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Achieving a high-density oleaginous yeast culture: Comparison of four processing strategies using *Metschnikowia pulcherrima*

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Abstract

Microbial lipids have the potential to displace terrestrial oils for fuel, value chemical and food production, curbing the growth in tropical oil plantations and helping to reduce deforestation. However, commercialisation remains elusive partly due to the lack of suitably robust organisms and their low lipid productivity. Extremely high cell densities in oleaginous cultures are needed to increase reaction rates, reduce reactor volume and facilitate downstream processing. In this investigation the oleaginous yeast *Metschnikowia pulcherrima*, a known antimicrobial producer, was cultured using four different processing strategies to achieve high cell densities and gain suitable lipid productivity. In batch mode, the yeast demonstrated lipid contents > 40 % (w/w) under high osmotic pressure. In fed-batch mode, however, high lipid titers were prevented through inhibition above 70.0 g L^{-1} yeast biomass. Highly promising were semi-continuous and continuous mode with cell recycle where cell densities of up to 122.6 g L^{-1} and maximum lipid production rates of 0.37 g L^{-1} h^{-1} (daily average), a nearly 2-fold increase from the batch were achieved. The findings demonstrate the importance of considering multiple fermentation modes to achieve high-density oleaginous cultures generally and indicate the limitations of processing these organisms under the extreme conditions necessary for economic lipid production.

Keywords: high cell density, oleaginous yeast, microbial lipids, fed-batch, semi-continuous, continuous

Introduction

Oils are one of the major commodities in human society, be it for fuels, food or personal care. Increased global consumption however, especially of palm oil, has led to vast deforestation and resulting global carbon emissions. To meet the growing demand for terrestrial oils, oleaginous microorganisms offer a promising alternative. These microbes are generally defined as producing over 20 % (w/w) lipids (Thorpe and Ratledge, 1972), though the cost associated with the bulk production of those single cell oils (SCOs) are still too high for commercial viability (Koutinas et al., 2014; Parsons et al., 2018).

Metschnikowia pulcherrima is a relatively under explored oleaginous yeast (Abeln et al., 2019; Canonico et al., 2016; Santomauro et al., 2014), however, the yeast is an ideal organism for industrial biotechnology due to:

- elevated inhibitor tolerance: specifically, formic acid, acetic acid, furfural, hydroxymethylfurfural (HMF) in concentrations up to 10 g L^{-1} (Fan et al., 2018; Sitepu et al., 2014);

- a wide substrate spectrum: range of C_5 and C_6 monosaccharides and oligosaccharides (Abeln et al., 2019; Fan et al., 2018; Santomauro et al., 2014; Sitepu et al., 2014);

- production of an array of secondary metabolites: most prominently 2-phenylethanol (2-PE), a valuable fragrance compound (Abeln et al., 2019; Chantasuban et al., 2018);

- its antimicrobial activity: secretion of antimicrobial agents such as 2-PE and iron sequestration through pulcherrimin production (Gore-Lloyd et al., 2019; Krause et al., 2018; Oro et al., 2014; Sipiczki, 2006; Türkel et al., 2014);

- being non-pathogenic: it is commonly used in wine fermentations as often found on grapes (Barbosa et al., 2018; Clemente-Jimenez et al., 2004; Oro et al., 2014).

These characteristics combined mean it can grow under non-aseptic conditions on a wide range of different substrates including inhibitor-rich lignocellulosic hydrolysates and waste streams (Abeln et al., 2019; Fan et al., 2018), making it an outstanding candidate for low-cost lipid production

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(Santomauro et al., 2014). Recently, promising improvements have also been made to enhance lipid production, whilst achieving beyond 40 % (w/w) lipids similar in composition to palm oil. However, the key issue with *M. pulcherrima* is its relatively slow growth cycle (Abeln et al., 2019; Chantasuban et al., 2018; Li et al., 2007; Pan et al., 1986; Santomauro et al., 2014; Zhang et al., 2011; Zhao et al., 2011): partly contingent upon a relatively low cultivation temperature promoting lipid synthesis (Santomauro et al., 2014), lipid productivities of up to only 0.05 g L^{-1} h^{-1} are reported in batch fermentation (Abeln et al., 2019). In order to yet facilitate economically feasible cultivation, the space-time yield requires improvement (Koutinas et al., 2014; Parsons et al., 2018). One promising approach could be the cultivation at high cell densities, which is proposed to facilitate higher rates of reaction, assuredly reduces reactor volume and facilitates downstream processing (Bunch, 1994).

So far, mostly fed-batch approaches have been pursued to achieve high cell densities of oleaginous yeasts. Via this operation mode, a cell density of 185.0 g L^{-1} (40.0 % w/w lipids) has been achieved with *Rhodutorula glutinis* under oxygen enriched air (Pan et al., 1986), 127.5 g L^{-1} (61.8 % w/w) with *Rhodosporidium toruloides* (Zhao et al., 2011) or 104.1 g L^{-1} (82.7 % w/w) with *Cutaneotrichosporon oleaginosus* (formerly *Cryptococcus curvatus*) (Zhang et al., 2011), all of which were cultured on glucose. But also other substrates have been utilised: *C. oleaginosus* has been cultured to high cell densities on glycerol (118.0 g L^{-1}, 25 % w/w) in fed-batch (Meesters et al., 1996), and also on whey permeate in continuous mode with cell recycle (91.4 g L^{-1}, 33 % w/w) (Ykema et al., 1988). An engineered strain of *Yarrowia lipolytica* has recently been cultured on acetate at 194 g L^{-1} (59.3 % w/w) in semi-continuous mode (Xu et al., 2017a).

Culturing at high cell densities inevitably requires fermentable substrate with high carbon concentrations. To that end, concentrated feed solutions such as crude glycerol (Papanikolaou and Aggelis, 2002), molasses (Johnson et al., 1995) or concentrated hydrolysates (Davis et al., 2013) are readily available. However, in batch mode their use is challenged by concomitant high osmotic pressure. *M. pulcherrima* has been previously described as osmophilic yeast (Rousseau and Donèche, 2001), but also other operational strategies as opposed to batch can be considered to achieve high cell

densities. For fed-batch, semi-continuous and continuous fermentation, *M. pulcherrima* is a promising candidate, as its antimicrobial activity is particularly valuable where multiple inlets and outlets are involved. In this study, we compared four different operational strategies to achieve a high-density oleaginous culture, revealing the unique physiology of this unusual yeast (Lachance, 2016).

Materials and methods

Chemicals were purchased from Sigma-Aldrich and Fisher Scientific and used without further purification. Centrifugations took place at 4667 × g and room temperature for 10 min (Rotina 380, Hettich). All cultivations took place at 20 °C (Abeln et al., 2019; Santomauro et al., 2014). Erlenmeyer flasks were filled at 20 % (v/v) working volume and placed on orbital shakers (Unimax 2010, Heidolph) at 180 rpm. Rates were calculated as average between two sample points (typically daily). The lipid flux was expressed as lipid production rate (r_P) over lipid-free biomass production rate (r_{X*}).

Organism, media, preculture

The utilised *M. pulcherrima* strain was evolved from the strain NCYC 2580 (National Collection of Yeast Cultures, Norfolk, UK) towards higher inhibitor tolerance (NCYC 4331[*]). It was kept at 20 °C on malt extract agar plates (MEA: agar 15 g L^{-1}; malt extract 30 g L^{-1}; mycological peptone 5 g L^{-1}) and sub-cultured every fortnight. A single colony was used to inoculate soy-malt broth (SMB: soy peptone 30 g L^{-1}; malt extract 25 g L^{-1}; pH 5) in 250 mL Erlenmeyer flasks, incubated for 24 h to an OD_{600} of around 24 (preculture). Basal medium for main cultures, inoculated with 2.5 % (v/v) preculture, was nitrogen-limited broth (NLB: KH_2PO_4 7 g L^{-1}; $(NH_4)_2SO_4$ 2 g L^{-1}; $NaHPO_4$ 1 g L^{-1}; $MgSO_4$ 7·H_2O 1.5 g L^{-1}; yeast extract 1 g L^{-1}; glucose 4 % (w/v); pH 5). NLB was modified in eight different ways as the media were scaled up to 48 % (w/v) glucose in steps of

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[*] This strain was evolved through adaptive laboratory evolution using NLB supplemented with 0.7 g L^{-1} furfural and acetic acid and 0.35 g L^{-1} formic acid and HMF. Cultures were transferred every 48 h for a total time of approximately 1000 h. Patent pending (reference number P124919GB).

4 % (w/v) (Table 1). Medium nomenclature includes its category (A, B, C, D, E, F, G & H) and glucose concentration (in % w/v), e.g. A4. Carbon-nitrogen (C/N) ratios were computed assuming 11 % (w/w) nitrogen and 12 % (w/w) carbon in yeast extract. The water activity (a_W) was calculated as outlined in the supplementary materials and methods (sec. S1). Media were prepared in deionised water on the day and not autoclaved or filter sterilised. For media containing ethanol, the evaporation rate was considered when determining the amount of assimilated ethanol (sec. S2).

Shake flask cultivations

Shake flask (SF) cultivations were performed batch-wise in 100 mL Erlenmeyer flasks for 5 days on media A-D with 4 to 40 % (w/v) glucose (Table 1). The yeast was also cultured on NLB with 40 g L^{-1} ethanol, glycerol, arabitol or arabitol/glucose (50:50 w/w), instead of glucose; as well as on NLB with spikes of ethanol, glycerol, arabitol, 2-PE or $(NH_4)_2SO_4$ on Day 1 (sec. S3).

Stirred tank reactor cultivations

Stirred tank reactor (STR) cultivations (Table 2) were performed in a 2 L FerMac 320 STR (Electrolab) equipped with off-gas analyser (sec. S4). With a working volume of 1 L (batch, fed-batch initial) or 2 L (semi-continuous, continuous), fermentation broths were maintained at pH 4 (2 M NaOH, 0.5 M HNO₃) and dissolved oxygen (DO) at 50 % (0.5 vvm aeration, 150-900 rpm agitation), unless indicated otherwise. Initially, 0.1 % (v/v) antifoam PPG 2,000 was supplied. Daily, aliquots of 5 mL were taken from the fermentation broth, and deionised water added to maintain a constant volume, with concentrations in the broth rectified accordingly.

In batch operation mode, media C4, C8, C40, E40 or F40 (Table 1) were fermented until glucose depletion. All fed fermentations were started as batch with medium C8. In fed-batch mode, medium E48 was fed (a) periodically after glucose depletion to reinstate 8 % (w/v) glucose (batch-wise feed), or (b) after 1 day at a rate of 0.97 g L^{-1} h^{-1} glucose (continuous feed). In semi-continuous mode, 25 % (v/v) of the fermentation broth was removed upon glucose depletion, centrifuged, the pellet resuspended in an equal amount of media E32, F32, G32 or H32, and the suspension fed back to the STR. With medium F32, additional experiments with the following modifications were

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performed: (a) oxygen supply at DO 80 % (0.9 vvm) throughout the cultivation, (b), acetate, furfural (2.8 g L^{-1}), formate and HMF (1.4 g L^{-1}) added to F32, (c) ethanol replaced glucose in F32, 6.25 % (v/v) broth exchange (instead of 25 % v/v). In a complementary experiment, 100 % (v/v) of the broth was removed, centrifuged, the cells resuspended in medium C8 and fed back to the STR. In experiments with F32, and the corresponding initial batch with C8, a different yeast extract was used compared to all other experiments (Table 2). In continuous mode, medium E32 was continuously fed to the bioreactor from Day 3 at 0.97 g L^{-1} h^{-1} glucose (0.067 d^{-1} dilution rate D). The broth was continuously removed at a rate of 4 × D, settled in a separation funnel and the bottom phase continuously fed back to the STR at 3 × D. The top phase was removed daily, centrifuged, the pellet resuspended in 5 mL supernatant and fed back to the funnel.

Analytical methods

Optical density OD$_{600}$ of the fermentation broth was assessed at 600 nm (Spectronic 200, Thermo Fisher Scientific), after appropriate dilution (Abeln et al., 2019). For determination of the DCW, the sample was centrifuged, the supernatant set aside for other analysis, the pellet resuspended in water and centrifugation repeated. For glucose concentrations ≥ 200 g L^{-1}, the middle fraction of the first centrifugate, accounting for 50 % (v/v) of the former, was removed before performing the second centrifugation. This step was necessary as due the high density of high sugar solutions (e.g. 1.17 g/cm^3 for C40 at 20 °C), a fraction of the cells was floating on the supernatant surface. The pellet was frozen (-80 °C) and lyophilised at -40 °C and 60 mbar (Modulyo, Thermo Savant) for 10 h and its weight gravimetrically assessed (B154, Mettler Toledo). Lipids were extracted from the dried yeast with chloroform/methanol (1:1, v/v) after cell disruption with 6 M HCl at 80 °C (Abeln et al., 2019). The extracted lipids were transesterified in methanol containing 1 % (v/v) sulfuric acid, and the relative proportions of fatty acid methyl esters determined through gas chromatography/mass spectrometry (GC-MS) with a 25 m x 0.25 mm CP-Sil (Agilent) capillary column (Abeln et al., 2019). Carbon contents of biomass and lipids were externally analysed through carbon, nitrogen and hydrogen (CHN) elemental analysis (Science Centre, London Metropolitan University, UK).

Molecular formulae of compounds in the filtered (0.22 µm) fermentation supernatant were ascertained with mass spectrometry (MS), either in loop injection or coupled with liquid chromatography (LC-MS). For the latter, a 2.1 x 50 mm ZORBAX Eclipse Plus (Agilent) C18 column was used, held at 50 °C and run at 0.3 mL min^{-1} with 50 % each methanol and water, both supplemented with 0.1 % formic acid. The compounds were detected through UV detection at 254 nm (1260 Infinity II LC System, Agilent). Compounds were ionised through Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) in negative mode and detected in the standard (3200 m/z) extended dynamic range (2 GHz) mode (6545 Q-TOF, Agilent). Confirmation of molecular formula was at an isotope match score > 60 % and -5 ppm < mass error < +5 ppm. Quantities relative to the highest observed concentration across multiple experiments were estimated through the compound peak height in the extracted ion chromatogram (EIC) (semi-quantitatively). Identity of glucose, glycerol, arabitol, ethanol and 2-PE were validated through high-performance liquid chromatography (HPLC) using a 300 x 7.8 mm Rezex™ RHM-Monosaccharide H+ (Phenomenex) ion exclusion column or 3 x 50 mm Poroshell 120 EC-C18 (Agilent) reversed-phase column with corresponding standard solutions, respectively, and their concentrations determined through 5-point calibration (Abeln et al., 2019). Total organic carbon (TOC) and total nitrogen (TN) in the fermentation broth were determined with an automated TOC-L analyser (Shimadzu) (Abeln et al., 2019). The ammonia nitrogen concentration of filtered (0.22 µm) supernatant sample was assessed with an ammonium cuvette test kit (LCK303, Hach) and the indophenol blue reaction photometrically evaluated in a DR 2800 Spectrophotometer (Hach).

Replication and statistical analysis

A systematic error was determined for SF and STR batch fermentations (triplicates), with further fermentations performed singularly. The strength and significance of a correlation were determined through Pearson's or Spearman's rank correlation coefficient and comparing the p-value to the significance level of $\alpha = 0.01$, respectively, all computed in Microsoft Excel.

Results & discussion

Batch with high initial substrate loading

Ideally, high cell densities are acquirable through applying high initial substrate concentrations, but the induced osmotic pressure typically inhibits cell growth. To investigate the osmotolerance of *M. pulcherrima*, its fermentation performance in four differently composed synthetic media A-D with a glucose concentration from 4 to 40 % (w/v) (Table 1) was assessed. These media were composed to establish the influence of pH, carbon-nitrogen (C/N) ratio and increased nutrition and salts on *M. pulcherrima* growth and lipid production under high osmotic pressure.

Yeast biomass peaked at a glucose concentration of 8 % (w/v) for all media except D, with the highest nutrient supplementation, peaking at 12 % (w/v). The corresponding DCW and lipid concentration were 27.2 g L^{-1} and 11.1 g L^{-1}, respectively (Fig. 1a+b). The lipid content substantially decreased with an initial glucose concentration $\geq$ 20 % (w/v) (Fig. 1b). Lipid droplets visibly filling the entire cell were obtained at 8 % (w/v) initial glucose constituting a lipid content of 41.6 % (w/w) (fig. S8). The lower lipid content with media D20 to D40 compared to other media (Fig. 1b) is presumably caused by the increased salt stress, and respectively lower oxygen concentration. Only a minor effect on the lipid content was induced by the C/N ratio (Fig. 1b, Table 1). Interestingly however, a higher C/N ratio, as with media C compared to A, did lead to a higher DCW (Fig. 1a), and therefore lipid concentration and yield (fig. S2). While it is possible that ammonia is inhibitory to cell growth, in spike experiments, where up to 10 g L^{-1} (NH$_4$)$_2$SO$_4$ was added after the first day of fermentation, no reduction in growth was notable (fig. S7). Biomass and lipid yields, peaking at 0.38 and 0.14 g g^{-1} (at 4 % w/v glucose loading), respectively, generally decreased with increasing initial glucose concentration (fig. S2). The only deviation is imposed by D4, where the low nutrient availability distinctly impaired growth. The lipid yields are competitive against other oleaginous yeasts, with *R. toruloides* reaching 0.12 g g^{-1} (Li et al., 2007) and *C. oleaginosus* 0.15 g g^{-1} (Zhang et al., 2011) in similar experiments, both at 6 % (w/v) glucose.

With both yeasts, *R. toruloides* and *C. oleaginosus*, similar patterns of biomass concentration and lipid content over glucose concentration have been observed, and highest lipid concentrations of 8.6 g L^{-1} (Li et al., 2007) and 8.9 g L^{-1} (Zhang et al., 2011) were achieved at 9 % (w/v) and 6 % (w/v) glucose, respectively. *M. pulcherrima* does not only exceed these limits, but concurrently at a higher glucose concentration. Thus, a higher osmotolerance is likely, presumably developed through its natural habitat on mature fruits. This argument is substantiated by *M. pulcherrima* growing to 10.1 g L^{-1} DCW even at 40 % (w/v) glucose (Fig. 1a), corresponding to a water activity (a_w) of 0.92 – a concentration at which *C. oleaginosus* is severely inhibited (producing only 1.0 g L^{-1}) (Zhang et al., 2011). More pronounced, STR fermentations at 40 % (w/v) initial glucose show that the yeast achieves far higher biomass yields on such concentrated solutions if sufficiently aerated (DO 50 %) (Fig. 2). Although overall biomass (0.17 g g^{-1}) and lipid yield (0.07 g g^{-1}) were significantly impaired from the high osmotic pressure, a considerable lipid content of 41.2 % (w/w) was recorded, with a similar fatty acid profile to palm oil (sec. S12). When supplying more macro- and micronutrients in the form of yeast extract, lipid production was not markedly compromised, but importantly, lipid productivity was improved up to 1.7-fold (0.13 g L^{-1} h^{-1}) with maximum lipid production rates of 0.27 g L^{-1} h^{-1} (daily average) (Table 2). Concurrently, the degree of fatty acid saturation decreased.

M. pulcherrima was termed osmophilic previously, when researchers demonstrated growth at an a_w of 0.88, better than six other yeast isolated from grapes, including the oleaginous yeast *R. glutinis* with an a_w threshold of 0.94 (Rousseau and Donèche, 2001); and even survived at 70 % (w/v) glucose loading ($a_w < 0.82$) (Mukherjee et al., 2017). High osmotolerance is known for *Candida* species (Rousseau and Donèche, 2001; Sipiczki, 2003; Zhuge et al., 2001) in close phylogenetic proximity to *M. pulcherrima* (Masneuf-Pomarede et al., 2016). Osmotic pressure typically leads to smaller cells (Maiorella et al., 1983), which was also the case with *M. pulcherrima* with increasing glucose concentration (fig. S9). Additionally, the pellet attained an increasingly red colour, which is presumably pulcherrimin, a red siderophore pigment produced by *M. pulcherrima* (Kántor et al., 2015; Roberts, 1946).

The yeast metabolised glucose at a rate exceeding 1.6 g L^{-1} h^{-1} (5-day average) (Fig. 1c). Highly buffered media (B & D) generally resulted in less acidic conditions favouring glucose uptake (Fig. 1c+d). Glucose consumption peaked at 20 % (w/v) initial glucose and decreased beyond this threshold. Rather than into biomass and lipids, the carbon was channelled into metabolite production indicating the yeast's energy requirement to maintain homeostatic and redox balance. Indeed, with increased osmotic pressure or oxygen limitation through the increased solutes, *M. pulcherrima* synthesised increasing amounts of alcohols (2-PE, ethanol), fatty acid ethyl esters (e.g. ethyl acetate, ethyl octanoate) and polyols (arabitol, erythritol, glycerol, mannitol). Further metabolites were identified, several of those not previously reported for *M. pulcherrima* in single culture (table S1). The full results and a thorough discussion regarding secondary metabolite production of *M. pulcherrima* are presented in the Supplementary results and discussion (sec. S5). Moreover, investigations on the toxicity and assimilation of selected *M. pulcherrima* metabolites (2-PE, arabitol, ethanol, glycerol) as well as media compounds (NH$_4$) are presented (sec. S6). In short, ethanol was produced up to 8.7 % (v/v) (0.40 g g^{-1} glucose) in SF fermentations (fig. S1), and *M. pulcherrima* shown to be inhibited by ethanol concentrations ≥ 5 % (v/v) and survive ≥ 15 % (v/v) (fig. S6). When providing oxygen in excess in STRs, ethanol production could be avoided, but glycerol and arabitol were produced up to 26.3 and 12.7 g L^{-1}, respectively (Table 2), both linked to osmotic pressure in other yeasts (Blomberg and Adler, 1992; Van Eck et al., 1989; Kayingo and Wong, 2005). However, through overflow mechanism ethanol may still be observed under high oxygenation (Table 2).

Overall, the high osmotolerance is beneficial to microbial competition in concentrated aqueous solutions, specifically against bacteria, of which most have higher a$_W$ growth limits. More importantly, it underlines the yeast's suitability for high density cultivations, in which highly concentrated culture regimes may be desired or required dependent on the cultivation mode and the substrate. However, it has been shown that, despite *M. pulcherrima*'s osmotolerance, biomass and lipids yields are low in batch operation mode. The yeast growth limitation at high substrate concentrations was not individually caused by the osmotic pressure, but also through oxygen transfer

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limitations and subsequently, product inhibition from ethanol. Thus, to achieve high cell densities, fed-batch, semi-continuous and continuous operation mode, both with biomass recycle, were approached in sufficiently aerated STRs (DO 50 %). The yeast was initially cultured batch-wise at 8 % (w/v) glucose, as simultaneously delivering high lipid content, concentration and yield (Table 2) as well as carbon assimilation (fig. S4). It should be noted at this stage, that lot-to-lot variation of yeast extract can have a large influence on *M. pulcherrima* growth performance, as observed in STR batch fermentations at 8 % (w/v) glucose (Table 2). Such variation is common when using complex nutrients as emphasised in other studies (Diederichs et al., 2014; Zhang et al., 2003). The feeding regime was set so that a_W would not fall below 0.98, including the produced metabolites. The increasing number of in- and outlets imposes an additional sterility risk, which has resulted in limited development of these operation modes to date. However, *M. pulcherrima* has a range of mechanisms to limit the impact of contamination, foremost the production of pulcherrimin (Gore-Lloyd et al., 2019; Oro et al., 2014; Sipiczki, 2006; Türkel et al., 2014) and volatile organic compounds such as ethyl acetate and 2-PE (Oro et al., 2018), which makes it a promising organism for these processing methodologies.

Fed-batch with concentrated feed

To achieve high cell densities in oleaginous yeast cultures, the fed-batch approach is commonly chosen as the mode of operation (Li et al., 2007; Pan et al., 1986; Ykema et al., 1988; Zhang et al., 2011; Zhang et al., 2016; Zhao et al., 2011). In this mode, the performance of *M. pulcherrima* was investigated using a batch-wise as well as continuous feeding strategy. A glucose-rich medium (48 % w/v) was added to obtain a total of 32 % (w/v) glucose fed based upon the final volume.

In fed-batch cultivation with batch-wise feed, a DCW of 70.0 g L^{-1} was achieved after 14 days (Fig. 3a). The highest lipid content was obtained on Day 10 at a DCW of 65.0 g L^{-1} (43.2 % w/w) – a lipid yield of 0.14 g g^{-1} and productivity of 0.12 g L^{-1} h^{-1}. Maximum lipid production rate (daily average) during the feeding stage was 0.22 g L^{-1} h^{-1} – an increase of 55 % (w/w) over the batch

(Table 2). However, the decreasing rate of biomass production from Day 7 despite higher biomass concentration, and the severely limited glucose uptake after the fourth feeding (Day 15), indicate inhibition or limitation. Product inhibition from glycerol, arabitol and 2-PE can be ruled out, as neither of the polyols are inhibitory at the obtained concentrations, and 2-PE only partially so (Fig. 3b & S7). The formation of ethanol of up to 3.7 g L^{-1} within the second and third feeding cycle could indicate limited respiratory capacities at this stage, despite sufficient oxygenation (DO 50 %). It was further confirmed that the growth inhibition was neither caused through short-term carbon starvation nor signal transduction induced by low glucose levels (Kim et al., 2013): when keeping the glucose concentration > 50 g L^{-1} through continuous feeding, similar profiles were obtained (sec. S9). The sustained nutrient availability led to glucose uptake rates of up to 1.98 g $L^{-1} h^{-1}$ (daily average), a 61.1 % (w/w) increase compared to batch-wise feeding, accompanied by lower instantaneous biomass yields, but similar ethanol formation of up to 3.1 g L^{-1}. Upon feed termination, both ethanol and 2-PE were re-metabolised despite further glucose uptake, indicating ease on the respiratory system. The underlying reason for the observed phenomena is unclear, but conceivable causes include physiological limits through the high density of cells, lipid-rich cells not taking part in cell proliferation, nitrosative stress, and/or inhibition through feed compounds (Maiorella et al., 1983) or unknown by-products.

A decline in biomass and lipid productivity, and glucose consumption over time has been observed in high-density fed-batch approaches with various oleaginous yeast (Li et al., 2007; Zhang et al., 2011; Zhao et al., 2011). Additionally, a lower lipid content in high-density fed-batch cultures compared to a batch has been observed before, for instance with *R. glutinis* (Pan et al., 1986). In that case, the lipid content was 40.0 % (w/w) compared to a batch with 60.0 % (w/w), arguably caused by high salt concentrations. These examples further demonstrate that a fed-batch approach is not necessarily the best solution to achieve high cell densities in oleaginous cultures. Indeed, with the applied strategies, fed-batch cultures were shown not to be suitable for *M. pulcherrima* to achieve biomass concentrations > 100 g L^{-1}. The challenges faced in the fed-batch approach could be

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remedied through cultivating in semi-continuous or continuous mode, where part of the broth is periodically or constantly removed and refreshed.

Semi-continuous operation with biomass recycle

Apart from removing possible inhibitory compounds, in semi-continuous fermentation with cell recycle, the entire reactor volume is utilised, potentially resulting in increased total growth rates and space time yield compared to fed-batch cultivation. In the typical semi-continuous fermentations herein (DO 50 %), three feeding cycles with 25 % (v/v) broth exchange were performed feeding a total of 32 % (w/v) glucose.

Applying the same C/N ratio in feed solution as in the batch medium (60.1 g g^{-1}), the yeast grew to a cell density of 111.8 g L^{-1} containing 44.4 g L^{-1} lipids (Fig. 4a). Most prominent fatty acids were oleic (C18:1, 48.0 % w/w) and palmitic (C16:0, 38.5 % w/w) acid. Therefore, the lipids show promising similarity to palm oil, though no linoleic acid (C18:2) was detected (sec. S12). Interestingly, the yeast also incorporated minor quantities of medium chain fatty acids (C6:0 to C10:0, 6.5 % w/w) into its lipids. When compared to the batch, overall lipid productivity could be enhanced by 40 % (w/w) (Table 2). More pronounced, a repeated batch system, in which only the 1st feeding cycle takes place, is advantageous over the batch, with lipid yields increased by 54 % (w/w) to 0.19 g g^{-1} (58 % w/w of the theoretical maximum (Ratledge, 1988)), and lipid productivity by 99 % (w/w) to 0.20 g L^{-1} h^{-1}, with a maximum lipid production rate of 0.21 g L^{-1} h^{-1} (daily average) – an enhancement of 46 % (w/w) compared to the batch (Table 2). The high lipid content of 42.3 % (w/w) was promoted by the increased residence time in the reactor (Fig. 4b). It could be observed that *M. pulcherrima* cells are frequently formed as lipid-rich cells during budding (fig. S11a). The decrease of ammonia nitrogen consumption relative to glucose uptake shows that the yeast does not require the same amount of nitrogen in the consecutive stages as in the batch and led to accumulation of ammonia nitrogen in the broth (fig. S14d).

Whilst shown to be suitable for reaching high cell densities, this feeding strategy may not be suitable for continued semi-continuous operation as it initiated a non-uniform process, rather than

simultaneous growth and lipid production as desired in a flow process. This is indicated by the increasing relative ammonia nitrogen consumption (fig. S14d), decreasing yields and productivities from the 2nd feeding cycle, and the changing trend of OD_{600}/DCW ratio and lipid content after the 3rd feeding (Day 11) (Fig. 4). To explore this behaviour, an additional feeding stage (4th feeding) was added to the process (fig. S14). The feeding resulted in a stage of prominent cell proliferation, evident in the rapidly deceasing NH_4 concentration (fig. S14d), but also through visual observation (fig. S11b+c). The rapidly changing OD_{600}/DCW ratio (fig. S14b), induced by the increased number of small cells, is a strong indicator for this behaviour. These cells converted the assimilated carbon very inefficiently into biomass and lipids, with respective yields in this stage only 0.13 g g^{-1} and 0.02 g g^{-1}. Such a shift in reproduction behaviour has not been observed in the fed-batch cultivations (fig. S15), but similarly, a switch from sexual to asexual reproduction has been reported with oleaginous *Debaryomyces etchellsii* previously, when switching from batch to continuous mode (Arous et al., 2015). When removing 100 % (v/v) of the broth, recycling the yeast biomass into fresh (batch) medium, small cell formation notable through the OD_{600}/DCW ratio occurred around the same time (fig. S15). As a higher glucose throughput had occurred up to this point mediated by increased glucose consumption rates of up to 2.35 g L^{-1} h^{-1} (daily average), presumably caused by the removal of previously secreted substances, this indicates that this issue could be age related. A relation to the glycerol metabolism, under which uptake generally more lipid-poor cells are formed (fig. S11d), or the formation of pyruvate (table S1) are also conceivable. It was furthermore postulated that this could be influenced by excess of nutrients, such as NH_4 and Mg^{2+}, playing a vital role in cell proliferation (Walker and Duffus, 1980). Consequently, to restrict cell proliferation, the feeding strategy should involve feeding with a higher C/N ratio than this of the batch.

Further semi-continuous fermentations were then carried out at higher C/N ratios (99.7 to 148.7 g g^{-1}, Table 1) to avoid nitrogen accumulation. The experimental results when increasing the C/N ratio are depicted in the Supplementary results and discussion (sec. S10) and summarised in Table 2. Briefly, when scaling NH_4 (and equally yeast extract) in the feed medium to meet the

nitrogen requirements observed in the repeated batch (2.3 mg NH_4-N g^{-1} glucose, fig. S14d) (C/N 120.0 g g^{-1}), the total nitrogen concentration stayed below 0.31 g L^{-1} throughout the semi-continuous part, but formation of small cells was still observed (fig. S11b). When using a feed medium high in yeast extract (C/N 148.7 g g^{-1}), the yeast produced lipids with a maximum rate of 0.37 g L^{-1} h^{-1} (daily average) – a 97.7 % (w/w) increase over the batch (Table 2). This elevated growth rate, achieved through increased micronutrient availability, was not sustainable though, as the yeast biomass decreased upon glucose starvation (fig. S17c), unlike with previous fermentations (Fig. 4a). This was presumably through low ammonia nitrogen availability, resulting in a slightly lower overall biomass yield (0.32 g g^{-1}). At a C/N ratio of 99.7 g g^{-1}, the highest maximum lipid production rate of 0.31 g L^{-1} h^{-1} (daily average) and overall lipid productivity of 0.18 g L^{-1} h^{-1} were achieved when fermenting at high dissolved oxygen concentrations (DO 80 %, Table 2).

With semi-continuous culturing successful to achieve high cell densities, the possibility to cultivate on other substrates was approached. Through growth on glucose in batch and ethanol in semi-continuous mode, a DCW of 88.8 g L^{-1} was obtained in 14 days (Fig. 5). The yeast achieved daily lipid yields of up to 0.34 g g^{-1} (63 % w/w of theoretical maximum (Ratledge, 1988)) due to the higher degree of reduction of ethanol compared to glucose. The % with respect to the theoretical maximum is similar to what was achieved with *M. pulcherrima* on glucose (63 % w/w, Fig. 4), and in the range of the maximum yields achieved with oleaginous yeast on ethanol so far (Papanikolaou and Aggelis, 2011). On the downside, the maximum lipid production rate (0.16 g L^{-1} h^{-1}) was only 57 % (w/w) of this under similar conditions on glucose (Fig. 5 & 6). No arabitol and glycerol were produced during the cultivation on ethanol. The appearance of small cells after 13 days cultivation (fig. S11e) shows that this phenomenon is not specific to the carbon source. Moreover, *M. pulcherrima* was cultured semi-continuously with four fermentation inhibitors (formate, acetate, furfural, HMF) to investigate (a) whether the sustained evolutionary pressure would reduce the

formation of small cells[†]; and (b) possible benefits of this culturing mode when using concentrated thermally processed lignocellulosic hydrolysates. The yeast was cultured to a DCW of 115.5 g L^{-1} in 13 days and achieved a maximum lipid production rate of 0.28 g L^{-1} h^{-1} (daily average) (Fig. 6). Importantly, the inhibitory effect was diminished through the course of semi-continuous culturing, presumably through the conversion of fermentation inhibitors facilitated by higher cell densities and cell memory. However, the formation of small cells remained (fig. S11f).

Continuous operation with biomass recycle

A continuous process has similar advantages to the previously discussed semi-continuous fermentation, however additionally continuous up- and downstream processing are required. In this mode, the yeast was cultured at a dilution rate of 0.067 d^{-1} with the biomass recycled, until a total of 32 % (w/v) glucose was fed.

Credentials of this fermentation were a DCW of 116.4 g L^{-1} with a maximum lipid production rate of 0.20 g L^{-1} h^{-1} (daily average) and productivity increase by 25 % (w/w) over the batch (Fig. 7, Table 2). The lipid content remained stable around 37 % (w/w), but the saturation of lipids generally decreased over fermentation time. Similar to semi-continuous fermentation, small cell formation was detected around Day 12 (fig. S15). Despite continuously removed, arabitol and glycerol concentrations increased up to 10.9 g L^{-1} largely due to the increased number of cells, as polyol production with respect to glucose uptake and DCW was stable (Fig. 7, sec. S11). With on average 8.3 % (w/w) of the glucose carbon channelled into both polyols in continuous operation, this manifests that, to increase lipid yields with *M. pulcherrima*, polyol metabolism needs to be further investigated and methodologies for its manipulation elaborated. A similar approach has already been followed to increase bioethanol production with *Saccharomyces cerevisiae* (Nissen et al., 2000; Tamás et al., 2003). Moreover, an attempted carbon balance indicates that there may be more bulk chemicals present in the fermentation broth, yet to be identified (sec. S11 & table S1).

[†] It was considered a possibility that the evolved strain was unstable and would (partly) revert to its progenitor (NCYC 2580), as the small cells have a similar morphology.

Comparison of the different operating modes

With the strategies employed in this study, cell densities > 100 g L^{-1} could be achieved in semi-continuous and continuous fermentation mode but neither in batch nor fed-batch (Table 2). *M. pulcherrima* achieved highest lipid production rates of up to 0.37 g L^{-1} h^{-1} (daily average) in flow. In all modes, lipid productivities can be superior over the (initial) batch process at 8 % w/v glucose, not yet considering downtime between batches. Whilst in a batch process with high initial glucose loading (40 % w/v) maximum lipid production rates and productivities were similar to those achieved in semi-continuous fermentation, the biomass and lipid yields were considerably impaired due to significant metabolite production and increased cell maintenance. Among the fed processes, concentrations of secondary metabolites arabitol and glycerol were the highest in continuous operation (Table 2), although in this mode the broth had the highest water activity (0.99 vs 0.98).

The distinct variability of the OD$_{600}$/DCW ratio (2.0 to 7.0 L g^{-1}) reveals that a fixed conversion factor between DCW and OD$_{600}$, as often applied in oleaginous yeast research (Back et al., 2016; Capus et al., 2016) is not a given for *M. pulcherrima* due to the pronounced cell size variation further osmotic pressure (fig. S9), but also metabolite production, lipid accumulation and reproduction behaviour (fig. S11). It is therefore emphasised that establishment of such a factor for oleaginous yeasts requires testing under multiple conditions and timescales. The OD$_{600}$/DCW ratio was shown to be useful in the determination of cell size variation (Fig. 4b). Moreover, its significant strong negative correlation with the cells' lipid content demonstrates it potential as indicator for lipid production in *M. pulcherrima* (Fig. 8a). Whilst a mid-OD$_{600}$/DCW ratio of 5 ± 0.5 L g^{-1} is associated with a wide possible lipid content (10.0 to 40.2 % w/w), for a low ratio of 2.5 ± 0.5 L g^{-1}, there is a 95 % probability that the lipid content is 40.6 ± 0.5 % (w/w) (n = 80) (Fig. 8b).

Compared to other oleaginous yeasts grown to high cell densities, *M. pulcherrima* achieves similar biomass yields, but lipid yields of up to 0.25 g g^{-1} with *C. oleaginosus* (Zhang et al., 2011) and productivities of up to 1.2 g L^{-1} h^{-1} with engineered *Y. lipolytica* have been reported in fed-batch cultivation on glucose (Table 3). Whilst the utilisation of (spent) yeast biomass towards animal feed

should be investigated for this species, particularly the kinetics require further improvement to enable eventual commercial production (Koutinas et al., 2014; Parsons et al., 2018). It is evident that, to fill the productivity gap to these organisms, further evolution/genetic modifications and improved oxygen transfer are likely required (Table 3). This should involve enablement of lipid production at higher temperatures, with the productivities herein achieved at a temperature comparably low for oleaginous yeasts (typically 28 to 30 °C). Furthermore, a beneficial effect of complex media compounds such as yeast extract, as often supplied in large quantities in reported fed-batch fermentations (Table 3), on *M. pulcherrima* lipid production kinetics needs to be ascertained.

Conclusions

Among batch (72.0 g L^{-1}), fed-batch (70.0 g L^{-1}), semi-continuous (115.5 g L^{-1}) and continuous mode (116.4 g L^{-1}), the flow processes were the most suitable to achieve high cell densities. *M. pulcherrima* can grow to a DCW > 120 g L^{-1} in semi-continuous cultivation with biomass recycle, with maximum lipid production rates improved up to nearly 2-fold compared to the batch – making this strategy a valuable tool to enhance productivities. Both in batch and semi-continuous mode, an oil with similar fatty acid composition to palm oil can be produced. However, changes in *M. pulcherrima* reproductive cell morphology were observed reducing high lipid production rates over prolonged periods of time. Further study is required to elucidate and bypass the underlying mechanisms of small cell formation through process development, further evolution and/or genetic engineering. Overall, the promising results herein call for more research in flow culture processes with cell retention, despite the additional technological means.

Further to the scope of this study, it was established that *M. pulcherrima* secretes arabitol, glycerol and ethanol in considerable amounts. In further study it is suggested to elucidate the mechanisms of *M. pulcherrima* biosynthesis and extracellular release of secondary metabolites arabitol and glycerol, and assess the potential of disrupting the involved genes and/or regulate their expression to increase the metabolic flux towards lipid production, such as with *S. cerevisiae* for

bioethanol production. Hereof, with *M. pulcherrima* achieving ethanol yields near those of *S. cerevisiae*, additionally responding well to stress factors such as osmotic pressure and fermentation inhibitors as present in concentrated lignocellulosic hydrolysates, and moreover considering its wide substrate spectrum and antimicrobial activity, this non-conventional yeast or its genes are worth exploring for second or third generation bioethanol production.

Acknowledgements

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Tables

Table 1. Synthetic media used in this study. Eight media were prepared based on NLB (Sec. Organism, media and preculture) with glucose concentrations scaled up to 48 % (w/v). The scaling factor f is the glucose concentration of the scaled medium c_S divided by the NLB glucose concentration $c_{S,NLB} = 4$ % (w/v). Medium B was prepared as 100 mM tartaric acid solution. Due to overlapping of media concentrations, it is C8 ≙ D8 ≙ E8 ≙ H8.

Medium	KH₂PO₄	(NH₄)₂SO₄	NaHPO₄	MgSO₄	Yeast extract	C/N ratio (g g⁻¹)		Associated cultures	
						Range	At f = 8	Vessel [†]	Operation mode [‡]
A	1	f	1	1	1	30.2-36.8	36.6	SF	B
B	1	f	1	1	1	30.2-36.8	36.6	SF	B
C	1	f/2	1	1	1	50.0-71.8	70.9	SF, STR	B
D	f/2	f/2	f/2	f/2	f/2	60.1	60.1	SF	B
E	1	f/2	1	1	f/2	60.1	60.1	STR	B, FB, SC, C
F	1	f/4	1	1	f/2	99.7	99.7	STR	SC
G	1	f/4	1	1	f/4	120.0	120.0	STR	SC
H	1	1	1	1	f/2	33.5-164.8	148.7	STR	SC

[†] SF: shake flask, STR: stirred tank reactor

[‡] B: batch, FB: fed-batch, SC: semi-continuous, C: continuous

Table 2. *M. pulcherrima* high-density cultures. Cells were cultured on synthetic glucose media (Table 1) using four different processing strategies in stirred tank reactors (Sec. Stirred tank reactor cultivation). Batch: values in brackets indicate the change compared to the batch C8. Fed-batch, semi-continuous and continuous cell recycle: values in brackets indicate the change compared to the initial batch. N/A: not applicable.

Cultivation mode and medium	Dry cell weight (g L⁻¹)	Max. lipid content (% w/w)	Lipid yield† (g g⁻¹)	Max. concentration of metabolites EtOH/ara/gly‡ (g L⁻¹)			Max. lipid production rate§ (g L⁻¹ h⁻¹)	Lipid productivity (g L⁻¹ h⁻¹)	Degree of fatty acid saturation∥ (% w/w)	Fig.	Comments/observations
Batch											
C4	17.7	35.2	0.15 (+24 %)	0.0	0.6	1.0	0.13 (-19 %)	0.09 (-16 %)	N/A	N/A	Yield increase due to lower sugar loading
C8	27.3	36.5	0.13	0.0	1.0	1.6	0.16	0.10	32.4	N/A	High repeatability: SE/mean = 6 % across depicted parameters (n = 3)
C8¶	33.5	35.1	0.15	0.0	0.6	1.9	0.24	0.16	N/A	N/A	Strong influence of yeast extract lot-to-lot variation; SE/mean = 4 % across depicted parameters (n = 3)
C40	63.9	41.2	0.07 (-46 %)	0.0	7.5	25.5	0.12 (-22 %)	0.08 (-27 %)	38.7	2	Ethanol formation avoided through controlled oxygen supply (DO 50 %)
E40	72.0	37.4	0.07 (-46 %)	13.8	7.7	22.9	0.27 (+69 %)	0.13 (+21 %)	17.9	S12	Increased kinetics due to high micronutrient supply; ethanol formation through overflow metabolism
F40	68.8	40.2	0.07 (-46 %)	12.0	12.7	20.3	0.25 (+55 %)	0.12 (+12 %)	17.0	S12	As above
Fed-batch											
E48, batch-wise feed	70.0	43.2	0.11 (-6 %)	3.7	3.1	7.5	0.22 (+55 %)	0.08 (-18 %)	26.3	3	Unexplained growth limitation possibly linked to ethanol production
E48, continuous feed	63.3	N/A	N/A	3.1	4.5	9.7	N/A	N/A	N/A	S13	As above
Semi-continuous cell recycle											
E32	111.8	42.3	0.15 (+20 %)	0.0	3.0	6.9	0.21 (+46 %)	0.14 (+40 %)	43.1	4	Formation of small cells after two weeks extensively investigated; medium chain fatty acids produced
F32, DO 80 %¶	89.2	44.1	0.12 (-18 %)	0.0	2.0	3.2	0.31 (+43 %)	0.18 (+9 %)	30.7	S16	Increased kinetics due to higher oxygenation
F32, ethanol¶	81.1	42.6	0.20 (+23 %)	N/A	0.6	2.0	0.16 (-43 %)	0.11 (-40 %)	24.0	5	Highest yield due to ethanol higher degree of reduction
F32, inhibitors¶	115.5	43.9	0.16 (-2 %)	0.0	2.1	10.5	0.28 (+10 %)	0.16 (-6 %)	27.1	6	Inhibitors not markedly affecting growth after 2nd addition
G32	80.4	N/A	N/A	0.0	8.5	5.9	N/A	N/A	N/A	S17	Lowest amount of yeast extract supplied
H32	99.6	45.1	0.12 (-9 %)	0.0	2.2	3.6	0.37 (+98 %)	0.12 (+15 %)	26.3	S17	Increased kinetics due to high micronutrient supply; but higher rate of cell death
Continuous cell recycle											
E32	116.6	38.5	0.13 (+12 %)	0.0	8.2	10.9	0.20 (+19 %)	0.12 (+25 %)	32.3	7	Degree of fatty acid saturation decreasing over time; 8.3 % (w/w) glucose carbon channelled into polyols

† With respect to consumed glucose

‡ EtOH: ethanol, ara: arabitol, gly: glycerol

§ Daily average

ˡ Percentage of total saturated fatty acids, detailed fatty acid profile in table S4

¶ Different yeast extract used, increased kinetics observed in (initial) batch

Table 3. Selected high-density cultures of oleaginous yeast grown in stirred tank reactors on glucose as carbon source. N/R: not reported.

Cultivation mode and yeast	Dry cell weight (g L^{-1})	Biomass yield (g g^{-1})	Lipid content (% w/w)	Lipid concen-tration (g L^{-1})	Lipid yield (g g^{-1})	Max. lipid production rate (g L^{-1} h^{-1})	Lipid productivity (g L^{-1} h^{-1})	Reference
Batch								
M. pulcherrima [†]	72.0	0.18	37.4	26.9	0.07	0.27	0.13	This study
Fed-batch								
C. oleaginosus [†]	104.1	0.30	82.7	86.1	0.25	0.94	0.47	(Zhang et al., 2011)
R. toruloides Y4 [†]	127.5	0.37	61.8	78.8	0.23	N/R	0.57	(Zhao et al., 2011)
R. toruloides Y4 [†]	106.5	0.35	67.5	71.9	0.23	N/R	0.54	(Li et al., 2007)
R. toruloides [‡]	118.4	0.29	75.6	89.4	0.22	N/R	0.62	(Zhang et al., 2016)
R. glutinis [§]	185.0	N/R	40.0	74.0	N/R	N/R	0.88	(Pan et al., 1986)
Y. lipolytica [‡]	148.0	0.40	66.8	98.9	0.27	N/R	1.20	(Qiao et al., 2017)
Y. lipolytica [‡]	89.3	0.31	81.4	72.7	0.25	0.97	0.61	(Xu et al., 2017b)
M. pulcherrima [†]	70.0	0.28	40.4	28.3	0.11	0.22	0.08	This study
Repeated fed-batch								
R. toruloides Y4 [†]	101.7	0.39	60.4	61.4	0.24	N/R	0.55	(Zhao et al., 2011)
Semi-continuous cell recycle								
M. pulcherrima [†]	115.5	0.36	43.9	50.6	0.16	0.28	0.16	This study
Continuous cell recycle								
M. pulcherrima [†]	116.6	0.34	38.5	44.8	0.13	0.20	0.12	This study

[†] Evolved

[‡] Engineered

[§] Oxygen enriched air
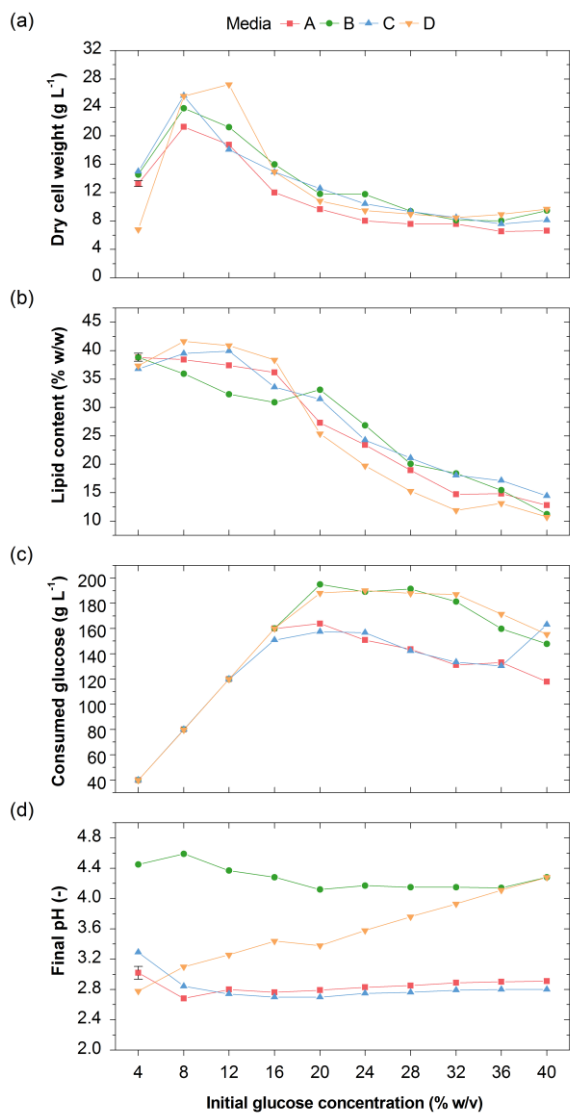
Fig. 1: Production of lipids by *M. pulcherrima* over increasing osmotic stress. Cultivation was performed batch-wise in shake flasks (oxygen limited) for 5 days on four different synthetic media A-D (Table 1) with increasing glucose concentration (n = 1). A systematic error was determined with medium A4 (n = 3, mean ± SE).
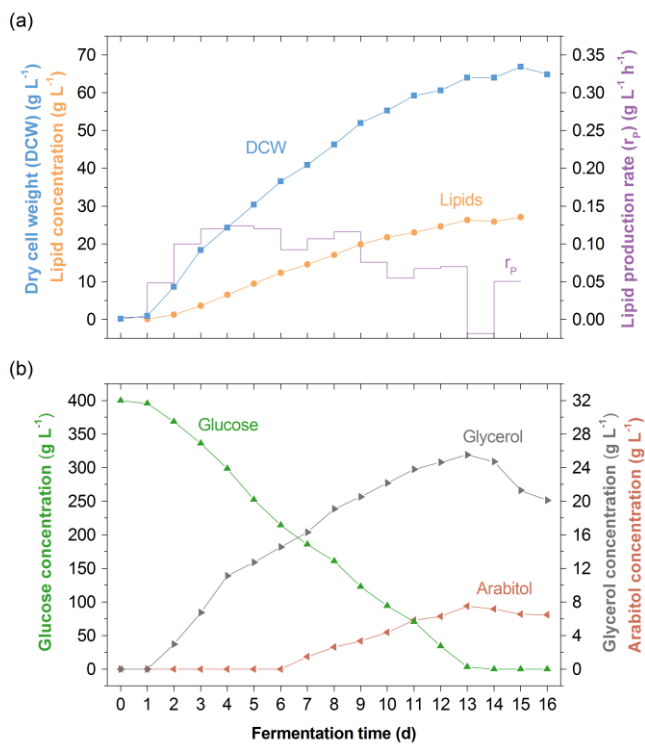
Fig. 2: *M. pulcherrima* batch cultivation on glucose-rich medium. (a) Profiles of dry cell weight, lipid concentration and daily lipid production rate, and (b) glucose, arabitol and glycerol concentration when cultured on synthetic medium (C40, Table 1) in a stirred tank reactor (DO 50 %) (n = 1).
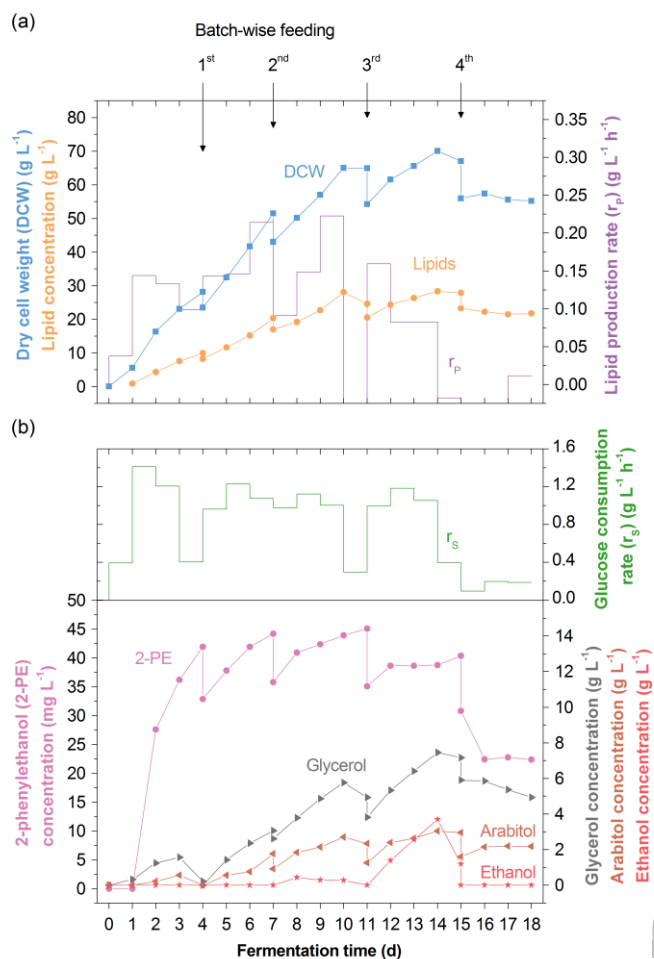
Fig. 3: *M. pulcherrima* fed-batch cultivation with batch-wise feed. Cells were initially cultured on synthetic glucose medium (C8, Table 1) and, periodically upon glucose depletion, fed batch-wise with glucose-rich medium (E48, C/N 60.1 $g\,g^{-1}$, Table 1) restoring 8 % (w/v) glucose in a stirred tank reactor (DO 50 %) (n = 1). (a) Profiles of dry cell weight, lipid concentration and daily lipid production rate, and (b) daily glucose consumption rate, 2-phenylethanol, arabitol, ethanol and glycerol concentration.
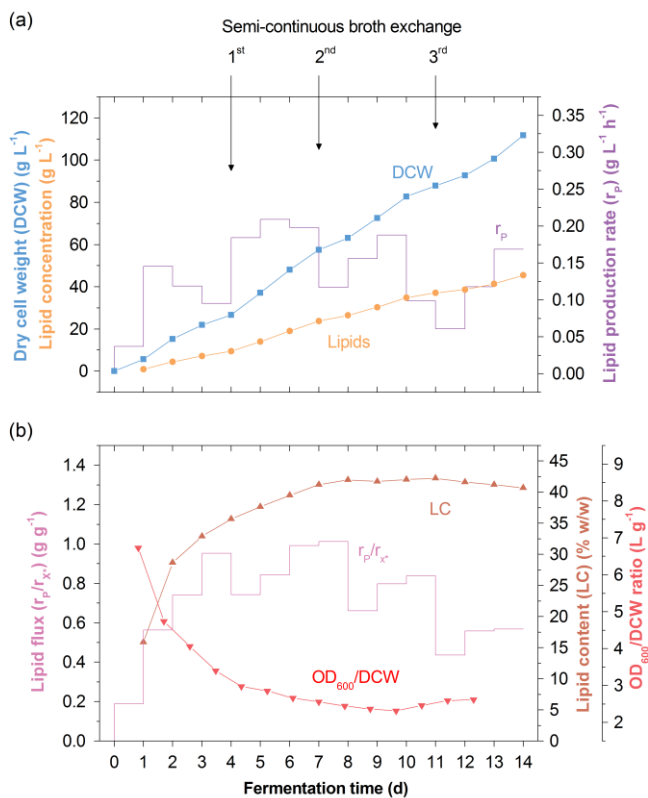
Fig. 4: *M. pulcherrima s*emi-continuous cultivation with biomass recycle. Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and, periodically upon glucose depletion (Day 4, 7 & 11), 25 % (v/v) of the broth was replaced by glucose-rich medium (E32, C/N ratio 60.1 g g^{-1}, Table 1), and the removed cells fed back to the stirred tank reactor (n = 1). (a) Profiles of dry cell weight (DCW), lipid concentration and daily lipid production rate, and (b) daily lipid flux, lipid content and OD$_{600}$/DCW ratio.
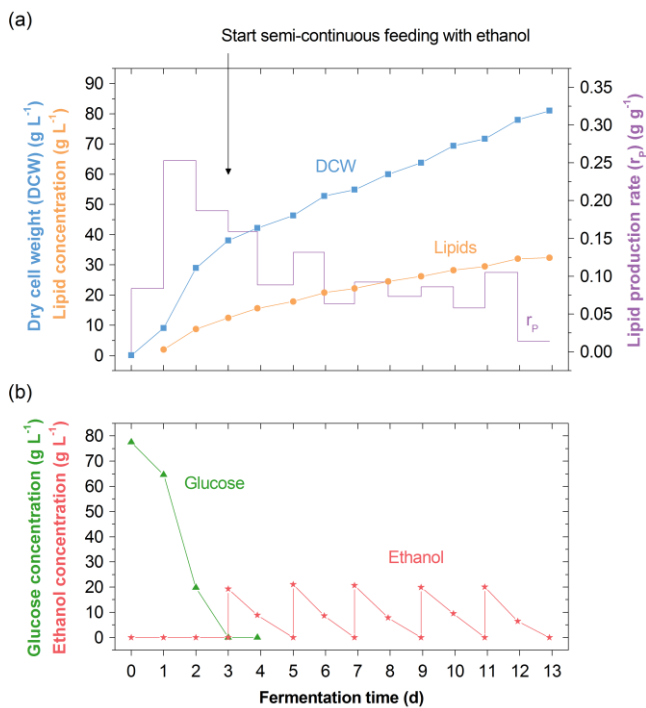
Fig. 5: *M. pulcherrima* semi-continuous cultivation with biomass recycle on ethanol. Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and, periodically upon glucose/ethanol depletion, 6.25 % (v/v) of the broth was replaced by ethanol-rich medium (F32, C/N ratio 99.7 g g^{-1}, Table 1), and the removed cells fed back to the stirred tank reactor (n = 1). (a) Profiles of dry cell weight (DCW), lipid concentration and daily lipid production rate, and (b) glucose and ethanol concentration.
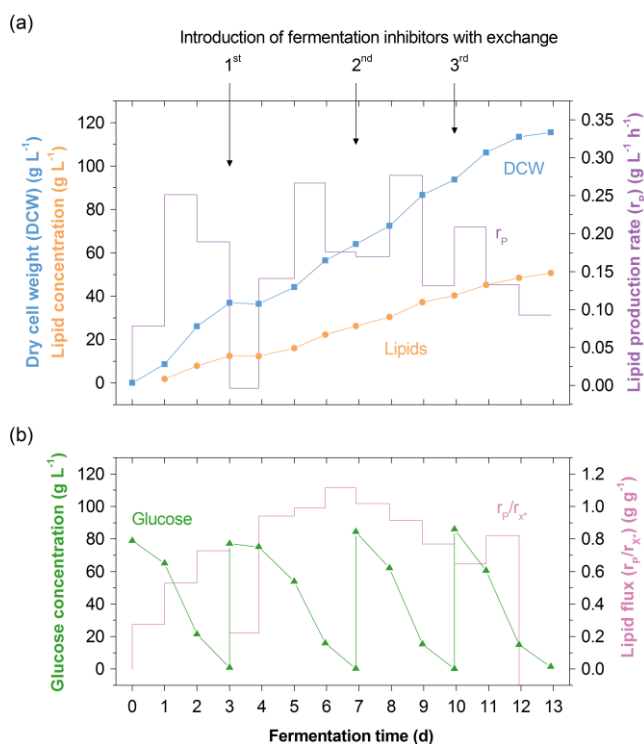
Fig. 6: *M. pulcherrima* semi-continuous cultivation with biomass recycle introducing fermentation inhibitors. Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and periodically upon glucose depletion, 25 % (v/v) of the broth was replaced by glucose-rich medium (F32, C/N ratio 99.7 g g^{-1}, Table 1) including acetate, furfural (2.8 g L^{-1}), formate and HMF (1.4 g L^{-1}), with the removed cells fed back to the stirred tank reactor (n = 1). (a) Profiles of dry cell weight, lipid concentration and daily lipid production rate, and (b) glucose concentration and daily lipid flux.
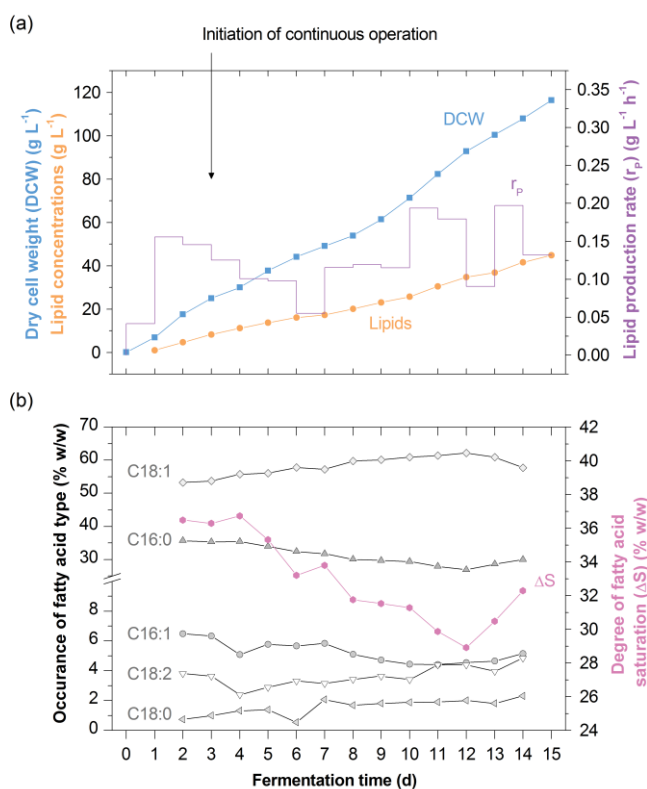
Fig. 7: *M. pulcherrima* continuous cultivation with biomass recycle. Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and on Day 3, continuous operation was initiated at a dilution rate of 0.067 d^{-1}, using glucose-rich medium (E32, C/N ratio 60.1 g g^{-1}, Table 1), the cells recycled back to the reactor (n = 1). (a) Profiles of dry cell weight, lipid concentration and daily lipid production rate, and (b) fatty acid profile of produced lipids.
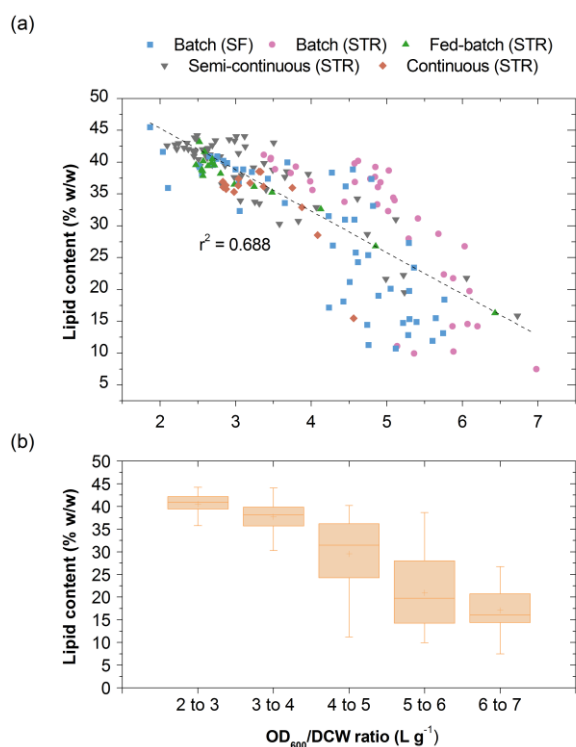
Fig. 8: The ratio of OD_{600} to dry cell weight (DCW) may be used as rough indicator for the lipid content in *M. pulcherrima*. The graph contains collated data from batch, fed-batch, semi-continuous and continuous cultivations in shake flasks (SF) and stirred tank reactors (STR) on glucose, glycerol and ethanol (Sec. Materials and methods). (a) A Spearman's rank correlation analysis found a statistically significant strong negative correlation (ρ = -0.808, n = 204, p = 4.2 × 10^{-53}) between lipid content and OD_{600}/DCW ratio. Largest deviations from a linear correlation (Pearson's r^2 = 0.688) were found with cells cultured at high glucose concentrations (> 300 g L^{-1}). (b) The box-plot of the data illustrates the suitability for lipid content estimation at low OD_{600}/DCW ratios. The plot shows 25th to 75th percentile including median, + the mean, the whiskers lower and upper values.

Appendix. Supplementary information

Supplementary information for

Achieving a high-density oleaginous yeast culture: Comparison of four processing strategies using *Metschnikowia pulcherrima*

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Supplementary materials and methods

Section S1: Calculation of water activity

The water activity $a_{W,S}$ (-) of a solution including a single solute S dissolved in water was estimated through combining equations for the osmotic potential π (Pa), derived from Van't Hoff (eq. S5.1) and Raoult-Lewis (eq. S5.2) law, yielding an equation for $a_{W,S}$ (eq. S5.3).

$$\pi = i\phi_S m_S RT\rho \qquad \text{(eq. S5.1)}$$

$$\pi = -\frac{RT}{V_W} \times \ln a_{W,S} \qquad \text{(eq. S5.2)}$$

$$a_{W,S} = \exp(-\rho v m_S \phi_S V_W) \qquad \text{(eq. S5.3)}$$

In these equations, R is the gas constant (8.3145 m³ Pa K⁻¹ mol⁻¹), T the absolute temperature (K), ρ the water density at temperature T (e.g. 998.2 kg m⁻³ at 20 °C), i the number of ions per molecule (e.g. i = 1 for non-electrolytes, i = 2 for 1:1 electrolytes), ϕ_S the osmotic coefficient (Robinson and Stokes, 1959) at molality m_S (mol kg⁻¹) and temperature T, and V_W the molar volume of water (e.g. 18.048×10⁻⁶ m³ mol⁻¹ at 20 °C). The water activity of the multicomponent solution $a_{W,T}$ was then estimated via the Ross equation (eq. S5.4).

$$a_{W,T} = \prod_S a_{W,S} \qquad \text{(eq. S5.4)}$$

In the given case, glucose, KH_2PO_4, $(NH_4)_2SO_4$, $NaHPO_4$ and $MgSO_4$ were considered as solutes contributing to $a_{W,T}$ in the synthetic medium.

Section S2: Ethanol evaporation

In cultivations where ethanol was used as carbon source, ethanol consumption was rectified according to its evaporation. For SF batch cultivations (sec. S3), the evaporation of ethanol was determined in control experiments where no cells were added, and for STR cultivations (Sec. Stirred tank reactor cultivations), the evaporation rate was determined from daily samples of an ethanol solution (initial 2.5 % v/v), aerated at 0.5 vvm, 450 rpm and 20 °C, reflecting (average) experimental conditions throughout the respective cultivations, for 20 days. In experiments were ethanol was

produced, the evaporation was not assessed, meaning that the yeast produced higher quantities than those reported.

Section S3: Supplementary shake flask spike experiments

Spike experiments in 100 mL Erlenmeyer flasks were started on NLB, and after one day either terminated (control) or spikes of arabitol, glycerol, ethanol or ammonium sulfate (2, 5, 10 g L^{-1}), 2-PE (20, 50, 100 mg L^{-1}), or ethanol (20, 40, 60, 80, 100, 120 g L^{-1}) created through addition of corresponding stock solutions (arabitol, glycerol 200 g L^{-1}, ethanol 800 g L^{-1}, $(NH_4)_2SO_4$ 80 g L^{-1}, 2-PE 2 g L^{-1}). The obtained concentrations were rectified according to the added liquid volume. Total cultivation time was 3 days, and 8 days where spikes exceeded 10 g L^{-1}.

Section S4: Off-gas analysis

The fermentation exhaust gas was dried through a drierite column and subsequently CO_2 and O_2 concentrations (% v/v) determined with the FerMac 368 gas analyser (Electrolab). Prior to analysis, the gas analyser was calibrated with certified gases (100 % N_2 and 20 % O_2, 8 % CO_2 in N_2) (BOC).

Supplementary results and discussion

Section S5: Metabolite production of *M. pulcherrima*

In this section the formation of secondary metabolites synthesised by *M. pulcherrima* when subjected to the experimental conditions used in this study (Sec. Materials and methods) is thoroughly discussed. These include the fully quantified compounds 2-phenylethanol (2-PE), arabitol, ethanol and glycerol, as well as several semi-quantitatively assessed compounds (table S1).

Table S1: Common secondary metabolites found in *M. pulcherrima* fermentation in addition to 2-phenylethanol, arabitol, ethanol and glycerol. The yeast was cultured in batch (B), fed-batch (FB), semi-continuous (SC) and continuous (C) operation modes in shake flasks (SF) and stirred tank reactors (STR) on synthetic medium with different (total) glucose loadings (Sections Shake flask cultivations and Stirred tank reactor cultivations) (n = 1). Absence (no detection) of the compound was set as 0 % (white) and the highest peak value across the experiments as 100 % (darkest green).

Chemical formula	Suspected compound	Relative concentration (across experiments)							
Fermentation mode		B	B	B	B	B	FB	SR	CR
Vessel type		SF	SF	SF	STR	STR	STR	STR	STR
Glucose loading (% w/w)		8	20	40	8	40	32	32	32
Fermentation time (d)		5	5	5	3	12	14	13	15
C_2H_4O	Acetaldehyde								
$C_3H_4O_3$	Pyruvic acid								
$C_3H_6O_2$	Ethyl formate								
$C_4H_6O_2$	Diacetyl								
$C_4H_8O_2$	Ethyl acetate								
$C_4H_{10}O_4$	Erythritol								
$C_5H_{10}O_2$	Ethyl propanoate								

$C_6H_8O_7$	Citric acid								
$C_6H_{12}O_2$	Ethyl butyrate								
$C_6H_{14}O_6$	Mannitol								
C_7H_8O	Benzyl alcohol								
$C_7H_{12}O_5$	Isopropylmalic acid								
$C_7H_{14}O_2$	Ethyl isovalerate								
$C_8H_{14}O_2$	Ethyl hexenoate								
$C_8H_{14}O_4$	Diethyl succinate								
$C_9H_{10}O_2$	Ethyl benzoate								
$C_{10}H_{20}O_2$	Ethyl octanoate								

An antimicrobial product synthesised by *M. pulcherrima* is 2-PE. In shake flask cultures with increasing initial glucose concentration (Sec. Shake flask cultivations), 2-PE production peaked at the point with the highest initial sugar loading where all the glucose was consumed (fig. S1a, Fig. 1c). With increasingly remaining glucose, extracellular 2-PE concentration dropped. Higher sugar concentration is known to elevate 2-PE production (Chantasuban et al., 2018), but the results show that it can be further enhanced by increasing the glucose concentration above the reported limit of 7.5 % (w/v).
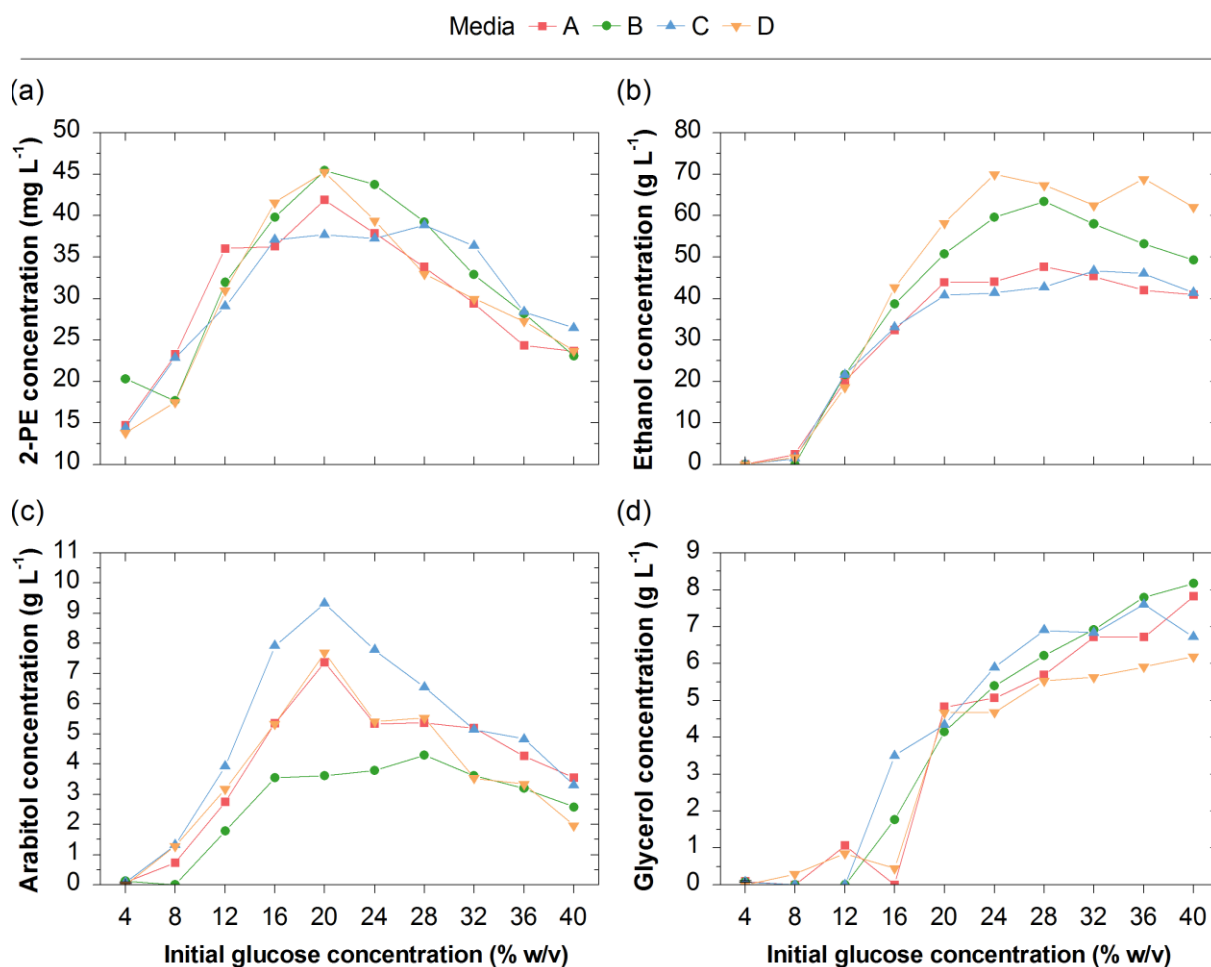
Figure S1: Production of secondary metabolites by *M. pulcherrima* over increasing osmotic stress. Cultivation was performed batch-wise in shake flasks (oxygen limited) for 5 days on four different synthetic media A-D (Table 1) with increasing glucose concentration (n = 1). Systematic error was determined with medium A40 (n = 3, mean ± SE).

Ethanol production was also elevated at higher glucose concentrations (fig. S1b), with an important factor being low oxygen availability through decreased oxygen solubility caused by increased solutes in the broth. Accordingly, highest production was with medium D, containing highest salt concentrations, amounting to 8.7 % (v/v) ethanol (fig. S1b), an ethanol yield of 0.40 g g^{-1} glucose (fig. S2) and 51.8 % w/w carbon efficiency (fig. S3c), neglecting the evaporated ethanol. This yield is promising, as *S. cerevisiae*, the major industrial ethanol producer typically achieves a practical yield around 0.46 g g^{-1} from glucose anaerobically, just below the theoretical maximum

(0.51 g g^{-1}) due to minor cell growth, metabolite formation and maintenance energy. Under fully aerated conditions in STRs (DO 50 %), ethanol formation was entirely avoided on glucose-rich medium (Fig. 2). The carbon was partly channelled into growth (fig. S4), which was not impaired by ethanol, resulting in a 3.9-fold biomass yield increase compared to respective SF experiments (Fig. 2). However, under these conditions ethanol formation may still take place through overflow metabolism when glucose consumption rates exceed 2.5 g L^{-1} h^{-1} contingent upon high nutrient availability (fig. S12). Under oxygen limited conditions, carbon conversion into ethanol appears to be more efficient than this into lipids under aerated conditions (fig. S4).

M. pulcherrima cultures produced both glycerol and arabitol (fig. 1c), as well as mannitol and erythritol (or respective isomers) in minor quantities (table S1). Polyol production, principally in the form of glycerol, but also arabitol, previously described as secondary osmolyte compared to glycerol (Van Eck et al., 1989; Kumdam et al., 2014), has been linked to osmoregulation in other ascomycetous yeasts (Blomberg and Adler, 1992) including *Candida* species (Kayingo and Wong, 2005; Zhuge et al., 2001), and has been suggested as criterion for osmotolerance (Van Eck et al., 1989). Besides its purpose as osmolyte, it is suggested that glycerol also played a role electron acceptor to enable NADH oxidation maintaining the intracellular redox balance, when the yeast undergoes alcoholic fermentation under (facultative) anaerobic conditions (Eriksson et al., 1995). Glycerol production has been reported with *M. reukaufii* (Nozaki et al., 2003), but so far only observed in microvinifications with *M. pulcherrima* (Clemente-Jimenez et al., 2004). Accumulation of arabitol, produced through the pentose phosphate pathway, has been reported with few *Metschnikowiae* (Koganti et al., 2011; Nozaki et al., 2003), including *M. pulcherrima* (Chantasuban et al., 2018; Sponholz et al., 1986), but the detailed mechanisms remain to be elucidated (Nozaki et al., 2003). Mannitol and erythritol on the other hand are less frequently linked to osmoregulation (Van Eck et al., 1993).
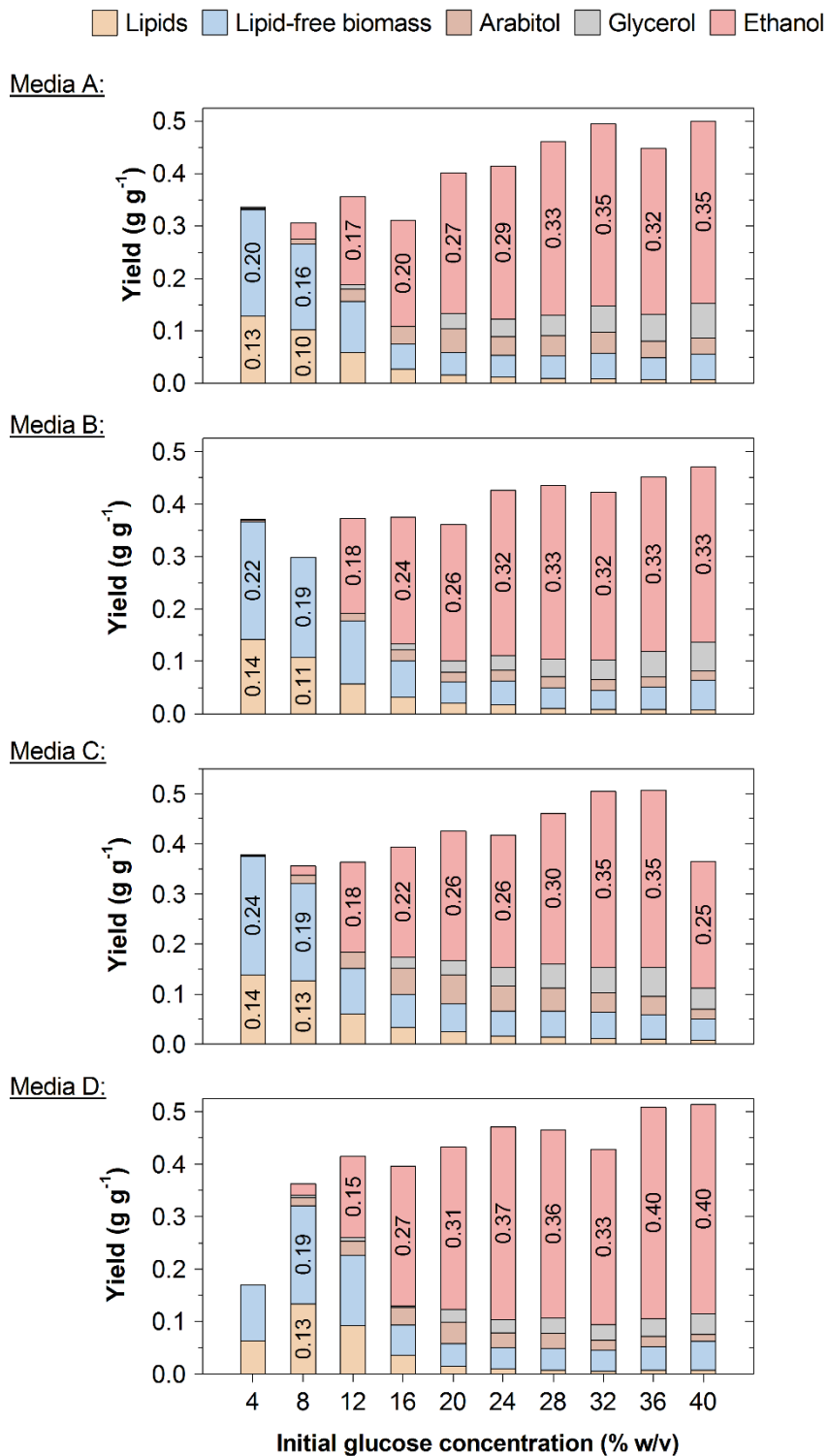
Figure S2: Yields of lipids, biomass and secondary metabolites produced by *M. pulcherrima* over increasing osmotic stress. Cultivation was performed batch-wise in shake flasks (oxygen limited) for 5 days on synthetic media A-D (Table 1) with increasing glucose concentration (n = 1), and yields calculated with respect to assimilated glucose.
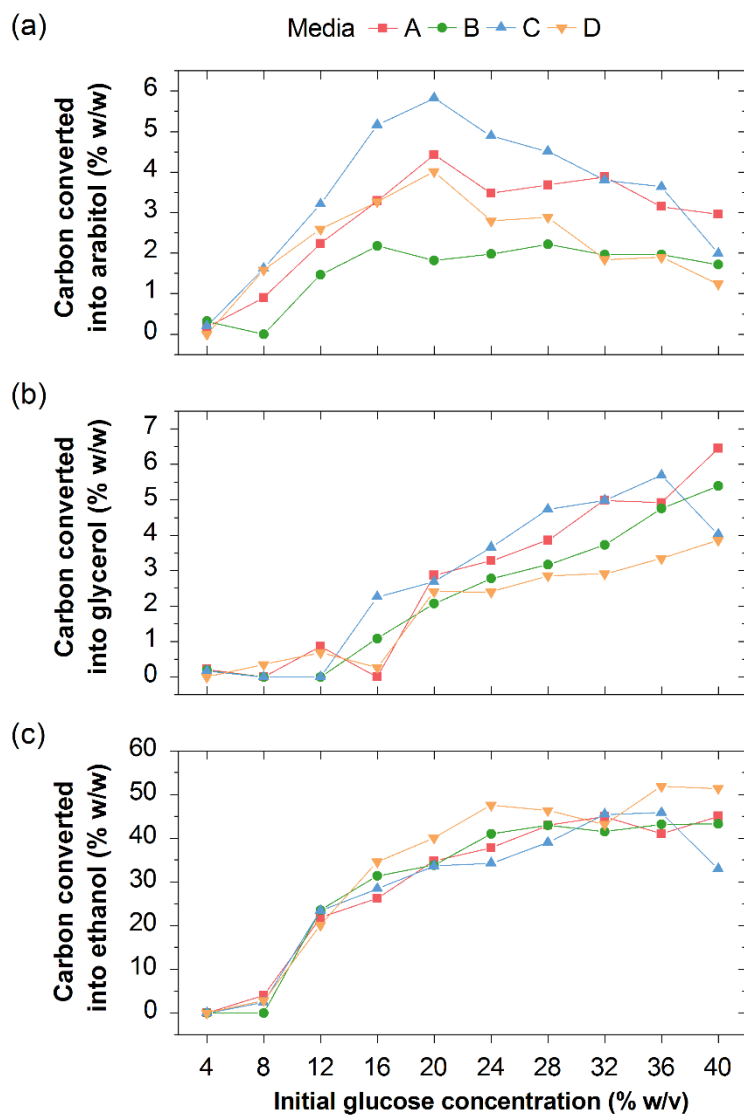
Figure S3: Carbon conversion into secondary metabolites produced by *M. pulcherrima* over increasing osmotic stress. Cultivation was performed batch-wise in shake flasks (oxygen limited) for 5 days on synthetic media A-D (Table 1) with increasing glucose concentration (n = 1), and percentage of carbon metabolised calculated with respect to assimilated glucose and supplied yeast extract (12 % w/w carbon).
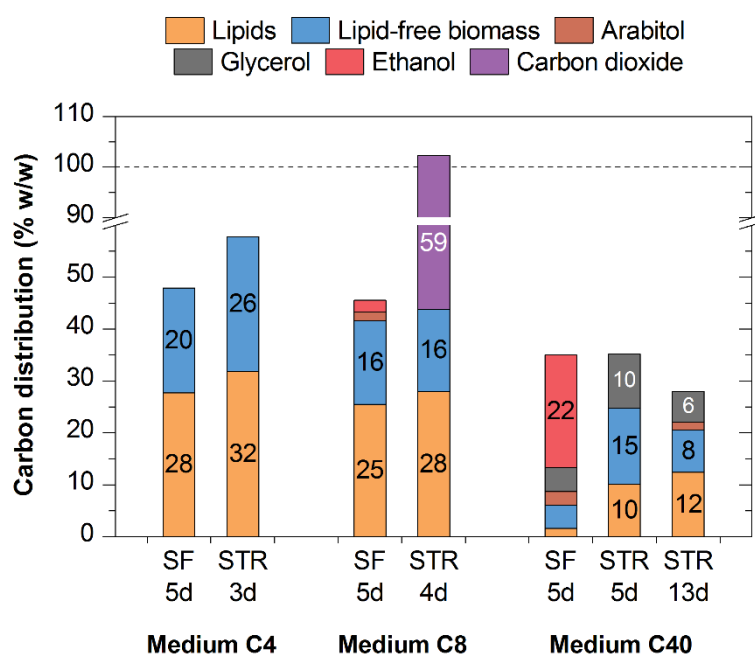
Figure S4: Carbon assimilation through yeast biomass and metabolites. Cells were cultured batchwise on synthetic media C (Table 1) with initial glucose concentrations of 4, 8 & 40 % (w/v) in shake flasks (SF) and stirred tank reactors (STR, DO 50 %) for the stated time (n = 1). Carbon distribution is denoted with respect to input glucose and yeast extract (at 12 % carbon). Data labels indicate the percentage of carbon converted into biomass and metabolites. Only for medium C8, STR 4d the carbon dioxide emissions were recorded.

The course of arabitol concentration is similar to this of 2-PE with its decline marked with glucose consumption (fig. S1c & Fig. 1c). Such a decrease from a certain water activity has been observed with other yeasts (Van Eck et al., 1989). It seems probable that the yeast releases intracellularly accumulated arabitol into the broth due to exposure to hypotonic conditions mainly induced by glucose consumption, the release being facilitated through channel proteins (Kayingo et al., 2001). This reasoning was substantiated through STR fermentations at 40 % (w/v) initial glucose, where considerate amounts of arabitol and glycerol were released, peaking at 12.7 and 26.3 g L^{-1}, respectively (Fig. 2 & S12a+c). Whilst glycerol was released from the start, arabitol was not until the glucose concentration was below approximately 20 % (w/v). Interestingly however, no arabitol had

been released at Day 5, where already 3.3 g L^{-1} were detected in SF (Fig. 2 & fig. S1c). This is presumably due to the low pH in uncontrolled SF fermentations (Fig. 1d) (Loman and Ju, 2014).

Glycerol, on the other hand, possesses a low molecular mass facilitating high membrane permeability, resulting in considerate passive diffusion of cytoplastic glycerol through the lipid bilayer, when compared to other polyols (Gostinčar et al., 2011; Vreeman, 1966). This resulted in its early-on extracellular release (Fig. 2), the high concentrations in general (Table 2), and the increasing glycerol over glucose concentration in the SF experiments (fig. S1d). Here, glycerol was not detected below 12 % w/v initial glucose, because glycerol, ethanol and under certain conditions also arabitol can be metabolised by the yeast (Fig. 2, S5 & S6, table S3), meaning that the presented values are not necessarily the peak values of each compound. Indeed, arabitol and glycerol can be detected before glucose depletion (table S2). Specifically, indicating high affinity towards *M. pulcherrima* metabolism (fig. S5), ethanol and glycerol are typically re-metabolised first after glucose exhaustion.

Table S2: Arabitol and glycerol detected at low osmotic pressure fermentation. *M. pulcherrima* was cultured batch-wise in stirred tank reactors on synthetic glucose media at 4 and 8 % (w/v) glucose loading (C4 & C8, Table 1) for 2 and 3 days, respectively.

Initial glucose concentration (% w/v)	Glucose concentration (g L^{-1})	Arabitol concentration (g L^{-1})	Glycerol concentration (g L^{-1})
4 (C4)	4.0	0.6	1.0
8 (C8)	7.6	0.6	1.4

It remains to point out that *M. pulcherrima* increasingly produced a range of other metabolites when subjected to high osmotic pressure or low oxygen availability (table S1).

Section S6: Toxicity and assimilation of *M. pulcherrima* metabolites and media compounds

The toxicity and assimilation of *M. pulcherrima* metabolites (2-PE, arabitol, ethanol, glycerol) and media compounds (NH_4) are depicted in this section. Additionally, the ethanol resistance of *M. pulcherrima* is discussed. In this respect, the yeast grew up to a DCW of 2.9 g L^{-1} when inoculated into nutrient-supplemented 5 % (v/v) ethanol, showing that even a small population is viable to deal with high ethanol toxicity (fig. S5). The formation of pseudohyphae, as occurring under anaerobic conditions (Kurtzman and Droby, 2001), was distinct (fig. S10), suggesting that the underlying reason for this effect are secreted metabolites in the first place.
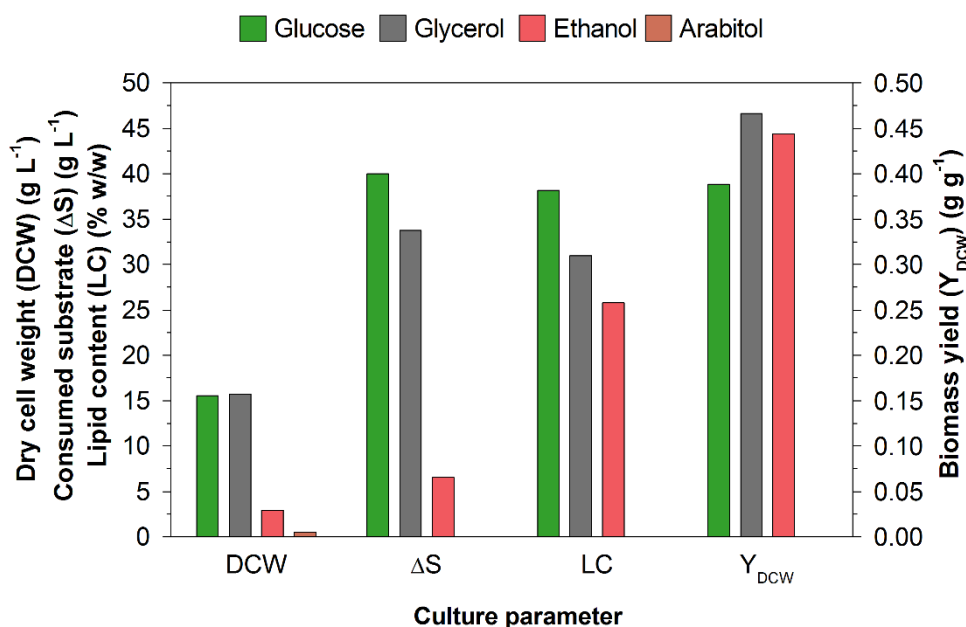
Figure S5: *M. pulcherrima* growth on glucose, glycerol, ethanol and arabitol. Dry cell weight (DCW), consumed substrate (ΔS), lipid content (LC) and biomass yield (Y_{DCW}) after 5-day batch cultivations on NLB medium with 40 g L^{-1} of either these substrates in shake flasks (n = 1).

Spike experiments further showed that the yeast is not inhibited by ethanol concentrations of up to 2.5 % (v/v), but survives concentrations exceeding 15 % (v/v) (fig. S6).
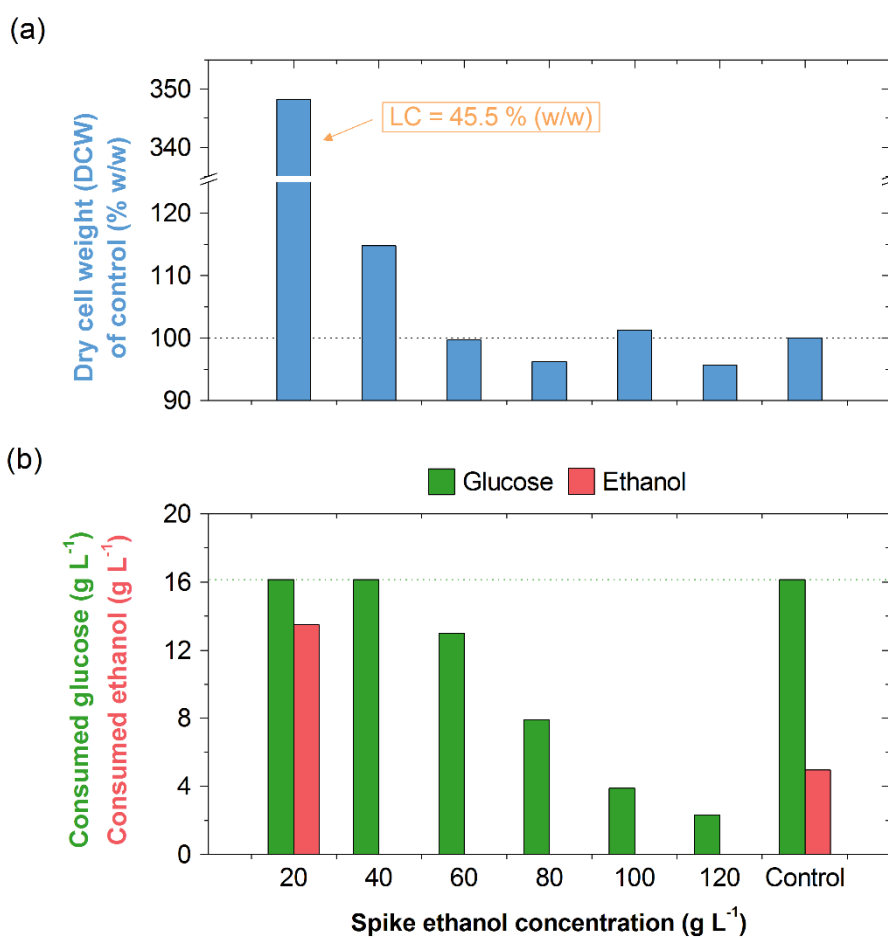
Figure S6: *M. pulcherrima* tolerance to ethanol. Cells were cultured in shake flasks on synthetic glucose medium (A4, Table 1) for 24 h, after which ethanol (spike) was added to the broth in the specified concentration. Total culturing time was 8 days. (a) Dry cell weight (DCW), rectified according to the added liquid volume and displayed with respect to a control (24 h, no spike, DCW 6.4 g L^{-1}); and (b) consumed glucose after ethanol addition and consumed ethanol, adjusted with evaporation. Ethanol in the control had been produced by the yeast itself. LC: lipid content.

This is similar to other *M. pulcherrima* strains (Barbosa et al., 2018; Mukherjee et al., 2017), and determines its suitability for wine fermentations (Clemente-Jimenez et al., 2004). Pseudohyphae were not observed in the spike experiments. Remarkably, ethanol in low concentrations appears to benefit lipid synthesis, with the lipid content of cultures spiked with 2.5 % (v/v) ethanol being 45.5 % (w/w), for instance, and therefore higher than this after growth at 4 or 8 % (w/w) glucose (fig.

S6a & 1b). Arguably, higher lipid yields, as 0.19 g g^{-1} in this case, may be expected with ethanol-tolerant yeast due to the higher degree of reduction of ethanol compared to glucose.
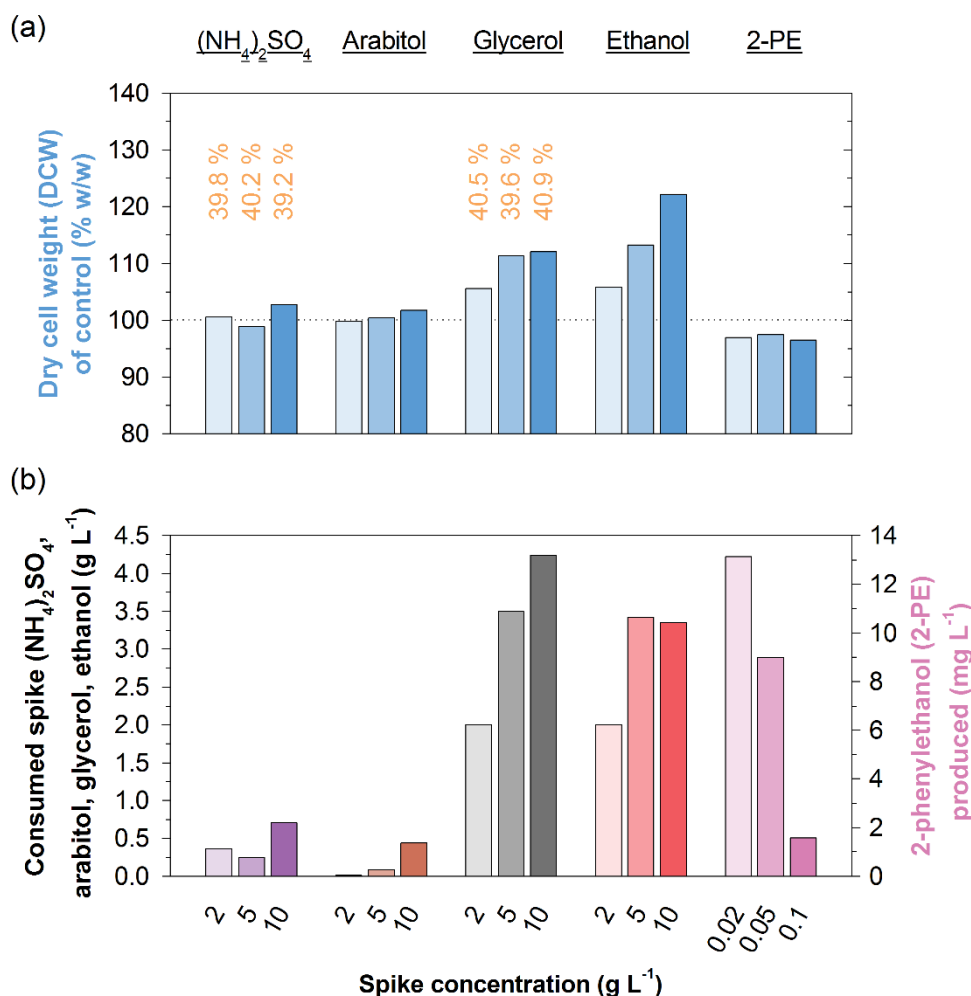
Figure S7: Influence of ammonia nitrogen, arabitol, glycerol, ethanol and 2-phenylethanol (2-PE) spikes on *M. pulcherrima* growth. Cells were cultivated batch-wise on synthetic glucose medium (A4, Table 1) in shake flasks and each compound was added to the broth after 24 h in the specified spike concentration. (a) Dry cell weight rectified according to the added liquid volume and displayed with respect to a control without spike, as well as selected lipid contents (% w/w); and (b) net concentrations of ammonia nitrogen, arabitol, glycerol and ethanol consumed, and 2-PE produced after 3 days total cultivation time.

Table S3: Arabitol assimilation in shake flask batch cultivations. *M. pulcherrima* was cultured on synthetic NLB (Section Organism, media, preculture) containing 40 g L^{-1} arabitol or mixed glucose

and arabitol (20 g L^{-1} each) for 5 days. The cultures were inoculated with 2.5 % (v/v) inoculum or with the cells equivalent to 100 % (v/v) preculture.

Initial arabitol concentration (g L^{-1})	Inoculum concentration (% v/v)	Optical density OD$_{600}$ (-)	Remaining arabitol concentration (g L^{-1})
40	2.5	4.7	40.1
40	100 [†]	12.0	40.1
20 [‡]	2.5	34.8	19.5

[†] preculture equivalent to 100 % (v/v) was centrifuged, the cells resuspended in 2.5 % (v/v) supernatant and the suspension used for inoculation

[‡] additionally, 20 g L^{-1} glucose

Section S7: Morphology of *M. pulcherrima*

The cell morphology of *M. pulcherrima* (NCYC 4331, evolved strain) is depicted in this section as influenced by the operation conditions used in this study (Sec. Material and methods).
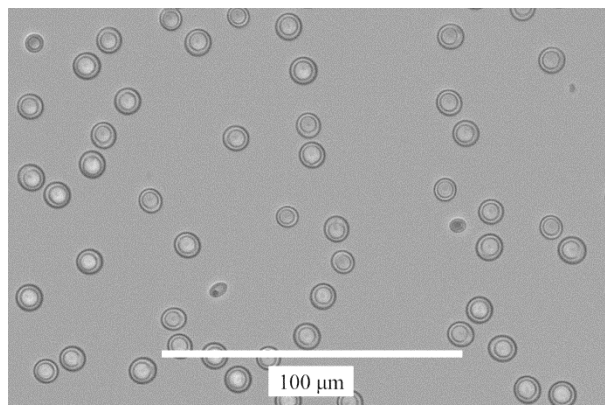
Figure S8: Lipid-rich *M. pulcherrima* cells. Cell morphology of *M. pulcherrima* after 5-day batch cultivation on synthetic glucose medium (D8, Table 1) in shake flasks.
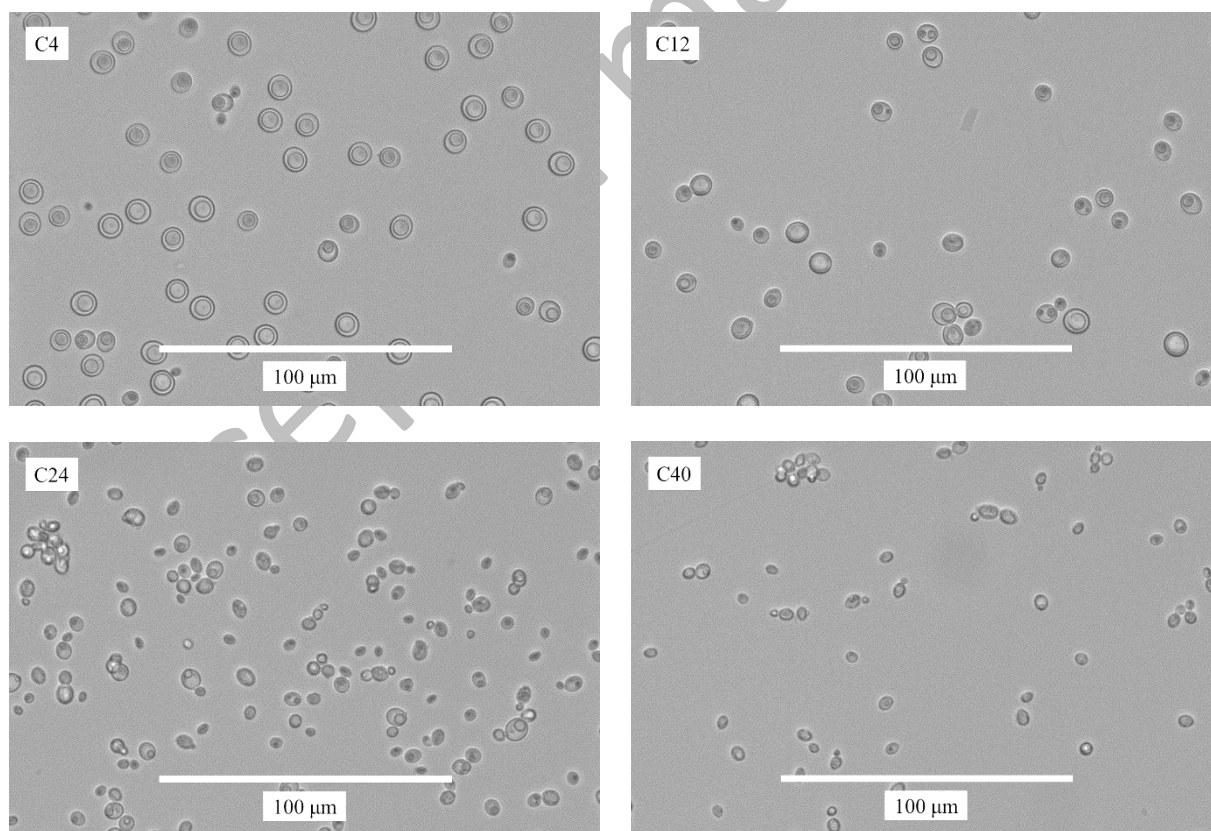
Figure S9: Variation of *M. pulcherrima* cell morphology with substrate loading. Cells were

cultivated batch-wise on synthetic media C (Table 1) with initial glucose concentrations of 4, 12, 24 and 40 % (w/v) in shake flasks (oxygen limited) for 5 days.
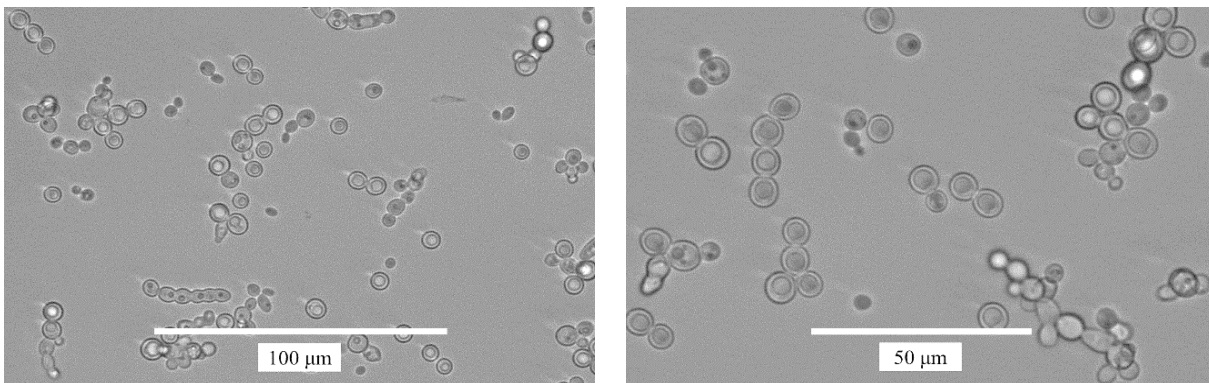
Figure S10: Effect of ethanol on cell morphology of *M. pulcherrima*. Cells were inoculated into NLB medium containing 5 % (v/v) ethanol and cultured for 5 days in shake flasks. The formation of pseudohyphae was distinct. Displayed are two different resolutions.
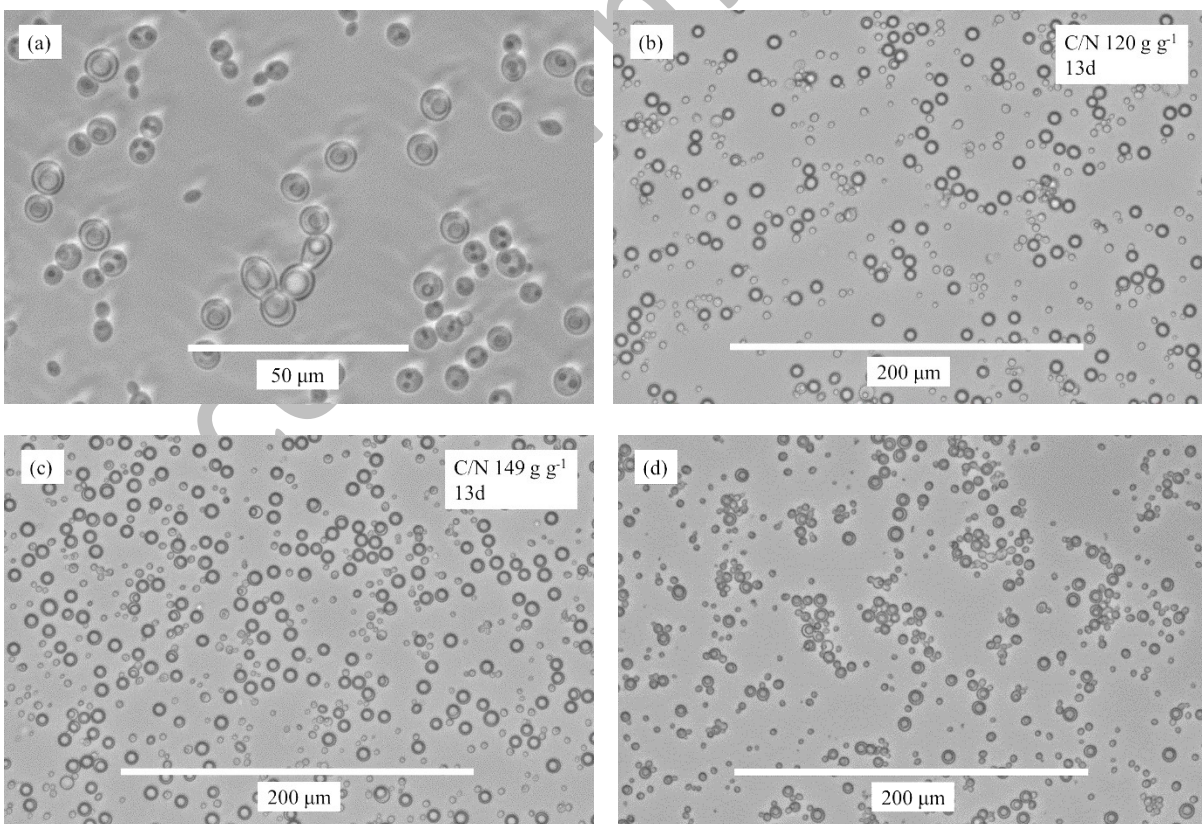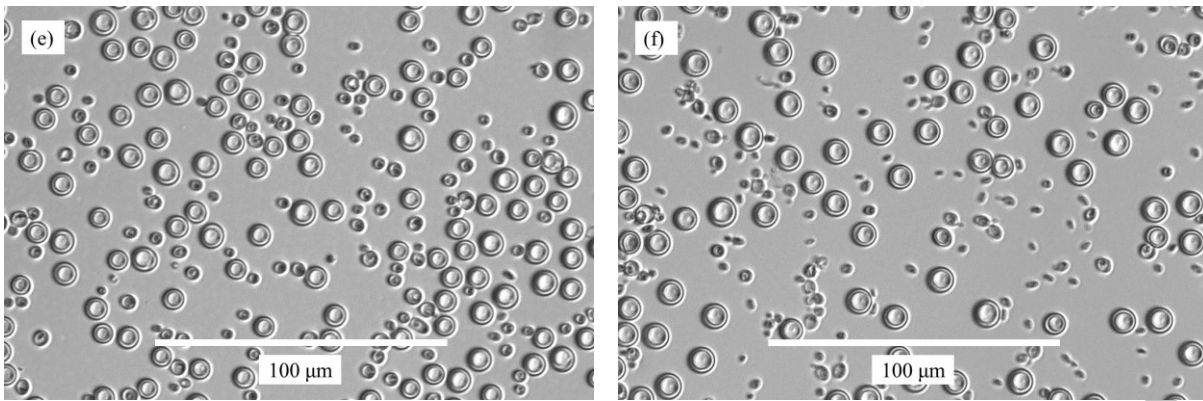
Figure S11: Types of *M. pulcherrima* cell morphology. (a) Budding of lipid-rich cells (Day 3), (b)+(c) formation of small cells (Day 13) when cultured semi-continuously on glucose with biomass recycle in stirred tank reactors (DO 50 %) at two different carbon/nitrogen (C/N) ratios, (d) cell population after 5-day batch-wise cultivation on 40 g L^{-1} glycerol in shake flasks, and (e)+(f) formation of small cells (Day 13) when cultured semi-continuously on ethanol (e) and with addition of fermentation inhibitors (f) in stirred tank reactors (DO 50 %).

Section S8: Additional information on batch cultivations

The additional data on batch cultivations includes fermentation parameters of two STR batch fermentations with high glucose and nutrient loading (media E40 & F40, Table 1).
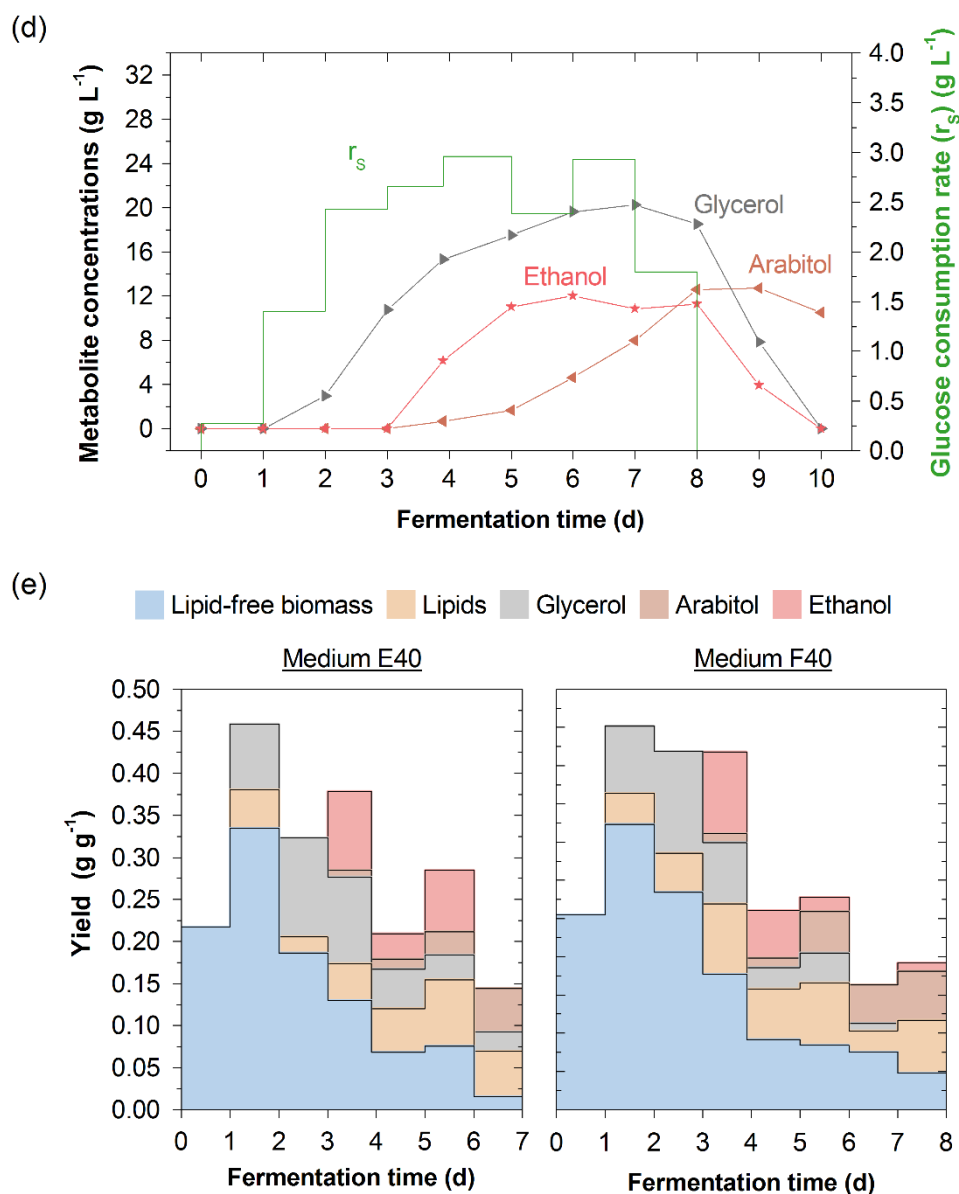
(a)

(b)

(c)
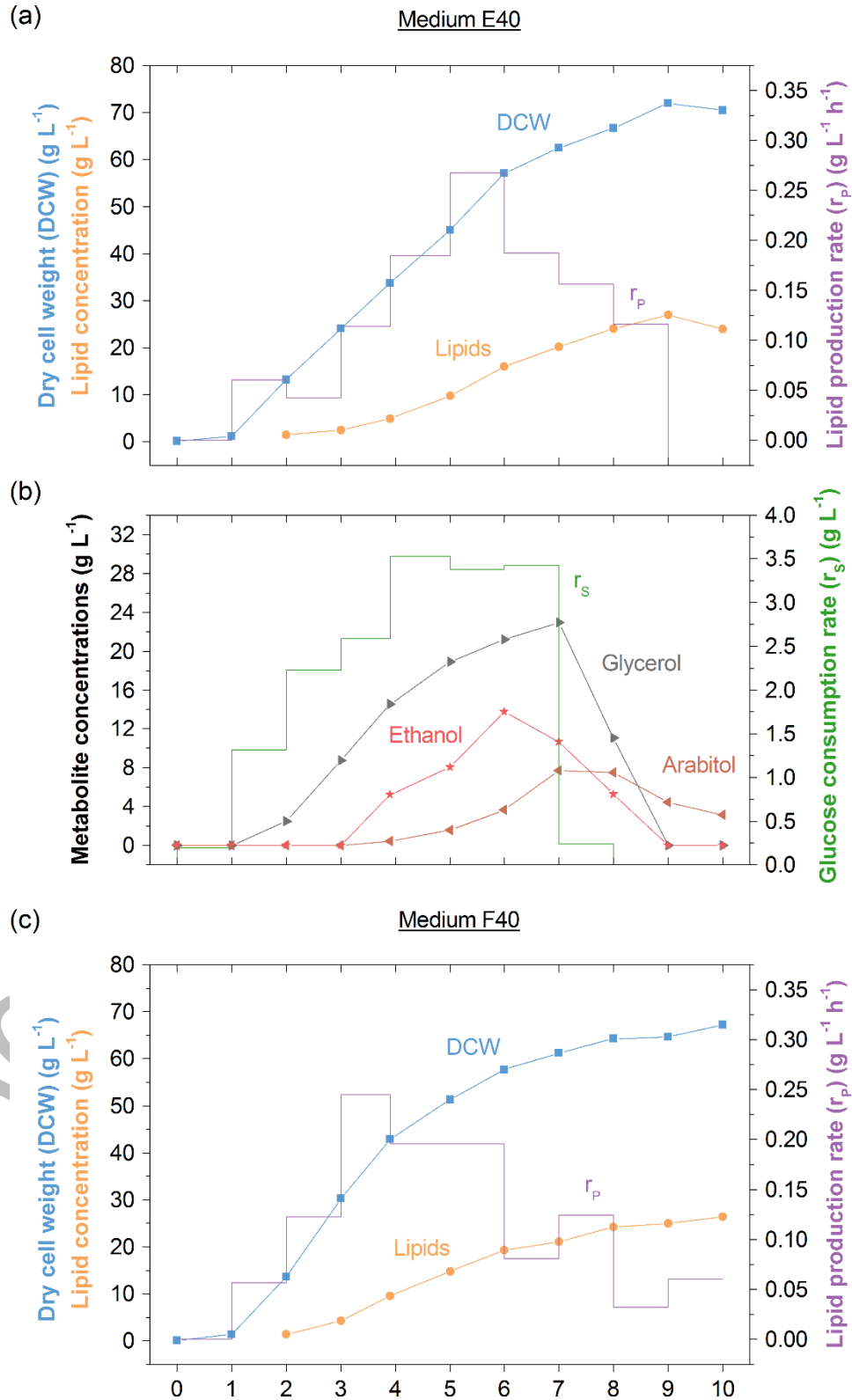
Figure S12: *M. pulcherrima* batch cultivation on glucose and nutrient-rich medium. (a)+(c) Profiles of dry cell weight, lipid concentration and production rate, (b)+(d) secondary metabolite (arabitol, ethanol, glycerol) concentrations and glucose consumption rate, (e) daily lipid-free biomass, lipid, glycerol, arabitol and ethanol yield with respect to glucose, when cultured on synthetic medium (E40 & F40, respectively, Table 1) in a stirred tank reactor (DO 50 %) (n = 1).

Section S9: Additional information on fed-batch cultivations

In this section, selected fermentation parameters of a fed-batch cultivation with continuous feed of a glucose-rich medium (E48, Table 1) are illustrated.
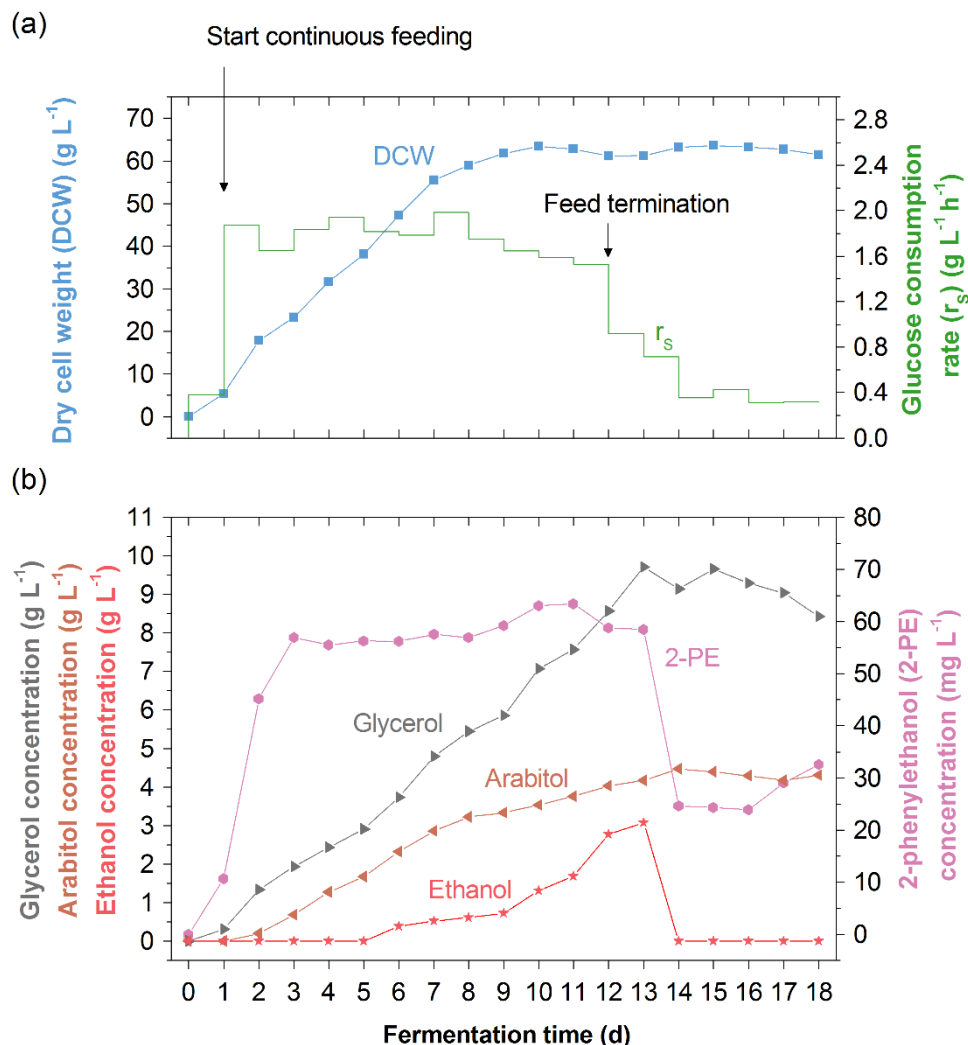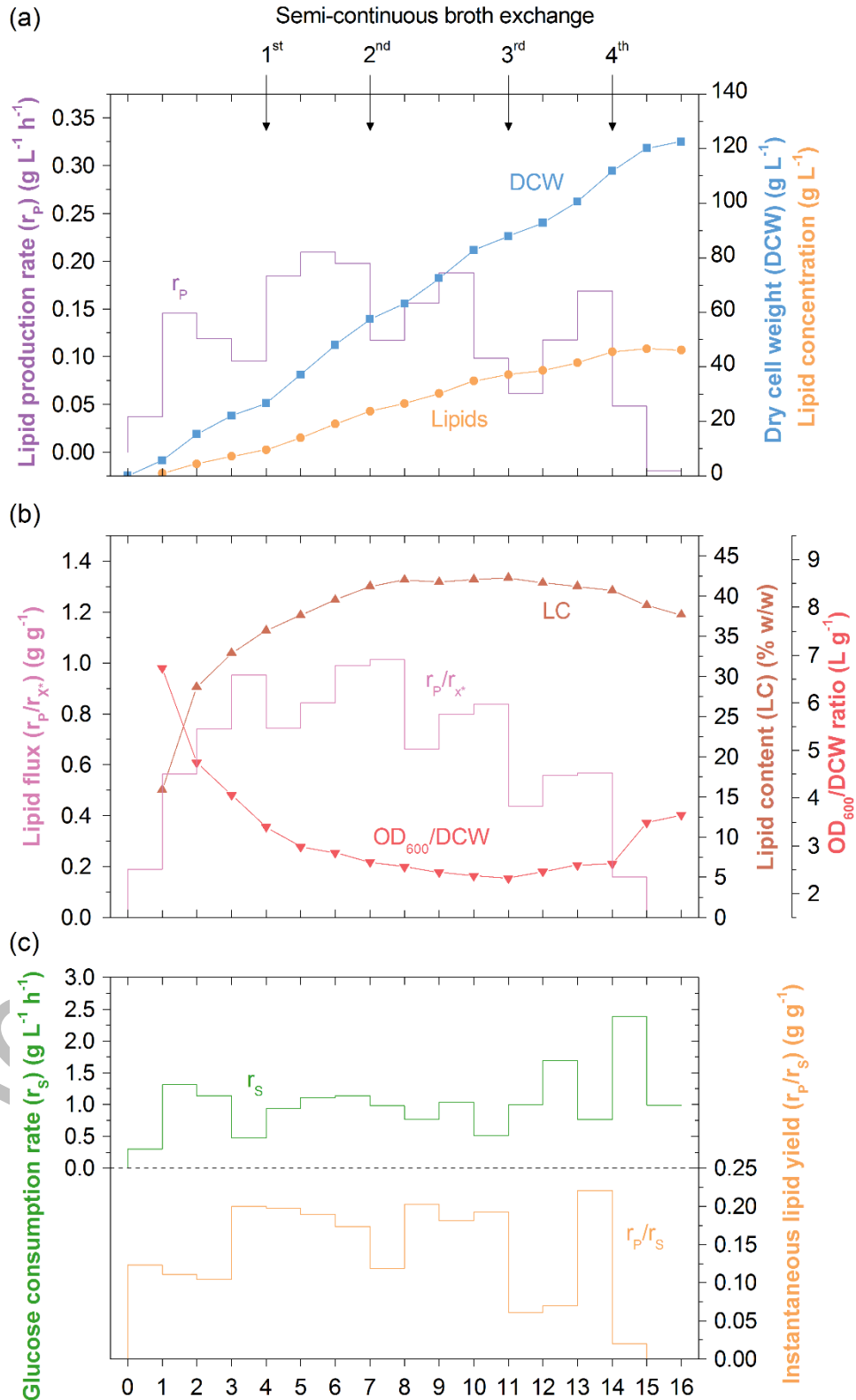

Figure S13: *M. pulcherrima* fed-batch cultivation with continuous feed. Cells were initially cultivated on synthetic glucose medium (C8, Table 1) and from Day 1 fed continuously with glucose-rich medium (E48, C/N 60.1 g g^{-1}, Table 1) keeping the glucose concentration > 50 g L^{-1} in a stirred tank reactor (DO 50 %) (n = 1). (a) Dry cell weight and daily glucose consumption rate, (b) glycerol, arabitol, ethanol and 2-phenylethanol concentrations. The feed was terminated on Day 12.

Section S10: Additional information on semi-continuous cultivations

Further information on semi-continuous cultivations is presented, the main themes being small cell formation, nitrogen accumulation in the broth and rate changes over time.
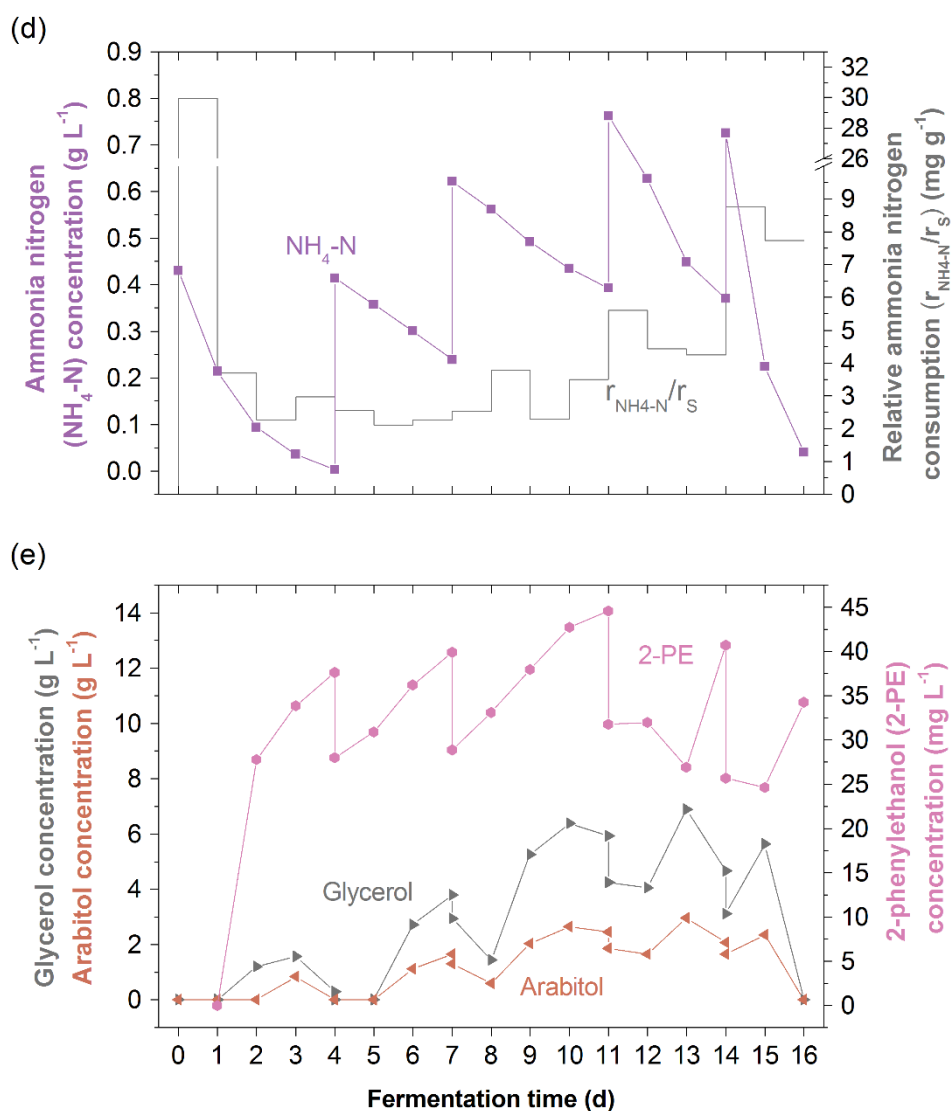
Figure S14: *M. pulcherrima* semi-continuous cultivation with biomass recycle (extension of Fig. 4). Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and, periodically upon glucose depletion (Day 4, 7, 11 & 14), 25 % (v/v) of the broth was replaced by glucose-rich medium (E32, C/N ratio 60.1 g g^{-1}, Table 1), and the removed cells fed back to the stirred tank reactor (n = 1). (a) Dry cell weight (DCW), lipid concentration and daily lipid production rate, (b) daily lipid flux, lipid content and OD$_{600}$/DCW ratio, (c) daily glucose consumption rate and lipid yield, (d) ammonia nitrogen concentration and daily ammonia nitrogen consumption relative to glucose uptake, (e) glycerol, arabitol and 2-phenylethanol concentrations.
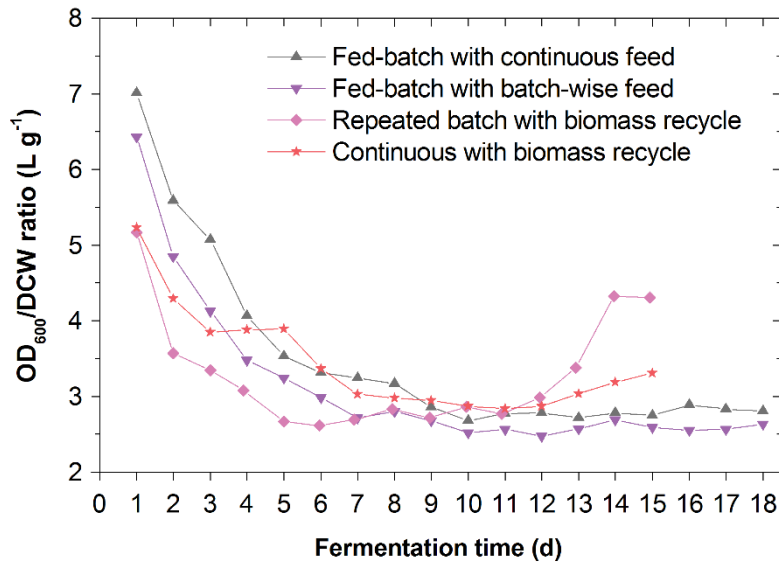
Figure S15: Change of OD$_{600}$/dry cell weight (DCW) ratio in *M. pulcherrima* fed-batch, repeated batch and continuous fermentations. Cells were cultured on synthetic glucose medium, and in repeated batch and continuous fermentation, removed cells were recycled to the reactor (n = 1, Sec. Stirred tank reactor cultivations).
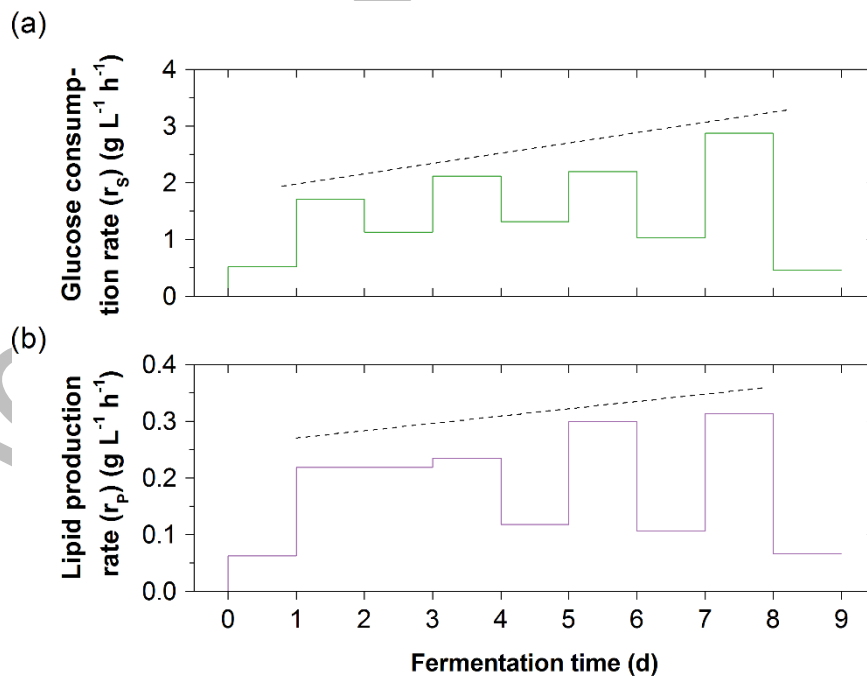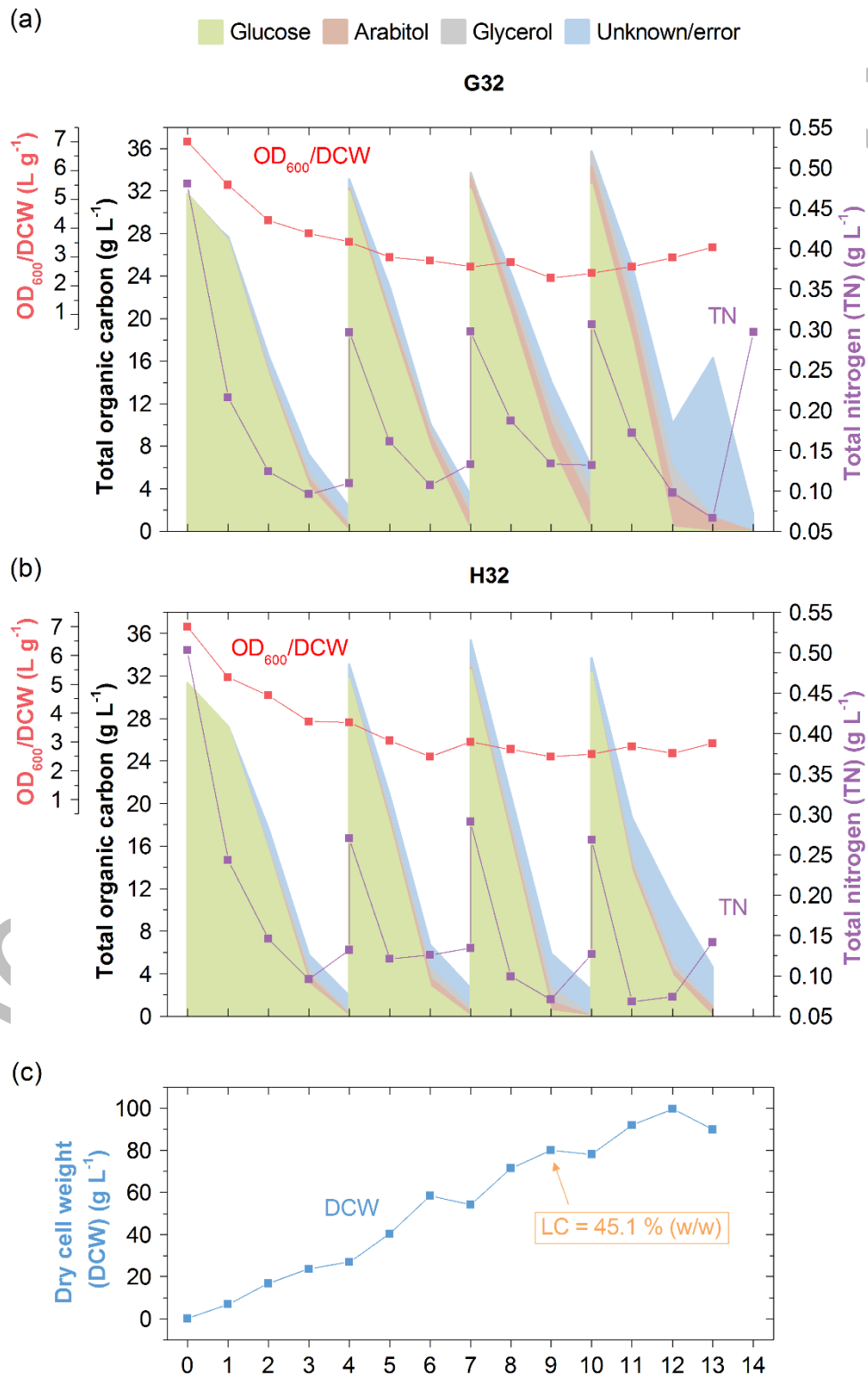

Figure S16: *M. pulcherrima* semi-continuous cultivation with biomass recycle at high oxygenation.

Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and, periodically upon

glucose depletion, 25 % (v/v) of the broth was replaced by glucose-rich medium (F32, C/N ratio 99.7 g g^{-1}, Table 1), and the removed cells fed back to the stirred tank reactor (DO 80%, n = 1). (a) Daily glucose consumption rate, (b) daily lipid production rate.
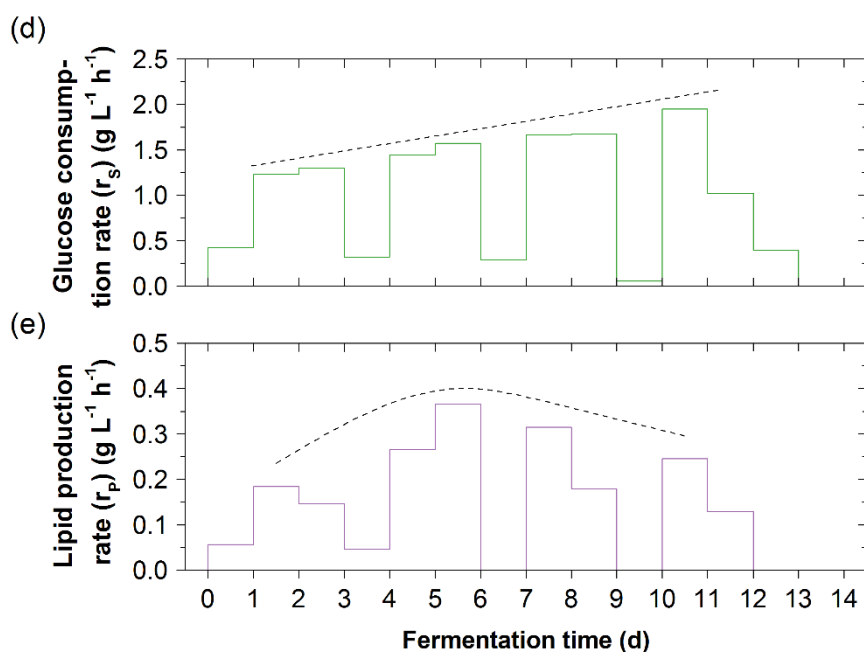
Figure S17: *M. pulcherrima* semi-continuous cultivation with biomass recycle on media with different carbon/nitrogen (C/N) ratios. Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and, periodically upon glucose depletion, 25 % (v/v) of the broth was replaced by glucose-rich medium (G32, C/N 120 g g^{-1}, or H32, C/N 148.7 g g^{-1}, Table 1), and the removed cells fed back to the stirred tank reactor (n = 1). (a)+(b) Total organic carbon (TOC) and total nitrogen (TN) in the broth, and OD$_{600}$/DCW ratio during three exchange cycles for both media. The fraction of unknown TOC constitutes the difference between measured TOC and this cumulatively derived from glucose, arabitol and glycerol concentrations; (c) dry cell weight (DCW), (d) daily glucose consumption rate, and (e) daily lipid production rate for medium H32. LC: lipid content.

Section S11: Additional information on continuous cultivations

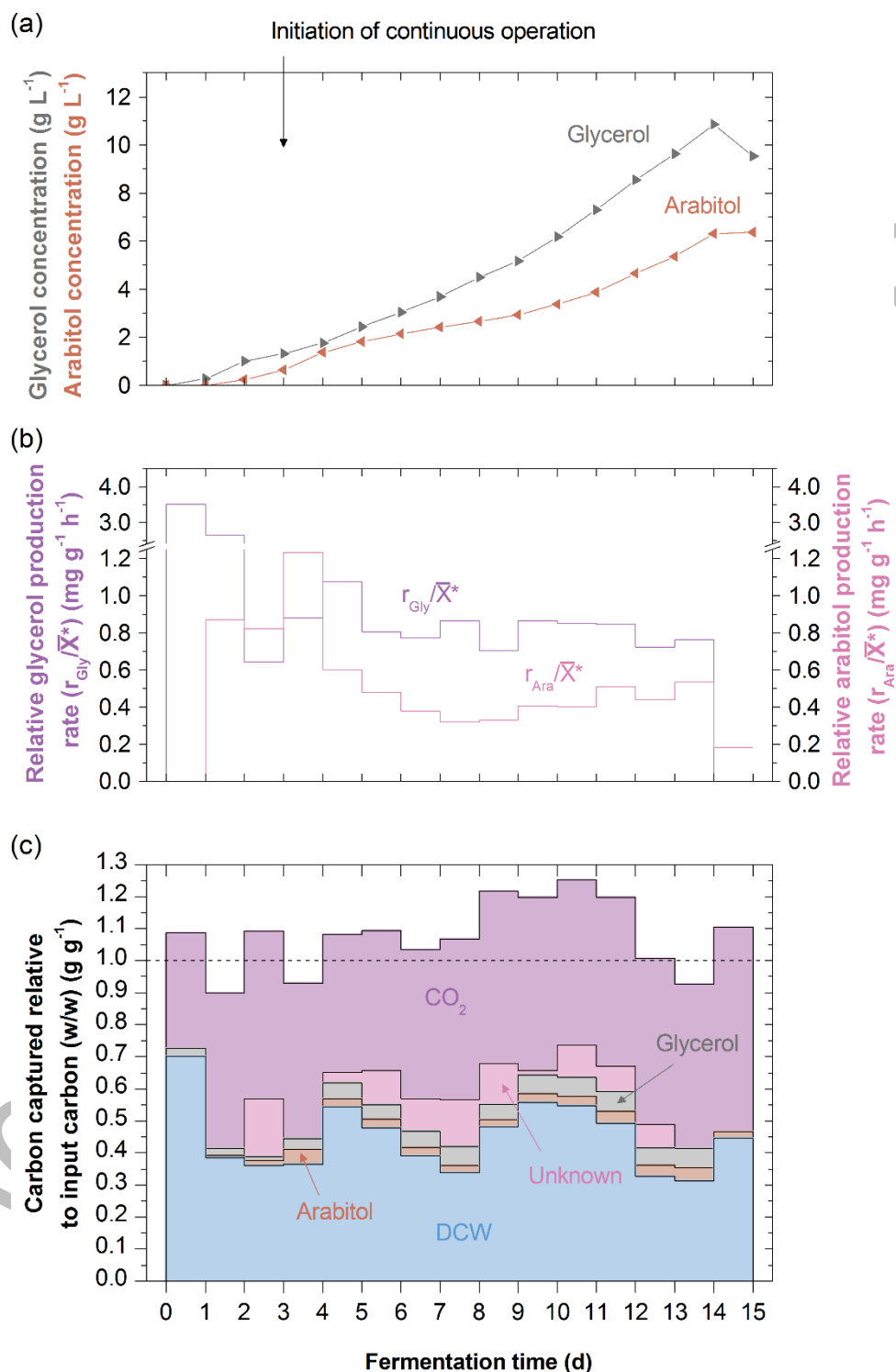Additional data on the continuous fermentation performed in this study (Fig. 7) is provided.

Figure S18: *M. pulcherrima* continuous cultivation with biomass recycle. Cells were cultured batch-wise on synthetic glucose medium (C8, Table 1) and on Day 3, continuous operation was initiated at a dilution rate of 0.067 d^{-1}, using glucose-rich medium (E32, C/N ratio 60.1 g g^{-1}, Table 1), the cells

recycled into the reactor (n = 1). (a) Glycerol and arabitol concentration, (b) daily glycerol and arabitol production rates with respect to averaged lipid-free biomass $\overline{X}^*$ between two sample points, and (c) carbon balance with respect to the input carbon (glucose, yeast extract).

Section S12: Fatty acid profiles

In this section, the fatty acid profiles of the lipids obtained in the various operation modes in STRs fermentations (Table 2) are depicted.

Table S4: Fatty acid profile in *M. pulcherrima* high-density cultures. Cells were cultured on synthetic media (Table 1) in batch, fed-batch, semi-continuous and continuous fermentation mode in stirred tank reactors (n = 1, Sec. Stirred tank reactor cultivations).

Cultivation mode and medium	Fatty acid composition (% w/w)					
	C16:0	C16:1	C18:0	C18:1	C18:2	Other
Batch						
C8	31.2	4.8	1.2	60.4	2.4	
C40	36.6	5.4	2.1	55.9	0.0	
E40	17.2	4.7	0.6	69.0	8.4	
F40	16.6	8.3	0.4	68.0	6.6	
Fed-batch						
E48, batch-wise feed	23.5	3.3	2.9	66.8	3.6	
Semi-continuous cell recycle						
E32	38.5	2.5	4.6	47.6	0.0	C6:0 (1.4), C8:0 (1.9), C9:0 (2.4), C10:0 (0.8)
F32, DO 80 % [†]	27.3	4.3	3.4	59.9	5.1	
F32, ethanol [†]	22.8	5.0	1.2	67.1	3.9	
F32, inhibitors [†]	23.6	5.1	3.5	61.6	5.3	
H32	23.1	4.5	3.2	61.9	6.6	
Continuous cell recycle						
E32	30.0	5.1	2.3	57.7	4.8	

[†] Different yeast extract used

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