Optimization of Carotenoids Production from *Camelina Sativa* Meal Hydrolysate by *Rhodosporidium Toruloides*

STEFANO BERTACCHI (s.bertacchi@campus.unimib.it)
University of Milano–Bicocca: Universita degli Studi di Milano-Bicocca https://orcid.org/0000-0002-8281-7625

Chiara Cantù
University of Milano–Bicocca: Universita degli Studi di Milano-Bicocca

Danilo Porro
University of Milano–Bicocca: Universita degli Studi di Milano-Bicocca

Paola Branduardi
University of Milano–Bicocca: Universita degli Studi di Milano-Bicocca

Research Article

Keywords: Biobased process, carotenoids, residual biomasses, Rhodosporidium toruloides, fermentation

Posted Date: June 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-607126/v1

Abstract

Background

Petrochemical synthetic dominates several markets, and carotenoids are not an exception. Since their applications in the food, feed and cosmetic sectors, carotenoids of natural origin are increasingly requested, but the production needs to be sustainable also in terms of initial feedstock. For these reasons we deployed the carotenogenic yeast *Rhodosporidium toruloides* to obtain such compounds from *Camelina sativa* meal, an underrated lignocellulosic biomass. As the process starts from hydrolyzed biomass, we separately optimized enzymatic and biomass loadings, to reduce the overall process costs.

Results

The best conditions (9% w/v biomass, 0.56% $w/w_{biomass}$ enzymes) were tested in different settings, in which fermentation was separate or co-current with the hydrolysis, showing similar carotenoids productions. The process was implemented in stirred-tank bioreactors, obtaining 3.6 ± 0.69 mg/L of carotenoids, and showing to be robust towards changes in different parameters.

Conclusions

These data pave the way to evaluate a possible industrialization of this bioprocess, considering the opportunity to optimize the use of different amounts of biomass and enzyme loading. In addition, the test in bioreactor is an additional step to further develop the proposed process.

Background

The widespread use of fossil resources is known to be detrimental for the environment as well as for human health [1], therefore alternatives as the development of bioprocesses based on renewable biomasses are desirable. Nonetheless, these processes are still not competitive towards the traditional petrochemical ones, despite the gap is not equal for all. Indeed, in the light of the principles of cascading, several commodities are requested by the market of reference in low amounts, such as compounds related to pharmaceutical, nutrition and cosmetic sectors [2–4]. Their high added value makes these molecules attractive for the scouting of biobased processes, especially from those originated from residual agro-industrial streams (often called lignocellulosic biomasses, LCBs). Such biomasses can be valorized often by the means of microbial cell factories, whose metabolism can transform carbon and energy sources of LCB origin into valuable compounds of interest [5].

In this scenario, we focused our attention on the exploitation of *Camelina* meal, the main by-product of oil extraction from *Camelina sativa* seeds [6–8]. This biomass is currently used as feed supplement: since its macromolecular components and relatively low cost ($0.25/kg) [9, 10], it is an attractive biomass for the development of sustainable bioprocesses. As we previously described in [11], it is possible to hydrolyse *Camelina* meal by the use of an enzymatic cocktail and provide it as sole component of the

growth medium to the yeast *Rhodosporidium (Rhodotorula) toruloides* for the production of carotenoids, by applying separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) processes [11].

R. toruloides is naturally able to accumulate carotenoids, such as β-carotene, torulene, and torularhodin [12–14], and productions were obtained also using residual biomasses as feedstock [15–17]. Recalling the cascading principles, carotenoids are high added value products, with a global market value expected to reach $2.0B by 2022 and mainly deployed in animal feed, cosmetic, food and dietary supplements [18–21].

In this work we propose process optimizations aimed to reduce materials (enzymes and substrate loading) and fermentation time of carotenoids production from *C. sativa* meal.

Enzymatic cocktails, although being valuable ally in the hydrolysis of LCBs and in the release of fermentable sugars, have costs that decisively impact second generation biorefineries, since the maximum hydrolysis yield is often not likely the economical optimum as well [22, 23]. Similarly, total solid loading is a parameter that could impact on production costs [24], therefore its optimization should be considered as well. Furthermore, the industrial implementation of a bioprocess needs a scale-up, which may bring to the rise of hurdles and complications [25, 26]: at lab scale, whenever possible, we need to acquire quantitative data in bioreactor under industrially relevant conditions.

In the present work, we considered separately enzymatic loading and the amount of total solid loading as variables of the hydrolysis to be modulated in order to release sufficient quantity of sugars for the growth of *R. toruloides*. Combining the two best options in a SHF process, *R. toruloides* specific productivity of carotenoids resulted increased if compared with the initial setting. With these data we ran fermentations at bioreactor scale and explored SSF and SHF to obtain quantitative data on carotenoids production by *R. toruloides* from *Camelina* meal, with the aim of promoting the industrial applications of this residual, yet underestimated, biomass.

Results And Discussion

Optimization of the enzyme loading for Camelina meal hydrolysis

Glycosidic enzymes are cost items when assessing the economics of a bioprocess, therefore reducing the loading of enzymatic cocktails can be crucial to reduce the overall costs. Nevertheless, the yield of the sugars released must be considered to evaluate the performances of the enzymatic hydrolysis itself and the subsequent cellular production of interest. In a previous work we hydrolyzed 15% (w/v) of *Camelina* meal with 11.9% w/w$_{\textit{Camelina}\ \text{meal}}$ of enzymatic cocktail for carotenoid production by *R. toruloides* [11]. In this work, enzyme loadings were gradually decreased from 11.9–0.56% w/w$_{\textit{Camelina}\ \text{meal}}$, pairing with the lower dose the specific usage indications by the manufacturer.

As shown in Fig. 1 (first set of bars, from the left), a significant reduction in sugar release could be measured when comparing 11.90% and 8.93% w/w$_{Camelina\ meal}$ to the lower enzymatic loadings. This can be related to the inhibitory effect of the biomass on enzymatic hydrolysis, both in terms of compounds released by the pre-treatment and the sequestration by LCBs on the enzymes themselves. Nevertheless, starting from 5.95% down to 0.56% w/w$_{Camelina\ meal}$ of enzyme loading, no significant difference could be observed, as also the lowest dose resulted in the release of about 15 g/L of total sugar.

Starting from the same samples, we considered only the sugars preferentially metabolized by *R. toruloides* (Figure S1, S2) [16, 27] as carbon and energy source (*i.e.* sucrose, glucose and fructose; Fig. 1, central set of bars). Despite we can see a similar trend, it is clear that higher amounts of enzymes mainly promoted the release of sugars such as arabinose and galacturonic acid, as starting from 5.95% of enzyme loading the sum of glucose fructose and sucrose does not significantly differ from the quantity released from the use of higher amounts of enzymes. Arabinose and galacturonic acid are not promptly consumed by *R. toruloides* when the other sugars are present (Figure S1) [11, 28]. Furthermore, when only glucose is considered, that is the first sugar to be consumed by *R. toruloides* and imposes catabolite repression on the other sugars (Figure S1, S2), its total release from the initial biomass is similar with all the different loadings (Fig. 1, right set of bars). Therefore, a reduction in the enzymatic loading by 95% (from 11.90–0.56% w/w$_{Camelina\ meal}$) provided a comparable amount of glucose (and fructose plus sucrose too) in the *Camelina* meal hydrolysate, thus suggesting a way for reducing the cost of the process, which should be further tested and implemented in terms of carotenoid production.

Effect of Camelina **meal solid loadings on the production of carotenoids by** R. toruloides

We previously demonstrated that *R. toruloides* was able to grow and accumulate carotenoids when provided with 15% w/v *Camelina* meal hydrolyzed with 11.9% w/w$_{biomass}$ of enzymatic cocktail [11]. Maintaining this enzyme loading, we here explored to increase the amount of solid loading, to understand the impact on sugar release, and how this would affect *R. toruloides* in a separated hydrolysis and fermentation (SHF) process.

We firstly increased the amount of *Camelina* meal up to 20% w/v, obtaining 35.1 ± 0.07 g/L of total sugars by hydrolysis, therefore maintaining a yield (17.5 ± 0.04%) very similar to the one obtained when starting from 15% w/v of biomass (16.4 ± 1.69% respectively).

Therefore, increasing the solid loading does not impair the enzymatic hydrolysis. Nonetheless, as shown in Fig. 2A, the growth of *R. toruloides* was reduced when provided with 20% w/v *Camelina* hydrolysate compared to 15% w/v in terms of OD. Arguably, this can be related to the presence of higher titers of growth inhibitors in the medium. In respect to the production of carotenoids, with 20% w/v *Camelina* meal it resulted significantly lower compared to the use of 15% w/v of initial biomass (Fig. 2B), suggesting that providing higher quantities of initial biomass is not beneficial for the obtainment of the product of interest.

Carotenoids are produced by cells as scavenger molecules in response to environmental and cellular stresses, such as the stationary phase. Therefore, we decided to move toward the opposite direction, namely lowering the amount of biomass, and, hence, of sugars initially provided to cells. In this context cells could experience precociously a starving situation, therefore accumulating carotenoids early and possibly in higher amounts. The tested solid loadings were 3%, 6%, 9% and 12% w/v, with a loading of the enzymatic cocktail NS22119 calculated in order to release increasingly amount of sugars [11]. The derived growth media were able to support the growth of *R. toruloides*, with a consistent anticipated entrance in stationary phase (Fig. 2A). Interestingly, the use of 6% w/v of substrate sustained a higher growth in terms of OD compared to 9% and 12% w/v, probably due to lower amounts of inhibitors in the media. Figure 2B shows the production of carotenoids over time, for each of the biomass amounts tested in SHF process. Carotenoids accumulation is quite low after 24 h, increasing with the respective entrance into stationary phases. In particular, the most interesting conditions are observed with 9% and 12% w/v, where carotenoids production obtained after 48 h of fermentation are comparable to the production obtained with 15% w/v, but only after 72 h of growth. Consequently, the best productivities were reached using 9% and 12% of *Camelina* meal hydrolysate ($p < 0.05$) (Fig. 2C). Figure 2D shows that the carotenoids yield on 9% w/v after 48 h resulted significantly higher compared to the yield on 12% w/v ($p < 0.05$), but not if compared with the yield with the 6% w/v, although the difference is at the limit of significativity (probably due to the uneven nature of the hydrolysates). Moving to specific productivity, after 48 h of growth this was significantly higher with 9% w/v compared to 6% w/v *Camelina* meal ($2.1 \pm 0.39 \times 10^{-5}$ h^{-1} and $0.5 \pm 0.11 \times 10^{-5}$ h^{-1}, $p < 0.02$), therefore supporting the inferior performance of the latter combination.

Therefore, considering *Camelina* meal solid loading as the variable parameter of the process, optimized conditions for carotenoids synthesis were the use of 9% w/v of biomass and 48 h of fermentation, with the following performances: production = 6.1 ± 0.85 mg/L, productivity = 0.13 ± 0.017 mg/L/h, specific productivity = $2.1 \pm 0.39 \times 10^{-5}$ h^{-1}, yield on CWD = 0.1 ± 0.02%, yield on consumed sugars = 0.1 ± 0.01 %, yield on total sugars provided = 0.02 ± 0.003%. This result highlights the importance to test different conditions when developing bioprocesses, changing single parameters and assessing their effect on the final product.

Combinatory effect of optimized enzymes and biomass titers on the production of carotenoids by R. toruloides

After testing the possibility to reduce the enzymatic loading and the solid loading of *Camelina* meal, we then combined the two strategies in a single process. Therefore, we run a SHF process in which *R. toruloides* was provided with 9% w/v of *Camelina* meal hydrolysed by NS22119 enzymatic cocktail 0.56% $w/w_{Camelina\ meal}$ as growth medium. The results in terms of OD, sugar consumption and carotenoids production are shown in Fig. 3: samples were collected until 48 h in the light of data shown in the previous section. *R. toruloides* did not consume all the sugars provided, arguably due to the depletion of fundamental micronutrients in the medium [11]. Furthermore, the reduction of enzymes and the consequent reduction in sugar titer contributed to anticipate the maximum carotenoid accumulation (2.2

± 0.33 mg/L) to 24 h. Although this value was inferior compared to that obtain from the use of 11.90% w/w$_{Camelina\ meal}$ at 48 h of fermentation, no significant difference could be observed in terms of productivity and specific productivity after 24 h when 0.56% w/w$_{Camelina\ meal}$ were used (0.1 ± 0.01 mg/L/h and 2 ± 0.3 x 10^{-5} h^{-1}, respectively). Despite a techno-economic analysis (including downstream processing and possible related issues) was not performed yet, it is reasonable to conclude that reduction in both process time and enzymatic loading would in turn reduce the overall cost of the process.

Therefore, optimized conditions for the synthesis of carotenoids by *R. toruloides* were the use of 9% (w/v) *Camelina* meal hydrolysed with 0.56% w/w$_{Camelina\ meal}$ of NS22119 as medium for 24 h of growth in a SHF process. Furthermore, in these conditions the specific growth rate (μ) of *R. toruloides* was calculated to be 0.25 h^{-1}, with a duplication time of 2.74 h (Figure S3). Entrance in exponential phase was evaluated to be reached after about 5 h from start, whereas the entrance in stationary phase after 21 h, coherently with what disclosed in Fig. 3.

We further explored the use of such conditions in SSF and SSF + presaccharification processes, to evaluate the effect of the presence of water insoluble components (WIS) as potential stressing agent triggering carotenoids production. Figure 4 shows that *R. toruloides* was able to consume sugars and to produce carotenoids in both settings, reaching the maximum after 48 h of fermentation time (4.6 ± 0.21 mg/L for SSF + presaccharification, 4.9 ± 0.39 mg/L for SSF). The delayed production compared to SHF may be related to the harsher conditions that cells had to face in the presence of WIS [29]. Overall, WIS do not significantly interfere with the production of carotenoids, and this fermentation conditions lead to a *Camelina* meal enriched in carotenoids, which may be directly used as enriched feed supplement.

Carotenoids Production In Batch Bioreactors

To test the reliability of the protocols in larger volume and to acquire data for quantitative analysis, we moved the process to stirred tank bioreactor. As starting point, the volume of the enzymatic hydrolysis was increased to 1 L: due to the uneven nature of lignocellulosic biomasses and their inhibitory effect towards enzymatic activity, scaling up this step may lead to a decrease in hydrolysis yield. Remarkably, after 6 h of hydrolysis of 9% w/v of *Camelina* meal by NS22119 0.56% w/w$_{Camelina\ meal}$ the amount of released sugars (7.2 ± 0.84 g/L, see T0 Fig. 3) was comparable with that obtained from lower volumes of hydrolysis, demonstrating the scalability of the first step of the process.

The obtained medium was used to test the production of carotenoids in batch bioreactors, where *R. toruloides* cells were inoculated at initial OD of 0.4, pH 5.6 ± 0.09 and oxygenation of 25%. Cells reached the stationary phase already after T16, as shown by the profile of the optical density and the cellular dry weight (Fig. 5A). Bioreactor cultivation permitted also to monitor pH and dissolved oxygen (Fig. 5A): the first increased over time to reach the value of 6.7 ± 0.03 at T48, whereas the second increased after T16 (up to 80%), witnessing a strong decrease in cellular growth, as from the reduction of oxygen consumption. The increase in pH could be related to the accumulation of ammonia in the medium,

initially consumed by cells (Fig. 5B), but then produced probably as a consequence of amino acid catabolism triggered in response to starvation. In fact, as shown in Fig. 5B, cells already consumed sugars and nitrogen (in the form of primary amines) at T16, although the incomplete consumption of both (that remained till the end of the fermentation) may be related to the depletion in the medium of micronutrients pivotal to cellular sustainment.

The exhaustion of nutrients, the increase in pH and pO_2 and the growth curve profiles (in terms of OD and CDW) suggest an early entrance in stationary phase, which is of interest as aforementioned for the production of secondary metabolites such as carotenoids.

Consistently, regarding the production of carotenoids, from T16 on there was no statistically significant increase in their accumulation, reaching 3.6 ± 0.69 mg/L after T24 (Fig. 5B), with a productivity (0.13 ± 0.03 mg/L/h) comparable with the one obtained in the same conditions in shake flasks (Fig. 3). Therefore, shake flask tests showed to be predictable on the behavior of *R. toruloides* in bioreactor.

The data here disclosed are the first reports of bioreactor scale fermentation of *Camelina* meal hydrolysate, and therefore they can pave the way for further optimization. To maximize the production of interest several modifications influencing lipid and carotenoid production in yeasts, like C/N ratio, initial CDW, pH and oxygenation [30–33], can be operated.

Conclusions

In the present work we demonstrate that the bioprocess involving the use of *Camelina* meal hydrolysate for the production of carotenoids by *R. toruloides* can be optimize in terms of different parameters. In fact, by dropping the enzymatic titer by 95% the hydrolysis was still efficient in releasing the main sugars consumed by this yeast in this medium (*i.e.* glucose, fructose, and sucrose). In parallel, we also modified the amount of total solid loading, exploring both higher and lower biomass titer than the original (15% w/v), in order to select the best option considering production, yield and productivity. Combining the optimized conditions for these two parameters, SHF was performed with 9% w/v *Camelina* meal hydrolysed by NS22119 0.56% $w/w_{Camelina\ meal}$, obtaining 2.2 ± 0.33 mg/L of carotenoids in 24 h, with a specific productivity of $2 \pm 0.3 \times 10^{-5}\ h^{-1}$. Furthermore, this SHF process in shake flask proved to be predictable of the behavior in SSF and SSF + presaccharification, and when the process was performed in batch bioreactors as well. The preliminary data from bioreactor fermentation pave the way for additional optimization of the process itself. Based on the logic of cascading [3, 34], the present work further fostered the use of *Camelina* meal as an alternative feedstock in second generation biorefineries exploiting microbial cell factories to produce fine chemicals. E-supplementary data of this work can be found in online version of the paper.

Materials And Methods

Camelina **meal hydrolysis**

Flanat Research Italia S.r.l., Rho, Italy, provided *Camelina* meal derived from plants cultivated and harvested in Lombardy in 2018 and 2019. *C. sativa* seeds were processed to collect the oil, while the leftover meal was delivered to the laboratory and stored at -20°C. Enzymatic hydrolysis of *Camelina* meal was performed using the enzyme mixture NS22119, kindly provided by Novozymes (Novozymes A/S, Copenhagen, Denmark) as described in [11]. Without drying the biomass, different quantities of *Camelina* meal were weighted to a concentration of 3%, 6%, 9%, 12%, 15% and 20% (w/v) into glass bottles, steeped in water with a final volume of 30 mL, and then autoclaved at 121°C for 1 h to both sterilize and pre-treat the biomass. Afterwards, enzymes were added directly to the biomass and incubated at 50°C in a water bath under mild agitation (105 rpm). The following enzyme concentrations were tested: 11.9%, 8.93%, 5.95%, 2.98%, 2.08%, 1.04%, and 0.56% $w/w_{Camelina\ meal}$. Three independent experiments were performed.

Microbial Strain, Media And Fermentations

R. toruloides (DSM 4444) was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, GmbH) and stored in cryotubes at -80°C in 20% glycerol (v/v). The composition of the medium for the pre-inoculum was as follows (per liter): 1 g yeast extract, 1.31 g $(NH_4)_2SO_4$, 0.95 g Na_2HPO_4, 2.7 g KH_2PO_4, and 0.2 g $Mg_2SO_4 \cdot 7H_2O$. The medium was supplemented with 15 g/L of glycerol as main carbon source and a 100× trace mineral stock solution consisting of (per liter): 4 g $CaCl_2 \cdot 2H_2O$, 0.55 g $FeSO_4 \cdot 7H_2O$, 0.52 g citric acid, 0.10 g $ZnSO_4 \cdot 7H_2O$, 0.076 g $MnSO_4 \cdot H_2O$, and 100 µL 18 M H_2SO_4. Yeast extract was purchased from Biolife Italia S.r.l., Milan, Italy. All other reagents were purchased from Sigma-Aldrich Co., St Louis, MO, USA.

For shake flasks fermentations, after plating on rich medium, a pre-inoculum was run until stationary phase. Then, cells were inoculated at 0.2 OD at 30°C and 160 rpm for both SHF and SSF processes (see below).

Separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF)

For SHF and SSF processes *R. toruloides* was grown in shake flasks supplemented with *Camelina* meal hydrolysate, with or without water insoluble components (WIS), which was prepared as follows. After 6 h of enzymatic hydrolysis at 50°C, the hydrolysate was centrifuged at 4000 rpm for 10 min to separate the water-soluble components from WIS. Then, for SHF, the liquid fraction was collected and transferred into a shake flask or a stirred tank bioreactor for microbial growth at 30°C. Alternatively, for the SSF + saccharification process, *Camelina* hydrolysate was provided directly to *R. toruloides* as growth medium, regardless of the presence of WIS. For the SSF process, *Camelina* meal was directly steeped and autoclaved in a shake flask, then supplemented with the enzymatic cocktail at 0.56% $w/w_{Camelina\ meal}$ and 0.2 OD of cells, and incubated at 30°C and 160 rpm. These conditions were also used to calculate specific growth rate of *R. toruloides*. Three independent experiments for each setting were performed.

Batch Bioreactor Fermentation

Enzymatic hydrolysis of 1 L of *Camelina* meal 9% w/v was performed in 2 L shake flasks by the action of NS22119 0.56% w/w$_{Camelina\ meal}$, for 6 h at 50°C and 130 rpm, in order to increase the homogenization of the solid component in the liquid. The hydrolysate was then centrifuged in an Avanti J-20 (Beckman Coulter Brea, California, USA), at 4°C and 8000 rpm for 10 min and the supernatant collected and stored at 4°C until use.

Regarding *R. toruloides* inoculum, from cryotubes cells were inoculated in 250 mL flasks with 50 mL of culture medium, as pre-seeding for the inoculation in 1 L flasks with 200 mL of the culture medium, and seed cultures were placed at 30°C and 160 rpm for 24 h. Exponential phase shake flasks cultures were used to inoculate bioreactors to a final optical density (OD_{600}) of 0.4. Briefly, cells were centrifuged at 6000 rpm for 10 min, washed twice with physiological solution (0.9% NaCl), and finally resuspended in 10 mL of sterilized water. The fermentations were conducted in 2 L stirred tank bioreactors (BIOSTAT® A plus, Sartorius Stedim Biotech GmbH, Goettingen, Germany) equipped with Visiferm DO ECS 225 for pO2 measurement and Easyferm Plus K8 200 for pH measurement (both from Hamilton Bonaduz AG, Bonaduz, Switzerland). The batch fermentation was carried out with 1 L of *Camelina* meal hydrolysate, and aeration rate, agitation, and temperature set to 1 vvm, 300 rpm (in cascade to 25% of dissolved oxygen), and 30°C, respectively. Three independent experiments were performed.

Carotenoids Extraction

Carotenoids were analyzed by acetone extraction from *R. toruloides* cells with a protocol adapted from [35]. Briefly, 1 mL of culture broth was collected and harvested by centrifugation at 7000 rpm for 7 min at 4°C, and the pellet was then resuspended in 1 mL acetone and broken using glass beads by thorough agitation with a FastPrep-24™ (MP Biomedicals, LLC, Santa Ana, CA, USA). Carotenoids were extracted in the acetone phase, the suspension was centrifuged, and the supernatant collected. The extraction was repeated with fresh acetone until the biomass was colorless. Carotenoid content was measured spectrophotometrically (see below).

Analytical Methods

HPLC analyses were performed to quantify the amount of glucose, sucrose, arabinose, fructose, galacturonic acid, and acetic acid. Briefly, 1-mL samples from each of the three different streams of production (enzymatically hydrolyzed *Camelina* meal, SHF or SSF) were collected and centrifuged twice (7000 rpm, 7 min, and 4°C), and then analyzed by HPLC using a Rezex ROA-Organic Acid column (Phenomenex, Torrance, CA, USA). The eluent was 0.01 M H_2SO_4 pumped at 0.5 mL min^{-1} and column temperature was 35°C. Separated components were detected by a refractive index detector and peaks were identified by comparing with known standards (Sigma-Aldrich).

Optical density (OD) of *R. toruloides* was measured spectrophotometrically at 600 nm. Cellular dry weight (CDW) was measured gravimetrically after drying 1 mL of cell culture (Concentrator 5301, Eppendorf AG, Germany).

Primary Amino Nitrogen Assay Kit (PANOPA) and Urea/Ammonia Assay Kit (K-URAMR, Megazyme International Limited, Bray, Ireland) ware used to determine the amount of primary amines, ammonia and urea in the *Camelina* meal hydrolysate.

The titer of carotenoids extracted in acetone from *R. toruloides* was determined spectrophotometrically (UV-1800; Shimadzu, Kyoto, Japan) based on the maximum absorption peak for β-carotene (455 nm). A calibration curve with standard concentration of β-carotene was obtained.

Calculations And Statistical Analyses

Carotenoids yield on consumed sugars (here $Y_{c/s}$) and carotenoids yield on maximum quantity of sugars per biomass (here $Y_{c/b}$) measured with acid hydrolysis in [11] were calculated by Equations 1 and 2, respectively. Specific productivity (q_p) was calculated by Eq. 3. Specific growth rate (μ) was calculated mathematically by equation obtained from plotting values of OD vs time on Excel (Figure S3). Duplication time (T_d) was calculated by Eq. 4.

1) $Y_{c/s} = {C_p}/{\Delta sug} \times 100$

2) $Y_{c/b} = {C_p}/{S_b} \times 100$

3) $q_p = {C_{p/t}}/{CDW}$

4) $t_d = {Ln2}/{\mu}$

Where Δsug corresponds to consumed sugars, S_b to maximum quantity of sugars in the biomass, C_p to carotenoids produced, and $C_{p/s}$ to carotenoids productivity.

For statistical analysis heteroscedastic two-tailed *t*-test was applied.

Abbreviations

CDW
cellular dry weight; HPLC:high-performance liquid chromatography; OD:optical density; SHF:Separate Hydrolysis and Fermentation; SSF:Simultaneous Saccharification and Fermentation; WIS:water-insoluble solids.

Declarations

Acknowledgments

This work was supported by the University of Milano-Bicocca with the FA (Fondo di Ateneo) to PB, the PhD fellowship, and funds “Sistemi Alimentari e Sviluppo Sostenibile: Creare sinergie tra ricerca e processi internazionali e africani” (SASS, 2016-NAZ-0228/L) and “Food Social Sensor Network” (FOODNET, 2016 - NAZ - 0143/A) to SB. The interdisciplinary cluster BEST4FOOD is also greatly acknowledged.

Author contribution

SB: Conceptualization, formal analysis, investigation, data curation, writing—original draft preparation, visualization. CC: formal analysis, investigation, data curation, writing—review and editing, visualization. PD: resources, supervision. PB: resources, writing—review and editing, supervision, project administration, funding acquisition.

Availability of supporting data

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors have no competing interest to declare

References

1. Jackson RB, Friedlingstein P, Andrew RM, Canadell JG, Le Quéré C, Peters GP. Persistent fossil fuel growth threatens the Paris Agreement and planetary health. Environ. Res. Lett. Institute of Physics Publishing; 2019. p. 121001.
2. Stegmann P, Londo M, Junginger M. The circular bioeconomy: Its elements and role in European bioeconomy clusters. Resour Conserv Recycl X Elsevier. 2020;6:100029.
3. IEA Bioenergy Task40. Cascading of woody biomass: definitions, policies and effects on international trade. IEA Bioenergy. 2016;71.
4. Vis M, Reumerman P, Gärtner S. Cascading in the wood sector. 2014.

5. Branduardi P. Closing the loop: the power of microbial biotransformations from traditional bioprocesses to biore fi neries, and beyond. Microb Biotechnol. 2020;0:1–6.
6. Zubr J. Carbohydrates, vitamins and minerals of *Camelina sativa* seed. Nutr Food Sci. 2010;40:523–31.
7. Murphy EJ. Camelina (*Camelina sativa*). Ind Oil Crop; 2016.
8. Dharavath RN, Singh S, Chaturvedi S, Luqman S. *Camelina sativa* (L.) Crantz A mercantile crop with speckled pharmacological activities. Ann Phytomedicine An Int J Marwah Infotech. 2016;5:6–26.
9. Cherian G. *Camelina sativa* in poultry diets: opportunities and challenges. Biofuel co-products as Livest Feed – Oppor challenges. 2012.
10. Li X, Mupondwa E. Production and value-chain integration of *Camelina sativa* as a dedicated bioenergy feedstock in the Canadian prairies. Eur Biomass Conf Exhib Proc. 2016.
11. Bertacchi S, Bettiga M, Porro D, Branduardi P. *Camelina sativa* meal hydrolysate as sustainable biomass for the production of carotenoids by *Rhodosporidium toruloides*. Biotechnol Biofuels BioMed Central. 2020;13:1–10.
12. Lee JJL, Chen L, Shi J, Trzcinski A, Chen WN. Metabolomic profiling of *Rhodosporidium toruloides* grown on glycerol for carotenoid production during different growth phases. J Agric Food Chem. 2014.
13. Kot AM, Błazejak S, Gientka I, Kieliszek M, Bryś J. Torulene and torularhodin: “New” fungal carotenoids for industry? Microb. Cell Fact. 2018.
14. Park Y-KK, Nicaud J-MM, Ledesma-Amaro R. The Engineering Potential of *Rhodosporidium toruloides* as a Workhorse for Biotechnological Applications. Trends Biotechnol. 2018.
15. Freitas C, Parreira TM, Roseiro J, Reis A, Da Silva TL. Selecting low-cost carbon sources for carotenoid and lipid production by the pink yeast *Rhodosporidium toruloides* NCYC 921 using flow cytometry. Bioresour Technol. 2014.
16. Singh G, Sinha S, Bandyopadhyay KK, Lawrence M, Paul D. Triauxic growth of an oleaginous red yeast *Rhodosporidium toruloides* on waste “extract” for enhanced and concomitant lipid and β-carotene production. Microb Cell Fact. 2018.
17. Wen Z, Zhang S, Odoh CK, Jin M, Zhao ZK. *Rhodosporidium toruloides* - A potential red yeast chassis for lipids and beyond. 20: FEMS Yeast Res. Oxford University Press; 2020. pp. 1–12.
18. Wang C, Zhao S, Shao X, Park J, Bin, Jeong SH, Park HJ, et al. Challenges and tackles in metabolic engineering for microbial production of carotenoids. Microb. Cell Fact. 2019.
19. Saini RK, Keum YS. Microbial platforms to produce commercially vital carotenoids at industrial scale: an updated review of critical issues. J. Ind. Microbiol. Biotechnol. Springer International Publishing; 2019. p. 657–74.
20. Yuan S-F, Alper HS. Metabolic engineering of microbial cell factories for production of nutraceuticals. Microb Cell Fact BioMed Central. 2019;18:46.

21. Nagarajan J, Ramanan RN, Raghunandan ME, Galanakis CM, Krishnamurthy NP. Carotenoids. Nutraceutical Funct. Food Components Eff. Innov. Process. Tech. Elsevier Inc.; 2017.
22. Pihlajaniemi V, Ellilä S, Poikkimäki S, Nappa M, Rinne M, Lantto R, et al. Comparison of pretreatments and cost-optimization of enzymatic hydrolysis for production of single cell protein from grass silage fibre. Bioresour Technol Reports. Elsevier Ltd; 2020. p. 9.
23. Aden A, Foust T. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. Cellulose. 2009;16:535–45.
24. Larnaudie V, Ferrari MD, Lareo C. Techno-economic analysis of a liquid hot water pretreated switchgrass biorefinery: Effect of solids loading and enzyme dosage on enzymatic hydrolysis. Biomass and Bioenergy. Elsevier Ltd; 2019. p. 130.
25. Humphrey A. Shake Flask to Fermentor: What Have We Learned? Biotechnol Prog. AIChE. 1998;14:3–7.
26. Crater JS, Lievense JC. Scale-up of industrial microbial processes. FEMS Microbiol Lett. 2018;365:138.
27. Gancedo JM. Carbon catabolite repression in yeast. Microbiol. Mol. Biol. Rev. 1998. p. 334–61.
28. Wiebe MG, Koivuranta K, Penttilä M, Ruohonen L. Lipid production in batch and fed-batch cultures of *Rhodosporidium toruloides* from 5 and 6 carbon carbohydrates. BMC Biotechnol BioMed Central. 2012;12:26.
29. Ask M, Olofsson K, Di Felice T, Ruohonen L, Penttilä M, Lidén G, et al. Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF. Process Biochem. 47: Elsevier Ltd; 2012. pp. 1452–9.
30. Júnnior H, Lopes S, Bonturi N, Lahtvee P, Kerkhoven EJ. C/N ratio and carbon source-dependent lipid production profiling in *Rhodotorula toruloides*. Appl Microbiol Biotechnol. Applied Microbiology and Biotechnology; 2020.
31. Dias C, Sousa S, Caldeira J, Reis A, Lopes da Silva T. New dual-stage pH control fed-batch cultivation strategy for the improvement of lipids and carotenoids production by the red yeast *Rhodosporidium toruloides* NCYC 921. Bioresour Technol Elsevier. 2015;189:309–18.
32. Bento TFSR, Viana VFM, Carneiro LM, Silva PA. Influence of Agitation and Aeration on Single Cell Oil Production by *Rhodotorula glutinis* from Glycerol. J Sustain Bioenergy Syst. 2019;9:29–43.
33. Pinheiro MJ, Bonturi N, Belouah I, Miranda EA, Lahtvee PJ. Xylose Metabolism and the Effect of Oxidative Stress on Lipid and Carotenoid Production in *Rhodotorula toruloides*: Insights for Future Biorefinery. Front Bioeng Biotechnol. 2020;8:1–15.
34. IEA Bioenergy Task42. Sustainable and synergetic processing of biomass into marketable food & feed ingredients, chemicals, materials and energy (fuels, power, heat). IEA Bioenergy. 2014;66.
35. Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T. Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochem Elsevier Ltd. 2011;46:210–8.

Figures

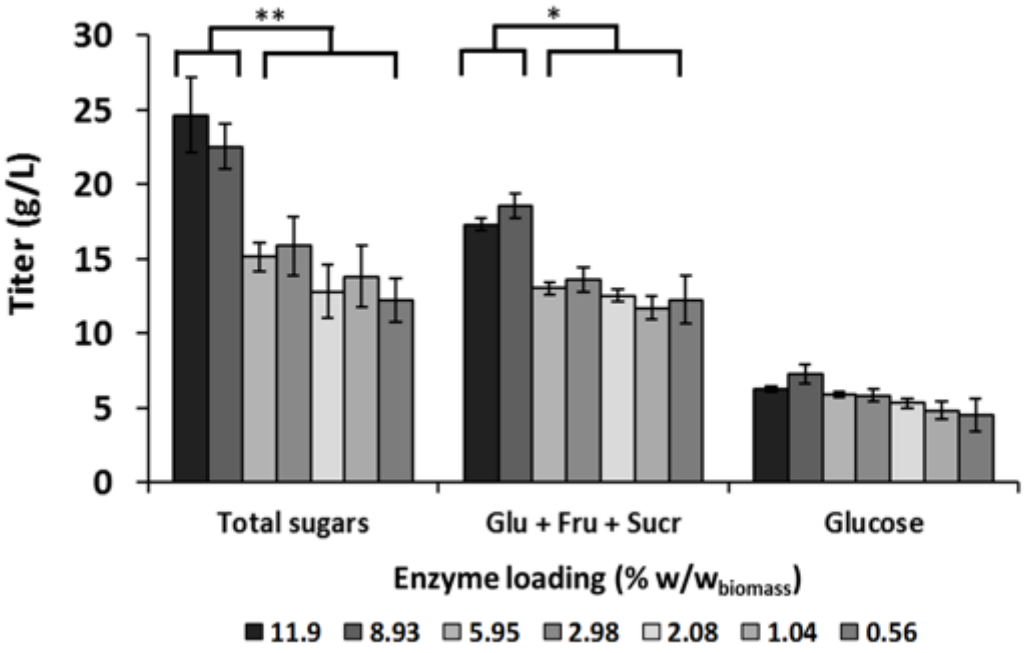

Figure 1

Sugar release from 15% w/v of Camelina meal by NS22119 enzymatic cocktail. The hydrolysis was performed at different loadings, and sugars released are shown in terms of total amount (intended as sum of sucrose, glucose, fructose, arabinose and galacturonic acid, first set of bars, from the left), sum of sucrose, glucose and fructose (central set of bars), and sole glucose (right bars). Values are the means of three independent experiments. ** $p < 0.02$, * $p < 0.05$

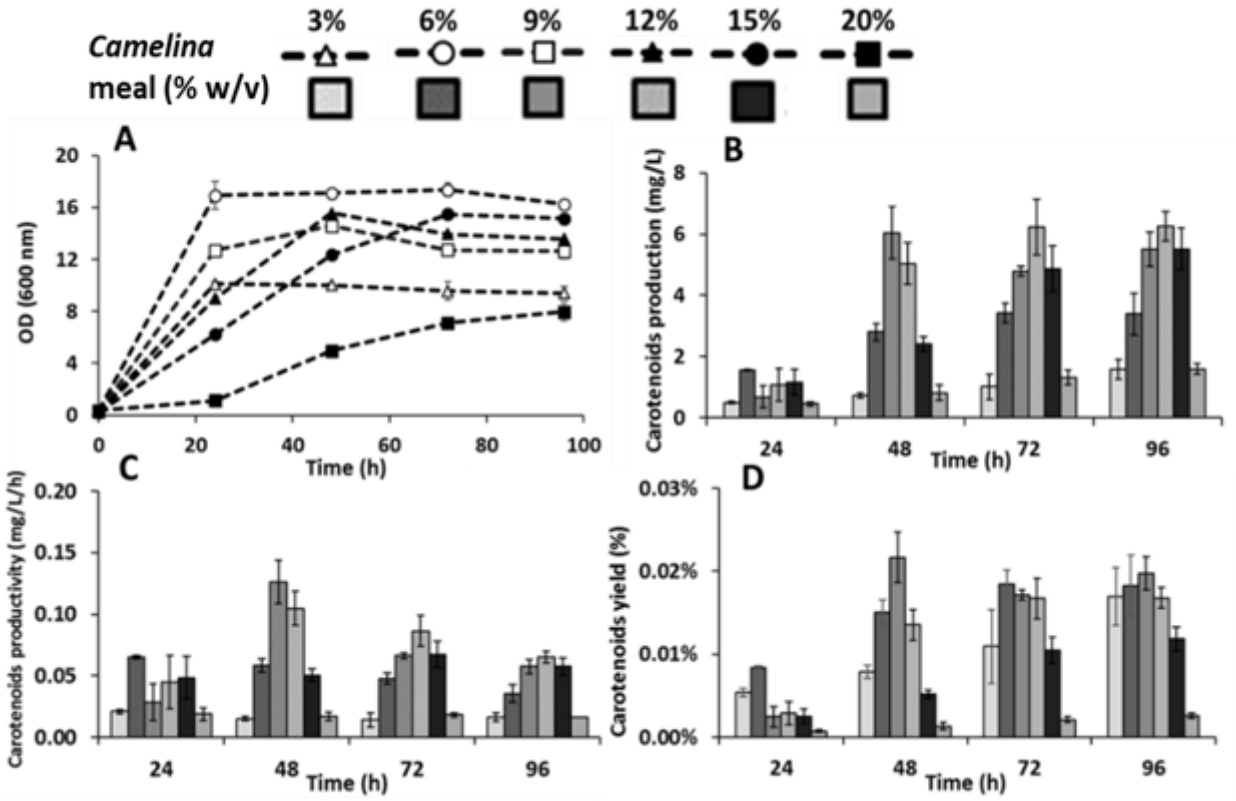

Figure 2

Production of carotenoids from different titers of Camelina meal. Modulation of solid loading of Camelina meal (from 3% to 20% w/v, hydrolysed with NS22119 11.9% w/wbiomass) and its effect on R. toruloides growth and carotenoids production. Growth in terms of OD (panel A), and carotenoids production in terms of titer (panel B), productivity (panel C) and yield (panel D) are given.

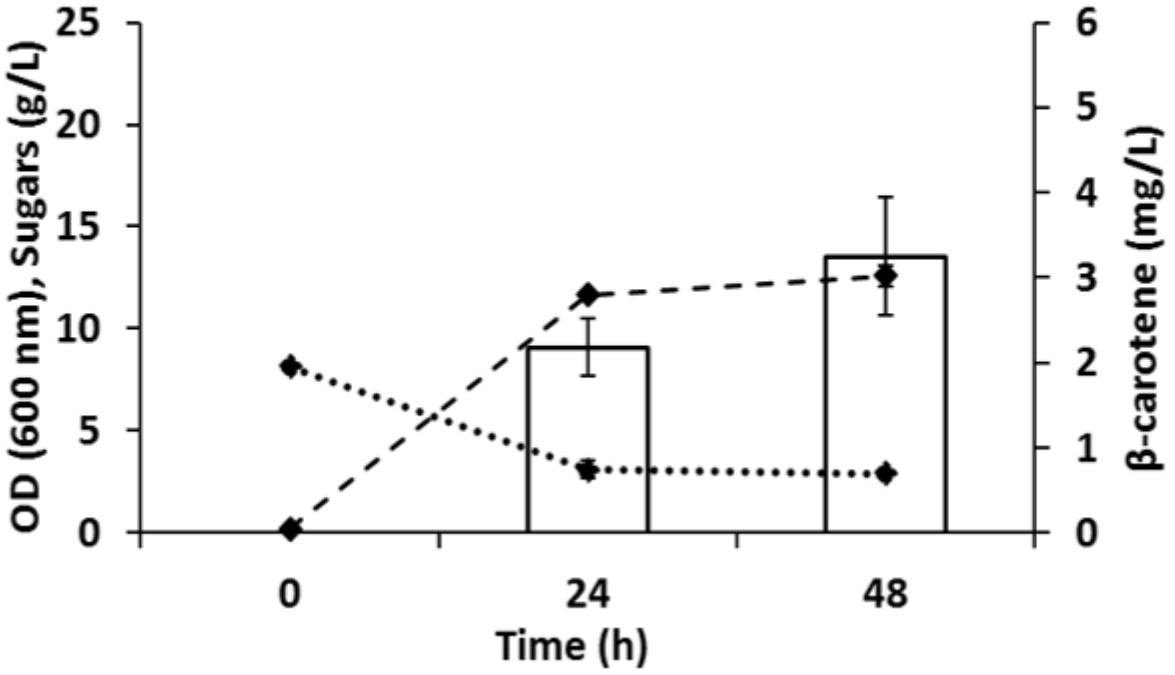

Figure 3

R. toruloides in SHF process with 9% (w/v) Camelina meal hydrolysed with 0.56% w/wCamelina meal of NS22119 as growth medium. OD (dashed line), sugar consumption (dotted line), carotenoids production (white bars). Values are the means of three independent experiments.

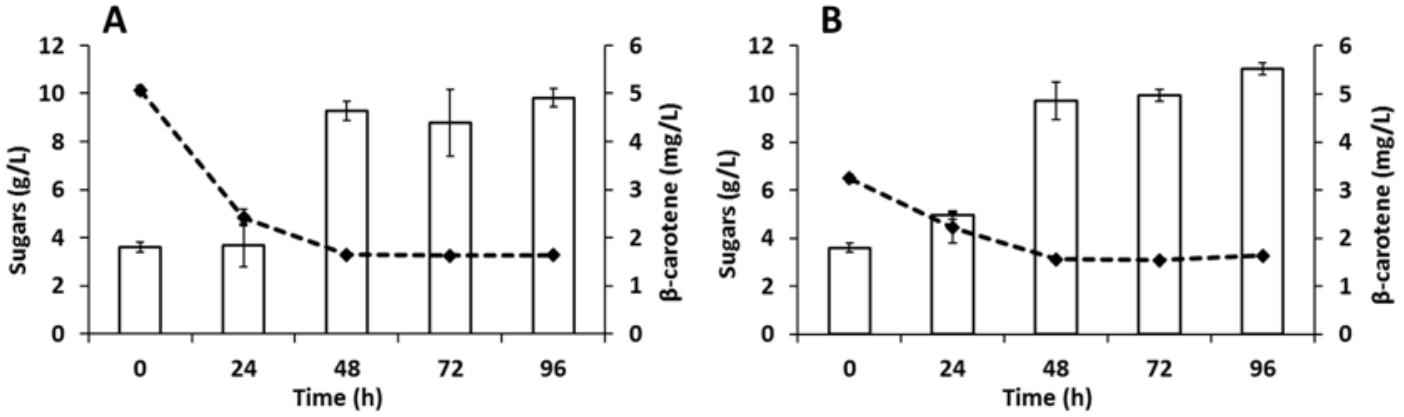

Figure 4

R. toruloides growth with 9% (w/v) Camelina meal hydrolysed with 0.56% w/wCamelina meal of NS22119 as growth medium in an SSF + presaccharification (panel A) and SSF (panel B) process. Sugar

consumption (dotted line), carotenoids production (white bars). Values are the means of three independent experiments.

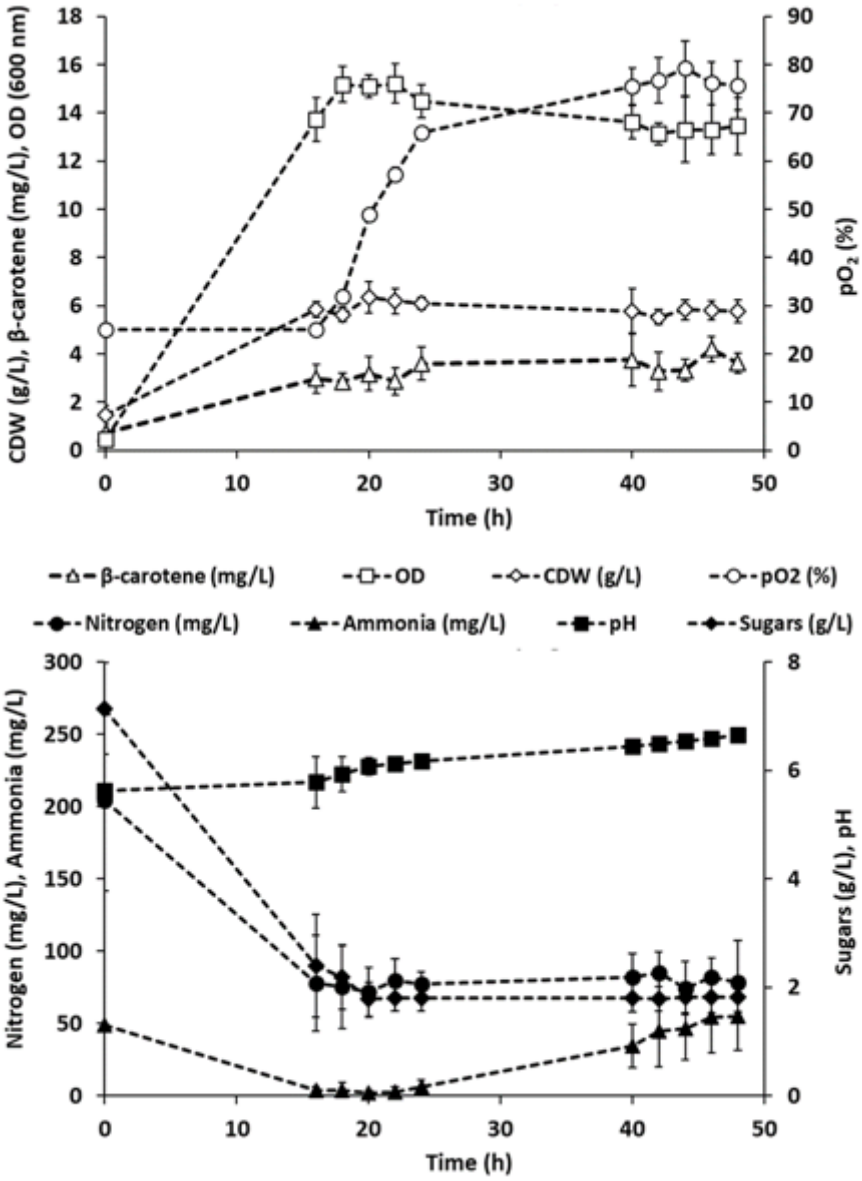

Figure 5

R. toruloides fermentation in stirred tank bioreactor (batch mode) in 9% (w/v) Camelina meal hydrolysed with 0.56% w/wCamelina meal of NS22119 as growth medium. Profile over time of β-carotene production (empty triangles), optical density (empty squares), cellular dry weight (empty diamonds), pO2 (empty circles) – panel A; nitrogen titer (full circles), ammonia titer (full triangles), sugars titer (full diamonds) and pH (full squares) – panel B.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Bertacchietal.Supplementaryfigures.pptx