Valorization of agricultural biomass residues towards the production of docosahexaenoic acid (DHA) by the heterotrophic dinoflagellate *Crypthecodinium cohnii*

S. Staikos[1], A. Karnaouri [1], S. D. Stefanidis[2], K.G. Kalogiannis[2], A.A. Lappas[2], E. Topakas[1]

[1]Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Str., Zografou Campus, 15780 Athens, Greece
[2]Chemical Process and Energy Resources Institute (CPERI), Centre for Research and Technology Hellas, 6th km Harilaou-Thermi Road, Thermi, 57001 Thessaloniki, Greece.
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Presenting author email: vtopakas@chemeng.ntua.gr

Abstract

The principal purpose of this work is to utilize the agricultural residual biomass obtained as a side stream from grain milling, towards the production of polyunsaturated omega-3 fatty acids (PUFAs) using the heterotrophic microalgae *Crypthecodinium cohnii*. Lignocellulosic biomass (wheat straw and bran) was initially pretreated with the OxiOrganosolv method using acetone and ethanol as organic solvents without any additional catalyst. The results showed that both the cellulose-rich solid pulp and the aqueous liquid fraction which contains hemicellulosic oligosaccharides can be efficiently hydrolyzed enzymatically after pretreatment, resulting in high yields of fermentable monosaccharides. Wheat straw biomass was assessed, which produced up to 7.10 mg DHA, for solid pulp pretreated with ethanol at 160 °C, and 1.41 mg DHA, for liquid fraction pretreated with ethanol at 175 °C, per g of untreated biomass. In order to enhance DHA productivity, a fed-batch approach was evaluated using the cellulose-rich wheat straw solid pulps; DHA yield increased from 38% to 61.45% in fed-batch culture compared to batch mode, using as a substrate wheat straw pulp pretreated with ethanol at 175 °C. Bran biomass was also evaluated as a source of sugars; the solid fraction hydrolysates led up to 11.35 mg TFA/ g biomass with a DHA content of 16.25 wt.% of total lipids, while the liquid fraction led to a production of 3.03 mg TFA/ g biomass with 6.59 wt.% DHA. The lower TFA and DHA yield in wheat bran may be attributed to the sugar composition of hydrolysates, namely the high presence of C5 together with C6 sugars.

Introduction

Lignocellulosic biomass residues comprise a widely available side stream that is mostly discarded without further valorization. However, the utilization of these residues towards the production of compounds of higher value is drawing increasing attention. Besides the abundance as a raw material, another advantage of biomass is its high versatility. After pretreatment and efficient biomass fractionation to its constituents, the different streams can be subjected to enzymatic hydrolysis to produce monosaccharides which can be used as carbon source for a variety of microbial fermentations, either as separate processes or as integrated parts of a biorefinery.

The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin. With the aim to tackle with the recalcitrant structure of raw biomass, achieve fractionation of its components and make further enzymatic hydrolysis more efficient, OxiOrganosolv pretreatment is an advantageous process (Kalogiannis et al., 2020). In OxiOrganosolv, aqueous solutions (50% *v/v*) of organic solvents, namely acetone (ACO) and ethanol (EtOH), can be used for the pretreatment, in the presence of pressurized oxygen. This method is preferred, owing to the almost complete delignification of raw materials without the use of any additional catalyst (Kalogiannis et al., 2020; Karnaouri, et al., 2021). The use of organic solvents results in the recovery of three streams; a cellulose-rich solid pulp, a hemicellulose-rich aqueous liquor and solid lignin, which is retrieved as a solid after solvent evaporation (Zhao et al., 2009). Both solid pulps and aqueous liquid streams can be subjected to enzymatic hydrolysis towards the production of fermentable monosaccharides; the latter can be supplied as carbon sources to fermentation processes for the production of value added products. One of these products is docosahexaenoic acid (22:6n-3, DHA), which can be produced by the heterotrophic microalgae *Crypthecodinium cohnii*.

DHA is a poly-unsaturated fatty acid (PUFA), essential for human health which cannot be synthesized in human body. The main source of DHA and other PUFAs is the fish oils. However, the isolation of these nutraceuticals

using eco-friendlier methods that protect the marine ecosystems and preserve the already diminished fish stocks is gaining great attention recently. One method of producing DHA is based on cultivation of heterotrophic microalgae fed with simple carbon sources such as monosaccharides (Ratledge, 2013). *C. cohnii* is a heterotrophic microalga which grows in a variety of substrates including organic acids (Chalima et al., 2020) and biomass-derived sugars (Karnaouri, et al., 2020; Karnaouri, et al., 2021), thus producing DHA in great yields. In this study, not only assessment of different feedstocks, namely straw and bran from wheat, as carbon sources for *C. cohnii* occurred, but also different cultivation modes were evaluated. Besides batch cultures, fed-batch mode was also employed in order to study whether the additional supply of carbon source in the culture medium under nitrogen limitation conditions can trigger the accumulation of fatty acids and increase the final DHA yield (de Swaaf et al, 2003).

Materials and methods

Biomass Pretreatment and Fractionation

The biomass feedstock used in this work was provided by Flourmills Thrakis S.A.; it was collected from wheat fields in Northern Greece in 2019. OxiOrganosolv pretreatment method was employed for the fractionation of the raw material, as described formerly. Approximately 25 g of biomass were pretreated, solid: liquid ratio was 1:20 and the liquid phase was an aqueous organic solvent mixture 50%v/v. The process took place in an autoclave reactor, which was pressurized with 100% O_2 gas to 16 bar, and then heated to the desired temperature. The t_0 (start point) of the reaction was considered as the point when the system reached the desired temperature. The organic solvents investigated in this work were acetone (ACO) and ethanol (EtOH), which are suitable for the production of nutraceuticals. Three reaction temperatures were studied, namely 150, 160 and 175 °C for straw samples and 130, 140, 150, 160 °C for bran biomass. Reaction time was kept constant at 120 min, except 130 °C that was 240 min. After the reaction, the cellulose-rich solid fraction was separated from the liquid with vacuum filtration. The organic solvent that was present in the liquid phase was removed using a rotary evaporator and lignin was collected as solid after solvent removal by vacuum filtration. Hence, a solid pulp rich in cellulose and a hemicellulose-rich liquor, both of them almost free of lignin, were obtained.

Enzymatic Hydrolysis of Solid Pulps

Wheat straw and bran solid pulps were subjected to enzymatic hydrolysis. Straw samples were both rich in cellulose (Figure 1), as expected, hence, these pulps were hydrolyzed enzymatically at 12% (*w/v*) solids loading, upon addition of 9 mg of Cellic CTec2 commercial cellulase cocktail (Novozymes A/S, Denmark) per g of substrate, for 72 h. Reaction took place at 500 mL flasks, keeping the reaction volume constant at 10% of the reactor volume. The hydrolysis conditions were 50 °C, 160 rpm, and constant pH level at 5.5 upon addition of 80 mM MES (2-N-morpholino-ethanesulfonic acid) buffer solution.

Wheat bran solid pulps, even after pretreatment, still contained hemicellulose, as well as resistant starch that has not been removed (Figure 2). For that matter, Cellic HTec2 (Novozymes A/S, Denmark) was also employed, with the same enzyme loading and with the same pH and temperature levels. Prior to any treatment with cellulases and hemicellulases, wheat bran samples were additionally treated with Spirizyme (Novozymes A/S, Denmark) for 30 min at 50 °C in order to break down any remaining starch and render cellulose and hemicellulose more accessible to the respective enzymes. For the assessment of hydrolysis yield, glucose and total reducing sugars (TRS) production was tracked down with the glucose oxidase/peroxidase (GOD/POD) (Raabo et al, 1960) and 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), respectively.

Detoxification, Hydrolysis and Compositional Analysis of Liquid Fraction

Prior to any enzymatic process, it is crucial to remove any lignin-derived phenol compounds present in the biomass liquor, due to their potential inhibition effects to microalgal growth. Detoxification of the liquid fractions with 5% *w/v* active carbon occurred and the concentration of phenolic compounds was measured with the Folin−Ciocalteu method (Singleto et al., 1999). Hydrolysis then took place with Cellic HTec2 (Novozymes A/S, Denmark), as described above. The sugar profile was determined after acid hydrolysis, following the protocol described by the National Renewable Energy Laboratory (Sluiter et al., 2008). The sugars that were quantified included glucose, xylose, mannose, galactose and arabinose using HPLC by isocratic ion-exchange chromatography,

with an Aminex HPX-87P column with a micro-guard column, at 85 °C (Bio-Rad Laboratories, Hercules, CA, USA), using water as a mobile phase at a flow rate of 0.6 mL/min. The solid pulps and the liquid fractions which showed the highest fermentable sugar yields were then used as a carbon source for *C. cohnii* (ATCC 30772) cultures.

Batch microalgal cultures

Microalgal cells were grown at 27 °C, pH 6.5 (MES buffer), 160 rpm in a linear shaker on medium containing -besides the carbon source- 25 g/L sea salts and 2 g/L yeast extract. All biomass hydrolysates were diluted 2-times prior to any fermentation experiment and initial sugar levels were 30-40 g/L depending on the hydrolysis yield, thus inhibition of microalgal cells growth due to high glucose concentration was evaded. The total culture volume was 30 mL in 100-mL shake flasks. Microalgal static precultures at 27 °C and pH 6.5, grown for 4 days with 9 g/L glucose, 25 g/L sea salts and 2g/L yeast extract were used as an inoculum for the shaken cultures. They were inoculated with 10% *v/v* inoculum and incubated for 120 h. Pure glucose was used at a concentration of 9% *w/v* as a carbon source for control cultures. At the end of cultivation, cells were harvested by centrifugation, washed thoroughly with 25 g/L sea salts water solution, lyophilized and weighed, so as to calculate the final biomass concentration. Total fatty acids (TFAs) were then extracted using a modified Folch method, were trans-esterified and the lipid profile was analyzed through gas chromatography (GC-FID) (Chalima et al., 2020; Karnaouri, et al., 2021)

Fed- batch microalgal cultures

Fed-batch cultures were carried out as had already been described until 120 h. At that point, cultures were fed with enough concentrated hydrolysate in order to reach a final sugar concentration of 20 g/L of culture medium (de Swaaf et al., 2003). A control culture with a mixture of glucose and xylose at a ratio of 85:15 as carbon source was used, in order to mimic the composition of wheat straw hydrolysates. At 240 h, cells were harvested and processed as mentioned.

Results and Discussion

OxiOrganosolv Pretreatment of Biomass/ Solid Pulps Characterization and Hydrolysis

All the different biomass were subjected to OxiOrganosolv pretreatment. Wheat straw exposed to higher temperatures (150, 160 and 175 °C), while bran samples were pretreated at 130, 140, 150, 160 °C. The compositional analysis of solid pulps after pretreatment of straw and bran samples are shown on **Figure 1** and **Figure 2**, respectively.

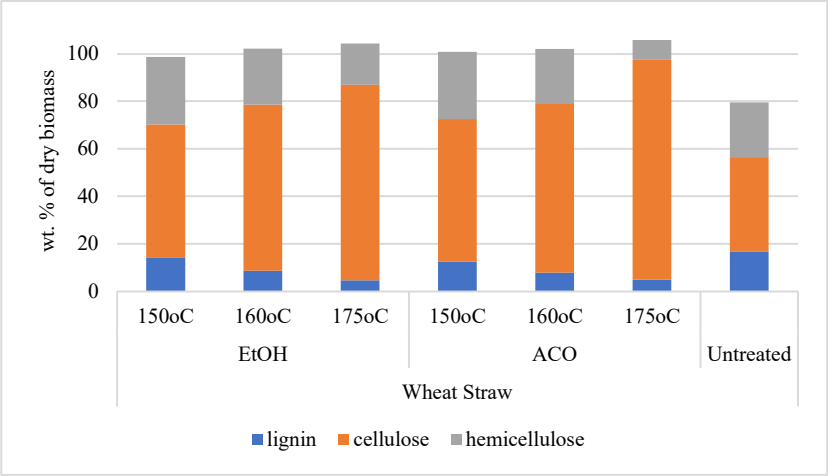

Figure 1. Compositional analysis of solid pulps of wheat straw pulps after OxiOrganosolv process. Standard error is ≤2.5% in all measurements.

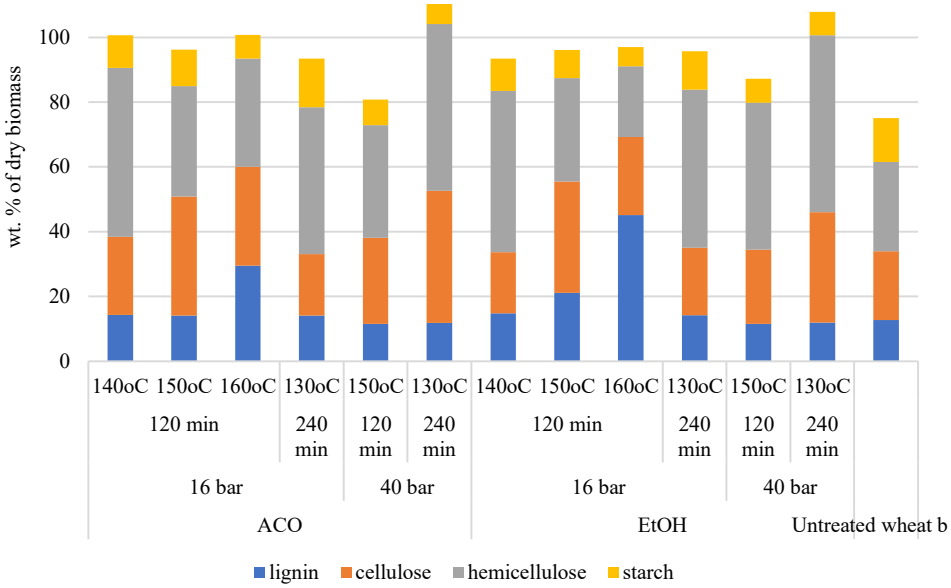

Figure 2. Compositional analysis of wheat bran pulps after OxiOrganosolv process. Standard error is ≤2.5% in all measurements

Regarding the straw samples, the higher the pretreatment temperature, the better the delignification yield. In all pretreated pulps, lignin content is significantly lower than that of the untreated biomass, verifying the efficiency of OxiOrganosolv. Moreover, even at the highest pretreatment temperature, cellulose remained intact and there was almost 100% cellulose recovery in solid pulps. Comparing the two solvents, both proved reliable in fractionation of biomass, yet ACO had a slightly better performance, as it produced cellulose richer pulps.

In case of bran samples, delignification yield peaked at 71% and 67.7% for ACO and EtOH, respectively, when the material was pretreated at 150 °C for 120 min under O_2 pressure of 40 bar, showing that lignin removal was undoubtedly lower than in the case of wheat straw. Moreover, a sugar loss of 37.7% and 33.5% was observed at these conditions, due to the removal of starch and hemicellulose sugars, thus indicating that higher lignin removal was combined with a significant sugar loss. On the contrary, when pretreatment took place at lower temperature (130 °C for 240 min under O_2 pressure of 16 bar or at 140 °C for 120 min under O_2 pressure of 16 bar), an almost 100% recovery of sugars was feasible. Furthermore, resistant starch is also present in these samples, even after pretreatment. The higher the pretreatment temperature the lower the starch present, namely 7.35% and 5.91% wt. of biomass for ACO and EtOH at highest severity of pretreatment conditions. For that reason, the addition of a treatment step with amylase was considered as crucial in the subsequent process in order to break down starch to fermentable sugars. Following solid pulps characterization, enzymatic hydrolysis took place whose results are displayed on **Figure 3** for wheat straw and **Figure 4** for wheat bran.

Regarding the hydrolysis of wheat straw pulps, almost entirely the total reducing sugars are comprised of glucose, due to the cellulose-rich nature of this solid fraction. Cellulose conversion and, hence, saccharification of wheat straw increased when the pretreatment temperature was at the highest points; at 175°C, cellulose conversion reached the highest values, namely 76.2% and 53.9% for ACO and EtOH, respectively, which corresponded to 783 and 488 mg of glucose/g of pretreated biomass. On the other side, wheat bran pulps produce less glucose, despite the high TRS levels. This outcome stems from the fact that wheat bran pulps contain also a significant hemicellulose content, thus a variety of sugars is produced.

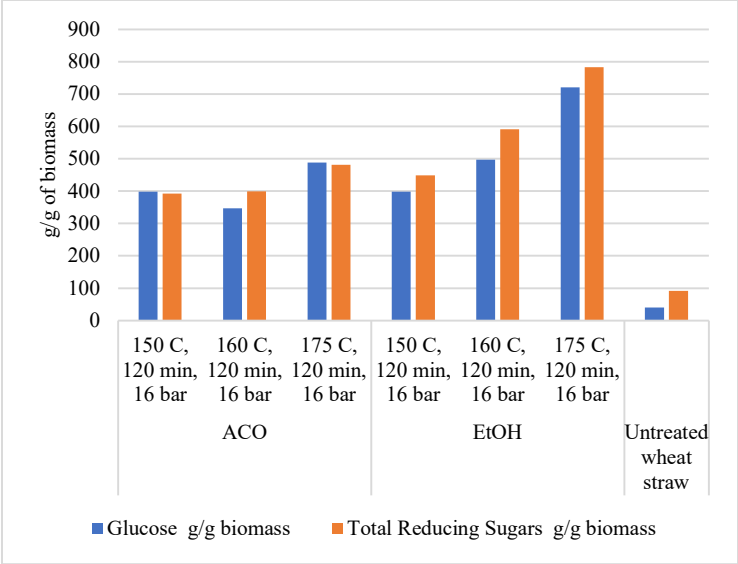

Figure 3. Hydrolysis yields for wheat straw pulps.

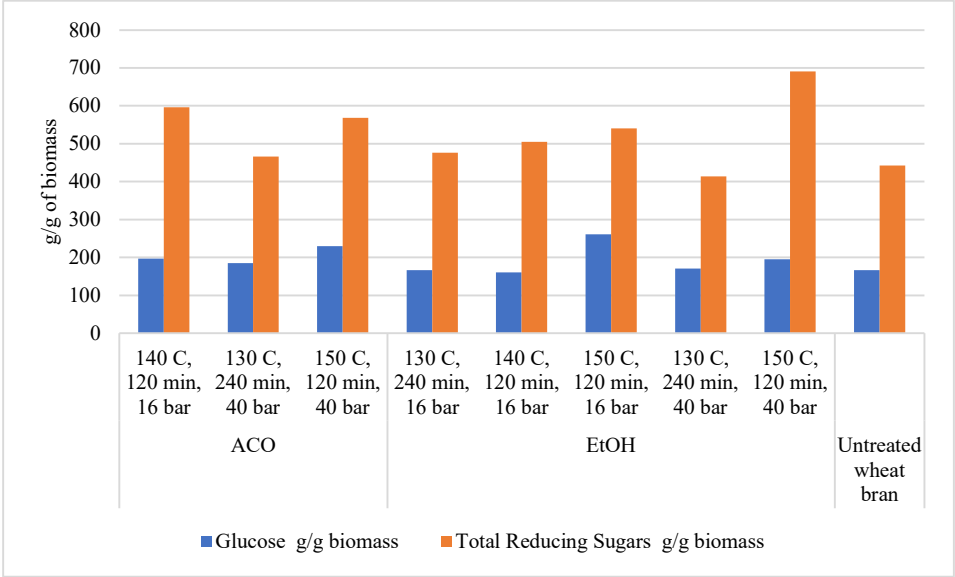

Figure 4. Hydrolysis yields for wheat bran pulps.

Liquid Fraction Characterization and Hydrolysis

Apart from the solid pulp, the hemicellulose-rich aqueous phase of the pretreatment liquor was also examined as a carbon source for the production of DHA. Prior the hydrolysis, detoxification and an initial compositional analysis took place, whose results are presented on **Figures 5 and 6**.

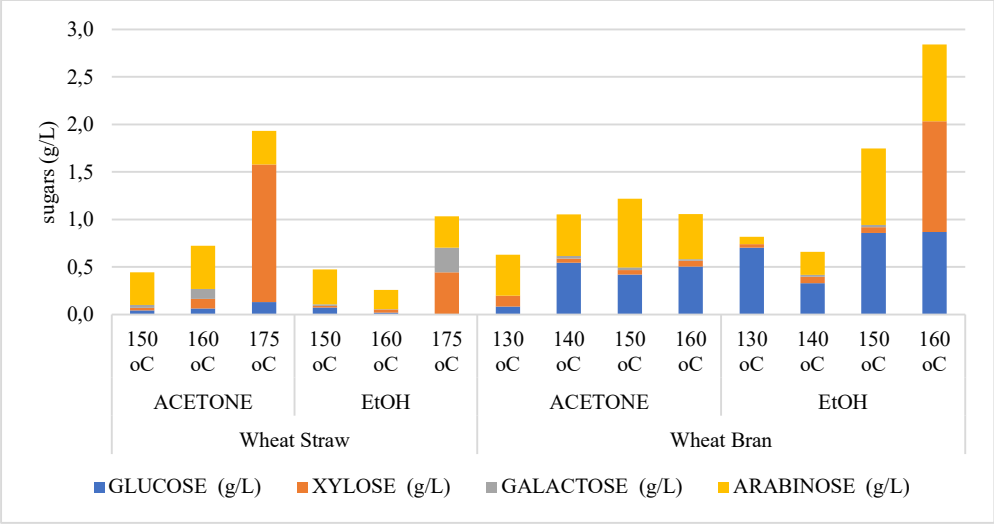

Figure 5. Monosaccharides sugar profile of wheat straw and bran aqueous liquid fraction after OxiOrganosolv process. Standard error is ≤2.5% in all measurements

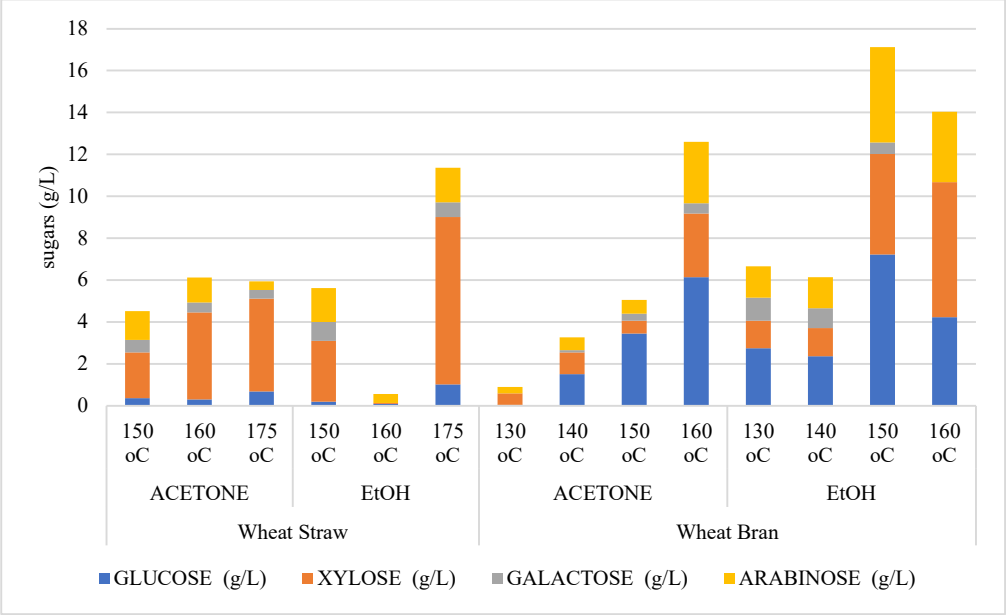

Figure 6. Oligosaccharides sugar profile of wheat straw and bran aqueous liquid fraction after OxiOrganosolv process. Standard error is ≤2.5% in all measurements

Comparing the two feedstocks, wheat straw liquid fraction is almost entirely composed of xylose and its oligomers, while in case of wheat bran the predominant sugar is xylose, followed by glucose and arabinose. Moreover, both mono- and oligo-saccharides concentration is higher in wheat bran samples, regardless of the milder pretreatment conditions that were applied. At higher temperature pretreatment, namely 150 or 160 °C for bran and 175 °C for straw, both mono- and oligo- saccharides levels are greater, leading to higher sugar levels in general and rendering the liquid fractions more promising to be used as carbon sources for potential fermentations. The three liquors with the greatest potential of each feedstock were subjected to enzymatic hydrolysis with the aim to produce fermentable sugars. The hydrolysis yields are displayed on **Table 1**.

Table 1. Total sugar levels before and after hydrolysis of wheat bran and straw liquors.

Sample	Biomass	Before hydrolysis		After hydrolysis	
		mono- (mg/mL)	oligo- (mg/mL)	mono- (mg/mL)	Hydrolysis yield
ACO/160°C	Wheat Straw	1.4	7.6	5.1	49.0%
ACO/175°C		3.6	9.2	10.3	73.4%
EtOH/175°C		2.0	12.6	8.3	50.2%
ACO/160°C	Wheat Bran	1.7	13.9	10.4	45.4%
EtOH/160°C		1.7	18.7	7.2	71.2%
EtOH/175°C		2.2	15.7	11.0	50.7%

Treatment with hemicellulases of the aqueous liquors rise steeply the levels of fermentable sugars, leading to hydrolysis yields up to 73.4% for wheat straw liquid fraction pretreated at 175 °C using ACO and up to 71.2% for wheat bran liquid fraction pretreated at 160 °C using EtOH. As presented in **Figure 7**, concerning wheat straw, glucose and arabinose-based oligosaccharides were mainly broken down, while hydrolysis of xylo-oligosaccharides took place to a lesser extent. On the contrary, wheat straw hydrolysates are rich in xylose rather than glycose. In any case, the sugar levels after hydrolysis are promising to be used towards the production of DHA.

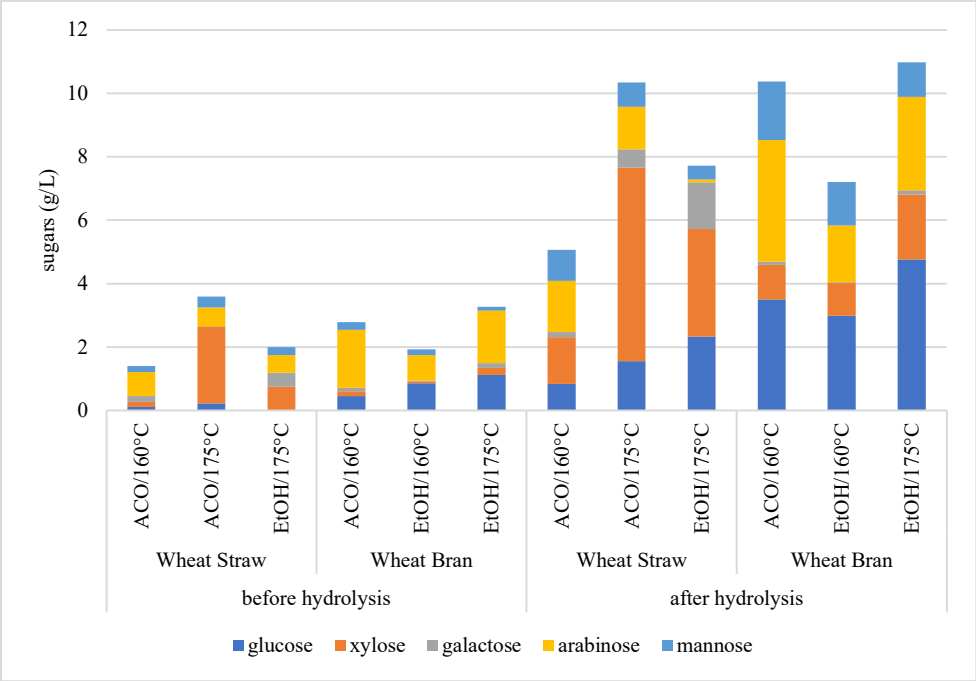

Figure 7. Monosaccharides profile before and after hydrolysis of the wheat straw and bran liquors.

Batch Cultures of Wheat Straw

Hydrolysates of the wheat straw pulps and liquors were assessed as carbon source towards the production of DHA using *C. cohnii*. Undoubtedly, pretreated wheat straw pulps achieved high lipid accumulation, which reached 70.3 *wt* % of harvested dried cell biomass and great DHA yield that reached up to 32.2% of the total lipids. A summary of the results is presented in **Table 2**. Expressing the results in mg/g of untreated biomass, when hydrolysate from biomass pretreated with EtOH at 160˚C was used as carbon source, 20.3 mg TFA/g of untreated biomass were

produced, corresponding to 7.1 mg DHA/g of untreated biomass, which was the highest achieved. These results, when compared to those of the untreated sample (3.8 mg TFA and 1.1 mg DHA/g of untreated biomass), were significantly higher, thus demonstrating the efficiency of the pretreatment and the fractionation for the downstream process yields. Liquid fractions resulted in lower yields, with the uppermost be 10.71 mg TFA and 1.41 mg DHA/g of untreated biomass. This outcome may spring from the different sugar profile of liquid fraction hydrolysates. Hemicellulose-rich streams can be valorized as carbon sources for *C. cohnii*; however, the different type of sugars affect not only lipid accumulation but also the DHA proportion.

Table 2. Cell biomass growth and accumulation of TFA on wheat straw hydrolysates. % TFA represent g of lipids per 100 g of cell biomass. % DHA refers to the percentagewise weight concentration of DHA in the *C. cohnii* total extracted lipids. The overall yields of total TFA and DHA yields per g of untreated biomass are also presented.

Pretreatment (organic solvent/ temperature)		Cell biomass (g/L)	TFA (%)	TFA (mg/g of untreated biomass)	DHA (%)	DHA (mg/g of untreated biomass)
ACO/150˚C	Solid	6.72 ± 0.67	37.04 ± 2.02	14.0 ± 1.09	38.82 ± 1.84	5.4 ± 1.09
ACO/160˚C		4.30 ± 0.11	38.35 ± 1.2	7.6 ± 0.35	33.78 ± 2.60	2.6 ± 0.35
ACO/175˚C		3.07 ± 0.06	39.02 ± 4.61	4.5 ± 0.22	33.50 ± 0.30	1.5 ± 0.22
EtOH/150˚C		5.01± 0.48	64.06 ± 2.98	18.4 ± 0.74	22.28 ± 0.83	4.1 ±0.74
EtOH/160˚C		6.23 ± 0.25	70.29 ± 5.6	20.3 ± 1.17	32.20 ± 3.40	7.1 ± 1.2
EtOH/175˚C		3.75 ± 0.97	60.55 ± 3.34	9.0 ± 0.93	29.19 ± 1.18	2.6 ± 0.93
untreated		4.29 ± 0.11	10.60 ± 2.73	3.8 ± 1.09	12.11 ± 1.83	1.1 ± 0.05
ACO/160˚C	Liquid	3.62 ± 0.34	48.50 ± 4.76	9.23 ± 1.77	9.68 ± 0.89	0.90± 0.23
ACO/175˚C		3.75 ± 0.25	22.45 ± 4.44	4.68 ± 1.23	2.21 ± 0.30	0.10 ± 0.05
EtOH/175˚C		4.04 ± 0.11	66.20 ± 4.73	10.71 ± 1.08	13.03 ± 1.41	1.41 ± 0.39

Fed- Batch Cultures of Wheat Straw

In order to enhance lipid productivity yields, fed- batch cultures were employed. This approach is considered as beneficial for lipid productivity, as it can lead to nitrogen limitation and increase DHA levels comparing to batch cultures; when the carbon source is added at the starting point of the culture, then the microalgae growth will be hindered due to high sugar concentration. This feeding strategy divides the process in two stages; a nitrogen richer first stage where cell growth is accelerated and a later carbon-richer stage dedicated to DHA formation. The results of this experiment are displayed in **Table 3.**

The results indicate that cell biomass growth was augmented in fed- batch systems, namely it has improved from 3.07 g/L to 7.00 g/L and from 3.75 g/L to 6.12 g/L for ACO and EtOH pretreated wheat straw pulps, respectively. Despite the apparent decrease of TFA percentagewise, due to the increase of cell biomass, TFA yield has improved when expressed in mg/g untreated biomass. The highest TFA accumulation was 10.4 mg/ g of untreated wheat biomass. Interesting is the fact that DHA purity has also amplified. DHA yield can be better observed in the lipid profile available on **Figure 8.** DHA productivity was improved in fed- batch cultures, as less palmitic (16:0) and oleic acid (18:1) are synthesized. DHA purity percentagewise reached 61.45% wt. of TFAs for the wheat straw pulp pretreated at 175 ºC with ethanol, which refers to 5.6 mg DHA/ g untreated biomass. However, the greatest performance observed in the ACO sample, namely 6.2 mg DHA/ g untreated biomass, because of the greater TFA accumulation.

Table 3. Cell biomass growth and accumulation of TFA on wheat straw hydrolysates in fed-batch cultures. For comparison the wheat straw batch cultures are also presented on the first two rows. % TFA represent g of lipids per 100 g of cell biomass. % DHA refers to the percentagewise weight concentration of DHA in the *C. cohnii* total extracted lipids. The overall yields of total TFA and DHA yields per g of untreated biomass are also presented.

Biomass-Pretreatment (organic solvent/ temperature)	Culture Method	Cell biomass (g/L)	TFA (%)	TFA (mg/g of untreated biomass)	DHA (%)	DHA (mg/g of untreated biomass)
Wheat-ACO/175°C	Batch	3.07 ± 0.06	39.02 ± 4.61	4.5 ± 0.3	33.50 ± 0.30	1.5 ± 0.2
Wheat-EtOH/175°C	Batch	3.75 ± 0.97	60.55 ± 3.34	9.0 ± 1.0	29.19 ± 1.18	2.6 ± 0.9
Wheat-ACO/175°C	Fed- Batch	7.00± 0.30	33.77 ± 3.83	10.4 ± 1.0	59.41± 1.20	6.2 ± 0.2
Wheat-EtOH/175°C	Fed- Batch	6.12 ± 0.39	38.00 ± 2.00	9.2 ± 0.5	61.45 ± 1.90	5.6 ± 0.2
Glucose/ Xylose (85%/15%)	Fed- Batch	5.30 ± 0.22	31.16 ± 2.52		61.06 ± 0.46	

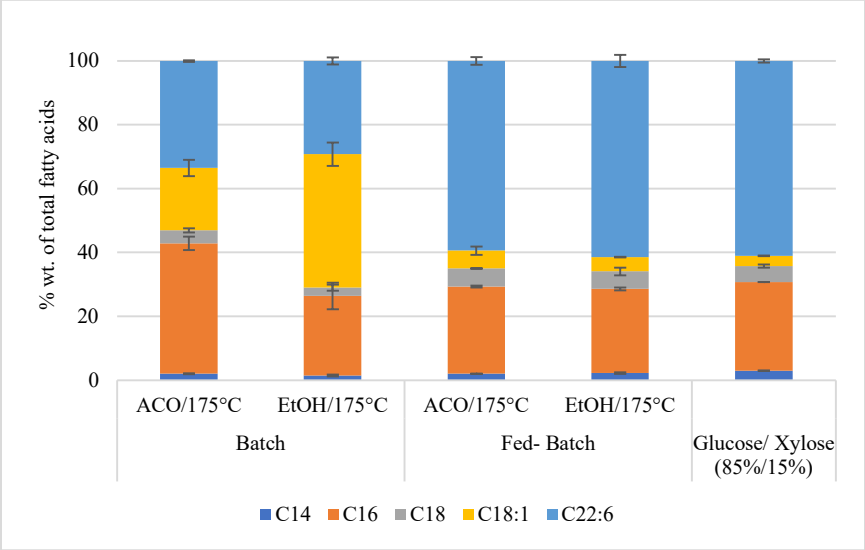

Figure 8. Fatty acid profile of oil extracted from fed-batch cultures on wheat straw pulps. Batch cultures are used for comparison.

Wheat Bran Cultures (Solid and Liquid Fractions)

Some of the wheat bran solid pulp hydrolysates, alongside the richer in sugars liquid fractions were assessed as well towards the production of DHA. The results are presented on **Table 4** and the lipid profile is demonstrated in **Figure 9**. Despite liquid fractions resulted in great lipid accumulation, with the highest being 25.59% TFA for solid pulp pretreated with ACO at 160 ºC and 70.98% TFA for liquor pretreated with EtOH at 160 ºC, DHA yields are significantly lower. The reason for the low DHA productivity may be the sugar composition of the hydrolysate, since the presence of C5 sugars together with the C6 may affect lipid synthesis at microalgal cultures, as more oleic acid is produced rather than DHA.

Table 4. Cell biomass growth and accumulation of TFA on wheat bran hydrolysates. % TFA represent g of lipids per 100 g of cell biomass. % DHA refers to the percentagewise weight concentration of DHA in the *C. cohnii* total extracted lipids. The overall yields of total TFA and DHA yields per g of untreated biomass are also presented

Pretreatment (organic solvent/ temperature)	Fraction	Cell biomass (g/L)	TFA (%)	TFA (mg/g of untreated biomass)	DHA (%)	DHA (mg/g of untreated biomass)
ACO/140˚C	Solid	6.14± 0.44	21.50 ± 2.18	7.51± 0.77	10.44 ± 0.68	0.79 ± 0.06
ACO/150˚C	Solid	5.68 ± 0.30	18.99 ± 2.24	3.82 ± 0.45	3.95 ± 0.28	0.15 ± 0.01
ACO/160˚C	Solid	7.71 ± 0.30	25.59 ± 1.40	11.35 ± 0.67	16.25 ± 0.09	1.84 ± 0.01
ACO/175˚C	Liquid	6.00± 0.10	18.01 ± 1.45	2.94 ± 0.22	2.94 ± 0.66	0.09 ± 0.01
EtOH/160˚C	Liquid	6.70 ± 0.02	70.98 ± 4.7	3.03 ± 0.20	3.81 ± 1.90	0.12 ± 0.01
EtOH/175˚C	Liquid	12.0 ± 0.76	54.50 ± 2.18	2.56 ± 0.10	6.59 ± 1.28	0.17 ± 0.01
untreated	-	8.36 ± 0.25	39.02 ± 4.61	16.74± 1.35	2.21 ± 1.23	0.37 ± 0.21

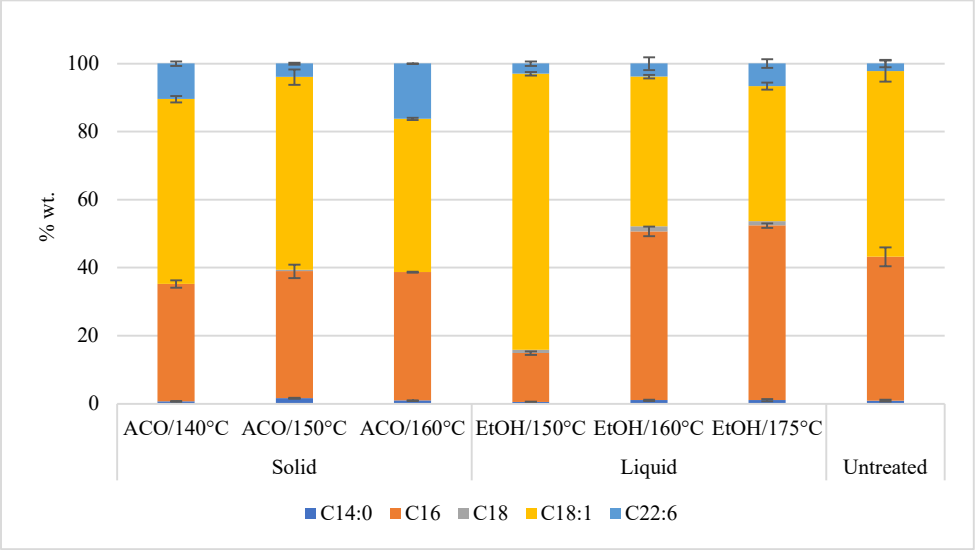

Figure 9. Fatty acids profile of oil produced from microalgal cells when grown on of wheat bran solid and liquid fractions.

Conclusions

Both wheat straw and bran feedstocks have been assessed regarding their potential to produce DHA. Wheat straw pulps were effectively used as carbon source in microalgal cultures, yielding great amounts of DHA. Furthermore, DHA productivity was enhanced when a fed-batch approach was evaluated. Thus, fed-batch cultures have great future potential in DHA production, especially in bioreactor systems where controlled conditions (pH and temperature levels, agitation and aeration) could be further beneficial. Moreover, fed-batch systems make feasible any possible scale-up attempts in the future. Despite the lower DHA yields in liquid fractions and wheat bran, it was a first attempted to valorize both streams retrieved from biomass pretreatment; those results can be helpful unravelling the drawbacks in DHA synthesis using C5 rich carbon sources and, thus, making possible the effective valorization of C5 sugars towards the production of DHA.

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References

Kalogiannis KG, Karnaouri A, Michailof C, Tzika AM, Asimakopoulou G, Topakas E, Lappas A.A. OxiOrganosolv: A novel acid free oxidative organosolv fractionation for lignocellulose fine sugar streams. Bioresour Technol. 313:123599 (2020)

Karnaouri A, Asimakopoulou G, Kalogiannis KG, Lappas AA, Topakas E.: Efficient production of nutraceuticals and lactic acid from lignocellulosic biomass by combining organosolv fractionation with enzymatic/fermentative routes. Bioresour Technol. 341:125846 (2021)

Chalima, A., Taxeidis, G., Topakas, E.: Optimization of the production of docosahexaenoic fatty acid by the heterotrophic microalga *Crypthecodinium cohnii.* Renew. Energy 152: 102-109. (2020)

Karnaouri A, Chalima A, Kalogiannis KG, Varamogianni-Mamatsi D, Lappas A, Topakas E.: Utilization of lignocellulosic biomass towards the production of omega-3 fatty acids by the heterotrophic marine microalga *Crypthecodinium cohnii* Bioresour Technol. 303:122899 (2020)

Zhao, X., Cheng, K., Liu, D.: Appl. Microbiol. Biotechnol. 82 (5): 815-827 (2009)

Ratledge, C.: Microbial production of polyunsaturated fatty acids as nutraceuticals. In: Microbial Production of Food Ingredients, Enzymes and Nutraceuticals, pp. 531–540. Woodhead Publishing Limited (2013)

de Swaaf M. E., Pronk J. T., Sijtsma L.: Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga Crypthecodinium cohnii on ethanol. Appl Microbiol Biotechnol. 61(1):40-3 (2003)

Raabo E, & Terkildsen T.C.: On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12 (4): 402-7 (1960)

Miller GL.: Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar Anal. Chem. 31: 426-8 (1959)

Singleton V.L., Orthofer R., Lamuela-Raventós R.M.: Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Meth. Enzymol. 299: 152-178 (1999)

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D.: Determination of Extractivesin Biomass NREL. TP-510-42619 (2008)