⁻0 met15⁻0 ura3⁻0</i>	Brachman <i>et al</i> , 1998
DHY213	BY4741	<i>SAL1+ HAP1+ CAT5(91M) MIP1(661T) MKT1(30G) RME1(INS-308A) TAO3(1493Q)</i>	Harvey <i>et al</i> , 2018
JHY686	DHY213	<i>prb1⁻ pep4⁻ ADH2p-npgA- ACSIt lys2⁻0</i>	Harvey <i>et al</i> , 2018
YCB03	JHY686	<i>YPRCTy1-2::iCas9::LEU2</i>	This study
YCB06	YRCB03	<i>ura3⁻0::ADH2p-CPR-ADH2Δ nte1⁻::ADH2p-lovA-ADH2Δ</i>	This study
YCB07	YCB06	<i>pyc2⁻::ADH2p-lovA-ADH2Δ</i>	This study
YCB09	YCB07	<i>HO::ADH2p-lovA-ADH2Δ</i>	This study
BJ5464-NpgA	S288C	<i>MATa ura3-52 trp1 leu2-1 his3- 200 pep4::HIS3 prb1-1.6R can1 GAL</i>	Ma <i>et al</i> , 2009
YRC01	BJ5464-NpgA	<i>ura3⁻::ADH2p-CPR-ADH2Δ</i>	Tang <i>et al</i> , 2015
YCB11	YRC01	<i>X-2::ADH2p-lovA-ADH2Δ</i>	This study
YCB19	YRC01	<i>pyc2⁻::ADH2p-lovA-ADH2Δ</i>	This study

Table 2:

Production plasmids used in this work

Production Plasmid	Description	Reference
pLovB	2 μ ; <i>AmpR</i> ; <i>URA3</i> ; <i>ADH2p-lovB</i> -6xHis- <i>ADH2</i>	YEplovB-6His from Xu, <i>et al</i> , 2013
pGC	2 μ ; <i>AmpR</i> ; <i>TRP1</i> ; <i>ADH2p-lovC</i> -6xHis- <i>ADH2</i> ; <i>ADH2p-lovG</i> -6xHis- <i>ADH2</i>	pSL05 from Xu, <i>et al</i> , 2013
pCB21	2 μ ; <i>AmpR</i> ; <i>TRP1</i> ; <i>ADH2p</i> -6xHis- <i>mlcG-ADH2</i> ; <i>ADH2p-lovG</i> -6xHis- <i>ADH2</i>	This study
pCB27	2 μ ; <i>AmpR</i> ; <i>HIS3</i> ; <i>ADH2p</i> -6xHis- <i>mlcG-ADH2</i> ; <i>ADH2p-lovG</i> -6xHis- <i>ADH2</i>	This study
pCB19	2 μ ; <i>AmpR</i> ; <i>LEU2</i> ; <i>ADH2p-lovA</i> -6xHis- <i>ADH2</i> ; <i>ADH2p</i> -6xHis- <i>lovD9-ADH2</i>	This study

Table 3:

Plasmid copy number characterization. The percent of cells carrying plasmids was calculated by comparing the colony forming units (CFU) on the appropriate selective media plates to the CFU on complex media plates. For selection markers, pLovB carried *ura3*, pCB21 carried *trp1*, and pCB19 carried *leu2*. The copy number averaged over all cells was calculated by qPCR using the standard curve method, and the copy number per cell carrying plasmid was calculated by taking the average copy number for all the cells and dividing it by the fraction of cells carrying the plasmid of interest.

	Time post inoculation	Percent of cells carrying plasmid	Copy number per cell carrying plasmid	Copy number averaged over all cells
pLovB	24 hours	39.3 ± 12.7	18.4 ± 5.7	6.8 ± 1.9
	48 hours	47.5 ± 12.2	22.8 ± 3.3	10.4 ± 1.0
	72 hours	44.1 ± 4.1	22.5 ± 5.3	9.7 ± 1.9
pCB21	24 hours	40.7 ± 13.5	75.6 ± 38.7	25.6 ± 8.4
	48 hours	43.3 ± 11.0	108.6 ± 20.7	45.1 ± 7.7
	72 hours	37.4 ± 5.0	81.5 ± 19.3	30.0 ± 5.5
pCB19	24 hours	51.7 ± 10.8	18.1 ± 3.1	9.5 ± 3.0
	48 hours	52.8 ± 12.7	25.2 ± 7.5	12.4 ± 0.7
	72 hours	44.7 ± 12.4	28.6 ± 6.8	12.1 ± 2.4