Utilization of organosolv pretreated lignocellulosic biomass for the production of omega-3 fatty acids by the marine microalgae *Crypthecodinium cohnii* and lactic acid by *Lactobacillus delbrueckii*

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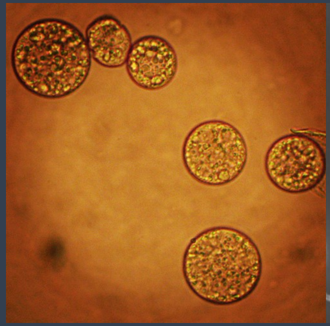
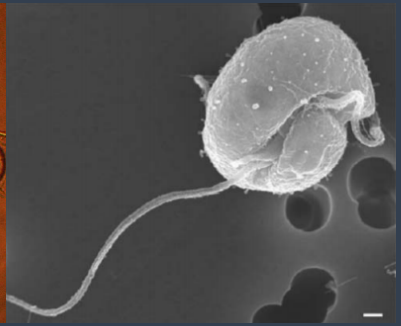
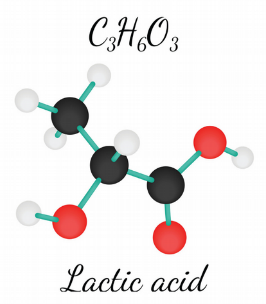
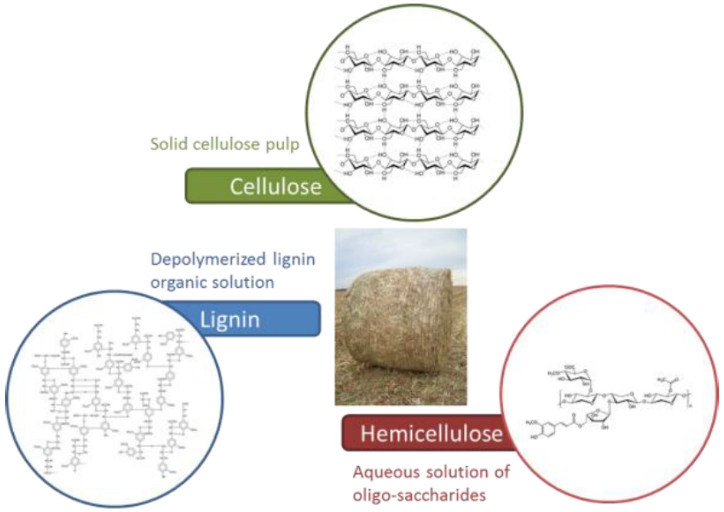






Valorization of lignocellulosic biomass on the forefront of sustainability and circular economy



- ✓ Greece has a developed ***agricultural sector*** that supports a big part of the economy.
- ✓ EU mandates about CO_2 emissions and sustainability of all production processes have given a significant boost to the ***biorefinery concept***.
- ✓ For biorefineries to be economically viable, they need to produce ***high added value products*** with **close to zero wastes**, satisfying niche markets such as the food and pharmaceutical ones.

NoWaste BioTech

"*Novel Conversion Technologies of Waste Biomass to Food additives and Fine Chemicals*", funded by Hellenic Foundation for Research & Innovation

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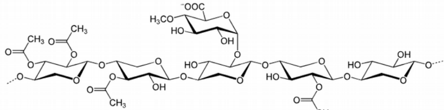
The overall scheme of NoWasteBioTech

corn stover

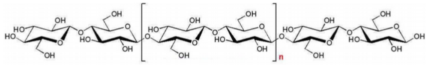
corn cob

Biomass

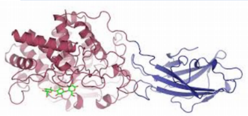
- solid **cellulose** pulp
- aqueous **hemicellulose** oligo- sugars



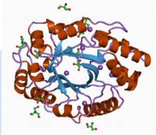
xylan structure



cellulose structure



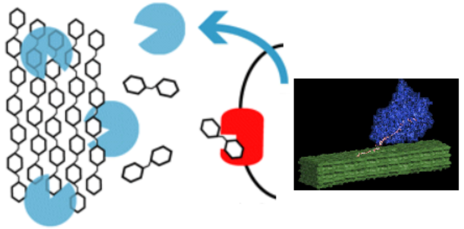
cellulase



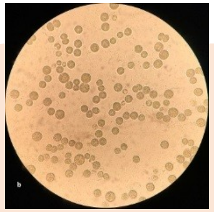
xylanase

Saccharification

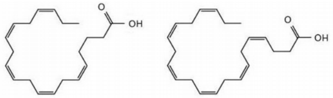
- **enzymatic production** of sugars
- **cellulases/he**



cellulose/hemicellulose hydrolysis



microalgae cells



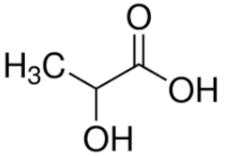
ω-3 fatty acids

Fermentation

- **ω-3 fatty**

c acid

lactic acid bacteria



lactic acid

Biomass pretreatment and efficient fractionation

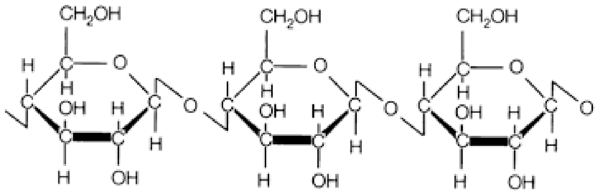
Organosolv delignification
mild wet oxidation in presence of organic solvents

- *Acetone*
- *Ethanol*
- *Tetrahydrofuran*

LIGNIN

HEMICELLULOS

Solid delignified, cellulose-rich fraction



CPERI
Chemical Process and Energy Resources Institute

Lignocel HBS 150/500 (beechwood)

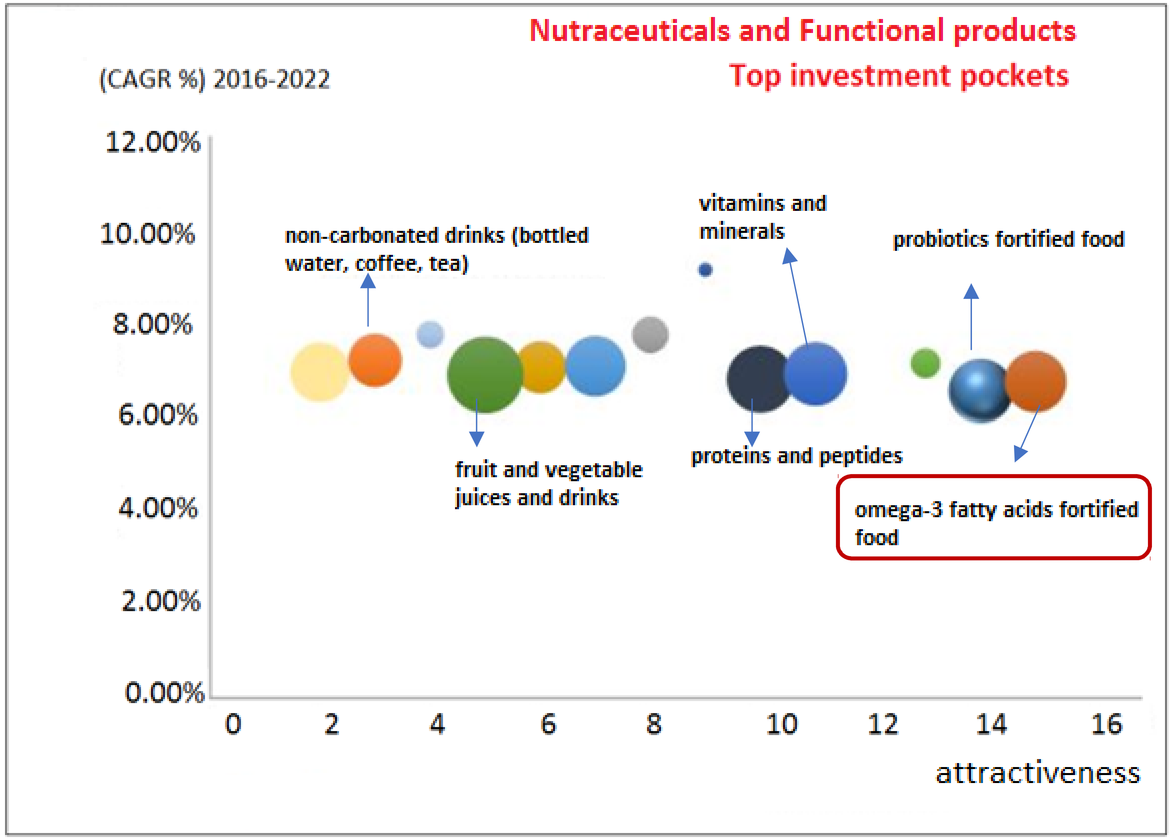
Composition	(% w/w)
Cellulose	47.6
Hemicellulose	21.2
Lignin	24.2
Extractives	3.7

Pretreatment conditions	% cellulose	% hemicellulose	% lignin
H_2O/THF, O_2 12 bar, 175^oC, 120min	80.11	11.39	5.77
H_2O/ACO, O_2 25 bar, 175^oC, 120min	80.28	12.26	1.58
H_2O/ACO, O_2 12 bar, 175^oC,			

Omega-3 fatty acids as important nutraceuticals

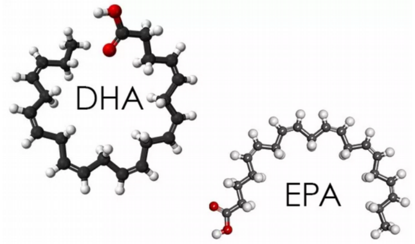
Functional products and Nutraceuticals:

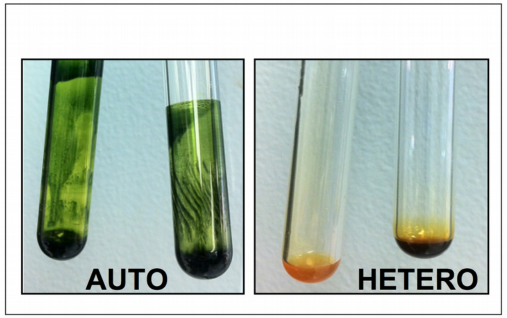
- ***Nutraceuticals*** are standardized grade of food sources derived from food sources, which contain one or more ***bioactive compounds***
- extra benefits in addition to basic nutritional value found in food (prevention or treatment of various diseases).
- addition of small amount of nutraceuticals 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, due to the increase in the cost

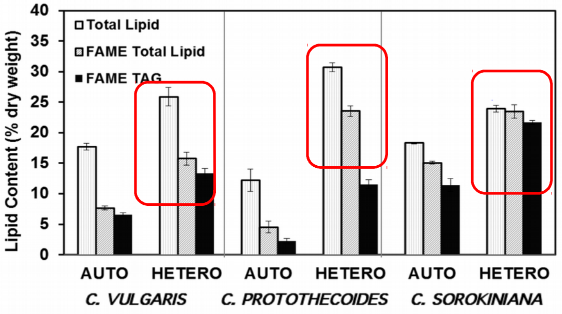


Production of Ω-3 fatty acids from marine microalgae

- 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
- microalgae oil is an attractive alternative to fish oil (its sustainable supply has been challenged due to the reduction of fish resources worldwide
- marine heterotrophic microalgae that belong to *Dinoflagellata*







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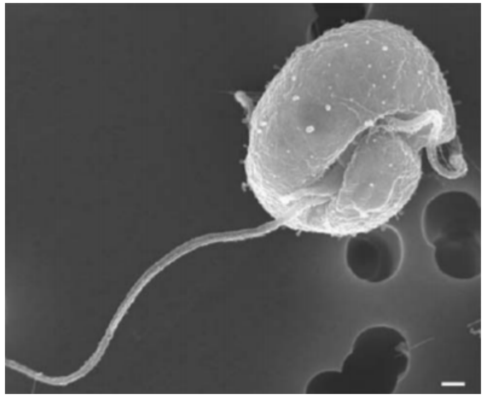
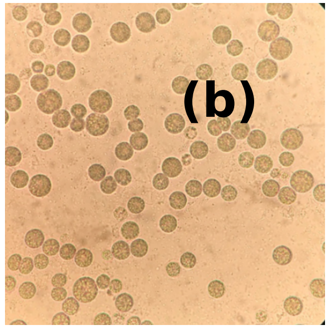
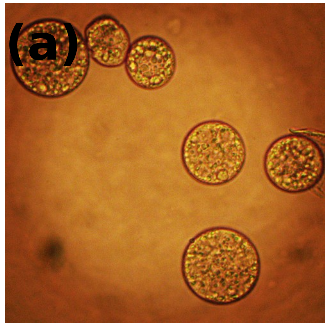
life'sDHA® is a vegetarian source of docosahexaenoic acid (DHA) omega-3 from algae that provides important brain and eye benefits throughout all stages of life. Most people incorrectly believe that fish produce their own DHA, when in fact it's the algae in their food chain. *life'sDHA®* goes straight to the source, resulting in a sustainable, high quality product rich in DHA omega-3.

The heterotrophic dinoflagellate *Crypthecodenium*

- ✓ *Crypthecodinium cohnii*, a flagellated marine microalga, is considered as a prolific DHA producer.
- ✓ The strain ATCC 30772 is able to grow utilizing a variety of different carbon sources, such as short chain fatty acids (acetic, propionic, butyric acid), ethanol, sugars (glucose, galactose, lactose)
- ✓ 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 *Crypthecodinium cohnii* for use in infant formula” (USA, 2001)







Κύτταρα του μικροφύκους C. cohnii x40 (a) και x100 (b)

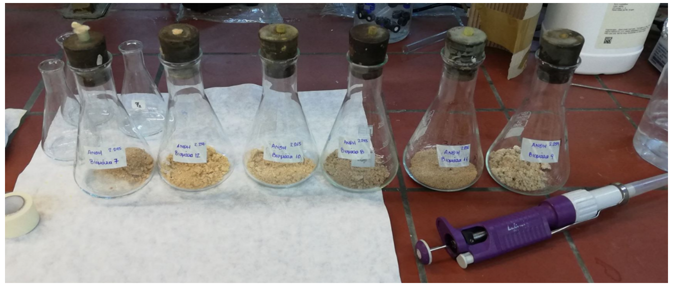
Efficient production of omega-3 fatty acids from *C. cohnii*

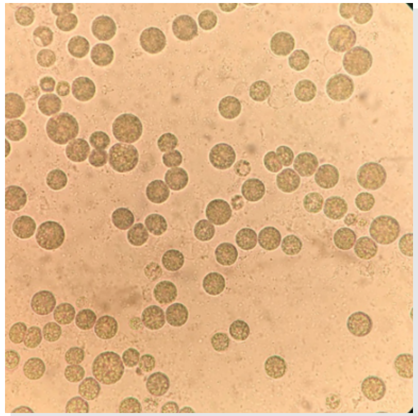
Cultivation strategy

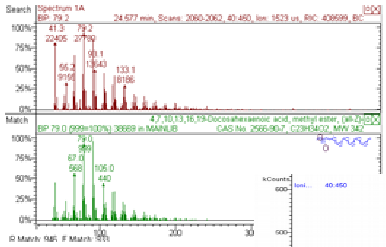
- ✓ Cultivation on shake flasks, 120h
- ✓ Nitrogen source: yeast extract 2 g/lt
- ✓ Sea salts 25 g/lt
- ✓ Carbon source: pure sugars, enzymatically hydrolyzed lignocellulosic biomass-derived sugars (*SHF process*)

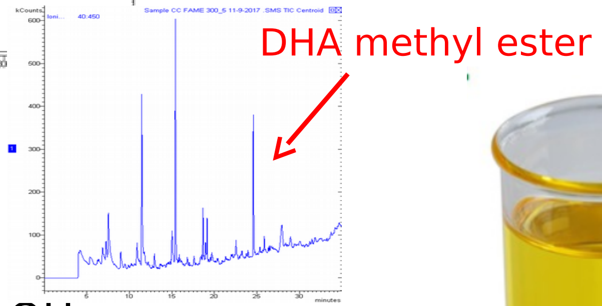
Analysis of ω-3 fatty acids

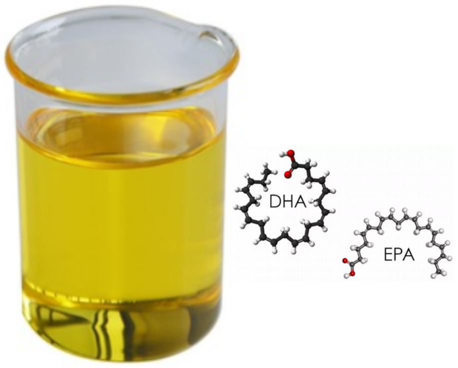
- ❖ Harvesting the microalgae cells – Freeze drying
- ❖ Determination of dry cell weight gravimetrically
- ❖ Extraction of fatty acids with modified Folch method ($CHCl_3$/MeOH 2:1 (*v/v*))
- ❖ Trans-esterification and analysis of FAMEs with GC-MS





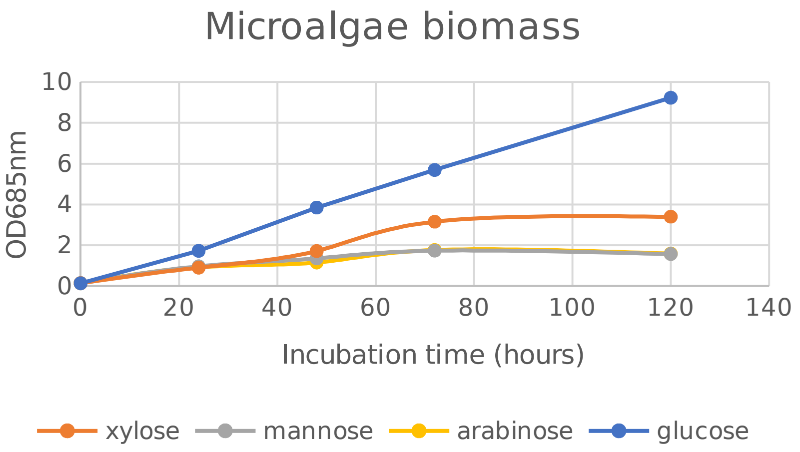


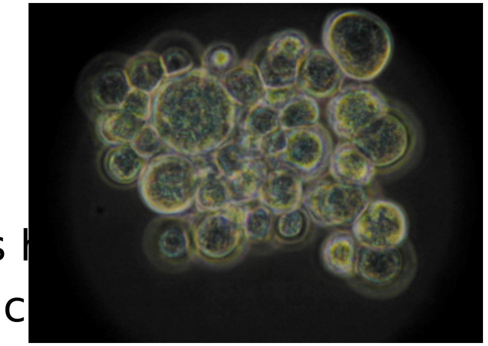




Pure sugars as carbon sources for *C. cohnii*

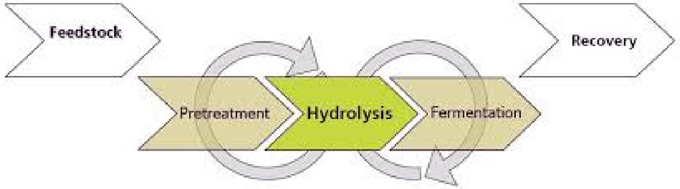
Sugar	Biomass g/lt	Biomass (mg/g consumed)	% TFA	TFA (g/lt)	% DHA	DHA (g/lt)
Glucose	9.67	306	43.03	4.16	29.98	1.25
Xylose	1.40	312	37.32	0.52	5.66	0.03
Mannose	1.23	187	45.40	0.56	17.78	0.10
arabinose	1.18	126	46.57	0.55	18.55	0.10
Glc/lignin *50:50	10.86	354	47.90	5.20	27.14	1.41
Glc/HMF	7.08	320	35.24	2.50	22.95	0.57
Glc/Xyl*	4.27	296	40.04	1.71	31.47	0.54

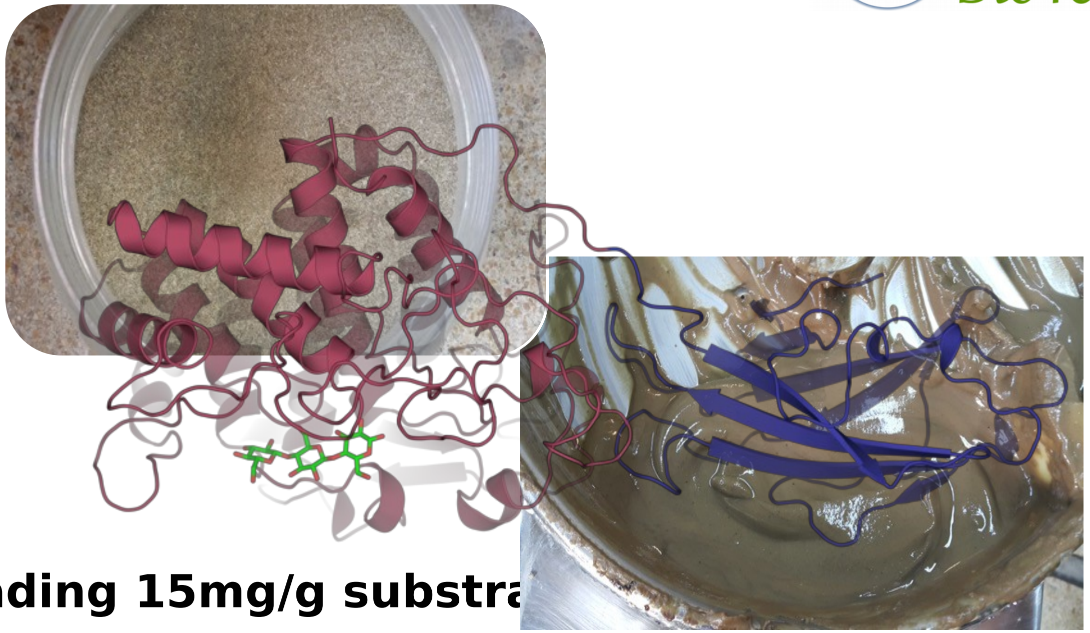




- ✓ *C. cohnii* prefers glucose as a carbon source over other sugars present in biomass h
- ✓ No catabolic repression by the simultaneous presence of hexose and pentose (gluc
- ✓ Presence of lignin can possibly affect positively the microalgae metabolism for enhanced DHA production
- ✓ Presence of furans inhibit cell growth and lipid accumulation

Enzymatic hydrolysis and Saccharification





- **Hydrolysis with Cellic Ctec2®, enzyme loading 15mg/g substra**
- **50ºC, MES bufffer 80mM pH 5.5, 160rpm agitation, 48 hours**

beechwood

pine

Pretreatment conditions	% cellulose	% hemicellulose	% lignin	% solubilization	% cellulose conversion
H_2O/THF (50/50%), O_2 12 bar, 175ºC, 120min	80.11	11.39	5.77	56.39	78.28
H_2O/ACO (50/50%), O_2 25 bar, 175ºC, 120min	80.28	12.26	1.58	67.05	68.52
H_2O/ACO (50/50%), O_2 12 bar, 175ºC, 60min	72.82	16.21	2.86	77.05	74.78

Biomass-derived sugars for the sustainable production of ω-3 fatty acids

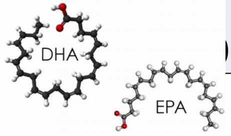
beechwood

pine

Pretreatment conditions	Biomass g/lt	biomass (mg/g consumed)	% TFA	TFA (g/lt)	% DHA	DHA (g/lt)
H_2O/THF (50/50%), O_2 12 bar, 175°C, 120min	7.39	320	44.18	3.27	23.89	0.78
H_2O/ACO (50/50%), O_2 25 bar, 175°C, 120min	7.71	312	38.22	2.95	23.15	0.68
H_2O/ACO (50/50%), O_2 12 bar, 175°C, 60min	7.76	304	33.45	2.60	27.14	0.70
Glucose	9.67	306	43.03	4.16	29.98	1.25
[illegible] 60min	8.72	325	39.06	3.41	26.85	0.91
H_2O/THF (50/50%), O_2 12 bar, 175°C, 60min	7.98	328	35.71	[illegible]	[illegible]	0.75
H_2O/EtOH (50/50%), O_2 16 bar, 175°C, 60min	5.47	294	38.66	[illegible]	[illegible]	[illegible].49

- ✓ *C. cohnii* is able to utilize lignocellulose-derived sugars to efficiently synthesize omega-3 fatty acids

Fatty acids composition
C14
C16
C18:0
C18:1
C22:0 (DHA)



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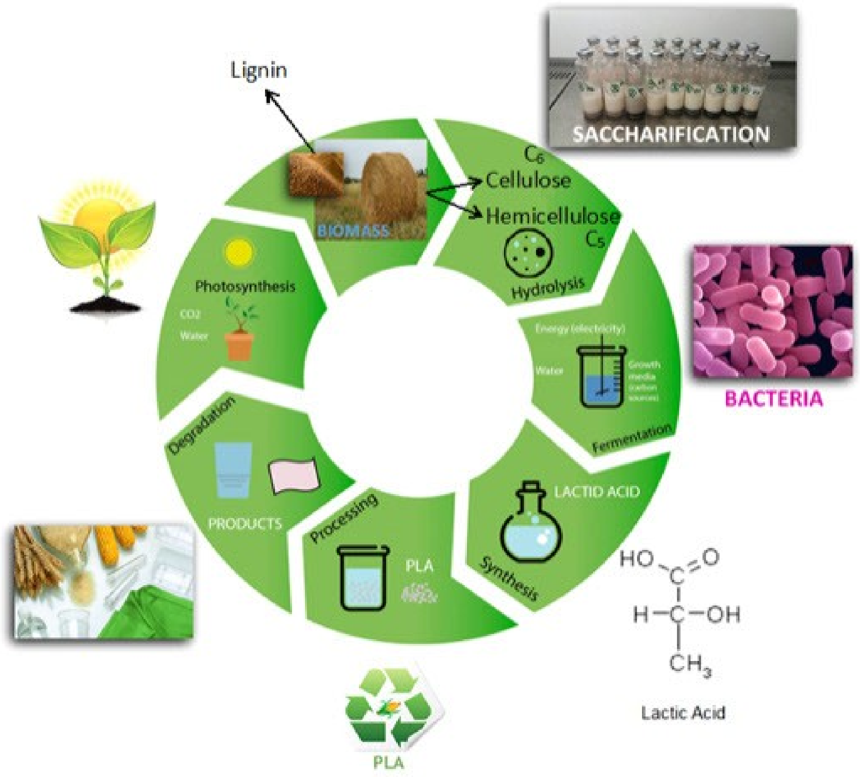
Lactic Acid

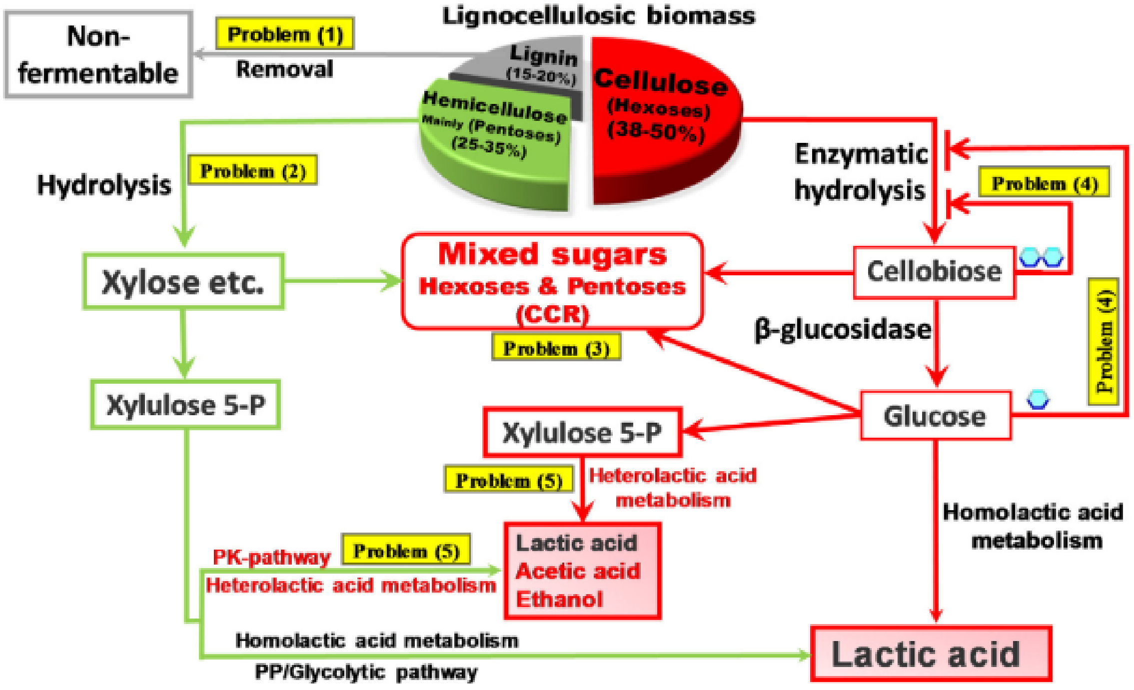
- a valuable chemical platform that has extensive applications: **food**, **cosmetics**, **textiles**, **pharmaceutical**, and **chemical industries**

- The raw substrate materials for LA production constitute 40–70% of the total production cost, which is a challenge for cost- effective LA fermentation

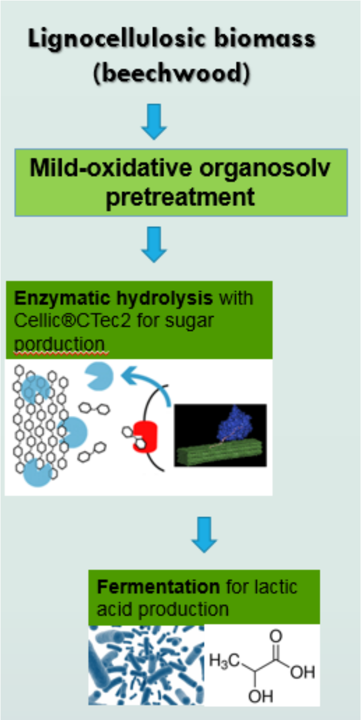
- there has been a growing interest in the use of lignocellulosic was… rials are abundant, rene… gar content without com…

Sustainable production of Lactic Acid from biomass





Lactic acid production by *Lactobacillus delbrueckii*



- *Lactobacillus delbrueckii* was able to utilize glucose, mannose and galactose to achieve high production yields of lactic acid

- During bioconversion, LA accumulation acidifies the fermentation broth, causing inhibition to bacteria metabolism and thus limiting the process efficiency and yield; addition of $CaCO_3$ can ***efficiently alleviate the adverse effects of low pH*** and boost the production of lactic acid.

sugar	***mg lactic/ g sugar (168h)***
glucose	939 ± 19
mannose	855 ± 2
arabinose	-
galactose	903 ± 26
xylose	-

Lactic acid yields produced by utilizing pure sugars (6% w/v) as carbon source

Lactic acid production by utilizing lignocellulosic biomass

LA production from *L. delbrueckii* and SSF using organosolv pretreated biomass at 45oC, 9% initial dry matter, with an enzyme loading of 9mg/ g of biomass, after 168h of fermentation. (*ACO: acetone, EtOH: ethanol, THF: tetrahydrofuran*)

Biomass pretreatment	Cellulose %	Hemicellulose %	Lactic acid (g/lt)	mg lactic acid/ g biomass	% theoretical yield
H_2O/ACO (50/50%), O_2 8 bar, 160°C, 120min	66.77	18,36	77.11	756	79.60 ± 8.16
H_2O/ACO (50/50%), O_2 16 bar, 160°C, 120min	76.63	13.32	61.07	678	67.67 ± 2.32
H_2O/ACO (50/50%), O_2 8 bar, 175°C, 120min	82.3	13.9	50.5	561	52.36 ± 1.01
H_2O/ACO (50/50%), O_2 16 bar, 175°C, 30min	79.74	15.69	64.56	717	67.43 ± 2.87
H_2O/EtOH (50/50%), O_2 16 bar, 160°C, 120min	72.96	16.03	52.5	583	58.72 ± 4.98
H_2O/EtOH (50/50%), O_2 16 bar, 175°C, 60min	81.28	13.99	67.1	745	70.21 ± 0.91
H_2O/THF (50/50%), O_2 16 bar, 150°C, 120min	73.09	13.3	78.93	837	86.97 ± 6.7
H_2O/ THF (50/50%), O_2 16 bar, 160°C, 120min	79.13	12.1	85.7	912	89.78 ± 10.1
H_2O/THF (50/50%), O_2 16 bar, 160°C, 60min	68.99	15.55	72.96	785	83.29 ± 1.14
H_2O/ THF (50/50%), O_2 16 bar, 175°C, 60min	85.28	10.82	53.23	591	55.25 ± 0.42

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Conclusions – Future work

- Lignocellulosic biomass can be used for the production of value-added products, such as omega-3 fatty acids and lactic acid, through ***environmentally friendly bioconversion processes***.
- ***Wet oxidation in presence of organic solvents*** is an efficient pretreatment method for the fractionation of lignocellulosic biomass.
- Fermentation of microalgae needs to be optimized to maximize the accumulation of fatty acids and boost the mechanism of DHA synthesis (fermentation strategy, effect of C/N ratio ect.)
- Utilization of pretreatment liquid fraction in order ***to achieve a zero-waste, holistic approach*** for the valorization of lignocellulosic biomass waste streams.

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Thank you for your attention!