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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Characterization of an evolved carotenoids hyper-producer of *Saccharomyces cerevisiae* through bioreactor parameter optimization and Raman spectroscopy

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Abstract An evolutionary engineering approach for enhancing heterologous carotenoids production in an engineered *Saccharomyces cerevisiae* strain was used previously to isolate several carotenoids hyper-producers from the evolved populations. β-Carotene production was characterized in the parental and one of the evolved carotenoids hyper-producers (SM14) using bench-top bioreactors to assess the impact of pH, aeration, and media composition on β-carotene production levels. The results show that with maintaining a low pH and increasing the carbon-to-nitrogen ratio (C:N) from 8.8 to 50 in standard YNB medium, a higher β-carotene production level at 25.52 ± 2.15 mg β-carotene g^{-1} (dry cell weight) in the carotenoids hyper-producer was obtained. The increase in C:N ratio also significantly increased carotenoids production in the parental strain by 298 % [from 5.68 ± 1.24 to 22.58 ± 0.11 mg β-carotene g^{-1} (dcw)]. In this study, it was shown that Raman spectroscopy is capable of monitoring β-carotene production in these cultures. Raman spectroscopy is adaptable to large-scale fermentations and can give results in near real-time. Furthermore, we found that Raman spectroscopy was also able to measure the relative lipid compositions and protein content of the parental and SM14 strains at two different C:N ratios in the bioreactor. The Raman analysis showed a higher total fatty acid content in the SM14 compared with the parental strain and that an increased C:N ratio resulted in significant increase in total fatty acid content of both strains. The data suggest a positive correlation between the yield of β-carotene per biomass and total fatty acid content of the cell.

Keywords Carotenoids · Yeast · Isoprenoids · Raman spectroscopy · Fermentation

Introduction

The metabolic engineering of microbial systems for the heterologous production of natural compounds is a growing area in industrial biotechnology. The ability to create biosynthetic pathways for non-native products in microorganisms enables new bio-based routes for the production of platform chemicals, pharmaceuticals, fuels, and nutraceuticals [5]. Extensive metabolic engineering efforts have converted *Saccharomyces cerevisiae* into a potential "cell factory" for production of a broad range of compounds [10]. Isoprenoids are a large and diverse class of over 30,000 naturally occurring organic compounds [19, 27]; they are essential for proper cellular function, but some of them also have industrial value as pharmaceuticals (e.g., artemisinin, taxol) [1, 26], pesticides (e.g., pyrethrins) [12], fragrance compounds [16], and food additives (e.g., carotenoids) [17, 21], and thus have been intensively targeted in metabolic engineering for microbial-based production.

Current approaches for introducing heterologous pathways in microorganisms generally focus on optimizing gene

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expressions and enzyme activities for the specific biosynthetic genes. Recent work demonstrated the effect of codon optimization of carotenoid genes for β-carotene production in *S. cerevisiae* [15]. Also, genes outside the direct biosynthetic pathway of the target compound may play a role in productivity. Indeed, prior studies have shown that genes such as *rssB* and *yjfP* that are outside of the isoprenoid biosynthesis pathway in *Escherichia coli* affected the heterologous production of lycopene in this organism [2]. To overcome the dependence on a priori knowledge of targets for rational metabolic engineering for carotenoids production, it was shown that carotenoid hyper-producing strains of *S. cerevisiae* could be obtained through a novel evolutionary engineering approach, utilizing oxidative stress as the driving force to select for mutants with enhanced carotenoid production [25]. The carotenoid biosynthesis genes *crtE*, *crtYB*, and *crtI* from *Xanthophyllomyces dendrorhous* [31] were integrated into *S. cerevisiae* to enable the yeast strain to produce downstream carotenoids products from GGPP such as phytoene, neurosporene, lycopene, and β-carotene [17]. The native hydrogen peroxide detoxification mechanism in yeast was removed by deleting the cytosolic catalase (*CTT1*) gene from the engineered carotenogenic strain of yeast; the resulting strain YLH2 was used in adaptive evolution experiments using periodic shock with hydrogen peroxide as the selective pressure. Using this strategy, a rapid increase in the average carotenoid production of the evolving populations was observed. Upon completion of the evolution experiment, mutants were isolated from the population, and isolate SM14 produced the highest yield of β-carotene [25].

In this work, the role of pH, aeration, and media composition on β-carotene production in bench-top batch bioreactors was investigated. The results showed that pH had a large effect on β-carotene production in defined medium, and aeration level influenced the rate of β-carotene production in the system. Gene expression differences between a β-carotene hyper-producer strain (SM14) and the ancestral strain isolated using the evolutionary engineering technique (YLH2) were analyzed previously. The data showed the expression of lipid biosynthesis genes to be upregulated and fatty acid beta-oxidation genes to be downregulated in SM14 compared with YLH2 [25]. Since carotenoids are lipophilic compounds, it was hypothesized that an increase in total lipid may lead to increased carotenoids production. To test this hypothesis, we also varied the carbon-to-nitrogen (C:N) ratio, which has been shown to impact lipid content of oleaginous yeasts [4], in defined medium in the bioreactor. A significant increase in β-carotene yield per biomass in the bioreactor at a higher C:N ratio was obtained. We also investigated whether Raman spectroscopy could be used to monitor β-carotene production in yeast and assess changes in cellular fatty acid composition, protein content, and metabolic activity when the cells are grown in a bioreactor. Raman spectroscopy is of interest, because it is fast (near real-time analysis), economical, and has been applied to large-scale bioprocesses [14, 22]. Also, recent research has demonstrated the ability of Raman spectroscopy to identify β-carotene [30], track changes in microbial phenotypes [3, 6, 8, 32], and discern changes in membrane lipid composition and fluidity [32]. Results from the Raman analysis helped to validate our hypothesis that the yield of β-carotene is directly correlated with total fatty acid content of the cell.

Table 1 *S. cerevisiae* strains used in this study

Strain	Description	Source
GSY1136	Matα, ura3–52, gal + in S288c background, YBR209 W::Act1p-GFP-Act1t-URA3	[11]
YLH2	Unevolved ancestral strain. GSY1136 with the carotenoid cassette *crt YB/I/E* integrated into the genome	[25]
SM12	Isolated hyper-producer from evolved population 1	[25]
SM14	Isolated hyper-producer from evolved population 1	[25]
SM22	Isolated hyper-producer from evolved population 2	[25]

Materials and methods

Yeast strains and plasmids

Saccharomyces cerevisiae strains SM14, SM12, SM22, and YLH2 (Table 1) [25], cultured in either yeast extract peptone dextrose (YPD) or yeast nitrogen base (YNB) (Amresco, USA) medium supplemented with 2 % (wt vol^{-1}) D-glucose at 30 °C, were used in this study.

Bioreactor studies

YPD or YNB media supplemented with 2 % (wt vol^{-1}) D-glucose were used for bioreactor studies. The seed cultures for fermentation were started using a 1-mL frozen culture stock (thawed) into 50 mL of media (same as that used in the bioreactor). Frozen culture starter stocks were created from an initial YPD bioreactor run. Seed cultures were grown for 48 h at 30 °C and 170 rpm. The 50-mL culture was used to inoculate 3 L of media in a 7-L bioreactor (Applikon®) at 30 °C with continuous air feed and online DO measurements. Bioreactor fermentations were run for 45–140 h. Bioreactor pH was maintained at set point using 2 M NaOH and 2 M HCl. Carotenoid quantification was performed as previously described [25]. Briefly, 500 μL of culture was sampled from the bioreactor, pelleted, followed

by removal of supernatant via aspiration and resuspended in 1 mL of dodecane with addition of approximately 250 μL of glass beads. Cells were disrupted via bead-beating, and 200 μL of the supernatant was transferred to a Corning® 96-well black-wall clear-bottom plate for quantification. A standard curve for β-carotene quantification was measured using commercially available β-carotene (Enzo Life Sciences) at OD_{454}, and intracellular β-carotene was quantified at an absorbance wavelength of OD_{454} [31]. For biomass measurements, OD_{600} was correlated with dry cell weight (dcw) measurements. To calculate β-carotene yield [mg g^{-1} (dcw)], the conversion equation used was: mg g^{-1} (dcw) = [10 × (mg L^{-1})]/(OD_{600} × 7.61985). 1 mL of the bioreactor sample was pelleted, and the supernatant was used for extracellular metabolite analysis with HPLC (Agilent Technologies®). HPLC analysis was carried out with an Aminex HPX-87H column (300 mm by 7.8 mm, Bio-Rad Laboratories, CA) with 0.005 M H_2SO_4 as the mobile phase and a flow rate of 0.6 mL min^{-1} at 50 °C using an RI detector (35 °C, for glucose, acetic acid, and ethanol).

Raman spectroscopy and data analysis

Raman spectroscopy was performed using an Agiltron PeakSeeker Pro attached to an upright laboratory microscope. Raman spectra were collected over the 200–2000 cm^{-1} spectral range with a 6 cm^{-1} spectral resolution using a laser excitation of 785 nm, 5 mW laser power, 1 s integration time, and a 10× microscope objective. Cells from 1 mL culture samples were pelleted by centrifuge (10,000*g* for 1 min) and resuspended in 1 mL phosphate-buffered saline. This procedure was repeated three times to remove culture media. A total of 0.5 μL of cell suspension was placed on an aluminum foil surface and air-dried. Cells were visualized under the microscope to obtain Raman spectra. This procedure is also described elsewhere [3]. A minimum of five Raman spectra were collected from different sections of the dried cell aggregate, and the results were averaged. Spectral data were analyzed in MATLAB (R2015B). The spectra were baseline-corrected before comparing band intensities. Vector normalization was applied to spectra as described previously [3, 32]; however, it was found that raw data, in absence of normalization, gave results consistent with other experimental findings.

Results

Impact of bioreactor parameters on carotenoid production

Hyper-producing strains of *S. cerevisiae* [25] were assayed for β-carotene production through bioreactor parameter analysis. The effect of bioreactor parameters on extracellular metabolite concentrations, biomass accumulation, and β-carotene production were studied. Extracellular metabolite analysis was used to quantify the rates of substrate consumption and product formation during different growth phases in the bioreactor. The evolved hyper-producing isolate SM14 was used in the majority of the bioreactor studies; the unevolved ancestral strain YLH2, from which SM14 evolved, was included for comparison. It is important to note that even though both strains accumulate a range of carotenoids, only absorbance at OD_{454} (the peak absorbance for β-carotene) was used for carotenoids measurements in our analysis. Our results showed that regardless of the fermentation parameters, the two main phenotypic differences between SM14 and YLH2 were biomass and β-carotene production levels (Fig. 1). During the previous adaptive evolution experiments, hyper-producers were isolated from evolved population samples based on large colony size and dark red color [25]. Thus, the observed higher final biomass yield and accumulation of β-carotene in the hyper-producer SM14 compared with YLH2 were not unexpected.

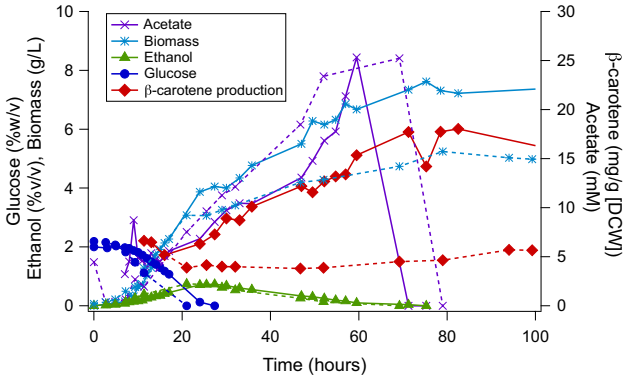

Fig. 1 Comparisons between β-carotene production, biomass, and extracellular metabolite profiles in 8.8 C:N YNB medium operated at 800 rpm and pH 4.0 in SM14 and YLH2 (ancestral strain). *Solid lines* SM14. *Dashed lines* YLH2. Data shown are a representative run

The effects of media composition, aeration, and pH on β-carotene production were determined in a bench-top bioreactor. For varying aeration, the agitation speed was varied between 400 and 800 rpm in YNB medium at pH 4.5. In the experimental conditions tested, the β-carotene production profiles differed between the two agitation speeds (Fig. 2). With a lower agitation speed of 400 rpm, β-carotene production remained at a low and constant level for the majority of the run and only peaked toward the very end of the run at around 70 h. When agitation speed was increased to 800 rpm, β-carotene production started to increase much earlier in the run (~30 h) and remained relatively constant for the remainder of the batch run. However, the kinetics of

biomass formation and final maximum titers of β-carotene produced were similar between the two agitation speeds at pH 4.5 (Fig. 2). Agitation speed affected the dissolved oxygen (DO) levels in the bioreactor (data not shown) and also the extracellular metabolite profiles (Fig. S1). When extracellular metabolite profiles were compared between the two aeration levels, only ethanol concentrations appeared to differ. At pH 4.5, for runs at both 400 and 800 rpm, ethanol concentration reached maximum at 12 h. At 800 rpm, ethanol was consumed within 36 h from the start of fermentation; however, ethanol, being a non-fermentable carbon source, took about twice as long to be consumed when agitation was at 400 rpm (Fig. S1).

The impact of pH was assessed between pH 4.0 and 5.0 at a constant agitation of 800 rpm in either YPD or YNB medium. In our experiments, pH was the variable that had the most effect on β-carotene production (Fig. 3, Fig. S2), glucose consumption, and extracellular metabolite concentrations (data not shown). However, this was only observed in defined media (YNB). In complex YPD media, pH had a negligible impact on carotenoid production. Carotenoids production in YNB media, however, was significantly affected by pH, and the results were consistent between replicate runs at the same conditions. For SM14 in YNB at 800 rpm, β-carotene production was 16.8 ± 1.8 mg g^{-1} at pH 4.0 but decreased to 10.7 ± 0.2 mg g^{-1} at pH 4.5 (Fig. 3). β-Carotene production in YNB media was highest at pH 4.0 and production decreased as pH increased (Fig. S2). Also, the extracellular metabolite profiles of SM14 differed between pH 4.0, 4.5, and 5.0 in YNB media. For SM14 growth in YNB media at pH 4.5 and 5.0, glucose exhaustion occurred around 12 h from the start of the run, and ethanol was fully consumed by 24–30 h from the start of the run (data not shown). For SM14 cultured in YNB at pH 4.0 (Fig. 1), extracellular metabolite analysis revealed a trend of slower glucose and ethanol consumption in comparison to the higher pH counterparts (data not shown). To determine whether the observed metabolic profiles of SM14 were also observed in other evolved carotenoids hyper-producers, SM12 (a mutant isolated from the same evolving population as SM14) and SM22 (a mutant isolated from an independently evolved population from SM14) were analyzed in the bioreactor. Interestingly, both SM12 and SM22 exhibited a similar trend in glucose and ethanol consumption in YNB media at 800 rpm and pH 4.0 (Fig. S3). This suggests that the metabolism at pH 4.0 in YNB medium at 800 rpm was similar across different mutant isolates.

Finally, the effects of media composition on carotenoids production and extracellular metabolite profiles in the bioreactor were analyzed at pH 4.0 and 800 rpm. YPD is a complete medium high in amino acids and nitrogen, which resulted in a high titer (mg β-carotene L^{-1}) in SM14 and

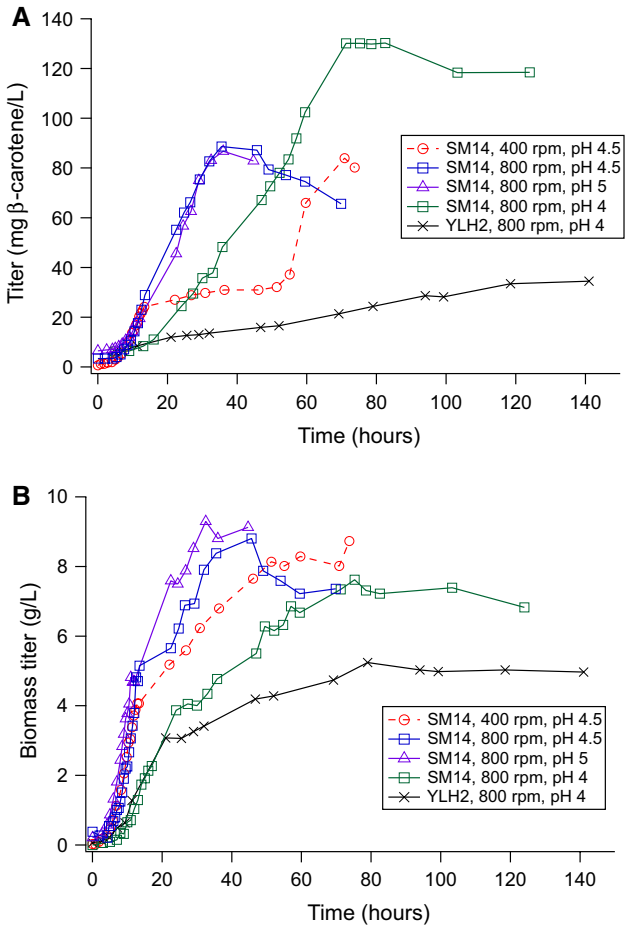

Fig. 2 **a** Effect of aeration and pH on SM14 and YLH2 β-carotene titer profiles in 8.8 C:N YNB medium. **b** Effect of aeration and pH on SM14 and YLH2 biomass titer profiles in 8.8 C:N YNB medium

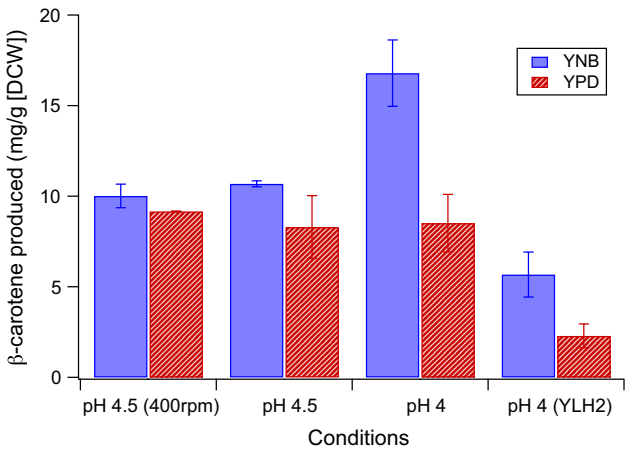

Fig. 3 Effect of aeration, medium, and pH on SM14 and YLH2 β-carotene production. Aeration is 800 rpm unless specified otherwise. Values represent the observed maximum β-carotene produced (normalized against amount of biomass at the time of sampling) during the duration of the run. *Error bars* represent standard deviation from a minimum of two independent runs

biomass (g L^{-1}) in both YLH2 and SM14; however, it also resulted in a lower yield [mg g $(dcw)^{-1}$] and performance variability, likely due to batch-to-batch variations in the yeast extract used in the culture medium (Figs. 3, S4). As expected, the use of defined YNB media led to higher reproducibility between runs. Interestingly, growth on YNB resulted in higher β-carotene production per cell [mg β-carotene per gram dry cell weight (dcw)] compared with growth on YPD. For example, at pH 4.0–800 rpm, the average β-carotene production in SM14 was 16.8 ± 1.8 mg β-carotene g^{-1} (dcw) in YNB and 8.5 ± 1.6 mg β-carotene g^{-1} (dcw) in YPD (Fig. 3). In YNB at 800 rpm and pH 4.0, β-carotene was produced during all growth phases, when glucose, ethanol, and acetate were consumed, and reached a maximum after all available carbon sources were depleted in both SM14 and YLH2 (Fig. 1).

In addition to β-carotene production, extracellular metabolite concentrations were also impacted by the bioreactor parameters. Out of the three extracellular metabolites quantified (glucose, ethanol, and acetate), acetate profiles varied the most between the two media tested (Figs. S5, S6). In YPD media with an agitation of 800 rpm and at both pH 4.0 and 4.5, acetate concentrations stayed around 2–4 mM until ~12 h from the start of the fermentation when it was consumed along with ethanol (Figs. S5, S6). There was not a very clear trend for correlating acetate to β-carotene production levels in YPD media. In YNB media at 800 rpm and the pH range between 4.0 and 5.0, the acetate concentration increased throughout the run during glucose and ethanol consumption. This trend of acetate production and consumption was observed in the three isolated single mutants tested (SM12, SM14, SM22) and the ancestral strain YLH2 (Fig. S6). The β-carotene production profiles were similar between strains SM12, SM14, and SM22 (Fig. S7).

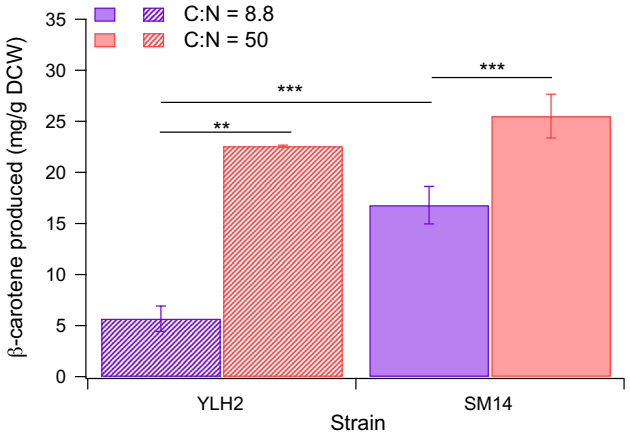

Fig. 4 Comparison of β-carotene production in SM14 and YLH2 (ancestral strain) in 50 C:N and 8.8 C:N YNB medium operated at 800 rpm and pH 4.0. YLH2: *hashed bars*. SM14: *solid bars*. Statistical significance calculated using Student's *t* test (**$p < 0.01$; ***$p < 0.001$)

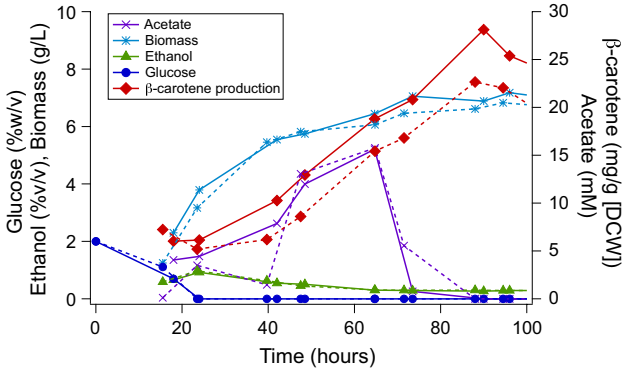

Fig. 5 Comparisons between β-carotene production, biomass, and extracellular metabolite profiles in 50 C:N YNB medium operated at 800 rpm and pH 4.0 in SM14 and YLH2 (ancestral strain). *Solid lines* SM14. *Dashed lines* YLH2

Impact of feed composition on β-carotene production

In some fatty yeasts, it has been shown that increasing the C:N ratio results in an increase in lipid contents [4, 24, 29]. Since carotenoids are generally hydrophobic in nature, increased lipid contents would potentially provide more hydrophobic areas for carotenoids to accumulate, possibly reducing previous physical limitations on carotenoid levels when grown in 8.8 C:N YNB medium [18]. Thus, it was hypothesized that changing the carbon and nitrogen composition of YNB media could increase carotenoid production of yeast in the bioreactor. The carbon-to-nitrogen (C:N) ratio in YNB was increased from 8.8 to 50 by keeping the total carbon constant while lowering the amount of ammonium sulfate used in media preparation. The effects of increasing the C:N ratio were analyzed in the bioreactor at pH 4.0 and at 800 rpm for SM14 and YLH2. β-carotene levels increased with increasing C:N ratio in both strains. In SM14, the average β-carotene production increased from 16.8 ± 1.8 mg β-carotene g^{-1} (dcw) to 25.52 ± 2.15 mg β-carotene g^{-1} (dcw) (Fig. 4). In YLH2, β-carotene production increased from 5.68 ± 1.24 mg β-carotene g^{-1} (dcw) to 22.58 ± 0.11 mg β-carotene g^{-1} (dcw), which is a 298 % increase and an amount higher than that produced by SM14 at a C:N ratio of 8.8 (Fig. 4).

In addition to an increase in overall β-carotene production, β-carotene, acetic acid, and biomass formation profiles for SM14 and YLH2 changed with growth in the higher 50 C:N YNB medium. β-carotene production for both SM14 and YLH2 steadily increased over the course of the run in medium containing C:N ratio of 50, whereas in 8.8 C:N medium, β-carotene production in YLH2 seemed to plateau early on in the fermentation (Figs. 1, 5). Acetic

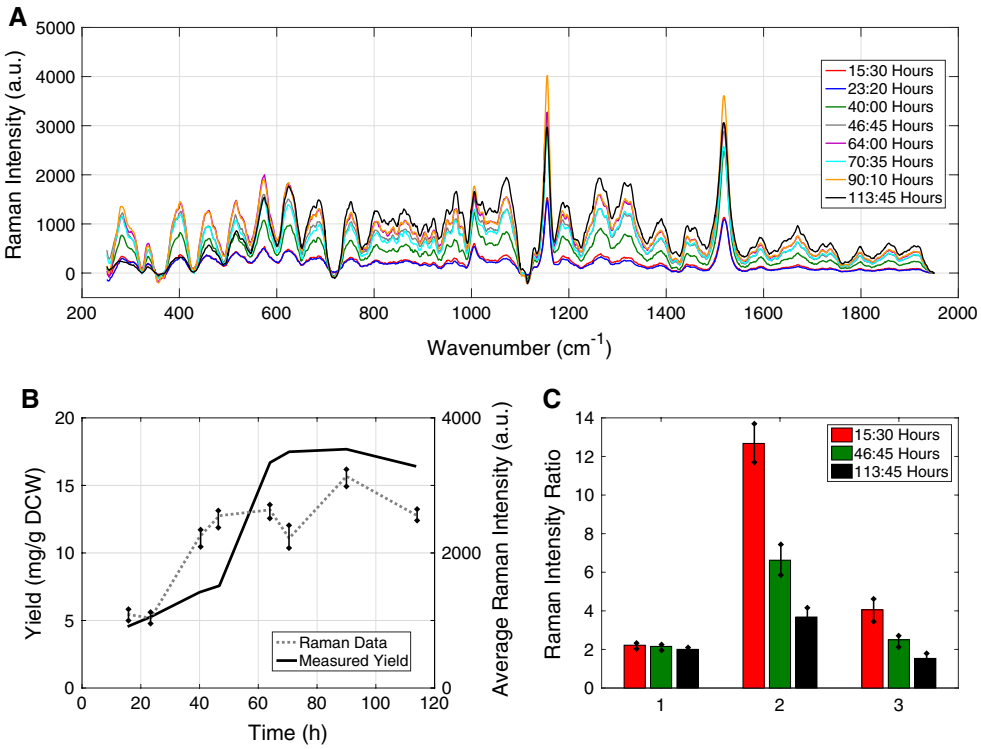

Fig. 6 Study of time-course SM14 growth and β-carotene production by Raman spectroscopy. **a** All raw Raman spectra obtained in the study at different time points. **b** The measured β-carotene yield and the average of Raman band intensities known to correlate with β-carotene (1006, 1156, and 1519 cm^{-1}). **c** The ratio of Raman band intensities corresponding to (1) unsaturated (1263 cm^{-1}) to saturated (1444 cm^{-1}) fatty acids, (2) β-carotene (1156 cm^{-1}) to total protein (1214 cm^{-1}), and (3) β-carotene (1156 cm^{-1}) to unsaturated fatty acids (1263 cm^{-1}). *Error bars* represent plus and minus 1 standard deviation

acid profiles for both SM14 and YLH2 were very similar in 50 C:N medium. The average maximum concentration of acetic acid was 15.7 mM in 50 C:N, whereas the average maximum concentration in 8.8 C:N YNB medium was 25.3 mM (Figs. 1, 5). The biomass formation profile of SM14 and YLH2 also changed with the higher C:N ratio. Toward the end of the run in 8.8 C:N medium, maximum amount of biomass was ~7.6 g L^{-1} for SM14 and ~5.2 g L^{-1} for YLH2 (Fig. 1). In 50 C:N medium, biomass reached a maximum of ~7 g L^{-1} for SM14 and ~6.4 g L^{-1} for YLH2 (Fig. 5).

Monitoring β-carotene production and culture phenotypes by Raman spectroscopy

Raman spectroscopy is of significant interest to cell cultivations, because productivity and cell phenotype information can be obtained in near real-time and without the use of chemical labels. Time course results for the SM14 strain in 8.8 C:N YNB medium and 800 rpm agitation are shown in Fig. 6. Baseline-corrected Raman spectra are shown in Fig. 6a. Immediately apparent are the strong β-carotene Raman bands, which occur at 1006, 1156, and 1519 cm^{-1} and are consistent with the literature data [30]. When monitoring yeast cells, it was found that no spectral normalization was necessary to demonstrate that Raman spectroscopy can monitor β-carotene production over a time course. The average of the three Raman band intensities is plotted along with experimentally determined yield in Fig. 6b, and a correlation coefficient between Raman and OD_{454} measurements of 0.8 was obtained. While Raman intensities are arbitrary units (au), the data in Fig. 6b show that the accumulation of β-carotene, when measured by Raman, is similar to what is obtained analytically (roughly threefold increase over 120 h). Also evident is that in addition to increasing β-carotene production, several other Raman bands increased over the time course. A more detailed analysis of lipid composition is given in Fig. 6c. Student's *t* test was used to determine statistical relevance. Here, the ratio of unsaturated to saturated fatty acids was investigated by Raman spectroscopy. Only a slight change in this ratio (~10 %) was observed over the time course, but this was found to not be statistically relevant. Typically, this value changes significantly in the presence of toxic chemicals, such as the solvent butanol [32]; however, β-carotene production does not appear growth-inhibitive due to toxicity, which was expected. Next, the ratio of β-carotene to total protein was found to decrease sharply over the time course. Data from all time points shown in Fig. 6c(2) are statistically different with 99 % confidence. This is of significant interest since protein-to-product ratios impact downstream separations cost, and it suggests that an optimal harvesting condition exists during the time course. Finally, the ratio of β-carotene to unsaturated fatty acids was investigated. Results showed a decrease in this ratio over time, which indicates the accumulation of unsaturated fatty acids during the time course. Student's *t* test also revealed these samples to be statistically different with 99 % confidence.

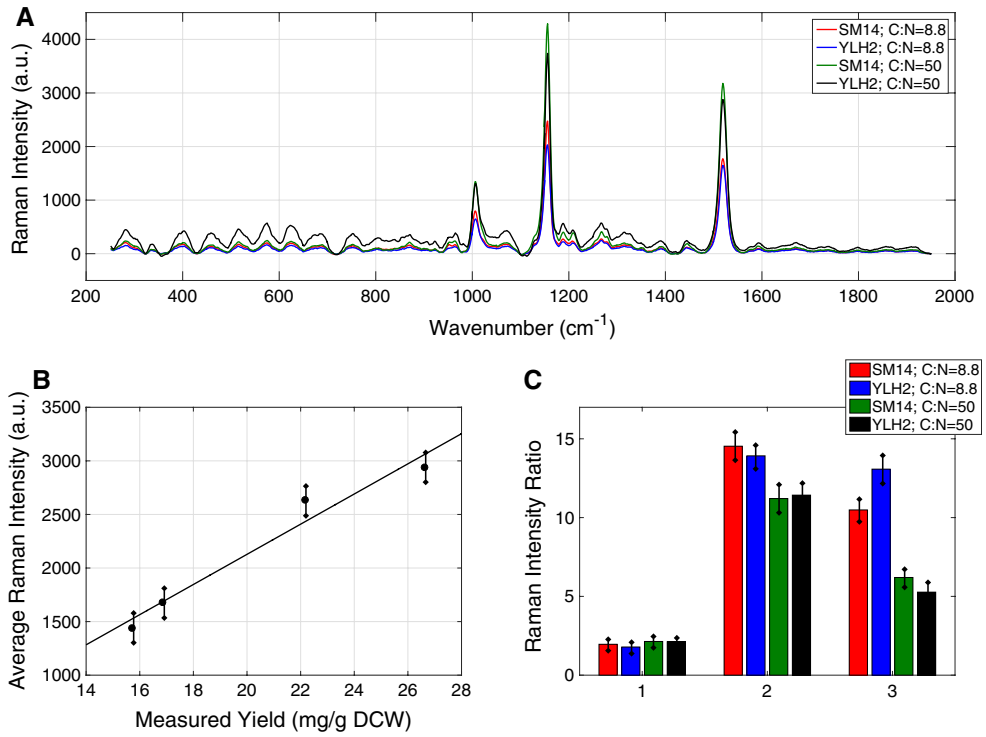

Fig. 7 Study of early-stage growth (<15 h) of SM14 and YLH2 at C:N ratios of 8.8 and 50 by Raman spectroscopy. **a** All raw Raman spectra obtained in the study. **b** Correlation between the measured β-carotene yield and the average intensity of corresponding Raman bands (1006, 1156, and 1519 cm^{-1}). **c** The ratio of Raman band intensities corresponding to (1) unsaturated (1263 cm^{-1}) to saturated (1444 cm^{-1}) fatty acids, (2) β-carotene (1156 cm^{-1}) to total protein (1214 cm^{-1}), and (3) β-carotene (1156 cm^{-1}) to unsaturated fatty acids (1263 cm^{-1}). *Error bars* represent plus and minus 1 standard deviation

The Raman comparison of SM14 to the parental YLH2 strain, at different C:N ratios of 8.8 and 50, is given in Fig. 7. Here, all strains were harvested during early growth (<15 h), so the β-carotene Raman bands dominate the spectra (Fig. 7a). Good correlation was observed between Raman band intensity and measured β-carotene yield, as shown in Fig. 7b. Raman results agreed with the previously presented results that the SM14 strain produces more β-carotene than YLH2, and the C:N ratio of 50 enhances production further. Virtually, no differences were seen in the unsaturated to saturated fatty acids ratios (Fig. 7c), but increases in overall fatty acids production are apparent (Fig. 7a). Statistical analysis confirmed that the unsaturated to saturated fatty acid ratios were not statistically different. Also of interest is the β-carotene-to-protein ratio, which is almost 20 % higher in the SM14 strain relative to YLH2. While the 50 C:N ratio boosted β-carotene production, it also resulted in a lower β-carotene to total protein ratio (Fig. 7c). With a C:N ratio of 8.8, the β-carotene-to-total protein ratios of the SM14 and YLH2 strains were statistically different using Student's *t* test at 90 % confidence, and these were not statistically different at a C:N ratio of 50. However, when comparing the C:N ratio of 8.8 and 50, the β-carotene-to-total protein ratio of both strains was significantly different at 99 % confidence. Also shown in Fig. 7c is the ratio of β-carotene to unsaturated fatty acids. At a C:N ratio of 8.8, the SM14 strain had a significantly lower ratio than the YLH2 strain, indicating that increased β-carotene production was accompanied by increased lipids production. This degree of difference between the strains was not observed at a C:N ratio of 50, however. For the β-carotene-to-unsaturated fatty acids ratios shown in Fig. 7c(3), all data points were found to be statistically different from one another at 99 % confidence using the Student's *t* test.

Discussion

In this work, previously isolated carotenoids hyper-producers and unevolved ancestral strain YLH2 were further characterized in a bench-top bioreactor. First, the growth conditions in the bioreactor were varied to determine which parameters had the most effect on β-carotene production. Three bioreactor parameters (media composition, agitation, and pH) were studied, and the resulting metabolic profiles were compared and correlated with β-carotene production in the cells. In our experiments, a dramatic increase in the

production of β-carotene was observed after the majority of the glucose substrate had been consumed (Fig. 1). A similar trend was seen in a study using *S. cerevisiae* for bisabolene production, which used the same pathway and precursor enzymes as those for β-carotene production [23]. Prior work has also correlated increase of ethanol and acetate concentrations to increased activity of the isoprenoid pathway in yeast [7, 20]; ethanol enhances the activity of HMG-CoA reductase and increases the amount of mevalonate in the cell. Acetate is the precursor to acetyl-CoA, which is the precursor of the MVA pathway [9]. An increase in acetyl-CoA formation likely leads to increased production of the MVA downstream products such as β-carotene. Despite the similar metabolic profiles between the evolved mutants and the ancestral strain, there was a considerable difference in β-carotene yields and productivities between the two strains in 8.8 C:N YNB medium. The β-carotene production [mg g^{-1} (dcw)] in the ancestral strain remained relatively constant within a 72-h fermentation. In the hyper-producers, however, there was a continued increase in the level of β-carotene accumulation in the cells throughout the run (Figs. 1, S7). β-carotene productivities for SM14 and YLH2 in 8.8 C:N YNB medium were 1.86 ± 0.74 and 0.42 ± 0.25 mg L^{-1} h^{-1}, respectively. This may suggest that our hyper-producers in 8.8 C:N YNB medium are more responsive to activation by ethanol and acetate metabolites (produced and secreted by the cell) in the culture medium; however, further experiments are needed to verify this hypothesis. When grown in 50 C:N YNB medium, both SM14 and YLH2 demonstrated continuous β-carotene production over the course of the fermentations (Fig. 5). For SM14, β-carotene productivity in 50 C:N YNB medium remained close to productivity levels in 8.8 C:N YNB medium at 1.90 ± 0.31 mg L^{-1} h^{-1}; however, YLH2 productivity in 50 C:N YNB medium increased by 291 % to 1.66 ± 0.07 mg L^{-1} h^{-1}. Overall, a production level of 25.52 ± 2.15 mg β-carotene g^{-1} (dcw) in 50 C:N YNB medium was obtained in a batch-mode benchtop bioreactor using strain SM14 (Fig. 4). The similarity between the metabolic profiles between SM14 and YLH2 at 50 C:N ratio suggests that mutations, which conferred the enhanced β-carotene production, in SM14 led to a similar phenotype as that observed from an increased C:N ratio in the parental YLH2 strain. The exact mutations responsible for the enhanced β-carotene production in SM14 and the mechanisms involved are of interest for further strain development and are under investigation.

Because β-carotene is a hydrophobic lipid-soluble molecule and generally accumulates within lipid bodies in a cell, it was hypothesized that potentially increasing the total lipid content in the cell would increase β-carotene production [18]. As an attempt to increase the intracellular lipid content and thus potentially increase the possibility for enhanced carotenoids storage capacity in the cell, a higher carbon-to-nitrogen ratio was used in the medium formulation by reducing the nitrogen content. There was an increase in β-carotene production in the higher carbon-to-nitrogen ratio medium (Fig. 4), and the total lipid content in the cell was quantified using Nile red (data not shown), which is a fluorescent lipophilic dye that is used for detection and quantification of intracellular neutral lipids in algae, yeast, fungi, and even macrophages. Previous studies have used Nile red to quickly and economically quantify the intracellular lipid content in yeast; however, Nile red quantification has also been reported to have several limitations [13, 28], and the results were inconsistent (data not shown). Further studies involving more detailed lipid analysis are needed to understand the lipid profile of the cell with respect to growth on different carbon-to-nitrogen ratios.

β-Carotene was produced in high amounts and displayed a significant Raman signal. Thus, it is conceivable to see how this technology could be used for bioprocess monitoring and online measurements for the detection of intracellular compound accumulation. It is noted that only β-carotene measurements were calibrated with the Raman signal intensity. All other measurements (e.g., protein and fatty acids) were relative observations (i.e., ratios of Raman bands) based on previous Raman band assignments [32], and more analytical work is necessary to discern total amounts of these macromolecules from the Raman data alone. However, this demonstrates for the first time that Raman spectroscopy can be used to monitor β-carotene production in engineered yeast while monitoring culture phenotype changes over the time course. It is hypothesized that this methodology could be used to identify optimal harvest conditions that minimize downstream processing costs. Raman data also suggested a higher fatty acid content in SM14 compared with YLH2 at 8 C:N ratio and an increase in fatty acid content in both strains at 50 C:N ratio (Fig. 7c(3)), which further warrants future investigation into the correlation between C:N ratio, lipid content, cell growth, and carotenoids production in *S. cerevisiae*.

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