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Chemical and enzymatic hydrolysis of waste wheat bran to sugars and their simultaneous biocatalytic conversion to valuable carotenoids and lipids

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Abstract:	Defatted wheat bran, an industrial waste of the food chain, represents a strategic renewable material for modern biorefinery schemes. Through a combination of chemical and biological catalysis, a cascade process was developed in water medium to produce high-value fine chemicals, such as carotenoids and lipids, from polysaccharide fraction. Due to the low lignin content and suitable particle size of defatted wheat bran, pretreatment steps are unnecessary, allowing the direct enzymatic or chemical hydrolysis of polysaccharide components (glucan, xylan, and arabinan) to give fermentable sugars. Regarding the biocatalytic approach, the optimisation of the main reaction parameters, such as enzyme dosage (15, 30, 45, 60 FPU Cellic®CTec 3 HS/g glucan) and biomass loading (5, 10, 15, 20 wt%), was performed to improve the monosaccharide yield. Regarding the chemical route, a microwave-assisted FeCl3-catalysed approach was optimised in terms of catalyst amount (1.0, 1.3, 1.6 wt%) and reaction time (2.5, 5, 10 min) to maximise the sugar yield, minimizing the formation of furanic derivatives which are strong inhibitors for the subsequent fermentation step. The biological conversion of sugars obtained by both enzymatic and chemical routes into carotenoids and lipids was then performed by adopting the commercial yeast Rhodosporidium toruloides DSM 4444. The simultaneous production of carotenoids and lipids was optimised by investigating the effect of the C/N ratio in the fermentation medium. Under the optimised process conditions (C/N 60), by fermenting hydrolysate obtained by chemical and enzymatic routes, carotenoid productions of 120 and 180 mg/L and lipids productions of 5.2 and 3.5 g/L were achieved, respectively. The highest carotenoids cell content achieved in this study (14.8 mg/g) is about 5 times higher than the maximum value to date reported in the literature for this yeast. Moreover, Rhodosporidium toruloides achieved the complete conversion of sugars into the desired bioproducts for both the biomass hydrolysates demonstrating the effectiveness of the two different catalytic approaches adopted for biomass hydrolysis.
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	Pedro J. Maireles Torres, Full Professor maireles@uma.es Professor Maireles Torres has outstanding experience on the catalytic conversion of biomass and on green chemistry approaches

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Pisa, 24/03/2024

Dear Editor,

enclosed you find our research article **"Chemical and enzymatic hydrolysis of waste wheat bran to sugars and their simultaneous biocatalytic conversion to valuable carotenoids and lipids"** for publication in the special issue of "**Catalysis Today**" entitled "Advances in Catalysis in Aqueous Media", upon the kind invitation of the Guest Editors. The submission is original, it is not under consideration for publication elsewhere and was not previously submitted to Catalysis Today. The English form has been revised by a native tongue speaker with expertise in the field. All the authors are aware of the submission and agree to its publication. This paper studies a very hot topic in the field of biomass exploitation for the industrial production of valuable chemicals: the valorization of defatted wheat bran, an industrial waste of the food chain, adopting an integrated biorefinery scheme. In fact, through the combination of chemical and biological catalysis, a cascade process was developed in water medium to produce high-value chemicals, such as carotenoids and lipids, from polysaccharide fraction. The low lignin content and suitable particle size of defatted wheat bran make pretreatment steps unnecessary, allowing the direct enzymatic or chemical hydrolysis of polysaccharide components (glucan, xylan, and arabinan) to give fermentable sugars. The biocatalytic approach, the optimisation of the main reaction parameters, such as enzyme dosage (15, 30, 45, 60 FPU Cellic®CTec 3 HS/g glucan) and biomass loading (5, 10, 15, 20 wt%), was performed to improve the monosaccharide yield. Regarding the chemical route, a microwave-assisted $FeCl_3$-catalysed approach was optimised to maximise the sugar yield, minimizing the formation of furanic inhibitors for the subsequent fermentation step. The biological conversion of sugars obtained by both enzymatic and chemical routes into carotenoids and lipids was then performed by adopting the commercial yeast *Rhodosporidium toruloides* DSM 4444. The simultaneous production of carotenoids and lipids was optimised by investigating the effect of the C/N ratio in the

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Sincerely yours,

Prof. Anna Maria Raspolli Galletti

Catalysis Today: Invitation to submit an article to a special issue entitled "Advances in Catalysis in Aqueous Media"

Georgios Papadogianakis <papadogianakis@chem.uoa.gr>

Dear Prof. Raspolli Galletti,

We would like to invite you to submit your research & overview article to a special issue of *Catalysis Today* we are guest editing on "*Advances in Catalysis in Aqueous Media*".

The subject matter of the research portion "*Aqueous-phase catalytic conversions of lignocellulosic biomass*" is of much interest and complementary to the other solicited contributions.

The manuscripts will be submitted from January 1, 2024. The deadline for submissions will be the March 31, 2024.

Please, would you let us know whether you accept this invitation at your earliest convenience.

Thank you and we look forward to hearing from you.

Yours sincerely,

Prof. Roger A. Sheldon

Prof. Paul J. Dyson

Prof. Bruce H. Lipshutz

Prof. Georgios Papadogianakis

Guest Editors

Highlights

- The innovative exploitation of waste defatted wheat bran was assessed.
- The microwave-assisted $FeCl_3$-catalysed hydrolysis of polysaccharides was studied.
- Enzymatic blend Cellic® CTec 3 was adopted as a biocatalyst for sugars production.
- The simultaneous production of high added-value carotenoids and lipids was optimised.
- The carotenoid content (14.8 mg/g) was the highest ever reported for *R. toruloides*.

Graphical Abstract (for review)

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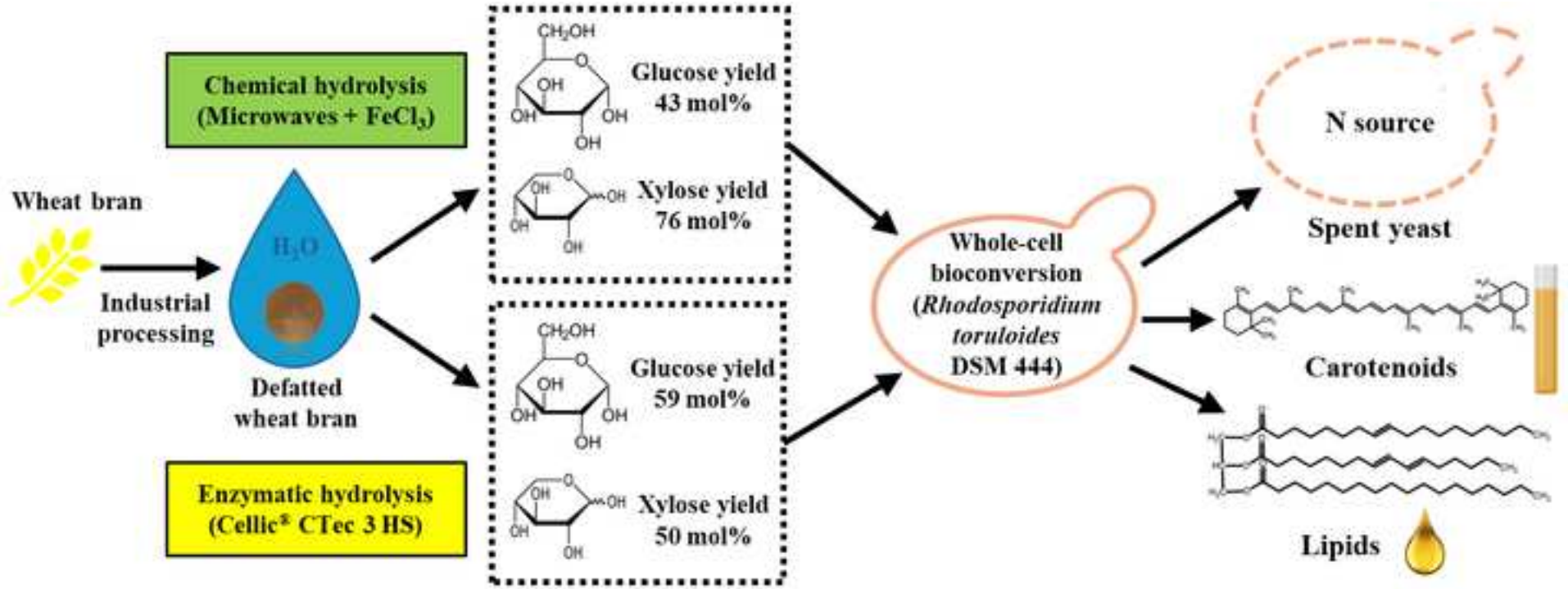

Chemical and enzymatic hydrolysis of waste wheat bran to sugars and their simultaneous biocatalytic conversion to valuable carotenoids and lipids

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Abstract

Defatted wheat bran, an industrial waste of the food chain, represents a strategic renewable material for modern biorefinery schemes. Through a combination of chemical and biological catalysis, a cascade process was developed in water medium to produce high-value fine chemicals, such as carotenoids and lipids, from polysaccharide fraction. Due to the low lignin content and suitable particle size of defatted wheat bran, pretreatment steps are unnecessary, allowing the direct enzymatic or chemical hydrolysis of polysaccharide components (glucan, xylan, and arabinan) to give fermentable sugars. Regarding the biocatalytic approach, the optimisation of the main reaction parameters, such as enzyme dosage (15, 30, 45, 60 FPU Cellic®CTec 3 HS/g glucan) and biomass loading (5, 10, 15, 20 wt%), was performed to improve the monosaccharide yield. Regarding the

chemical route, a microwave-assisted $FeCl_3$-catalysed approach was optimised in terms of catalyst amount (1.0, 1.3, 1.6 wt%) and reaction time (2.5, 5, 10 min) to maximise the sugar yield, minimizing the formation of furanic derivatives which are strong inhibitors for the subsequent fermentation step. The biological conversion of sugars obtained by both enzymatic and chemical routes into carotenoids and lipids was then performed by adopting the commercial yeast *Rhodosporidium toruloides* DSM 4444. The simultaneous production of carotenoids and lipids was optimised by investigating the effect of the C/N ratio in the fermentation medium. Under the optimised process conditions (C/N 60), by fermenting hydrolysate obtained by chemical and enzymatic routes, carotenoid productions of 120 and 180 mg/L and lipids productions of 5.2 and 3.5 g/L were achieved, respectively. The highest carotenoids cell content achieved in this study (14.8 mg/g) is about 5 times higher than the maximum value to date reported in the literature for this yeast. Moreover, *Rhodosporidium toruloides* achieved the complete conversion of sugars into the desired bioproducts for both the biomass hydrolysates demonstrating the effectiveness of the two different catalytic approaches adopted for biomass hydrolysis.

Keywords: wheat bran; enzymatic hydrolysis; chemical saccharification; lipids; carotenoids.

1. Introduction

The transition from a linear economy based on the exploitation of fossil resources to a bio-based circular economy represents an urgent global goal. Annually, 181.5 billion tons of lignocellulosic biomass are produced globally. To date, only 8.2 billion tons per year are used, indicating that the amount of untapped raw material in various application areas is still extremely high [1]. Therefore, the exploitation of waste biomasses into added-value bio-chemicals within innovative biorefinery schemes is strongly encouraged. Lignocellulosic biomass consists mainly of cellulose,

hemicellulose and lignin. Other minority components are extractives, proteins and ash. Cellulose and hemicellulose, i.e., the constituent polysaccharides with structural functions for the plant, are linked together by hydrogen bonds, while lignin and hemicellulose are linked by ether-type bonds [2]. Cellulose and hemicellulose are polysaccharides that can be depolymerised into simple sugars that can be used as platform compounds to obtain high added-value molecules such as bioethanol, biobutanol, fumaric, itaconic, lactic, citric and levulinic acids, furfural, carotenoids and lipids [3-9]. On the other hand, lignin is a biopolymer rich in aromatic components [10]. The relative abundance of the three main components, within the chemical composition of a biomass, greatly varies depending on the type of biomass, the cultivation conditions and the season in which the biomass is harvested [11]. The main problem in using lignocellulosic biomasses is their high recalcitrance to subsequent chemical and/or biological treatments so often a pretreatment step to increase their reactivity is necessary [12, 13]. Among lignocellulosic biomasses, defatted wheat bran represents a strategic food-chain waste since it is a by-product of milling and is characterised by a high content of polysaccharides and a very low content of lignin. These properties make this substrate highly reactive and allow it to be used without any pre-treatment for subsequent chemical or enzymatic saccharification processes. This provides significant economic savings because usually pretreatment technologies require suitable equipment, chemicals and process control [14]. Moreover, according to the producing company, the defatted wheat bran used as feedstock in the present study cannot be used for animal feed due to its low content of proteins and other nutritional elements. For these reasons, an innovative cascade biorefinery in water medium was designed, developed and optimised to first produce fermentable sugars by chemical or enzymatic hydrolysis and then to convert them into high added-value products, namely carotenoids and lipids, by fermentation, adopting the safe commercial yeast *Rhodosporidium toruloides*.

Chemical hydrolysis of polysaccharidic fractions of biomasses has the advantage of being faster than enzymatic hydrolysis and can be used for both the pretreatment of biomass and its direct

conversion to bioproducts. The most commonly usedcatalysts are strong homogeneous acids, such as H_2SO_4 or HCl, adopted in concentrated or dilute form [15, 16]. The adoption of concentrated acids has some advantages over the use of dilute ones, such as higher yield and selectivity to sugars using lower temperatures, causing the formation of low percentages of sugar degradation products, such as 5-hydroxymethylfurfural(5-HMF) and furfural. However, the adoption of concentrated acids involves significant plant corrosion. In addition, the process of separating and recycling the homogeneous acid is energy-intensive [15]. Moreover, the adoption of acid-catalysed hydrolysis in a biorefinery scheme in which products of interest are obtained by fermentation technology requires a trade-off between the hydrolysis of cellulose and hemicellulose fractions to give glucose and xylose, respectively, and the formation of inhibitory by-products. Chemical hydrolysis is hindered by the cellulose crystallinity and its limited solubility in water. The temperature increase promotes hydrolysis but also the subsequent dehydration of glucose and xylose to give 5-HMF and furfural inhibitors, respectively [17].

Recent studies have proposed an innovative catalytic approach for the chemical saccharification of biomass using inorganic salts characterised by both Lewis and Brønsted acidity [18-20]. The use of inorganic salts has several advantages with respect to the adoption of strong acids: lower reactor corrosion, similar costsand higher selectivity towards sugars, reducing the production of inhibitors for the subsequent fermentation process [19, 21]. Moreover, metal chlorides can be recovered as metal hydroxides through ultrafiltration [22] and then reconverted in the starting salt using the conjugated acid (e.g. HCl). Among the inorganic salts, $FeCl_3$ appears to be one of the most efficient catalysts for biomass hydrolysis to monosaccharides [19]. Furthermore, if this salt is adopted for the hydrolysis in the perspective of the subsequent biological valorisation of the sugars-rich hydrolysate by oleaginous yeasts, there is no need to remove the Fe^{3+} ions as they do not harm cell duplication but also promotes lipids biosynthesis [23]. The ability of $FeCl_3$ to hydrolyse hemicellulose has been demonstrated in various studies where it has been used in the pretreatment of various biomasses,

such as miscanthus, olive tree, poplar and giant reed [17, 24-27]. Our previous work demonstrated that $FeCl_3$ can efficiently catalyse the selective hydrolysis of the hemicellulose fraction of *Arundo donax* to xylose using mild reaction conditions ($FeCl_3$ 1.6 wt%, 2.5 min, 150 °C and biomass loading 9 wt%) and then the cellulose-rich solid residue rich to give glucose by adopting harsher reaction conditions ($FeCl_3$ 2.7 wt%, 36 min, 155 °C, biomass loading 9 wt%) [17, 27]. On the other hand, enzymatic hydrolysis is one of the most studied catalytic approaches for obtaining simple sugars from biomasses. This is a viable alternative to chemical hydrolysis because it allows the use of milder reaction conditions (pH ≈ 5, T ≈ 35-50 °C), reduces the environmental impact of the process increasing sustainability, and achieves very high sugar selectivity, avoiding the formation of undesired by-products. However, from a process scale-up perspective, the use of enzymatic mixtures for the hydrolysis reaction of biomass is often limited by the costs of enzymes, long process times and difficult catalyst recycling. Moreover, the performance of enzymatic catalysis is affected by the 3D structure of biomass, its surface area, porosity, presence of lignin and crystallinity degree of cellulose. For lignocellulosic biomasses, pretreatment is usually necessary to increase the effectiveness of enzymatic hydrolysis because the native structure of the biomass prevents the enzyme accessibility to the polysaccharide component [28]. Enzymatic mixtures mainly consisting of cellulases and hemicellulases are commonly employed for the enzymatic hydrolysis of lignocellulosic biomass. Cellulases are the class of enzymes responsible for the hydrolysis of β-1,4-glycosidic bonds. A large number of enzymes belong to this class, such as endoglucanase, exoglucanase, cellobiohydrolase and β-glucosidase. Endoglucanases catalyses the random hydrolysis of 1,4-glycosidic bonds within the amorphous region of cellulose. Exoglucanases catalyse the hydrolysis of reducing or non-reducing ends of cellulose releasing cellobiose. β-glucosidases hydrolyse cellobiose or cellulose oligomers with a degree of polymerisation less than 7 to give glucose [29]. The commercial enzymatic mixture Cellic® CTec 3 HS used in the present investigation consists of cellulases, endo- and exo-cellobiohydrolases, bacterial β-glucosidases and hemicellulases [30].The monosaccharides obtained by chemical or

biological hydrolytic routes were then converted into carotenoids and lipids by whole-cell biocatalysis based on the use of the red oleaginous yeast *Rhodosporidium toruloides*. Carotenoids are lipophilic 40-carbon-atom pigments with antioxidant properties which find commercial applications in food, pharmaceutical, nutraceutical and cosmetic sectors as well as in the field of innovative materials for electronics, such as organic-based transistors. The structural diversity of carotenoids arises from the action of various enzymes on the 40-carbon-atom chain during their biochemical synthesis, such as cyclase, hydroxylase, ketolase and other enzymes. Lutein and zeaxanthin are part of the xanthophylls category and have hydroxyl groups (Figures 1A and 1B). β-Carotenes are the most common carotenoids; structurally they have two rings connected by long conjugated alkyl chains (Figure 1C). In the case of astaxanthin and canthaxanthin, there is the addition of a ketone group with or without a hydroxyl group to the β-carotene structure [31, 32] (Figures 1D and 1E).

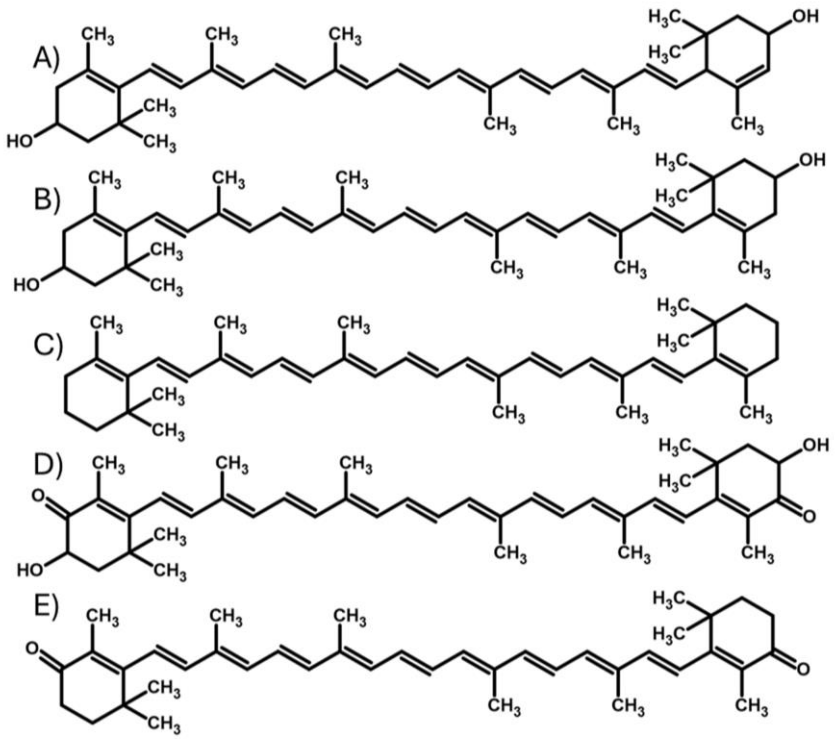

Figure 1. Chemical structure of various carotenoids: A) lutein; B) zeaxanthin; C) β-carotene; D) astaxanthin; E) canthaxanthin.

The effect of key process parameters such as C/N ratio, inoculum age, type and concentration of carbon source, type of nitrogen source, and presence of osmotic and/or oxidative stress-inducing compounds, on carotenoid biosynthesis is still not fully understood. Therefore, with a view to a possible process scale-up for commercial applications of carotenoids, it is important to study and optimise the main process parameters in order to maximise the intracellular accumulation of carotenoids.

Similarly, lipids are important industrial platform chemicals to produce biofuels (e.g., biodiesel), animal feed (e.g. for fishes in aquaculture), bioplastics, biosurfactants, additives and lubricants. Carotenoids and lipids production is affected by several parameters of the fermentation process, such as the nature and the concentration of the carbon source (e.g. glucose and/or xylose), temperature, C/N ratio, pH and the presence of additional micronutrients, such as Fe^{3+}[31, 33].

The microorganism *Rhodosporidium toruloides* is a yeast able to simultaneously produce both lipids and carotenoids in high amounts as intracellular bioproducts. It was first isolated in 1922 from pine wood pulp in the Dailan area of China [34]. It can accumulate up to 60 wt% of its dry cell weight as lipids [35] and, in the best case reported to date in the literature, up to 2.9 mg/g about of carotenoids with respect to the dry cell weight[36]. This yeast can grow at different temperatures (25-35 °C) and pH values (4.4-7.5) [37], fermenting different carbon sources, such as glucose, xylose and glycerol to give lipids and carotenoids [38]. In previous studies reported in the literature, this yeast was grown in media consisting of real and non-detoxified hydrolysates obtained from various biomasses, such as cinnamon waste [39], tea waste [40], *Camelina sativa* flour waste [8], brewery secondary wastewater and sugarcane molasses [41], demonstrating its high versatility in the perspective of biotechnological processes of industrial interest.

In this context, the goals of the present study were (i) the optimisation of both microwave (MW)-assisted chemical hydrolysis and biological hydrolysis of defatted wheat bran to give glucose, xylose and arabinose and the comparison between the two catalytic approaches; (ii) the

optimisation of the biological conversion of the hydrolysates obtained from the two catalytic processes to give carotenoids and triglycerides.

2. Materials and methods

2.1. Materials

Defatted wheat bran was provided by the Italian company Casillo Next Gen Food (Corato, Bari, Italy). The particle size of the biomass was 0.5 mm. For enzymatic hydrolysis, the enzymatic mixture Cellic® CTec 3 HS was supplied by Novozymes company (Denmark). For the fermentation process, the commercial and non-pathogenic yeast strain *Rhodosporidium toruloides* DSM 4444 was purchased by Leibniz Institute DSMZ (Germany).

Sulfuric acid (95%), hydrochloric acid (33%), $FeCl_3 \cdot 6H_2O$ (97%), glucose (99.5%), xylose (95%), arabinose (95%), dimethyl sulfoxide (95%), 2-methyl tetrahydrofuran (95%), citric acid (99.5%), yeast extract, $CaCl_2$ (96%), $MgSO_4 \cdot 7H_2O$ (≥98%), $(NH_4)_2SO_4$ (99%), Na_2HPO_4 (99%), KH_2PO_4 (99%) and β-carotene (99%) were purchased from Merck and used as received.

2.2. Chemical composition analysis of defatted wheat bran

The chemical composition of defatted wheat bran was evaluated through the standard NREL protocols [42-45]. The procedure involves a preliminary hydrolysis of the biomass sample (300 mg) with 3 mL of 72 wt% sulfuric acid solution, performed at 30 °C for 1 h, followed by a second acid hydrolysis of the obtained slurry with a 4 wt% sulfuric acid solution, carried out at 121 °C for 1 h. At the end of the second reaction, the slurry was filtered through a ceramic crucible, and the filtered liquid phase was analysed by HPLC in order to determine the composition of the structural C5 and C6 carbohydrates. Regarding the acid-insoluble residue recovered after the filtration, it was dried up to constant weight at 105 °C, enabling the gravimetric quantification of the Klason (acid-insoluble)

lignin. The ash content was determined as the percentage of residue recovered after dry oxidation of the starting biomass at 550 °C for 24 h. The determination of the extractive content was performed on 2 grams of oven-dried biomass samples, which were extracted in a Soxhlet apparatus for 24 h, with 200 mL of absolute ethanol. All the analyses were performed in triplicate.

2.3. Quantitative analysis of the reaction products

Quantitative and qualitative analysis of sugars (glucose, xylose, arabinose) and by-products (HMF, furfural, acetic acid, formic acid, levulinic acid) were performedaccording to previously described methods and operating conditions[17].

Molar yields of glucose (G), xylose (X) and arabinose (A) were calculated according to Equations 1, 2 and 3.

$$\text{G yield (mol\%)} = [(mol_G/mol_{glucan})] \times 100 \quad (1)$$

$$\text{X yield (mol\%)} = [(mol_X/mol_{xylan})] \times 100 \quad (2)$$

$$\text{A yield (mol\%)} = [(mol_A/mol_{arabinan})] \times 100 \quad (3)$$

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of defatted wheat bran was carried out by using a commercial enzymatic mixture Cellic® CTec 3 HS, having an activity of about 165 FPU/g_{glucan}. The desired amounts of biomass, enzymatic mixture and sodium citrate buffer (0.05 M, pH 4.9) were placed into a 150 mL Erlenmeyer flask. The total weight of the reaction system was fixed at 70 g. The enzymatic reactions were conducted in an incubator equipped with an orbital stirring system at 50 °C and 180 rpm for 96 h. The effect of different biomass loadings (5, 10, 15 and 20 wt%) and enzyme dosages (15, 30, 45, 60 FPU/g glucan) on the sugar yield was investigated.

Biomass loading was calculated according to Equation 4.

$$\text{Biomass loading (wt\%)} = m_b/(m_{H2O} + m_b) \quad (4)$$

where m_b is the mass of defatted wheat bran employed for each reaction and m_{H2O} is the mass of water.

The enzyme dosage (in grams) was calculated according to Equation 5.

$$\text{Cellic}^{®} \text{ CTec 3 HS (g)} = (m_{glucan} \times activity_r)/activity_{stock\_solution} \quad (5)$$

where m_{glucan} is the amount (in grams) of glucan present in the feedstock, $activity_r$ is the enzymatic activity selected for each reaction (e.g. 15, 30, 45, 60 FPU/g glucan) and $activity_{stock\_solution}$ is the enzymatic activity of the stock solution provided by the producer company.

2.5 Microwaves-assisted biomass saccharification catalysed by $FeCl_3$

MW-assisted hydrolysis of defatted wheat bran was carried out by using a monomodal microwave reactor CEM Discover S-class System. $FeCl_3 \cdot 6H_2O$ was used as a homogeneous acid catalyst, and its loading was calculated hrough Equation 6.

$$FeCl_3 \text{ (wt\%)} = [(m_{FeCl3})/(m_{H2O} + m_{FeCl3 \cdot 6H2O}] \quad (6)$$

Biomass (9 wt%), water (10 mL) and $FeCl_3$were added into the glass high-pressure tube (35 mL), adopting the proper catalyst amount. The system was stirred for 10 min to promote homogenisation and then heated in the MW reactor at 150 °C for the selected time under magnetic stirring. At the end of the reaction, the pressure tube was rapidly cooled at room temperature through an external airflow.

2.6 Yeast cultivation

The yeast strain *Rhodosporidium toruloides* DSM 4444 was maintained at 4 °C on a solid medium, containing yeast extract 2.6 g/L, glucose 50 g/L, $(NH_4)_2SO_4$ 1 g/L, agar 20 g/L (pH 4.9), and re-plated every month. Yeast was expanded in a preculture medium consisting of yeast extract 2.6 g/L, glucose 50 g/L, 1 g/L $(NH_4)_2SO_4$ (pH 4.9) kept stirring at 30 °C and 150 rpm on an orbital shaker placed in an incubator. The inoculum was prepared by centrifuging 100 mL of preculture medium for 5 min at 4000 rpm, after which the cells were washed twice with sterile deionised water

and resuspended in about 5 mL of sterile deionised water. 500 μL of suspension was dried in an oven at 80 °C until reaching a constant weight for dry cell weight (DCW) determination. A proper volume of cell suspension was used as inoculum in the fermentation medium to obtain a DCW concentration of 1 g/L at the start of the fermentation process.

Fermentations of defatted wheat bran hydrolysates were carried out in triplicate in 100 mL Erlenmeyer flasks using a working volume of 20 mL. The nitrogen source (yeast extract and $(NH_4)_2SO_4$) was properly added into the fermentation medium as a function of the sugar concentration (i.e., the carbon source) obtained in the hydrolysis reaction in order to reach the selected C/N ratio of 20, 40 and 60. These values were selected according to previous studies reported in the literature for *Rhodosporidium toruloides*[46]. The micronutrients $CaCl_2$ 0.13 g/L, $MgSO_4$ 0.73 g/L, Na_2HPO_4 2 g/L and KH_2PO_4 7 g/L were added according to the study of Dias et al. [33]. Fermentations were carried out at 30 °C, pH 4.9 and 180 rpm until the almost complete conversion of sugars. At the end of the fermentation, the cells were recovered by centrifugation at 4000 rpm for 5 min and then washed twice with sterile deionised water. The cell suspension was then lyophilized for 24 h and stored in a desiccator for the successive extraction of triglycerides and carotenoids.

2.7. Elemental analysis

Elemental analysis (C, H, N, S) of defatted wheat bran and yeast samples was performed by an automatic analyser Vario MICRO Cube (Elementar), equipped with a thermal conductivity detector. The oxygen content was calculated by Equation 7.

$$O\ (wt\%) = 100\ (wt\%) - (C\ (wt\%) + H\ (wt\%) + N\ (wt\%) + S\ (wt\%) + ash\ (wt\%)) \quad (7)$$

2.8. ATR-FTIR analysis of raw material

Fourier Transform-Infrared (FT–IR) characterisation of wheat bran was carried out with a Perkin-Elmer Spectrum-Two spectrophotometer, equipped with an Attenuated Total Reflectance

(ATR) accessory, working in the wavenumber range between 4000 and 450 cm^{-1}. 24 scans were acquired with a nominal resolution of 8 cm^{-1}.

2.9. Carotenoids extraction and quantification

A modified version of one of the most common methods reported in the literature was adopted in the present study [47, 48]. 150 mg of lyophilised cells were suspended in 6 mL of DMSO and the suspension was stirred for 1 h at 55 °C to favour the cell lysis. Then, the cells were precipitated by centrifugation at 4000 rpm for 5 min. The liquid fraction was removed and separately collected. 6 mL of fresh DMSO was added to the cells for their resuspension and the mixture was heated at 55 °C for 10 min under magnetic stirring to enhance the carotenoid extraction. This step was repeated 2-3 times until the cells appeared colourless. After collecting all the DMSO fractions in a separatory funnel, hexane was added (3:1 v/v with respect to DMSO) for a liquid-to-liquid extraction. Then, the hexane and DMSO phases were separately collected in an amber glass bottle and stored at 4 °C until carotenoids quantification.

Carotenoids were quantified spectrophotometrically by using a Jasco UV-Vis spectrophotometer working at a wavelength of 450 nm, according to the literature [49]. Quantification of carotenoids was performed by using commercial β-carotene calibration curves from 1 to 5 ppm. The concentration of carotenoids was calculated through Equation 8, while the intracellular content of carotenoids was calculated by using Equation 9.

$$\text{Carotenoids concentration (mg/L)} = (A/\varepsilon)\times MW_{\beta\text{-carotene}}\times 1000 \quad (8)$$

$$\text{Carotenoids cell content (mg/g)} = ([\text{carotenoids}]\times V_{extraction})/m_{cell} \quad (9)$$

where A is the absorbance,ε is the molar extinction coefficient, $MW_{\beta\text{-carotene}}$ is the molecular weight of β-carotene and m_{cell} is the amount (in grams) of lyophilised cell used for the extraction protocol.

Carotenoids production and productivity were calculated through Equations 10 and 11.

Carotenoids production (mg/L) = (Carotenoids cell content×DCW_f) (10)

Carotenoids productivity (mg/L/h) = (Carotenoids production/time) (11)

where DCW_f is the final dry cell weight value achieved at the end of each fermentation, and time (in hours) is the duration of each fermentative process.

2.10 Lipids extraction and characterisation

A modified version of the traditional chemical method for cell lysis and lipids extraction was adopted in the present work [6]. Approximately 300 mg of lyophilised cells were suspended in 20 mL of a 4 M HCl solution in a borosilicate glass vessel. To promote cell lysis, the vessel was subjected to heating for 1 h at 60 °C, under magnetic stirring, within the single-mode CEM MW reactor. Subsequently, 2-methyltetrahydrofuran (2-MeTHF) was added to the aqueous cell suspension with a ratio of 1:1 v/v and mixed under magnetic stirring for 1 hour at room temperature. Then, the biphasic system was transferred in a separatory funnel and the organic phase containing lipids was recovered. A second extraction was performed by adding fresh 2-MeTHF in a 1:1 v/v ratio with respect to the aqueous phase. The organic solvent containing lipids was evaporated through a rotavapor at 60 °C under vacuum. The bio-oil was then weighed for its gravimetric quantification and stored at 4 °C until its characterisation. The lipids cell content was calculated according to Equation 12.

Lipids cell content (wt%) = m_{oil}/m_{cell} (12)

where m_{oil} is mass in grams of bio-oil extracted from yeast cells.

Lipids production and productivity were calculated through Equations 13 and 14.

Lipids production (mg/L) = (Lipids cell content×DCW_f) (13)

Lipids productivity (mg/L/h) = (Lipids production/time) (14)

For the conversion of triglycerides into FAMEs, 40-50 mg of each SCO sample was treated as described by standard ISO 12966-2:2017. To extract the FAMEs from the reaction mixture, hexane was added with a ratio of 0.04:1 w/v concerning the starting amount of lipids. A saturated NaCl solution was then added to aid phase separation. The upper phase was recovered and diluted 1:2 v/v. The sample was then stored at 4 °C until the GC-MS analysis.

The FAMEs profile was determined by GC-MS analysis using the instrument Agilent 7890B-5977A, equipped with an MSDHP 5977 detector and with a GC column HP-5MS column (5% diphenyl-95% dimethyl polysiloxane stationary phase, column length 30 m, inner diameter 0.25 mm and thickness of the stationary phase 0.25 μm), in splitless mode. The transport gas was helium 5.5 and the flow was 1 mL/min. The temperature of the injection was set at 250 °C, carrier pressure at 100 kPa. The oven was heated according to the following operating conditions: (i) 60 °C for 1 min; (ii) 10 °C/min from 60 to 200 °C; (iii) 200 °C for 2 min; (iv) 5 °C/min from 200 to 220 °C; (v) 220 °C for 20 min; (vi) 10 °C/min from 220 to 270 °C; (vii) 270 °C for 2 min. The area of each peak was divided by the total area of the peaks in the chromatogram to calculate the relative abundance of each FAME.

3. Results and discussion

Defatted wheat bran represents a strategic starting material because, according to the producing company (Casillo Group, Italy), there is currently no profitable value chain for it due to its chemical composition, which will be discussed in more detail below. This residual biomass derives from the industrial processing of wheat bran to produce wheat flour, wheat bran oil and proteins for food applications using proprietary technologies. As a result, the residual defatted wheat bran has poor nutritional properties and is also unsuitable for animal feed. In the perspective of the circular economy and to valorise this negative-value biomass, in this study, both glucan, xylan and arabinan fractions of the starting feedstock were simultaneously hydrolysed in a one-pot approach to give fermentable monosaccharides by adopting two catalytic approaches: the first one

based on the use of the inorganic salt $FeCl_3$ (chemical catalysis) and the second one based on the use ofthe commercial enzymatic mixture Cellic® CTec 3 HS (biological catalysis). Then, the biomass hydrolysates rich in glucose and xylose obtained from the two catalytic approaches were fermented by using the oleaginous yeast *Rhodosporidium toruloides* to obtain triglycerides and carotenoids, which are two high-value platform chemicals. The scheme of the biorefinery process designed and developed in the present work is reported in Figure 2.

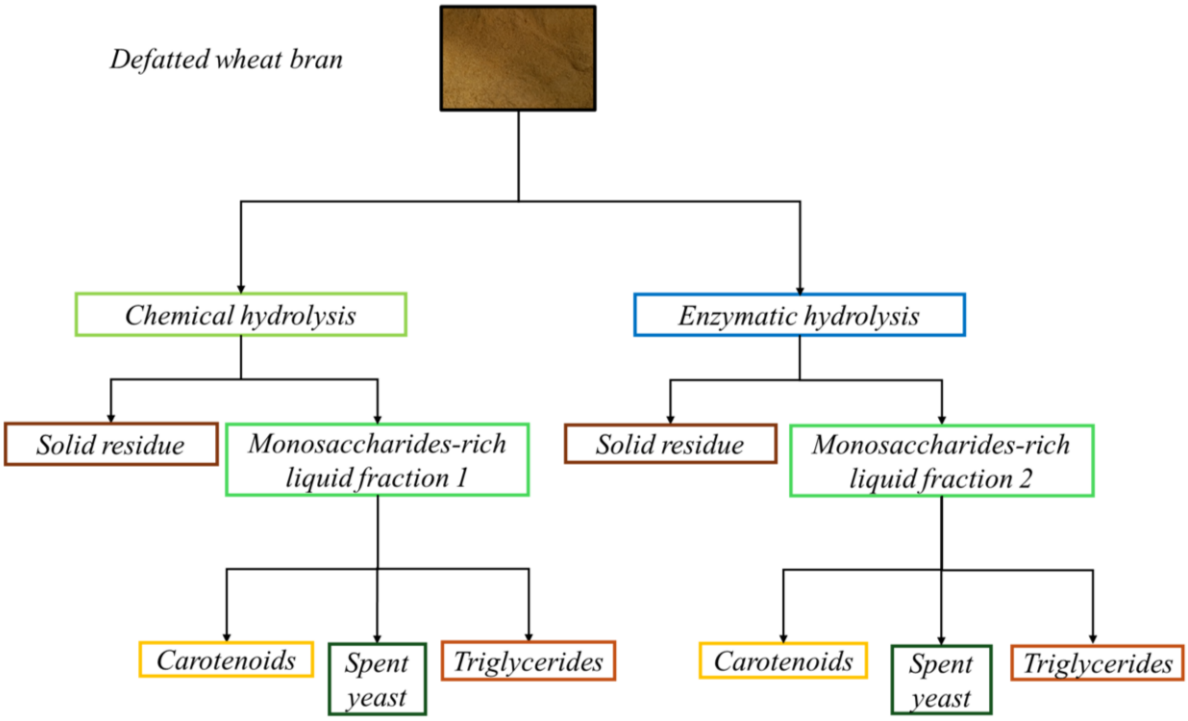

Figure 2. Flow diagram of the biorefinery scheme developed for the exploitation of defatted wheat bran.

The catalytic performances of $FeCl_3$ and Cellic® CTec 3 HS were evaluated and optimised by studying the effect of the main process parameters, such as temperature, reaction time, biomass loading and catalyst amount, on the sugar yield. In the case of chemical hydrolysis, the aim was not only the maximisation of monosaccharides production but also the minimisation of the formation of by-products which strongly hamper the following fermentation step.

The fermentation process was optimised by investigating the effect of the carbon/nitrogen ratio on the yeast growth, namely the dry cell weight production, and the biosynthesis of lipids and carotenoids. Moreover, carotenoids and triglycerides are intracellular bioproducts, thus the downstream processing of yeast cells generates solid waste represented by spent yeast (i.e., yeast extract), which could in principle be recycled as a cheap nitrogen source within the same fermentation process or employed in anaerobic digestion processes.

3.1. Chemical characterisation of defatted wheat bran

The characterisation of the starting feedstock according to the standard NREL protocols [42-45]is reported in Table 1.

Table 1. Composition (wt% on dry basis) of defatted wheat bran.

Fraction	**Relative abundance (wt%)**
Glucan	37.2 ± 0.9
Xylan	27.5 ± 0.7
Arabinan	12.2 ± 0.3
Acetyl groups	3.0 ± 0.7
Acid insoluble lignin	2.0 ± 0.1
Acid soluble lignin	0.1 ± 0.0
Proteins	12.5 ± 0.6
Extractives	4.4 ± 0.2
Ash	3.5 ± 0.1
Total	102.4 ± 3.6

Remarkably, polysaccharide fraction represents about 75 wt% of the biomass while lignin is only about 2 wt%. The ascertained glucan, xylan and arabinan contents of defatted wheat bran were

higher than those reported in the literaturefor pristine wheat bran (glucan: 24.9-25.4%, xylan: 15.2-20.3%, arabinan: 7.8-9.3%) [50, 51]. This result agrees with the enrichment of biomass in polysaccharides due to the selective oil and proteins removal performed in the transformation process.

Figure 3 shows the ATR-FTIR spectrum of the starting feedstock.

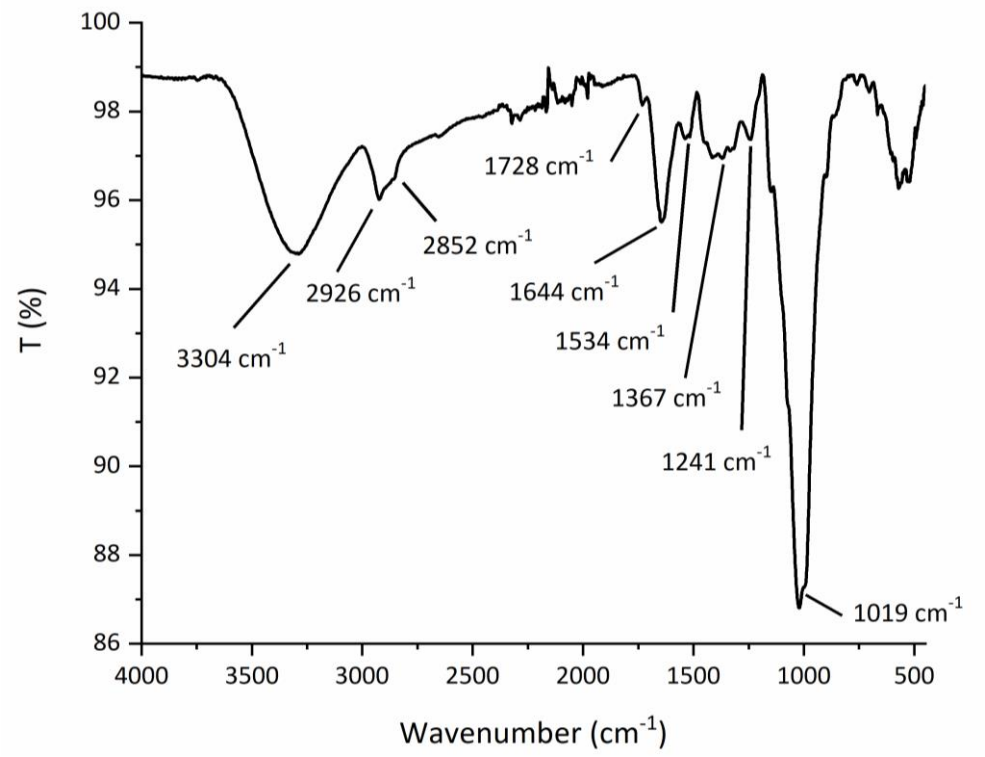

Figure 3. FTIR-ATR spectrum of defatted wheat bran

The absorption peak at 3304 cm^{-1}can be attributed to the stretching vibration of the O-H bonds of the polysaccharides. The peaks at 2926 and 2852 cm^{-1} are due to the -CH_2 and -CH_3 groups bending of the different organic components. The peak at 1728 cm^{-1} is assigned to the stretching of the C=O bond of the acetyl groups of hemicellulose (consisting of xylan, arabinan and acetyl groups). The band at 1644 cm^{-1} corresponds to the stretching vibration of the C-O and C-N bonds typical of proteins. Similarly, the absorption peak at 1534 cm^{-1} corresponds to the bending of the N-H bond, while the signals in the 1360-1410 cm^{-1} region result from the bending of the C-N bond, both

attributable to the presence of proteins. The absorption bands at 1241 and 1019 cm^{-1} are due to the stretching of the C-O-C bond of the polysaccharides.The ATR-FTIR analysis confirmed the abundance of the polysaccharidic fractions according to the previous chemical composition determination (Table 1).

The elemental analysis of defatted wheat bran was carried out: the carbon, hydrogen, nitrogen, sulphur and oxygen contents were 43.2, 7.0, 2.0, 0.2 and 44.1 wt%, respectively.The value of the higher heating value (HHV) calculated by the equation reported byChanniwala et al. [52]was 18.7 MJ/kg, which is low for direct energy applications of this biomass [53, 54]. The typical HHV values of biomasses and biomass-derived residues suitable for direct combustion for energy production range from 20 to 35 MJ/kg. Based on this, the selected defatted wheat bran can therefore be strategically valorised through chemical and/or biological catalytic approaches. Moreover, the very high content of sugars and the low content of lignin make the defatted wheat bran an ideal substrate for direct biomass hydrolysis without any pretreatment. The absence of pretreatment in the proposed biorefinery significantly increases the techno-economic and environmental sustainability of the process.

3.1 Microwave-assisted biomass saccharification catalysed by $FeCl_3$

Up to now, the hydrolysis reaction of biomass polysaccharides in the presence of $FeCl_3$ for the selective production of C5 and C6 sugars has been poorly studied[24-26, 55]. In our previous studies, we optimised the selective cascade fractionation of xylan and glucan of the lignocellulosic biomass giant reed (*Arundo donax* L.) to give xylose and glucose, respectively, using MW as an efficient and energy-saving heating system[17, 27]. Differently, in the present work, considering the significant differences in the chemical composition of the starting biomass, especially in terms of low content of lignin, a chemical or biological one-pot approach was tested to simultaneously hydrolyse glucan, xylan and arabinan to give glucose, xylose and arabinose, respectively. As

reported in the literature, in water $FeCl_3$ acts as a Lewis acid, functioning as an electron pair acceptor that can engage with a Lewis base to create a Lewis adduct. Román-Leshkov and Davis proposed a catalytic mechanism wherein the metal cation (Fe^{3+}) coordinates with six water molecules as a monodentate ligand[56]. These coordinated water molecules, associated with the hydrated cation, interact with the glycosidic oxygen atom, leading to the cleavage of glycosidic linkages and the formation of glucose, xylose or arabinose by acting as nucleophiles. In addition, similar to common Brønsted acids,the dissociation of $FeCl_3$in water favours the formation of H_3O^+ ions, which catalyse the depolymerisation of polysaccharides into monosaccharides[19, 57]. Moreover, in the presence of Brønsted and Lewis acidity of metal salts, glucose can be further dehydrated to 5-HMF and finally converted to LA and formic acid, while xylose and arabinose can be further dehydrated to furfural [58]. In this context, the role of the reaction parameters is crucial to promote the selectivity of the reaction towards sugars while minimising the formation of furanic derivatives. In order to optimise chemical saccharification, the effect of reaction time and catalyst loading on sugars yield and by-products formation was investigated. The temperature was fixed at 150 °C based on our previous studies on the MW-assisted $FeCl_3$-catalysed hydrolysis of hemicellulose fraction [17]. In fact, by increasing the temperature over 150 °C both the hydrolysis of cellulose and the dehydration of C5 sugars to furfural are promoted, thus, to minimise furfural formation the temperature was controlled. Moreover, adopting a reaction time of 2.5 min, the $FeCl_3$ amount was increased from 1.0 to 1.6 wt% to investigate the effect of catalyst loading on sugars yield. Figure 4 shows the obtained results in terms of sugars concentration and yield.

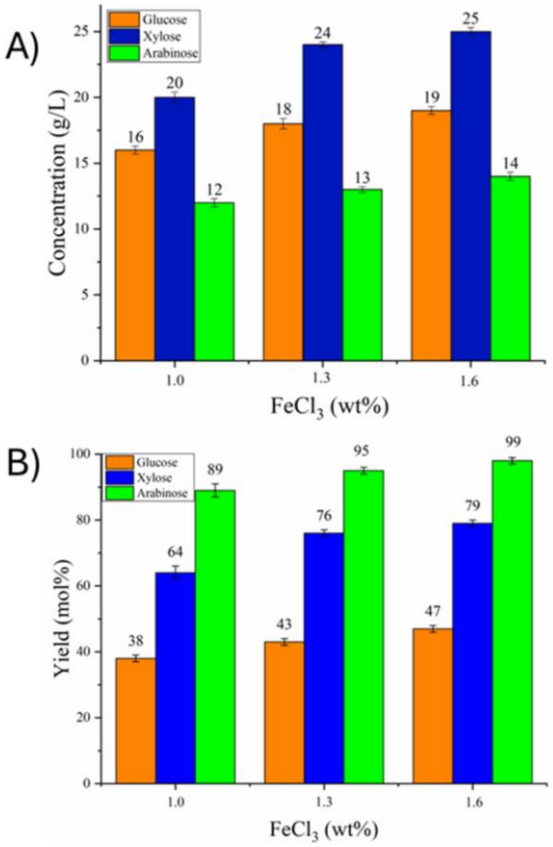

Figure 4. Glucose, xylose and arabinose concentrations (A) and molar yields (B) as a function of $FeCl_3$ amount. Reaction conditions: MW heating, 2.5 min, 150 °C, biomass loading 9 wt%.

Sugar yields increased as a function of the increase in the catalyst amount. In particular, xylose yield increased from 64 to 76 mol% while arabinose yield from 89 to 98 mol%. On the other hand, glucose yield showed a slighter increment (from 38 to 40 mol%) than that observed for xylose and arabinose, but this agreed with the mild reaction conditions adopted, which mainly favour the selective hydrolysis of the hemicellulose fraction. However, the glucose yield achieved in the one-pot hydrolysis was similar to the value (39.9 mol%) reported in the literature for the two-step MW-assisted $FeCl_3$-catalysed hydrolysis of the cellulose-rich solid residue of giant reed performed under harsher reaction conditions (155 °C, 34 min, $FeCl_3$ 2.7 wt%, biomass loading 9 wt%)[27], confirming the higher chemical reactivity of defatted wheat bran. As shown in Figure 5, no significant increase in 5-HMF, furfural and levulinic acid was observed by increasing the amount of

catalyst from 1.0 to 1.3 wt%. However, a further increase of the $FeCl_3$ concentration to 1.6 wt% did not improve the sugar yield but resulted in a significant increase in by-product concentrations up to 2 g/L for furfural and 0.8 g/L for 5-HMF.

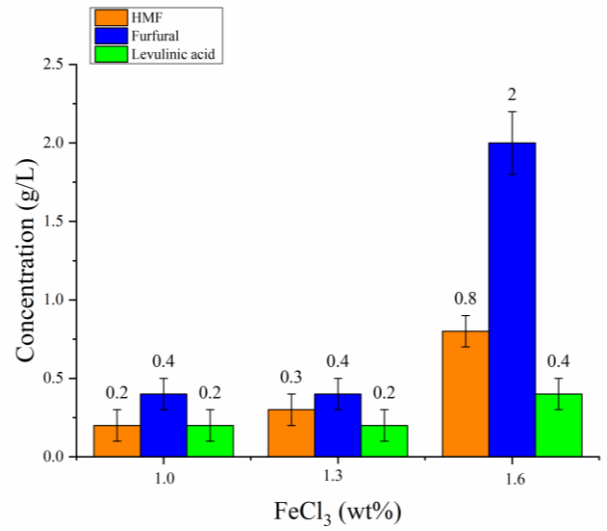

Figure 5. HMF, furfural and levulinic acid concentration as a function of $FeCl_3$ amount. Reaction conditions: MW heating, 2.5 min, 150 °C, biomass loading 9 wt%.

Yeast fermentation processes require low concentrations of furanic derivatives (usually < 0.5 g/L) to avoid inhibition of biocatalyst growth[59]. For this reason, in the proposed catalytic approach, a compromise between sugar and by-product production was reached by the adoption of the $FeCl_3$ amount of 1.3 wt%. Then, the effect of reaction time on the production of sugars and by-products was studied by extending the reaction time to 5 and 10 min, keeping the other process parameters constant. The trends of sugar concentration and yield are shown in Figure 6.

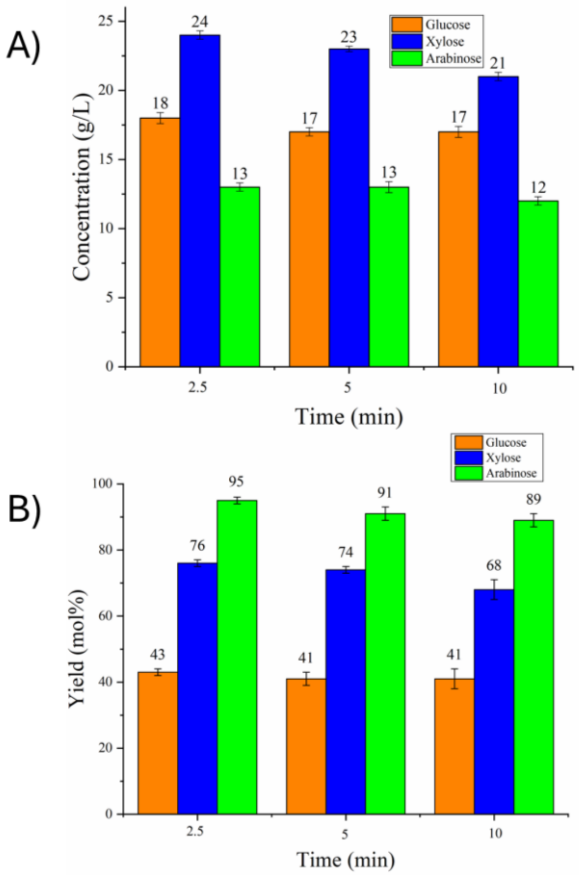

Figure 6. Glucose, xylose and arabinose concentrations (A) and molar yields (B) as a function of the reaction time. Reaction conditions: MW heating, $FeCl_3$ 1.3 wt%, 150 °C, biomass loading 9 wt%.

The increase in reaction time slightly affected the sugar production. Glucose yield remained almost constant, whereas the xylose and arabinose yields decreased from 76 to 68 mol% and from 95 to 89 mol%, respectively, due to the progressive formation of inhibitors as evidenced in Figure 7.

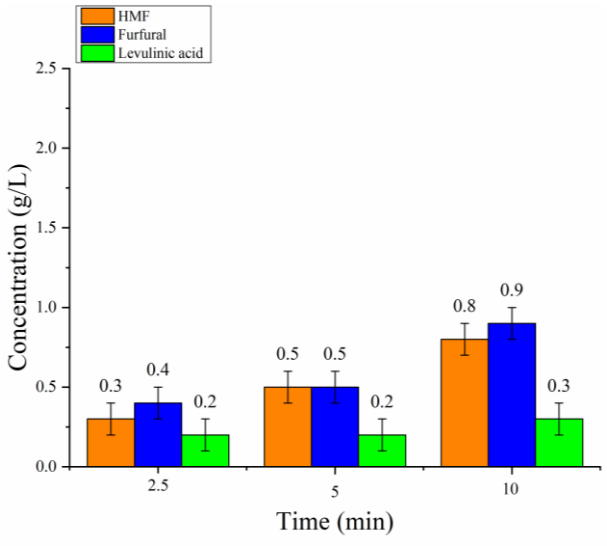

Figure 7. HMF, furfural and levulinic acid concentration as a function of the reaction time. Reaction conditions: MW heating, $FeCl_3$ 1.3 wt%, 150 °C, biomass loading 9 wt%.

Therefore, the optimised reaction conditions for the chemical saccharification of defatted wheat bran were as follows: $FeCl_3$ 1.3 wt% and reaction time of 2.5 min. Under these conditions, approximately 42 g/L of fermentable sugars were obtained in the hydrolysate, a suitable value for the whole-cell biocatalytic production of carotenoids and lipids in batch mode fermentation[33].

3.2 Enzymatic saccharification of defatted wheat bran

Biological catalysis based on the use of cellulolytic enzymes is a common alternative to chemical catalytic approaches for the hydrolysis reaction of polysaccharides to monosaccharides. In the present study, the commercial enzymatic mixture Cellic® CTec3 HS was used as biocatalyst.This system represents one of the recent and most advanced enzymes formulations industrially used for lignocellulosic biomass exploitation due to its high enzymes activity (expressed as filter paper units per gram of enzyme) and relative composition of enzymes optimised for the hydrolysis of lignocellulosic biomass [60]. This enzymatic mixture contains cellulases, endo- and exo-cellobiohydrolases, bacterial β-glucosidase, and hemicellulases and is still poorly

studied with respect to its previous version Cellic® CTec 2. As reported in the literature, the activity and the protein content of Cellic® CTec3 HS are higher than those of Cellic® CTec2 [30].

The catalytic performances of the Cellic® CTec 3 HS in the exploitation of defatted wheat bran was investigated by preliminarily studying the effect of the enzyme dosage (15, 30 and 45 FPU/g glucan) on sugars yield using a biomass loading of 5 wt% and the other reaction conditions previously optimised for the hydrolysis of pretreated defatted cardoon and giant reed [30, 61]. Figure 8 shows the variation of sugars concentration and molar yield as a function of Cellic® CTec 3 HS dosage.

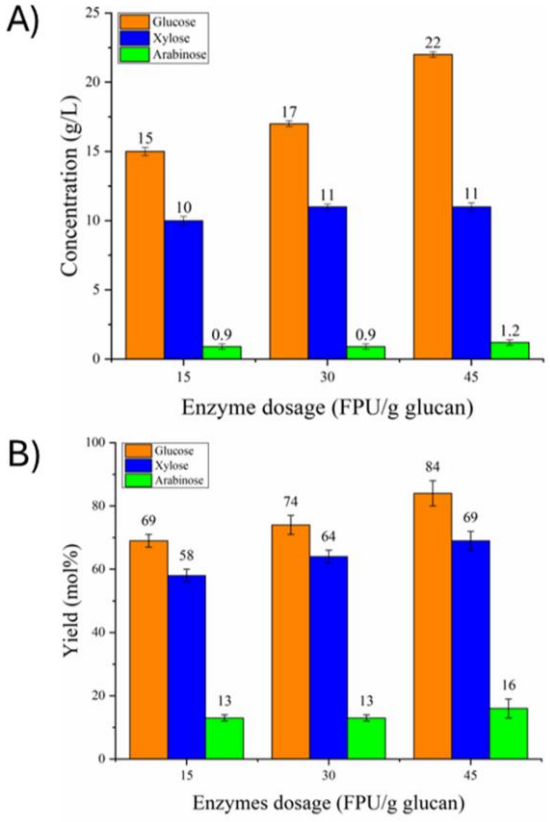

Figure 8. Glucose, xylose and arabinose productions (A) and molar yields (B) as a function of the enzyme blend dosage. Reaction conditions: 50 °C, 180 rpm, pH 4.9, 96 h, biomass loading 5 wt%.

The increase of the enzyme dosage favoured the hydrolysis of glucan and xylan but did not affect

the hydrolysis of arabinan as Cellic®CTec 3 HS has not specific enzymes for the hydrolysis of this polysaccharide. By increasing the enzyme dosage from 15 to 30 FPU/g glucan the glucose yield increased from 69 to 74 mol% while the xylose yield from 58 to 64 mol%. In the presence of 45 FPU/g glucan the maximum glucose and xylose yields of 84 and 69 mol% were achieved, respectively. Under this last condition, the total sugars concentration (sum of glucose and xylose concentrations) was 33 g/L due to the low biomass loading used (5 wt%). In order to increase the sugars concentration over 40 g/L for favouring the productivity of the subsequent biosynthesis of lipids and carotenoids, the effect of enzyme dosage was investigated by increasing the biomass loading to 10 wt%, i.e. a value similar to that adopted in the chemical catalytic approach.

Figure 9 shows the variation of sugars concentration and molar yield in the presence of different enzyme blend dosages.

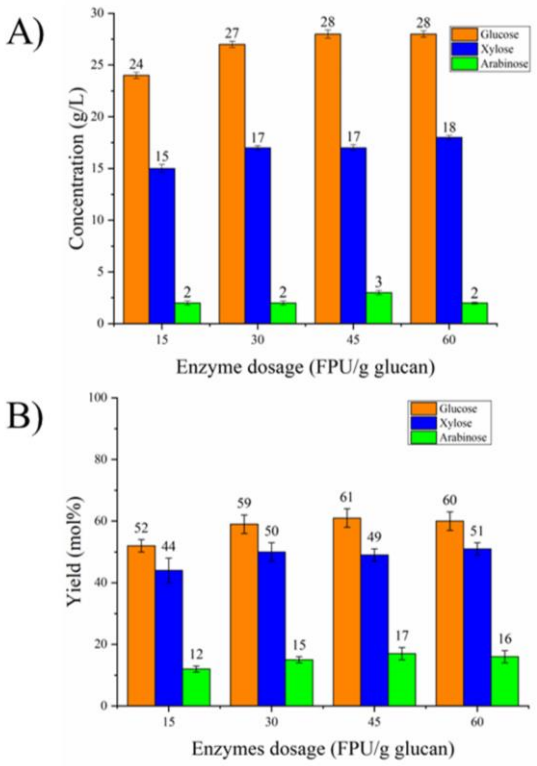

Figure 9. Glucose, xylose and arabinose productions (A) and molar yields (B) as a function of the enzyme blend dosage. Reaction conditions: 50 °C, 180 rpm, pH 4.9, 96 h, biomass loading 10 wt%.

As expected, the sugars molar yields ascertained at higher biomass loading decreased due to the worsening of the stirring efficiency of the reaction system and the diffusion limits of matter (compare Figure 9 with Figure 8). Also in this case, an interesting increase in sugars yield was observed by increasing the enzyme amount from 15 to 30 FPU/g glucan. In particular, glucose yield varied from 52 to 59 mol% and xylose yield from 44 to 50 mol%, but a further increase of Cellic®CTec 3 HS up to 60 FPU/g glucan did not significantly increase the sugars yield. Arabinose yield was not characterised by a relevant variation as a function of the biocatalyst amount. Considering the glucose and xylose yields and concentrations, the enzyme dosage of 30 FPU/g glucan was selected for the following investigation of the biomass loading effect, being a reasonable compromise between catalyst concentration, which affects up to 25-30% of the overall process cost [28], and a suitable sugars productivity for the subsequent biocatalytic upgrading to give valuable fine chemicals. Moreover, the effect of the defatted wheat bran loading on sugars production was investigated by increasing the biomass loading from 5 to 20 wt% in the presence of the Cellic®CTec 3 HS dosage of 30 FPU/g glucan (Figure 10).

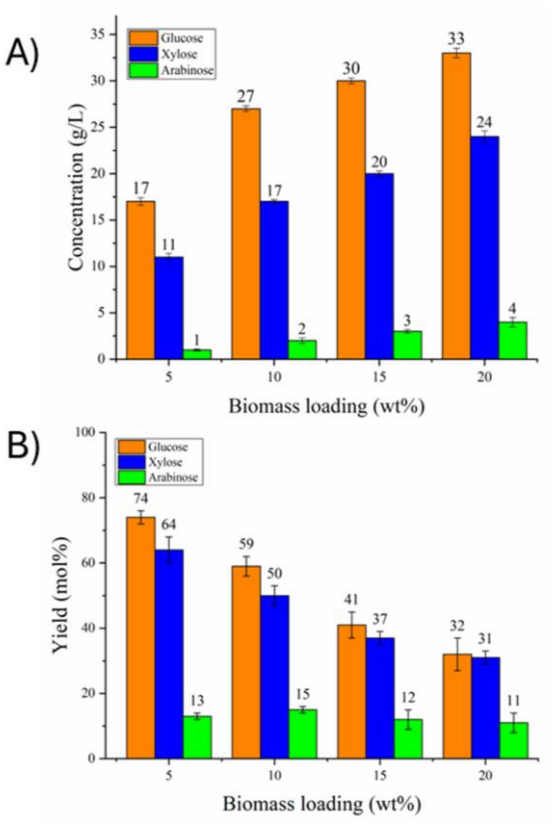

Figure 10. Glucose, xylose and arabinose productions (A) and molar yields (B) as a function of the biomass loading. Reaction conditions: 50 °C, 180 rpm, pH 4.9, 96 h, Cellic®CTec 3 HS 30 FPU/g glucan.

Increasing the defatted wheat bran loading from 5 to 20 wt% was associated with a significant decrease in glucose and xylose yields, which ranged from 74 to 32 mol% and from 64 to 31 mol%, respectively. In particular, by comparing the results obtained at biomass loading of 5 and 20 wt%, it can be observed that quadrupling the amount of enzyme mixture used in the reaction only doubles the sugar concentration. In fact, glucose and xylose concentrations ranged from 17 to 33 g/L and 11 to 24 g/L, respectively. This trend agreed with those reported in the literature for other types of lignocellulosic biomasses[30, 62].Taking into account the obtained results regarding the effect of biomass loading and enzyme dosage on the one-pot hydrolysis of defatted wheat bran, the most suitable conditions resulted: biomass loading of 10 wt% and enzyme dosage of 30 FPU/g glucan.

Under these reaction conditions, a total fermentable sugar concentration of 44 g/L was achieved, which is suitable for the following fermentation process.

In the literature, there are a few studies on the use of Cellic® CTec3 HS for hydrolysis reaction of biomasses, while no study on the enzymatic saccharification of wheat bran is reported. The total sugars concentration achieved in the present study is higher than that (28 g/L) obtained by Gomes et al. [6] for the Cellic® CTec 3 HS-catalysed hydrolysis of pretreated bagasse, but in this case, the biomass (9 wt%) was pretreated for about 1.5 h at 100°C with an aqueous solution of citric acid (6 wt%). Kim et. al [63] studied the enzymatic hydrolysis of empty palm oil shells, previously pretreated through a hydrothermal treatment (190 °C, 15 min), using a solid loading between 20 and 30 wt% and an enzyme dosage between 20 and 60 FPU/g glucan. Under the optimised reaction conditions (60 FPU/g glucan, biomass loading of15 wt%)a glucose yield of 99 mol% was achieved, confirming the high catalytic efficiency of this commercial enzymatic mixture especially when pretreated biomasses are employed.Fockink et al. [62] optimised the enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse (195 °C, 7.5 min) by varying the enzyme dosage between 7 and 40 FPU/g glucan and the biomass loading between 10 and 20 wt%. Under the optimised reaction conditions (40 FPU/g glucan, biomass loading of 10 wt%), a glucose yield of 99 mol% was obtained. Campos et al. [64]used Cellic® CTec 3 HS to hydrolyse sugarcane bagasse firstly pretreated for 75 min at 120 °C with a 1.5 wt% H_2SO_4 solution and a biomass loading of 9 wt%. After this pretreatment, the obtained solid residue was treated for 3 h at 80 °C with a 7.5 v/v% H_2O_2 solution and a biomass loading of 5 wt%. The solid residue obtained after these two pretreatments was enzymatically hydrolysed to produce sugars. Under the optimised reaction conditions (biomass loading of 10 wt%, enzyme dosage of 30 FPU/glucan, 120 h) a glucose yield of only 20.2 mol% was obtained by the authors.

3.3 Fermentation of defatted wheat bran hydrolysates

For the profitable valorisation of defatted wheat bran within the innovative biorefinery scheme proposed in the present study (Figure 2), the two sugars-rich hydrolysates obtained by the chemical and biological hydrolytic routes were directly fermented without any intermediate detoxification step in order todecrease the steps of the process and increase its sustainability. Both the hydrolysates were fermented by the yeast *Rhodosporidium toruloides* which can simultaneously synthesise carotenoids and lipids, namely two strategic high added-value products with several commercial applications in different industrial fields. To maximise the production of bio-oil and carotenoids, the effect of the C/N ratio, deriving from the concentration of monosaccharides and nitrogen sources added in the medium (yeast extract and $(NH_4)_2SO_4$), and the composition of the carbon source (i.e., the relative abundance of glucose and xylose) on the production of the target bioproducts was investigated and optimised. The C/N ratio is a key parameter for the biochemical regulation of carotenoids and lipids production [9, 32]. Relatively low C/N values ($\leq$ 60) were selected for the present study in order to favour the intracellular biosynthesis of carotenoids, which represent the product of primary interest in this work due to their much higheraddedvalue. Moreover, the nature of the carbon source can also influence the production and the chemical profile of carotenoids and triglycerides [38]. It is important to note that not all biocatalysts used in modern biomass refineries are able to convert C5 sugars, such as xylose and arabinose, thus limiting the simultaneous profitable exploitation of the hemicellulose fraction. Therefore, from an industrial perspective, it is crucial to select microorganisms capable of converting the hydrolysed sugars into the bioproducts of interest.The selection of *R. toruloides* for the present cascade process was also based on this criterion, as it is known to efficiently convert xylose into bio-oil and carotenoids[36, 46].

The defatted wheat bran hydrolysate obtained by the chemical route was fermented at C/N ratios of 20, 40 and 60. For each fermentation, the sugar and DCW concentrations were monitored daily throughout the process. Figure 11 shows the kinetics of sugar consumption and DCW increase during the fermentation carried out on the $FeCl_3$ hydrolysate medium.

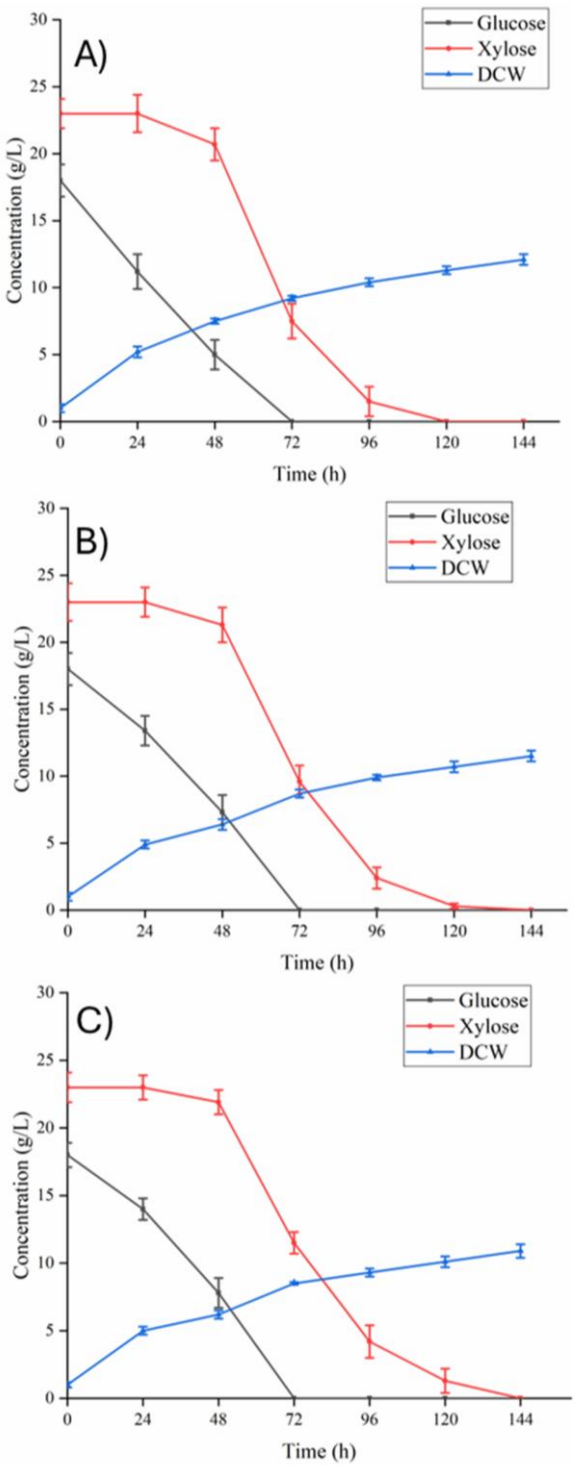

Figure 11. Profiles of xylose, glucose and DCW concentrations of the fermentation of $FeCl_3$-derived hydrolysate at different C/N ratios: A) 20; B) 40; C) 60.

In all the processes, no significant lag phase was observed as DCW concentration increased from 1 to about 5 g/L in the first 24 h of fermentation with the simultaneous consumption of glucose. After

72 h cell growth rate decreases reaching the stationary phase after around 144 h. At the end of each fermentation, the DCW concentrations were 12.1, 11.5 and 10.9 g/L for C/N ratios of 20, 40 and 60, respectively. These trends can be explained by considering that the decrease in microbial biomass production usually occurs as the C/N ratio increases because nitrogen is essential for the synthesis of structural and catalytic proteins required for cell replication. Moreover, the DCW values achieved by fermenting this wheat bran hydrolysate agreed with those reported in the literature for the same biocatalyst grown on other types of lignocellulosic hydrolysates[8]. Regarding the kinetics of monosaccharides conversion, the preferential consumption of glucose with respect to the xylose was observed in the first part of the fermentation, according to the biochemical phenomenon of glucose repression typical of many yeasts and eukaryotic organisms [9]. In all the fermentations, glucose was completely consumed within 72 h, while the xylose conversion started when glucose concentration was lower than about 7 g/L. The total conversion of glucose and xylose occurred in 120 h for the C/N ratio of 20 and in 144 h for the ratios of 40 and 60, according to the literature [8].

The results obtained in the fermentation of the wheat bran hydrolysate in the presence of Cellic® CTec 3 HS at different C/N ratios are reported in Figure 12.

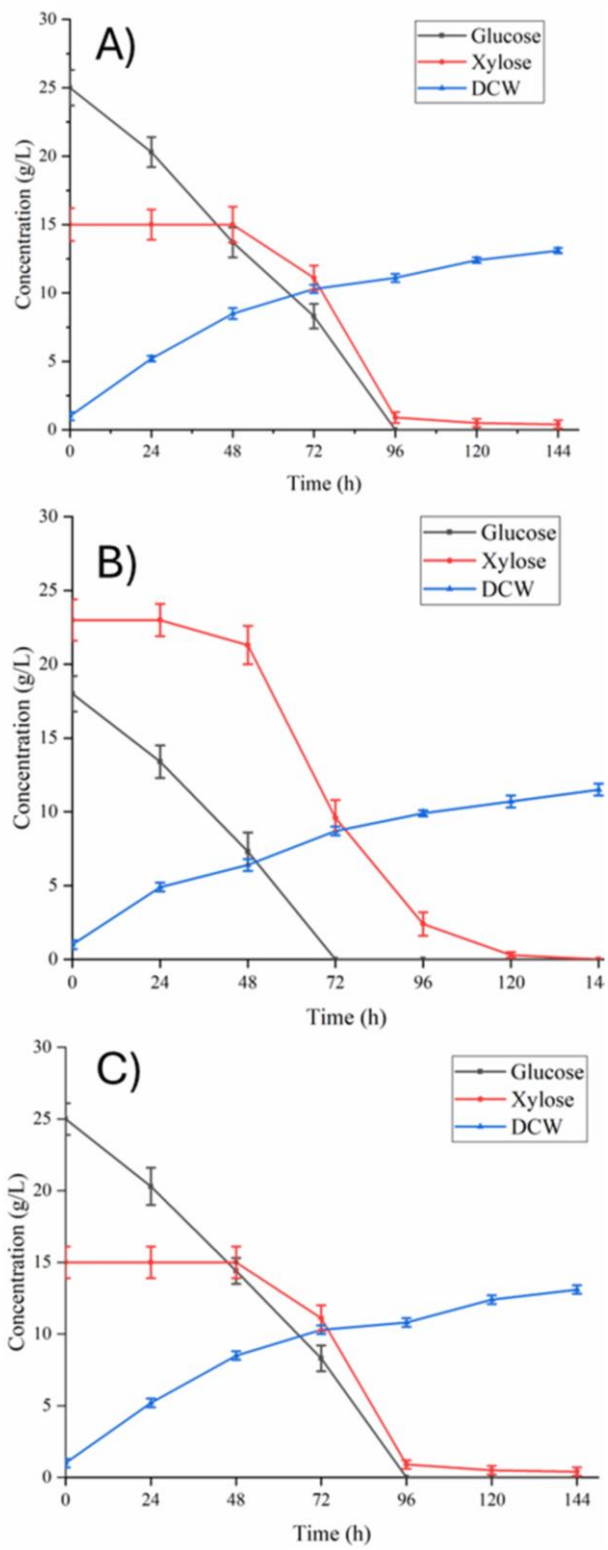

Figure 12. Profiles of xylose, glucose and DCW concentrations of the fermentation of Cellic® CTec 3 HS-derived hydrolysate at different C/N ratios: A) 20; B) 40; C) 60.

Also in this case, no appreciable lag phase was present in the first day for all the media, observing an increase of about 5-folds of DCW from 1 to 5 g/L. This behaviour was similar to that observed in the fermentation of biomass hydrolysate obtained by the chemical hydrolytic route (Figure 11). The kinetic profiles of the yeast growth weresimilar tothose observed in the previous fermentations, confirming the high reproducibility of the proposed bioconversion processes. At the end of fermentations, the DCW values of 13.1, 13.0 and 11.9 g/L were achieved for C/N ratios of 20, 40 and 60, respectively. These values agreed with those previously discussed (12.1, 11.5 and 10.9 g/L for C/N ratios of 20, 40 and 60, respectively), confirming that increasing the C/N ratio (obtained by reducing the nitrogen source added to the fermentation medium) adversely affects yeast growth and DCW production.

Differently from the kinetics reported in Figure 11, *Rhodosporidium toruloides* consumed all the glucose in the medium in 96 h instead of 72 h, due to its higher initial concentration in the hydrolysate (Figure 12). In fact, the two hydrolysates contained a different relative ratio of glucose and xylose concentration. In the one obtained by $FeCl_3$-catalysed hydrolysis the glucose and xylose concentrations were 18 and 24 g/L, respectively, whereas in the one obtained by enzymatic hydrolysis, the sugars concentrations were 27 and 17 g/L, respectively. As shown in Figure 12, xylose consumption started after 72 h in all processes and ended after about 120 h.

Figure 13 shows the trends of the intracellular content of carotenoids and lipids in *Rhodosporidium toruloides* as a function of the C/N ratio in all the fermentations carried out.

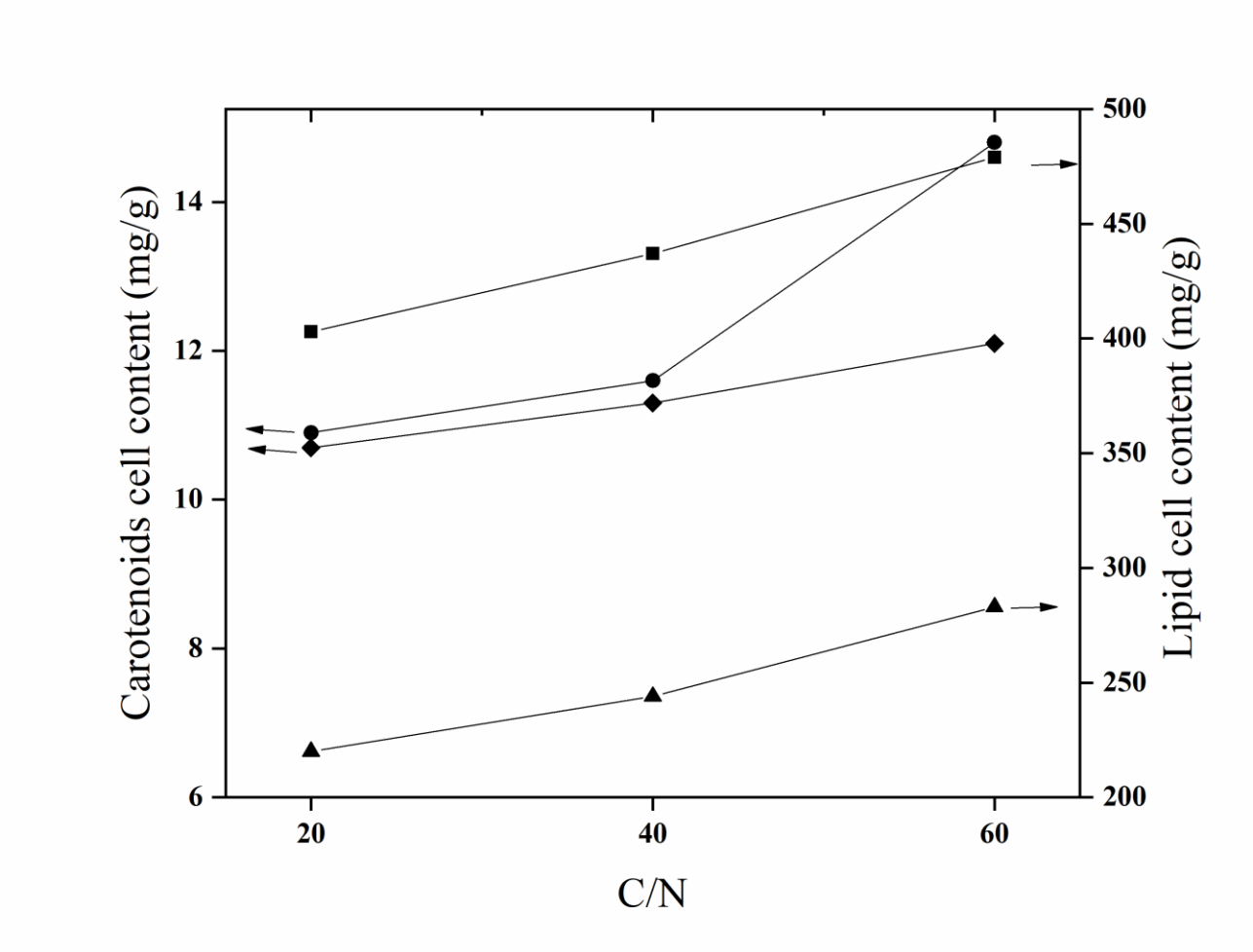

Figure 13. Variation of carotenoids and lipids content per cell (mg/g) as a function of C/N ratio:indicates the carotenoids cell contents obtained from the fermentation of the hydrolysate obtained by the chemical catalytic route;indicates the carotenoids cell content obtained from the fermentation of the hydrolysate obtained by the biological catalytic route; indicates the lipids cell contents obtained from the fermentation of the hydrolysate obtained by the chemical catalytic route; indicates the lipids cell content obtained from the fermentation of the hydrolysate obtained by the biological catalytic route.

Increasing the C/N ratio from 20 to 60 resulted in an appreciable increase in the carotenoid cell content, which ranged from 10.7 to 12.1 mg/g for the culture medium obtained by chemical hydrolysis of defatted wheat bran. A similar trend was observed for carotenoid accumulation in the culture medium obtained by enzymatic hydrolysis, reaching the highest value of 14.8 mg/g at a C/N

of 60 (Figure 13). According to the literature, the lipid content increased with increasing C/N ratio, ranging from 220 to 283 mg/g for the fermentations carried out in the culture medium obtained by enzymatic hydrolysis and from 403 to 479 mg/g for the fermentation carried out in the medium obtained by chemical hydrolysis. The higher bio-oil production obtained by fermenting the latter hydrolysate could be due to the presence of Fe^{3+} ions, which have been shown to favour lipid synthesis with respect to Mn^{2+}, Ca^{2+} and Zn^{2+} [23]. This is consistent with the choice of $FeCl_3$ as a homogeneous acid catalyst in the chemical catalytic route for the one-pot saccharification of defatted wheat bran. Moreover, the oil content values obtained in the present work for *R. toruloides* agree with those reported in the literature for the same biocatalyst [46].

Table 2 resumes the main results of all the fermentation processes carried out by using the two hydrolysates of defatted wheat bran obtained in the present work.

Table 2. Overview of the fermentation results.

Parameter	**Fermentation of enzymatic hydrolysate**			**Fermentation of chemical hydrolysate**		
	C/N ratio			C/N ratio		
	20	40	60	20	40	60
Dry cell weight production (g/L)	13.1	13.0	11.9	12.1	11.5	10.9
Lipids content per cell (wt%)	22.0	24.4	28.3	40.3	43.7	47.9
Lipids production (g/L)	2.9	3.2	3.5	4.9	5.1	5.2
Lipids productivity (mg/L/h)	20.1	22.2	24.3	34.1	35.4	36.1
Carotenoids content per cell (mg/g)	10.9	11.6	14.8	10.7	11.3	12.1
Carotenoids production (mg/L)	142.8	150.8	176.1	129.4	129.9	131,9
Carotenoids productivity (mg/L/h)	1.0	1.0	1.3	0.9	0.9	0.8

For the carotenoids production, the best result was obtained from a culture medium deriving from enzymatic hydrolysis of defatted wheat bran, using a C/N ratio of 60. Under these conditions, carotenoids and lipids production were 176.1 mg/L and 3.5 g/L, respectively. On the other hand, the best result for lipids production was reached using the hydrolysate obtained by the chemical saccharification of defatted wheat bran and the C/N ratio of 60. Under these conditions, the lipids production of 5.2 g/L and the carotenoids production of 131.9 mg/L were reached.To the best of our knowledge, up to now, very few studies have been reported in the literature on carotenoids production from both synthetic media and real biomass hydrolysatesby using the yeast *Rhodosporidium toruloides*, thus confirming the novelty of the proposed biorefinery scheme. Table 3 summarises all the literature studies on fermentation using *Rhodosporidium toruloides* and the process performance achieved for each substrate in terms of carotenoids content per cell (mg/g), carotenoids production (mg/L) and carotenoids productivity (mg/L/h).

Table 3. Literature review of the research studies on carotenoids production by the yeast species *R. toruloides*.

Entry	Species	Fermentation medium	Carotenoids content per cell (mg/g)	Carotenoids production (mg/L)	Carotenoids productivity (mg/L/h)	References
1	*R. toruloides* DSM 4444	*Camelina sativa* hydrolysate	0.3	13.0	0.10	[8]
2	*R. toruloides* DSM 4444	Cinnamon waste hydrolysate	1.0	2.0	0.02	[39]
3	*R. toruloides* ATCC 204091	Mandi waste	0.5	62.0	0.60	[47]
4	*R. toruloides* NCYC 921	Primary brewery wastewater	0.1	0.2	n.a.	[49]
5	*R. toruloides*	Cane molasses	0.3	n.a.	n.a.	[65]

	Z11					
6	*R. toruloides* NRRL Y-1091	Synthetic	2.9	8.3	0.07	[36]
7	*R. toruloides* NRRL Y-1091	Wheat straw hydrolysate	2.6	21.4	0.20	[36]
8	*R. toruloides* C23	Synthetic	0.8	21.4	0.10	[66]

n.a. = data not reported by the authors.

As shown in Table 3, the intracellular carotenoids content of 14.8 mg/g obtained under the optimised process conditions adopted in the present study is about 5 times higher than the best result reported to date in the literature for this biocatalyst (2.9 mg/g) andobtained by fermenting a synthetic medium containing only xylose as a carbon source with the C/N ratio of 25 [36]. Liu et al. [36] performed the fermentation of washed wheat straw hydrolysate with the same yeast used in this work to obtain lipids and carotenoids. The initial glucose and xylose concentrations were 46.4 and 11.4 g/L, respectively. The DCW production obtained after 120 h with C/N 50 was 8.4 g/L, lipids and carotenoids accumulationswere 249 and 2.45 mg/g, respectively. Lipid accumulation was similar to that obtained in this work using the hydrolysate obtained enzymatically with C/N 40.

Dias et al. [49]carried out the fermentation of primary brewery wastewater in the presence of *R. toruloides*to produce carotenoids and lipids by adding sugarcane molasses and urea as the organic source of nitrogen. In this study, the effect of the concentration of sugarcane molasses was investigated in the range of 10-280 g/L, corresponding to a C/N range between 5 and 144. Under the optimised conditions(C/N ratio of 52, similar to that found in the present work), the maximum lipidsand carotenoids contentswere 289 mg/g and 63.6 μg/g, respectively[49].

Bertacchi et al. [8, 39]studied the fermentation process of the sugars-rich hydrolysatesobtained from cinnamon waste and *Camelina sativa*for the sole production of carotenoids, employing the same yeast. The two hydrolysates used as media contained the total sugar concentration of 12 and 20 g/L,

respectively. The DCW production achieved after 96 h was 2 and 15 g/L, respectively. Differently from the present work, the fermentation process was stopped at the beginning of the stationary phase of the cell growth curve, leading to a low intracellular carotenoids content, whichwas 1 mg/g for the fermentation of the cinnamon waste hydrolysate and 0.3 mg/gfor the fermentation of *Camelina sativa* hydrolysate. Singh et al. [47] fermented a water extract of a mixture of wastes deriving from orange peels, sweet lemon, banana, pea pods and cauliflower stem, whose total sugar concentration was 20 g/L. The fermentation process was carried out at a C/N ratio of 30 for 96 h, namely up to the complete consumption of sugars in the medium. At the end of the process, the carotenoids content was 0.5 mg/g.Sanchez et al. [67] studied the lipids production by *R. toruloides*by fermenting a corn straw hydrolysate in a 6 L bioreactor. The hydrolysate consisted of 58 g/L glucose and 35 g/L xylose, and the fermentation was carried out until the total sugar consumption that occurred after 120 h. The DCWreached at the end of the process was 30 g/L and the lipids content was 608 mg/g, which is higher than that obtained in the present work due to the very high C/N ratio of 120 used for maximising the lipids production. Yu et al. [68] fermented a wheat straw hydrolysate consisting of 3.7 g/L glucose and 14 g/L xylose for bio-oil production using a C/N ratio of 20. Fermentation was stopped when all sugars were consumed (144 h) achieving the DCW concentration of 9.9 g/L and a lipid content of 246 mg/g. This last value is lower than thoseachieved in the present work due to the low C/N ratio that favours yeast growth but disfavours lipids accumulation.

3.4 Use of spent yeast as a possible nitrogen source

In a circular economy perspective, the spent yeast obtained as residue from the extraction of carotenoids and lipids was dried and analysed by elemental analysis to determine the residual nitrogen content, in order to propose it as a potential nitrogen source for other fermentation processes and/or anaerobic digestion. To validate this hypothesis, elemental analysis was carried out

on yeast cells before and after the fermentation process and after the extraction of bioproducts. The results obtained are presented in Table 4.

Table 4. Elemental analysis of *R. toruloides* cellsbefore and after fermentation and after the extraction of lipids and carotenoids.

Sample	C (wt%)	H (wt%)	N (wt%)	S (wt%)
Yeast before fermentation	45.2	6.9	6.7	0.2
Yeast after fermentation	52.7	8.0	3.6	0.1
Yeast after carotenoids and lipids extraction	43.6	6.7	4.8	1.2

After fermentation, an increase in carbon and hydrogen content and a decrease in nitrogen content were observed,due to the accumulation of lipids and carotenoids in the yeast cells and the simultaneous consumption of amino acids during yeast growth. After the selective extraction of carotenoids and lipids, the carbon and hydrogen contents returned to their initial levels, while the nitrogen content of the spent yeast was about 5 wt%. This value is not significantly different in order of magnitude from the nitrogen content of commercial yeast extract (10 wt%) used as a nitrogen source in fermentation processes, making the obtained spent yeast a potential low-cost substitute for yeast extract within the same biorefinery model. In this way, the obtained spent yeast, namely an organic waste, could be recycled as a nitrogen source for lipids and carotenoids production or in other biotechnological processes, such as the anaerobic digestion for biogas production [69], as well as for the production of versatile nitrogen-rich biochars [70].

3.5 Triglycerides characterisation

In order to define the suitable commercial applications of bio-oils obtained by the yeast *R. toruloides*, the chemical profile of fatty acid methyl esters obtained from yeast lipids was

characterised. Table 5 reports the FAMEs profile obtained under the optimised process conditions of fermentation of both hydrolysates obtained by chemical and biological routes.

Table 5. FAMEs profile of SCOs obtained by *R. toruloides* by fermenting the two defatted wheat bran hydrolysates under the optimised process conditions (C/N 60).

Sample	FAMEs profile (%)[e]								Unsaturated FAMES (%)
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C22:0	C24:0	
SCO A[a]	1.8	31.9	0.5	10.7	53.8	0.2	0.5	0.7	54.5
SCO B[b]	1.6	32.1	0.7	11.3	52.9	0.3	0.5	0.6	53.9
SCO C[c]	1.2	21.6	0.9	17.8	54.5	3.2	-	-	58.6
Rapeseed oil[d]	0.9	40.1	0.1	4.1	43.0	11.0	-	-	54.1
Palm oil[d]	1.5	36.7	0.1	6.6	46.1	8.6	0.1	0.1	54.8

[a] FAMEs profile of SCO obtained from the fermentation of the hydrolysate obtained by the chemical route. [b] FAMEs profile of SCO obtained from the fermentation of the hydrolysate obtained by the biological route.[c]Typical FAMEs profile of SCO obtained from *R. toruloides* reported in the literature [70].[d] FAMEs profile of typical vegetable oils used for commercial applications reported by Sutanto et al.[71].
[e]The percentage value was calculated as the ratio between the area of a single peak and the total area of the peaks.

The FAMEs profile obtained from *Rhodosporidium toruloides* was very similar to that reported by Liu et al. for the same yeast [70]. Moreover, about 54% of the fatty acids in this microbial oil are unsaturated, and this value is very similar to those reported in the literature for rapeseed and palm oils[71], which are two of the most widely used vegetable oils for industrial applications. The chemical composition of this SCO is suitable for the production of new generation biodiesel because the high content of unsaturated fatty acids favours the cold properties of this biofuel, such as cloud point, pour point and cold filter plugging point. On the other hand,unsaturated fatty acids reduce the oxidation stability of biodiesel. The presence of high levels of monounsaturated fatty

acids (C18:1) and low levels of polyunsaturated fatty acids (C18:2, C18:3) could balance these two properties [72]. Based on the obtained results, it can be stated that the lipid profile obtained from the yeast used in this work is very similar to that of the main oils used for biofuel production, making it a good alternative to the use of vegetable oils for energy purposes and, more generally, for industrial purposes not related to the food chain. Furthermore, oleic acid represents 54% of the lipids produced in this process, which could be applied for the synthesis of azelaic acid, which is one of the monomers used for the synthesis of polyamides [73]. The multiple potential applications of this new generation bio-oil obtained from an industrial waste, such as defatted wheat bran, can be implemented once the economics of the process and its scale-up have been further optimised. The economic sustainability of the proposed biorefinery model should be enhanced by the simultaneous production of high added-value carotenoids and lipids.

4. Conclusions

For the first time, an integrated biorefinery model was developed to convert a residue of the flour industry, namely the defatted wheat bran, to sugars (glucose, xylose, arabinose), high-added value triacylglycerols and carotenoids, and nitrogen-rich yeast biomass leftover.The MW-assisted chemical hydrolysis of defatted wheat bran catalysed by $FeCl_3$ was optimised as well as the enzymatic hydrolysis catalysed by the commercial blend Cellic® CTec3 HS. Under the optimisedreaction conditions of the chemical hydrolysis (1.3 wt% $FeCl_3$, 150 °C, 2.5 min, 9 wt% biomass loading) the yields of glucose, xylose and arabinose were 43, 76, and 95 mol%, respectively, and a total sugar concentration of 57 g/L was achieved. In the second catalytic approach, under the optimised reaction conditions (30 FPU/g glucan, 96 h, 10 wt% biomass loading) the yields of glucose, xylose and arabinose were 59, 50 and 15 mol%, respectively, corresponding to a total sugars concentration of 42 g/L. *Rhodosporidium toruloides* was used for the whole-cell biocatalysis in order to simultaneously convert glucose and xylose to carotenoids and lipids. Working with the optimised C/N value of 60, the carotenoids and lipids production values

were 176.1 mg/L and 3.5 g/L for the enzymatic hydrolysate and 131.9 mg/L and 5.2 g/L for the chemical hydrolysate. The obtained carotenoids cell contents are the highest ever reported for a fermentation process, a remarkable result given the extremely high added value of these products. Finally, the single cell oil profile was similar to that of traditional vegetable oils used for commercial applications.This novel approach to the integrated production of these valuable chemicals makes the proposed biorefinery scheme extremely interesting from an economic sustainability perspective.

CRediT authorship contribution statement

Nicola Di Fidio: Conceptualization, Investigation, Data Curation, Writing – original draft. **Leonardo Carmassi**: Investigation, Data Curation, Writing – original draft. **Getari Kasmiarti**: Investigation, Data Curation. **Domenico Licursi**: Methodology, Writing – review & editing. **Sara Fulignati**: Methodology, Writing – review & editing. **Claudia Antonetti**: Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Anna Maria Raspolli Galletti**: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Funding acquisition.

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Figure 1

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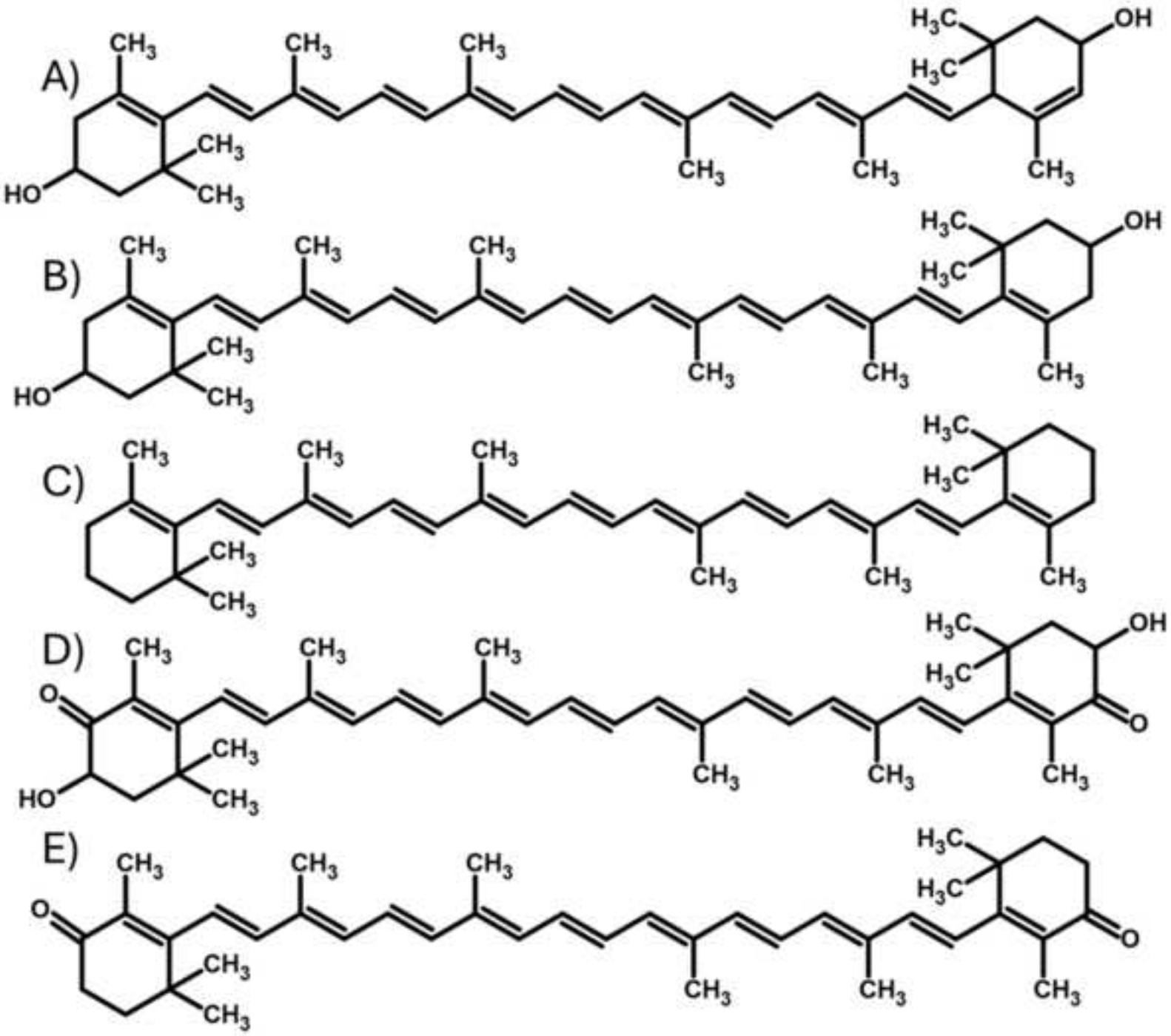

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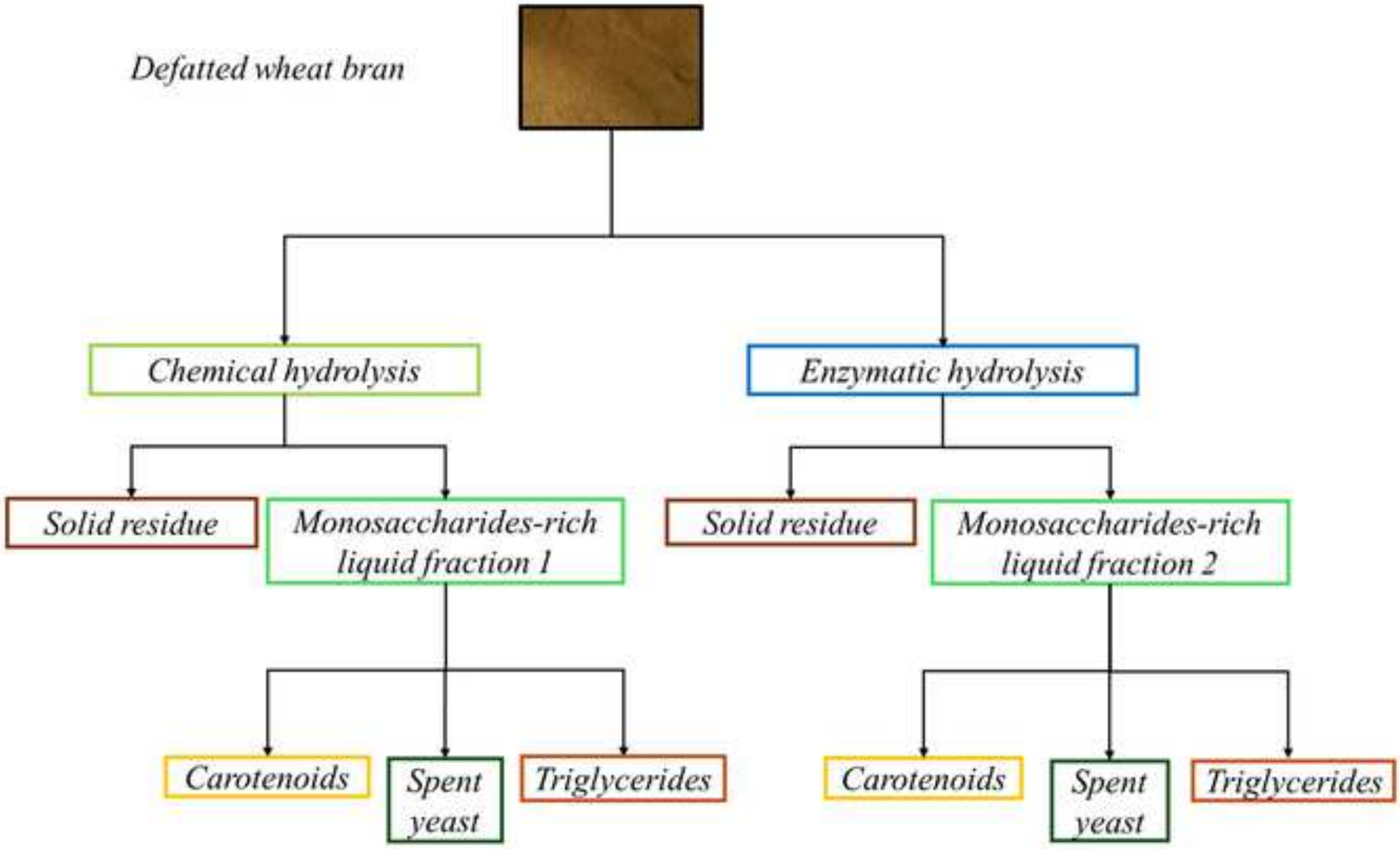

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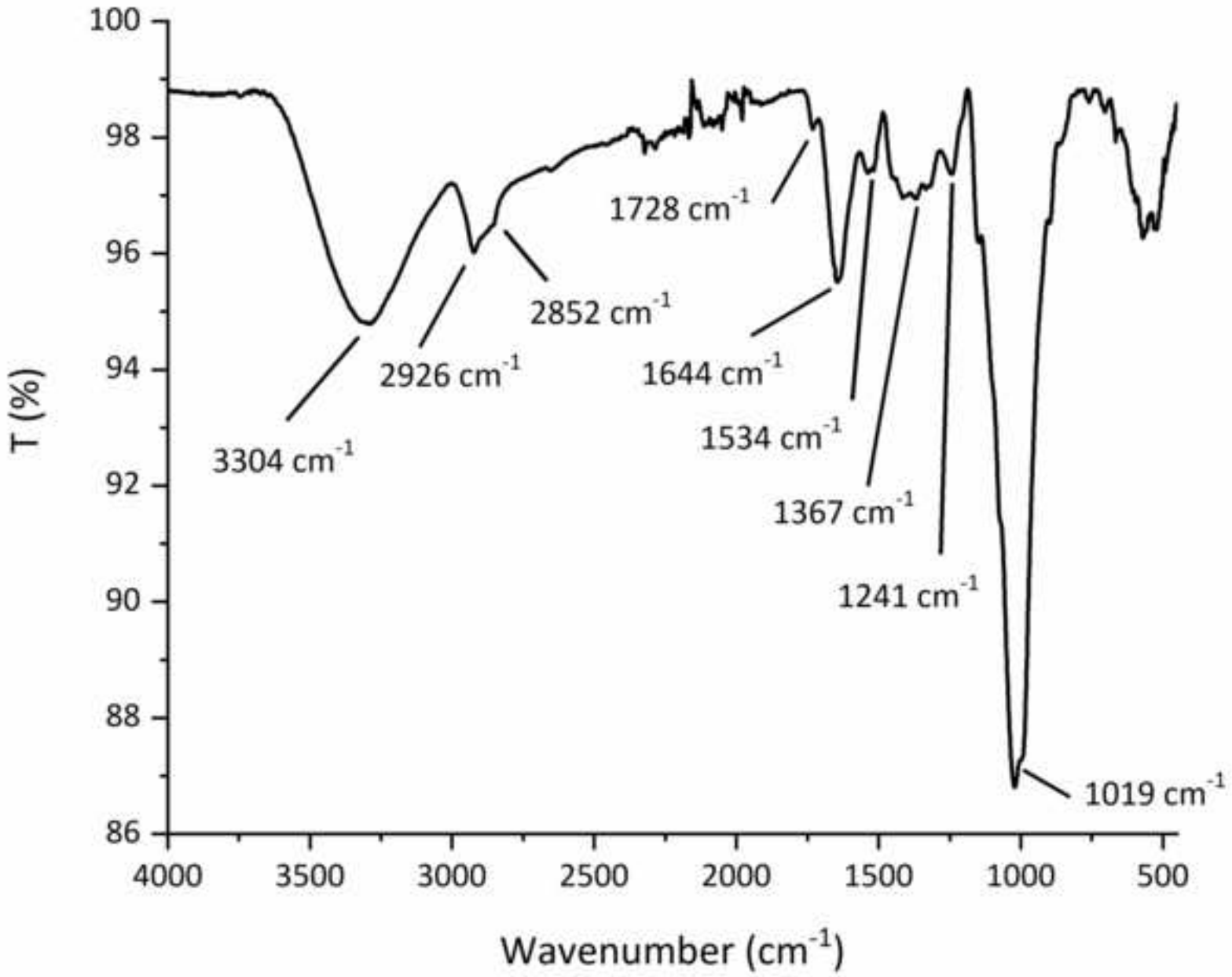

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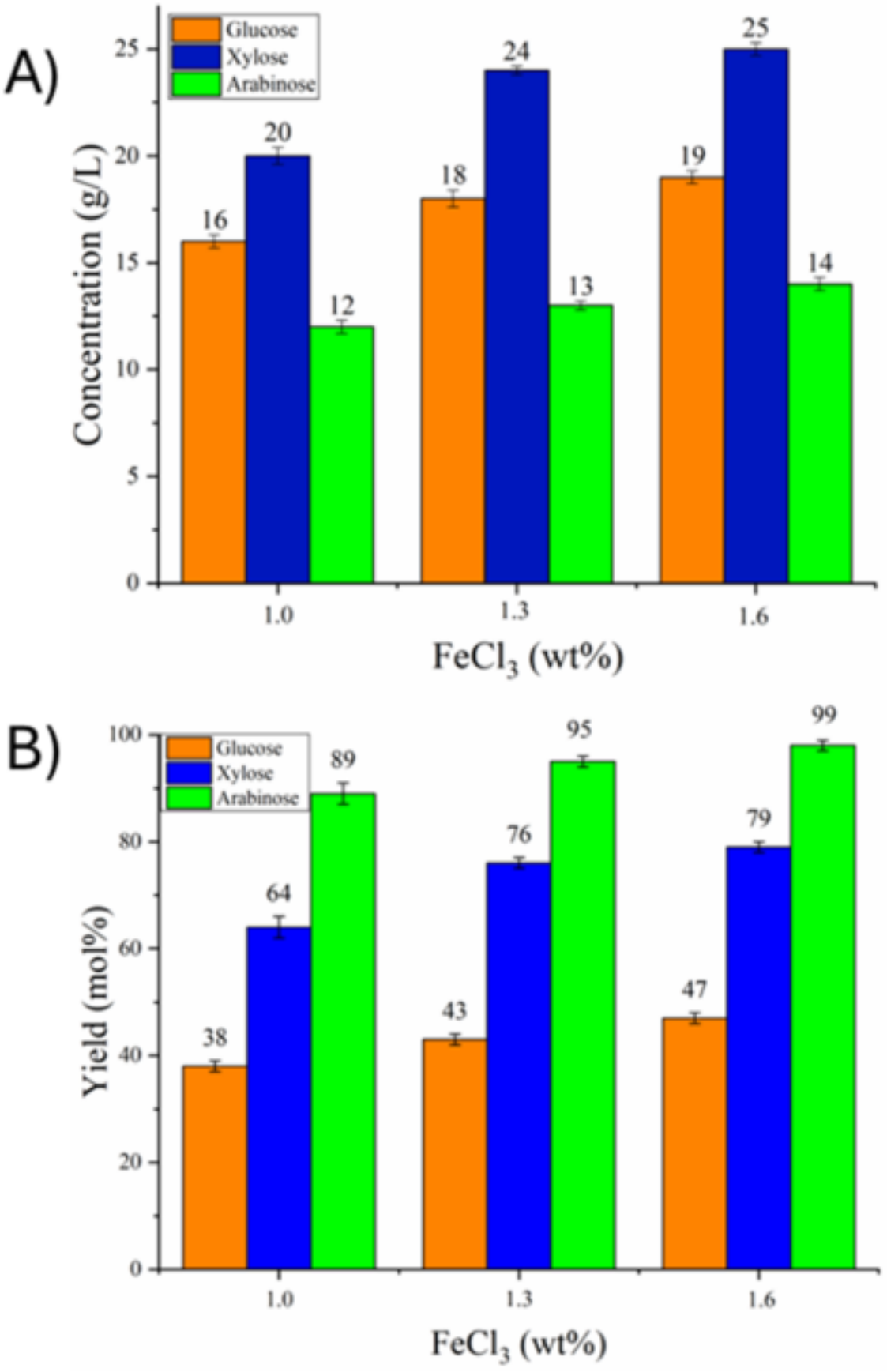

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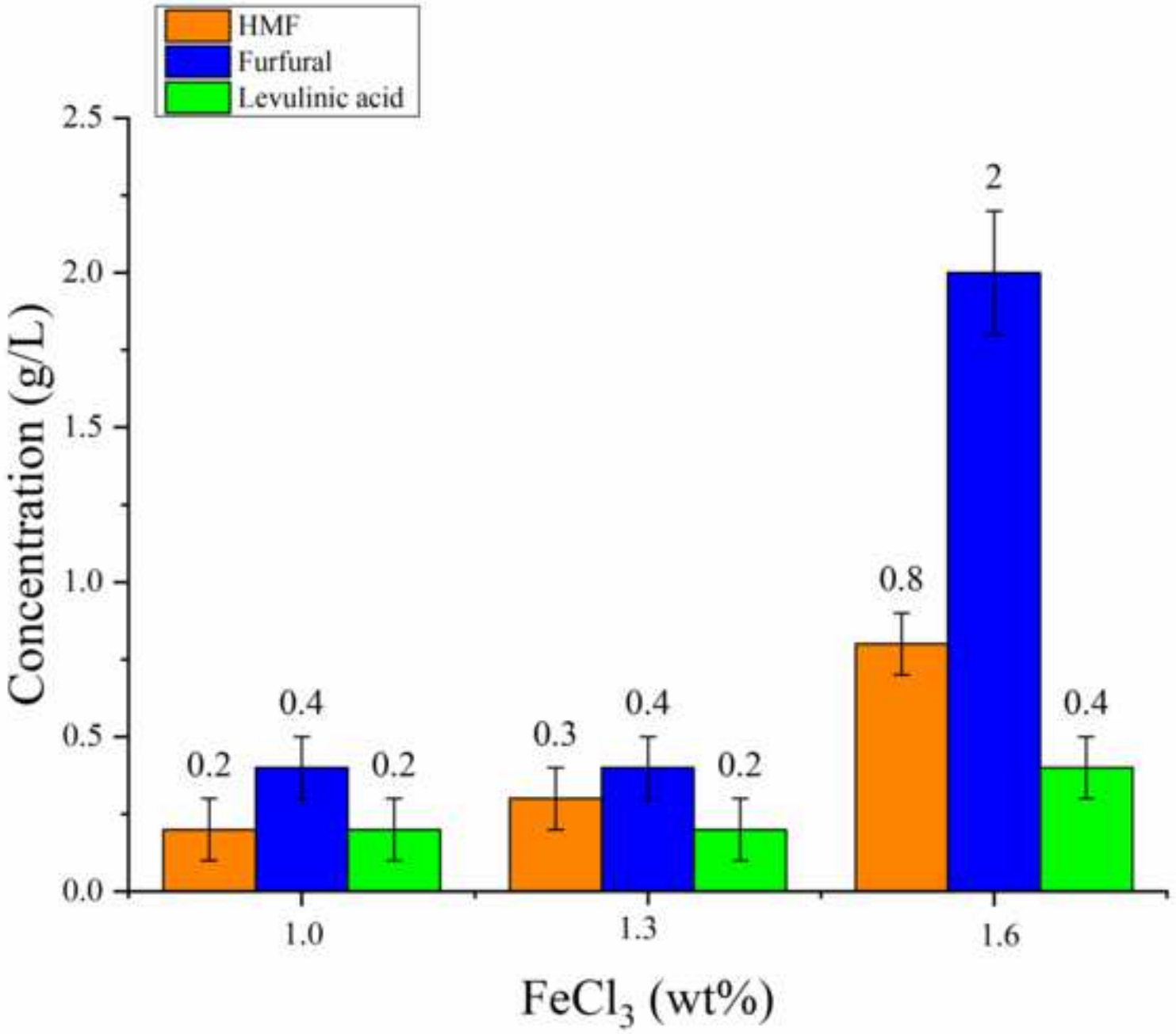

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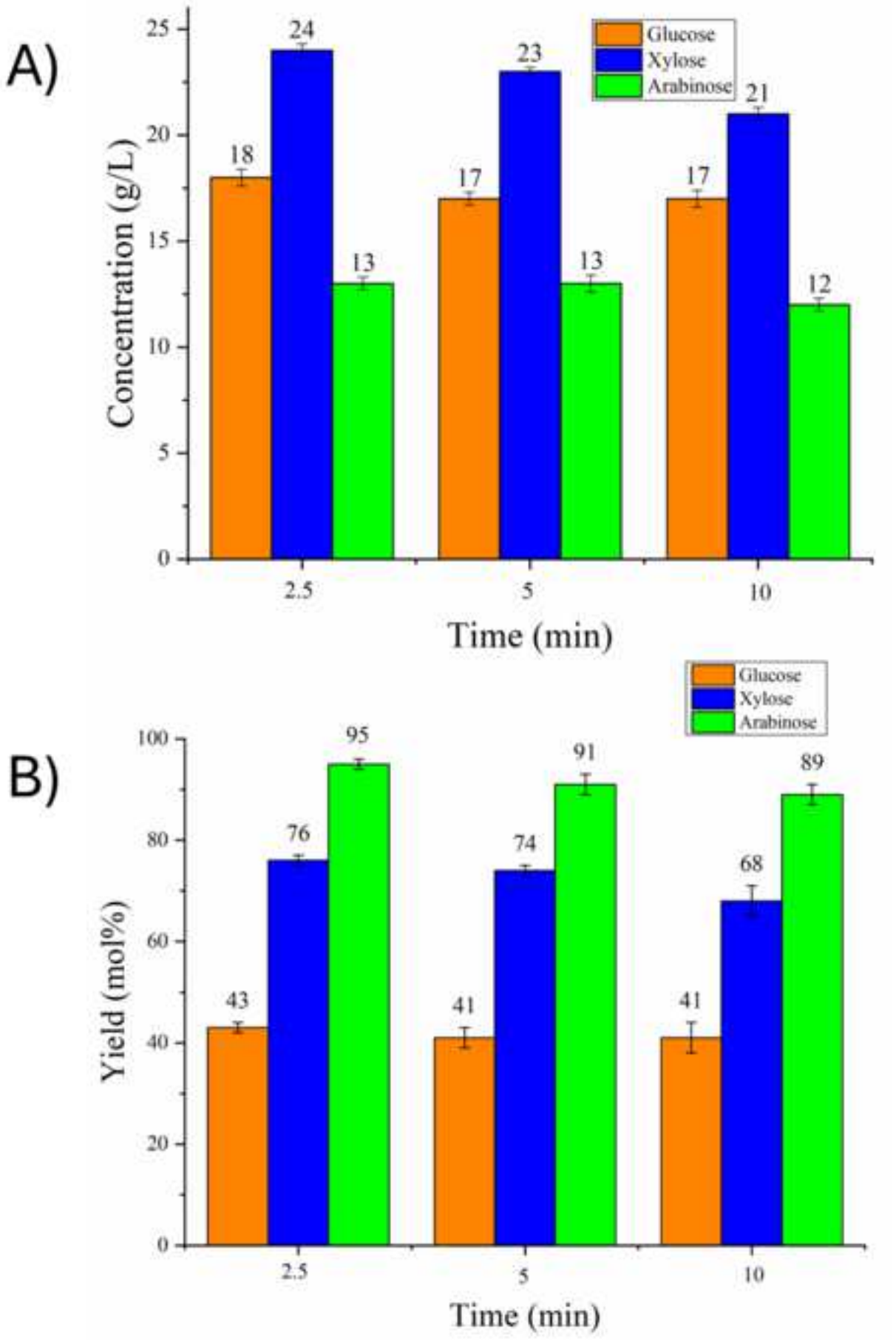

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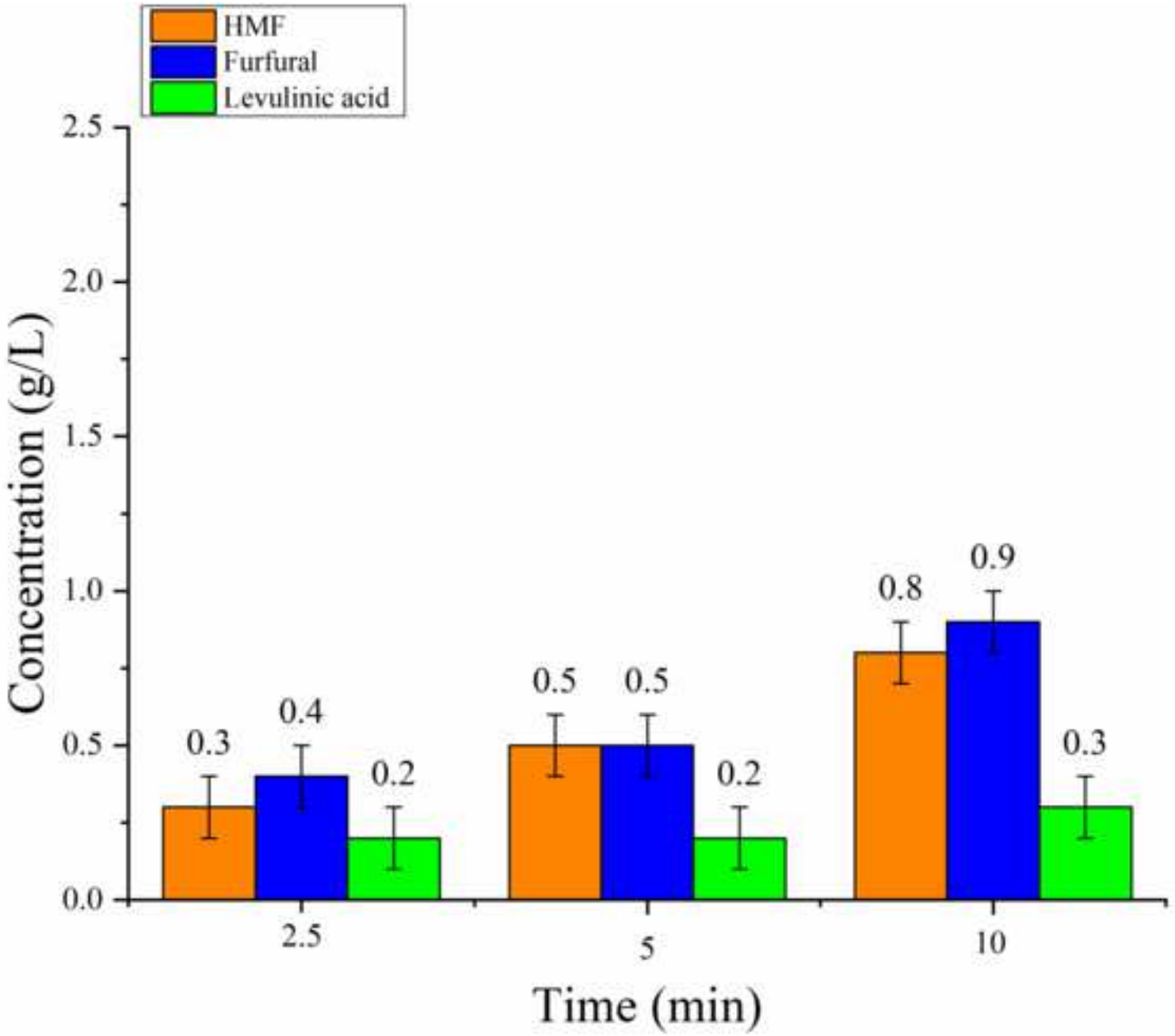

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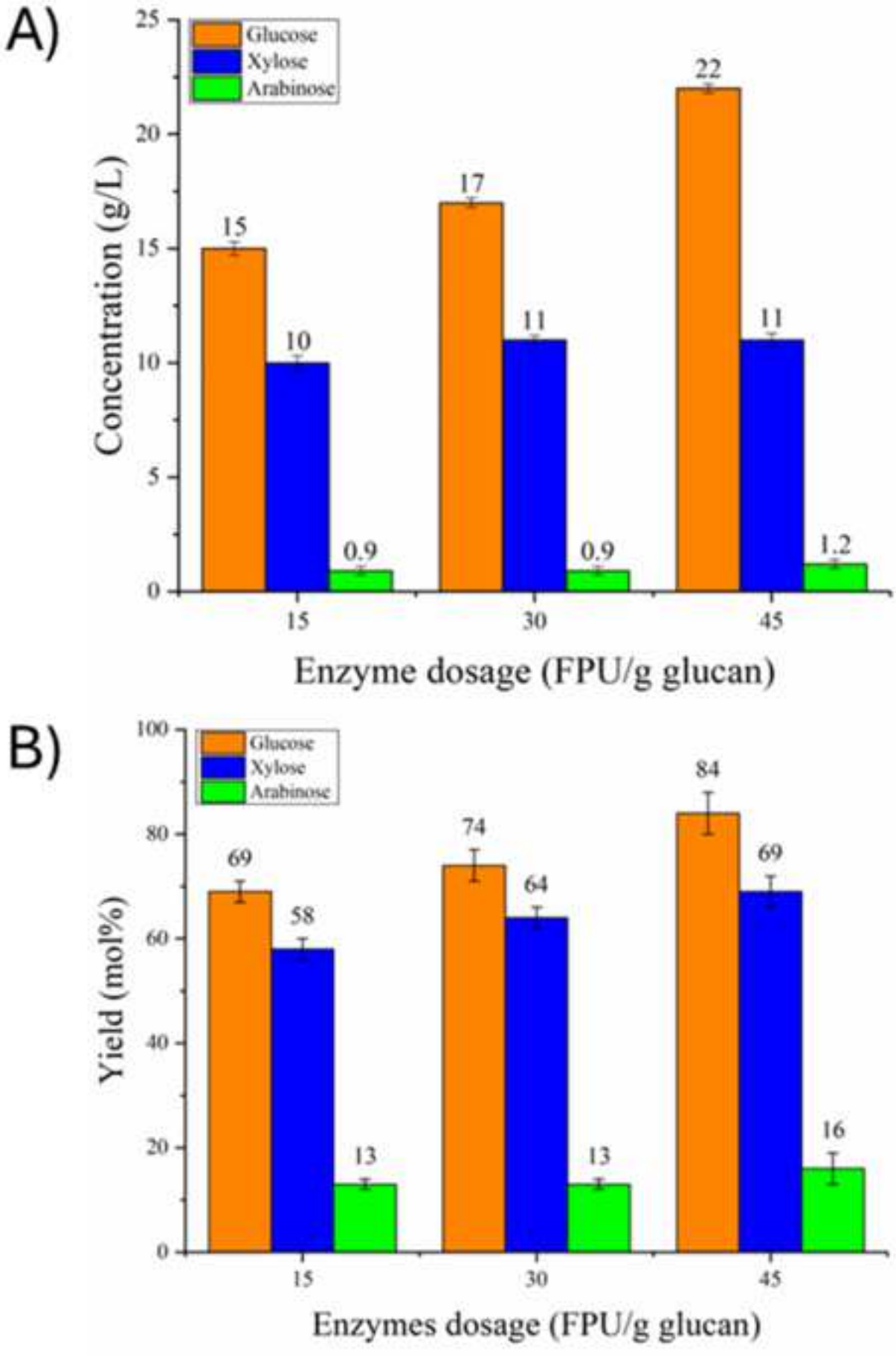

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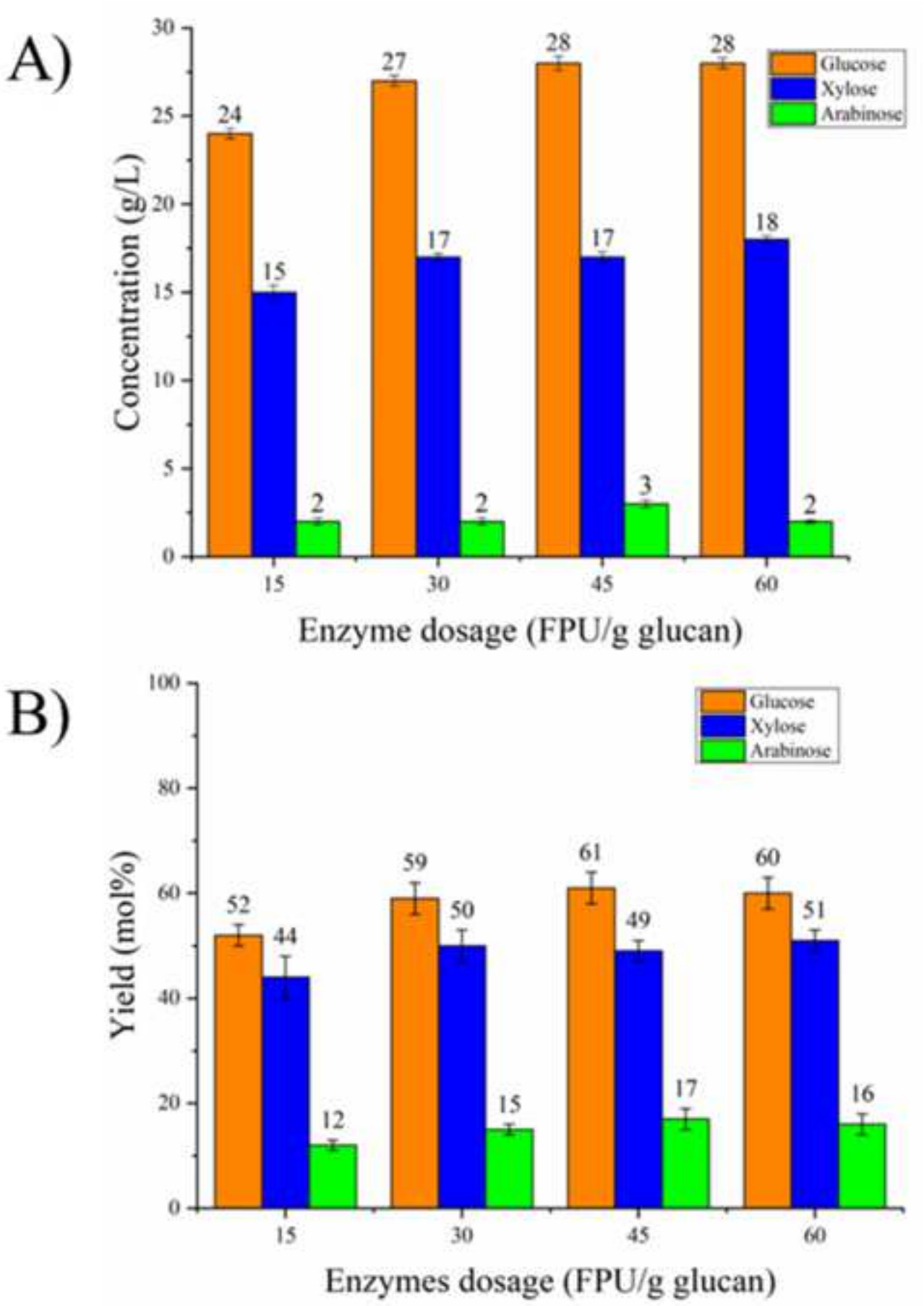

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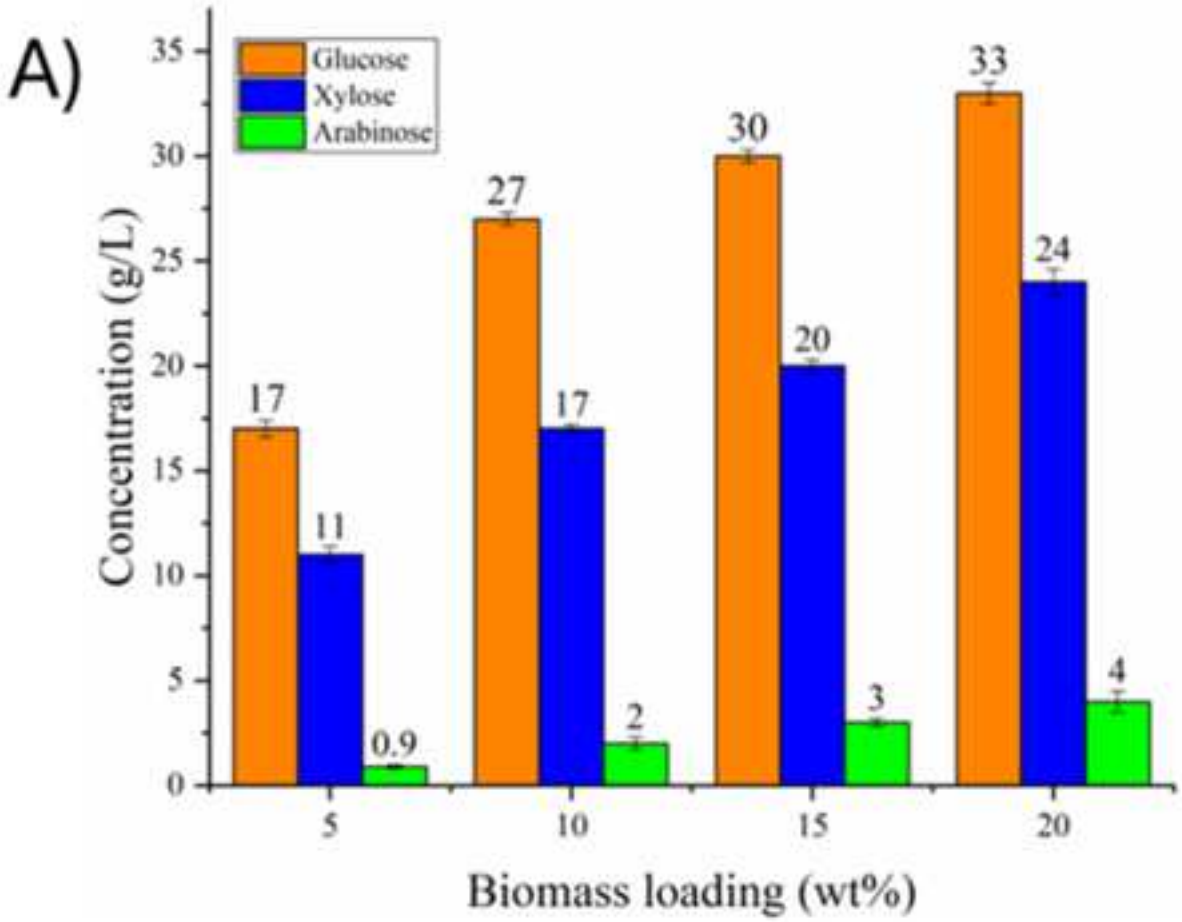

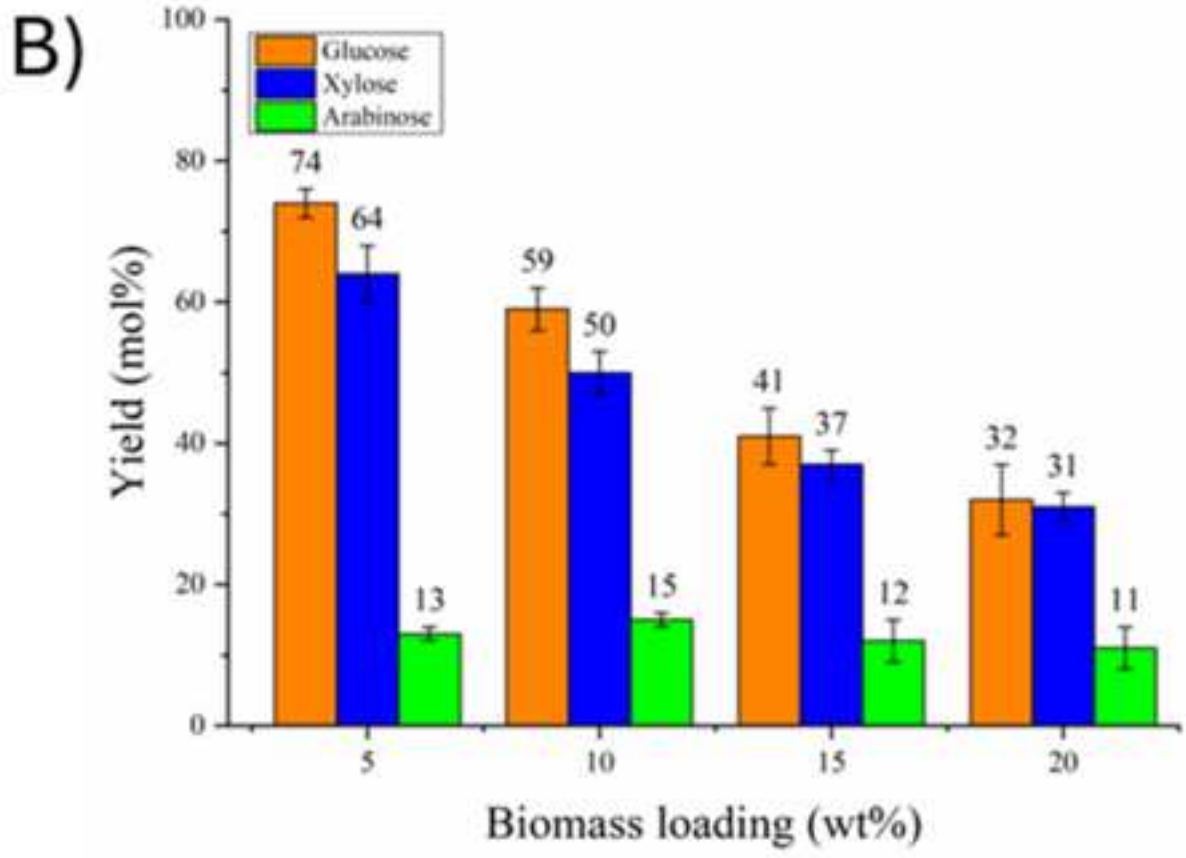

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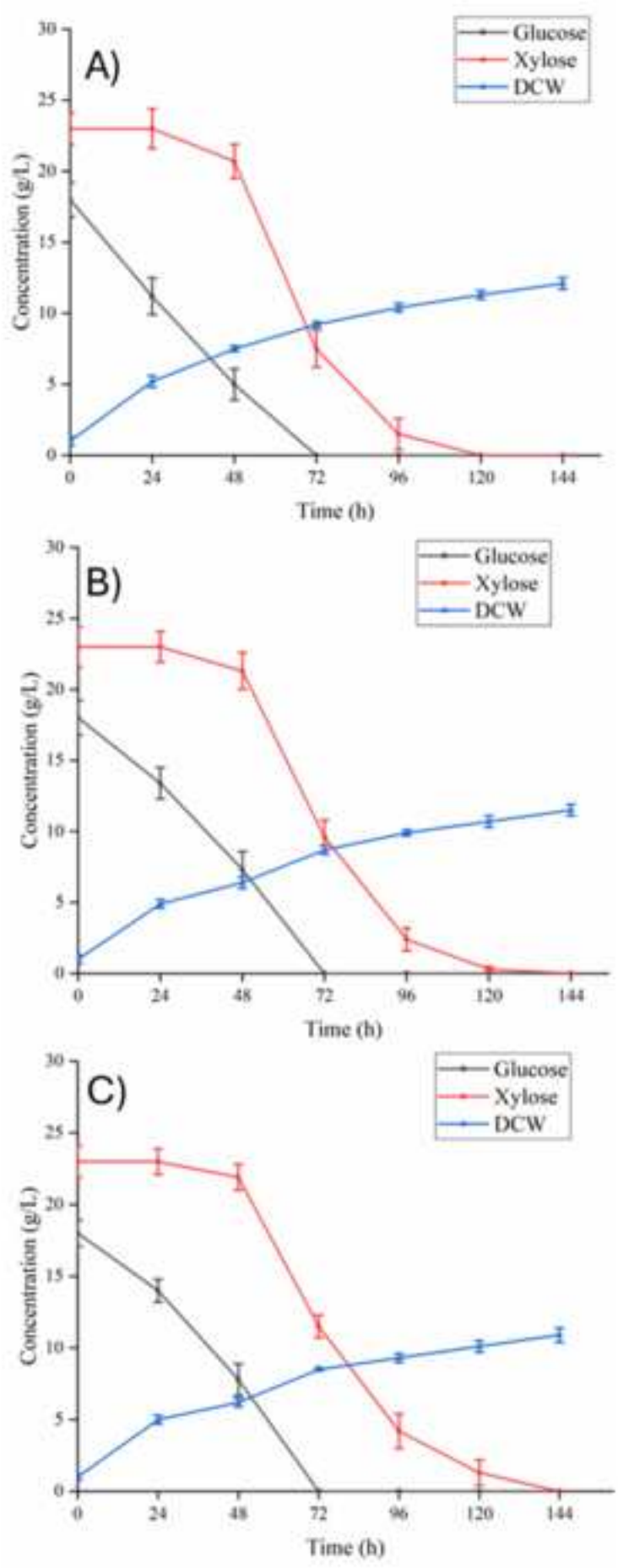

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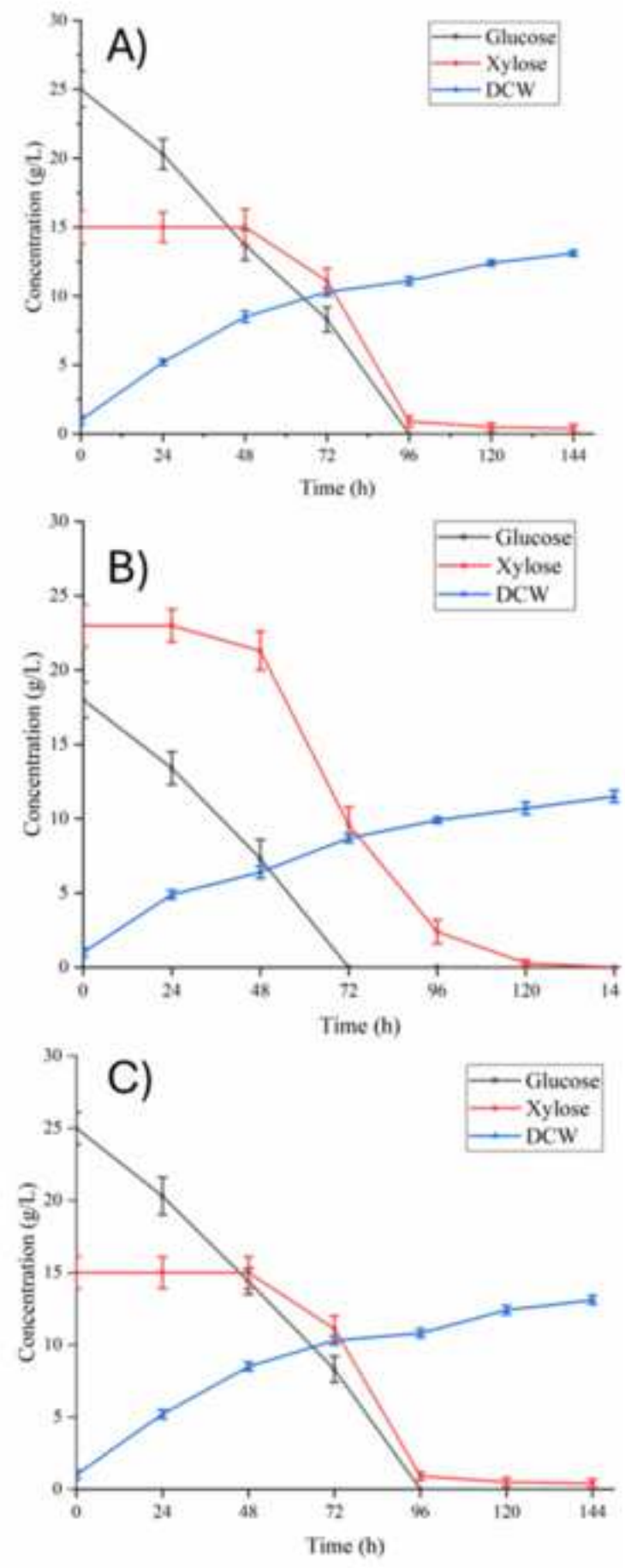

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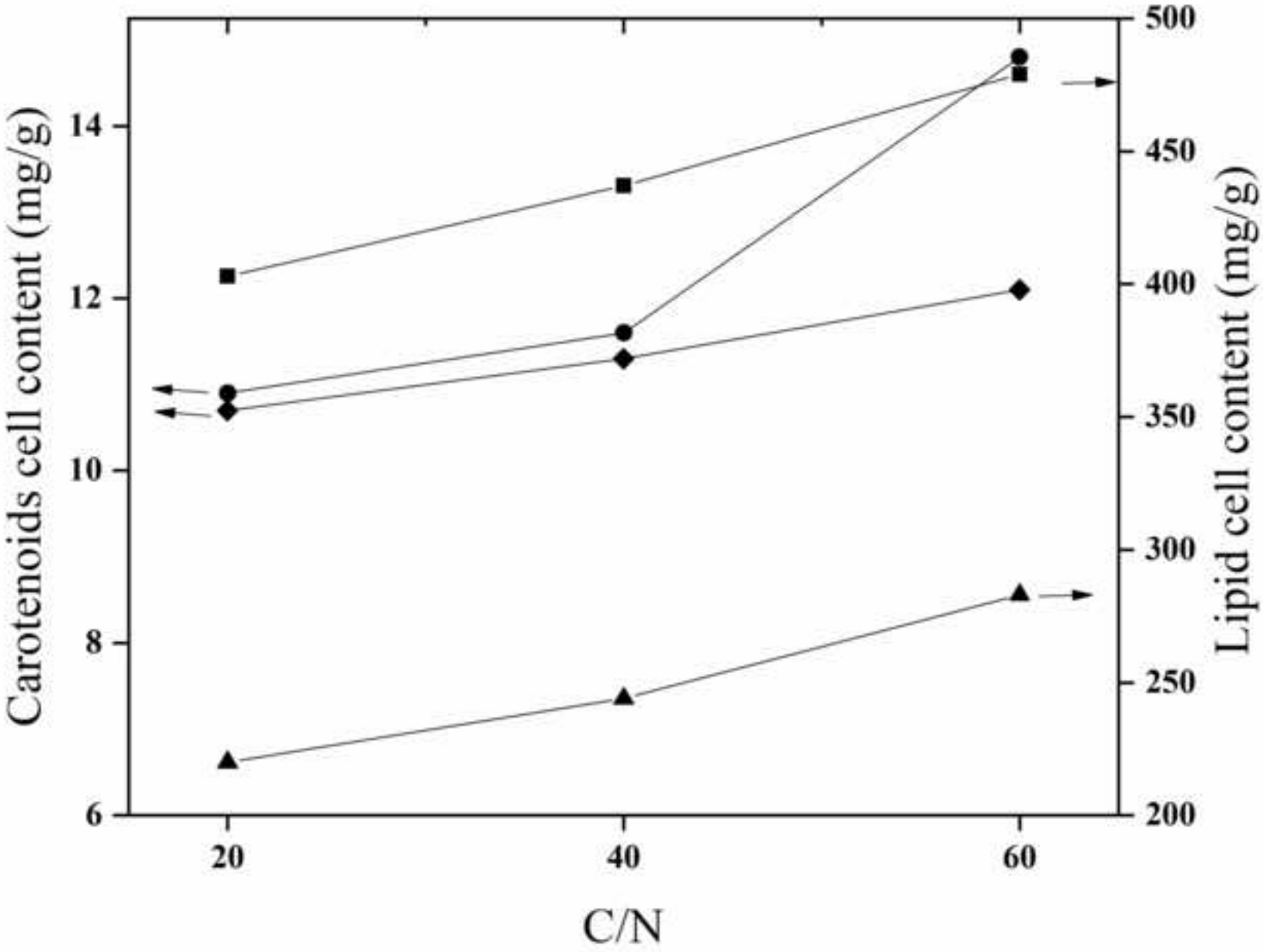

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Anna Maria Raspolli Galletti reports financial support was provided by Ministry of University and Research. Nicola Di Fidio reports financial support was provided by PON R&I FSE-REACT EU. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.