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Microaerobic fermentation of algae biomass residuals for mixed alcohol production and nutrient recycling

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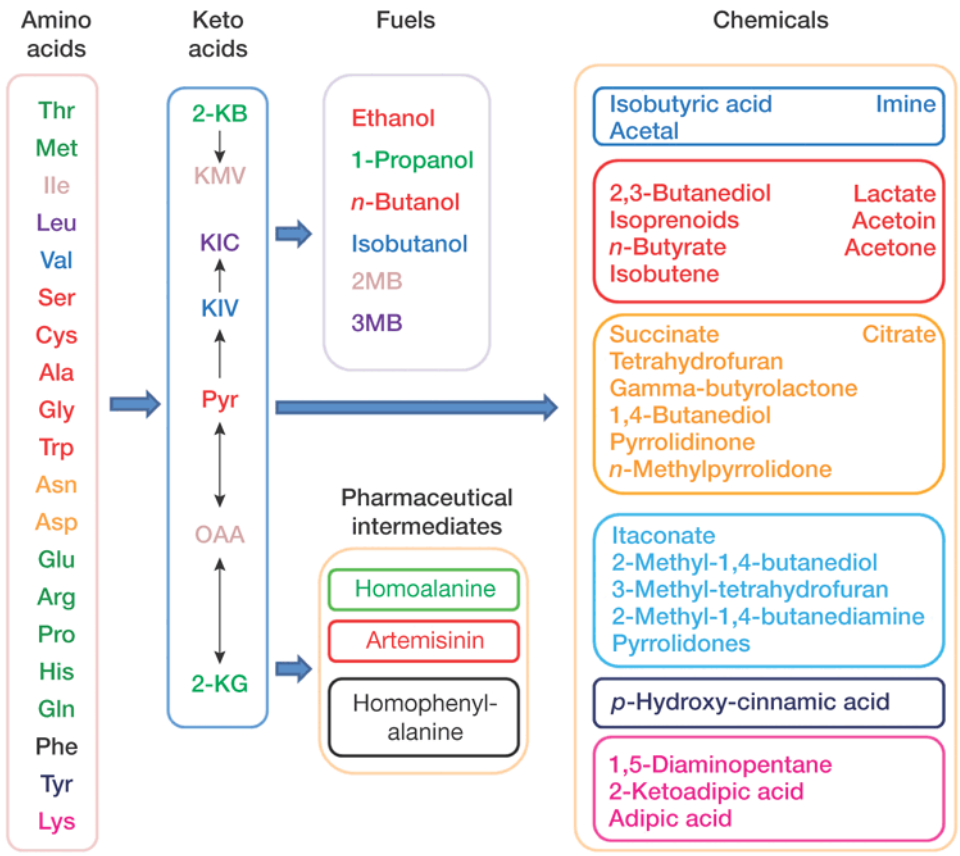
Conversion of amino acids to fuels, industrial chemicals, and pharmaceuticals

Chemical composition of algae

Strain	Protein	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Scenedesmus quadricauda	47	-	1.9	-
Scenedesmus dimorphus	8-18	21-52	16-40	-
Chlamydomonas rheinhardii	48	17	21	-
Chlorella vulgaris	51-58	12-17	14-22	4-5
Chlorella pyrenoidosa	57	26	2	-
Spirogyra sp.	6-20	33-64	11-21	-
Dunaliella bioculata	49	4	8	-
Dunaliella salina	57	32	6	-
Euglena gracilis	39-61	14-18	14-20	-
Prymnesium parvum	28-45	25-33	22-38	1-2
Tetraselmis maculata	52	15	3	-
Porphyridium cruentum	28-39	40-57	9-14	-
Spirulina platensis	46-63	8-14	4--9	2-5
Spirulina maxima	60-71	13-16	6-7	3-4.5
Synechoccus sp.	63	15	11	5
Anabaena cylindrica	43-56	25-30	4-7	-

Source: Becker, (1994)



Consolidated Bioprocessing of Algae Biomass Residuals

Algae biomass is composed of roughly equal fractions of lipids, carbohydrates, and proteins

- Protein content is significantly higher than most terrestrial & multicellular organisms
- Protein compositional variation is low

Conversion of proteins to alcohols shows promise for increasing fuel yields, with significant potential for side benefits, including boosting octane and reducing particulate emissions via fuels blending

Ehrlich Mechanism

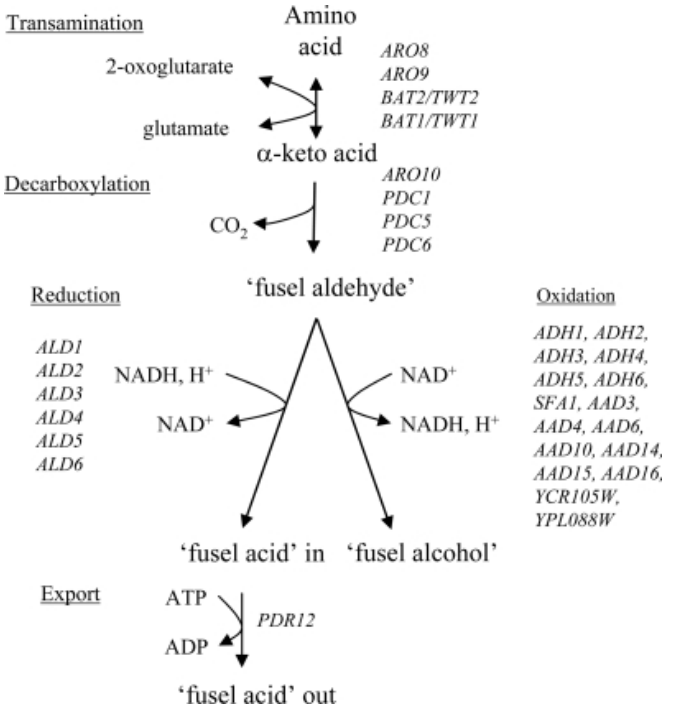
Pathway identified in *Saccharomyces* supporting amino acid metabolism

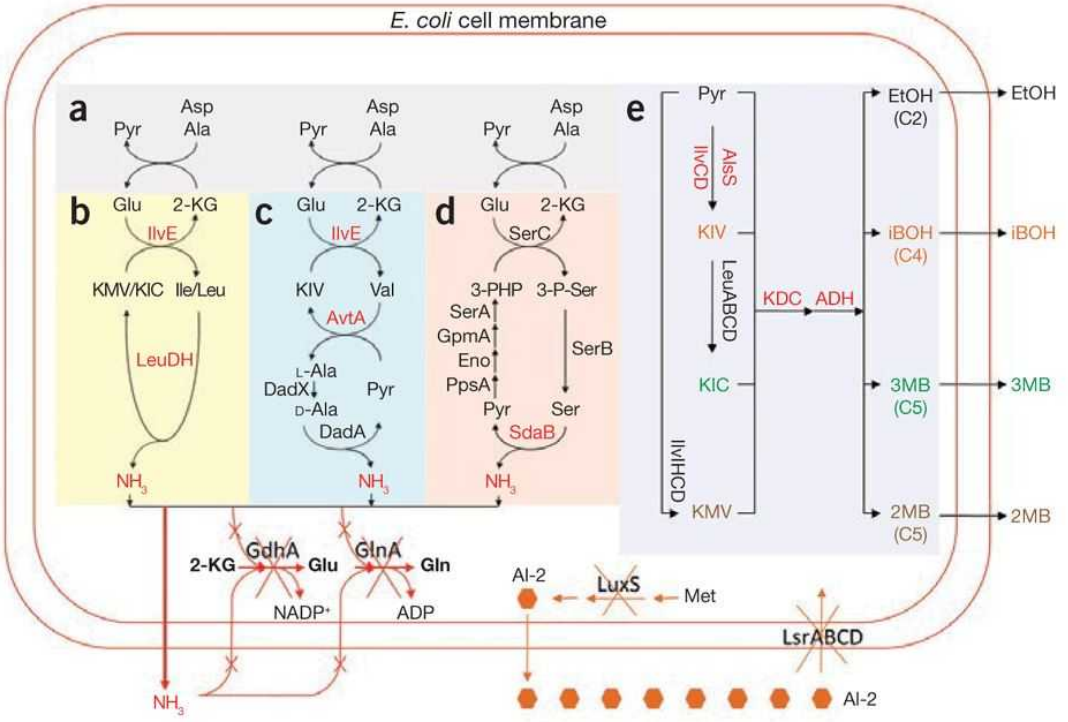
- Low yield, slow kinetics
- Inhibited by presence of fermentable sugars, etc.

Ehrlich pathway intermediates[a] (from *Hazelwood, et al. Appl Environ Microbiol 2008)*

Amino acid	α-Keto acid		Fusel alcohol	
	Systematic	Traditional	Systematic	Traditional
Leu	4-Methyl-2-oxo-pentanoate	α-Ketoisocaproate	3-Methylbutanol	Isoamyl alcohol
Val	3-Methyl-2-oxo-butanoate	α-Ketoisovalerate	2-Methylpropanol	Isobutanol
Ile	3-Methyl-2-oxo-pentanoate	α-Ketomethylvalerate	2-Methylbutanol	Active amyl alcohol
Phe	3-Phenyl-2-oxo-propanoate	Phenylpyruvate	2-Phenylethanol	
Tyr	3-(4-Hydroxyphenyl) 2-oxopropanoate	*p*-Hydroxyphenylpyruvate	2-(4-Hydroxyphenyl) ethanol	*p*-Hydroxyphenylethanol or tyrosol
Trp	3-(Indol-3-yl)-2-oxopropanoate	3-Indole pyruvate	2-(Indol-3-yl) ethanol	Tryptophol
Met	4-Methylthio-2-oxobutanoate	α-Keto-γ-(methylthio) butyrate	3-(Methylthio) propanol	Methionol

Biochemical pathway & metabolic engineering





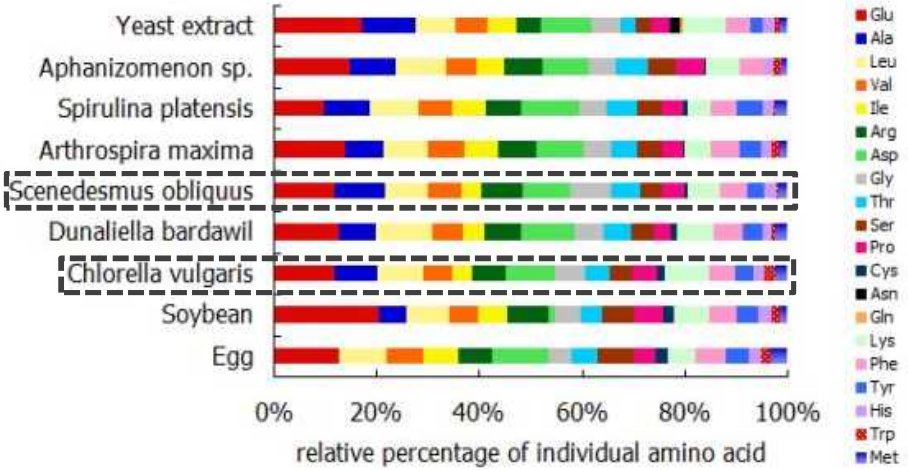
Pretreatment and fermentation conditions matrix

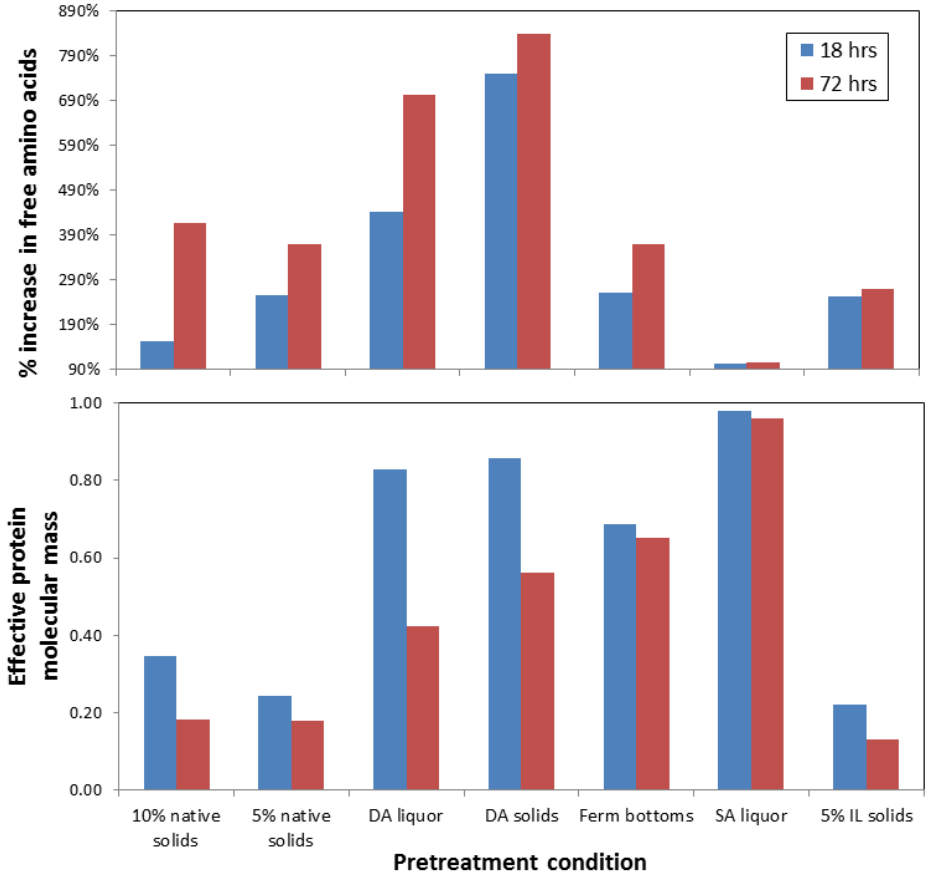
	Native SD 10% solids	Native SD 5% solids	DA SD liquor	DA SD solids	DA CZ solids	DA CZ liquor	SA SD liquor	DA, distilled light ferm bottoms	DA, distilled heavy ferm bottoms	IL prot-carb CZ
+ pronase	X	X	X	X	X	X	X	X	X	X
- pronase	X	X	X	X	X	X	X	X	X	X
+ IPTG	X	X	X	X	X	X	X	X	X	X
- IPTG					X	X				
pH 7	X	X	X	X	X	X	X	X	X	X
pH 5.2									X	
+ vitamins					X	X				

SD = *Scenedesmus sp.*
CZ = *Chlorella sp.*
DA = dilute acid hydrolysis
SA = strong acid hydrolysis
IL prot-carb = ionic liquid extracted protein/carbohydrate fraction

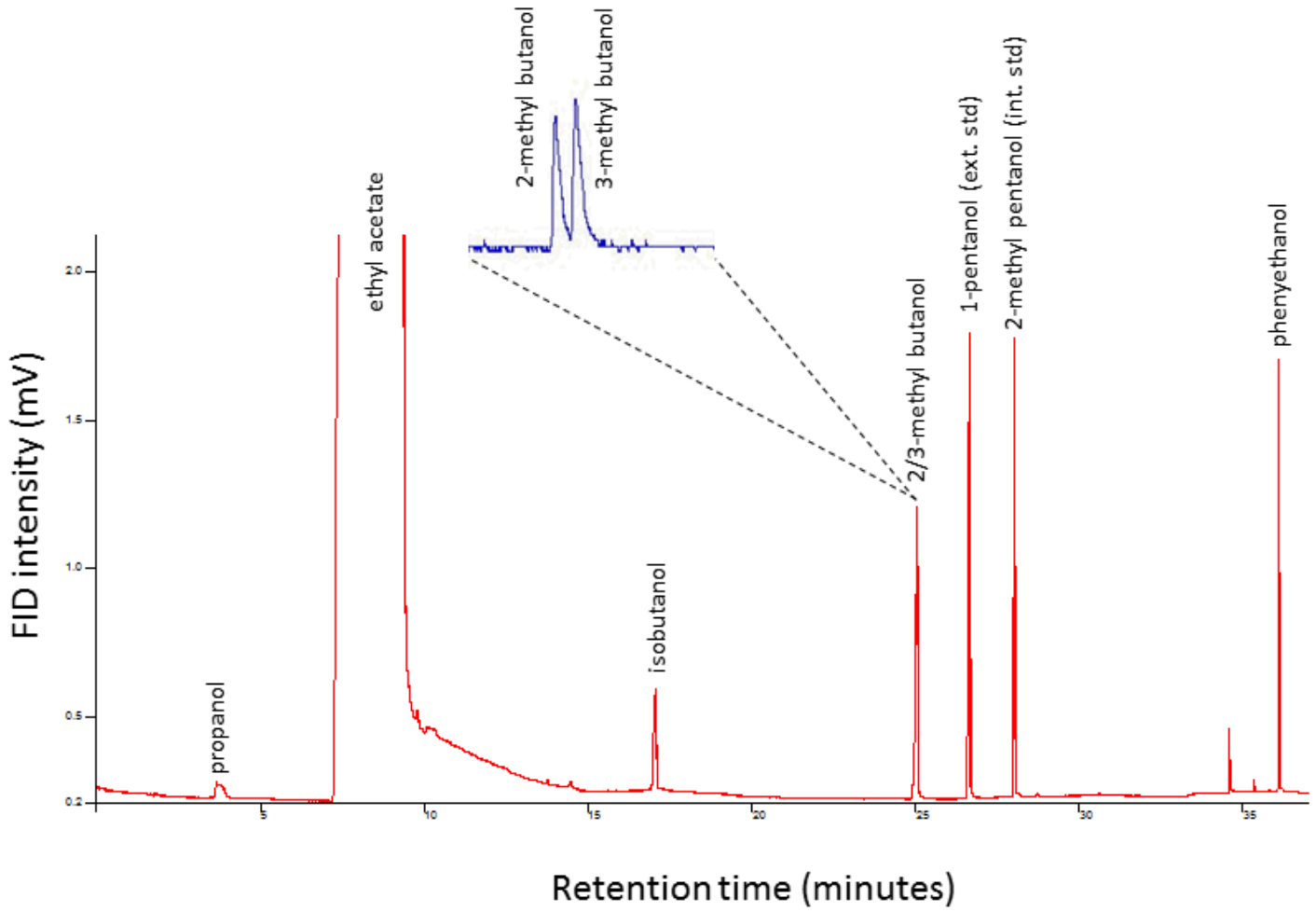
Hydrolysis of *Scenedesmus* and *Chlorella* biomass

- Dilute acid hydrolysis converts majority of carbohydrates to glucose (~90%)
- Proteins are only ~40% hydrolyzed to amino acids
- Strong preference for amino acids for conversion to mixed alcohols
- Enzymatic (pronase) digestion yields ~80% protein hydrolysis





Chromatographic analysis of alcohol production

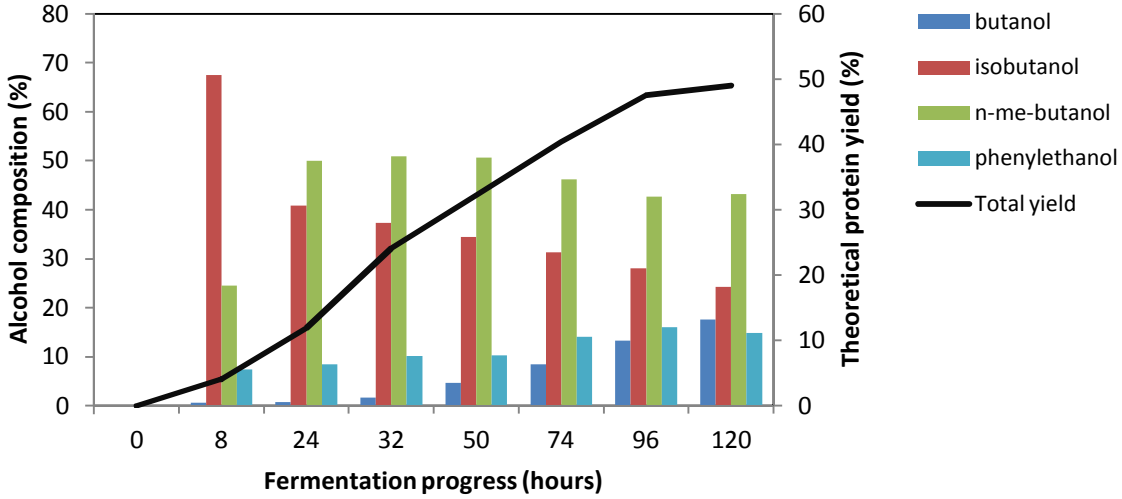


Microaerobic fermentation of algae biomass residuals for fuel production and nutrient recycling

Goal: Develop process to maximize conversion of microalgal residuals to fuels and liberate N/P

Strategy: Subject pretreated microalgae biomass to microaerobic fermentation using a metabolically engineered *E. coli* strain, developed by J. Liao and co-workers (Xin Huo, et al *Nature Biotech*, 2011)

- Alcohol tolerance
- Assimilation of nitrogenous carbon sources
- Knock-down of quorum sensing



Results Overview

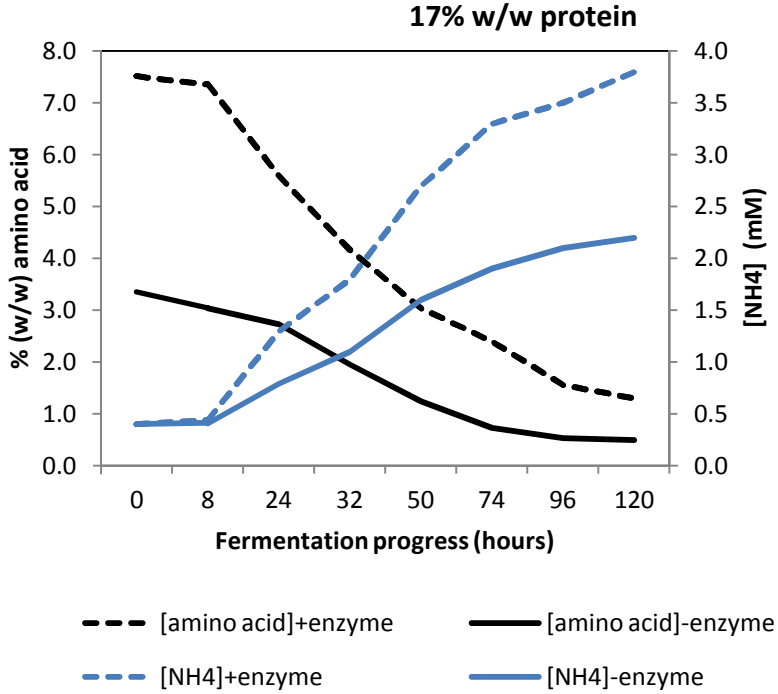
1) Mixed alcohols = 50% protein yield using 5-step process:

   dilute acid pretreatment -> ethanolic fermentation -> distillation -> enzymatic digestion (proteins) -> microaerobic fermentation (37°C, 96-120 hrs)

2) Alcohol components do not significantly vary with biomass type
3) Accumulation of alcohols proceeds in distinct temporal phases:

   isobutanol -> n-methyl-butanol -> phenylethanol and n-butanol

4) Ammonium is accumulated in fermentation liquor as amino acid breakdown product (stoichiometric with alcohol production)



Nuances, Challenges, & Opportunities

1.) Evidence for metabolic inhibition by some chemical components of algae biomass slurry: more aggressively lipid extracted biomass provides increased higher alcohol yields; ionic liquid pretreatment outperforms dilute acid for alcohol production: ammonium + alcohol products, TAG/phospholipids, sugars/sugar degradation products are likely to be inhibitory

2.) Different catalysis rates for different amino acids -or- are products re-entering metabolic pathways?

→ **Optimization necessary for removing both desired and undesired products:**

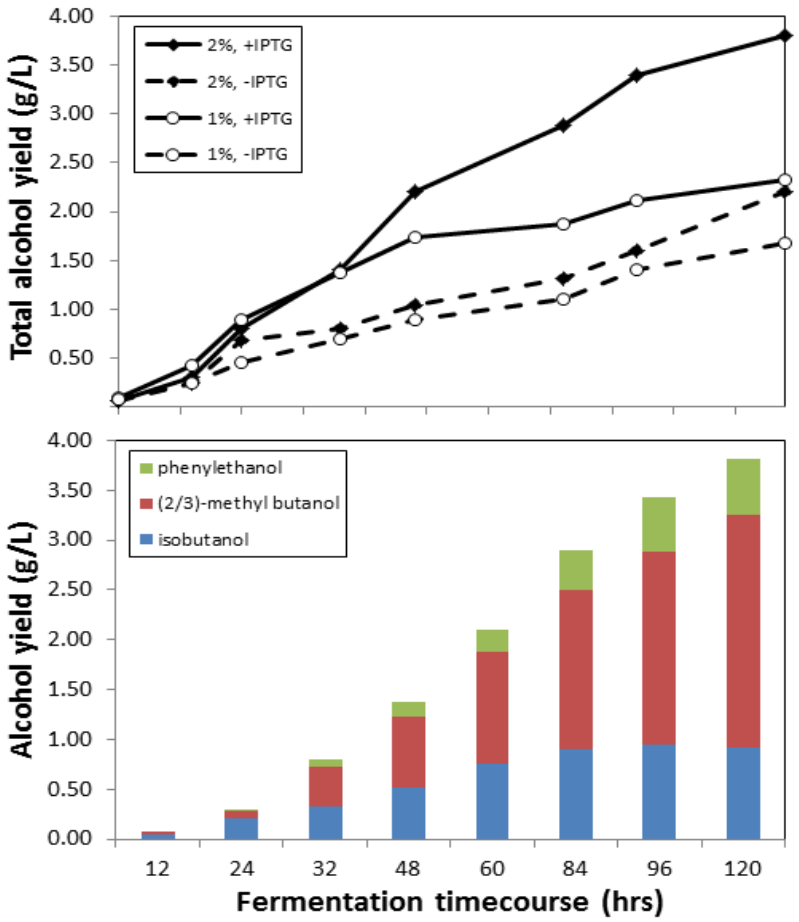
- Thermal trapping of alcohols during fermentation
- Precipitation of accumulated ammonium as phosphate mineral salts

3.) How to eliminate dependence on enzymatic treatment?
Observation: NO alcohol production in dilute acid pretreated samples without enzymes; however we do observe alcohol production on native (untreated biomass), and biomass that has undergone preliminary ethanolic fermentation. In most cases, enzymes increase the total alcohol yield by **2x.** Furthermore, we observe release of distinct intermediates (dialcohols/ketones) with/without enzymatic treatment

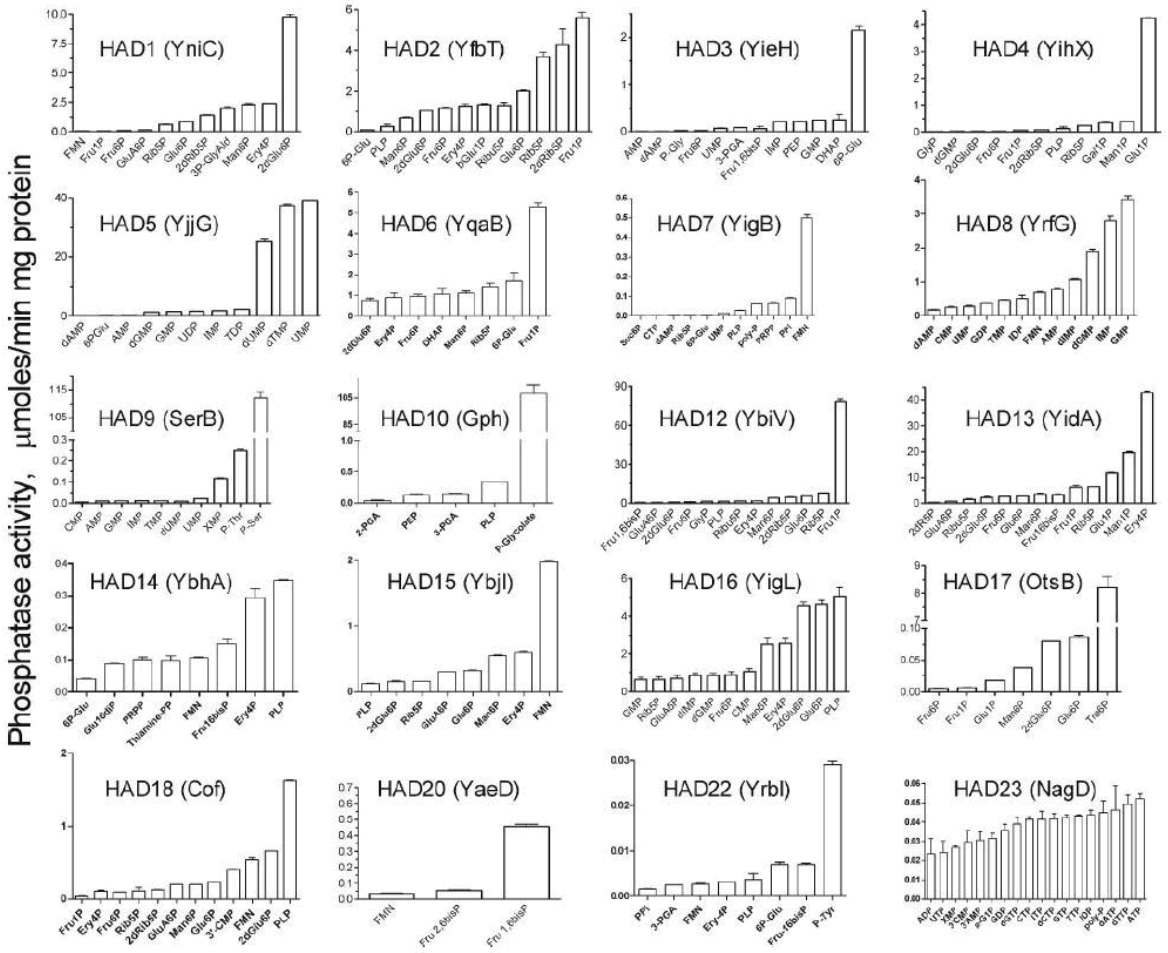
→ **Steps toward maximal consolidation of bioprocess:**

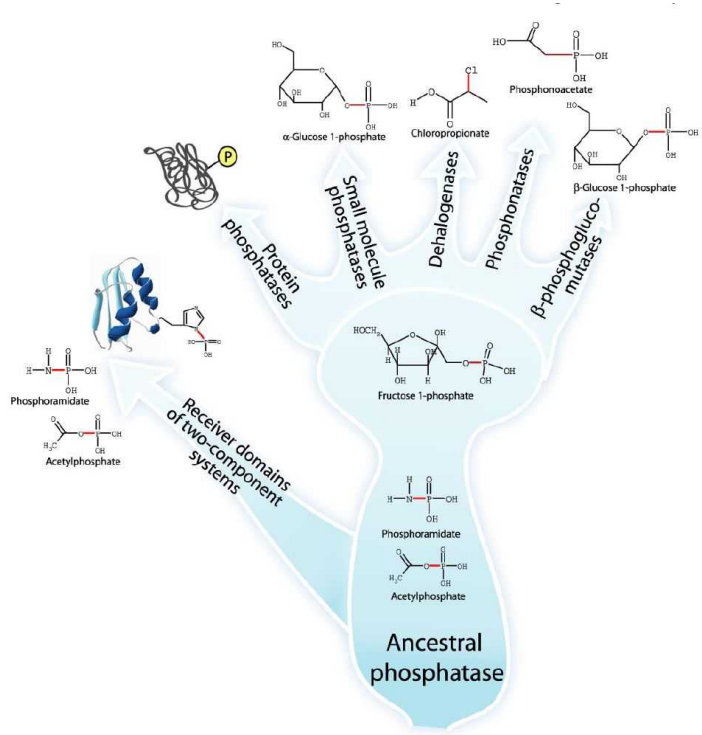
- Co-fermentation of sugars & proteins (*Saccharomyces + E. coli YH83*, with thermal ramp)
- Heterologous expression of proteases in *Saccharomyces and/or E. coli YH83*
- Combine metabolic carbohydrate and protein -> mixed alcohol pathways in *E. coli YH83*
- Triggered protease expression based on carbon souce, stabilization of expressed pathways
- Engineered intermediates via keto-isovalerate pathway up-regulation & thermophilicity (J. Takasumi)

Contribution of induced vs constituatively expressed genes to total mixed alcohol yield



E. coli Haloacid Dehalogenase-like Phosphatases





Conclusions

- Conditions identified for optimal hydrolysis of algae biomass
- High yield of mixed alcohols from hydrolyzed algae biomass protein residuals
- Significant release of ammonium and phosphate for nutrient recycling
- Non-aerobic conditions offer significant cost reduction for biomass processing
- No need for co-factors (vitamins, trace elements)