Camelina sativa meal hydrolysate as sustainable biomass for the production of carotenoids by *Rhodosporidium toruloides*

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Research

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Abstract

Background: The sustainability of biorefineries is strongly related to the origin, the availability and the market of the biomass used as feedstock. Moreover, one of the pillars of circular economy aims at reducing waste, ideally to zero. These considerations well justify the increasing industrial interest in exploiting many and diverse residual biomasses. This work focuses on the valorization of the leftover from Camelina sativa oil extraction, named Camelina meal. Despite Camelina meal is used as animal feed, there is an increasing interest in further valorizing its macromolecular content or its nutritional value.

Results: Here we valorized Camelina meal hydrolysates by using them as nutrient and energy source for shake-flask fermentations where Rhodosporidium toruloides , a yeast natural producer of carotenoids, accumulated these pigments as desired product. Initially, by total acid hydrolysis we determined that in Camelina meal carbohydrates account for a maximum of 30.8 ± 1.0 %. However, since the acid hydrolysis is not optimal for subsequent microbial fermentation, an enzymatic hydrolysis protocol was assessed, obtaining a maximum sugar recovery of 53.3%. Having stated that, by Separate Hydrolysis and Fermentation, with or without water insoluble solids (SHF, SHF+WIS), or Simultaneous Saccharification and Fermentation (SSF) we obtained 5.51 ± 0.67, 12.64 ± 2.57, and 15.97 ± 0.67 mg/L of carotenoids, respectively, from Camelina meal hydrolysate. Significantly, the presence of WIS, possibly containing microbial inhibitors, correlates with a higher titer of carotenoids, which can be seen as scavengers.

Conclusions: The proposed study paves the way for the development of bioprocesses based on the exploitation of Camelina meal, scarcely investigated in the field before, as feedstock. The processes depicted provide an example of how different final products of industrial interests can be obtained from this leftover, such as pure carotenoids and carotenoid-enriched Camelina meal for the feed industry, without diminishing but possibly increasing its initial value. These data provide valuable basis for the economic evaluations necessary to assess the feasibility of a bioprocess based on Camelina meal to obtain high-value added products.

Background

The increasing demand for fossil resources is known to be a worldwide problem. Therefore, the need for new alternative sources of energy, chemicals and materials is urgent. The development of biorefineries is indeed a driving force in this scenario, and biotechnology is a key enabling technology when microbial cell factories operate the transformation of biomass. The sustainability of biorefineries is strongly related to the origin, availability and market of the biomass. For example, edible crops have been already exploited since decades as feedstocks for the production of several fine and bulk chemicals. However, environmental and social issues, as the direct or indirect competition with the food sector, discourages these processes for large productions of commodities, since the sustainability would be very poor if not absent [1]. On the other hand, the logic of “take, make, dispose”, which is still driving our dominant linear economy, is generating a large amount of waste, comprising organic matter. For these reasons,

biorefineries based on residual biomasses are of increasing scientific and industrial interest. In spite of the attractiveness of biorefineries based on microbial cell factories, conventional pretreatments and saccharification processes of residual biomasses often release toxic compounds that might impair both the microbial growth and the synthesis of the target product [2]. All these remarks have to be taken into account to develop robust biorefineries, ideally able to produce high-value molecules from low-cost substrates.

The increasing use of oilseed crops for food and biofuel sectors is leading to a surplus of process leftovers that are currently mainly used for animal feed [3] because of their protein content, together with carbohydrates and fibers. A good example of these leftovers is *Camelina* meal (or cake), main by-product of oil extraction from *Camelina sativa* seeds [4–8]. *Camelina* meal is commonly provided as component of cattle and poultry diet: however, its composition and its relatively low market price ($0.25/kg; [9]), is an element of interest for the development of sustainable bio-based processes, either for further valorizing the macromolecular components therein, or for augmenting the nutritional value of this animal feed. Nevertheless, *Camelina* meal has never been considered for these purposes, except for the study of Mohammad *et al.* [10], where *Saccharomyces cerevisiae* was provided with this substrate mixed with other *Camelina* derived sugars for the production of bioethanol, a low value-added molecule. In order to increase the viability of the process, the present study focuses on the microbial biotrasformation of *Camelina* meal into carotenoids as high value-added products.

The global market value for carotenoids was estimated to be $1.5 bln in 2017, expecting to reach $2.0 bln by 2022, with a compound annual growth rate (CAGR) of 5.7% [11–13]. The main field of application for carotenoids is animal feed (41% of the total revenue share), followed by supplements for food and dietary, thanks to their beneficial effect on human health [12,14]. For instance, ruminants are entirely dependent on feed as source of carotenoids, since they are not able to produce them by themselves [3]. Chemical manufacture of carotenoids from synthetic resources satisfies 80%-90% of the market, but the increasing demand for naturally produced molecules started the quest for new sources, mainly vegetal [12]. β-carotene alone has a market value of $246.2 mln, and the natural product can be extracted from carrots and fruits of palm oil. However, the direct competition with the food sector promoted the commercial production of β-carotene from microbial cell factories, like the microalga *Dunaliella salina* and the filamentous fungus *Blakeslea trispora* [12]. Nevertheless, algal carotenoid production is generally expensive and needs large areas for cultivation [15,16], whereas filamentous fungi are frequently characterized by a slow growth and pluricellular nature that may impair the productivity of the processes [17]. Studies regarding other cell factories are therefore desirable for increasing the overall sustainability of the process: in this scenario, yeasts can be considered suitable candidates. In particular, the oleaginous yeast *Rhodosporidium (Rhodotorula) toruloides*, also called “pink yeast”, naturally accumulates carotenoids, both carotenes and xanthophylls, such as β-carotene, torulene and torularhodin [16,18,19]. *R. toruloides* can use different sugars as main carbon sources, such as glucose, cellobiose, sucrose, mannose, xylose, arabinose and galacturonic acid [20]. In addition, *R. toruloides* converts complex matrixes, like carob pulp syrup, sugarcane bagasse, corn stover and food wastes, into lipids

and/or carotenoids [21–24]. This yeast is therefore a good candidate for the development of 2nd generation biorefineries.

With the objective of producing carotenoids in *R. toruloides*, in this work *Camelina* meal was first saccharified by an optimized enzymatic hydrolysis. Then the released sugars were used as feedstock in Separate Hydrolysis and Fermentation (SHF). Alternatively, a Simultaneous Saccharification and Fermentation (SSF) process was also assessed, resulting in the highest measured carotenoid production of 15.97 ± 0.67 mg/L. In conclusion, these data will promote further investigations on the optimization of process conditions, and indicate that *Camelina* meal and *R. toruloides* can be used for developing a novel bio-based process for carotenoids production.

Results And Discussion

Evaluation of total sugar content in *Camelina* meal and optimization of the enzymatic hydrolysis

The content of water, insoluble components, acetic acid and sugars was quantified in *Camelina* meal. As shown in Table 1, total acid hydrolysis (see Materials and Methods) revealed that almost one third (31%) of *Camelina* meal is composed by sugars: by HPLC analysis it was determined that the main fraction of carbohydrates is represented by hexoses, namely glucose and fructose, accounting for more than 2/3 of the total. Despite acid hydrolysis can be used for saccharification, it poses limitations related to the conditions of the reaction itself: the low final pH, which needs to be neutralized prior to provide sugars to the cells, and the release of inhibitory compounds such as furfurals [2,25]. Taken together, these issues can limit the sustainability of the overall biorefinery process, for example considering the use and disposal of acid solutions [25]. Therefore, to release monomeric sugars from their polymeric form an enzymatic instead of an acid hydrolysis was performed, testing different conditions (see below) and comparing the fraction of the released sugars with the value of 31%, considered as 100%. The other main components of *Camelina* meal, as reviewed by [7], are crude proteins, which account for 35.2-46.9% of the total biomass, and crude fats, spanning from 4.9 to11.9%. In addition, micronutrients such as vitamins are present in *Camelina* meal [7]. Overall, *Camelina* meal is a potential good substrate for the growth of *R. toruloides* and the consequent production of carotenoids, never reported in literature before.

The optimization of the enzymatic hydrolysis, whose mild operative parameters are generally more compatible to the subsequent growth of microbial cell factories and that can take advantage from the broad range of commercial available enzymatic cocktails [26,27], was performed with the cocktail NS22119 (Novozymes A/S), to release both hexose and pentose sugars. Initial different concentrations of *Camelina* meal were tested in order to determine the effect of solids loading on sugar release. After autoclaving, the measured pH was 5.5, which is compatible with the enzymatic catalysis, since NS22119 would display up to 90% of its maximum activity, according to the indication of the producers. Remarkably, the pH remained constant during time until the end of the hydrolysis. Both the economic and environmental impact of the procedure can be therefore reduced, since neutralization or addition buffer are both avoidable. As shown in Figure 1A, pre-treatment of the biomass by autoclaving resulted in an

initial concentration of released sugars spanning from 1.8 ± 0.03 g/L to 9.27 ± 0.32 g/L, where the increase in sugar concentration is essentially due to the concentration of the initial loaded biomass. After enzymes treatment (11.9% w/w$_{Camelina\ meal}$), the concentration of free sugars doubled (at least), compared to the initial amount, independently from the quantity of the loaded biomass (Figure 1A). No additional release of sugar was detectable over time from negative control samples, were 3% or 15% of the initial biomass were incubated in shaking water bath at 50°C, but no enzymes were added (Additional File 1, Figure S1). Sugar titer increased during time until 24 hours, with a linear proportionality (R^2=0.98, p < 0,001, calculated with R) in respect to the initial quantity of biomass; therefore, the yield of sugars released by enzymatic hydrolysis was constant regardless of the concentration of the provided *Camelina* meal (Figure 1B). The maximum yield of sugars over total biomass was 20% after 24 hours from the start; considering the original amount of carbohydrates, a sugars recovery of 65% was calculated, which is in accordance with common reported values for lignocellulose enzymatic hydrolysis [28,29].

Given these data, the successive experiments were performed using *Camelina* meal at the maximum tested solids loading (15%). To test if it was possible to further improve the sugar recovery, considering a possible inhibition of the enzymatic activity determined by the released products or by the biomass itself, two different strategies were designed. In one setting, the initial quantity of the enzymes was doubled, in a second setting a second pulse of enzyme, resulting in a double total amount, was added after 6h of hydrolysis. When the hydrolysis was performed in one batch with a double amount of enzymes (23.8% w/w$_{Camelina\ meal}$), no significant increase in the quantity of sugars released from *Camelina* meal was observed (Figure 2A), and therefore this strategy was not further considered. Similarly, also the addition of another aliquot of enzymatic cocktail (11.9% w/w$_{Camelina\ meal}$) after 6 hours of hydrolysis did not lead to an increase in the final sugar titer (Figure 2B). Overall, the data seem to indicate that the incomplete saccharification is more related to the intrinsic accessibility of polysaccharides in the biomass, rather than to some limit in the catalytic activity. These gimmicks suggest to adhere to the initial procedure, limiting the overall cost of the process in terms of use of enzymes. Furthermore, it is also evident that the action of the enzymatic cocktail is mostly concentrated during the first hours of hydrolysis: increasing the incubation time up to 24 hours improved sugar titer of only 20% in 18 hours. Therefore, the optimized conditions for the enzymatic hydrolysis of *Camelina* meal are as follows: *i*) 15% w/w solids loading *ii*) 11.9% w/w$_{Camelina\ meal}$ of enzymatic cocktail NS22119 *iii*) 6 hours reaction time *iv*) operative temperature 50°C *v*) initial 5.5 pH. As underlined before, the pH of the reaction mixture remains unvaried over time, without the need of neutralization. This pH value is closer to the optimum reported in literature for carotenoids accumulation in *R. toruloides* (pH 5), rather than for lipid production (pH 4) [30], being therefore a favorable condition for the aim of this work.

With this setting, about 25 g/L of monomeric sugars were released, with a recovery of 53.3%. Notwithstanding this, the fraction of residual carbohydrates not hydrolyzed could be considered as an added value to the final product: in fact, a feed with *Camelina* meal enriched in carotenoids by fermentation of *R. toruloides* would still contain fibers of nutritional value.

At the best of our knowledge, these are the first data reporting an enzymatic hydrolysis protocol for *Camelina* meal to make sugars accessible for a subsequent microbial biotransformation.

Inhibitory compounds in *Camelina* meal hydrolysate

Biomass hydrolysis is efficacious in releasing sugars from lignocellulose, minimizing the accumulation of high content of inhibitory compounds. Nevertheless, there are some drawbacks related to other compounds detached from these complex matrixes (Jönsson and Martín, 2016; Sitepu *et al.*, 2014). Acetic acid is the most common inhibitor released by hydrolysis of the hemicellulose fraction composing lignocellulosic biomasses: it can easily impair microbial growth and metabolism due to its generic and specific toxicity [31], therefore reducing the Key Performance Indicators (KPI) of the production process [2,31,32]. Nevertheless, it is important to underline that the toxicity of acetic acid is sharpened at low pH: extracellular pH values higher than its pKa (4.76) reduce its diffusion across the membrane and, therefore, the cellular damages that it can trigger [33,34]. As aforementioned, the operative pH (5.5) is higher than the pKa of acetic acid, lowering the detrimental effect of this molecule towards cells. Moreover, it has been previously described that *R. toruloides* can withstand acetic acid as additive to defined media or even as sole carbon source up to 20 g/L at pH 6 [35–37]. Figure 3 shows that acetic acid titer increases during time, due to the action of the enzymatic cocktail, reaching 1.80 ± 0.01 g/L after 24 hours from start. This amount has been described as bearable by diverse yeasts [32,35,36], including *R. toruloides*.

Given these data, the hydrolysate of *Camelina* meal has all the trumps to be a suitable feedstock for establishing yeast cell factory-based biorefineries, exploiting yeasts biodiversity and possible engineering strategies for obtaining different products of interest.

Carotenoids production from *Camelina* meal hydrolysate in SHF and SSF processes

Having established a protocol for obtaining *Camelina* meal hydrolysate, the carotenoids production was investigated by mean of the Separate Hydrolysis and Fermentation (SHF). The first experiment considered as medium the sole clarified supernatant, collected after 6 hours of enzymatic hydrolysis: it resulted to sustain the growth of *R. toruloides*, as indicated by the accumulation of biomass and sugars consumption (Figure 4A, dotted and dashed lines, respectively). The accumulation of carotenoids increased over time, reaching 5.51 ± 0.67 mg/L after 96 hours of fermentation (Figure 4A, white bars). These data are in accordance to the previous observation that *R. toruloides*, and carotenogenic microorganisms in general, produces carotenoids mainly in response to stressful or sub-optimal conditions, such as the stationary phase of growth [17,18,38,39]. Time lapse of the fermentation was first chosen as 96 hours mainly to compare this work with similar ones from the literature, when *R. toruloides* was provided with defined media or other/different residual biomasses (Table 2). Indeed, after 96 hours we observed a reduction of extracted carotenoids from the cells (Additional File 1, Figure S2): this could correlate to the export/release of the molecules from the cells o to an imbalance of nutrients that may promote their consumption/corruption. The production achieved in the here described SHF process are

competitive compared to shake flasks carotenoid productions that have been reported for *R. toruloides* provided with other complex matrixes (Table 2).

In order to overcome the need to clarify the media after enzymatic hydrolysis, we provided to the cells the entire *Camelina* meal hydrolysate, comprising also the water-insoluble solid (WIS) fraction remained after the enzymatic hydrolysis. WIS may be troublesome for microorganisms, both considering the uneven homogenization of the liquid media, due to the presence of solid components, and the toxicity of some of the molecules there comprised [28]. These two aspects combined might impair microbial growth and production. In the conditions that here we named “SHF with WIS”, *R. toruloides* was not only able to consume sugars and to produce carotenoids (Figure 4B), but remarkably it was observed a higher titer of intracellular carotenoids, reaching 12.64 ± 2.57 mg/L after 96 hours from the inoculum. It is important to underline that the carotenoids measured in this as in the following experiments in the presence of WIS are due to microbial metabolism: indeed, the measured amount of carotenoids extracted from *Camelina* meal with and without the addition of enzymes, at the beginning and at the end of the reaction, remained constant over time (Additional File 1, Figure S3).

Often proposed as alternative to SHF, the Simultaneous Saccharification and Fermentation (SSF) displays a single and co-current step of hydrolysis and microbial fermentation. The two processes have several pros and cons compared to each other, considering parameters such as efficiency, time, presence/release of inhibitory molecules and downstream of the final product [40,41]. SHF and SSF have been proposed and compared for several 2nd generation biorefineries, based for an example on *Arundo donax*, grass or wheat straw [28,42,43]. A potential drawback of incubating enzymes and cells in the same environment is the compromise to be reached between the optimum conditions for them both. In the present study, since 50°C was not a viable temperature for *R. toruloides*, 30°C was selected as operative temperature, while an increased shaking was imposed with the aim to partially compensate for the reduced activity by augmenting the probability of interactions between the matrix and the enzymes. Remarkably, the release of sugars in these conditions was comparable with the previous setting (Additional File 1, Figure S4). Figure 4C shows that after the first 6 hours of hydrolysis the amount of sugars in the media was lower compared to the amount of the SHF, very likely due to the initial growth (and therefore sugar consumption) of *R. toruloides*. After 24 hours from the start, there is a clear consumption of sugars, which in parallel led to an accumulation of carotenoids. After 96 hours, the concentration reached is 15.97 ± 1.93 mg/L. Therefore, SSF process seems to be even more effective than “SHF with WIS”. Significantly, the higher amount of carotenoids was registered when WIS was left into the medium. Diverse environmental variables can influence carotenoids production, and sub-lethal concentrations of insoluble solids might from the one hand still impair microbial growth but on the other hand can trigger the accumulation of metabolites that natural producers, such as microalgae and yeasts, synthetize for their own defense [39,44]. A more specific example was reported, where β-phenol can trigger carotenoid production in yeast [45].

The titers reached from SSF process also indicate an efficacious concurrent hydrolysis and fermentation, suggesting that a simplified procedure involving a single vessel can be used. The final productivity

remains similar, suggesting that the initial sugar release in the presence of cell do not speed the overall process. Overall, data from SSF and SHF+WIS reveal that with this residual biomass, the often mandatory detoxification step, also indicated for *R. toruloides* [46], is avoidable. Moreover, the final product obtained by both SHF with WIS and SSF is a *Camelina* meal enriched with carotenoids, which has the potential for being directly used in the feed industry.

Therefore, as outcomes to the proposed processes different products can be recovered, such as pure carotenoids and carotenoids-enriched *Camelina* meal. In particular, the latter would be an innovative product in the market, since carotenoids are commonly added to animal feed for nutritional and organoleptic reasons [3,12]. In addition, the production of carotenoids from a residual biomass of lower value may increase the economic attractiveness of the proposed process. Therefore, the present work, according to the logic of cascading [47,48], paves the way for an alternative use of *Camelina* meal as feedstock in second generation biorefineries exploiting microbial cell factories to produce fine chemicals.

Conclusions

Here we demonstrated that *Camelina* meal could be considered as residual biomass for the potential development of novel biorefineries. After an optimized hydrolysis, this biomass was provided to the oleaginous yeast *R. toruloides* as sole nutritional and energy source, resulting in carotenoids production. When SHF (with or without WIS) and SSF processes were compared, *R. toruloides* resulted to produce the maximum titer of carotenoids (15.97 ± 1.93 mg/L) under SSF fermentation mode. The presence of WIS seemed to play a positive role in this scenario, triggering the accumulation of the desired product and showing how common foes of biorefineries can turn into possible allies. At the best of our knowledge, this is the first report of using *Camelina* meal hydrolysate to sustain microbial growth and to produce carotenoids by any microorganism. To further investigate the pliancy of this study, we will analyze the titer of concurrently accumulated carotenoids (e.g. torulene and torularhodin) and their relative ratio, and we will test alternative microbial cell factories to produce other high-value added molecules (as, for example, flavors). Moreover, the biotransformation will be extended from shake flasks to bioreactor scale, to provide data useful to calculate the competitiveness of a potential industrial process intended to further valorize *Camelina* meal, following the logic of cascading.

Materials And Methods

Camelina meal composition

Flanat Research Italia S.r.l., Rho, Italy, provided *Camelina* meal derived from plants cultivated and harvested in Lombardy in 2018 and 2019. *Camelina sativa* seeds were internally processed to collect the oil: the leftover meal was delivered to the laboratory and stocked at -20°C. To measure the water percentage of *Camelina* meal, 0.9 g and 4.5 g of biomass were dried out at 160°C for 3 h, and then the biomass was weighted again to calculate the amount of evaporated water. To measure mainly the saccharides content of *Camelina* meal, the biomass was treated following instructions for the analysis of

structural carbohydrates and lignin in biomass from the National Renewable Energy Laboratory (NREL https://www.nrel.gov/docs/gen/fy13/42618.pdf) with some modifications as follows. 300 mg of biomass were diluted in 3 mL H_2SO_4 72%, and then incubated at 30°C for 1 h, stirring thoroughly every 10 minutes. The solution was diluted to 4% by adding 84 mL of distilled water; mix by inversion and autoclave it (121°C, 1 h). The hydrolysis solution was vacuum filtered through one of the previously weighted filtering crucibles, and the insoluble components were measured gravimetrically on the filter paper. The filtered liquid was neutralized with NaOH until pH 5-6 and then the samples were analyzed at the HPLC (as described below) after filtration. Three independent experiments were performed.

Pretreatment and Enzymatic hydrolysis of *Camelina* meal

Enzymatic hydrolysis of *Camelina* meal was performed using the enzyme mixture NS22119, kindly provided by Novozymes (Novozymes A/S, Copenhagen, Denmark). Different quantities of *Camelina* meal were weighted to a concentration of 3%, 6%, 9%, 12% and 15% into glass bottles and steeped in water with a final volume of 30 mL, then autoclaved (121°C, 1 h) in order to both sterilize and pre-treat the biomass. Afterwards, enzymes were added directly in the bottles and incubated at 50°C in a water bath under agitation. 1 mL of sample was collected every 2, 4, 6 and 24 hours from the start, and the sugar content was analyzed by HPLC (see below). Enzyme concentrations tested were 11.9% and 23.8% $w/w_{Camelina\ meal}$; alternatively, the digestion started with a concentration of enzyme of 11.9% $w/w_{Camelina\ meal}$ then the same amount of enzymes was again added after 6 hours. Three independent experiments were performed.

Microbial strain and media

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 (vv^{-1}). The composition of the medium for the pre-inoculum was (per liter) as here described: 1 g of yeast extract, 1.31 g of $(NH_4)_2SO_4$, 0.95 g of Na_2HPO_4, 2.7 g of KH_2PO_4, 0.2 g of Mg_2SO_4 $7H_2O$. The medium was supplemented with a 100X trace mineral stock solution consisting of (per liter): 4 g $CaCl_2$ $2H_2O$, 0.55 g $FeSO_4$ $7H_2O$, 0.52 g citric acid, 0.10 g $ZnSO_4$ $7H_2O$, 0.076 g $MnSO_4$ H_2O, and 100 µL 18 M H_2SO_4. Yeast extract was provided by Biolife Italiana S.r.l., Milan, Italy. All the others reagents were provided by Sigma-Aldrich Co., St Louis, MO, USA. After plating, a pre-inoculum was run in rich media until stationary phase: then cells were inoculated at 0.2 OD in both SHF and SSF processes (see below). Growth condition for *R. toruloides* in shake flasks: 30°C, 160 rpm. Optical density (OD) of *R. toruloides* was measured by spectrophotometric analysis at λ = 600 nm.

Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF)

R. toruloides was grown in shake-flasks mimicking SHF and SSF processes, providing *Camelina* meal hydrolysate with or without water insoluble solids (WIS) as follows. After 6 hours of enzymatic hydrolysis at 50°C, the hydrolysate was centrifuged at 4000 rpm for 10 minutes, to separate the soluble components

from WIS. Afterwards, the liquid fraction was collected and transferred into a shake flask for microbial growth at 30°C, in a SHF process. Alternatively, *Camelina* hydrolysate was directly provided to *R. toruloides* as medium for growth, regardless of WIS presence. For the SSF process, *Camelina* meal was directly steeped and autoclaved in a shake flask, then added with 11.9% $w/w_{Camelina\ meal}$ of enzymatic cocktail and 0.2 OD of cells: afterwards, shake flasks were incubated at 30°C, 160 rpm. Three independent experiments for each setting were performed.

Carotenoids extraction

Carotenoids were analyzed through acetone extraction from *R. toruloides* cells with an adapted protocol from [49]. 1 mL of brodoculture was collected and harvested by centrifugation at 7000 rpm for 7 minutes at 4°C, and the pellet was then resuspended in 1 mL acetone and broken using glass beads by thorough agitation with FastPrep-24™ (MP Biomedicals, LLC). 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 and acetic acid. 1 mL samples from each of the three different streams of production (enzymatic hydrolyzed *Camelina* meal, SHF or SSF) were collected and centrifuged twice (7000 rpm, 7 min, and 4°C), then analyzed at the HPLC using a Rezex ROA-Organic Acid (Phenomenex). 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, St Louis, MO, USA).

The pH was measured with indicator strips at the beginning and at the end of the enzymatic hydrolysis, in order to assess if the initial conditions were suitable for the enzymatic hydrolysis and to foresee possible toxic effect of the final media.

The titer of carotenoids extracted in acetone from *R. toruloides* was determined spectrophotometrically accordingly to the maximum absorption peak for β-carotene (455 nm); similarly, a calibration curve with standard concentration of β-carotene was obtained. The instrument used for these analyses was a UV-1800 Shimadzu spectrophotometer.

Supplementary Information

Additional file 1: Figure S1. Effect of enzymatic hydrolysis conditions on different concentrations of *Camelina* meal without the addition of NS22119 cocktail. **Figure S2.** *R. toruloides* production of carotenoids from 15% *Camelina* meal hydrolysate. Profile of OD (dotted line), sugars consumption

(dashed line) and β-carotene producion (white bars) by *R. toruloides* during SHF processes. **Figure S3.** Carotenoids extraction from *Camelina* meal hydrolysate.

List Of Abbreviations

OD: optical density; SHF: Separate Hydrolysis and Fermentation; SSF: Simultaneous Saccharification and Fermentation; WIS: water insoluble solids.

Declarations

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Authors' contribution

SB performed the experimental work, analyzed the data and wrote the manuscript. SB

and PB designed the experiments. PB and MB helped in data analysis and in drafting the

manuscript. PB and DP helped for funding acquisition. All authors have read and approved the final manuscript.

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Availability of supporting data

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to submit the work to the journal.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1: *Camelina* meal hydrolysed composition by acid treatment. Values are the means of three independent experiments.

Total acid hydrolysis of *Camelina* meal	
Measured component	**Percentage**
Water	9.0 ± 1.8 %
Acetate	10.7 ± 1.4 %
Insoluble fraction	13.2 ± 1.4 %
Sugars	30.8 ± 1.0 %
of which	
glucose	15.6 ± 0.9 %
fructose	8.3 ± 0.0 %
arabinose	6.9 ± 0.0 %

Table 2: Carotenoids production by *R. toruloides*. Comparison of data obtained from different media and fermentation modes.

R. toruloides strain	Substrate	Time	β-carotene (mg/L)	Reference
ATCC 204091	MM-5% NaCl[1]	172 h	54.868	[38]
ATCC 204091	WE[2]	72 h	62 ± 1.70	[23]
	MM	100 h	57 ± 2.18	
NCYC 921 (alias ATCC 10788)	Glucose (fed-batch)[3]	87 h	25.23	[30]
ATCC 10788	YPG[a]	288 h	3.6	[16]
AS 2.1389			4.3	
CBS 5490			6.8	
CCT 0783	SCBH[b]	72 h	1.2 ± 0.1	[21]
	cSCBH	94 h	2.18 ± 0.2	
NCYC 921 (alias ATCC 10788)	CPS100[c]	48 h	0.41	[22]
	CPS75		0.47	
	SCM100[d]		0.04	
	SCM75		0.18	
DSM 4444	CM SHF[e]	96 h	5.51 ± 0.67	This study
	CM SHF+WIS		12.64 ± 2.57	
	CM SSF		15.97 ± 1.93	

[1] MM = minimal media with 5 g/L glucose [2] WE = waste extract from mandis, [a] YPG = 20 g peptone, 10 g yeast extract, 60 g glycerol; [b] SCBH = sugarcane bagasse hydrolysate, cSCBH = SCBH concentrated; [c] CPS = carob pulp syrup; [d] SCM = sugarcane molasse; [e] CM = *Camelina* meal
[1-3] bioreactor, [a-d] shake flasks.

Figures

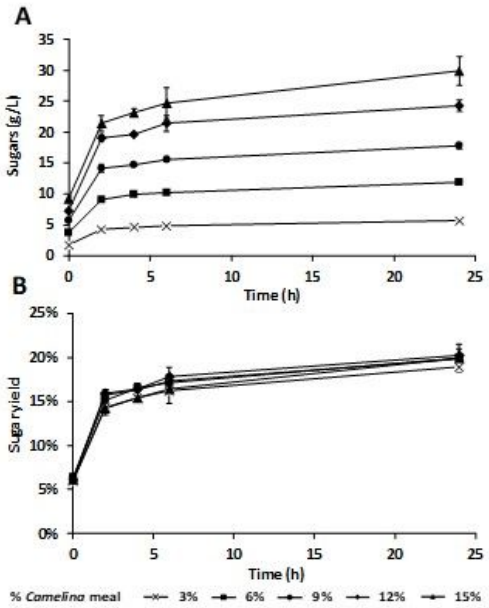

Figure 1

Effect of enzymatic hydrolysis on different Camelina meal concentrations by NS22119 cocktail 11.9% w/wCamelina meal. Sugar released (Panel A) and sugar yield from the biomass (Panel B) during time. The values are the means of three independent experiments.

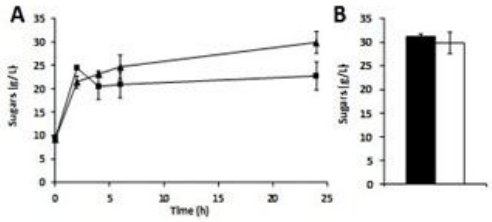

Figure 2

Enzymatic hydrolysis of 15% Camelina meal. Panel A, sugars released by NS22119 cocktail 11.9% w/wCamelina meal (triangles) or 23.8% w/wCamelina meal (squares). Panel B, supplement of additional 11.9% w/wCamelina meal of NS22119 cocktail after 6 hours of hydrolysis: sugar released after 24 hours from the start with the addition (black bar) and without (white bar). The values are the means of three independent experiments.

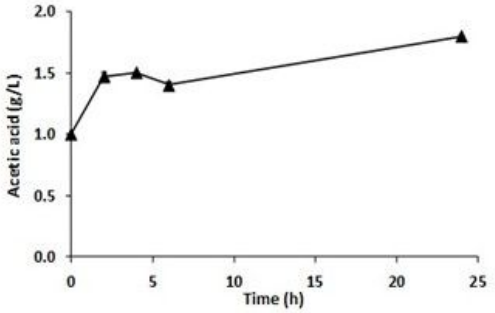

Figure 3

Acetic acid released during enzymatic hydrolysis. The concentration of acetic acid released from 15% Camelina meal by treatment with NS22119 cocktail 11.9% w/wCamelina meal was evaluated during time. The values are the means of three independent experiments.

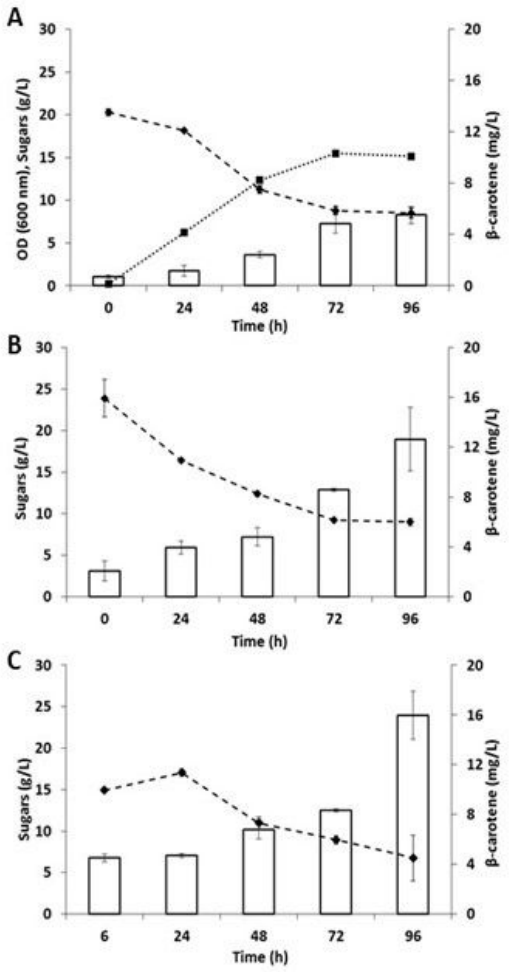

Figure 4

R. toruloides production of carotenoids from 15% Camelina meal hydrolysate. Profile of OD (dotted line), sugars consumption (dashed line) and β-carotene producion (white bars) by R. toruloides different processes: SHF (panel A), SHF with WIS (panel B) and SSF (panel C). The values are the means of three independent experiments.

Supplementary Files

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