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Engineering Terpene Biosynthesis in *Streptomyces* for Production of the Advanced Biofuel Precursor Bisabolene

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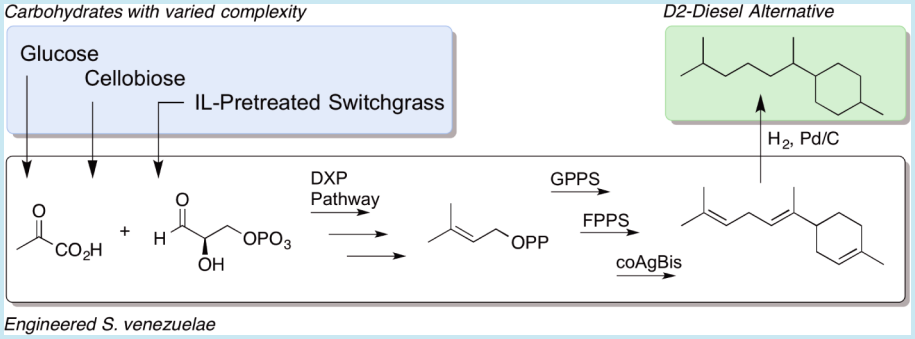
Supporting Information



ABSTRACT: The past decade has witnessed a large influx of research toward the creation of sustainable, biologically derived fuels. While significant effort has been exerted to improve production capacity in common hosts, such as *Escherichia coli* or *Saccharomyces cerevisiae*, studies concerning alternate microbes comparatively lag. In an effort to expand the breadth of characterized hosts for fuel production, we map the terpene biosynthetic pathway in a model actinobacterium, *Streptomyces venezuelae*, and further alter secondary metabolism to afford the advanced biofuel precursor bisabolene. Leveraging information gained from study of the native isoprenoid pathway, we were able to increase bisabolene titer nearly 5-fold over the base production strain, more than 2 orders of magnitude greater than the combined terpene yield in the wild-type host. We also explored production on carbon sources of varying complexity to, notably, define this host as one able to perform consolidated bioprocessing.

KEYWORDS: *Streptomyces, isoprenoid, terpene, biofuel, consolidated bioprocessing*

Throughout modern history petroleum has played a key role in providing low-cost energy and simple commodity chemicals to numerous sectors of the world economy. In the face of global climate change and the serious threat it represents, recent efforts to meet our global energy needs have shifted away from merely identifying additional fossil fuel stores to developing and improving sources of renewable energy.[1] One of many viable alternatives, biologically derived fuels offer a cost-effective and environmentally benign source of energy that, importantly, fits with our current infrastructure. Accordingly, recent focus has centered on the improvement of biobased fuel and commodity chemical production using natural and modified biosynthetic schemes.[2−6]

Traditionally thought of as fragrances and therapeutic agents, many terpenes, for example, pinene and bisabolene, have been touted as potential fuel replacements.[4,5] A recent study demonstrated bisabolane **2** (reduced bisabolene **1**) to have favorable fuel properties, comparable, and in some cases superior, to that of D2 Diesel fuel.[5] Similarly, dimerized pinene has been established as a suitable replacement for JP-10 jet fuel.[4,7] While examples of engineered, high-titer production of terpenes in organisms such as *Escherichia coli*[4,8] and *Saccharomyces cerevisiae*[5] are well established, the use of other, genetically tractable organisms remains relatively unexplored. Investigation of new hosts for biofuel production could provide bacterial chassis that allow similar production yields with the added benefits of consolidated bioprocessing (CBP),[9,10] carbon fixation[6,11] or uncommon sugar utilization.[12,13]

Streptomyces have long been targets of investigation due to their complex secondary metabolism;[14,15] in fact, over half of all known polyketides (PK) and nonribosomal peptides (NRP) originate from this genus. Recent focus has looked beyond the PK- and NRP-generating pathways to uncover a rich and largely untapped cache of isoprenoids.[16,17] Outside the immense range

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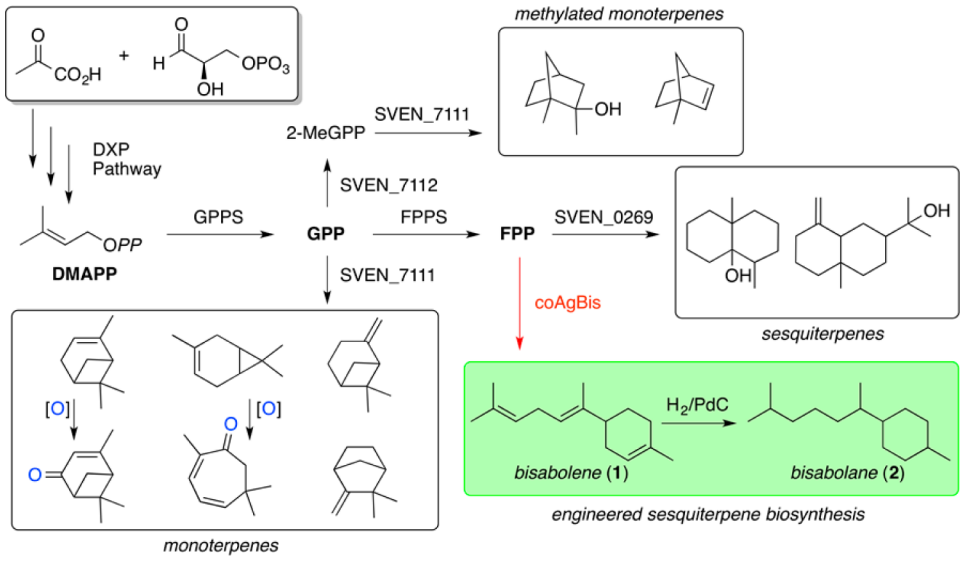

Figure 1. Biosynthetic routes to predominant isoprenoids produced by endogenous (black arrows) and heterologous (red arrow) terpene synthases in *S. venezuelae*.

of secondary metabolites, traits such as the ability to use multiple hexose and pentose sugars[12,13] and consume lignocellulosic biomass[18,19] position these bacteria well for efforts toward the engineered biosynthesis of low-cost fuel and commodity chemicals. Because of genetic tractability, rapid doubling time (compared to other *Strepotmyces*) and the absence of mycelial clumping during growth, *Streptomyces venezuelae* presents an ideal actinobacterial chassis from which to investigate modified terpene production and assess the native ability to use varying glycosidic polymers, a trait integral to efficient CBP.[9] Herein we define the isoprenoids endogenous to *S. venezuelae* ATCC 10712 (hereafter referred to solely as *S. venezuelae*) and detail efforts to produce bisabolene, increase titer and explore the ability of this organism to catabolize glucose and higher order carbohydrate polymers.

Two principal biosynthetic routes, the mevalonate (MEV) and deoxyxylulose-5-phosphate (DXP), are responsible for producing isoprenoids. Interestingly, given the choice of either path, *Streptomyces* are known to almost exclusively use the DXP pathway to generate dimethylallyl pyrophosphate (DMAPP) and its isomer isopentenyl pyrophosphate (IPP); the first committed intermediates in the production of higher isoprenoids. The joining of DMAPP and IPP provides the C_{10} building block geranyl pyrophosphate (GPP): further condensation of IPP and GPP provides farnesyl pyrophosphate (FPP) (Figure 1). Identity of the downstream terpene cyclase dictates the size and structure of the resultant product. Modification of the DXP/MEV pathway, GPP/FPP synthase or the terpene cyclase can, and has been demonstrated to, drastically alter titer or structure of the generated terpene.[5,20]

Of the many possible isoprenoid metabolites *Streptomyces* are widely known as producers of the "earthy-odorants" geosmin[21,22] and the unique C_{11} terpene 2-methylisoborneol (2-MIB).[23] While these compounds are generated by many actinobacteria, genes responsible for their genesis and the associated biosynthetic paths have only recently been elucidated.[21,22,24] Efforts to identify additional bacterially produced terpenes, beyond the two canonical examples, have exposed a vast, overlooked collection of terpene synthases and corresponding compounds. Accordingly, prior to manipulation of terpene biosynthesis within *Streptomyces*, a sound understanding of existing biosynthetic paths is essential.

Preliminary analysis of *S. venezuelae*-specific terpenes revealed the anticipated production of geosmin and 2-MIB, as well as trace amounts of both monoterpenes and complex aliphatic sesquiterpenes (Figure 1). Search of the *S. venezuelae* genome using antiSMASH[25] identified four hypothetical terpene cyclases corresponding to geosmin (SVEN_0269) and 2-MIB (SVEN_7111) biosynthesis and an additional two, putatively assigned as a generic terpene cyclase (SVEN_0552) and a hopene cyclase (SVEN_6451). In support of SVEN_7111 functioning as the 2-MIB cyclase, a characteristic methyltransferase (SVEN_7112) known to be required for 2-MIB biosynthesis was identified immediately downstream of the SVEN_7111 open reading frame.[23,24]

As it is difficult to predict the function of any given terpene cyclase *a priori* we opted to disrupt each of the four suspected terpene cyclases and the 2-MIB synthase-associated methyltransferase to determine the effect on the overall metabolite profile. Knockout of both the presumed geosmin and 2-MIB cyclases by gene disruption revealed, in fact, that these genes were correctly identified. SVEN_0269, required for geosmin biosynthesis, was also found to produce related sesquiterpenes putatively identified by MS as β-eudesmol and germacrene C (Supporting Information, Figure S1). SVEN_7111 solely formed the C_{11} terpene 2-MIB with roughly 15% of the alcohol degraded to 2-methyl-2-bornene, likely derived from the thermodynamically favorable elimination of water. Interestingly, while SVEN_7111 clearly prefers methyl-geranyl pyrophosphate (meGPP) as its substrate, it was also found to produce trace amounts of monoterpenes and oxidized derivatives from GPP. This substrate promiscuity mirrors results from *in vitro* studies concerning a homologue of this enzyme.[11] Knockout of the associated SVEN_7112 methyltransferase resulted in the abolition of C_{11} terpene production and the increased amounts of monoterpenes, predominantly pinene isomers, camphene, 2-carene and oxidized derivatives (Supporting Information, Figure S2), thus confirming the activity of this enzyme in the formation of meGPP (Figure 1).

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Table I. Relevant Genotypes and Descriptions of *S. venezuelae* Mutants Generated in This Study

strain	genotype	description
w.t.		w.t. ATCC 10712
T1K	ΔSVEN_0269::*aac(3′)IV*	disruption of geosmin synthase by in frame insertion of apramycin resistance gene
T2K	ΔSVEN_0552::*aac(3′)IV*	disruption of sesquiterpene cyclase by in frame insertion of apramycin resistance gene
T3K	ΔSVEN_6451::*aac(3′)IV*	disruption of putative hopene cyclase by in frame insertion of apramycin resistance gene
T4K	ΔSVEN_7111::*aac(3′)IV*	disruption of 2-MIB synthase by in frame insertion of an apramycin resistance
T4mK	ΔSVEN_7112::*aac(3′)IV*	disruption of meGPP synthase by in frame insertion of an apramycin resistance gene
T1D-T4K	ΔSVEN_0269; ΔSVEN_7111::*aac(3′)IV*	T1K with disruption of the 2-MIB synthase by in frame insertion of an apramycin resistance gene
T1-4D	ΔSVEN_0269; ΔSVEN_7111	deletion of both the geosmin and 2-MIB synthase genes
SZ01	pSV-coAgBis	wild-type strain harboring pSV-coAgBis
SZ02	ΔSVEN_0269::*aac(3′)IV*; pSV-coAgBis	T1K containing pSV-coAgBis
SZ03	ΔSVEN_0269::*aac(3′)IV*; ΔSVEN_7111; pSV-coAgBis	T1D-T4K containing pSV-coAgBis
SZ04	ΔSVEN_0269; ΔSVEN_7111; pSV-coAgBis; SVEN_0055::attP	T1-4D containing pSV-coAgBis and FPPS1 under control of ermEp* promoter inserted at the aatP chromosomal site
SZ05	ΔSVEN_0269; ΔSVEN_7111; pSV-coAgBis; SVEN_4268::attP	T1-4D containing pSV-coAgBis and FPPS2 under control of ermEp* promoter inserted at the aatP chromosomal site
SZ06	ΔSVEN_0269; ΔSVEN_7111; pSV-coAgBis; SVEN_4915::attP	T1-4D containing pSV-coAgBis and FPPS3 under control of ermEp* promoter inserted at the aatP chromosomal site
SZ07	ΔSVEN_0269; ΔSVEN_7111; pSV-coAgBis; SVEN_6450::attP	T1-4D containing pSV-coAgBis and FPPS4 under control of ermEp* promoter inserted at the aatP chromosomal site

As studies revealed monoterpenes to be acutely toxic to *S. venezuelae*, disruption of SVEN_7112 produced a recombinant strain with severely compromised growth characteristics. This phenotype is presumably due to increased metabolic flux toward biosynthesis of toxic monoterpenes.

Beyond the predominant isoprenoids generated in *S. venezuelae* two cyclases remained that could potentially be responsible for low-titer production of the remaining aliphatic sesquiterpenes. It was discovered that disruption of SVEN_0552 caused the disappearance of minor products (longifolene, cubene, *etc.*), while knockout of SVEN_6451 had no discernible effect on the metabolite profile. The latter result was not surprising as the gene was expected to code for a hopene cyclase, a class of compounds that would have not have been identified in our GC/MS analysis. Neither disruption appreciably changed the viability of the resultant recombinant strains.

With regard to wild-type terpene production, we were able to estimate overall titers (combined monoterpenes and sesquiterpenes) to be 30 μg/L (Supporting Information, Table S1). We determined that the most active cyclase was that responsible for 2-MIB synthesis, roughly accounting for 75% of the total isoprenoids detected. The majority of remaining terpene-based compounds were created by geosmin synthase (SVEN_0269) with sesquiterpene synthase SVEN_0552 only providing trace amounts of product.

Beyond efforts to catalog the biosynthesis of isoprenoids, a second thrust aimed to determine the ability of *S. venezuelae* to generate the biofuel precursor bisabolene **1**, as it (i) is a precursor to the D2 diesel alternative bisabolane and (ii) demonstrated low toxicity to *S. venezuelae* in external-supplementation growth experiments (Supporting Information, Table S6). As no dedicated bisabolene synthase has been isolated from a bacterial source we turned to alternate organisms for the cyclase of interest. A recent screening effort examined five different bisabolene synthases isolated from plant sources and assessed the effect of codon optimization and cyclase identity on overall production.[5] The *Abeis grandis* (Grand Fir) bisabolene cyclase (AgBis), optimized for expression in *S. cerevisiae*, vastly outperformed alternate bisabolene synthases *in vivo*. Taking a cue from this study, we synthesized a codon-optimized variant of AgBis (coAgBis) for expression in *Streptomyces*. This gene was inserted into the multicopy expression plasmid pUWL-oriT[26] under control of the strong constitutive promoter *ermE*p*[27] to yield pSV-coAgBis. The resultant plasmid was introduced in wild-type (w.t.) *S. venezuelae* to generate a new strain expressing coAgBis (*S. venezuelae* SZ01, Table I). This strain was initially tested with a variety of media to optimize production. The highest titer with *S. venezuelae* SZ01 was determined to be approximately 2.5 mg/L when grown in modified PM-4 medium (see Supporting Information). Bisabolene production appeared to begin about 12 h prior to 2-MIB, although production for both compounds peaked at 72 h (Supporting Information, Figure S3). Encouraged by the success of our initial effort we reasoned that elimination of competition for intermediates in isoprenoid biosynthesis, chiefly, GPP and FPP, would increase titers beyond what was observed in *S. venezuelae* SZ01.

Preliminary attempts to increase bisabolene production focused on the removal of genes competing with coAgBis for intracellular resources, namely, alternate terpene cyclases. Our initial mapping of the isoprenoid biosynthetic network in *S. venezuelae* revealed the 2-MIB and geosmin synthase formed the majority of observed terpenes; therefore, we opted to focus on a single and pairwise knockout strategy in an effort to boost yield. On the basis of the biosynthetic scheme outlined (Figure 1) we reasoned that the main competition for coAgBis with regards to FPP would be geosmin synthase (SVEN_0269); hence, we opted to create a SVEN_0269 deletion strain using gene replacement (see Materials and Methods). Introduction of pSV-coAgBis in the ΔSVEN_0269 mutant yielded recombinant strain *S. venezuelae* SZ02 (Table I). To further reduce competition for isoprenoid precursors, we next generated a deletion mutant of the 2-MIB synthase SVEN_7111 in the *S. venezuelae* SZ02 background to construct a mutant deficient for

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the two most active terpene cyclases. A bisabolene production strain *S. venezuelae* SZ03 was generated by introducing pSV-coAgBis into the double deletion mutant.

Bisabolene production results in *S. venezuelae* SZ02 and SZ03 were unexpected, as no discernible increase in bisabolene titer, compared to SZ01 strain, was observed (Figure 2). Contrary to

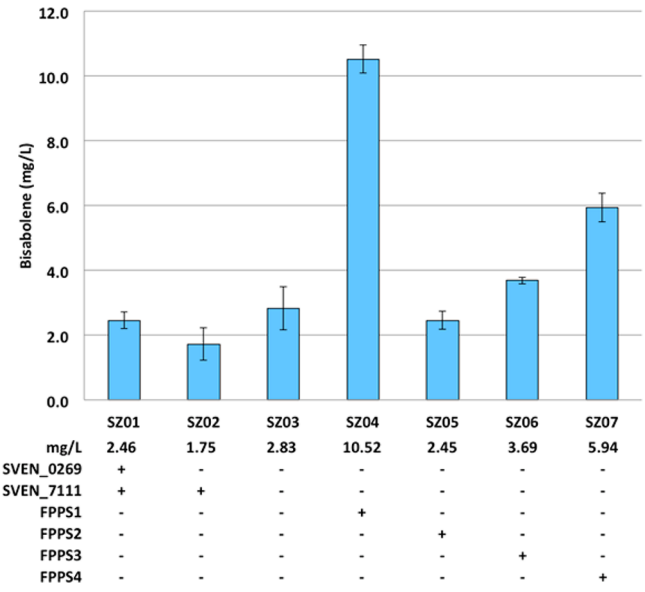

Figure 2. Bisabolene production by engineered *S. venezuelae* variants in modified PM-4 media at 72 h. Bottom table indicates presence (+) or absence (−) of a specific gene in the listed strain.

our expectations, *S. venezuelae* SZ02 produced approximately 30% less bisabolene when compared to that of wild-type. Closer investigation revealed the decrease in yield was the result of a growth defect imparted on strains possessing the single geosmin synthase knockout and carrying pSV-coAgBis. Growth comparison of SZ01 to SZ02 and SZ03 revealed decreased cell mass for SZ02 and a corresponding reduction in bisabolene production, while pairwise deletion of the 2-MIB and geosmin synthases in SZ03 restored growth to w.t. levels (Supporting Information, Table S4). This result implicated the 2-MIB synthase as at least partially contributing to the impaired phenotype of the geosmin synthase knockout. It is possible that as terpene biosynthesis is redirected in our engineered strains flux is increased toward generation of toxic monoterpenes, as observed in the SVEN_7112 knockout. Removal of the 2-MIB synthase restored w.t. growth and increased bisabolene yields 15% over that of the base strain SZ01. Unfortunately, efforts to increase bisabolene yield through removal of both competing enzymes was only moderately successful and in the case of SZ02 counterproductive. Aggregate results hint at additional regulatory elements or enzymatic bottlenecks contributing to an inability to increase FPP pools, despite elimination of competing enzymes.

Results from single and pairwise knockouts of the geosmin and 2-MIB synthases demonstrated cyclase competition was not the sole limiting factor in bisabolene biosynthesis. Review of data concerning native terpene biosynthesis provided a clue as to one of the possible factors restricting improvement of bisabolene titers. As methylated monoterpenes compose the majority of products generated in w.t. cells, it is highly likely that flux toward FPP is restricted to provide ample, but not excessive precursor concentrations for required sesquiterpene formation.

To test our hypothesis we first identified four putative FPP synthases (FPPS) in the *S. venezuelae* genome (Table I), which were subsequently cloned and inserted into an integrative expression vector under control of *ermE**p promoter. The four candidate FPPS genes were introduced into a double knockout strain isophenotypic to SZ03 to generate new strains overexpressing each of the identified FPP synthases and carrying pSV-coAgBis (SZ04−07). Resultant recombinant strains were grown under shake flask conditions and bisabolene production was determined. Titers could not be normalized to cell mass due to the insoluble nature of our production medium, but we did determine all cell lines, except SZ02 and SZ05, which were ∼30% reduced compared to w.t., to have similar growth characteristics and provide comparable cell mass when grown over 48 h in tryptic soy broth (Supporting Information, Table S4). While two of the FPP synthases, FPPS2 (SZ05) and FPPS3 (SZ06), improved bisabolene yields little, significant increases were observed in strains over-expressing FPPS1 (SZ04) and FPPS4 (SZ07). These two synthases increased bisabolene titers approximately 4- and 2-fold, respectively, over the improved double knockout strain, SZ03, with SZ04 producing in excess of 10 mg/L (Figure 2).

With regard to the outcome of the FPP upregulation experiments it is clear that the native synthase represents one bottleneck in the efficient overproduction of sesquiterpenes. The two seemingly inactive synthases, FPPS2 and FPPS3, were cloned from *S. venezuelae*, therefore, it is unlikely that the limited improvement was due to improper expression or folding. Rather, two more plausible explanations would be that either these genes were improperly identified by bioinformatics and are not functional FPP synthases, or, more likely, kinetic parameters of the new FPPSs are poor and do not support an increase of intermediates over the native FPPSs. Trials concerning FPPS1 and FPPS4 clearly demonstrate their efficacy and pinpoint one step in *S. venezuelae* terpene biosynthesis that can be modified to increase flux toward products.

In addition to the vast array of secondary metabolites produced by *Streptomyces*, this genus has also been documented to use numerous hexose and pentose sugars, and in some isolated instances consume cellulose. While use of alternative sugars has been demonstrated with *S. venezuelae*, the ability to consume higher order carbohydrate polymers has not yet been established. To define the innate ability of *S. venezuelae* to catabolize carbohydrate polymers we challenged our best strain, SZ04, to synthesize bisabolene from glucose, cellobiose and cellulose; the latter two being dimerized and polymerized glucose, respectively. Furthermore, as microcrystalline cellulose (Avicel) has rarely been identified as a carbon source for *Streptomyces* we opted to assess bisabolene production on ionic liquid-pretreated switchgrass. Pretreatment of switchgrass enables better access to single glucan polymers (*e.g.*, cellulose and hemicellulose) that would otherwise be blocked due to the crystalline nature of refined cellulose, thus providing a more accurate reflection of (hemi)cellulase activity. In addition to the presence of glucan in the pretreated switchgrass (∼47%) a significant fraction of our sample contained additional carbohydrate polymers, primarily xylan (∼22%). Because of the presence of putative xylanases within the *S. venezuelae* genome and its known ability to consume xylose it is possible that any activity realized on the switchgrass may be attributed to consumption of both glucan and xylan polymers. Regardless

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of preference for glucan or xylan, synthesis of bisabolene using this carbon source would establish *S. venezuelae* as a chassis capable of performing CBP (Figure 3).

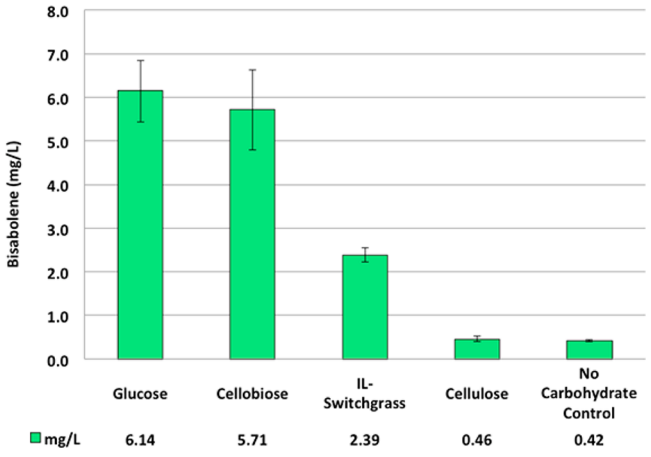

Figure 3. Assessment of bisabolene production on varying carbon sources in minimal media with *S. venezuelae* SZ04 at 72 h.

Results clearly demonstrate the ability of *S. venezuelae* to produce bisabolene on glucose polymers of varying length, albeit with differing efficiencies. Comparison of the glucose and cellobiose trials establishes the presence of sufficient active cellobiohydrolases to the extent that observed titers from the sugar monomer and dimer were essentially equivalent. While production on IL-pretreated switchgrass was less than that of glucose, bisabolene yields in excess of 2 mg/L from cellulosic biomass are a significant achievement. Biosynthesis of bisabolene at this concentration compares well to alternate, highly engineered hosts (*e.g.*, *E. coli*) that have been demonstrated to produce the related monoterpene pinene at similar levels on comparable feedstocks.[10] Moreover, as this host is unmodified with respect to CBP, bisabolene yields could conceivably be increased to match those realized with glucose given a minor investment in upregulating or heterologously expressing improved (hemi)cellulases or xylanases. It is not surprising that production from microcrystalline cellulose was largely unsuccessful as the compact nature of this carbon source often precludes breakdown of cellulose polymers and is incapable of supporting cell growth. Combined, these results define the ability of *S. venezuelae* to grow and produce terpenes on alternative carbohydrate polymers and identify an important bottleneck (*i.e.*, polysaccharide depolymerization) that prevents pretreated switchgrass from competing with more highly refined carbon sources, such as cellobiose or glucose.

In a departure from traditional efforts concerning *Streptomyces* our studies went beyond merely cataloging novel secondary metabolic pathways and to demonstrate how a detailed understanding of terpene biosynthesis in *S. venezuelae* could be leveraged for successful metabolic engineering. Building on our investigations to map terpene biosynthesis we were able to heterologously express a codon optimized bisabolene synthase from *A. grandis* and demonstrate significant bisabolene production. Identification of catalytic bottlenecks helped increase yields almost 5-fold over our base engineered strain (SZ01), 350 times greater than cumulative terpene yields realized in wild-type. While these production levels are an approximate order of magnitude off bisabolene production titers found in *S. cerevisiae*,[4] they compare well to pinene production in *E. coli*,[5] both on simple and complex carbohydrates.[10] In a further effort we were able to investigate the production of bisabolene on carbohydrate polymers and demonstrate this bacterium as one capable of catabolizing IL-pretreated switchgrass, thus establishing the innate CBP capabilities of *S. venezuelae*. While we have demonstrated the ability to generate bisabolene at titers comparable to alternate systems using cellulosic biomass, this study further establishes the promise of this genus for future applications and sets the stage for numerous engineering endeavors to further improve traits for high-titer biofuel or commodity chemical biosynthesis.

MATERIALS AND METHODS

Inactivation of Genes Involved in Terpene Biosynthesis. Internal gene fragments for SVEN_0269, SVEN_7111, SVEN_0552, SVEN_6451, and SVEN_7112 were PCR-amplified from the *S. venezuelae* genomic DNA and joined with the PCR-amplified portion of pSOK201[28,29] to generate gene disruption plasmids pT1K, pT2K, pT3K, pT4K and pT4mK, respectively. Oligonucleotides used as primers for amplification of the joining parts were designed using j5 online software package,[30] and are listed in Table S2 (Supporting Information). After verification, the gene disruption plasmids were introduced into *S. venezuelae* using conjugation from *Escherichia coli* ET12567 (pUZ8002), and transconjugants verified for the correct insertion of the plasmids using PCR (data not shown).

For the in-frame deletion of the genes encoding geosmin and 2-methylisoborneol synthases (SVEN_0269 and SVEN_7111, respectively), the left and right flanking regions for these genes were amplified from the genomic DNA of *S. venezuelae* and seamlessly fused with one another and a part of the pSOK201 vector using Gibson assembly. Oligonucleotides used as primers for amplification of the joining parts were designed using j5 online software package, and are listed in Table S2 (Supporting Information). The gene deletion plasmid pTD1 for SVEN_0269 was introduced into *S. venezuelae* by conjugation, and transconjugants verified by PCR for correct insertion of the construct *via* single crossover. After 3 rounds of subculturing in TSB medium (Oxoid, UK) at 30 °C, 250 rpm and without antibiotic selection, the culture was inoculated on ISP4 plates and grown until sporulated at 28 °C. Spores were plated out in dilutions on ISP4, and resulting colonies were replica plated on ISP4 supplemented apramycin. Genomic DNAs from the sensitive clones were tested for the correct deletion of the gene *via* the second crossover using PCR (data not shown). The resulting mutant designated T1D was used as a host for the gene deletion plasmid pTD4 for SVEN_7111, and the deletion of the latter gene was achieved using the same procedure. The double deletion mutant was designated T1-4D.

Generation of Bisabolene and Farnesyl Pyrophosphate Synthase Expression Vectors. The gene for bisabolene synthase AgBis was synthesized (see Supporting Information) as a codon-optimized version based on *Streptomyces* codon usage, flanked by sequences with recognition sites for endonucleases *Hin*dIII (5′ end) and *Xba*I (3′ end). The *Hin*dIII-*Xba*I DNA fragment with codon-optimized AgBis was cloned, using corresponding sites, into the pUWL-oriT vector[31] under control of the *ermE**p promoter, yielding plasmid pSV-coAgBis.

For expression of farnesyl pyrophosphate synthase (FPPS) genes, an integrative vector pSOK806 was constructed *via* fusion of a PCR-amplified vector pSOK804 with the *ermE**p

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promoter using Gibson assembly. The four putative FPPS genes (SVEN_0055, SVEN_4286, SVEN_4915, and SVEN_6450) were PCR-amplified from the *S. venezuleae* chromosome and cloned into the pSOK806 vector under control of the *ermE**p promoter using Gibson assembly. Primer design for the above experiments was done using j5, and primer sequences are presented in Table S3 (Supporting Information).

Generation of Bisabolene and Farnesyl Pyrophosphate Synthase Expression Strains. To obtain the first-generation bisabolene-producing strains, the plasmid pSV-coAgBis was introduced into the T1D and T1-4D deletion mutants, yielding strains SZ02 and SZ03, respectively. The second generation bisabolene-producing strains were generated based on the T1-4D mutant, where the plasmids for overexpression of four putative FPPS genes were inserted into the genome. Finally, the pSV-coAgBis was introduced into the four resulting recombinant strains each overexpressing putative FPPS genes SVEN_0055, SVEN_4286, SVEN_4915, and SVEN_6450, yielding recombinant strains SZ04, SZ05, SZ06 and SZ07, respectively.

Bisabolene Production in *Streptomyces venezuelae*. The appropriate *S. venezuelae* strain was streaked on glycerol-arginine agar and let grow at 30 °C until fully sporulated, typically 72 h. Fresh spores were collected and used to inoculate an overnight preculture in tryptic soy broth, which was grown at 30 °C with 200 rpm shaking. A 3% inoculum from the overnight culture was used to seed 50 mL of PM-4 or carbon utilization media (see Supporting Information), again in baffled flasks. Following inoculation, a 10 mL overlay of dodecane was applied and the cultures were let grow at 30 °C with shaking. After 72 h the dodecane layer was collected and diluted 1:4 in ethyl acetate to give a final concentration of 1× with respect to the initial culture. Bisabolene production was analyzed on a Hewlett-Packard 6890 series GC fitted with an Agilent 5973Network mass detector with a 30 m × 0.25 mm DB-5MS column (Agilent). Samples were injected at 100 °C, held at that temperature for 0.75 min, and then ramped to 150 °C at 40 °C/min and further to 300 °C at 60 °C/min, at which point the temperature was held for 1.0 min and returned to the initial temperature. Samples were compared to an authentic bisabolene standard curve, all repeated in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1−S6 and Figures S1−S4, as well as primer and coAgBis sequences. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

R.M.P and S.B.Z. conceived the study. All authors helped design experiments. R.M.P, O.N.S., and S.B.Z. executed the experiments. R.M.P. prepared the manuscript, which was critically read and revised by all.

Notes

The authors declare no competing financial interest.

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