MACROFUELS

MacroFuels

Task 2.3 Enzymatic hydrolysis of macroalgal polysaccharides

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2nd Project Meeting – 10th January 2017 Wageningen

TEKNOLOGISK INSTITUT · ECN · FOOD & BIOBASED RESEARCH WAGENINGEN UR · avantium · SAMS · SIOEN INDUSTRIES

ERM · AARHUS UNIVERSITY DEPARTMENT OF BIOSCIENCE · EURIDA Research Management · matís · FERMENTATIONEXPERTS AS –for your health

Task 2.2Storage and pre-treatment by biological and chemical ensiling (DTI; FERMENTATION-EXPERTS AS, MATIS OHF; M6-44)

Task 2.2.3 **Biological pre-treatment** (MATIS OHF, DTI)

Robust and efficient alginate degrading bacteria strains will be constructed by MATIS OHF for use in the pre-treatment of seaweed prior to biofuel production (WP3) and to instigate alginate hydrolysis during storage and the downstream pre-treatment steps. Three different microbial systems will be established and examined for this purpose. They are based on *Bacillus subtilis*, *Lactobacillus sp.*, and the thermophilic marine bacterium *Rhodothermus marinus*. The organisms will be optimized for maximum sugar liberation and a minimal viscosity.

Biomass used

- In-house (for method development)
- from SAMS/DTI
  - to be analyzed for quality
  - for methodology comparisons between groups
  - For fuel generation

Recombinant enzymes

Recombinant enzymes

- For analysis and degradation
- Brown alagae: *Saccharina latissima*
  - Laminarinases
    - 939 & Amo176
  - Alginate lyases
    - Alg1-4.
- Red algae: *Palamaria palmata*
    - Xylanases

Production status of recombinant enzymes

Enzyme	Substrate	System	Date due	Application	Volume	Cell yield
Alginase 3	Alginate	*E. coli*	March.16	For development of quantitative assay	**10**	
939 Laminarinase	Laminarin	*E. coli*	March.16	For development of quantitative assay	~~**10**~~	
Pustulanase	Laminarin	*E. coli*	March.16	For development of quantitative assay	**5**	
Alginase 4	Alginase	*E. coli*	Mai 2016	For development of quantitative assay	**10**	
Alginase 3	Alginate	*E. coli*	Jun-16	Preprocessing	**100**	**4 kg**
Alginase 4	Alginate	*E. coli*	Jun-16	Preprocessing	**100**	**5 kg**
939 laminarinase	Laminarin	*E. coli*	Jun-16	Preprocessing	~~**30**~~	
Sulfatase	Fucoidan/ Galactan	*E. coli*	sep.16	Preprocessing	~~**10**~~	

Laminarin (ases)

For Brown algae: Saccarhina

MATIS

Laminarinases:

(**Lam1:** active on 1.3; **Lam2** active on 1,3 and 1,6 linkages:

&

Laminaribiosidase:

Highly thermostable: Active on **cellobiose** and **laminari-biose**

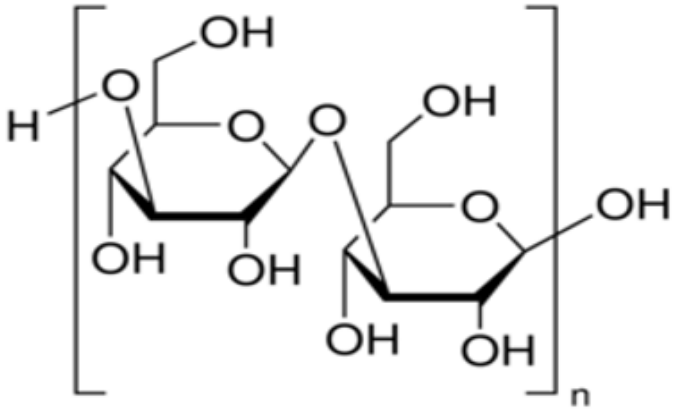
Lam1:

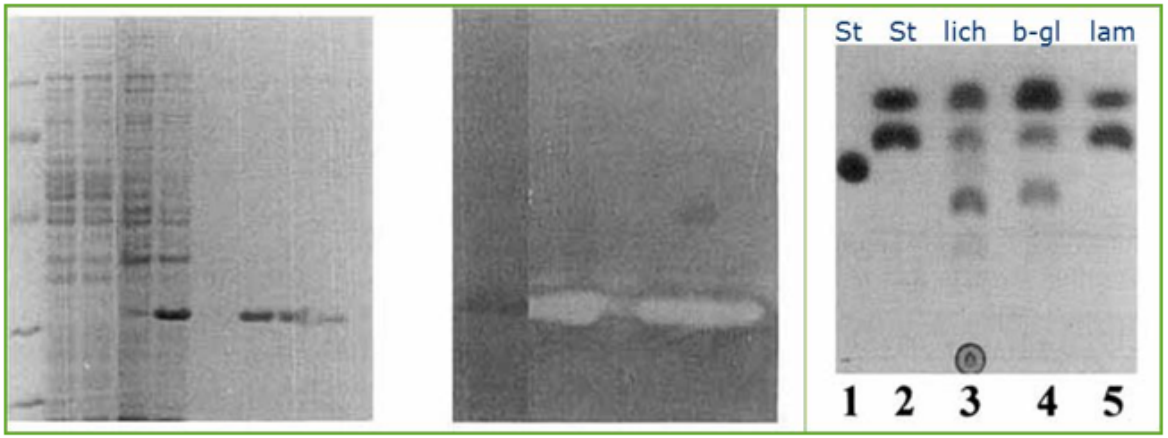
Expression:
20-30% of the total cell protein.

Degree of hydrolysis (16hrs):
74% barley beta-glucan,
55% lichenan
83% laminarin

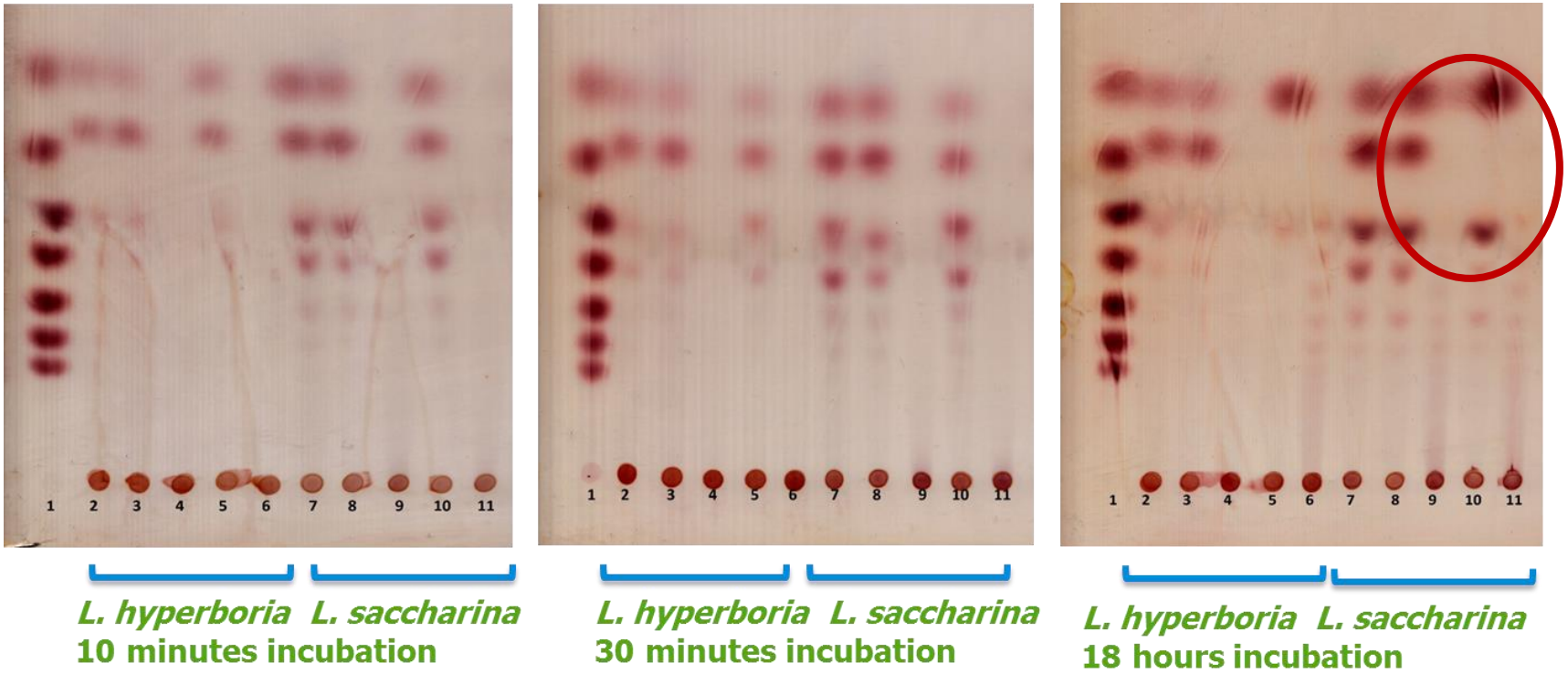
Optima: pH 7, 80°C

Stability:
Retained 100% of its activity after incubation at 80°C for 16 hrs





Laminarinases: 939 & Amo176



- Incomplete degradation- Products: Glucose, laminaribiose, and oligosacharides
- Product inhibition?
- Add a pustulanase (Cel136) and/or laminari-exoenzymes (GH3 or GH1)?
- **Enzyme cocktail for complete hydrolysis?**

Alginate (lyases)

For Brown algae: Saccarhina

Alginate utilization

Alginate lyases

Depolymerize alginate through a β-elimination

- Form a double bond between the C4 and C5 carbons
- Giving an unsaturated non-reducing terminal residue

Matis has identified and characterized the **first thermostable alginate lyases** (T_{opt}>60°C)

- Four different enzymes
- Endo and exo activity
- Different specificities towards mannronate, guluronate or mixed blocks
- Thermostability > 16 h at 60°C
- Combination of two enzymes (AlyRm3 and AlyRm4) gives complete conversion (99%) of alginic acid into unsaturated monosugars

Patent pending

Task 2.3.1 Development and test of robust of thermophilic and marine origins

MACROFUELS

- Alginases

Enzyme	AlyRm1	AlyRm1ΔC	AlyRm2ΔN	AlyRm2ΔNC	AlyRm3	AlyRm4
T opt (°C)	87	68	81	81	75	81
T stability (enzyme half-life)[1]	5 h at 70°C >16 h at 60°C	12 h at 60°C	16 h at 70°C >16 h at 60°C	>16 h at 70°C	8 h at 70°C >16 h at 60°C	>16 h at 70°C
pH opt	7.2	8.0	6.5	6.5	5.5	6.5
NaCl opt.[2] (mM) Up to 2.1%	0-600	200-1000 Up to 3.5%	0-800 Up to 2.8 %	0-800 Up to 2.8 %	0-800 Up to 2.8 %	0-600 Up to 2.1%
Enzyme type	Endo & Exo	Endo & Exo	nd	Endo	Endo	Exo
Major activity	M-G	M-G, G-G	nd	M-G	All	M-M
Minor activity	G-G, M-M	M-M	nd	M-M		G-G

Combination of AlyRm3 and AlyRm4 gives near complete conversion (99%) of alginic acid into unsaturated monosugars

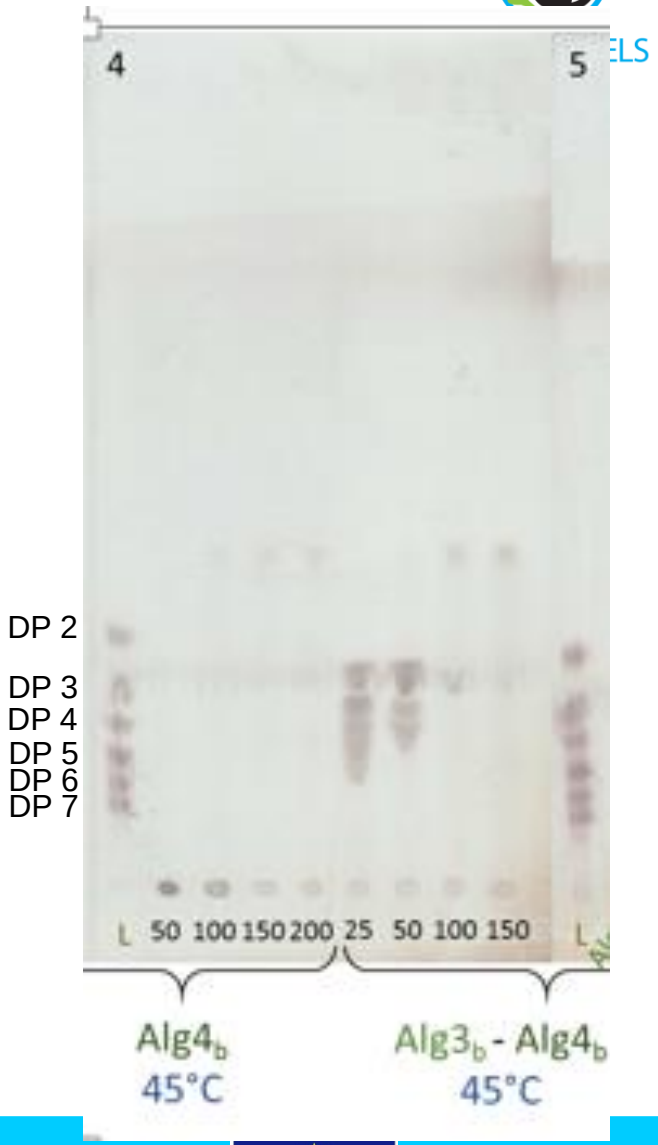
TLC – Degradation of Alginic acid 1% Low viscosity by Alg3 and Alg4

Migration with Butanol, Acid Acetic and H_2O as solvent

Development using Sulfuric Acid and Ethanol (*Da Mao Wang, 2014*) for detection of monosaccharides

Volume of enzyme from 25µl to 200µl in 800µl of alginic acid.

Hydrolysation made for 48h at 45°C



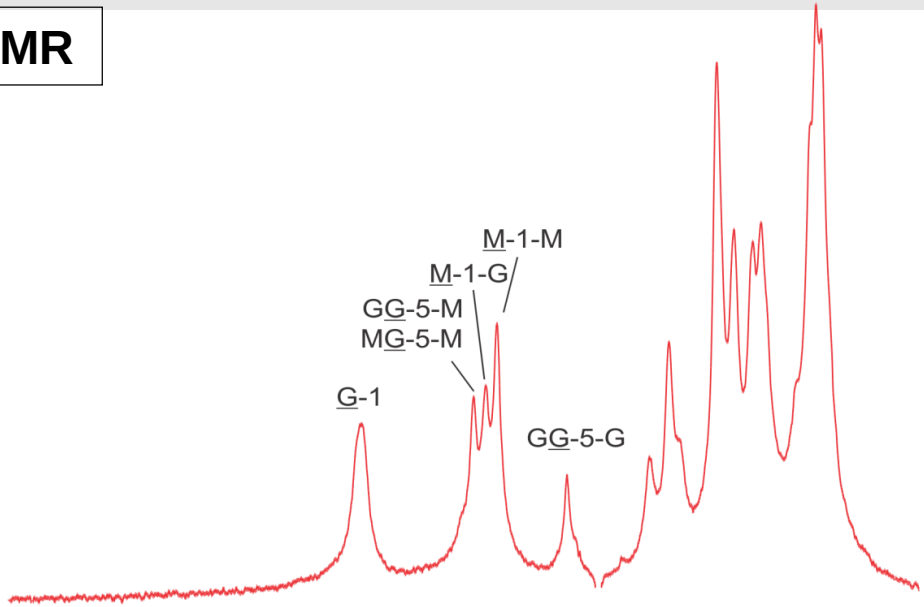
DP = Degree of polymerization
L = Laminarin Ladder (2 to 7 sugars)
S = Substrat

$Alg3_b$ - $Alg4_b$ = batch from December 2016

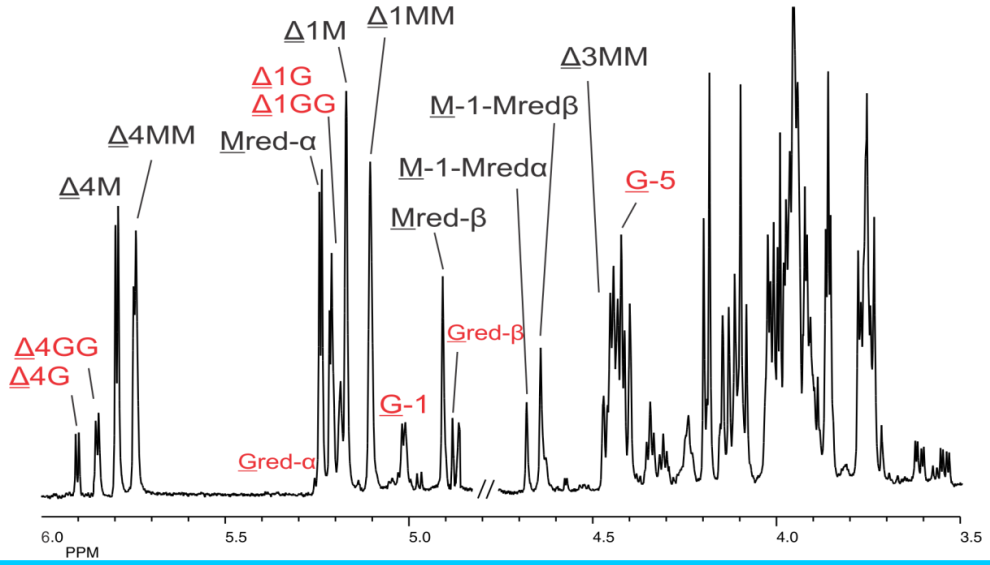
Activity of Alg3 on alginic acid

1D ^{1}H NMR

0h



24h



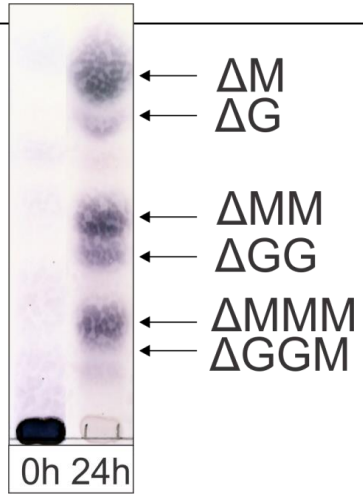
Enzyme incubations:

80µL 12,5mg/mL Alginic acid

10µL 0,5M phosphate buffer pH 5.5

10µL Enzyme (10U/mL)

T 60 °C



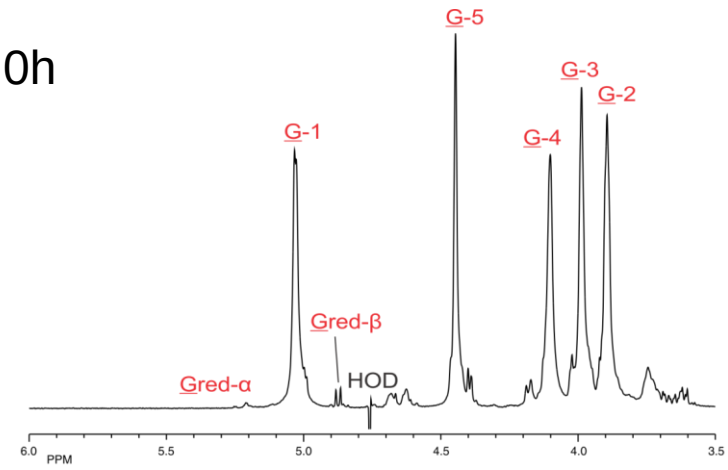
- G-block is gone
- MG-block is gone
- M-block is gone

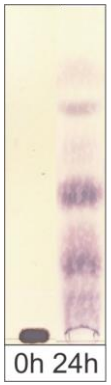
Activity of Alg3 on G-block and on M-block

1D ^{1}H NMR

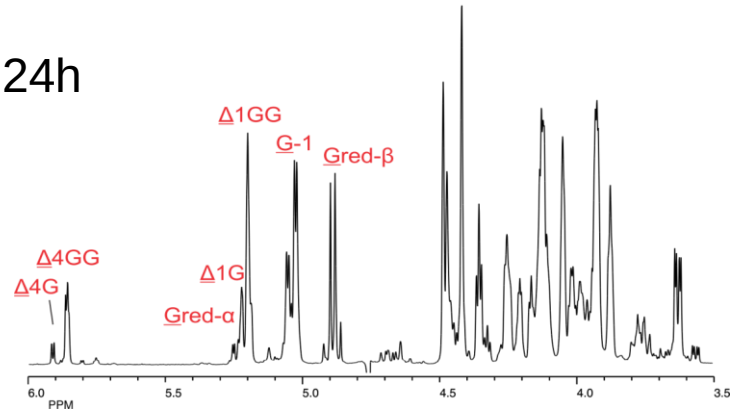
G-Block

0h



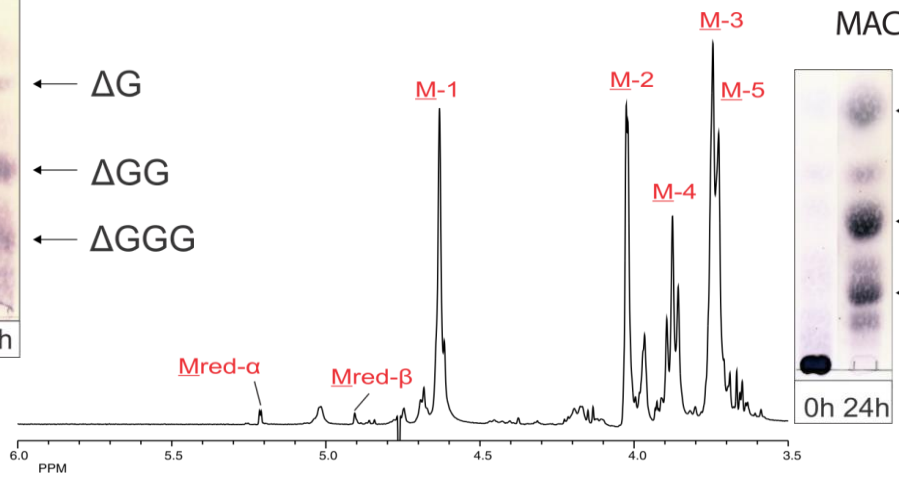


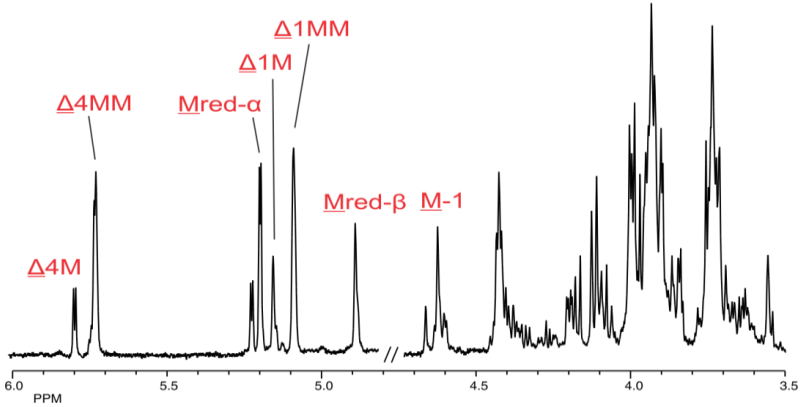
24h



- high activity on G-G bonds
- endolytic activity

M-Block

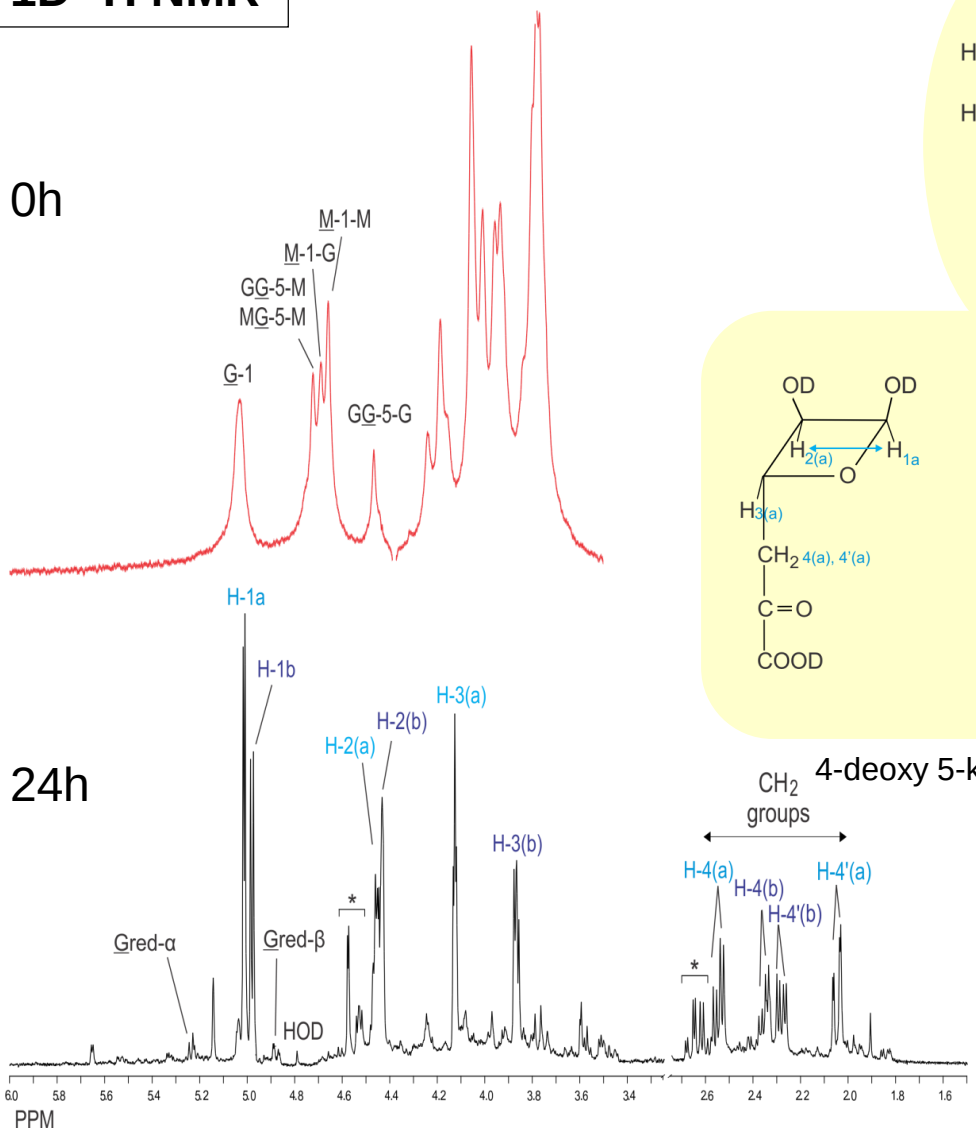


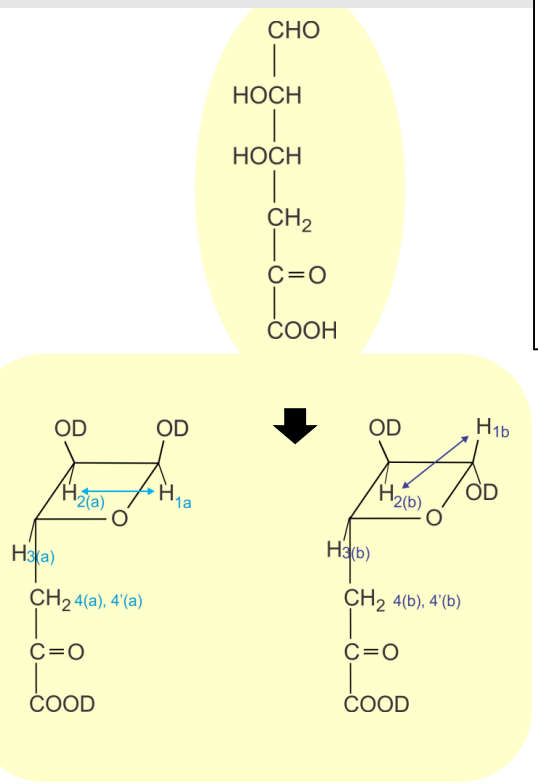


- high activity on M-M bonds
- endolytic activity

Activity of Alg4 on alginic acid

1D ^{1}H NMR





4-deoxy 5-keto uronic acid

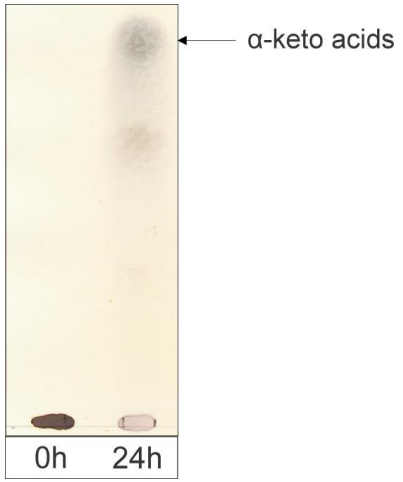
Enzyme incubations:

80µL 12,5mg/mL Alginic acid

10µL 0,5M phosphate buffer pH 5.5

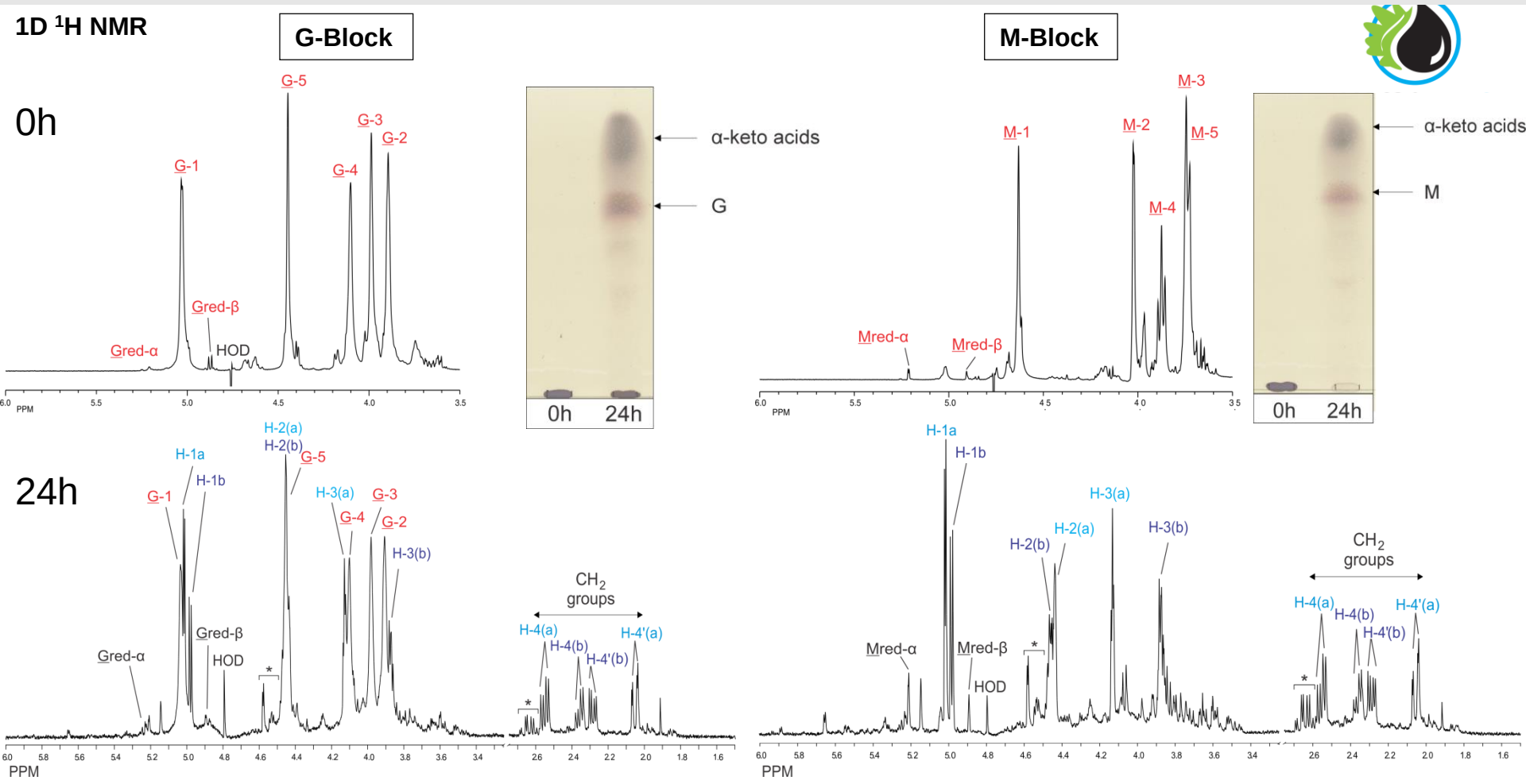
10µL Enzyme (10U/mL)

T 45 °C



- G-block is gone
- MG-block is gone
- M-block is gone
- (mainly) exolytic activity (2.75-2.0ppm)
- 4-deoxy 5-keto uronic acid present

Activity of Alg4 on G-block and on M-block

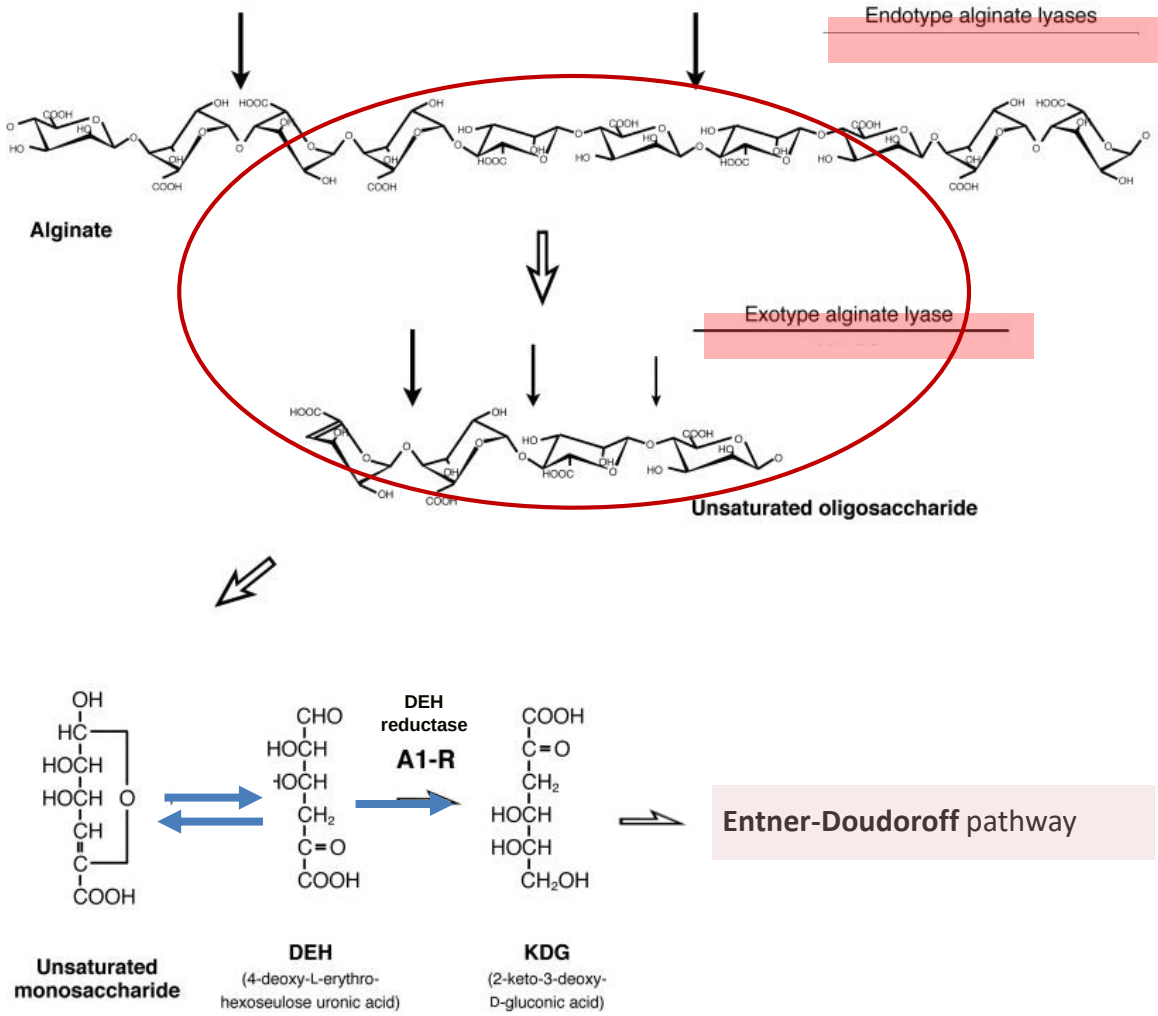


- high activity on G-G bonds
- exolytic activity
- 4-deoxy 5-keto uronic acid present

- high activity on M-M bonds
- exolytic activity
- (mainly) 4-deoxy 5-keto uronic acid present

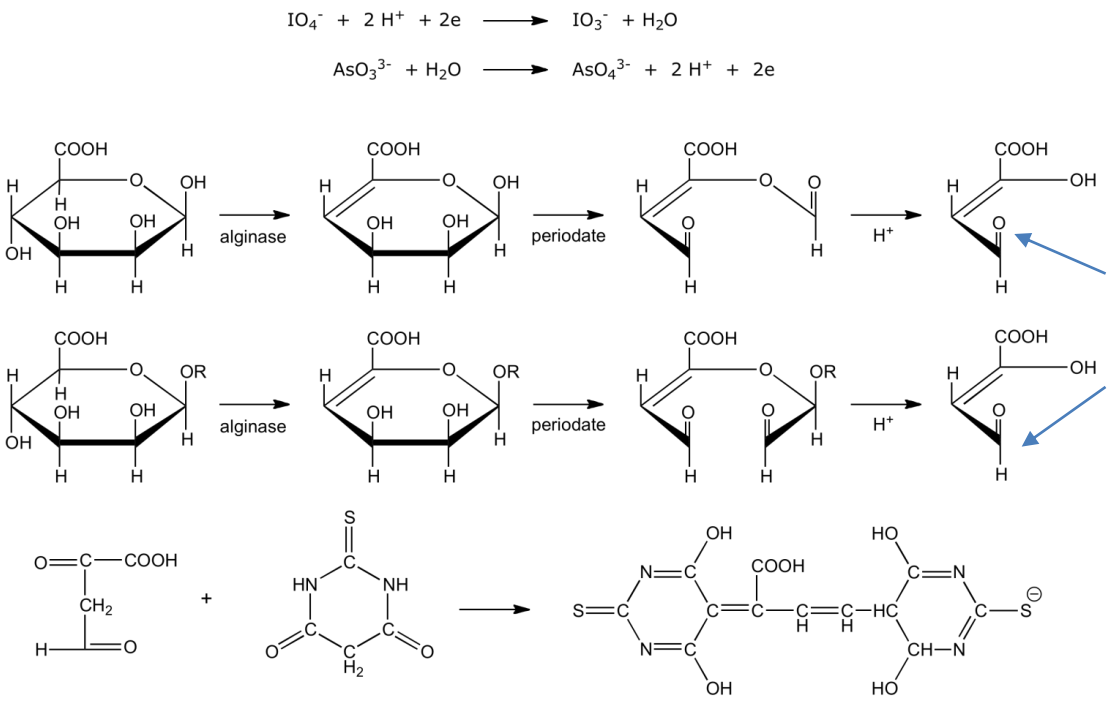
Assey development

Alginate lyases

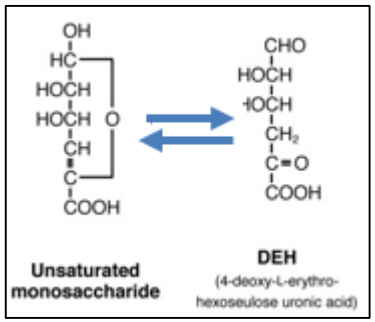


Adapted from Takase *et al.,* 2010. Biochim Biophys Acta; 1807(9): 1925-36

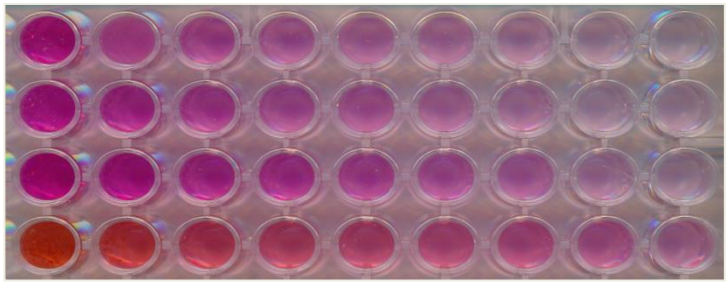
Determination of the concentration of unsaturated alginate products



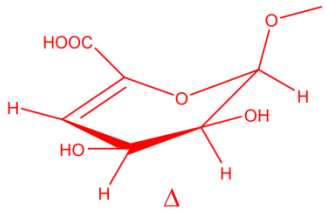
β-formylpyruvate

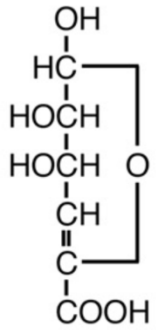


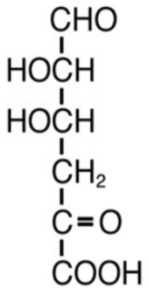
0.01 µmol β-formylpyruvate gives optical density reading of 0.29 at 548 nm [ε = 2.9 x 10^4 $M^{-1}cm^{-1}$])

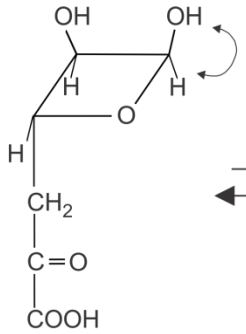


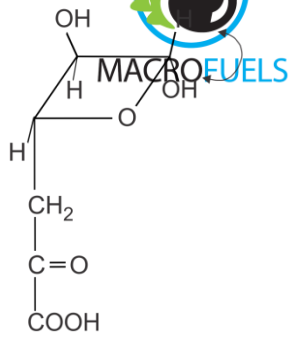
Influence of high temperature and pH on unsaturated monomer



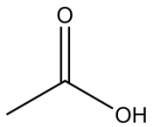






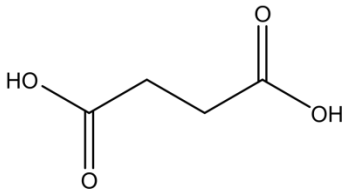


Monocarboxylic acids



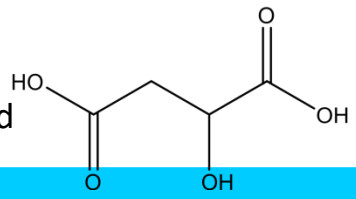
acetic acid

Dicarboxylic acids



succinic acid

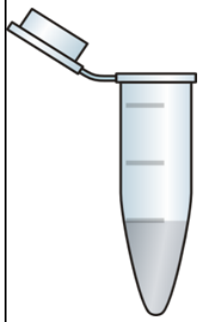
malic acid



Signals at 7.4 ppm and 8.6 ppm suggest the presence of aromatic compunds

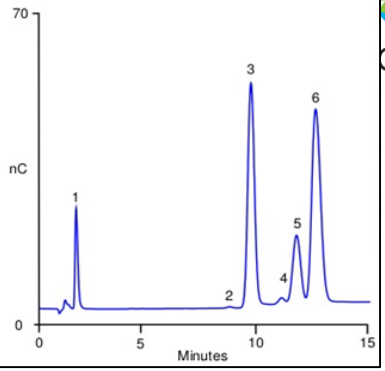
dark brown colour

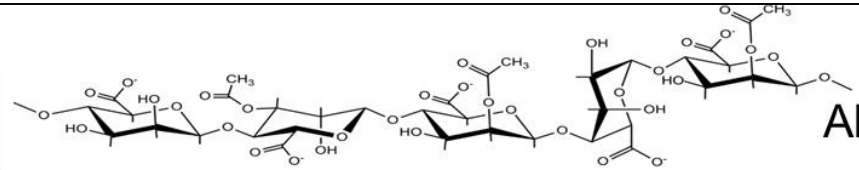
Alginate quantitative analysis



Sample with alginate
+ alginases (Alg3+Alg4)
+ reductase

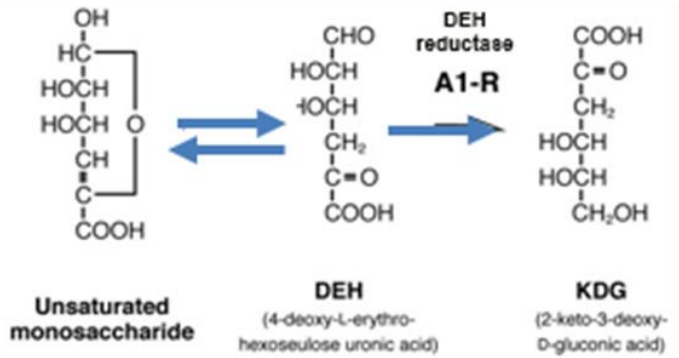
Incubate at 50°C for 6 hours – run sample on HPAEC – PA20

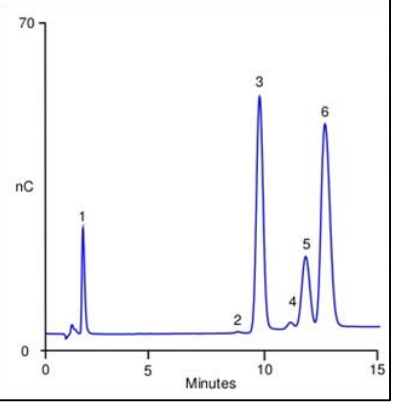




Alginate

(Alg3+Alg

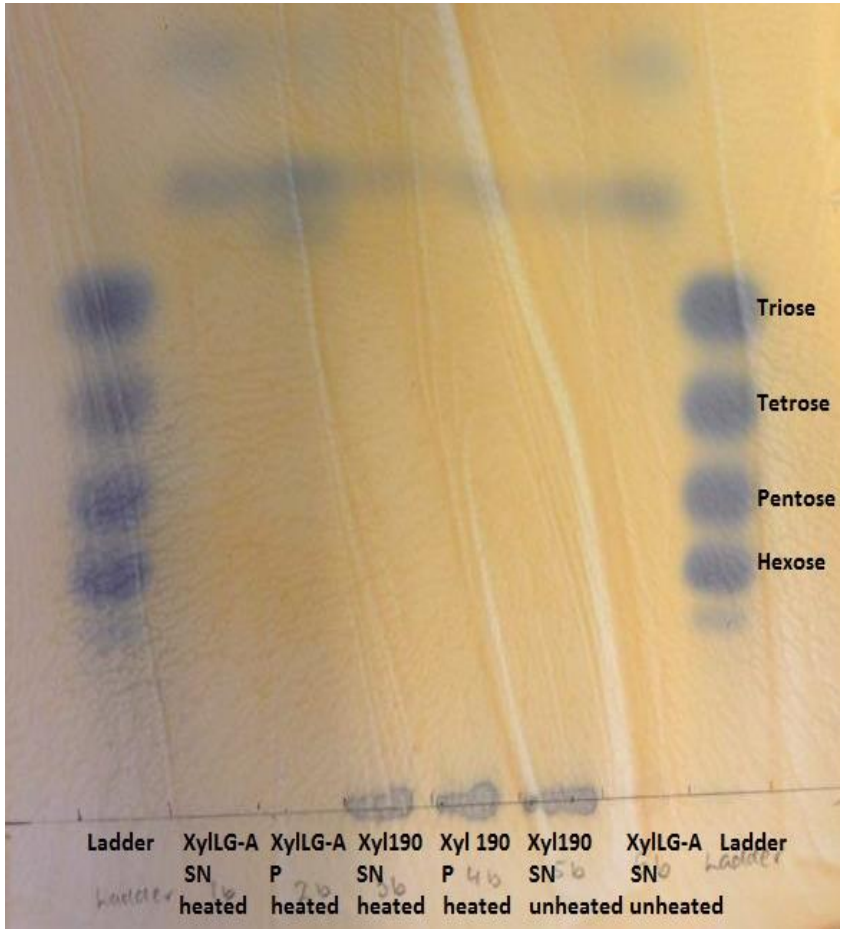




Red algae: Palmaria

Xylanase

- Number of enzymes were screended for activity on xylan from Palmaria
- An efficient thermostable enzyme has been idenified that works well on crude Palmaria lysates
- Forms xylose and xylobiose
- Enzyme cocktail needed for complete degradation to xylose

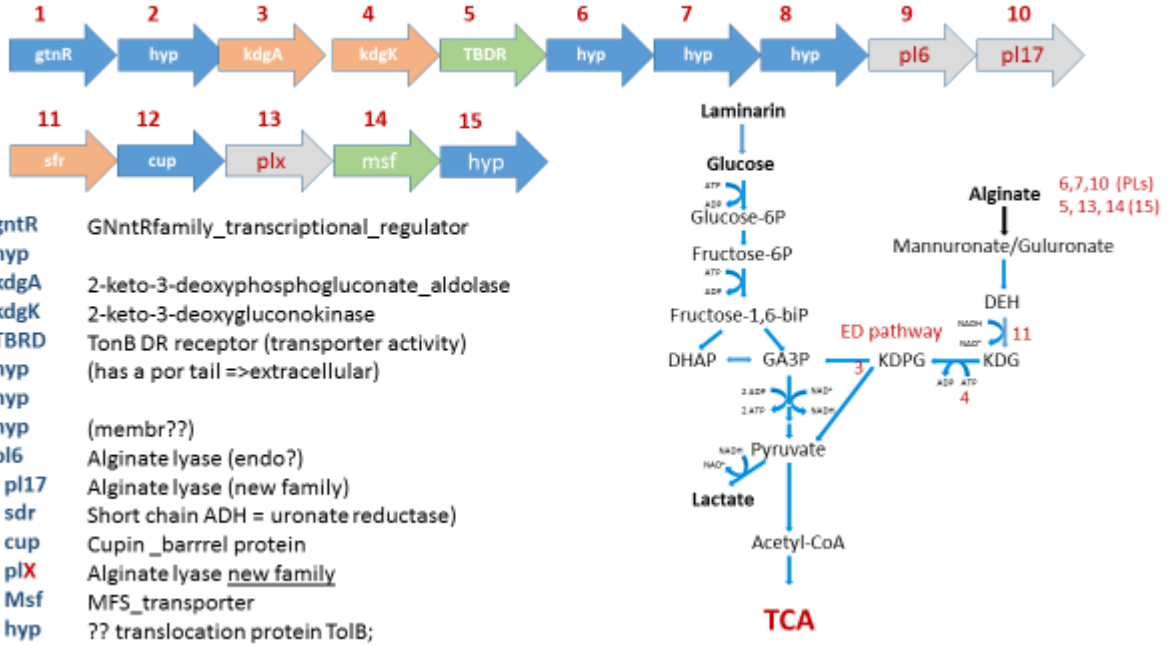


Task 2.2.3 **Biological pre-treatment**

"Preprocessing microbes"

- Srains will e develope that can hydrolyse alginate growing on („fermenting „) seaweeds
  - *Rhodothermus marinus* (aerobic)
  - *Bacillus subtilis* (aerobic)
  - *Lactobacillus reuterii* (anaerobic)

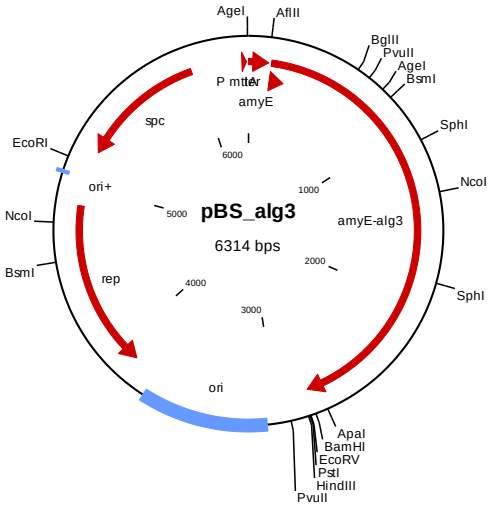
R. marinus



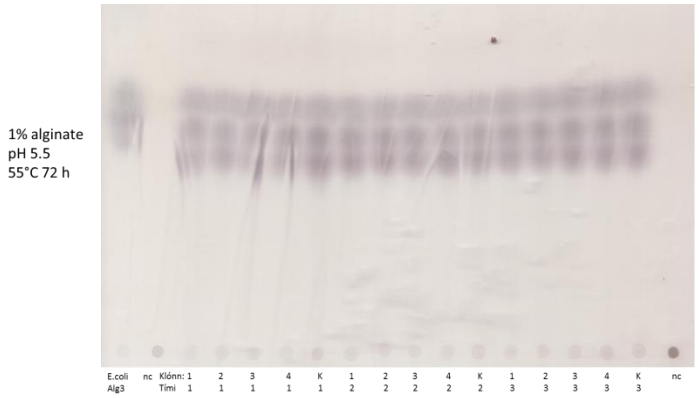
1. gntR	GNntRfamily_transcriptional_regulator
2. hyp	
3. kdgA	2-keto-3-deoxyphosphogluconate_aldolase
4. kdgK	2-keto-3-deoxygluconokinase
5. TBRD	TonB DR receptor (transporter activity)
6. hyp	(has a por tail =>extracellular)
7. hyp	
8. hyp	(membr??)
9. pl6	Alginate lyase (endo?)
10. pl17	Alginate lyase (new family)
11. sdr	Short chain ADH = uronate reductase)
12. cup	Cupin _barrrel protein
13. plX	Alginate lyase new family
14. Msf	MFS_transporter
15. hyp	?? translocation protein TolB;

B. subtilis

- Alginate degrading bacteria strains
  - *Bacillus subtilis* – recombinant (mannitol induction)



Alginase activity of *Bacillus* clones



L. reuterii

- Alginate lyase(s) with endo-activity will be cloned and expressed in L. reuterii. The strani will be able to grow on mannitol, express and cleave alginate, thereby decreasing viscosity pof the seaweed biomass at later stages

- Work starts in February

Plan next 6 months:

- Finish development of an assay for alginate quantification and to measure extent of degradation by alginae lyases
- Evaluate the efficency of enzymes for degradation of alginate
  - purfied
  - & in crude seaweed biomass
- Finish development of laminarin assay
  - Enzyme cocktail
- Evaluate the efficency of enzyme cocktail for degradation of laminarin
  - purfied
  - & in crude seaweed biomass

- Develop a xylanase assay
  - Enzyme cocktail
- Evaluate the xylanase cocktail for degradation of laminarin
  - purfied
  - & in crude sea
  - weed biomass

- Produce enzymes for scaled up enzyme hdyrolysis

Acknowledgements

Biotechnology, Matis, Iceland
- Olafur H. Fridjonsson
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- Solveig Olafsdottir
- Lilja Björk Jónsdóttir
- Justine Vanhalst
- Antoine Moenaert

Lund University
- Eva Nordberg-Karlsson

Universities of Groningen Utrecht
- Hans Kamerling
- Justyna Dobrochovka

Universities of Stuttgart
- Josef Altenbuchner
- Hildegard Watzlawick

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Composition from ECN in SAMS samples

Seaweed	Supplier	Glc	Xyl	Gal	Fuc	Rham	Glycerol	Mannitol	Gal-A	Gul-A	Glc-A	Man-A	Idu-A	Protein	SO_4	Ash
Gracilaria [a]	AU (July)	8.5[a]	1.2	24.1[a]	2.2		2.1			[a]	[a]	[a]		9.2	9.0	25.7
Fucus	AU (July)	5.6	1.2	1.1	9.9			6.5	0.3	2.6	2.3	3.2		6.6	7.8	20.4
Ulva (fresh)	SAMS (Aug)	10.6	2.5	0.4		4.7					2.0		0.8	8.4	14.5	39.2
Ulva (air-dried)	SAMS (Aug)	11.5	3.0	0.4		6.2					2.6		ND	10.6	16.4	34.9
Palmaria (fresh)	SAMS (Aug)	5.5	32.7	11.5	0.3		6.4							12.8	2.3	22.6
Palmaria (air-dried)	SAMS (Aug)	4.1	31.1	15.1	0.4		7.4							12.3	2.1	22.0
Saccharina (fresh)[b]	SAMS (Aug)	4.5	0.4	0.7	1.4	0.5		1.4		3.1	1.0	5.1		13.1	3.7	47.1
Saccharina (air-dried)[b]	SAMS (Aug)	4.8	0.4	0.7	1.5	0.3		6.5		3.6	1.0	6.2		13.3	3.8	43.0

a. Analytical protocol applied for carbohydrates needs to be further optimized / adjusted for Gracilaria. First, too large duplicate differences were found for galactose and glucose. Second, there are two significant unknown peaks in Gracilaria Dionex spectrum. Probably galactose varieties (methyl galactose, anhydrous galactose, ...) that are part of agar. Third, strongly varying contents for uronic acids were identified, albeit low.

b. The high ash content in addition to the low content of mannitol and reducing carbohydrates of Saccharina reflect its bad condition (especially of the fresh material).

Icelandic samples

- Season important: September in Iceland best for Saccharina
- Glucose comparable – reported in litt. up to 18% dW
- Ash always high, from 25%-40%