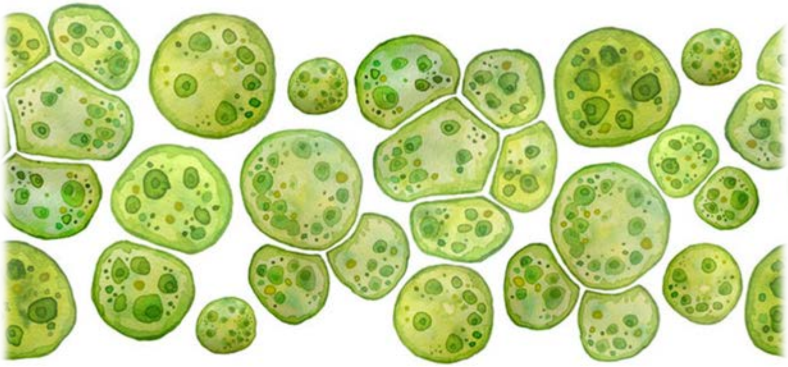  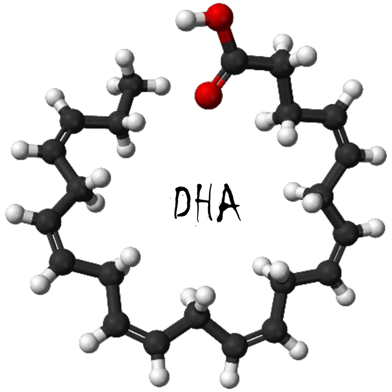

Valorization of forest and agricultural biomass residues towards the production of docosahexaenoic acid (DHA) by the heterotrophic dinoflagellate *Crypthecodinium cohnii*

Savvas Staikos[1], Anthi Karnaouri[1], Stylianos D. Stefanidis[2], Konstantinos G. Kalogiannis[2], Angelos A. Lappas[2], Evangelos Topakas[1]

[1]Industrial Biotechnology & Biocatalysis Group, School of Chemical Engineering, National Technical University of Athens, Greece

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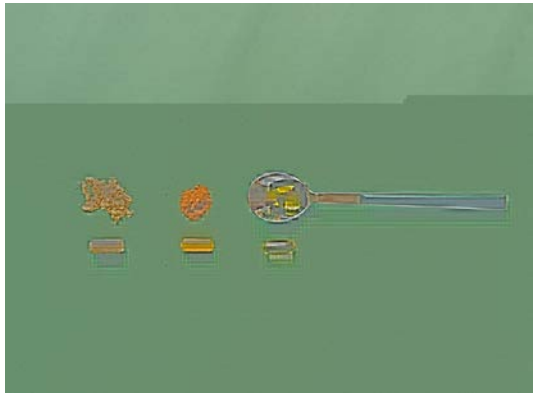
9th International Conference on Sustainable Solid Waste Management
Corfu, Greece, 15 - 18 June 2022

Omega-3 fatty acids as important nutraceuticals



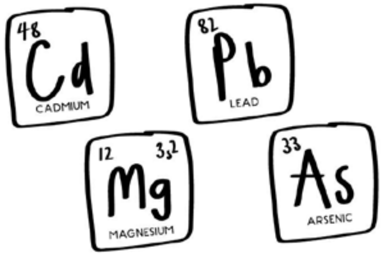
Functional products and Nutraceuticals:

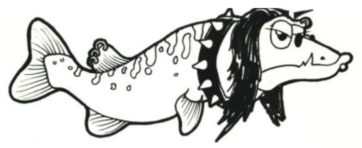
- ***Nutraceuticals*** are standardized grade of food sources → contain ***bioactive compounds***
- Extra benefits in addition to basic nutritional value found in food (prevention or treatment of various diseases).
- Addition of small amounts in foods (1-5%) adds ***higher value to the final products***.
- the demand for functional foods and beverages, especially for ***omega-3 fatty acid fortified products***, has increased significantly in the past few years

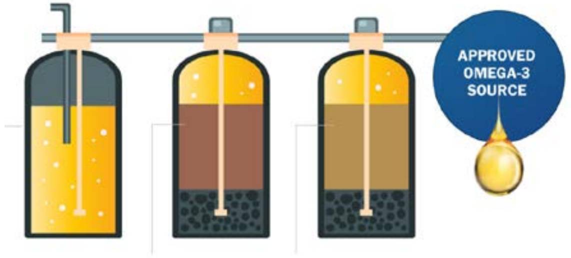
Conventional sources of Ω-3 fatty acids in marine environments

Fishes and other marine organisms are abundant sources of Ω-3 fatty acids…

But…

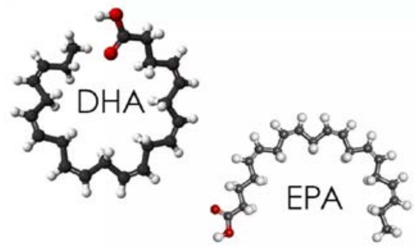






Production of Ω-3 fatty acids from marine microalgae

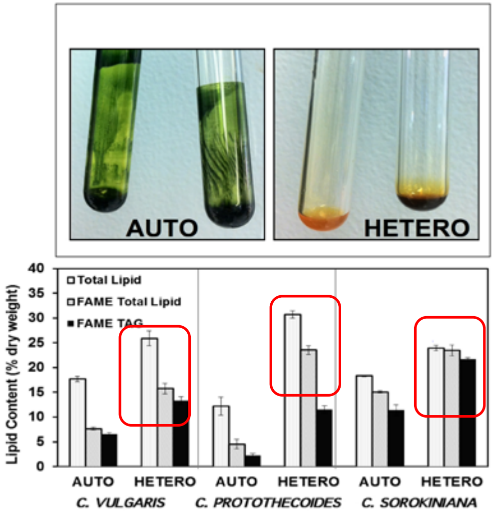
- Microalgae oil as an attractive alternative to fish oil!
- Production of ***Ω-3 polyunsaturated fatty acids (PUFAs***), especially those with with ***long chain (LC-PUFAs)***
- Microalgae oil is rich in **EPA (20:5n-3) and DHA (22:6n-3)** → recognized as bioactive compounds of pivotal importance



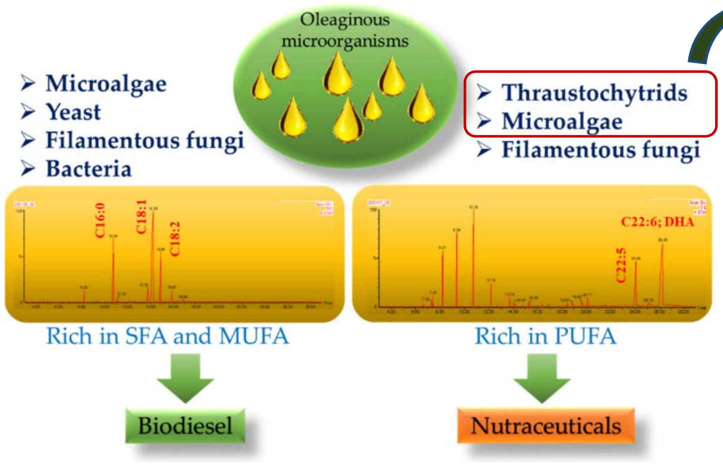
3 benefits of omega-3 oil from microalgae!

The Dark Side of Microalgae Biotechnology!

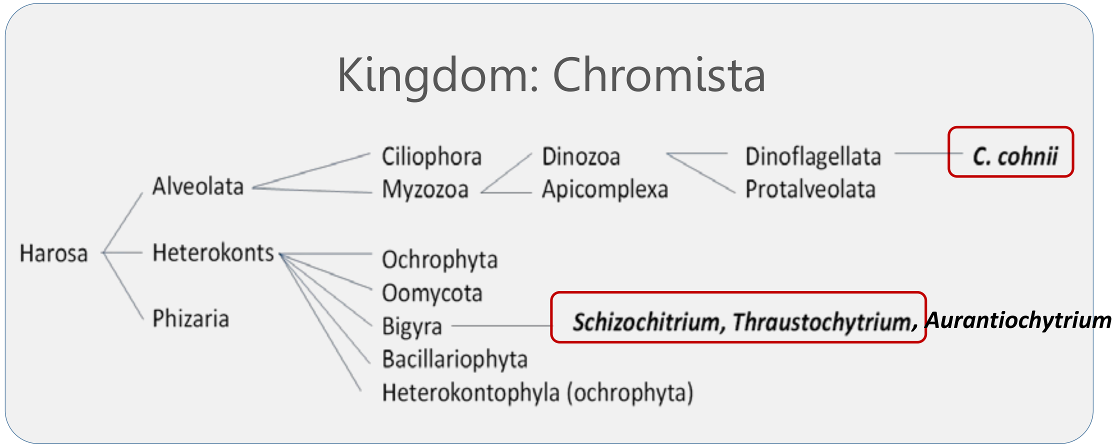
Heterotrophic Platforms Directed to ω-3 Rich Lipid Production



- Non-seasonality, non-dependence on climatic conditions, non-need of arable land
- Utilization of low-cost substrates (organic wastes/residues) to produce PUFAs
- Marine heterotrophic microalgae that belong to *Dinoflagellata*

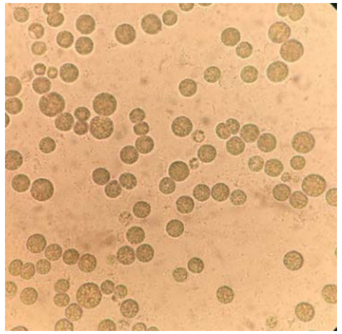


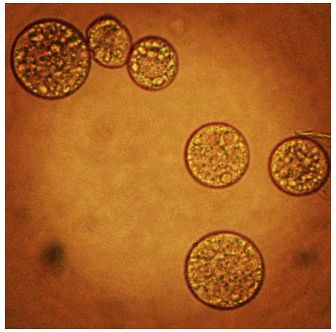
Feedstock	Microorganism	EPA/DHA Production
Food waste hydrolysate	*Schizochytrium mangrovei Chlorella pyrenoidosa*	85.5 ± 11.2 $mg \cdot g^{-1}$ DHA
Sweet sorghum juice	*Schizochytrium limacinum*	273 $mg \cdot g^{-1}$ DHA1.1 $mg \cdot g^{-1}$ EPA
Carob pulp syrup	*C. cohnii* CCMP 316	1.99 $g \cdot L^{-1}$ DHA45.2 $mg \cdot g^{-1}$ DHA
Rapeseed meal hydrolysate + crude waste molasses	*C. cohnii* ATCC 30772	8.72 $mg \cdot L^{-1}$ DHA22–34 % $w \cdot w^{-1}$ DHA of TFA
Cheese whey + Corn steep liquor	*Crypthecodinium cohnii* CCMP 316	8.5–27% $w \cdot w^{-1}$ DHA of TFA

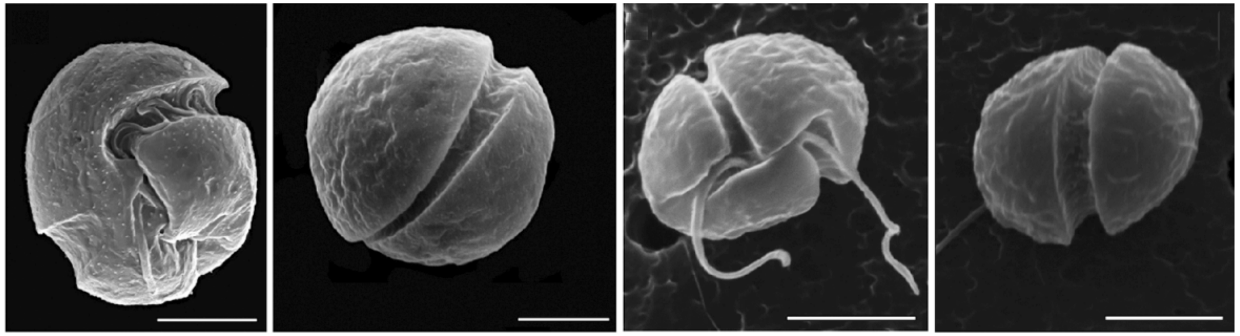


The heterotrophic dinoflagellate *Crypthecodenium conhii*

- ✓ *C. cohnii* is able to grow utilizing a variety of different carbon sources, such as **short chain organic acids** (acetic, propionic, butyric acid), **ethanol, sugars** (glucose, galactose, lactose, xylose)
- ✓ The accumulation of lipids reaches up to **45-50%** of dry cell weight, with DHA to comprise up to **60%** of total fatty acids
- ✓ **GRN 41; “DHASCO** (docosahexaenoic acid-rich single-cell oil) from *C. cohnii* used in infant formula” (USA, 2001)

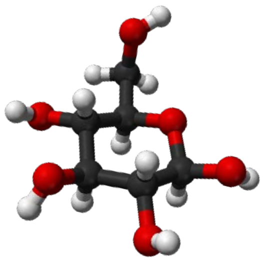






Oil fatty acid composition
C14
C16
C18:0
C18:1
C22:0 (DHA)

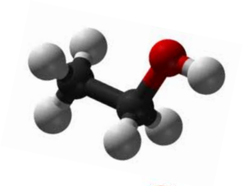
Valorization of different carbon sources for the production of omega-3 fatty acids

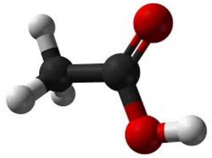


glucose 16 € kg^{-1}

ethanol 1.82 € kg^{-1}

acetic acid 0.45 € kg^{-1}





Are there other available carbon sources in order to reduce the costs of the overall process and develop a sustainable bioeconomy?

low-cost substrates that have been explored...

- ✓ VFAs from anaerobic digestion effluent
- ✓ crude glycerol
- ✓ sugarcane molasses
- ✓ vinegar effluent
- ✓ liquid fraction of exhausted olive pomace
- ✓ date syrup

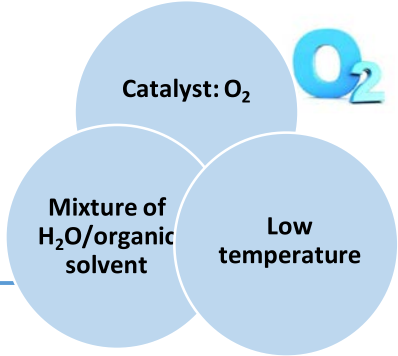
Lignocellulosic biomass?

- ✓ Valorization of underutilized sugar streams
- ✓ Integration in biorefineries

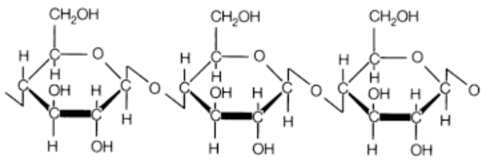
OxiOrganosolv: Biomass pretreatment and fractionation

Organosolv delignification → mild wet oxidation in presence of organic solvents

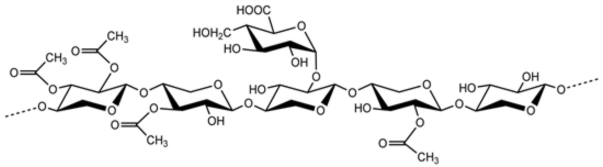
- o *Ethanol*
- o *Acetone*
- o *Isobutanol*
- o *Tetrahydrofuran*



Solid delignified, cellulose-rich fraction



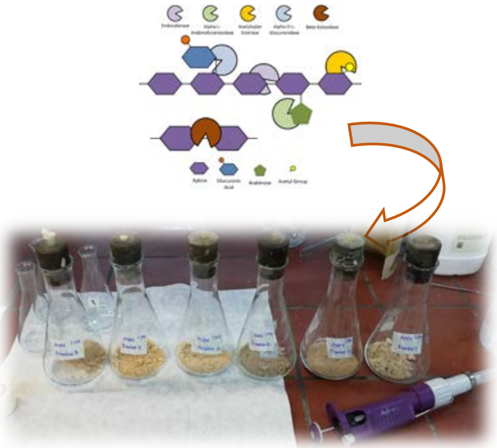
Hemicellulose-rich liquid fraction

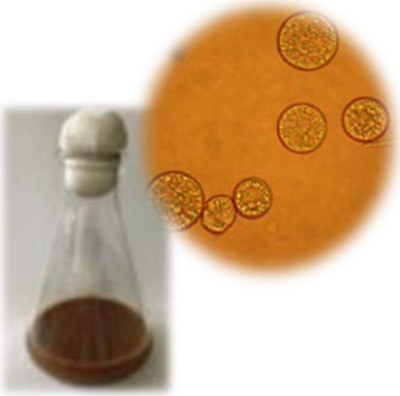


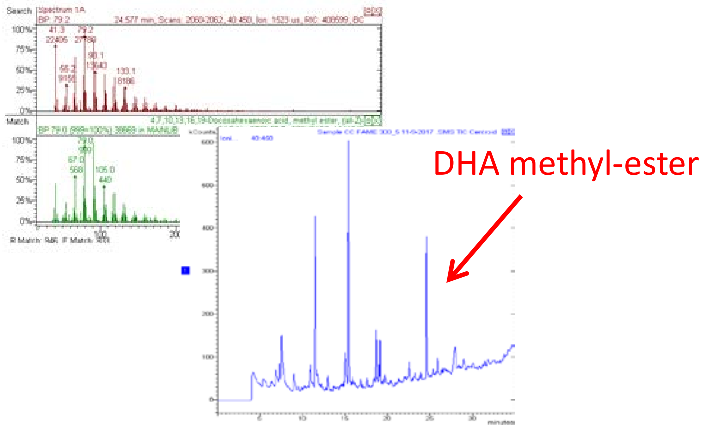
Substrate/Pretreatment conditions	% delignification	% cellulose recovery in solid pulp
Beechwood, H_2O/EtOH, O_2 12 bar, 175°C, 60min	94.2	99.1
Beechwood, H_2O/ACO, O_2 12 bar, 175°C, 120min	97.0	94.2
Pine, H_2O/EtOH, O_2 12 bar, 175°C, 60min	87.6	98.5
Wheat straw, H_2O/EtOH, O_2 12 bar, 175°C, 120min	86.8	97.6

Kalogiannis K., Karnaouri A., Michailof C. et al. Bioresource Technology, 2020, 313, 123599

Efficient production of omega-3 fatty acids from *C. cohnii*







Prehydrolysis

- Cellic®CTec2 and HTec2 (when needed)
- 9% (w/v) DM
- 15 mg /g enzyme loading
- 72 h, 50°C, 160 rpm
- Buffer MES, pH=5.5

Cultivation in biomass-derived hydrolysate

- 30 mL final volume
- 25 g/L sea salts
- 2 g/L yeast extract
- 120 h, 23°C,160 rpm

Cells harvesting

- Centrifuge
- Freeze drying
- Determination of dry cell weight gravimetrically

Extraction of fatty acids

- Extraction of fatty acids with modified Folch method ($CHCl_3$ / MeOH 2: 1 (*v/v*))

Identification of fatty acids profile

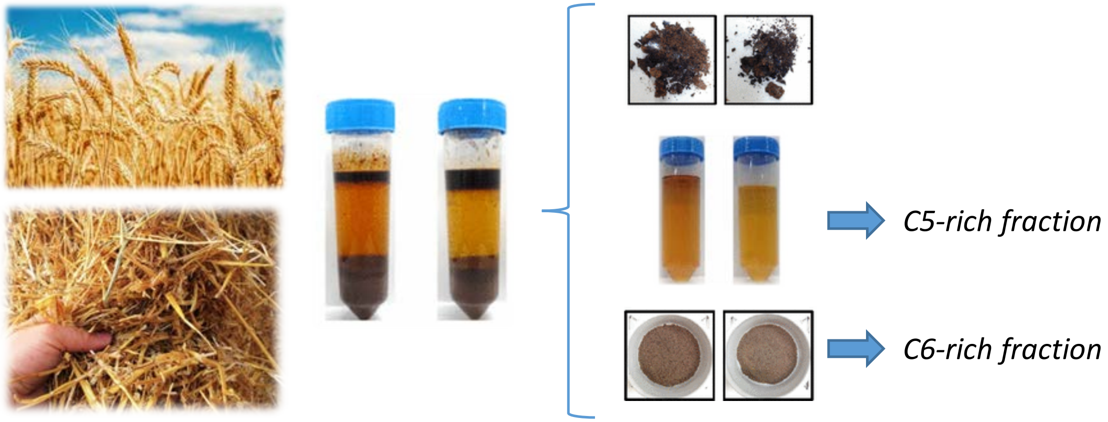
- Trans-esterification
- Analysis of FAMEs with gas chromatography

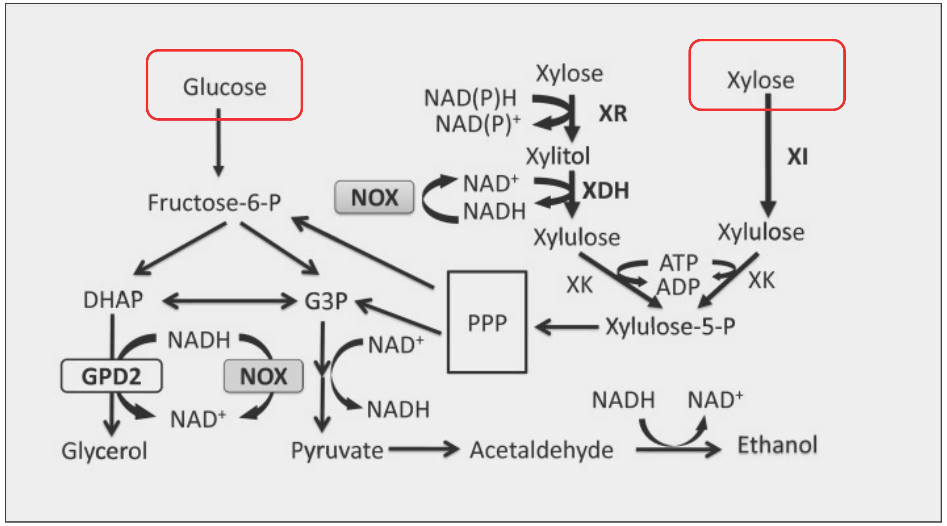
Forest pulps-derived hydrolysates as carbon sources for the production of DHA

	Pretreated Biomass	Microalgae biomass (g/L)	Microalgae biomass (wt.% of total sugars consumed)	% TFA (w/w)	TFA (g/L)	% DHA (w/w)	DHA (g/L)
Beechwood	H_2O/THF (50/50%), O_2 8 bar, 175°C, 120 min	8.74	35.3	61.76	5.40	26.62	**1.44**
Beechwood	H_2O/ACO (50/50%), O_2 12 bar, 175°C, 120 min	6.48	36.0	45.93	2.97	27.69	0.82
Beechwood	H_2O/EtOH (50/50%), O_2 12 bar, 175°C, 120 min	8.57	33.6	54.81	4.70	28.62	1.35
Beechwood	H_2O/THF (50/50%), O_2 12 bar, 175°C, 120min	7.39	31.9	44.18	3.27	27.23	0.89
Beechwood	H_2O/ACO (50/50%), O_2 25 bar, 175°C, 120min	7.71	31.1	38.22	2.95	21.99	0.65
Beechwood	H_2O/ACO (50/50%), O_2 12 bar, 175°C, 60min	7.76	30.4	33.45	2.60	28.96	0.75
Beechwood	H_2O/EtOH (50/50%), O_2 12 bar, 175°C, 60min	8.72	32.5	39.06	3.41	28.24	0.96
Beechwood	H_2O/THF (50/50%), O_2 12 bar, 175°C, 60min	7.98	32.8	35.71	2.85	**29.50**	0.84
Beechwood	H_2O/THF (50/50%), O_2 16 bar, 150°C, 120 min	7.90	37.3	54.30	4.29	29.40	1.26
Beechwood	H_2O/EtOH (50/50%), O_2 16 bar, 175°C, 60 min	8.60	34.5	47.98	4.13	29.51	1.22
Pine	H_2O/EtOH (50/50%), O_2 16 bar, 175°C, 60min	5.47	29.4	38.66	2.12	24.76	0.52
	Cellulose (Avicel)	5.57	35.5	23.33	1.30	45.68	0.59
	Untreated beechwood	0.99	49.1	35.96	0.36	14.70	0.05
	Glucose	9.67	30.6	43.03	4.16	34.88	**1.45**

Karnaouri A., Chalima A., Kalogiannis K. et al. Bioresource Technology, 2020, 303, 122899

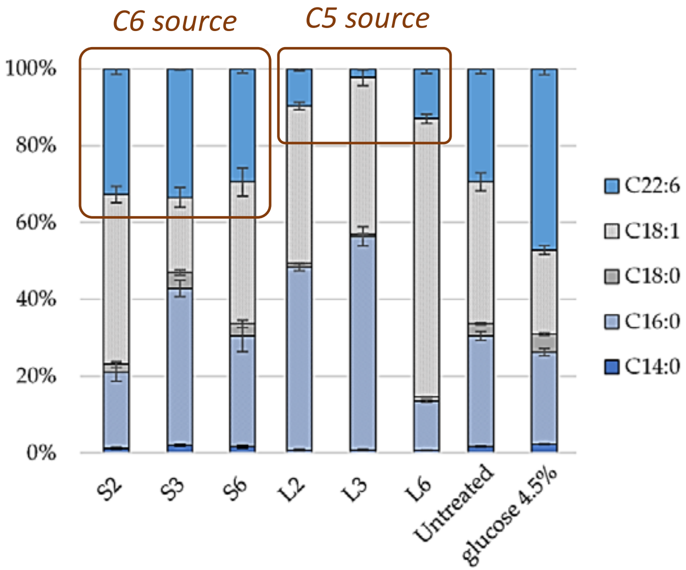
Utilization of both C5 and C6 sugars from wheat straw





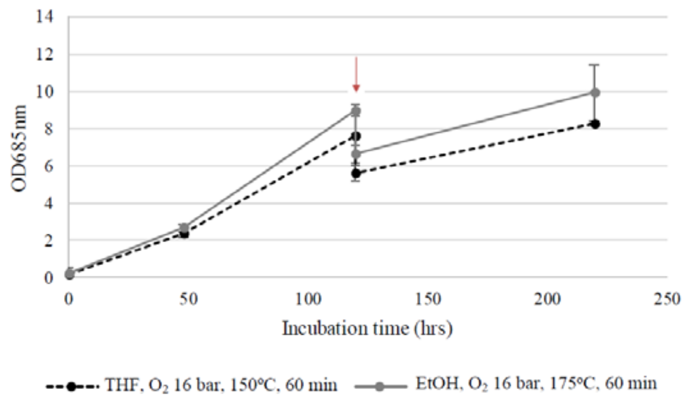
			TFA (mg/g pr. biomass or mg/mL liquor)	TFA (mg/g of untreated biomass)	DHA (mg/g pr. biomass or mg/mL liquor)	DHA (mg/g of untreated biomass)
C6-rich fraction	S2	ACO, 160°C, 120 min	**13.76**	**7.6**	**4.65**	**2.6**
	S3	ACO, 175°C, 120 min	**9.97**	**4.5**	**3.34**	**1.5**
	S6	EtOH, 175°C, 120 min	**18.93**	**9.0**	**5.53**	**2.6**
C5-rich fraction	L2	ACO, 160°C, 120 min	1.95	9.23	0.19	0.9
	L3	ACO, 175°C, 120 min	0.94	4.68	0.02	0.1
	L6	EtOH, 175°C, 120 min	2.97	10.71	0.39	1.4
		untreated	*3.69*	*3.8*	*0.46*	*1.1*

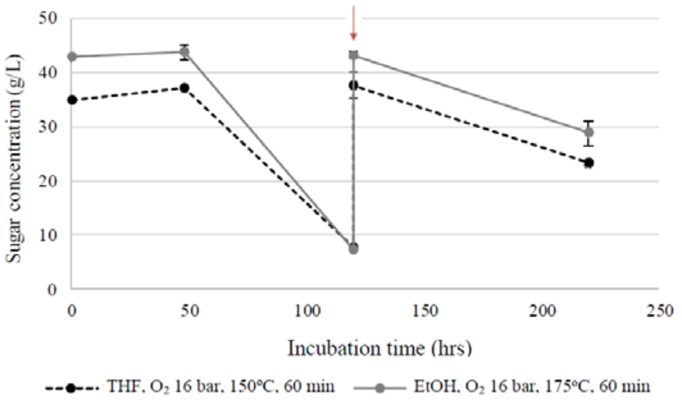
4 mg/g



Asimakopoulou G., Karnaouri A., Staikos S. et al. Fermentation, 2021, 7(4), 219

Employment of fed-batch strategy with biomass hydrolysates





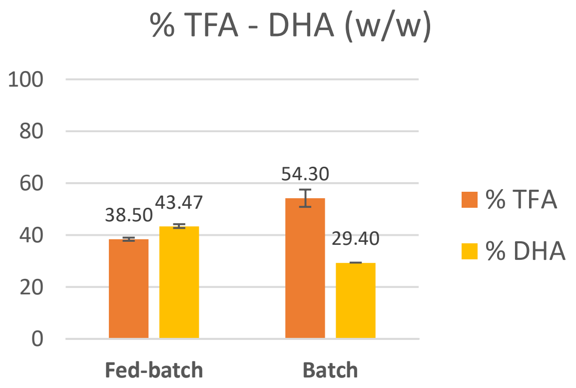
Experiments in shaking flasks

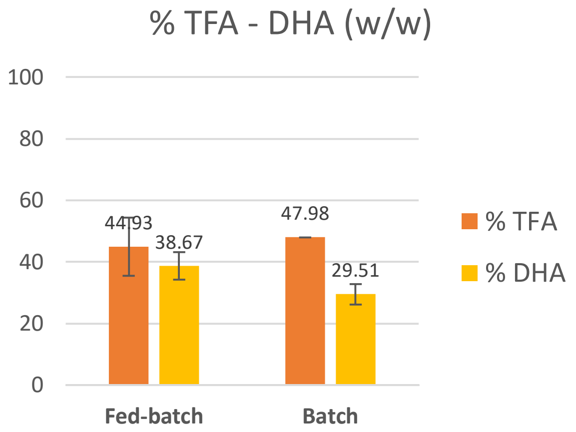
Substrate: H_2O/THF (50/50%), O_2 16 bar, 150°C, 120 min

Strategy	Biomass (g/lt)	% TFA (w/w)	TFA (g/lt)	%DHA (w/w)	DHA (g/lt)
Batch	7.90	54.30	4.29	29.40	1.26
Fed-batch	10.28	38.50	3.96	43.47	1.72

Substrate: H_2O/EtOH (50/50%), O_2 16 bar, 175°C, 60 min

Strategy	Biomass (g/lt)	% TFA (w/w)	TFA (g/lt)	%DHA (w/w)	DHA (g/lt)
Batch	8.60	47.98	4.13	29.51	1.22
Fed-batch	12.71	44.93	5.71	38.67	2.21

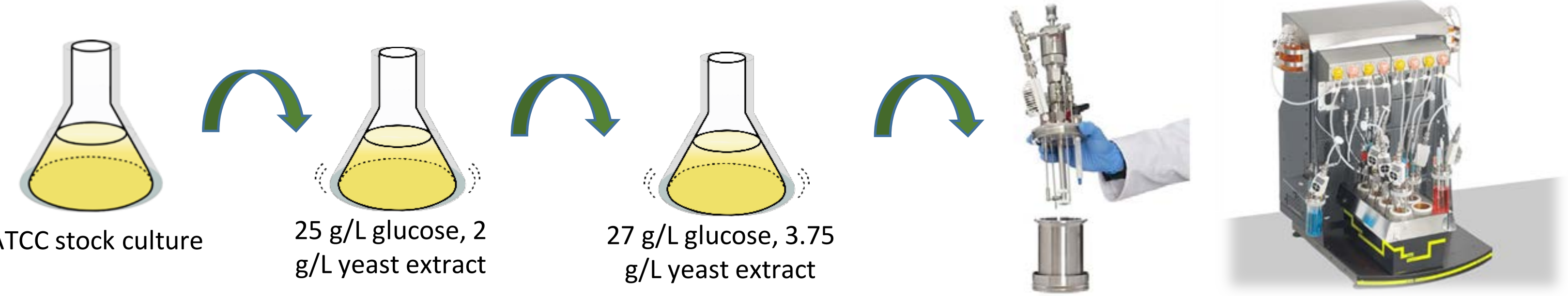




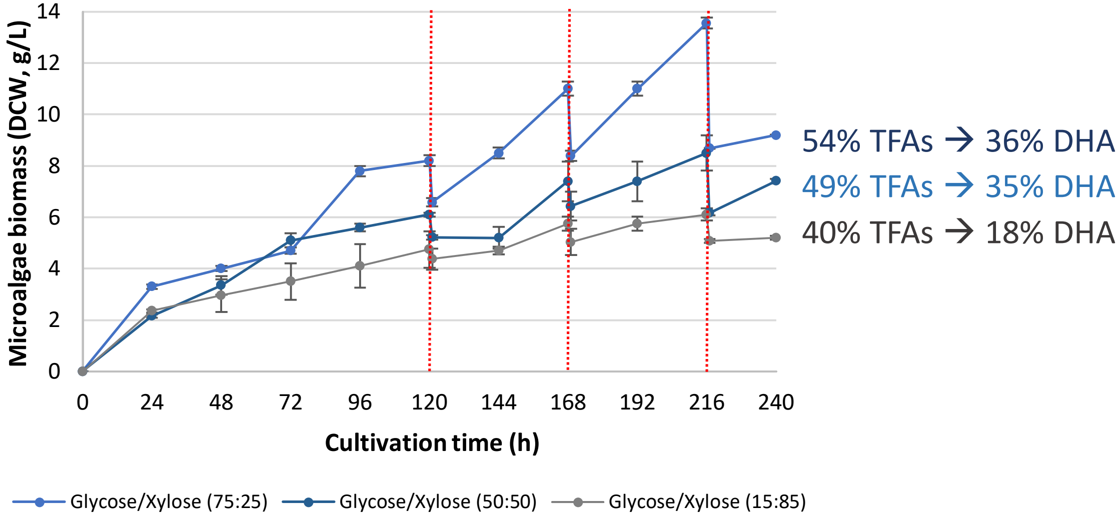
Karnaouri A., Chalima A., Kalogiannis K. et al. Bioresource Technology, 2020, 303, 122899

Fed-batch strategy in bioreactors

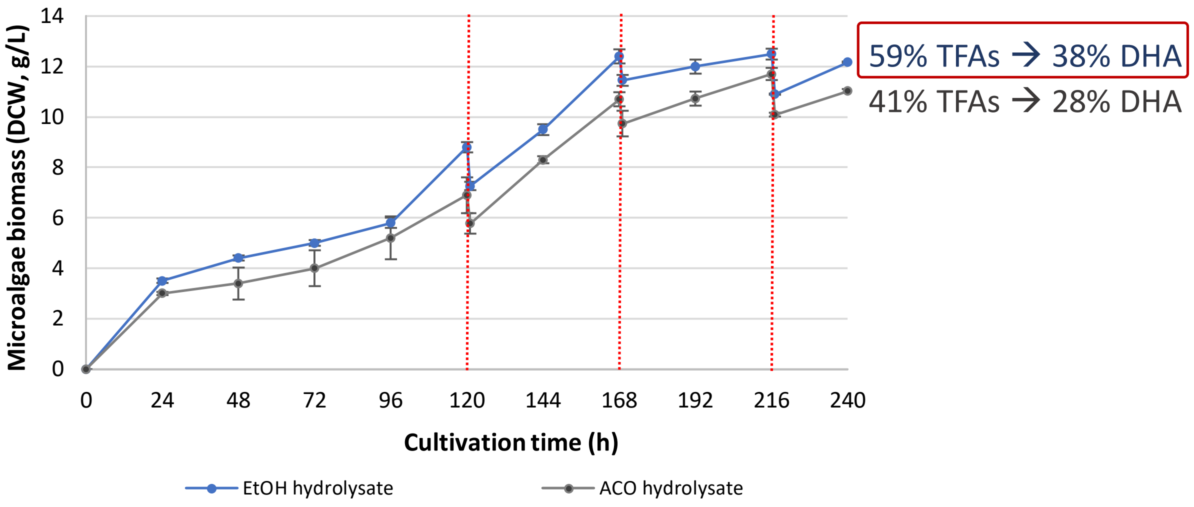
- BioXplorer 100 | bench-top, parallel 8 bioreactor platform
- Constant agitation (250 rpm) aeration (1 vvm)
- pH set at 6.5 (control with HCl)
- 25 g/L sugars, 7.5 g/L YE, 25 g/L sea salts
- Carbon source feeding at 120, 168, 216 h



Experiments with pure sugars



Experiments with beechwood hydrolysate



Conclusions and Future perspectives

What we have learned so far…

- OxiOrganosolv is an efficient pretreatment method for biomass delignification – agnostic process
- Utilization of both pentose and hexose sugars for DHA production by *C. cohnii*
- Fermentation in batch-mode has several limitations

What we are interested to explore further…

- Addressing the challenges of liquid fraction utilization
- Optimization of culture conditions – Fermentation in bioreactors with continuous and fed-batch mode of feeding
- Performing technoeconomical studies of the process

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Dr. Konstantinos Kalogiannis
Dr. Stelios Stefanidis
Anna-Maria Tzika

CPERI Chemical Process and Energy Resources Institute

Thank you for your attention!

NoWasteBioTech: "Novel Conversion Technologies of Waste Biomass to Food additives and Fine Chemicals"

H.F.R.I. Hellenic Foundation for Research & Innovation

AMALTHYA: "Valorisation of Agricultural Residues by Transformation in Cascade of Bio- and Thermo- Chemical Routes to Food Additives of High Added Value" - EPAnEK 2014-2020 Operational Programme, Competitiveness-Entrepreneurship-Innovation

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