High-throughput colorimetric assays optimized for detection of ketones and aldehydes produced by microbial cell factories

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Summary

Randomized strain and pathway engineering are critical to improving microbial cell factory performance, calling for the development of high-throughput screening and selection systems. To facilitate this effort, we have developed two 96-well plate format colorimetric assays for reliable quantification of various ketones and aldehydes from culture supernatants, based on either a vanillin-acetone reaction or the 2,4-dinitrophenylhydrazine (2,4-DNPH) reagent. The vanillin-acetone assay enabled accurate and selective measurement of acetone titers up to 2 g l⁻¹ in a minimal culture medium. The 2,4-DNPH-based assay can be used for a wide range of aldehydes and ketones, shown here through the optimization of conditions for 15 different compounds. Both assays were implemented to improve acetone production from different substrates by an engineered Escherichia coli strain. The fast and user-friendly colorimetric assays proposed here open the potential for iterative rounds of (automated) strain and pathway engineering and screening, facilitating the efforts towards further boosting production titers of industrially relevant ketones and aldehydes.

Introduction

With an increased pressure on society to replace fossil-based production with more sustainable alternatives, the interest in biomanufacturing is at an all-time high. Ketones and aldehydes are a class of valuable molecules to consider for biomanufacturing as they have widespread applications in industry. Their solvent properties and high evaporation rates play an important commercial role in the fragrance, flavour, textiles and agrochemical industries (Smit et al., 2009; Park et al., 2012; Scognamiglio et al., 2013), as well as components in pharmacological and plastics syntheses (Rodriguez and Atsumi, 2014; He and Hartwig, 2019). To date, the supply of these compounds predominantly relies on chemical production based on the oil-derived industry. Replacement of these processes with biosynthesis through cell factory engineering is gaining a growing interest as demonstrated by the production of various ketones (May et al., 2013; Walker and Mills, 2014; Srirangan et al., 2016; Yuzawa et al., 2018) and aldehydes (Kunjapur and Prather, 2015) using metabolic engineering approaches in different hosts. Promising bioproduction of acetone from acetate, for example, resulted in titers up to 122 mM acetone in 48-h Escherichia coli cultures (Yang et al., 2019). The highest production yield of methyl ketones reported to date has been achieved through rational strain engineering of the non-conventional host Pseudomonas taiwanensis, leading to titers of 9.8 g l⁻¹ and highlighting the potential for high titre production of these compounds (Nies...
et al., 2020). While initial strain development is often done in a rational, human-designed manner, novel approaches relying on computer-aided strain design and randomized strain engineering allow tapping into the space of non-understood metabolism through a systems metabolic engineering approach, pushing yields and productivities further (Choi et al., 2019).

Through iterative rounds of systems analysis and engineering, possibly combined with machine learning, significant strain optimization aimed at achieving near-theoretical maxima and industrial applicability can be achieved (Zhou et al., 2018). Critical to such strategies, reliant on the generation of large libraries of strains, is an effective method to screen or select improved strains from baseline starting strains. Various methods have been developed to this end, relying on intracellular response elements such as biosensors or high-throughput analysis (Zeng et al., 2020) of produced metabolites, such as high-throughput high-pressure liquid chromatography (HPLC). Though these approaches may be effective, the development of biosensors is complex, whereas high-throughput HPLC has a heavy reliance on expensive hardware, making its use impractical. As an alternative to equipment-heavy analytical techniques, spectrophotometric methods can provide quantitative determination in various sample types of interest, for the determination of a wide range of target molecules such as metabolites, nucleotides and proteins. In the early 20th century, several spectrophotometric methods to determine ketone and aldehyde concentrations were developed before gas or liquid analytical chromatography became available in most laboratories. These methods include the use of 2,4-dinitrophenylhydrazine (2,4-DNPH) (Lappin, 1951) and vanillin (Amlathe and Gupta, 1990). Here, we optimized two spectrophotometric methods of ketone and aldehyde determination with further potential for high throughput screening of microbial production strains. We demonstrate the use of vanillin- and 2,4-DNPH-dependent assays for quantifying the amounts of various ketones and aldehydes in cell culture supernatants, allowing for easy screening of strain performance during iterative rounds of cell factory optimization.

Results and discussion

Tailoring the vanillin assay for specific detection of acetone

To specifically quantify acetone, we adapted a spectrophotometric method, originally used in 10 mL flasks for acetone determination in clinical and biological samples (Amlathe and Gupta, 1990), to a more sensitive high-throughput 96-well plate format. The reported protocol for the vanillin-based colorimetric analysis of acetone (Amlathe and Gupta, 1990) included sodium bisulfite, which was omitted in our assay, as its influence on the colour development was found to be negligible. To match a 96-well plate format, the overall volume of the assay was scaled down 50-fold, to a final 200 μl. Additionally, the incubation temperature was changed to 60°C, to allow for faster colour development. We observed that the concentration of sodium hydroxide is essential for the sensitivity and can be adapted depending on the expected product yield; increasing the amount of base allows for higher sensitivity. Therefore, compared with the original study, we use a 10-fold increased amount of sodium hydroxide to fit the sensitivity range expected from microbial production. Similarly, the amount of vanillin added to the assay was increased by 3-fold. Reliant on the specific reaction of acetone with vanillin in the base solution, the resulting optimized protocol is presented in Fig. 1A. When using fermentation samples, the cell culture should be centrifuged to remove cells and culture supernatants used as a sample. Then, (i) an aliquot of the sample supernatant (100 μl) with an unknown concentration of acetone is carefully mixed with 60 μl of 130 mM vanillin, followed by the addition of 40 μl of 5 M sodium hydroxide. (ii) The plate with the resulting solution is transferred to 60°C for 10 min, and afterwards cooled to room temperature for 10 min. Acetone reacts with vanillin in an alkaline medium forming 4-(4-hydroxyphenyl-3-methoxy)-3-buten-2-one (Fig. 1B), a yellow compound visible by eye (Fig. 1C). (iii) The absorbance can be measured at 430 nm in a plate reader and remains unchanged for 15 min, and is reliable for at least 1 h. The reagent blank (prepared with water, buffer or production medium, depending on the type of experiment), as well as a suitable calibration curve, should be done in parallel. The blank showed negligible absorbance values, which can be extracted from the absolute value of the sample for precise determinations. With the proposed protocol, sensitivity ranges from 50 to 2000 mg l⁻¹ were observed, which is suitable for evaluating microbial strain performance.

Additionally, we have verified that the developed vanillin assay is specific to acetone and observed no formation of colourful product(s) with neither 2-butanolone, 2-pentanone, 2-nonanone nor acetaldehyde (Fig. S1). This enables specific detection of acetone, especially useful during microbial production in vivo (Yang et al., 2019), where the promiscuous activity of enzymes and cell metabolism may result in the production of a range of other compounds, specifically ketones.

The vanillin assay can be used for vanillin detection

Considering that vanillin itself is an industrially relevant aldehyde, we established a protocol with optimized conditions for vanillin quantification, which enables sensitive
detection of this product. The procedure is described in Fig. 1, with the difference that acetone is now used at a fixed concentration. (i) An aliquot of the sample supernatant (100 μl) with an unknown concentration of vanillin is carefully mixed with 60 μl of 100 mM acetone, followed by the addition of 40 μl of 5 M sodium hydroxide. (ii) The plate with the resulting solution is transferred to 60°C for 10 min, and afterwards cooled to room temperature for 10 min. Acetone reacts with vanillin in an alkaline medium forming 4-(4-hydroxyphenyl-3-methoxy)-3-buten-2-one (Fig. 1B), a yellow compound visible by the naked eye. (iii) The absorbance can be measured at 430 nm in the plate reader and remains unchanged for 10 min and reliable for 30 min. The calibration of this assay for vanillin detection with the acetone assay is presented in Fig. S2. The reagent blank (with water, buffer, or production medium, depending on the type of experiment), as well as the calibration curve, should be done in parallel. The protocol established here can for example be used during strain development for vanillin production and characterization (Banerjee and Chattopadhyay, 2019) or protein engineering (Luziatelli et al., 2019). The assay allows for accurate detection of vanillin concentrations between 80 and 2100 mg l⁻¹.

Detection of acetone with an optimized 2,4-DNPH assay

Though the vanillin assay is well suited for the specific detection of acetone, to expand the scope of ketones and aldehydes that can be detected, an optimized assay based on 2,4-dinitrophenylhydrazine (2,4-DNPH) was developed. 2,4-DNPH contains a benzene ring, two nitro groups and a hydrazine functional group that is dissolved in a solution of phosphoric acid and can be used for the detection of ketones and aldehydes in solution. 2,4-DNPH does not react with amides, esters, carboxylic acids or alcohols, making it sufficiently selective in a complex fermentation broth, yet flexible enough to detect a variety of ketones and aldehydes with (Lawrence, 1965; Lewis et al., 2018).

To allow effective use in 96-well plate format, optimization of the setup was performed leading to a suitable protocol for detection in the cell culture broth. Fixing the total assay volume at 200 μl, the concentration and volume of the 2,4-DNPH reagent (whilst maintaining the same absolute amount of 2,4-DNPH added) were optimized to allow linear detection with a maximum resolution. Additionally, the most suitable wavelength for the detection of acetone was found through spectral

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scanning from 400 to 600 nm wavelengths with a 10 nm step, examples of which are depicted in Fig. S3. The wavelength was selected based on the potential for the largest linear detection range when acetone was dissolved in Milli-Q water. Though this cannot be considered an optimal wavelength, due to the high background absorbance of the 2,4-DNPH reagent, it forms a compromise to allow the best estimation of ketone or aldehyde concentrations in the assay. The optimized protocol describing key steps for acetone determination (as an example) is presented in Fig. 2A: (i) An aliquot of the sample supernatant of interest (195 μl) with an unknown concentration of acetone is carefully mixed with 5 μl of 200 mM 2,4-DNPH in a phosphoric acid solution (Sigma–Aldrich, St. Louis, MO, USA). (ii) The plate with the resulting mixture is incubated for 2 min at room temperature (RT). Acetone reacts with 2,4-DNPH forming acetone 2,4-dinitrophenylhydrazone (Fig. 2B), an orange compound visible by eye. (iii) The absorbance can be measured at 490 nm (suitable for acetone) in a plate reader and remains reliable for 30 min (Fig. 2C). The reagent blank (with water, buffer, or production medium, depending on the type of experiment) should be done in parallel as well as with a calibration curve. The developed 2,4-DNPH assay presented here shows a linear range of 25–500 and 10–200 mg mL⁻¹ in Milli-Q and rich (LB) medium respectively. Though the response differs depending on background media, this can be accounted for through appropriate calibration curves. This also indicates that the presence of complex media components in a rich medium, often present in the fermentation broth, does influence but does not prohibit quantifiable output.

One consideration when working with 2,4-DNPH is its low solubility in pH-neutral solutions. Therefore, when adding the reagent to approximately neutral fermentation supernatants, precipitation of the hydrazine substrate and hydrazone product may result. This can cause larger deviations in technical replicates, and it is the reason why the assay should be read out within 30 min of adding the sample. The precipitation can be prevented by working in acidic conditions, which in turn increases the sensitivity and detection range (Zhou et al., 2016). However, this would imply additional assay steps and

Fig. 2. 2,4-Dinitrophenylhydrazine assay guideline with optimized conditions.
A. Scheme of the protocol. The sample supernatant (195 μl) is carefully mixed with 5 μl of the reagent (0.2 M 2,4-DNPH solution in phosphoric acid). The mixture is incubated for 2 min at room temperature (RT) and the absorbance of the analysed samples is measured at 490 nm.
B. The chemical reaction between acetone and 2,4-dinitrophenylhydrazine, leading to the formation of a colourful 2,4-dinitrophenylhydrazone adduct under acidic conditions.
C. Calibration of the assays using acetone as a standard in lysogeny broth (LB) medium and Milli-Q deionized water (MQ). Regression over the linear range is shown, with R² values indicated for each condition. Presented values are averages of three technical triplicates, with standard deviation represented as error bars.

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reagents which is not beneficial for high throughput screening. Considering that the assay still proved reliable, though within a narrower concentration range, it is still suitable for the purpose of high-throughput strain screening, where exact, absolute determination of titers is not required. Under these conditions, resolving high and low producers is the target, with the added advantage of short processing time and low reagent and equipment cost. After strain screening with the proposed assays and the selection of a few promising candidates, analytical techniques such as HPLC should be used to accurately estimate fermentation titers of ketones and aldehydes other than vanillin or acetone. Yet, for analysing large sets of strains or production conditions, the ease of use, cheap reagents and fast reaction times of the 2,4-DNPH assay come at an advantage, albeit at the cost of lower specificity and precision.

The 2,4-DNPH assay allows for the detection of a variety of aldehydes and ketones

Contrary to vanillin, the condensation reaction between 2,4-DNPH and the functional ketone or aldehyde group is insensitive to the rest of the groups present in the molecule, thus allowing this assay to be used for quantification of other ketones and aldehydes of interest. Depending on the ketone or aldehyde present, the resulting 2,4-dinitrophenylhydrazone conjugate will have different absorbance wavelengths, also visible as a different colour by the eye ranging from yellow to orange or red. To enable the 2,4-DNPH assay described here to be used for the detection of a panel of 7 different ketones and 8 different aldehydes, for the first time we have reported suitable detection wavelengths and corresponding linear ranges per compound, presented in Table 2, with spectral scanning used to determine the wavelengths shown in Fig. S3. This highlights the wide applicability of such an assay to use in the optimization of cell factories to produce a wide range of compounds, including cyclic ketones and lactones. Conversely, when cell factories produce large amounts of mixed ketones and aldehydes, it could result in the possibility of interference. To investigate this issue, we show the quantification of acetone in the presence of 2-pentanone (Fig. S4). Some sensitivity and dynamic range were lost in these experiments; however, acetone was still linearly detected within a specific range. Therefore, unless equimolar amounts of mixed ketones or aldehydes are produced, the 2,4-DNPH assay is a valuable tool. Especially when considering that cell factories are often engineered to produce one specific product, to facilitate higher titers and more favourable product purification in downstream processing, large amounts of mixed ketones or aldehydes are not to be expected.

Application of assays for in vivo production of acetone

To demonstrate the ease of use and application of the assays, they were used to evaluate microbial strain performance during the in vivo production of acetone in E. coli. The biological production of acetone predominantly relies on the canonical (c) pathway from Clostridium acetobutylicum ATCC 824. In this pathway (Fig. 3A): (i) acetate is first converted into acetyl-CoA through acetyl-CoA synthetase (AcS). Then, (ii) 2 mol of acetyl-CoA are condensed by thiolase (Thl) to generate 1 mol of acetoacetyl-CoA. (iii) Acetoacetyl-CoA transferase (CtfAB) transfers the CoA moiety from acetoacetyl-CoA to acetate, and forms acetoacetate, which is (iv) catalysed by acetoacetate carboxylase (Adc) to form acetone and carbon dioxide. This natural canonical pathway has previously been simplified through the establishment of a synthetic alternative to one-step: using an acetoacetyl-CoA transferase (AtODA) from E. coli MG1655 to replace the C. acetobutylicum acetoacetyl-CoA transferase (CtfAB), where the concentration of acetone after 24 h of fermentation in modified E. coli strain reached up to 20 mM from 120 mM of acetate, used as a sole carbon source (Yang et al., 2019) (Fig. 3A).

The SEVA (Standard European Vector Architecture) format to build and collect plasmids was created in 2013, allowing their assembly in a unified, pre-formatted fashion (Silva-Rocha et al., 2013). Combination of functional DNA modules (replication origins, antibiotic resistance genes and cargo elements) are easy to exchange, allowing standardized manipulation in Gram-negative hosts and represented by a constantly updating number of vectors (Martinez-Garcia et al., 2020). To this end, we constructed vector pSEVA4318 (Table 1), contributing to the collection with the tightly controlled rhamnose inducible expression system RhamRS/P_rbaBAD (Calero et al., 2016). For acetone biosynthesis, using the previously shown standard acetone bioproduction pathways, plasmids pS4318-MKc were created (Table 1), harbouring genes encoding the canonical (c, Thl, CtAB, Adc from C. acetobutylicum) or synthetic (s, Thl, Adc from C. acetobutylicum and AtODA from E. coli) acetone production pathway, with a detailed vector scheme illustrated at Fig. 3B. To validate the pathway performance, we inoculated E. coli DH5α cultures carrying the constructed empty, canonical or synthetic vector and performed 24 h fermentation in 10 mL of minimal M9 medium containing 10 g L⁻¹ of glucose as a carbon source, streptomycin at 100 µg mL⁻¹, and 1 mM of rhamnose for the pathway induction. After 24 h, cultures were cooled down at 4°C for 1 h, and then centrifuged at 5,000 × g for 15 min. We evaluated acetone concentration in the resulting supernatant using the vanillin
We observed a 3.5-fold improvement of acetone synthesis in the strain containing the synthetic pathway compared with the canonical pathway (Fig. 3C), which corresponds well to previously published data (Yang et al., 2019). Thus, having confirmed previous observations of the better performing pathway, we proceeded to further validate the assay performance using this synthetic pathway for acetone bio-production.

Since E. coli MG1655 is considered a workhorse host strain for industrial bioprocesses (Soupene et al., 2003; Igonina et al., 2020), this K-12 strain was used for the next round of fermentation experiments to better mimic microbial bioproduction, and hence validate the performance of the proposed assays. To this end, vector pS4318-MKs encoding the best performing pathway (Fig. 3C) was transformed into E. coli MG1655, and the resulting strain was used for demonstrating the

**Table 1. Bacterial strains and plasmids used in this study.**

| Bacterial strain | Relevant characteristics | Reference or source |
|------------------|-------------------------|---------------------|
| Escherichia coli |                         |                     |
| DH5α             | Cloning host; F− ΔendA1 glnX44(AS) thiE1 recA1 relA1 spoT1 gyrA96(NalR) rfbC1 deoR rupG−80(lacZΔM15) Δ(argF-lac)U169 hsdR17(k− m−) | Meselson and Yuan (1968) |
| MG1655           | Wild-type strain; F− ΔendA1 rph-1 | Blattner et al. (1997) |
| Plasmid          |                         |                     |
| pSEVA4318        | Standard expression vector carrying a rhamnose-inducible expression system; oriV(pBR1); rhaR, rhaS, P_{rhaBAD}, SmR/SpR | This work |
| pS4318-MKc(s)    | Derivative of vector pSEVA4318 harbouring the genes encoding the canonical acetone production pathway from C. acetobutylicum; RhaRS/P_{rhaBAD}→thlCa, ctfAB^{Ca}, adc^{Ca}; SmR/SpR | This work |
| pS4318-MKs       | Derivative of vector pSEVA4318 harbouring the genes encoding a synthetic MK production pathway; RhaRS/P_{rhaBAD}→thf^{Ca}, atoD^{Ec}, adc^{Ca}; SmR/SpR | This work |

a. Antibiotic markers and abbreviations are identified as follows: Nal, nalidixic acid; Sm, streptomycin; Sp, spectinomycin. The source of relevant genes is indicated with a superscript as follows: Ca, Clostridium acetobutylicum; and Ec, Escherichia coli.
application of the vanillin- and the 2,4-DNPH-based assays by comparison with HPLC detection. To validate the sensitivity of the proposed assays to variations in pathway expression levels and so product titers, the MG1655 strain harbouring the pS4318-MKs plasmid was cultured with varying rhamnose concentrations (Fig. 4A, B). As E. coli has the potential to catabolize rhamnose as the sole carbon source, a control without supplemented glucose was added (Eagon, 1961). Acetone concentrations were determined using the vanillin assay, the 2,4-DNPH assay and HPLC for validation of quantification potential (Fig. 4A), where final culture OD and glucose consumption were also determined (Fig. 4B). Though slight growth of E. coli is observed on the low amounts of rhamnose added, no production of acetone is observed using any of the analytical methods under these conditions. Thus, the added rhamnose does not confound the amount of acetone produced. Culturing of the wild-type MG1655 strain highlights the absence of production of any natural compounds that interfere with acetone detection in either the vanillin or 2,4-DNPH assays. Both the vanillin and 2,4-DNPH assay resolve the increasing acetone produced with increasing rhamnose concentration, as validated by HPLC data, highlighting the resolving power of these assays for microbial fermentation purposes. Even though the 2,4-DNPH assay can react with various aldehydes and ketones, in vivo insignificant amounts of other compounds are produced that interfere with the assay in such a way that resolution between production levels is hampered.

A common practice for further optimization of bioproduction lies in investigating microbial strain performance with alternative carbon sources, for example, to decrease reliance on expensive, less-sustainable carbon sources such as glucose. To facilitate the green transition, acetate is an interesting carbon source due to its low cost and its potential to be efficiently produced sustainably through CO₂-fixing acetogens (Lemaire et al., 2020). The production of acetone in an engineered E. coli strain from either glucose or acetate as a carbon source was previously shown (Yang et al., 2019). In a wild-type E. coli, aerobic growth on acetate is achieved through the ATP demanding conversion into acetyl-CoA and assimilating this further into energy and biomass respectively. Here, we investigated if the co-feeding of glucose and acetate would improve acetone yields, through the rationale that energy can be generated through glucose metabolism, allowing a more efficient conversion of acetate into acetone. As we previously observed near complete glucose consumption (Fig. 4B), here we fed higher levels of glucose to the production strain in either minimal or rich medium (Fig. 4C). Both the vanillin- and 2,4-DNPH-based assays accurately capture the different production levels of acetone, though the 2,4-DNPH assay shows higher levels of variation between biological replicates. As expected, we observe no acetone accumulation when fed sole acetate, as this is expected to be used for energy and biomass assimilation. Interestingly, when 2 g l⁻¹ acetate is added as co-substrate to 10 g l⁻¹ glucose, we observed a 2.2-fold increase in acetone titre when cultured in an M9 medium compared with production on sole glucose. In LB medium, we observed a higher level of acetone production on glucose as substrate than in minimal medium, and as a result, the increase of acetone production when acetate is co-fed is less pronounced in LB medium (Fig. 4C). The varying additional nutrients found in LB can facilitate additional metabolic pathways for energy generation and anabolism, hypothesized to result in less advantage of co-feeding on acetone yield. The difference in production highlights the variation in strain performance that can be observed upon modifying carbon source or media and emphasizes the advantage of having high throughput colorimetric assays that function in both rich and minimal media. It also shows the potential to use such assays for medium optimization, though downstream production setup can introduce variations into absolute production titers observed.

Taken together, the developed approaches proved useful for the accurate detection of acetone in both minimal M9 medium through the vanillin assay, as well as in rich LB medium through the 2,4-DNPH assay, thereby resolving high, medium and low levels of production similarly to HPLC results.

Conclusion

Microbial strain optimization to move cell factories from lab-scale titers to industrially relevant productivities depend on the development of high throughput and systems-level technologies, facilitated through computer-aided design and automation. However, a critical factor often hampering the application of these approaches is an effective strain screening or selection strategy. In this work, we have presented high-throughput strain screening approaches specifically for (i) either acetone or vanillin detection (vanillin-based assay) and (ii) various ketones and aldehydes screening (2,4-DNPH-based assay). These assays can be easily implemented on fermentation supernatants.

The vanillin-acetone assay can be used to determine the acetone or vanillin production titers with high precision, comparable to HPLC detection. With high specificity towards acetone and vanillin, this assay can be used to accurately distinguish acetone or vanillin concentration in minimal media, in a range of 50–2000 mg l⁻¹. This feature can be especially relevant
Fig. 4. Assay application for acetone biosynthesis by engineered E. coli.
A. Overview of biosynthesis of acetone, dependent on the inducer (rhamnose) concentration, as detected by either HPLC, vanillin assay or 2,4-DNPH assay. Varying concentrations of inducer were tested in the presence or absence of 2 g L\(^{-1}\) glucose as a carbon source.
B. OD\(_{600}\) and glucose consumption in E. coli MG1655 containing the selected pathway encoded by plasmid pS4318/MKS, for varying rhamnose concentrations, with and without glucose supplemented.
C. Experiment simulating carbon source screening. Acetone production and determination after fermentation of E. coli MG1655 containing the synthetic pathway borne by plasmid pS4318/MKS. In these experiments, 10 g L\(^{-1}\) of glucose, 2 g L\(^{-1}\) of acetate, or a combination of 10 g L\(^{-1}\) of glucose and 2 g L\(^{-1}\) of acetate, was added to the medium as a carbon source. Assay performance was evaluated by comparison to HPLC acetone determinations (see Experimental Procedures). (Left) Vanillin assay, where bacterial growth was performed in M9 minimal medium. (Right) 2,4-DNPH assay where bacterial growth was performed in LB medium. The bars represent average values from biological triplicates.

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when other ketones or aldehydes (potentially cross-reacting with other reagents) are produced by the same cell factory.

When the product of interest is another ketone or aldehyde, the presented 2,4-DNPH assay can be used. Optimized for a total of 15 structurally diverse ketones and aldehydes (Table 2), this shows the versatility of the proposed assay, with the application likely being translatable to an even wider number of compounds. Additionally, the 2,4-DNPH assay works even in the presence of complex culture media, is often used in further production steps and is beneficial to screen small-scale productivity. Previously, methods for ketone (Mei et al., 2020; Zhou et al., 2016) and aldehyde (Ressmann et al., 2019) determination were described based on other chemical reactions (fluorescence and 2-aminobenzamidoxime [ABAO] respectively). Additionally, the 2,4-DNPH-based assay was previously shown for various, highly similar prochiral ketones in a 96-well format, at only one wavelength (500 nm; Zhao et al., 2016). Advantages and limitations of these previously published assays, as well as the assays presented here, are summarized in Table 3. All the previously available assays allow screening of purified enzymes or whole resting cells to evaluate specific enzyme activity in a highly sensitive manner. However, a resulting impediment is a strong dependency on a clear enzymatic buffer as background with little interfering with other components. As a result, this introduces additional steps in the process, preventing the fast and easy screening of microbial cell supernatants in the complex fermentation broth. Considering that expected supernatant titers far exceed the detection range of the previous, highly sensitive in vitro assays, assay optimization with the final application in mind is important. Though optimizing specific enzymatic conversions in vitro is critical to improving production pathways (Zhao et al., 2016), complex microbial metabolism additionally requires reflection of in vivo pathway performance. This provides more translatable titers when moving to fermentation settings. Therefore, the lower sensitivity yet higher detection range of the optimized assays presented here, together with the lower dependency on background components allows easy evaluation of whole strain bioproduction. Thus, we foresee the specific, in vitro assays being synergistic with the assays presented here, to push pathway and strain performance forward.

From the available protocols, solely the vanillin-acetone assay allows for specific detection of one ketone (acetone) or aldehyde (vanillin) in the presence of other similar chemicals. This hampers the use of the assay for detecting other ketone and aldehyde products of interest, yet is advantageous when cell factories are expected to produce large amounts of side products, specifically other ketones and aldehydes. To detect other ketones and aldehydes of interest, 2,4-DNPH or ABAO-dependent assays can be used. However, a consequence of their unspecific detection can result in the convergence of the reaction with other present aldehydes and ketones produced by the cell. Contrary to the previously proposed ABAO and 2,4-DNPH assays reliant on one sole wavelength, in the 2,4-DNPH assay proposed here, tailoring of the detection wavelength can limit the interference of other reacted ketones or aldehydes, when present. Additionally, with the advancement of synthetic biology and metabolic engineering, microbial production hosts are designed to predominantly produce one single compound, as this facilitates further downstream processing and overall productivity of a process. This is reflected by the data presented here, when simple engineering of E. coli through sole plasmid-based pathway introduction results in the production of acetone, quantifiable through both the vanillin and 2,4-DNPH assay, regardless of any other aldehydes or ketones that may be produced by the cell factory. HPLC analysis confirms that no other aldehydes or ketones have been detected, and so we expect that for the purpose of cell factory engineering, the wide reactivity of the 2,4-DNPH assay with various ketones and aldehydes is not of concern when the target molecule is overproduced. Though to truly estimate the yields and validate the compound detected, results for the best-selected strains should be verified through more precise analytical methods such as HPLC, which is nevertheless considered an important follow-up step after the high-throughput screening.

Table 2. Overview of selected wavelengths, with corresponding linear detection ranges and observed fit for various ketones and aldehydes detected with the 2,4-DNPH assay in a Milli-Q water background.

| Compound          | $\lambda_{opt}$ (nm) | Linear detection range (mg ml$^{-1}$) | Fit ($R^2$) |
|-------------------|----------------------|--------------------------------------|-------------|
| **Aldehydes**     |                      |                                      |             |
| Acetaldehyde      | 500                  | 50–500                               | 0.99        |
| Formaldehyde      | 550                  | 10–100                               | 0.77        |
| D-Glyceraldehyde  | 470                  | 10–1000                              | 0.84        |
| Isobutyraldehyde  | 500                  | 10–500                               | 0.97        |
| 2-Phenylacetdehyde| 500                  | 10–200                               | 0.98        |
| Propionaldehyde   | 510                  | 25–500                               | 0.92        |
| Vanillin          | 570                  | 10–750                               | 0.98        |
| **Ketones**       |                      |                                      |             |
| Acetone           | 490                  | 10–500                               | 0.79        |
| Acetyl-acetone    | 490                  | 10–200                               | 0.83        |
| 2-Butanone        | 520                  | 75–1000                              | 0.92        |
| γ-Butyrolactone   | 420                  | 10–200                               | 0.88        |
| Cyclohexanone     | 520                  | 25–200                               | 0.91        |
| 2-Nonanone        | 420                  | 25–500                               | 0.77        |
| 2-Pentanone       | 520                  | 10–500                               | 0.92        |
| 3-Pentanone       | 510                  | 10–750                               | 0.97        |

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Finally, to demonstrate how the presented assays can be applied to explore strain productivity, *E. coli* was equipped with an inducible synthetic acetone production pathway. Varying inducer concentrations show the sensitivity of both proposed assays to varying pathway expression levels, seen as readout through acetone titers. Additionally, acetone titers in response to glucose, acetate and the combination were determined through both assays and HPLC, in both minimal and rich medium. The assay titers showed good correlation to the HPLC titers, for low, medium and high production ranges, making it possible to distinguish the best microbial producers from others in a 96-wells format. The availability of an assay to determine acetone concentration through simple culture supernatant analysis opens the possibility for further genome and pathway engineering in randomized approaches. Additionally, it can be used to further optimize media components of substrates, as indicated through the co-supply of glucose and acetate to further improve acetone titre. Altogether,
this facilitates the use of fully automated pipelines for the iterative improvement of production strains and conditions, allowing society to transition towards a truly green biomanufacturing basis.

**Experimental procedures**

**Bacterial strains, plasmids and culture conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* cultures were incubated at 37°C. For cloning procedures and during genome engineering manipulations, cells were grown in a lysogeny broth (LB) medium (10 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract and 10 g l\(^{-1}\) NaCl; solid culture media additionally contained 15 g l\(^{-1}\) agar). All cultures were agitated at 200 rpm (MaxQ\(^{TM}\) 8000 incubator; ThermoFisher Scientific, Waltham, MA, USA). Streptomycin (Str) was added whenever needed at 100 μg ml\(^{-1}\). The optical density measured at 600 nm (OD\(_{600}\)) was recorded in a Genesyss 20 spectrophotometer (Thermo Fisher Scientific) to estimate bacterial growth. During physiological characterization of engineered strains, growth kinetics were followed at OD\(_{600}\) with light path correction in a Synergy\(^{TM}\) MX microtiter plate reader (BioTek Instruments Inc., Winooski, VT, USA).

**General cloning procedures and construction of plasmids and mutant strains**

All plasmids, oligonucleotides and gene fragments used in this work are listed in Tables 1, S1 and S2. Unless stated otherwise, uracil-excision (USER) cloning (Cavaleiro et al., 2015) was used for the construction of all plasmids. The AMUSER tool was employed for designing oligonucleotides (Genee et al., 2015). Phusion\(^{TM}\) U high-fidelity DNA polymerase (ThermoFisher Scientific) was used according to the manufacturer’s specifications in amplifications intended for USER cloning. For colony PCR, the commercial OneTaq\(^{TM}\) master mix (New England BioLabs, Ipswich, MA, USA) was used according to the supplier’s instructions. *E. coli* DH5α λpir (Table 1) was employed as a host for general cloning purposes. Chemically competent *E. coli* cells were prepared and transformed with plasmids using the Mix and Go\(^{TM}\) commercial kit (Zymo Research, Irvin, CA, USA) according to the manufacturer’s indications. The sequence of all used plasmids and strains was verified by Mix2Seq sequencing (Eurofins Genomics, Ebersberg, Germany).

**Characterization of engineered *E. coli* strains for acetone biosynthesis**

Overnight pre-cultures of *E. coli* MG1655 transformed with plasmids pSEVA4318 (non-production control), pSEVA4318 MKc (canonical acetone production pathway) or pSEVA4318 Mks (synthetic acetone production pathway) were grown in 5 ml of M9 minimal medium added with Str at 100 μg ml\(^{-1}\) and 10 g l\(^{-1}\) glucose as a carbon source. These cultures were used as the inoculum for acetone production experiments by dilution in fresh medium at a starting OD\(_{600}\) = 0.05. Cells were grown for 24 h at 37°C with agitation at 200 rpm in 10 ml of either M9 minimal medium or LB medium, as indicated, in 50 ml Falcon tubes with Str at 100 μg ml\(^{-1}\), and with varying concentrations of rhamnose for pathway induction. For experiments with varying rhamnose concentrations, 2 g l\(^{-1}\) glucose was added to a minimal medium, as indicated. In the experiments with varying carbon sources, 10 g l\(^{-1}\) glucose, 2 g l\(^{-1}\) sodium acetate (pH = 7) or their combination were added to the medium as a carbon source. After 24 h, cells were incubated under the assay conditions for 1 h at 4°C to allow any formed volatile products to condense, then centrifuged at 5000 g for 15 min, and the supernatant was analysed by HPLC or colorimetry assays for the acetone content.

**HPLC analysis of acetone biosynthesis**

The concentration of acetone was measured in a Dionex UltiMate 3000 HPLC system equipped with an Aminex HPX-87X ion exclusion (300 × 7.8 mm) column (BioRad, Hercules, CA) coupled to RI-150 refractive index and UV (260, 277, 304 and 210 nm) detectors. The column was maintained at 30°C, the mobile phase comprised of 5 mM H\(_2\)SO\(_4\) in Milli-Q water at a flow rate of 0.6 mL min\(^{-1}\), with a run length of 30 min. The eluted compounds were detected after 24.0 min by an HPLC Waters 481 UV–visible detector at 214 nm. This detector was connected in series to an RI detector (model 410). HPLC data were processed using the Chromeleon\(^{TM}\) chromatography data system software 7.1.3 (Thermo Fisher Scientific). The detection of acetone was monitored at RI and compound concentrations were calculated from peak areas using a calibration curve prepared with acetone (99% HPLC standard, Sigma–Aldrich Co.).

**Data analysis and visualization**

The Prism 9 software (GraphPad Software Inc., San Diego, CA, USA) was used to plot obtained results. Chemical reactions were crafted using ChemDraw Professional 20.1 software, and Adobe Illustrator\(^{21}\) was used for the remaining data visualization. We recommend working with vector-format figures and using dedicated software to improve the quality of graphical overviews.
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Author contributions

E.K.: Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing – original draft and editing. V.M.: Investigation, Methodology, Data curation, Validation, Writing – original draft and editing. P.I.N.: Supervision, Resources, Funding acquisition, Writing – review and editing. A.T.N.: Supervision, Resources, Funding acquisition, Project administration, Writing – review and editing.

References

Amlathe, S., and Gupta, V.K. (1990) Spectrophotometric determination of acetone using vanillin. Analyst 115: 1385–1387.
Banerjee, G., and Chattopadhyay, P. (2019) Vanillin biotechnology: the perspectives and future. J Sci Food Agric 99: 499–506.
Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M. et al. (1997) The complete genome sequence of Escherichia coli K-12. Science 277: 1453–1462.
Calero, P., Jensen, S.I., and Nielsen, A.T. (2016) Broad-host-range ProUSER vectors enable fast characterization of inducible promoters and optimization of p-coumaric acid production in Pseudomonas putida KT2440. ACS Synth Biol 5: 741–753.
Cavaleiro, A.M., Kim, S.H., Seppälä, S., Nielsen, M.T., and Nerholm, M.H. (2015) Accurate DNA assembly and genome engineering with optimized uracil excision cloning. ACS synth Biol 4: 1042–1046.
Choi, K.R., Jang, W.D., Yang, D., Cho, J.S., Park, D., and Lee, S.Y. (2019) Systems metabolic engineering strategies: integrating systems and synthetic biology with metabolic engineering. Trends Biotechnol 37: 817–837.

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Eagon, R.G. (1961) Bacterial dissimilation of L-fucose and L-rhamnose. J Bacteriol 82: 548–550.
Genée, H.J., Bonde, M.T., Bagger, F.O., Jespersen, J.B., Sommer, M.O.A., Wernersson, R., and Olsen, L.R. (2015) Software-supported USER cloning strategies for site-directed mutagenesis and DNA assembly. ACS synth Biol 4: 342–349.
He, Z.T., and Hartwig, J.F. (2019) Enantioselective alpha-functionalizations of ketones via allylic substitution of silyl enol ethers. Nat Chem 11: 177–183.
Igonina, O., Samsonov, V., Ublinskaya, A., Hook, C., Malych, E., Kozaeva, E. et al. (2020) A novel one-step method for targeted multiplication of DNA fragments from the Escherichia coli chromosome mediated by coordinated functioning of lambda and phi80 bacteriophage recombination systems. J Microbiol Methods 170: 105842.
Kunjapur, A.M., and Prather, K.L. (2015) Microbial engineering for aldehyde synthesis. Appl Environ Microbiol 81: 1892–1901.
Lappin, G., and Clark, L. (1951) Colorimetric method for determination of traces of carbonyl compounds. Anal Chem 23: 541–542.
Lawrence, R.C. (1965) Use of 2,4-dinitrophthalhydrazine for estimation of micro amounts of carbonyls. Nature 205: 1313–1314.
Lemaire, O.N., Jespersen, M., and Wagner, T. (2020) CO2 fixation strategies in energy extremophiles: what can we learn from acetogens? Front Microbiol 11: 486.
Lewis, S.A., Connatser, R.M., Olarte, M.V., and Keiser, J.R. (2018) Determining aromatic and aliphatic carboxylic acids in biomass-derived oil samples using 2,4-dinitrophthalhydrazine and liquid chromatography-electrospray injection-mass spectrometry/mass spectrometry. Biomass Bioenergy 108: 198–206.
Luziatielli, F., Brunetti, L., Picca, A.G., and Ruzzi, M. (2019) Maximizing the efficiency of vanillin production by biocatalyst enhancement and process optimization. Front Bioeng Biotechnol 7: 279.
Martinez-Garcia, E., Goni-Moreno, A., Bartley, B., McLaughlin, J., Sanchez-Sampedro, L., Pascual Del Pozo, H. et al. (2020) SEVA 3.0: an update of the standard European vector architecture for enabling portability of genetic constructs among diverse bacterial hosts. Nucleic Acids Res 48: D1164–D1170.
May, A., Fischer, R.J., Maria Thum, S., Schaffer, S., Versek, S., Durre, P., and Bahl, H. (2013) A modified pathway for the production of acetone in Escherichia coli. Metab Eng 15: 218–225.
Mei, Z., Zhang, K., Qu, G., Li, J.K., Liu, B., Ma, J.A. et al. (2020) High-throughput fluorescence assay for ketone detection and its applications in enzyme mining and protein engineering. ACS Omega 5: 13588–13594.
Meselson, M., and Yuan, R. (1968) DNA restriction enzyme from E. coli. Nature 217: 1110–1114.
Nies, S.C., Alter, T.B., Noting, S., Thiery, S., Pan, A.N.T., Drummen, N. et al. (2020) High titer methyl ketone production with tailored Pseudomonas taiwanensis VLB120. Metab Eng 62: 84–94.
Park, J., Rodriguez-Moya, M., Li, M., Pichersky, E., San, K.Y., and Gonzalez, R. (2012) Synthesis of methyl...
ketones by metabolically engineered *Escherichia coli*. *J Ind Microbiol Biotechnol* 39: 1703–1712.

Ressmann, A.K., Schwendenwein, D., Leonhartsberger, S., Mihovilovic, M.D., Bornscheuer, U.T., Winkler, M., and Rudloff, F. (2019) Substrate-independent high-throughput assay for the quantification of aldehydes. *Adv Synth Catal* 361: 2538–2543.

Rodriguez, G.M., and Atsumi, S. (2014) Toward aldehyde and alkane production by removing aldehyde reductase activity in *Escherichia coli*. *Metab Eng* 25: 227–237.

Scognamiglio, J., Letizia, C.S., and Api, A.M. (2013) Fragrance material review on cyclohexyl methyl pentanone. *Food Chem Toxicol* 62 (Suppl 1): S138–S143.

Silva-Rocha, R., Martinez-Garcia, E., Calles, B., Chavarria, M., Zeng, W., Guo, L., Xu, S., Chen, J., and Zhou, J. (2020) Zhao, H., Sun, Y., Peters, J.M., Gross, C.A., Garner, E.C., and Helmann, J.D. (2016) Depletion of unde-
caprenyl pyrophosphate phosphatases disrupts cell envelope biogenesis in *Bacillus subtilis*. *J Bacteriol* 198: 2925–2935.

Zhou, J.Y., Xu, G.C., Han, R.Z., Dong, J.J., Zhang, W.G., Zhang, R.Z., and Ni, Y. (2016) Carbonyl group-dependent high-throughput screening and enzymatic characterization of diaromatic ketone reductase. *Cat Sci Technol* 6: 6320–6327.

Zhou, Y., Li, G., Dong, J., Xing, X.H., Dai, J., and Zhang, C. (2018) MiYA, an efficient machine-learning workflow in conjunction with the YeastFab assembly strategy for combinatorial optimization of heterologous metabolic pathways in Saccharomyces cerevisiae. *Metab Eng* 47: 294–302.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Fig. S1. Vanillin-based detection of acetone, 2-butanone, 2-pentanone, and acetaldehyde, during absorbance measurement at 430 nm, Milli-Q water was used for sample preparation. The average of three independent experiments is shown along the individual measurements. Bars represent standard deviations.

Fig. S2. Acetone-based detection of vanillin. The average of three independent experiments is shown along the individual measurement. Bars represent standard deviation. (Top) Calibration plot in the linear range. (Bottom) Acetone specificity comparison for different aldehydes as a validation for detection conditions.

Fig. S3. Spectral scanning done to determine most suitable detection wavelengths for all presented compounds in MilliQ. The average over three technical triplicates are shown, with standard deviations shown as shaded grey areas. The dotted line indicates the selected optimum wavelength for each compound, as stated in Table 2.

Fig. S4. 2,4-DNPH-based detection of acetone in the presence of 2-pentanone, absorbance measurement at 490 nm. The average of three independent experiments is shown along the individual measurement. Bars represent standard deviations.

Table S1. Oligonucleotides used in this work.
Table S2. Gene fragments used in this work.
Table S3. Composition of the M9 minimal medium.