ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Decoupling Production From Growth by Magnesium Sulfate Limitation Boosts De Novo Limonene Production

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ABSTRACT: The microbial production of isoprenoids has recently developed into a prime example for successful bottom-up synthetic biology or top-down systems biology strategies. Respective fermentation processes typically rely on growing recombinant microorganisms. However, the fermentative production of isoprenoids has to compete with cellular maintenance and growth for carbon and energy. Non-growing but metabolically active *E. coli* cells were evaluated in this study as alternative biocatalyst configurations to reduce energy and carbon loss towards biomass formation. The use of non-growing cells in an optimized fermentation medium resulted in more than fivefold increased specific limonene yields on cell dry weight and glucose, as compared to the traditional growing-cell-approach. Initially, the stability of the resting-cell activity was limited. This instability was overcome via the optimization of the minimal fermentation medium enabling high and stable limonene production rates for up to 8 h and a high specific yield of $\geq$50 mg limonene per gram cell dry weight. Omitting $MgSO_4$ from the fermentation medium was very promising to prohibit growth and allow high productivities. Applying a $MgSO_4$-limitation also improved limonene formation by growing cells during non-exponential growth involving a reduced biomass yield on glucose and a fourfold increase in specific limonene yields on biomass as compared to non-limited cultures. The control of microbial growth via the medium composition was identified as a key but yet underrated strategy for efficient isoprenoid production.

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Introduction

The fermentative production of valuable compounds from renewable resources with genetically engineered or synthetic pathways in microorganisms relies on microbial metabolism and therefore on the integrity of intracellular anabolic and catabolic reactions (Lee et al., 2012; Paddon and Keasling, 2014; Zhang et al., 2011). Operational regeneration of redox and energy equivalents is often mandatory. Therefore, the use of growing cells is advantageous with respect to the regeneration of cells as well as the efficient synthesis of energy carriers (Orth et al., 2011; Schrewe et al., 2013). This might be of special importance, when production conditions cause stress, for example, when substrates and products are toxic for the microorganisms applied (Willrodt et al., 2015b). Consequently, most current in silico optimization strategies for microbial cell factories producing such compounds aim at maximizing coupling of compound production to microbial growth using biomass objective functions (Campodonico et al., 2014).

On the other hand, approaches based on non-growing, also called resting, cells decouple growth and biocatalyst synthesis from the production phase. The resting state of microorganisms is a natural phenomenon arising under conditions of nutrient limitation (Harder and Dijkhuizen, 1983). In a laboratory setting, the resting state can be induced by the depletion of an element compulsory for growth, for example, nitrogen. Resting but metabolically active cells were shown to be beneficial for redox biotransformations in terms of both specific reaction rates (Bühler et al., 2008; McIver et al., 2008; Panke et al., 2002) and practical concerns, such as reusability (Barghini et al., 2007). However, biocatalytic activities of non-growing recombinant cells were found to decrease steadily, for example, due to limited protein stability (Gottesman, 2003), altered product inhibition profiles (Julsing et al., 2012), or altered gene expression profiles (Ishihama, 1997). Decoupling of growth and product formation is even more beneficial for fermentative de novo syntheses of valuable compounds as it reduces the use of resources and energy for by-product formation (biomass, fermentation products) (Chubukov and Sauer, 2014; Julsing et al., 2012). The

physiological and practical advantages of resting cells were, for instance, harnessed for the efficient homologous production of volatile metabolites with *Lactococcus lactis* (van de Bunt et al., 2014) or ethanol, acetate (Cotter et al., 2009), and butanol (Förberg et al., 1983; Loyarkat et al., 2013) with different *Clostridium* species.

The potential of physiological advantages of resting cells has not been explored systematically for the de novo formation of isoprenoids. The efficient fermentative production of isoprenoids requires the engineering of complex, partly heterologous, biosynthesis pathways involving, for example, the yeast mevalonate pathway and terpene cyclases (Martin et al., 2003). Research on heterologous terpene production in *E. coli* has mainly focused on improving the supply of terpene precursors via optimization of promotor strength (Anthony et al., 2009), codon usage (Redding-Johanson et al., 2011), translation (Nowroozi et al., 2014), and gene selection (Ma et al., 2011; Yang et al., 2012). This resulted in high titers for various terpenes. Typically, the production of terpenes, such as limonene (Alonso-Gutierrez et al., 2013; Willrodt et al., 2014), pinene (Sarria et al., 2014), or sclareol (Schalk et al., 2012) was based on growing *Escherichia coli* cells, co-producing the terpene alongside major quantities of by-products (e.g., biomass). Resting cells only have been applied for a mixed strain approach to produce perillyl acetate (Willrodt et al., 2015a).

We exploit the microbial biosynthesis of the monoterpene limonene with recombinant *E. coli* under growth-prohibiting and growth-limiting conditions. Limonene has received much attention as scent or flavor component for perfume and food industries (Thomas and Bessiere, 1989) and as precursor for a variety of fine chemicals such as perillyl alcohol or carvone (Marmulla and Harder, 2014). More recently, limonene also gained attention as jet fuel additive (Ryder, 2009; Tracy et al., 2009). We demonstrate that limonene formation profits from both a resting-cell biocatalyst configuration and a reduction of microbial growth by omitting or restricting essential nutrients. Characterization of the limonene producing *E. coli* strain under growth-prohibiting conditions guided the successful optimization of limonene production and resulted in significantly increased limonene yields.

Materials and Methods

Chemicals, Oligonucleotides, and Bacterial Strains

Anhydrous D-glucose was obtained from Alfa Aesar (Karlsruhe, Germany). Custom synthesized oligonucleotides were purchased from Sigma–Aldrich (Steinheim, Germany). All other chemicals were obtained from AppliChem (Darmstadt, Germany), Sigma–Aldrich, or Carl Roth (Karlsruhe, Germany) at the highest purity available. For all experiments, *E. coli* BL21 (DE3) (Studier and Moffatt, 1986) harboring the plasmids pTAC:LS:AGPPS2 (limonene synthase (LS) and geranyl diphosphate synthase (GPPS)) and pMVAidi (mevalonate pathway genes) was applied (Willrodt et al., 2014).

Cultivation of *E. coli*

E. coli BL21 (DE3) (pTAC:LS:AGPPS2, pMVAidi) was cultivated as described before (M9* medium, baffled Erlenmeyer flasks, liquid: gas phase ratio of 1:4) (Willrodt et al., 2014). Cultures were incubated at 30°C (200 rpm, 2.5 cm amplitude, Multitron, Infors, Bottmingen, Switzerland), if not stated otherwise. Recombinant gene expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Ampicillin and chloramphenicol were added at concentrations of 100 and 34 mg L^{-1}, respectively. Cell growth was monitored by measuring the optical density of the aqueous phase at 450 nm (Libra S11, Biochrom Ltd., Cambridge, UK, $OD_{450} = 1$ equals 0.166 g_{cdw} L^{-1} (Blank et al., 2008)). For two-liquid phase fermentations with diisononyl phthalate (DINP, organic:aqueous phase ratio 1:4) as organic carrier solvent, cells were washed once with potassium phosphate buffer (0.1 M, pH 7.4) prior to OD_{450} determination, in order to circumvent an OD_{450} increase due to remaining DINP.

Growing-Cell Fermentation

Eighty milliliters of M9* medium (0.5% (w/v) glucose) were inoculated at an optical density (OD_{450}) of 0.2 using an appropriate volume of a M9* pre-culture. The culture was overlaid with 20 mL DINP upon induction of gene expression in the early exponential growth phase (OD_{450} 0.6–0.8) and incubation was continued. Aliquots of the two-liquid phase fermentation were retrieved from the culture at regular intervals and subjected to centrifugation (4°C, 4618*g*, 10 min) for phase separation. One hundred microliters of the organic phase were diluted in 900 μL diethyl ether (with 0.2 mM dodecane as internal standard) and dried over anhydrous Na_2SO_4. The aqueous phase was analyzed for the determination of glucose, acetate, and pyruvate concentrations and the cell density was assessed by measuring the OD_{450}. Aqueous phase concentrations of glucose, acetate, and pyruvate and organic phase concentrations of (*S*)-(−)-limonene (LIM) were determined via HPLC or GC, respectively, as described previously (Willrodt et al., 2014). An exponential function was iterated to the limonene data points in the exponential production range (4–14 h) by the least squares method (coefficient of determination: 0.98) and used for the calculation of specific activities.

Resting-Cell Fermentation

Cells were grown as described above, without the addition of DINP upon induction of gene expression. Cells were harvested by centrifugation (4°C, 4618*g*, 20 min), washed 1-2 times with 0.1 M potassium phosphate buffer pH 7.4, and resuspended to approximately 1 g_{cdw} L^{-1} in desired media, that is, 0.1 M potassium phosphate buffer pH 7.4, nitrogen-free M9* ($M9^*_{-N}$), or M9* medium lacking magnesium sulfate ($M9^*_{-MgSO4}$) at different time points after induction. Twenty milliliters of the cell suspension were added to a baffled Erlenmeyer flask (250 mL total volume) and incubated at 30°C for 10 min in a rotary shaker (200 rpm, 2.5 cm amplitude). Subsequently, the culture was overlaid with 5 mL DINP and limonene formation was initiated by the addition of 0.4 mL 50% (w/v) glucose. Absence of growth was deduced from OD_{450} measurements, which were occasionally confirmed via the determination of colony forming units. The ΔpH over the time course of the resting cell fermentations with low cell densities (approximately 1 g_{cdw} L^{-1}) was ≤0.1. Aliquots of the two-liquid phase fermentation were retrieved at defined time points and processed as reported for the quantitation of metabolites (Willrodt et al., 2014).

Results

Limonene Formation in Growing Cells Is Not Limited by Heterologous Gene Expression

The achievable biomass concentration during batch cultivations is typically limited by the amount of glucose in the medium. In addition, oxygen mass transfer into the medium and release of toxic by-products (e.g., acetate) can also restrict microbial growth and prevent the formation of high biomass concentrations in batch cultures. The formation of limonene from glucose in recombinant *E. coli* is connected to cell growth (Willrodt et al., 2014) and might thus be limited by the above mentioned factors as well. Furthermore, the carbon source is utilized for both limonene and biomass/by-product formation.

The limonene formation capacity of resting cells was determined to investigate whether limonene synthesis competes with microbial growth. Resting cells were prepared by harvesting cells from growing cultures at different time points during cultivation, followed by resuspension in glucose-containing phosphate buffer. The capacity was compared with the limonene formation activity of growing cells. For this, *E. coli* BL21 (DE3) harboring the plasmids pTAC:LS:AGPPS2 (encoding the enzymes limonene synthase and GPP synthase) and pMVAidi (encoding the enzymes of the mevalonate pathway) was grown in shake flasks with M9* containing 0.5% w/v glucose. The specific limonene formation rate of growing cells was highest (0.21 U g_{cdw}^{-1}) within the first 2 h after induction and decreased to 0.15 and 0.06 U g_{cdw}^{-1} in the late exponential phase and during the transition to the stationary phase, respectively (Fig. 1). Compared to the specific production rate observed during glucose-limited fed-batch cultivation (75 mU g_{cdw}^{-1}) (Willrodt et al., 2014) the maximal rate achieved during unlimited batch growth was 2.8 times higher. The decrease in limonene formation rates during the exponential phase is unexpected, especially considering the longer time available for heterologous gene expression. The limonene formation activities were assessed in short-term (<2 h) resting cell assays with cells retrieved from a growing culture in order to investigate whether the specific limonene formation rate decreased due to hampered enzyme synthesis or physiological constraints such as limiting precursor (e.g., acetyl-CoA) availability. Resting cell activities transiently increased depending on the expression time and, after 9 h of gene expression, were almost threefold higher (0.44 vs. 0.15 U g_{cdw}^{-1}) than the activity of their growing counterparts (Fig. 2A). The estimated specific glucose uptake rates of both growing and resting cells were similar (during the first 2 h, data not shown), suggesting at least initially similar metabolic capacities of the cells in both biocatalyst configurations.

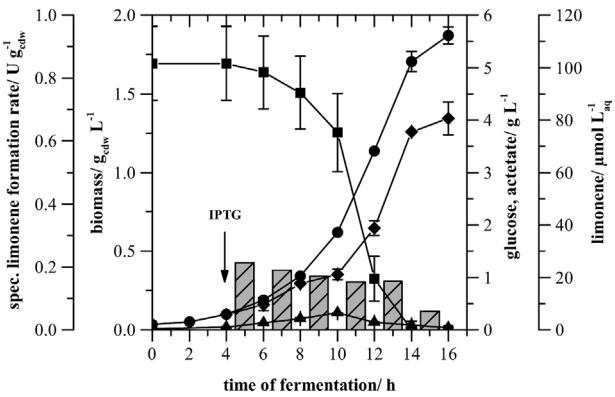

Figure 1. Limonene production profile in batch cultivation of *E. coli* BL21 (DE3) harboring pMVAidi and pTAC:LS:AGPPS2 from 0.5% (w/v) glucose. Biomass (●), limonene (◆) glucose (■), and acetate (▲) concentrations were monitored throughout the cultivation. The specific limonene formation rate of growing cells is presented as grey bars. The error bars refer to the standard deviations retrieved from two independent cultivations (biological replicates).

The highest specific limonene formation activity of resting cells (0.47 U g_{cdw}^{-1}) was observed 12 h after induction of gene expression. This was also the case when the exponential growth phase was prolonged by adding 2% (w/v) glucose in a pH controlled and aerated bioreactor (data not shown), where this resting-cell activity remained constant until glucose depletion. Thus, under the applied conditions of batch cultivation, the use of growing cells appeared to be sub-optimal for efficient limonene synthesis from glucose. We hypothesize that the higher specific limonene formation activities of non-growing cells might reflect a reduced competition with growth demands as glucose fueled the formation of both biomass and limonene.

Pathway Gene Expression and Limonene Synthesis Rate Depend on Temperature

The functional production of heterologous proteins is not only governed by genetic and cellular aspects, such as genetic regulatory elements and the intracellular environment of the heterologous host, but also by exogenous factors (e.g., temperature). Lowering the expression temperature has frequently been shown to improve the synthesis of soluble heterologous proteins (Rosano and Ceccarelli, 2014). The production of soluble GPPS was shown to profit from a reduced expression temperature, although the improved solubility of synthesized GPPS did not increase limonene titers accumulated by growing cells (Willrodt et al., 2014). *E. coli* BL21 DE3 (pTAC:LS:AGPPS2, pMVAidi) was cultivated at different temperatures to modulate the heterologous expression of limonene biosynthesis pathway genes. The limonene formation capacity of resting cells derived from these cultures after different expression times was assessed. The resting-cell fermentations were performed at 30°C to ensure comparability and exclude effects of intrinsic enzyme activities at different temperatures. Although the initial glucose uptake rates during the resting-cell fermentations were similar in either case (data not shown), cells grown at 30°C showed significantly higher maximal resting-cell limonene formation rates as compared to biocatalyst production at 20, 25, and 37°C (Fig. 2B, Fig. S1). Additionally, the maximal rates were reached faster at higher expression temperatures (Fig. 2B, Fig. S1). The tendency observed for limonene formation at different expression temperature resembles the earlier finding of maximal limonene yields at 30°C with growing cells (Willrodt et al., 2014). Here, an expression temperature of 30°C combined with an expression duration of 12 h can thus be considered optimal for the balanced synthesis of active GPPS, LS, and mevalonate pathway enzymes.

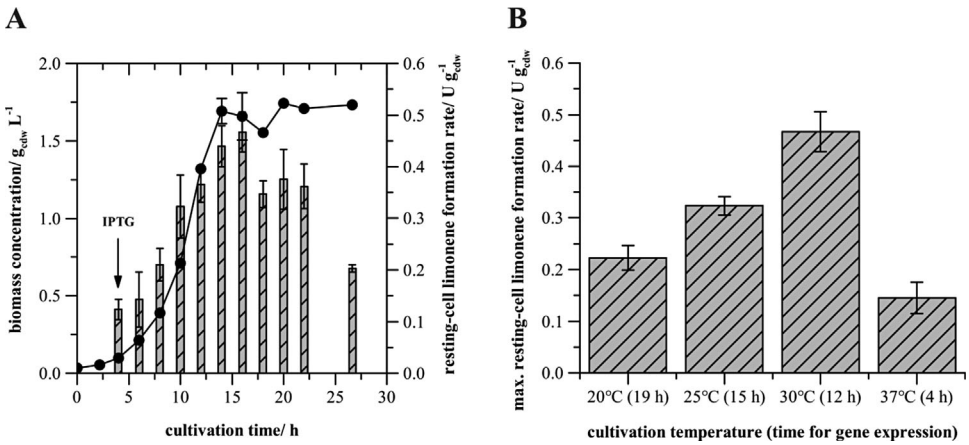

Figure 2. (**A**) Specific resting-cell limonene formation activities (grey bars) of *E. coli* BL21 (DE3) (pMVAidi, pTAC:LS:AGPPS2) at 30°C in dependency of the expression time and microbial growth phase. Resting cells were retrieved from cultures actively growing at 30°C (●) by harvesting the cells at indicated time points of growth and subsequent resuspension in potassium phosphate buffer (0.1 M, pH 7.4) supplemented with 1% (w/v) glucose. (**B**) Maximal limonene formation activities of resting cells in dependency of the temperature during cultivation and expression. The time points after induction, at which maximal activities were reached, are given in parentheses. To ensure comparability, all resting-cell fermentations were conducted at 30°C. The limonene formation rates were obtained by linear regression of data points within the initial linear production phase (~2 h). The error bars refer to standard deviations obtained from two independent cultures (biological replicates).

Enzyme Instability Under Operational Conditions Limits Limonene Production by Resting Cells

The resting-cell fermentations reported above were performed to assess the maximal initial limonene formation rate during a limited time window of maximally 2 h with low cell concentrations. However, up-scaling of both cell concentration and production period is required for productive limonene formation. The resting cell-approach was well scalable regarding biomass concentration (Fig. S2). An increase in biomass concentration up to 10 g_{cdw} L^{-1} increased the initial productivity and final yields proportionally. However, these fermentations suffered from limited biocatalyst stability. Resting-cell fermentations, for which the cells were obtained under the above deduced optimal conditions, were performed at different temperatures (25, 30, 37°C) and sampled for 8 h to investigate this instability in detail. The initial limonene formation rate was highest (0.55 U g_{cdw}^{-1}) for fermentation at 37°C and lowest (0.25 U g_{cdw}^{-1}) at 25°C (data not shown). The final limonene concentrations were similar in all cases, despite these differences in initial specific activities (Fig. 3A). The limonene formation rate at 37°C decreased faster as compared to 30°C or 25°C (Fig. 3B). The glucose uptake rate was most stable at 37°C (Fig. 3C). This might be attributed to higher glycolytic flux at this temperature. This indicates, in combination with the decreasing limonene yield over time at 37°C, instability of the heterologous limonene formation pathway rather than instability of the metabolic capacity of resting cells. In contrast, the specific limonene yield on glucose increased during the fermentation performed at 25°C (Fig. 3D) indicating an improved pathway stability at lower temperatures, which is in good agreement with the decreasing glucose uptake rate.

Resting cells were incubated at 30°C in presence or absence of glucose to investigate the reason for the rapidly decreasing specific limonene formation rates (Fig. 3B). The specific limonene formation rate of the cells incubated in absence of glucose remained almost unaffected when glucose was added after an incubation time of 6 h (~0.3 U g_{cdw}^{-1}, Fig. 4). The limonene formation rate after 6 h of continuous fermentation, however, was threefold lower (Fig. 4). This suggests that the stability under production conditions rather than thermal/temporal inactivation of one or more enzymes of the limonene formation pathway restricts limonene formation by resting cells.

Limonene Formation and Biocatalyst Stability as Function of Medium Composition

The resting-cell fermentations in glucose containing potassium phosphate buffer were supplemented with 1 mM IPTG to overcome instability. The initial reaction rate was slightly increased by approximately 10% after addition of the inducer leading to 1.6-fold increased limonene concentrations after 9 h of fermentation (Fig. 5A). This supports the hypothesis of an operational instability of the heterologous enzymes, but also indicates (limited) capability of protein synthesis of heterologous proteins under growth-prohibiting conditions. However, protein re-synthesis is likely limited by the availability of free amino acids and only facilitated by the turnover of intracellular resources under growth-prohibiting conditions induced by the lack of nitrogen (Mandelstam, 1958). An additional 1.3-fold increase in limonene titer after 9 h of fermentation was observed when the resting-cell fermentation medium was supplemented with the two antibiotics used during normal growing-cell cultivation (Fig. 5A).

Besides carbon and oxygen, which constitute 45 and 30% (w/w) of the dry biomass of *E. coli* B strains (Bauer and Ziv, 1976), respectively, nitrogen is the most important element for the synthesis of essential cellular macromolecules, such as nucleic acids and proteins. In accordance with that, the utilization of a resting-cell

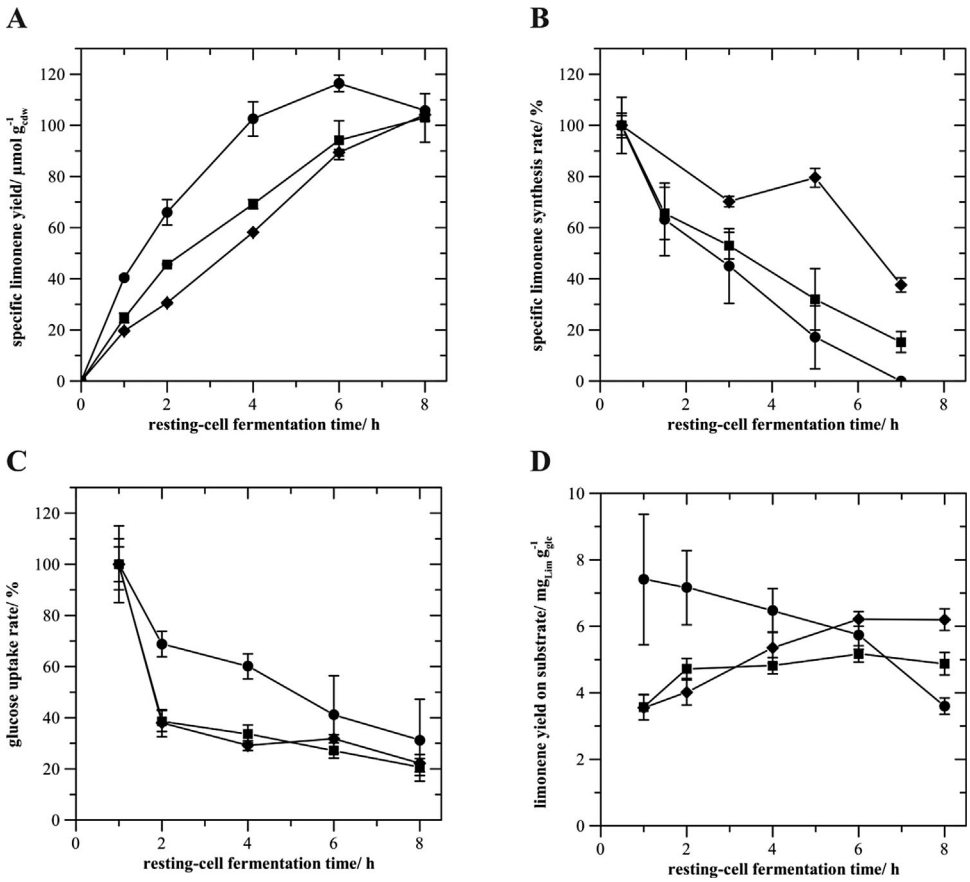

Figure 3. Time course of resting-cell fermentations in dependency of the temperature. Limonene yields on biomass (**A**), relative specific limonene synthesis rates (**B**), relative glucose uptake rates (**C**) and limonene yields on consumed glucose (**D**) are shown. The temperatures investigated were 25°C (◆), 30°C (■), and 37°C (●). The error bars refer to standard deviations obtained from two independent cultures (biological replicates).

fermentation medium containing all essential elements except nitrogen (M9*$_{-N}$) did not result in higher final limonene yields on biomass compared to potassium phosphate buffer supplemented with glucose (Fig. 5A and C).

The activity was less stable, though the observed initial rate for the nitrogen-deficient fermentation was higher (0.67 U g_{cdw}^{-1} vs. 0.48 U g_{cdw}^{-1}, both fermentations supplemented with IPTG and antibiotics). The glucose consumption and acetate production profiles were similar in both fermentations (Fig. 5B and D, media supplemented with IPTG and antibiotics). Low amounts of pyruvate accumulated additionally to acetate in the fermentation based on the nitrogen-free M9* medium. This indicates either a saturated or inhibited activity of pyruvate dehydrogenase with respect to acetyl-CoA formation.

As a next step, magnesium sulfate was omitted from the fermentation medium to obtain non-growing cells, which then might be able to synthesize amino acids and proteins more efficiently as nitrogen is present. The initial specific limonene formation rate was very similar compared to the rate observed in nitrogen-free medium (0.7 vs. 0.68 U g_{cdw}^{-1}). Strikingly, limonene formation was much more stable and linear for more than 7 h and decreased only slightly afterwards (Fig. 5E). The high and stable specific limonene formation rates were accompanied by increased rates for glucose uptake and pyruvate accumulation (Fig. 5F). Pyruvate accumulation possibly occurred as a consequence of the increased glucose uptake rate and a resulting glycolytic pathway overflow.

In conclusion, we consider the application of magnesium sulfate deficient medium as being optimal for limonene formation by non-growing cells. It features the highest specific limonene yield on both glucose and biocatalyst after 9 h of fermentation (Table I). These high yields and stable activities correlated with the increased and stabilized glycolytic activity (glucose uptake rate, overflow metabolite formation), possibly because of facilitated regeneration of both metabolic and heterologous proteins.

Limonene Formation Benefits From Magnesium Sulfate Limited Growth

The optimized production system shows numerous advantages, despite the fact that the generation of resting cells for production purposes involves additional working steps. It is therefore desirable to combine the simplicity of a typical batch cultivation and the superior potential of non-growing cells. Growth of *E. coli*

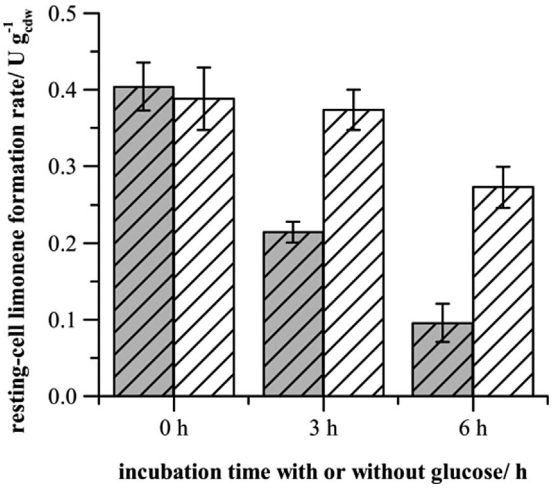

Figure 4. Initial (~2 h) specific resting-cell limonene formation rate after different times of incubation at 30°C in the presence (grey bars) or the absence of glucose (white bars). Resting-cell fermentations were performed in 0.1 M potassium phosphate buffer (pH 7.4) supplemented with 1% (w/v) glucose. The error bars refer to standard deviations obtained from two independent cultures (biological replicates).

in batch cultures is typically terminated by the depletion of the carbon and energy source, frequently glucose. Limonene formation during the subsequent stationary phase is thus substrate limited. Glucose remains available for limonene formation, but is barely consumed for biomass formation when another element, for example, magnesium or sulfate, is depleted. Hence, resting cells are obtained in situ and can be applied for fermentative limonene production.

The amount of magnesium sulfate in M9* was decreased to discrete concentrations between 2 mmol L^{-1} and 0 mmol L^{-1} and the biomass yield on magnesium sulfate was determined. A constant yield was observed for magnesium sulfate concentrations up to 160 μmol L^{-1} (0.008 $g_{biomass}$ μmol_{MgSO4}^{-1}) (Fig. 6A). This empirically determined yield was used to limit microbial growth by $MgSO_4$ (118 μmol L^{-1} magnesium sulfate was added) to obtain biomass concentrations, at which oxygen availability was not limiting (approximately 1 g_{cdw} L^{-1}). The growth profile did not show an exponential pattern and entered a stationary phase only after glucose depletion between 18 and 29 h fermentation time. Limonene accumulation, however, appeared to profit from $MgSO_4$-limitation, as the highest specific limonene formation rate was determined at 0.67 U g_{cdw}^{-1} (Fig. 6B). This rate was not only 3.2-fold higher than the maximal rate observed for exponentially growing cells (0.21 U g_{cdw}^{-1}), but also comparable to the rate observed during resting-cell fermentation in $MgSO_4$-deficient M9* medium (Fig. 5E). Limonene is produced at a reduced rate, putatively from storage compounds, after glucose depletion. Finally twofold and fourfold higher limonene yields on glucose and biomass, respectively, as compared to growing cells (Willrodt et al., 2014) were obtained (Table I). Thus, limonene formation profits from the sub-optimal growth conditions and the $MgSO_4$ limitation, putatively by increased availability of carbon and/or energy resources or reduced detrimental effects of $MgSO_4$ on the molecular level.

Discussion

Productivity of Resting Cells is Limited by Microbial Physiology and Operational Enzyme Stability

A critical selection and evaluation of the appropriate whole-cell biocatalyst configuration is key for developing an efficient production process (Kuhn et al., 2010; Willrodt et al., 2015b; Woodley, 2006). As a first step, the maximal specific limonene formation rate was found to be 2.8 times higher during unlimited batch growth as compared to carbon-limited fed-batch cultivation (Willrodt et al., 2014). Further, this study focused on fermentative production with cells (resting or nutrient-limited) subjected to different types of growth limitations. The resting-cell configuration has not been systematically exploited for de novo syntheses involving recombinant microorganisms. Exceptions are rhamnolipid (Wittgens et al., 2011) and perillyl acetate (Willrodt et al., 2015a) production with *Pseudomonas putida* and *E. coli*, respectively.

A linear antiproportional correlation between the specific terpene (amorphadiene) production rate and the specific growth rate was observed in constraint-based flux analyses of *E. coli* harboring a heterologous terpene formation pathway (Kim et al., 2014). In the present study, $MgSO_4$-limited growth of *E. coli* BL21 (DE3) (pTAC:LS:AGPPS2, pMVAidi) was found to increase the biomass-specific limonene production yield fourfold as compared to non-limited conditions (Table I). This and the decreased maximal biomass yield on glucose in $MgSO_4$-limited fermentations (0.1 vs. 0.17 $g_{biomass}$ $g_{glucose}^{-1}$) (Willrodt et al., 2014) point to a competition for resources between the heterologous limonene formation pathway and cellular growth or maintenance under the conditions investigated. On the other hand, limited supply of $MgSO_4$ itself might be hypothesized as the primary reason for increased limonene yields. However, limonene yields and specific activities of non-growing cells in nitrogen-free, but $MgSO_4$ containing, M9* medium are also significantly increased as compared to the application of growing cells (Table I). Therefore, it appears valid to attribute the increased yields to the absence or reduction of growth rather than to limited supplementation of $MgSO_4$. Nevertheless, superimposed beneficial effects of $MgSO_4$ limitation on the molecular level, such as altered enzyme inhibition profiles, cannot be excluded. Consequently, a $MgSO_4$-based feeding strategy supplying limiting amounts of $MgSO_4$ in a bioreactor might be a promising alternative to the typical carbon-limited feeding approaches that previously led to significantly lower specific limonene production rates of maximally 75 mU g_{cdw}^{-1} as indicated above (Willrodt et al., 2014). The results obtained in this study applying low cell densities in shake flasks are well transferable to bioreactor environments as long as cell densities are kept low. As a next step, high cell density conditions and respective effects related to mass transfer, shear forces, and nutrient/metabolite gradients, which might influence both stabilities and activities, need to be evaluated on bioreactor scale.

Limonene production in truly resting cells without any growth suffered from low stability of the pathway enzymes for limonene

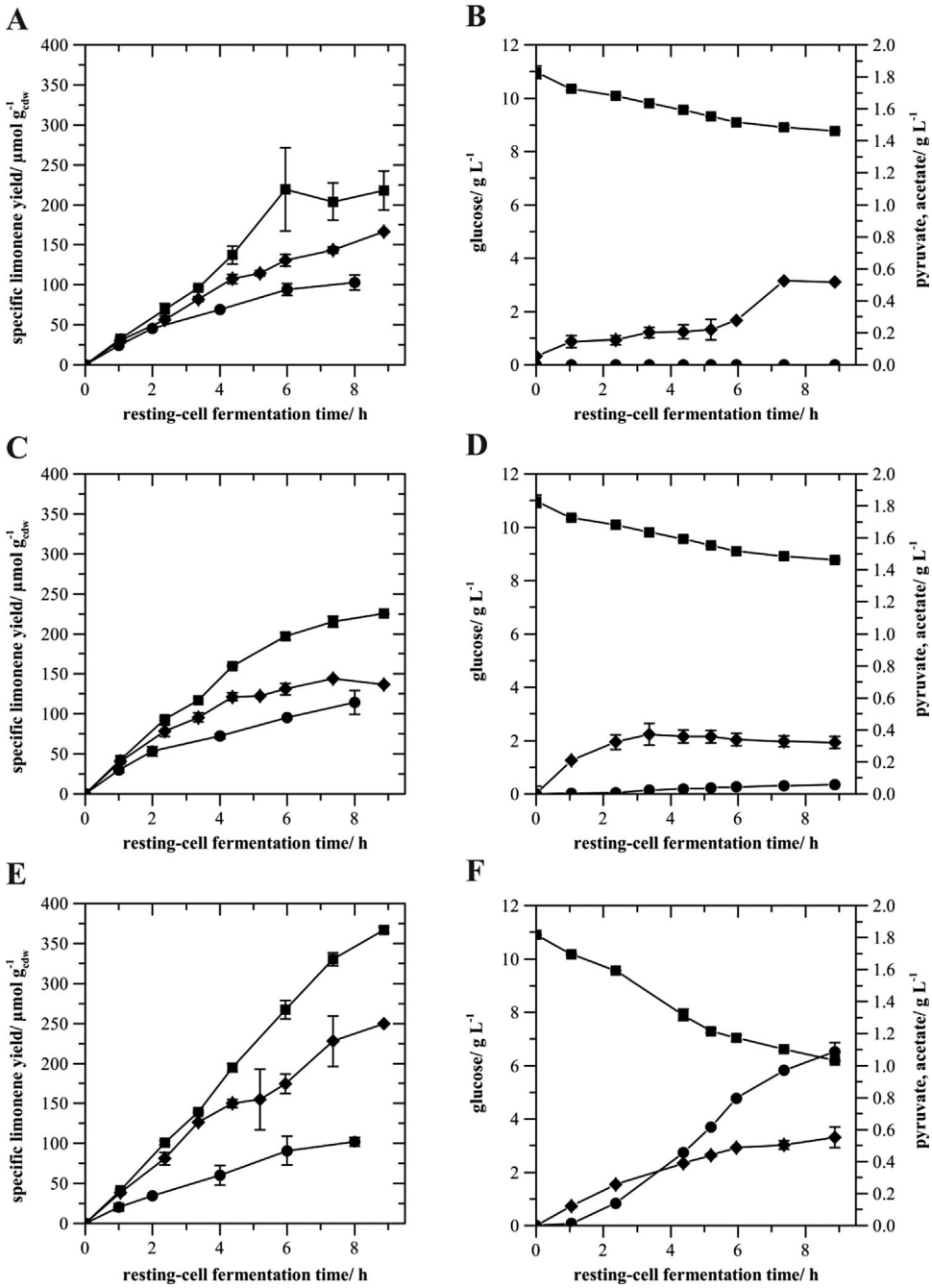

Figure 5. Resting-cell fermentations for limonene production from 1% (w/v) glucose under different nutrient limitations in media either supplemented with IPTG (◆) or IPTG, chloramphenicol, and ampicillin (■), or without supplementation (●). Experiments were performed either in potassium phosphate buffer (**A**, **B**), M9* medium lacking nitrogen (**C**, **D**), or M9* medium lacking magnesium sulfate (**E**, **F**). Panels **A**, **C**, and **E** show the course of limonene formation during these resting-cell fermentations, whereas panels **B**, **D**, and **F** illustrate respective glucose (■), acetate (◆), and pyruvate (●) concentration profiles of the resting-cell fermentations supplemented with IPTG and antibiotics. The error bars refer to standard deviations obtained from two independent cultures (biological replicates).

formation. This could partially be relieved by the addition of IPTG and antibiotics. The effect of IPTG addition can be readily retraced to facilitated heterologous protein biosynthesis. The plasmid maintenance of resting cells, although only minor plasmid loss was observed during resting-cell fermentations in KPi buffer without supplementation of antibiotics (Fig. S3), and/or the intracellular levels of guanosine tetraphosphate (ppGpp) might provide explanations for the positive effects of antibiotics supplementation. ppGpp accumulates in *E. coli* during nutrient starvation (Irr, 1972) and represses global cellular processes such as rRNA transcription (Cashel and Gallant, 1969), translation (Svitil et al., 1993), and DNA replication (Maciag et al., 2010). Bokinsky et al. showed in a recent

Table I. Characteristics of limonene biosynthesis in growing and non-growing *E. coli* BL21 (DE3) harboring pTAC:LS:AGPPS2 and pMVAidi under different experimental conditions.[a]

Condition/biocatalyst configuration	c_{lim}/μmol L_{aq}^{-1}	c_{lim}/mg L_{aq}^{-1}	$Y_{p/s}$/mg_{lim} g_{glc}^{-1}	$Y_{p/x}$/mg_{lim} g_{cdw}^{-1}
Growing cells[b]	111.5 ± 17.7	15.2 ± 2.4	1.5 ± 0.2	9.5 ± 1.5
$MgSO_4$ limited growing cells[b]	200.5 ± 0.8	27.3 ± 0.1	2.7 ± 0.0	38.2 ± 3.9
Resting cells (KP_i)[c]	164.8 ± 18.6	22.4 ± 2.5	11.6 ± 1.3	29.7 ± 3.4
Resting cells ($M9^*_{-N}$)[c]	170.1 ± 0.1	23.2 ± 0.0	10.6 ± 1.2	30.7 ± 0.0
Resting cells ($M9^*_{MgSO4}$)[c]	277.1 ± 2.4	37.8 ± 0.3	8.0 ± 0.1	50.0 ± 0.4

The values for growing cells refer to data from (Willrodt et al., 2014).
[a]All data refer to experiments involving IPTG and antibiotic supplementation.
[b]The values for growing (Willrodt et al., 2014) and $MgSO_4$ limited growing cells refer to the data obtained after depletion of 1% (w/v) glucose.
[c]Values refer to concentrations and yields obtained after 9 h of resting-cell fermentation.

study that ppGpp indeed inhibits RNA and DNA synthesis (Bokinsky et al., 2013). The addition of chloramphenicol led to 60% decreased ppGpp levels within 15 min and thus an onset of RNA and DNA synthesis (Bokinsky et al., 2013). The exact mechanism of the chloramphenicol-mediated ppGpp degradation remains unclear, although this effect has been described in the early 1970s (Lund and Kjeldgaard, 1972). However, the degradation of ppGpp might have led to improved protein synthesis and thus increased limonene formation rates of resting *E. coli* (pTAC:LS:AGPPS2, pMVAidi) supplemented with chloramphenicol.

Resting Cells as a Tool to Study Environmental Perturbations

Environmental perturbations frequently affect both microbial growth and heterologous pathway activity. This makes it difficult to discriminate between different effects when growing cells are applied. Here, growth (biocatalyst synthesis) and terpene production were assessed and optimized individually. An expression temperature of 30°C rather than 20, 25, or 37°C was optimal for the balanced synthesis of all heterologous pathway enzymes in an experiment using the temperature as variable for gene expression (resting-cell fermentations were performed at 30°C). Low temperatures during gene expression have been reported to enhance soluble expression of GPPS (Willrodt et al., 2014). Nevertheless, increasing the expression temperature to 30°C also increased the specific limonene formation rate of resting cells. This observation confirms that the GPPS availability does not limit the total limonene formation rate, as hypothesized recently (Willrodt et al., 2014). The rapid decrease of the specific limonene formation rates indicated instability of one or more enzymes of the limonene formation pathway (Figs. 3 and 4). Operational enzyme instability thus constitutes an optimization target for improving terpene production with resting as well as growing cells, although the reason for this instability remains to be elucidated. In addition, a resting-cell configuration can also be applied to individually dissect and exploit the effects of perturbations/modifications such as medium additives to enhance expression (Zhou et al., 2012), carbon sources (Yoon et al., 2009), or (pathway) enzyme inhibitors/promoters, for example acetate, on growth and product formation.

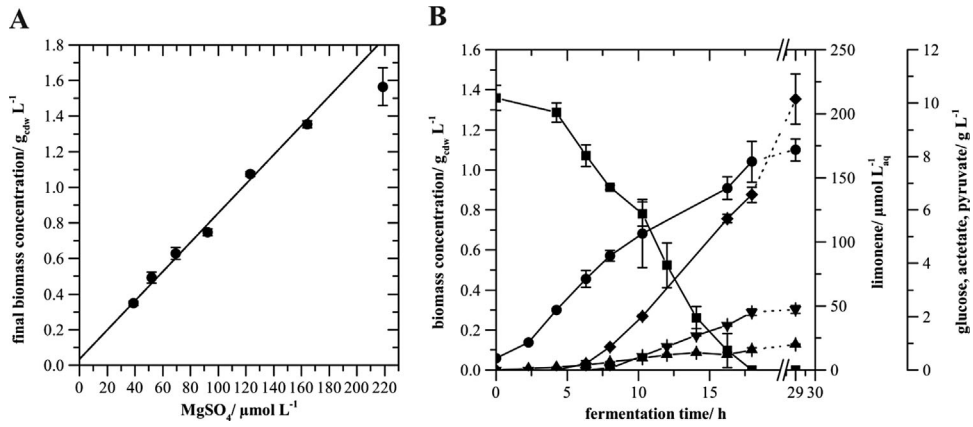

Figure 6. Specific biomass concentration (●) as a function of the $MgSO_4$ amount added to M9* minimal medium (**A**) and fermentation profile of *E. coli* BL21 (DE3) harboring the limonene formation pathway in M9* medium supplemented with 1% (w/v) glucose and 118 μmol $MgSO_4$ per liter (**B**). The biomass concentration (●), the limonene yield on biomass (◆), as well as glucose (■), acetate (▲), and pyruvate (▼) concentrations are given. The error bars refer to standard deviations obtained from three independent cultures (biological replicates).

Physiological Advantages and Constraints of Resting Cells for Terpene Production

The transition of conditions where microbes face an excess of all essential nutrients to conditions where one or more of these nutrients are depleted is accompanied by severe changes in the microbial physiology with respect to intracellular metabolite concentrations and fluxes (Brauer et al., 2006). The altered pattern of metabolites and fluxes also affects the performance of heterologous biosynthesis pathways, which are fueled by these metabolites. The type of nutrient limitation (e.g., nitrogen, sulfur, or phosphate) provokes distinct phenotypes. Nitrogen limitation in *E. coli* for instance results in significantly lower glucose uptake rates compared to magnesium limitation (Chubukov and Sauer, 2014). Concomitantly, a very high pyruvate secretion rate was observed for magnesium-limited cultures, which was hypothesized to be the result of an impaired functioning of the Mg^{2+}-dependent pyruvate dehydrogenase complex (Chubukov and Sauer, 2014; Kale et al., 2007). In our experiments, recombinant *E. coli*, starved for magnesium and sulfur entirely, also showed a similar profile with higher glucose uptake, pyruvate and acetate excretion rates as compared to nitrogen-limiting conditions. Obviously, limonene synthesis substantially profited from these facts with respect to the stability and final (specific) product concentration.

The observed accumulation of the overflow products pyruvate and acetate may also occur due to a reduced activity of the tricarboxylic acid cycle leading to increased availability of acetyl-CoA, the starting material for terpene biosynthesis. In accordance with that, elevated acetyl-CoA availability was recently hypothesized to be the result of an altered transcription profile in response to nitrogen limitation in cultures of *E. coli* BW25113 (Marzan and Shimizu, 2011). However, the gradual transition into the non-growing state (stationary phase in batch cultivation) has to be differentiated regarding physiology and gene expression from the active generation of resting cells (harvesting from an actively growing culture and resuspension in limiting medium), where cells are suddenly facing depletion of an essential nutrient. The observed limonene production rates are relatively low in comparison to turnover rates of the reactions of the central carbon metabolism. This raises the question whether limonene biosynthesis can at all be limited by the availability of intermediates of the central carbon metabolism. However, gene expression and thus flux via the heterologous pathway may not be balanced, which may result in uncoupling of the consumption of cellular resources from limonene formation. It thus seems reasonable to conclude that the availability of intracellular resources was improved by optimization of the resting-cell medium from simple potassium phosphate buffer to a magnesium sulfate deficient medium.

Conclusion

A yet underrated potential for optimizing the de novo synthesis of valuable chemicals with resting recombinant *E. coli* was revealed by successfully demonstrating that the decoupling from microbial growth boosts the formation of the monoterpene limonene. Evaluation of resting cells under different medium conditions and growth-limitations allowed the development of an effective limonene production system in which microbial growth is limited by magnesium sulfate availability. The system is characterized by superior terpene production capacities, reduced formation of biomass as a side product, and a more efficient utilization of resources. In conclusion, controlled reduction of growth by careful optimization of the medium composition rather than maximization of microbial growth was identified as a key strategy for efficient monoterpene production. Such approaches involving limited growth or resting cells, complemented by genetic pathway and host engineering strategies, may well develop into promising process design strategies with relevance for the production of valuable fine chemicals (e.g., oxygenated terpenoids) at scale.

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