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Screening for improved isoprenoid biosynthesis in microorganisms

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Abbreviations

*Aa*ADS: *Artemisia annua* amorpha-4,11-diene synthase *Aa*FS: *Artemisia annua* β-farnesene synthase, CAT: chloramphenicol acetyltransferase, DMAPP: dimethylallyl diphosphate, DXP: deoxyxylulose-5-phosphate, FCM: fluorescence-activated cell sorting by flow cytometry and magnetic cell sorting, FPP: farnesyl diphosphate, GPP: geranyl diphosphate, GGPP: geranylgeranyl diphosphate, GGPPS: GGPP synthase, IPP: isopentenyl diphosphate, HTS: high-throughput screening, MEV: mevalonate, PPi: inorganic diphosphate, TEAS: *Nicotiana tabacum* 5-*epi*-aristolochene synthase

Abstract

The production of isoprenoids in recombinant microbes for flavor & fragrance, pharmaceutical, agricultural or fuel applications is a booming research field. Isoprenoid extraction from natural resources and chemical synthesis is frequently neither ecological nor commercially profitable. However, recombinant microbes also show severe limitations in specific isoprenoid synthesis. Therefore, diverse directed evolution strategies have been developed for recombinant microbes. The focus has been laid either on the overall engineering of recombinant hosts or on the improvement of isoprenoid synthases. Currently, the most prominent and advanced approaches are based on carotenoid-producing strains, which can be screened by simple colorimetric readout. Other screening strategies are based on spectrophotometric analyses of colored by-products, fluorescence applications, growth selection and, to a minor extent, the use of biosensors indicating the pool of isoprenoid precursors. Although the number of approaches is still small, we observe a trend towards rigorous and highly creative assays that, however, often rely on the indirect detection of the evolved enzyme activities or host strains. We conclude that the use of whole-cellular systems is clearly favored over cell extracts and predict that next-generation screening assays need to be developed towards broader applicability and more direct assessment of isoprenoid production levels.

Introduction

Progress in molecular biotechnology heavily depends on directed enzyme or strain evolution strategies and a variety of applications is taking profit from optimized enzyme or strain performance (Dalby, 2011; Davids et al., 2013; Goldsmith and Tawfik, 2012). Most importantly, the heart and prerequisite of each directed evolution approach is the availability of a stable and reliable high-, or at least medium-throughput assay. But what if the screening assay needs to be established on challenging substrates and products that are, among others, water-insoluble, volatile and toxic to the production host? A commercially interesting substance class that combines all of these characteristics is isoprenoids. These compounds are also referred to as terpenoids for their first isolation from conifer turpentine secretions, but may be summarized as isoprenoids since they are metabolically derived from five-carbon isoprene units (Croteau et al., 2000). Volatile isoprenoids are primarily produced by plants, and, to a lower extent, by microorganisms (Quin et al., 2014; Yamada et al., 2015) and insects (Dewick, 2002; Dubey et al., 2003; Sobotník et al., 2010). Isoprenoid isolation from natural sources may be rather unsatisfactory on a commercial scale due to low abundances, contaminations with (isoprenoid) impurities and, thus, unpredictable seasonal variations in product quality (Ishida and Chapman, 2009; Lapkin et al., 2006). Therefore, isoprenoid overproduction with recombinant microbes has been established as attractive alternative (Keasling, 2010). Isoprenoid biosynthesis is initiated by the condensation of the two 5-carbon precursors isopentenyl diphosphate (IPP) and its isomer dimethyl allyl diphosphate (DMAPP) by prenyl diphosphate synthases, e.g. geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) synthases. Eukaryotes and prokaryotes have developed distinct pathways for the generation of IPP and DMAPP, namely the mevalonate-dependent (MEV) pathway (Buhaescu and Izzedine, 2007) and the mevalonate-independent or deoxyxylulose-5-phosphate (DXP) pathway (Wanke et al., 2001). Plants use both, the MEV pathway and the DXP pathway (Eisenreich et al., 2004; Rohmer, 1999). The condensation products of IPP and DMAPP termed polyprenyl diphosphates, e.g. GPP, FPP and GGPP, are converted to more complex isoprenoid structures by isoprene synthases (Dewick, 2002; Tholl, 2006)

and enzymatic hydroxylation and oxidation reactions (Figure 1). Hence, isoprene synthases are important regulators at metabolic branch points and often compete with other metabolic enzymes for the prenyl diphosphate pool. For example, FPP is the precursor to many cellular molecules including squalene (sterols), dolichols, ubiquinones and the heme cofactor. Isoprene synthases form a multitude of isoprene carbon skeletons and their reaction mechanisms and product specificities have been heavily studied (Cane et al., 1997; Gao et al., 2012; Mathis et al., 1997; Miller and Allemann, 2012; Segura et al., 2003). Isoprene synthases are slow enzymes with turnover rates of less than 0.5 per second (Cane, 1990; Liang, 2009) and, therefore, often constitute the bottlenecks of isoprenoid metabolic pathways. Given that prenyl diphosphates are essential for diverse cellular processes, this is hardly surprising. Cells will most likely not be viable if isoprene synthases extensively deplete the prenyl diphosphate pool. Additionally, isoprenoid synthesis can be limited by substrate inhibition (Crock et al., 1997) or the incompatibility of the engineered microbial hosts with their hydrophobic products (Brennan et al., 2013; Liu et al., 2013). Compared to the 55.000 natural isoprenoids that have been identified, the number of characterized isoprene synthases is surprisingly small (Christianson, 2008). As sequence homology of isoprene synthases of all species - plants, bacteria, fungi and insects - is very low, the search for unknown synthase genes based on sequence homology is rather unsatisfactory (Miller and Allemann, 2012; Quin et al., 2014; Yamada et al., 2015). Therefore, the chase for suitable and, ideally, more active isoprene synthases is ongoing.

There are basically two concepts of how to improve isoprenoid biosynthesis in recombinant microbial hosts. Either the recombinant microbial cell factory is engineered overly regarding availability of isoprenoid precursors or isoprene synthase activities are improved. Since isoprene synthesis will most often be the limiting factor of the overall metabolic flux to a desired isoprenoid, there are enormous benefits to be expected from improving the catalytic efficiency of isoprene synthases. Targeted approaches are limited by lack of fundamental knowledge of enzymatic, and host cell, functionalities, which is why directed evolution is of great interest. However, the evolutionary approach may be

cumbersome for isoprenoid biosynthesis given a shortage of straight-forward, efficient high-throughput screening strategies for these compounds. Nevertheless, several groups managed to come up with excellent and highly creative ideas of how to efficiently screen for enhanced isoprenoid production. Within this review we provide an overview of current advances, discuss their challenges and pitfalls, and prospect possible future development in this field.

Screening assays: To be high-throughput, or not

Ideally, a screening assay should meet the following criteria: medium to high-throughput, robustness, high specificity and sensitivity to discern improvements in performance, easy readout and, especially in the field of isoprenoid biosynthesis, compatibility with whole-cell microbial systems. The demand for proliferating microbes can easily be explained by the fact that isoprenoid building blocks are either produced by endogenous metabolic pathways of the host or by a cascade of reactions transplanted from other cell types (Keasling, 2010). The alternative use of cell extracts involves an additional cell-disruption step that scales down sample throughput and might unnecessarily harm enzyme activity. Additionally, screening assays that deal with whole cells provide the opportunity to screen for pathway engineering. On the downside, the expression of heterologous genes, e.g. isoprene synthases, in a microbial host can lead to metabolic imbalance or the accumulation of toxic metabolites (Brennan et al., 2013; Emmerstorfer et al., 2015; Liu et al., 2013). Therefore, it is particularly important to validate whether isoprenoid production harms the viability of the host. Generally speaking, ‘high-throughput’ has been defined as the testing of 10,000 to 100,000 compounds per day, accomplished with mechanization that ranges from manual operations to fully automated robotic systems (Inglese et al., 2007; Wölcke and Ullmann, 2001; Wunder et al., 2008). By this criterion, there are currently only a few isoprenoid screening procedures that can truly be considered as ‘high-throughput’. In most cases, the key limitation in the engineering of microbes for improved isoprenoid production still is a dependence on low-throughput, chromatography-based screens

for compound detection (Behrendorff et al., 2013; Emmerstorfer et al., 2015; Wang et al., 2014; Wriessnegger et al., 2014). Colorimetric screens would be amenable to high-throughput scenarios, but most isoprenoid compounds are colorless and require indirect optical readouts, e.g. sensor compounds that fluoresce. Only carotenoids can be identified and quantified based on color development. To date, the majority of screening approaches have been established on the basis of carotenoid colony pigmentation (Table 1) including fluorescence-based cell sorting by flow cytometry and magnetic cell sorting (FCM) (An et al., 1991). In fact, these assays are the ones that come closest to the term 'high-throughput'. By definition, most of the other strategies need to be classified as low- to medium-throughput only. For example, specific by-products of isoprenoid biosynthesis, e.g. phosphate or methanol (Figure 1), have been converted to colored compounds that were quantified spectrophotometrically in 96-well plates and were correlated to isoprenoid productivity. We provide a concise overview of all classes of isoprenoids that have been screened for in (recombinant) microbes and also list possible other applications of the published assays in Table 1.

1. Screening for carotenoid pigmentation

By far the most popular screening strategy is to visualize enhanced isoprenoid productivities by a colorimetric readout. Hence, many isoprenoid screening assays take advantage of microorganisms that produce carotenoids as this provides a visual readout for fast and easy assessment of isoprenoid production levels (Alper et al., 2005; Misawa, 2011; Park et al., 2008). Carotenoids are not only natural colorants but also potent antioxidants and, thus, interesting high-value compounds for pharmaceutical, cosmetic and food industries (Fiedor and Burda, 2014; Igielska-Kalwat et al., 2015). Especially astaxanthin is a most valuable substance (Ambati et al., 2014). Therefore, it is not surprising that many groups aim to engineer recombinant microbial systems to increase carotenoid yield or to mutate carotenoid backbone synthases to either obtain enzymes with higher product specificity or to derive novel products (Sandmann,

2015). To date, more than 700 naturally occurring carotenoids have been identified as products of the C_{30} and C_{40} carotenoid biosynthetic pathways. C_{40} carotenoids are much more common than their C_{30} counterparts (Hornero-Méndez and Britton, 2002; Wang et al., 2007). *E. coli* is the most frequently used host for the recombinant production of carotenoids (Harada and Misawa, 2009). In 1994, the so-called 'red-white screening' method has been the first assay published based on carotenoid pigmentation of an *E. coli* strain that expressed carotenoid biosynthesis genes, phytoene synthase (*crt*B) and phytoene desaturase (*crt*I), of *Erwinia uredovora*. Since carotenoids are synthesized from GGPP, a molecule that is not naturally present in *E. coli*, the assay was used to screen a genomic DNA library from *Sulfolobus acidocaldarius* for GGPP synthase (GGPPS) activity (Ohnuma et al., 1994). In case GGPP was synthesized, *crt*B and *crt*I converted it to lycopene and colonies changed their color from white to red. Shortly thereafter, an FPP synthase gene of *Bacillus stearothermophilus* was randomly mutagenized by $NaNO_2$ treatment and the library was screened with the same 'red-white screening' method (Ohnuma et al., 1996). Indeed, specific mutations of the FPP synthase were shown to favor the production of GGPP over FPP. Several years later, the endogenous *E. coli* geranyl diphosphate synthase IspA was evolved towards GGPP formation (Lee et al., 2005). Leonard and co-workers applied a saturation mutagenesis approach to isolate improved *Taxus canadensis* GGPP synthase variants (Leonard et al., 2010). In both cases, 'red-white screening' was employed.

Not only the flux towards the GGPP building block has been engineered, but also novel carotenoids have been synthesized. In 2002, the Arnold group inserted mutant libraries of the C_{30} carotene synthase *crt*M from *Staphylococcus aureus* into a C_{40} pathway and, *vice versa*, the C_{40} carotene synthase *crt*B from *E. uredovora* into a C_{30} pathway to assess whether unusual carotenoid compounds are formed in recombinant *E. coli* (Umeno et al., 2002). Differences in the pigmentation patterns of the resulting colonies proved that novel compounds could be generated. The strategy was brought to the next level by not only mutating the carotene synthase genes but also other genes of the C_{30} and C_{40} biosynthetic pathways and, thereby, diversifying carotenoid production. Strikingly, also non-natural C_{35}, C_{45}, and C_{50} carotenoid

pathways could be constructed (Umeno and Arnold, 2004). A specific variant of a C_{30} carotenoid backbone synthase, *crtM*$_{F26A}$, was shown to be responsible for forming C_{40} carotenoids (Umeno et al., 2002), and random mutagenesis of *crtM*$_{F26A}$ brought to light three new amino acid exchanges, H12A, D27A and I240F, that affected C_{30} and C_{40} carotenoid synthesis (Furubayashi et al., 2014b). Additionally, a gene-shuffling approach was described to screen for phytoene desaturase and lycopene cyclase activities to produce new carotenoid compounds (Schmidt-Dannert et al., 2000). Error-prone PCR was used to boost the activity and broaden the substrate scope of a carotenoid desaturase homolog from *S. aureus* (CrtOx) (Mijts et al., 2005). Excellent reviews sum up the achievements in the field of synthetic diversification and over-production of carotenoid compounds (Lee and Schmidt-Dannert, 2002; Umeno et al., 2005; Wang et al., 2007). A carotenoid-based screen has also been applied for the selection of yeast Erg20p variants capable of catalyzing all three IPP additions to DMAPP forming GGPP and alleviating a major bottleneck for diterpenoid biosynthesis in yeast cells (Ignea et al., 2015). Thereby, not only the production of carotenoids but also of sclareol, *cis*-abienol and abietadiene was enhanced several hundred-fold.

Differences in cellular pigmentation can also be used to specifically screen for higher activities of single pathway enzymes, provided that the enrichment of certain intermediate products of the C_{40} carotenoid pathway leads to the formation of unique carotenoid chromophores. For example, β-carotene ketolases are involved in the conversion of β-carotene to canthaxanthin, zeaxanthin and asthaxanthin, provoking a color change from yellow to orange and red, respectively. By taking advantage of this phenotypical changes, the activity of β-carotene ketolases were engineered by transforming mutant libraries into *E. coli* and screening for the most reddish colonies (Tang et al., 2007; Tao et al., 2006; Ye et al., 2006). However, another enzyme that is involved in the carotenoid biosynthesis pathway, β-carotene hydroxylase, could not be evolved as easily, since its activity does not result in a color change apparent to the naked eye (Scaife et al., 2012). Based on the principle that β-carotene and its hydroxylation product zeaxanthin are both yellow but show slightly different absorbance spectra, a wavelength-dependent screening assay was established to screen for β-carotene hydroxylase variants with higher activities.

Carotenoid-producing strains have further been used to engineer diverse isoprene synthases or even whole cellular pathways for high-level production of isoprenoids. Such screening systems are usually based on the following principle: An isoprene synthase competes with carotenoid backbone synthesis for the FPP pool (Figure 1). The higher the activity of the isoprenoid synthase, the lower are intracellular precursor levels for carotenoid biosynthesis thereby reducing the pigmentation of the host cells. Along these lines, mutant libraries of taxadiene synthase (TXS), tobacco 5-*epi*-aristolochene synthase (TEAS) and geraniol synthase (GES) were screened in carotenoid-producing *E. coli* cells for higher activities (Furubayashi et al., 2014a). Aiming for enhancing carotenoid biosynthesis pathways without any mutational modification of the respective biosynthetic genes, the following approaches might be interesting. Özaydin et al. (2013) transformed a plasmid encoding carotenoid biosynthesis (Verwaal et al., 2007) into a S. *cerevisiae* knockout library and screened for cells with higher pigmentation. Many of the targeted metabolic engineering efforts described to date exclusively focus on improving the mevalonate pathway (Asadollahi et al., 2010; Ro et al., 2006; Scalcinati et al., 2012; Westfall et al., 2012). In the work of Özaydin et al. (2013), gene deletions known to be involved in mevalonate pathway action have intentionally been disregarded to enable the identification of unexpected targets. Thereby, twenty-four deletion strains that showed higher cellular pigmentation were chosen to be further tested for improved activity of bisabolene synthase from *Abies grandis* (*Ag*BIS). Indeed, four gene deletions (*rox1*, *prb1*, *yjl064w*, and *ypl062w*) did improve bisabolene titers in bioreactor cultivations up to 20-fold (Özaydın et al., 2013). Similarly, a heterozygous deletion screen was applied to identify genes that restrict carotenoid, and potentially diterpene, productivity (Trikka et al., 2015). Through an iterative application of this approach, six deletions were identified which led to a 12-fold increase in sclareol titer over wild type cells, without any obvious effects on yeast fitness.

Screening assays that depend on carotenoid-based pigmentation allow for screening of whole cells and pathway engineering, which is a clear advantage of this strategy. Thereby, not only enzyme activities can

be improved but also whole cells can be screened for additional supporting features, e.g. specific gene knockouts or knock-ins. However, especially if isoprenoid synthases other than carotenoid synthases are to be evolved, the system harbors some risks. The interpretation of a loss-of-function assay is not always easy and favorable, i.e. false-positive targets might be picked up that reduce cell viability or somehow influence carotenoid production thereby reducing pigmentation without any beneficiary effect on isoprenoid biosynthesis. In turn, as all isoprenoids are synthesized from the overall IPP-pool, the assay can be broadened to all isoprene synthases and does not show any limitations in substrate scope.

2. Screening by fluorescence

A screening assay that is based on the principle of detecting water immiscible compounds via fluorescence dyes such as Nile Red and BODIPY has been patented by Amyris, Inc. in 2012 (Frenz and Ubersax, 2012) (Figure 1). The assay has specifically been established to screen for isoprene synthases with evolved functionalities, but the method may be generally applied to detect other industrially useful compounds, including polyketides, fatty acids, and derivatives thereof. The authors and inventors have screened for improved farnesene production in recombinant *S. cerevisiae* cells cultivated and induced in 96-well plates followed by incubation with Nile Red. Afterwards, stained cells were measured spectrophotometrically and spectral conditions were optimized so that fluorescence from Nile Red bound to farnesene was uninfluenced by background fluorescence of biomass. A farnesene specific read was obtained with excitation at 290 nm and emission at 550 nm, followed by a biomass specific read obtained with excitation at 350 nm and emission at 490 nm. Ultimately, a farnesene to biomass ratio was calculated.

Moreover, fluorescence-activated cell sorting by flow cytometry and magnetic cell sorting (FCM) was implemented to screen for high-level production of astaxanthin in the basidiomycetous yeast *Xanthophyllomyces dendrorhous* (telemorphic state of *Phaffia rhodozyma*) (Brehm-Stecher and Johnson,

2012; Ukibe et al., 2008) (Figure 1). Previously, an FCM method to screen for carotenoid overproducers among mutagenized cells of *P. rhodozyma* had been established (An et al., 1991). This technique was optimized to the effect that the fluorescence signals measured reflected the content of astaxanthin or other carotenoids more accurately (Brehm-Stecher and Johnson, 2012; Ukibe et al., 2008). *X. dendrorhous* cells were mutagenized with 3% ethyl methanesulfonate and were analyzed by FCM. Cells that emitted strong fluorescence at around 675 nm were collected within the first screening round as interesting candidates, rescreened and astaxanthin amounts were quantified by HPLC. The best mutant accumulated 3.8-fold more astaxanthin than the wild type (Ukibe et al., 2008). A further study compared four different mutagenesis methods (UV radiation, benomyl, ethyl methanesulfonate and ethidium bromide treatment, respectively) for the efficiency in generating asthaxanthin-hyperproducing *X. dendrorhous* strains. Mutagenesis with benomyl resulted in the highest number of stable astaxanthin overproducers (Stachowiak, 2013).

In principle, the two fluorescence-based screening methods described are suitable for pathway and synthase optimization. However, the measurement of fluorescence signals emitted by biological samples needs to be meticulously fine-tuned and optimized due to disturbing background signals. The Nile Red assay is a promising approach as it is universally applicable for numerous expression hosts and offers an extremely broad substrate range. On the downside, the product specificity of the approach is low and fluorescent dyes are costly. Until now, the FCM method has only been tested for chemically mutagenized yeast strains. Classical chemical mutagenesis displays several drawbacks such as spontaneous reversion or introduction of secondary mutations that may hamper strain fitness, but the simplicity of the method is highly attractive (An et al., 1991; Rodríguez-Sáiz et al., 2010). Importantly, the use of cell sorting by flow cytometry facilitates an extremely high throughput. However, the method cannot be properly applied on non-fluorescent isoprenoids and, hence, has a very narrow scope of application.

3. Screening for colored by-products

A different type of screening assay is based on the formation of by-products that can be quantified by a colorimetric readout and can be directly traced back to altered enzyme activities. Two outstanding and unique assays of that category were published recently (Figure 1). The first assay uses a specially modified substrate, a vinyl methyl ether isoprenoid derivative, referred to as 'substrate 3' (Lauchli et al., 2013). Substrate 3 shows close structural similarity to FPP, which is the natural substrate of sesquiterpene synthases. Upon cyclization of substrate 3, methanol is released and immediately converted by an alcohol oxidase added to the system (Figure 1). The resulting formaldehyde further reacts with Purpald® to form a purple product. With this approach, the thermostability of the sesquiterpene synthase from *Botrytis cinerea Bc*BOT2 was increased by 12°C. The technique also enabled rapid optimization of conditions for expression and stabilization of another isoprene synthase, SSCG_02150, which catalyzes the formation of (−)-δ-cadinene. The second isoprenoid screening assay that relies on a colorimetric output is the malachite green assay (Vardakou et al., 2014). The principle behind the assay is as simple as smart. Isoprene synthases do not only produce the isoprenoid of interest from FPP, but liberate inorganic diphosphate (PPi) as by-product. Diphosphatases are used to break down PPi to monophosphates that further react with molybdate and malachite green. A turquoise complex is formed that can be then quantified by standard UV spectrometers. Three mechanistically distinct but well studied enzymes were successfully used as models to test the assay, namely *Artemisia annua* β-farnesene synthase (*Aa*FS), *A. annua* amorpha-4,11-diene synthase (*Aa*ADS), and *Nicotiana tabacum* 5-*epi*-aristolochene synthase (TEAS).

A third assay has been described to take advantage of a special dye, 2,2-diphenyl-1-picrylhydrazyl (DPPH), that had commonly been used to estimate the antioxidant capacity of complex plant oils, which naturally contain high amounts of monoterpenoids (Behrendorff et al., 2013). The so-called modified DPPH radical-scavenging assay was used to screen for the monoterpenoid (+)-limonene using n-dodecane as an extractant. Upon transferring an electron to the C-C double bond of the monoterpenoid, DPPH changes its

color from purple to green. The assay detected standard preparations of myrcene and γ-terpinene dissolved in dodecane at concentrations as low as 10 and 15 μM, respectively, and limonene as low as 200 μM. However, the system could not be optimized to be used quantitatively, but definitely has promising potential for future applications, since it is one of a few assays that directly screen for isoprenoid production level.

All three assays are highly innovative and unique. The malachite green and the Purbald® assay are remarkable, since they are among the few published examples to specifically screen for synthase activities. They might also be useful to tackle a diverse range of challenges for isoprene synthases like overcoming product inhibition by inorganic diphosphate, increasing activity on native or alternative substrates, altering cofactor preferences and tolerance to organic solvents. However, cells need to be disrupted for activity measurements, which necessitates an additional preparation step. As a consequence thereof, the assay is not applicable for high throughput approaches, e.g. for pathway engineering. Another limitation of the Purbald®-assay is that it depends on 'substrate 3', which needs to be specially synthesized and – currently - limits the assay to screening sesquiterpene synthases. The malachite green assay, on the contrary, is rather inexpensive and can be applied for any type of isoprene synthase.

4. Screening by selection

Screening approaches that are based on distinct growth advantages, or disadvantages, are easy, fast and most efficient. Unfortunately, this type of assay can hardly be established to directly screen for isoprenoid productivities due to the non-essential nature of these compounds. Nevertheless, a couple of assays have been described to profit from selection for pathway or enzyme engineering as well as for the identification of new isoprene synthases. One promising approach was based on the finding that *E. coli* cells engineered with a heterologous mevalonate pathway show reduced viability due to the accumulation of the

isoprenoid precursors DMAPP, IPP and FPP (Martin et al., 2003). Co-expression of an isoprene synthase that converted these prenyl diphosphate precursors restored normal growth (Figure 1). Hence, the *E. coli* producing prenyl diphosphates was used to screen a genomic library of *Bacillus subtilis* for genes encoding isoprene synthesis activity. The expression library was enriched in several rounds by supplementing cultures with mevalonate, thus greatly reducing the amount of clones to be analyzed further. This strategy led to the identification of two proteins, YhfR and NudF, that produce the prenyl alcohol isopentenol. These two enzymes were the first shown to be involved in the isoprenoid secondary metabolism of *B. subtilis* (Withers et al., 2007). Interestingly, the cytotoxic effect of DMAPP and IPP has also been described for *B. subtilis*, indicating a possible applicability of this strategy for host organisms apart from *E. coli* (Sivy et al., 2011).

A very different but also effective approach has been described by Jay Keasling and co-workers, who were looking for a chemical agent that could act as a selection agent for enhanced sesquiterpene production levels in yeast cells (Kirby et al., 2014). Sesquiterpenes such as α-bisabolene have been shown to be predominantly cell associated when produced in the absence of an extractant such as dodecane (Özaydın et al., 2013). This may lead to altered membrane composition and, presumably, increased tolerance of membranes to disrupting agents. As a proof-of-principle, α-bisabolene was found to comprise cell protective properties against the membrane-disrupting agent Tween 20 (Kirby et al., 2014) (Figure 1). Since mutagenesis of the active site of α-bisabolene synthase failed to result in higher enzyme activity, the 3' region was randomly mutagenized to focus on improved mRNA stability. The library was enriched by growing cells in media containing Tween 20 and a mutant was isolated that yielded 30% higher α-bisabolene production levels. In a different approach, adaptive laboratory evolution in *S. cerevisiae* was applied by using a periodic hydrogen peroxide shocking strategy to select for better producers of carotenoids (Reyes et al., 2014). The hypothesis was that increased yields of carotenoids would result in

increased resistance to oxidative stress. Thereby, a *S. cerevisiae* strain was evolved to produce 3-times higher titers of carotenoids.

Random mutagenesis methods will inevitably produce a significant number of nonsense mutations or mutations that cause insolubility of the target protein. This does not matter much for high-throughput assays, but it matters for low-throughput methods such as GC that can hardly analyze more than 100-200 clones per day. Therefore, the elimination of insoluble proteins prior to analysis via GC is highly desirable. The fusion of chloramphenicol acetyltransferase (CAT) to the mutagenized target gene can be used as a screening tool for expression of soluble proteins, as fusion protein insolubility reduces CAT activity and thus the cells' resistance to chloramphenicol (Maxwell et al., 1999). By fusing mutagenized (+)-δ-cadinene synthase to CAT, the library was enriched for soluble proteins. Subsequent screening with GC-MS revealed several muteins with altered product selectivity (Yoshikuni et al., 2006). Another screening approach was based on the finding that improved thermostability of a protein correlated with increased resistance to proteolysis (Parsell and Sauer, 1989). The effect of computationally suggested mutations on thermostability of the tobacco 5-epi-aristolochene synthase (TEAS) was tested by subjecting the phage-displayed TEAS to chymotrypsin digestion at elevated temperatures. Thereafter, intact c-*myc* tagged TEAS muteins were selected using an antibody. This strategy resulted in a TEAS mutein that was still active at 65°C, which is approximately 30°C higher than the maximum temperature at which the wild type enzyme retained activity (Diaz et al., 2011).

Selection-based approaches provide an easy readout and are highly useful since they help to enrich a library and, thus immensely reduce the number of clones that need to be analyzed further. Although it is only possible to indirectly screen for isoprene synthase activities, concepts such as prenyl diphosphate precursor toxicity enable a fast and easy screening of genomic libraries for new, yet unknown enzymes without being limited to sequence similarities. However, it remains to be clarified, if the sensitivity of this assay is high enough to be used for pathway engineering or mutagenesis experiments. Yet, the use of

Tween 20 would also be applicable for pathway engineering to select for high α-bisabolene producers, since it allows to discriminate even low differences in isoprene production levels. A similar selection mechanism for other isoprenoids seems conceivable and, hence, the Tween 20 assay presents a powerful tool for future applications. Screening strategies that target isoprene synthase-specific properties, such as proper folding and temperature stability, allow a wide range of applications. While fusion of isoprene synthases to CAT and subsequent selection on chloramphenicol is a high-throughput concept, the screening for improved thermostability of phage-displayed proteins consists of several steps that scale down efficiency. What definitely needs to be considered is that screening for solubility or increased stability of proteins does not necessarily lead to muteins that harbor the desired level of catalytic activity.

5. Screening for mevalonate levels

Biosensors have started to emerge as valuable tools for high-throughput screenings (Schallmey et al., 2014). To date, only two examples of biosensors have been applied to screen for high mevalonate levels. As mevalonate is the ubiquitous precursor needed by every cell to produce isoprenoids, we consider these two biosensor approaches important concepts for efficient pathway engineering towards improved isoprenoid production. One very promising HTS assay is based on the construction of a biosensor strain that senses extracellular mevalonate levels (Pfleger et al., 2007). The biosensor, an *E. coli* strain auxotrophic for mevalonate, was engineered to produce green fluorescent protein constitutively, which allows correlating growth rate with fluorescent readout. To test its applicability, the strain was grown in media containing different levels of mevalonate and the determination of the Z-score classified the assay as excellent (Iversen et al., 2006; Zhang et al., 1999). For the screening, a heterologous mevalonate pathway operon with varying intergenic regions was expressed in a standard *E. coli* strain in 96-well plates. The transformant library secreted varying levels of mevalonate into the culture supernatants that, in turn, were used to cultivate the *E. coli* mevalonate biosensor. Depending on the level of mevalonate produced

by the library, the biosensor gave different levels of fluorescence and the mevalonate production level of the best hit was found to be improved seven-fold (Pfleger et al., 2006). Another example of how to use a biosensor to screen for mevalonate was described some years later (Tang and Cirino, 2011). In this approach, the *E. coli* AraC protein, a transcriptional repressor of the P_{BAD} promoter, was engineered to be regulated by mevalonate instead of arabinose. This mevalonate-responsive AraC variant was expressed in a strain containing a chromosomal *lacZ* (β-galactosidase activity) under the control of P_{BAD}. A library of randomly mutagenized ribosomal binding site (RBS) sequences of the truncated hydroxymethylglutaryl-CoA reductase (tHMGR) was transformed into this strain and screened for increased mevalonate production indicated by dark blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Utilization of this reporter protein screening system led to RBS variants with up to 3.5-fold higher mevalonate production levels.

Both biosensor approaches developed to detect varying levels of mevalonate demonstrate vast potential as they enable fast assays with easy, optical readouts. However, such auxotrophic biosensors are limited to pathway metabolites that are essential for the respective strain, e.g. mevalonate, and will most likely not be expandable to more sophisticated isoprenoid precursors that are dispensable for biosensor viability.

Conclusion and outlook

Strategies to screen for enhanced isoprenoid production in (recombinant) microbes are highly diverse (Figure 1) and, due to the challenging character of this substance class, are based on ingenious strategies. Especially whole-cell assays have clearly been favored over cell extract-based assays, although both approaches comprise positive and negative aspects. Whole-cell assays are efficient and allow for pathway engineering but will always have to deal with metabolic imbalances as a consequence of over-regulation of isoprene synthases or other pathway genes. The often quoted first law of directed evolution states: 'You

get what you screen for' (Schmidt-Dannert and Arnold, 1999). Yet, if the screening is targeted towards isoprenoid levels that irrefutably lead to the death of the production host, the best producing strains will never be selected for. Therefore, it is very important to accurately evaluate your system before considering directed evolution. It might be advisable to boost cellular metabolic pathways producing needed building blocks, e.g. IPP, DMAPP and/or FPP, so that cells do not 'starve' upon one-dimensional engineering of isoprene synthases. If one is exclusively interested in engineering synthase activities, and not in engineering the pathway in general, the use of assays that are based on cell extracts, like the malachite green assay or the Purbald® assay, might be attractive. Those assays also provide the possibility to screen isoprene synthases for diverse other properties, e.g. changed co-factor specificity or tolerance to organic substances, which would not be easily realized in whole cells.

It is obvious that there is still plenty of room for further improvement and development regarding easy, and broadly applicable assays to screen for enhanced isoprenoid productivities in a high-throughput fashion. Table 1 underscores the clear trend that colorimetric approaches are the most favored and obviously easiest to be established. A major drawback of most current screening strategies applied for isoprenoid production is that they are based on indirect readouts, which always goes hand in hand with complex assay constructions, unilateral applicability and a certain error-proneness. Direct screening assays are for example all assays used to improve carotenoid production, the Nile Red screening approach patented by Amyris (Frenz and Ubersax, 2012) or the DPPH radical-scavenging assay (Behrendorff et al., 2013). Although promising, the radical-scavenging assay has not been further developed towards better applicability so far. Still, the whole isoprenoid field is longing for direct screening possibilities that provide a simple, preferably optical readout for isoprenoid quantity.

One sector that has definitely not been fully exploited yet is biosensors, who represent promising key players in screening for next-generation drugs or orphan GPCRs. Literature only holds *E. coli* sensors applied to boost mevalonate productivity (Pfleger et al., 2006; Pfleger et al., 2007; Tang and Cirino, 2011)

but did not come up with any biosensors used for the screening of directly. There have been interesting approaches of how to apply a yeast GPCR-based biosensor to detect hydrophobic substances such as medium chain fatty acids (Mukherjee et al., 2015) or the environmental toxin dinitrotoluene (DNT) (Dhanasekaran et al., 2009; Radhika et al., 2007). As most isoprenoids are high-value fragrances that stimulate olfactory receptors in the human nose, the so-called 'smelling yeast' might be an interesting biosensor (Dhanasekaran et al., 2009; Emmerstorfer et al., 2014; Minic et al., 2005; Pajot-Augy et al., 2003). Upon cellular engineering of *S. cerevisiae*, an effector (isoprenoid) docking to a heterologous GPCR actuates a MAP kinase cascade ultimately leading to optical readouts or complementation of auxotrophies (Dowell and Brown 2009; Ladds et al. 2005). However, we assume that such systems may still have to be cured from diverse limitations like difficult fine-tuning of cellular response to a stimulus and the fact that only about half of all heterologously expressed GPCRs couple to the pheromone signalling pathway of *S. cerevisiae*.

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Figure legend

Figure 1: Overview of screening strategies applied to screen for higher microbial and enzymatic isoprenoid production levels.

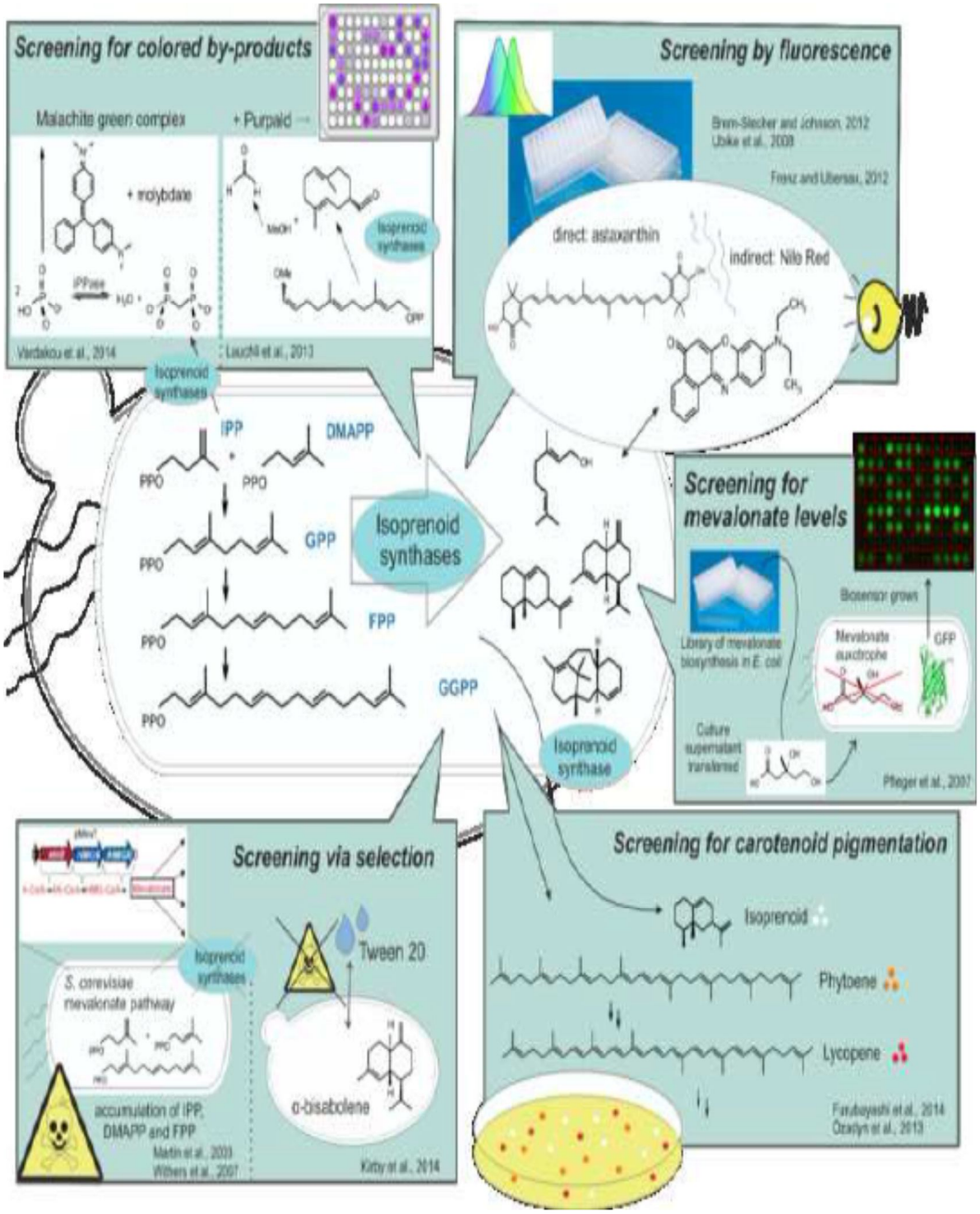

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Table 1: Overview of screening assays established for different types of terpenoids providing an overview of the screening approach that has been applied [1: carotenoid pigmentation based, 2: colored by-products, 3: fluorescence-based, 4: selection, 5: biosensors for mevalonate levels] and other possible application

Terpenoids	Type of screening assay					Strategy expandable to	References
	1	2	3	4	5		
Precursors							
mevalonate					x	Terpenoid precursors	(Pfleger et al., 2007)
					x	Terpenoid precursors	(Tang and Cirino, 2011)
GGPP	x					Terpenoid precursors	(Ohnuma et al., 1994)
	x					Terpenoid precursors	(Ohnuma et al., 1996)
	x					Terpenoid precursors	(Lee et al., 2005)
	x					Terpenoid precursors	(Ignea et al., 2015)
Hemiterpenoids (C_5)							
isopentenol				x		Terpene synthases	(Withers et al., 2007)
Monoterpenoids (C_{10})							
geraniol	x					C_{10}-C_{30} terpenoids	(Furubayashi et al., 2014a)
β-farnesene		x				C_{10}-C_{40} terpenoids	(Vardakou et al., 2014)
			x			Water-immiscible compounds, including terpenoids, polyketides, fatty acids, and derivatives thereof	(Frenz and Ubersax, 2012)
limonene		x				Monoterpenoids, terpenoids with double bonds	(Behrendorff et al., 2013)
myrcene		x				Monoterpenoids, terpenoids with double bonds	(Behrendorff et al., 2013)
γ-terpinene		x				Monoterpenoids, terpenoids with double bonds	(Behrendorff et al., 2013)
Sesquiterpenoids (C_{15})							
5-*epi*-aristolochene	x					C_{10}-C_{30} terpenoids	(Furubayashi et al., 2014a)
		x				Terpenoid synthesis with PPi as co-product	(Vardakou et al., 2014)
				x		Terpenoid synthases in general	(Diaz et al., 2011)
botrydial		x				Limited to sesquiterpenes	(Lauchli et al., 2013)
(−)-δ-cadinene		x				Limited to sesquiterpenes	(Lauchli et al., 2013)
(+)-δ-cadinene				x		Proteins in general	(Yoshikuni et al., 2006)
bisabolene	x					C_{10}-C_{40} terpenoids	(Özaydın et al., 2013)
				x		Proteins in general	(Kirby et al., 2014)
amorpha-4,11-diene		x				Terpenoid synthesis that produce PPi as co-product	(Vardakou et al., 2014)
Diterpenoids (C_{20})							
taxadiene	x					C_{10}-C_{30} terpenoids	(Furubayashi et al., 2014a)
sclareol	x					C_{10}-C_{40} terpenoids	(Trikka et al., 2015)
	x					All terpenoidssynthesized from GGPP	(Ignea et al., 2015)
levopimaradiene	x					All terpenoidssynthesized from GGPP	(Lee et al., 2005)
	x					IPP pathway engineered, suitable for C_{10}-C_{30} terpenoids	(Leonard et al., 2010)
2-abietadiene	x					IPP pathway engineered, suitable for C_{10}-C_{30} terpenoids	(Leonard et al., 2010)
sandaracopimaradiene	x					IPP pathway engineered, suitable for C_{10}-C_{30} terpenoids	(Leonard et al., 2010)
Tetraterpenoids (C_{40})*							
β-carotene	x					carotenoids	(Umeno et al., 2002)
				x		carotenoids	(Reyes et al., 2014)
canthaxanthin	x					carotenoids	(Tang et al., 2007)
zeaxanthin	x					carotenoids	(Scaife et al., 2012)
astaxanthin			x			carotenoids	(Ukibe et al., 2008)
			x			carotenoids	(Brehm-Stecher and Johnson, 2012)

* Not all synthetically generated carotenoids are listed due to their large number. For details please see chapter 1 and several excellent reviews (Lee and Schmidt-Dannert, 2002; Umeno et al., 2005; Wang et al., 2007).