Chapter 14

Isolation of Carotenoid Hyperproducing Mutants of *Xanthophyllomyces dendrorhous (Phaffia rhodozyma)* by Flow Cytometry and Cell Sorting

Byron F. Brehm-Stecher and Eric A. Johnson

Abstract

Approaches for improving astaxanthin yields in *Xanthophyllomyces dendrorhous* include optimization of fermentation conditions and generation of hyperproducing mutants through random mutagenesis using chemical or physical means. A key limitation of classical mutagenesis is the labor-intensive nature of the screening processes required to find relatively rare mutants having increased carotenoid content, as these are present against a high background of low-interest cells. Here, flow cytometry is described as a high-throughput, single-cell method for primary enrichment of mutagenized cells expressing high levels of astaxanthin. This approach improves the speed and productivity of classical strain selection, enhancing the chances for isolating the carotenoid hyperproducing mutants (CHMs) needed to enable high-titer, economical production of natural astaxanthin.

Key words: *Xanthophyllomyces dendrorhous*, *Phaffia rhodozyma*, Astaxanthin, Mutant, Flow cytometry, Cell sorting

1. Introduction

Carotenoid biosynthesis is a fundamental process in plants, algae, photosynthetic bacteria, and certain fungi. Carotenoids play important roles as photoprotectants or precursors to hormones, and they may have as of yet undiscovered functions in producer organisms. In addition to their biological roles, carotenoids also have high economic value and are used in agriculture as vitamin precursors and colorants in feeds, particularly in aquaculture (1). Since many carotenoids are excellent antioxidants and quench singlet oxygen and other reactive oxygen species, there is also great interest in their use in nutraceutical or cosmeceutical applications to prevent various human diseases and to slow the aging process.

José-Luis Barredo (ed.), *Microbial Carotenoids From Fungi: Methods and Protocols*, Methods in Molecular Biology, vol. 898,
DOI 10.1007/978-1-61779-918-1_14,

An example of a high-value carotenoid at the nexus of several industrial markets (agriculture, food, nutraceutics, cosmetics, etc.) is astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione). Although it is a natural product, much of the astaxanthin used today in applications such as aquaculture is produced synthetically from petrochemical feedstock (1, 2). The red yeast *Xanthophyllomyces dendrorhous* (the teleomorphic state of *Phaffia rhodozyma*) produces astaxanthin as its principal carotenoid. Along with the microalgae *Haematoccoccus pluvialis*, *X. dendrorhous* is a promising natural source of astaxanthin. However, in order to become competitive with synthetic production, the astaxanthin titer from microbial fermentations must be increased substantially (e.g., ~10–50-fold) (1, 3).

The current market value of synthetic astaxanthin is estimated to be $2,500 US per kg, but naturally produced astaxanthin can command a higher market price (1). This and additional factors, such as consumer preference for natural products and "greener" production methods, are fueling a resurgence of interest in methods for improved microbial production of this carotenoid (1). Approaches for improving astaxanthin yields in *X. dendrorhous* have included optimization of fermentation conditions, generation of hyperproducing mutants through random mutagenesis using chemical or physical means, and, more recently, metabolic engineering of microbial carotenoid pathways (1, 3). Despite the potential drawbacks of classical mutagenesis, such as spontaneous reversion or introduction of secondary mutations that may reduce strain fitness, the simplicity of these methods is attractive, especially when used in conjunction with optimized fermentation conditions (1, 3). However, a key limitation of classical mutagenesis is the labor-intensive nature of the screening processes required to find the "needle(s) in the haystack"—the relatively rare cells having increased carotenoid content that may be present against a high background of low-interest cells. High-throughput, single-cell methods for primary enrichment of mutagenized cells expressing high levels of astaxanthin would improve the speed and productivity of classical approaches and would enhance the chances for isolating the carotenoid hyperproducing mutants (CHMs) needed to enable high-titer, economical production of natural astaxanthin.

This chapter outlines a general approach for using flow cytometry and cell sorting (FCCS) to isolate CHMs of the red yeast *P. rhodozyma* (*X. dendrorhous*) after chemical mutation. The first description of this technique was published approximately 20 years ago (4). Since then, there have been many advances in instrumentation, including more sensitive detectors, more capable and user-friendly analysis software, and enhanced (approximately tenfold) sorting capacity. In 2008, Ukibe et al. revisited this original approach and made some improvements to the technique (5). Although the underlying concept remains the same, the method has been refined, with particular reference to the wavelength of

light chosen to monitor astaxanthin fluorescence for the isolation of astaxanthin hyperproducing mutants. These refinements are reflected here.

2. Materials

2.1. Yeasts

1. *X. dendrorhous* ATCC 24230 (American Type Culture Collection, Manassas, Virginia, USA) (4) (see Note 1).
2. *X. dendrorhous* ATCC 96815 (American Type Culture Collection, Manassas, Virginia, USA) (see Note 1).
3. *X. dendrorhous* ATCC 74218 (American Type Culture Collection, Manassas, Virginia, USA) (see Note 1).

2.2. Culture Media and Mutagenesis

Complex media routinely used for the growth of yeasts are used.

1. YM: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L dextrose.
2. YM solid: YM and 20 g/L agar.
3. Ethyl methanesulfonate (EMS).
4. Phosphate buffer 0.05 M, pH 7.0.
5. Phosphate buffer 0.1 M, pH 7.0.

2.3. Carotenoid Extraction and Analysis

1. Carotenoid standards (Sigma-Aldrich Co., St. Louis, MO, USA).
2. Phosphate-buffered saline: 0.1 M potassium phosphate, 0.1 M NaCl, pH 7.4.
3. AMINCO French press (American Instrument Company, Silver Spring, MD, USA).
4. Acetone and hexane 1:2 (vol/vol).
5. N_2 gas.
6. HPLC (Rainin Instrument Co., Emeryville, CA, USA).
7. C18 reverse-phase column (250×4.6 mm, 5 μm particle size) (Alltech Econosphere, Grace, Deerfield, IL, USA).
8. Solvent A: 85% acetonitrile and 15% methanol.
9. Solvent B: 100% dichloromethane.
10. Dynamax UV-1 UV/vis detector (Rainin Instrument Co., Emeryville, CA, USA).

2.4. Flow Cytometry and Cell Sorting

1. Sorting instrument.
2. Potassium phosphate buffer 10 mM, pH 7.4.
3. Polystyrene tube (5-mL tube fitted with a cell-strainer cap) (Becton Dickinson, Franklin Lakes, NJ, USA).
4. Polystyrene tube (5-mL tube with a CellTrics® in-tube filter) (Partec GmbH, Görlitz, Germany).

3. Methods

Best practices for carrying out basic research should be followed, including use of analytical-grade reagents, ultrapure (18 MΩ) deionized water, and proper handling and disposal of biological and chemical wastes in accordance with existing regulations or institutionally approved practices.

3.1. Growth and Harvest of Yeast Cells

1. Seed flasks (100–300 mL) containing YM broth (20–30 mL) with isolated colonies from YM plates.
2. Incubate the flasks at 18–20°C and 200 rpm until the desired growth phase is reached:
   - 2.1. For mutagenesis, grow the cells for 2 days, harvest them by centrifugation and treat them as described below (see Subheading 3.2).
   - 2.2. For traditional isolation of CHMs, inoculate mutagenized cells onto YM plates, grow for 7–10 days and screen visually for the desired pigmentation. Then, grow isolated colonies for 5–7 days in flasks prior to carotenoid extraction and analysis via HPLC.
   - 2.3. For screening of CHMs via FCCS, inoculate mutagenized cells into fresh YM broth and grow for as little as 2 days after treatment with EMS, followed by cytometric screening (see Subheading 3.4).

3.2. Yeast Mutagenesis

As described elsewhere (3, 4), various chemical or physical methods for inducing mutations may be used for generation of carotenoid mutants in addition to the EMS method described here. These include UV light, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), ethidium bromide, and acriflavine.

1. For EMS mutagenesis, harvest 2-day-old cultures of flask-grown cells via centrifugation (800 × *g*, 2 min) and wash them with potassium phosphate buffer (0.1–0.05 M, pH 7.0).
2. Adjust cell suspensions to an optical density at 660 nm of 0.3–0.4.
3. Resuspend in the same buffer containing 3–4% EMS (see Note 2).
4. Incubate treated suspensions with shaking at yeast growth temperatures (18–20°C) or simply allowed to stand for a period after treatment.
5. Add sodium thiosulfate to inactivate EMS (see Note 3).

3.3. Carotenoid Analysis

There are various approaches for recovery and analysis of cellular carotenoids (5, 6). The relatively straightforward approach reported by Echavarri-Erasun and Johnson (7) is described below. Astaxanthin and other carotenoid standards are commercially available.

1. Grow cells for 5 days in 30 mL YM broth (see Subheading 3.1), harvest via centrifugation and wash with distilled water.
2. Resuspend cells in phosphate-buffered saline and chill on ice for at least 10 min prior to lysis.
3. Lyse chilled cell suspensions at 1,500 psi pressure in a French press. Monitor microscopically the completeness of cell lysis.
4. Add 1:2 (vol/vol) mixture of acetone and hexane to each sample and mix samples vigorously for 2 min.
5. Separate aqueous and organic phases via centrifugation and remove the organic supernatant to a clean 10-mL glass tube (wrapped in aluminum foil to exclude light).
6. Repeat extractions until the cellular debris pellet is colorless, indicating complete extraction of carotenoids.
7. Dry the organic phase under N_2 and store the extract at −20°C until analyzed.
8. Carry out the quantitative carotenoid analysis via HPLC using a C18 reverse-phase column. Use a flow rate of 1 mL/min for each 18-min run, with the solvent gradient as follows: (a) 100% solvent A, 1 min, (b) linear gradient over 1.5 min to 32% solvent B; isocratic conditions with 32% solvent B for 11 min, (c) decreasing linear gradient of solvent B to 100% solvent A for 30 s, and (d) system reequilibration with 100% solvent A for 4 min.
9. Monitor astaxanthin absorption at 474 nm using an UV-1 UV/vis detector. Collect astaxanthin peaks eluting at 3.85 min, dry and store as described above.

3.4. Flow Cytometry and Cell Sorting

Flow cytometry technology is continually evolving and several sorting instruments are produced commercially. Because different cytometers will undoubtedly be available to individual researchers, no recommendation for specific instrumentation is given here, other than that the system used must be capable of high-throughput analysis and physical sorting of target cells based on their physical or physicochemical characteristics, specifically high astaxanthin content. Available sorting instruments include those from BD Biosciences, Beckman Coulter, iCyt, and Partec, with some instruments delivering sort rates of 100,000–200,000 cells/s. Other instruments, while no longer commercially manufactured, may still be available in academic labs. As noted above, FCCS-based screening can be carried out on yeast populations after as little as 2 days growth following mutagenesis.

1. Wash cells with and resuspend in 10 mM phosphate buffer pH 7.4.
2. Filter cell suspensions through a 40-μm mesh and collect into a 5-mL polystyrene tube. For example, a 5-mL tube fitted with

a cell-strainer cap (Becton Dickinson) or with a CellTrics® in-tube filter (Partec) may be used (see Note 4).

3. Adjust the concentration of the cell suspension to be analyzed to achieve quality sorting of CHMs (see Note 5).
4. Use a sorting flow cytometer equipped with a 488-nm excitation laser. Commercial sheath fluid or 0.1 M sodium phosphate, pH 7.0 may be used as sheath fluid (see Note 6). Calibrate flow cytometers daily using commercially available fluorescent bead kits and associated software (see Note 7).
5. Analyze cells in log/log mode and collect multiple parameters, including forward scatter (FSC), side scatter (SSC), and fluorescence. Use factory default band-pass filters designed to transmit light centered at specific wavelengths (see Note 8).
6. Use defined control cultures to facilitate proper definition of sort regions and to demonstrate experimentally that the approach is capable of detecting and isolating CHMs having high astaxanthin content (see Note 9).

3.5. Isolation of Carotenoid Hyperproducing Mutants via FCCS

1. Open an acquisition plot of FL4 (675 nm) versus FL1 (525 nm).
2. Run samples of wild-type and astaxanthin hyperproducer control strains, examining where each population falls on this plot.
3. Draw four contiguous rectangular gates as shown in Fig. 1, so that when a 1:100 mixture of CHM to wild-type strains is examined, the greatest number of events is centered in gate A and those events having the highest fluorescence fall into gate C.
4. Apply the sort regions established above to the examination of mutagenized cell populations grown for ~2 days, as described above.
5. Sort those events falling into region C (or region D, if applicable) and collect these into glass tubes containing sterile YM broth. With accurate sorting, this process should yield an approximately tenfold physical enrichment for *X. dendrorhous* cells having high astaxanthin content (see Note 10).
6. Collect cells having increased fluorescence at 675 nm via FCCS and deposit into YM broth (a primary screen).
7. Plate cells onto YM agar and screen as in the traditional approach. This step represents a secondary screen carried out on the FCCS-enriched population.
8. Grow cells for 7–10 days and examine visually for highly pigmented colonies.
9. Grow colonies of interest for 5–7 days in flasks prior to carotenoid extraction and analysis via HPLC.

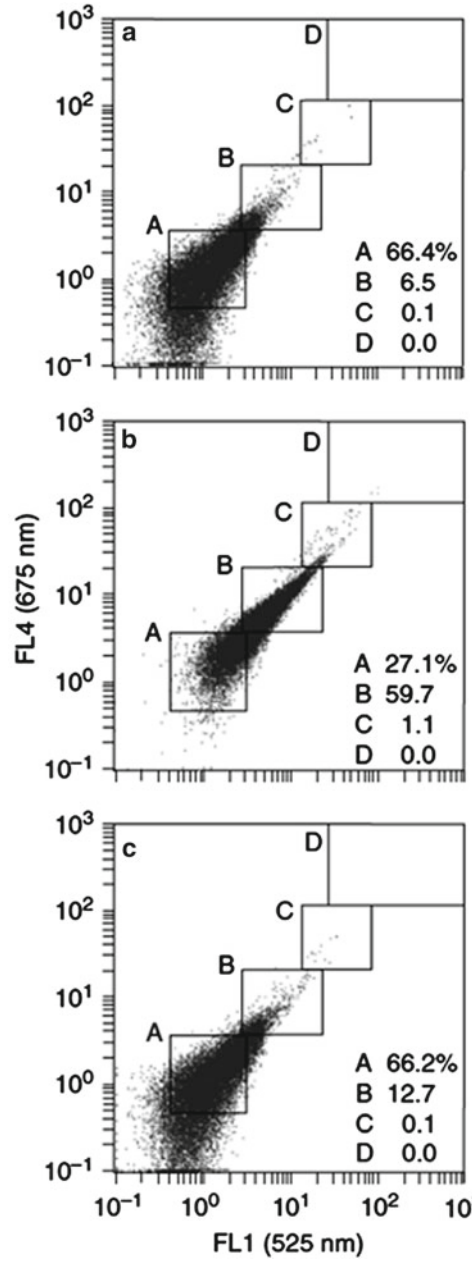

Fig. 1. Gating solution for sorting of *Xanthophyllomyces dendrorhous* based on cellular astaxanthin content. This figure shows an experimental validation of a gating solution for sorting of *X. dendrorhous* cells with increased astaxanthin content. Log/log plots of FL4 (675 nm) and FL1 (525 nm) fluorescence were used to analyze three different samples: (**a**) A wild-type strain (Y989), (**b**) a CHM strain (Y2342), and (**c**) a 1:100 mixture of CHM and wild-type strains. Four contiguous rectangular gates were drawn based on the FL4/FL1 responses of the wild-type and CHM strains when assessed individually. For the 1:100 cell mixture, gate *A* contained the bulk of the events and gate *C* contained the most highly fluorescent events. Cells from gates *A–D* were sorted and plated onto YM agar. Plates inoculated with cells sorted from gate *C* were enriched ninefold for the CHM strain (Y2342). The same approach was applied to EMS-mutagenized cells, enabling the authors to select for mutants producing between 1.5 and 3.8-fold more astaxanthin than parent strains. The overall efficiency of the FCCS-based method was five times greater than for the traditional plating method, allowing isolation of 39 CHM strains versus only seven for plating. Figure reproduced from Ukibe et al. (5), with permission from John Wiley & Sons.

4. Notes

1. Wild-type strains of *X. dendrorhous* used in the lab for basic research or for process development may be used as parent strains for mutagenesis and selection of CHM's. These strains are typically well characterized and possess known and desirable attributes, such as sufficient growth rates, cell yield, good basal level of expression for the carotenoid of interest, etc. Examples of yeasts used as the basis for generation and FCS-based screening for CHMs include natural isolates such as *X. dendrorhous* ATCC 24230 (4) or industrial strains (6). Defined carotenoid mutants (albino strains such as ATCC 96815 and CHMs isolated via traditional methods, such as ATCC 74218 and related strains) are used as negative and positive controls when setting up and validating FCCS gating strategies (4, 6) (see Subheading 3.5).
2. EMS is a hazardous compound, is an inhalational and contact hazard, and is toxic by ingestion. Exposure to EMS must be avoided through proper containment procedures and use of appropriate personal protective equipment. Refer to the manufacturer's Material Safety Data Sheet (MSDS) for additional information. As with EMS, alternative mutagens should be considered hazardous and treated with the same care as described here for use of EMS.
3. Although EMS may be inactivated with the addition of sodium thiosulfate, this treatment may further reduce yeast viability. Alternatively, EMS may be removed using successive water washes. EMS exposure parameters should be chosen to yield a 60–70% survival rate after treatment. Variables expected to affect yeast survival rate include the strain used, EMS dosage, cell density, and incubation conditions. Initial experiments should be performed to achieve the dosage conditions producing the desired survival rate.
4. This step is important because the assay is intended to screen for and physically isolate individual cells having higher astaxanthin content. Therefore, the presence of cell aggregates could be a potentially confounding factor for this single-cell technique. Filtered cell suspensions are diluted further in the same buffer and diluted to an optimal concentration for sorting.
5. An et al. (3) analyzed a suspension at ~10^7 CFU/mL and Ukibe et al. (5) used a suspension at ~2×10^6 CFU/mL. However, optimal sorting concentrations may differ among various instruments. Therefore, initial experiments should be performed to optimize this parameter. As an example, with the BD FACSAria III, the best sort rates are typically achieved by

balancing the cell concentration and the sample pressure settings, with the highest possible cell concentration and lowest possible sample pressure yielding optimal results. Appropriate control populations should be used to determine and validate the optimal sorting parameters.

6. Although An et al. (3) used a laser operating at 200 mW, other instruments equipped with lower-power lasers may be used, such as the 20-mW laser used in the Becton Dickinson FACSAria III instrument or the 15-mW laser used in the Beckman Coulter EPICS Elite ESP instrument (5). A 60- to 70-μm nozzle is used, depending on the instrument used. Commercial sheath fluid is formulated with sodium azide and surfactants. No information on the impact of azide on *X. dendrorhous* recovery after sorting is available. If desired, azide-free 0.1 M phosphate buffer, pH 7.0 may be used.
7. Because flow cytometers are inherently variable, absolute scatter or fluorescence values may differ from instrument to instrument, or even from day to day on the same instrument. Therefore, instruments are calibrated daily using commercially available fluorescent bead kits and associated software, according to manufacturer's instructions. Fluorescent beads are also used to set the sort delay prior to sorting of cells.
8. For example, the ranges reported by Ukibe et al. (5) for their instrument were FL1 (490–550 nm), FL2 (570–580 nm), FL3 (600–620 nm), and FL4 (655–685 nm). Although "factory presets," or default filter sets are useful for selection of *X. dendrorhous* CHMs, custom filters are available for use, if deemed warranted. These are available from custom vendors such as Chroma Technology Corporation (Bellows Falls, VT, USA). Threshold settings for FSC and SSC, detector voltages and fluorescence compensation settings will differ among instrument makes and among individual instruments. These are therefore determined locally for each experiment. Typically, microorganisms such as bacteria and yeasts are thresholded on SSC. SSC threshold settings are obtained by examining both unstained yeasts and buffer without yeasts. The setting that provides clear SSC signals from yeasts while minimizing background noise (checked using buffer control) is used.
9. Flow cytometry is a valuable tool that investigators can use to study complex populations according to specific cell traits. It is expected that differences in fluorescence staining among cell populations can be linked to underlying biological phenomena. In order to demonstrate such linkage, sound experimental design is required, including use of appropriate controls. These controls are critical to data collection and interpretation. Both albino and CHM strains are available through the American Type Culture Collection (ATCC). Albino strains available

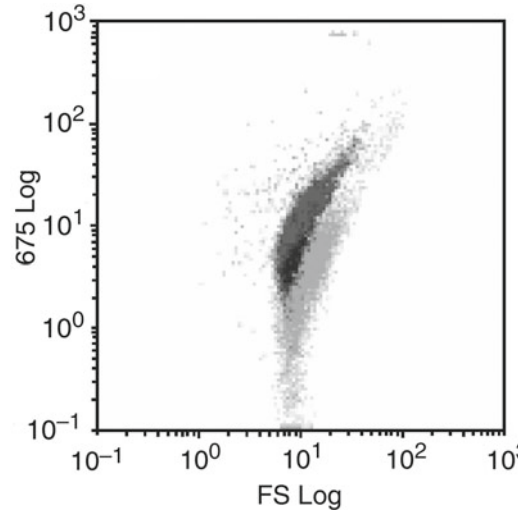

Fig. 2. Astaxanthin-related differences in fluorescence of *Xanthophyllomyces dendrorhous* cells can be detected by flow cytometry. An astaxanthin-overproducing strain of *X. dendrorhous* (Y2342) was grown in YM with (a) the addition of 50 μM diphenylamine, an inhibitor of astaxanthin biosynthesis (*light gray* population) or (b) without added diphenylamine (no inhibition of astaxanthin, *darker* population). Plotting of fluorescence in FL4 (675 nm) against FSC allows clear differentiation between astaxanthin-producing cell populations and those in which astaxanthin synthesis was chemically inhibited. These results suggest that similar differentiation of CHM and wild-type cells, based on astaxanthin content, can be carried out via cytometric analysis of chemically mutagenized *X. dendrorhous* populations. Figure reproduced from Ukibe et al. (5), with permission from John Wiley & Sons.

include ATCC 96815. CHM strains include ATCC 74218, ATCC 74219, ATCC 74220, and ATCC 74221. When screening for *X. dendrorhous* CHMs, appropriate controls include *X. dendrorhous* strains representative of the lower-, middle-, and upper-tier responses for astaxanthin-related fluorescence. Positive and negative control cultures (e.g., astaxanthin CHMs and albino strains), as well as wild-type strains, are used. These strains should be analyzed alone and in defined mixtures. In the absence of defined mutant strains, chemical inhibition of carotenoid synthesis may also be used to generate cells having different carotenoid content. Figure 2 illustrates differences seen between an astaxanthin-overproducing strain of *X. dendrorhous* (Y2342) grown with 50 μM diphenylamine (an inhibitor of astaxanthin biosynthesis, gray population) or with 0 μM diphenylamine (no inhibition of astaxanthin, black population). When these strains were examined as a function of fluorescence in FL4 (675 nm), the astaxanthin-overproducing strain was notably shifted toward increased fluorescence in FL4 (5). For reporting purposes, experimental details should meet agreed upon standards for the minimal information required of flow cytometry experiments (MIFlowCyt) (8).

10. Other options for sorting, according to the instrument used, include sorting into 96-well plates or onto solid agar media.

Acknowledgments

The methods described here are based on the work of Gil-Hwan An, whose doctoral work in the Johnson laboratory was focused on "Improved astaxanthin production from the red yeast *P. rhodozyma*" (4), and on the work of Ukibe et al. (5) who recently revisited and revised our original protocol for FCCS-based selection of CHMs.

References

1. Rodríguez-Sáiz M, de la Fuente JL, Barredo JL (2010) *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin. Appl Microbiol Biotechnol 8:645–658
2. Johnson EA (2003) *Phaffia rhodozyma*: colorful odyssey. Int Microbiol 6:169–174
3. An GH, Bielich J, Auerbach R et al (1989) Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. Biotechnology (N Y) 9:70–73
4. An GH (1991) Improved astaxanthin production from the red yeast *Phaffia rhodozyma*. PhD thesis, University of Wisconsin, Madison
5. Ukibe K, Katsuragi T, Yoshiki T et al (2008) Efficient screening for astaxanthin-overproducing mutants of the yeast *Xanthophyllomyces dendrorhous* by flow cytometry. FEMS Microbiol Lett 286:241–248
6. An GH, Schuman D, Johnson E (1989) Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl Environ Microbiol 56:2944–2945
7. Echavarri-Erasun C, Johnson EA (2004) Stimulation of astaxanthin formation in the yeast *Xanthophyllomyces dendrorhous* by the fungus *Epicoccum nigrum*. FEMS Yeast Res 4:511–519
8. Lee JA, Spidlen J, Boyce K et al (2008) MIFlowCyte: the minimum information about a flow cytometry experiment. Cytometry A 73A:926–930