253rd ACS National Meeting

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# One-pot bioconversion of distillers' grains hydrolysate to advanced biofuels

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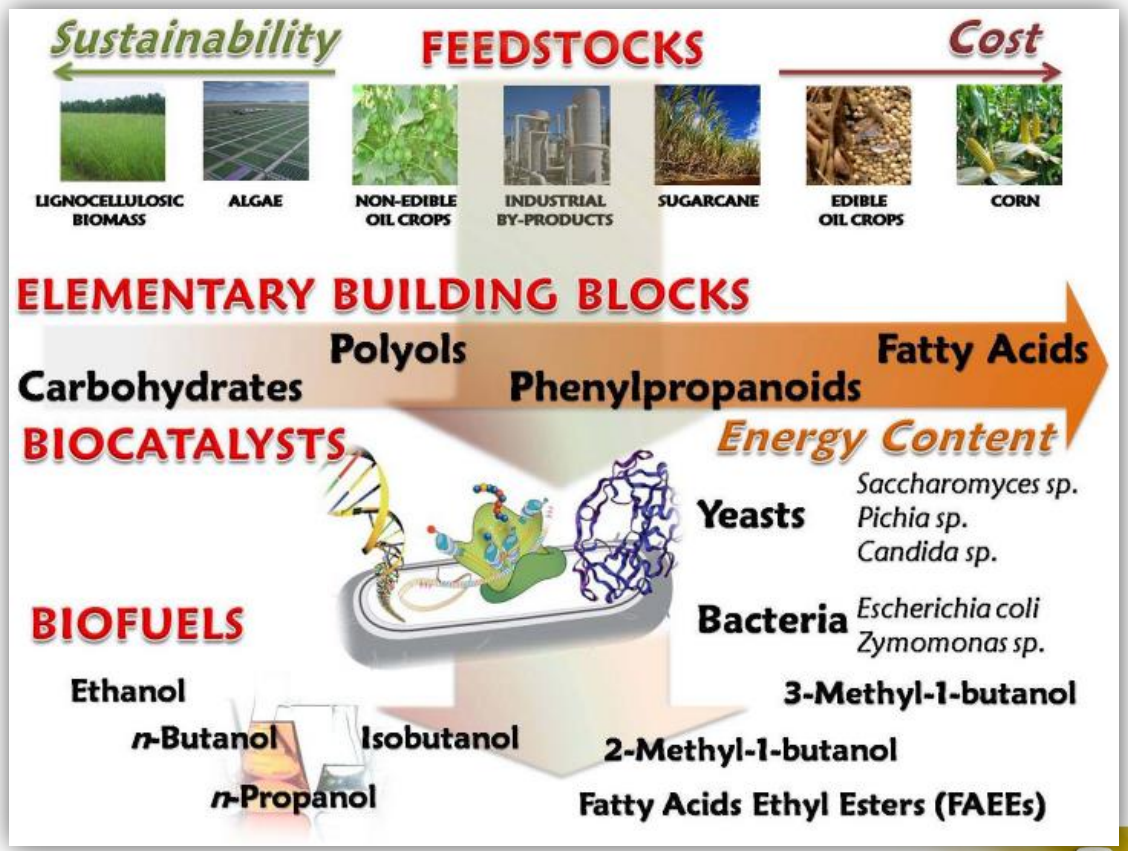
# Production of fuels from renewable feedstocks

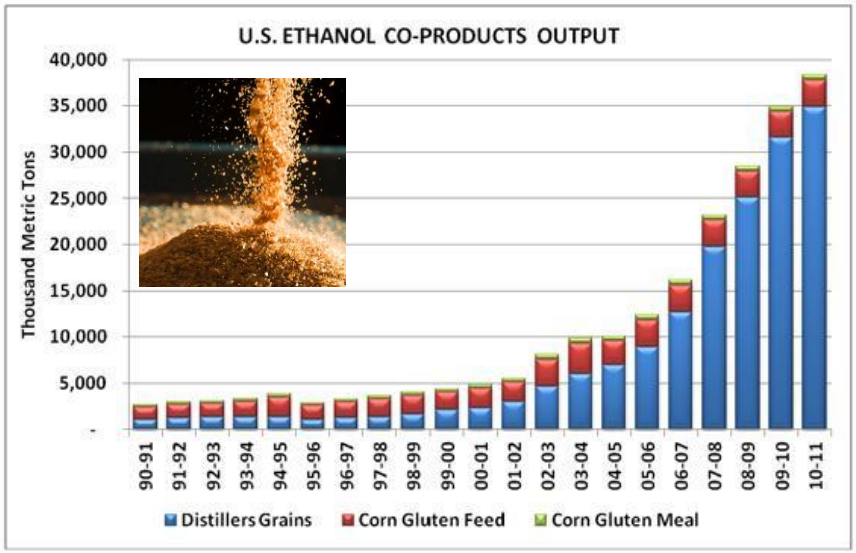
- Recent advances in synthetic biology, metabolic engineering, and systems biology, have enabled the construction of microbial factories for the synthesis of biofuels and other chemicals.
- Renewable feedstocks:
  - Edible and non-edible crops
  - Waste streams (e.g. bagasse from sugar manufacture, industrial by-products)
  - Agricultural lignocellulosic residues
  - Algae



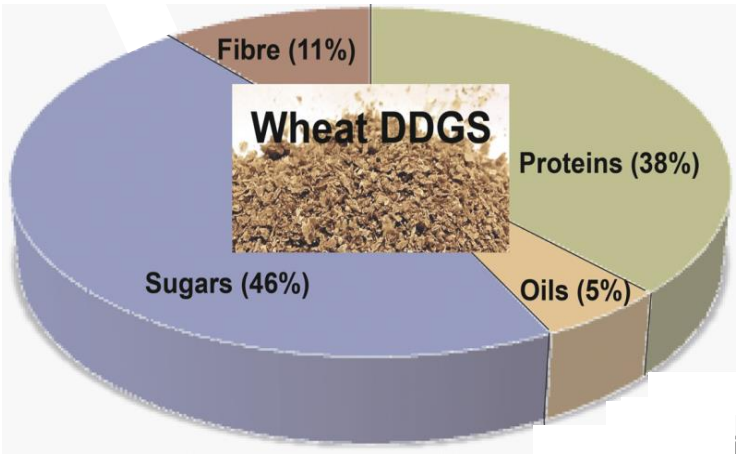

( Dellomonaco et al. *Microbial Cell Factories*. 2010,9:3 )

# Distillers' grains with solubles (DGS)

- 1st generation bioethanol production from corns generates a massive supply of DGS as coproduct.
- The total amount produced in 2011 was 35 million metric tons in the US.
- DGS are rich in cellulosic polysaccharides and protein.
- Risk of using as animal feed: mycotoxins, antibiotic residues, sulphur content and introducing bacterial pathogens.
- Efficient valorization of DGS to produce petroleum replacements will significantly improve the techno-economic feasibility of the established starch bioethanol process.




(Source: http://www.biofuelscoproducts.umn.edu/general-information/overview)




( Villegas-Torres, et al, *New Biotech*, 2015, 32(6):606-611 )

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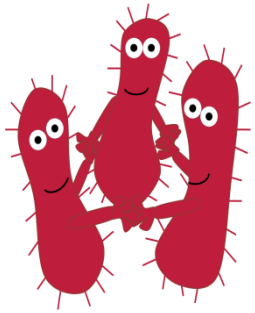
# Consortia-based cell factory

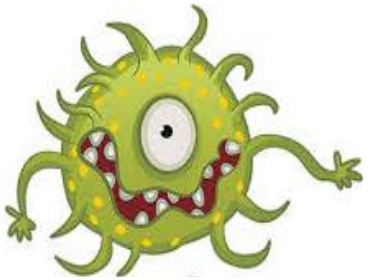
- Engineering a single microbe to simultaneously optimize multiple metabolic tasks represents a major challenge under most situations.
- Microbial consortia: enhanced productivity, stability or metabolic functionality.
- Consortial interaction motifs:
  - Division of labor
  - Commensalism
  - Resource exchanges or cross-feeding



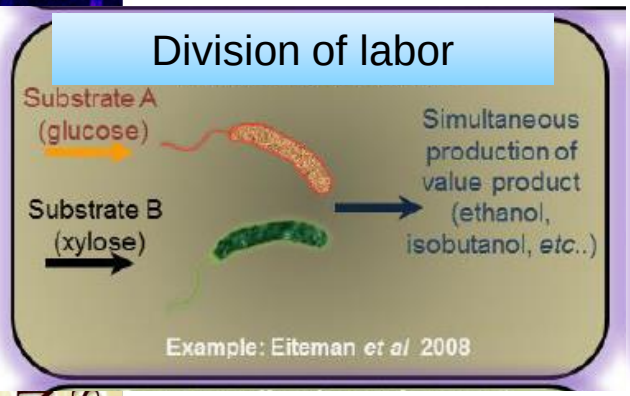
**Microbial consortium**

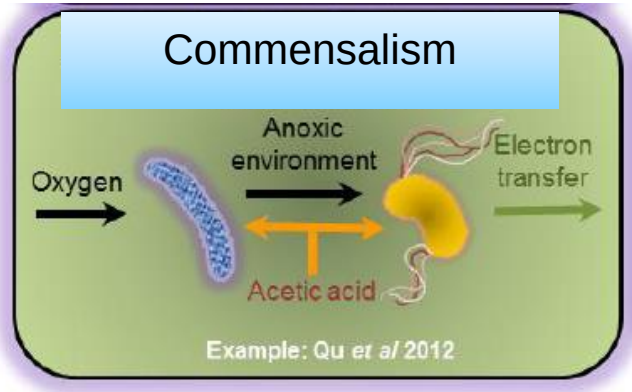
**??**

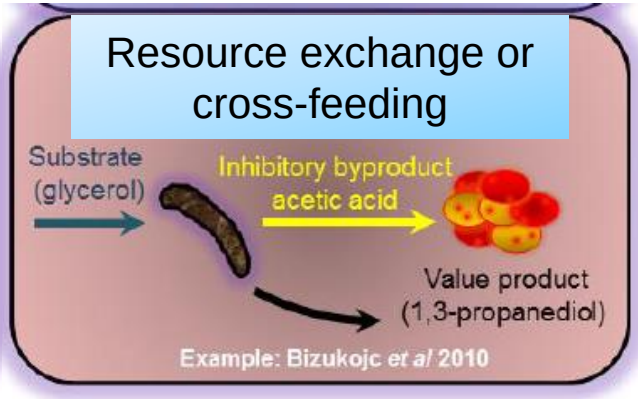
***vs***



**Superbug**



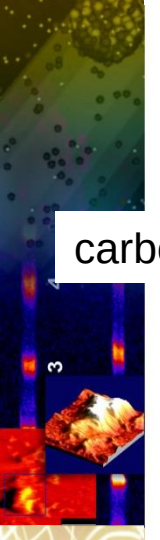




( Bernstein et al. *Comput Struct Biotechnol J*. 2012, 3(4): e201210017 )

# Engineering an *E.coli-E.coli* co-culture system for fusel alcohol production

- Sugar fermentation strain: convert sugars to isobutanol and other fusel alcohols.
  Protein fermentation strain: convert amino acids to C4 and C5 alcohols.
- Use of an *E. coli* - *E.coli* co-culture would minimize problems of dominance of one species and culture instability.
- It allows the “one-pot” bioconversion of the protein and carbohydrate fractions of the DGS hydrolysates into fusel alcohols.



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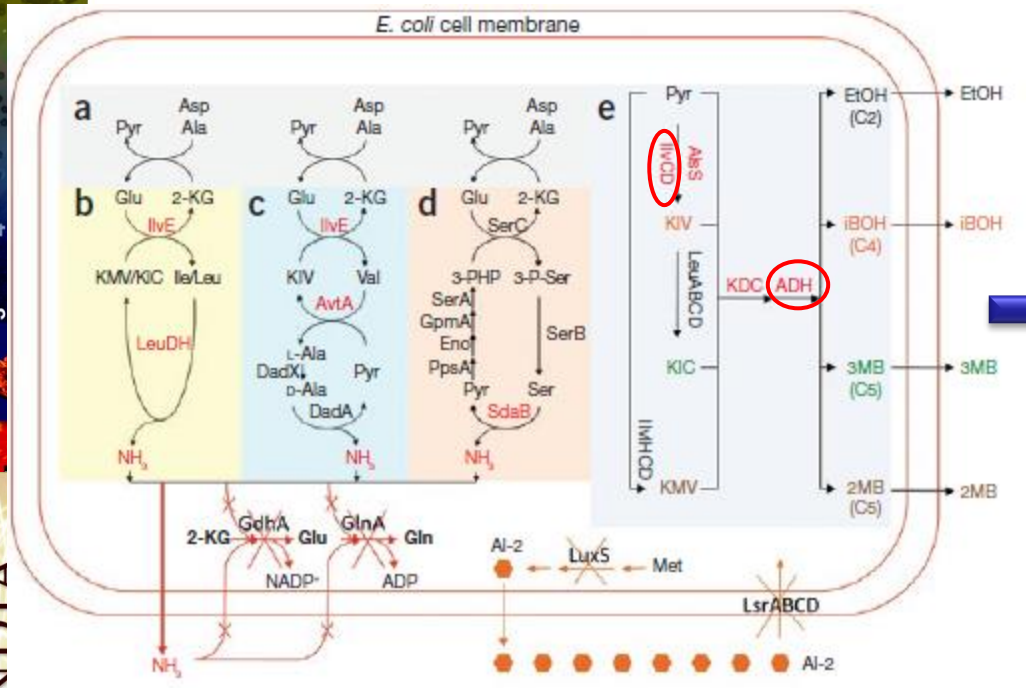
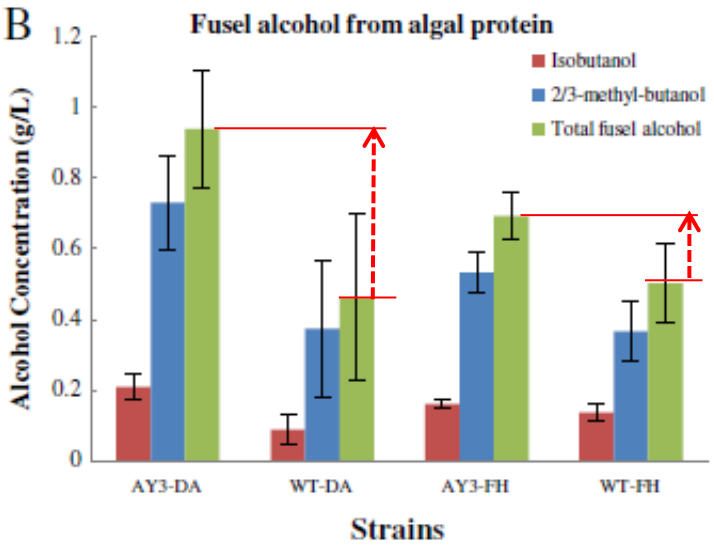
# Protein conversion strain

- Protein utilization strain
  - deaminate protein hydrolysates and convert proteins to C4 and C5 alcohols

- Improved strain: *E. coli* AY3
  - by modifying the cofactor specificity of two key enzymes (IlvC and YqhD) from NADPH to NADH



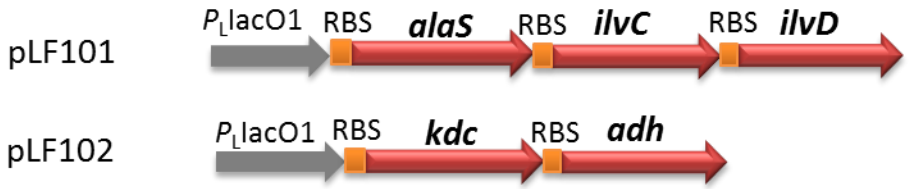

( Huo et al, *Nat. Biotech,* 2011, 29(4): 346-352 )



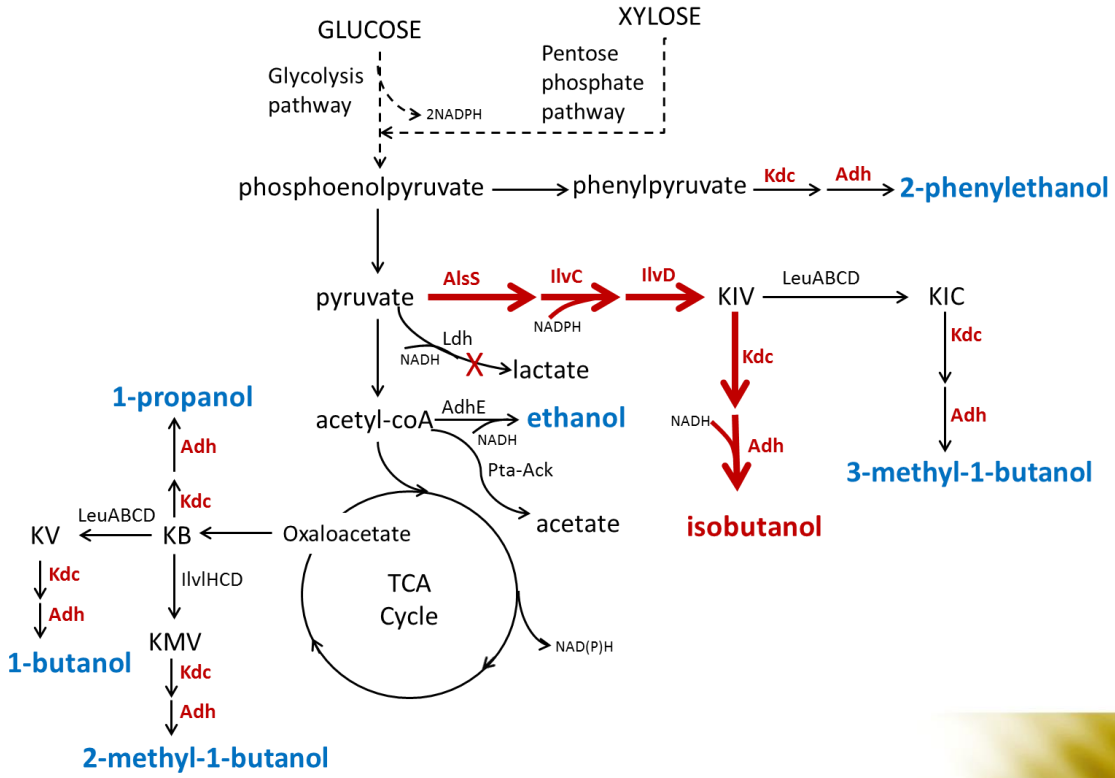

( Wu et al, *Algal Research,* 2016,19: 162-167 )

# Engineering *E. coli* strain B for fusel alcohol production using carbohydrates

- *E. coli* ATCC11303 (Luria strain B) as wild type strain for engineering: has great ability to metabolize hexose and pentose sugars.
- Deleted *ldh* gene from the chromosome and replaced with chloramphenicol resistance gene ($Cm^R$).
- Cloned the 2-keto acid pathway to strain B → production strain: *E.coli* BLF2.




*E. coli* BLF2

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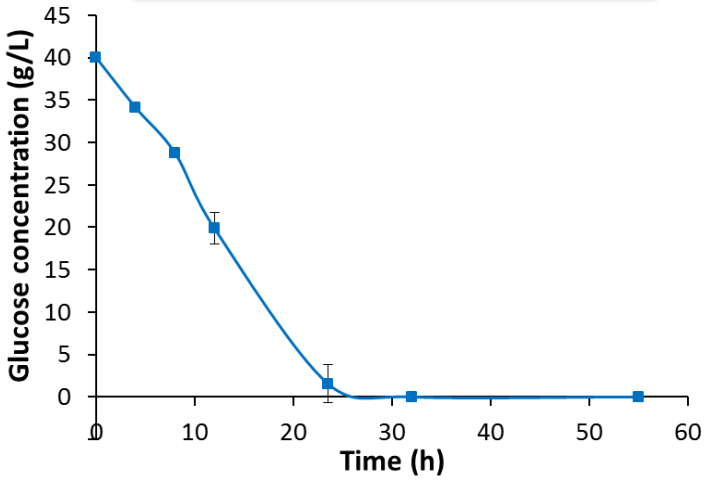
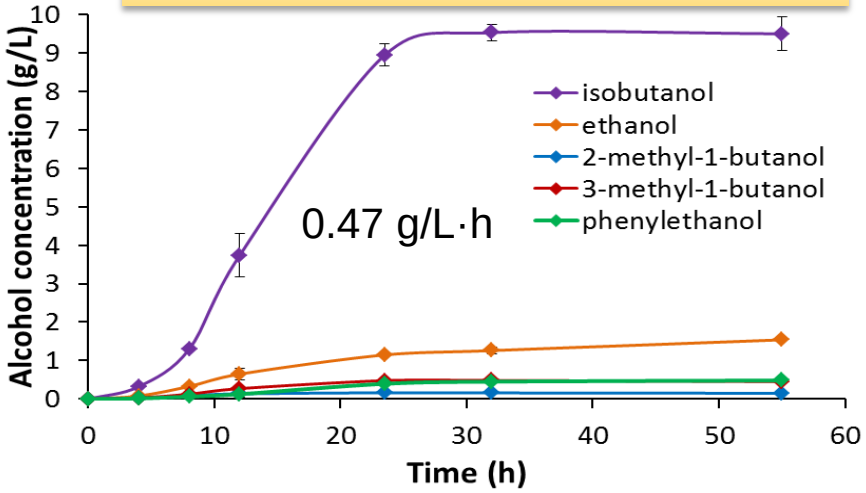
# Alcohol production from glucose and xylose by *E. coli* BLF2

- The volumetric productivity of the total fusel alcohols produced from xylose was 30% lower than from glucose.

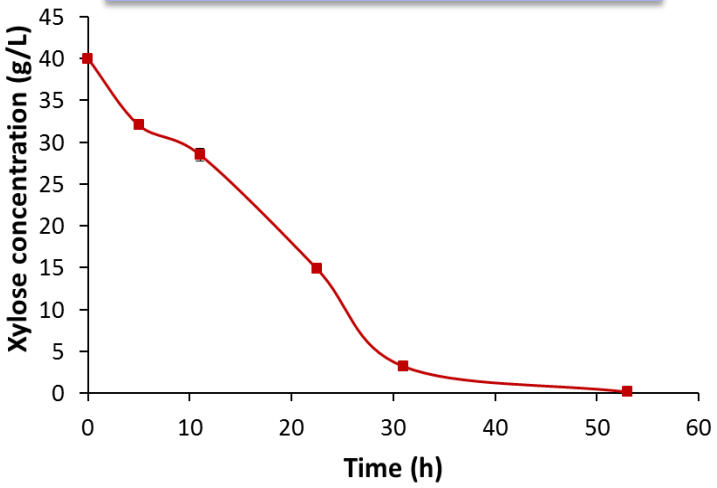
Glucose consumption



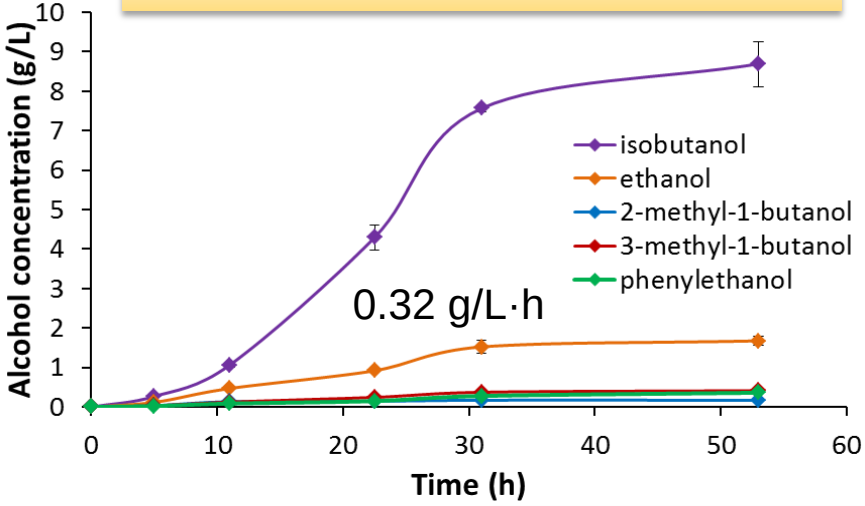

Alcohol production from glucose




Xylose consumption




Alcohol production from xylose

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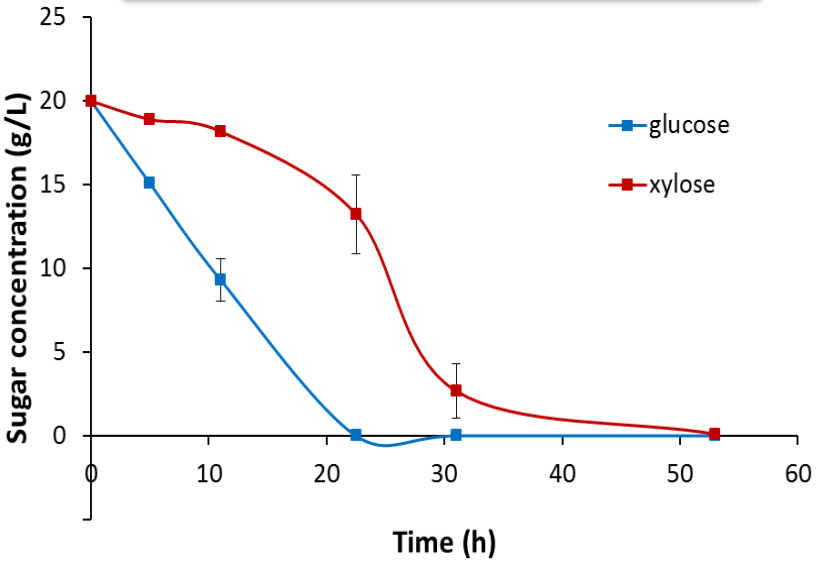
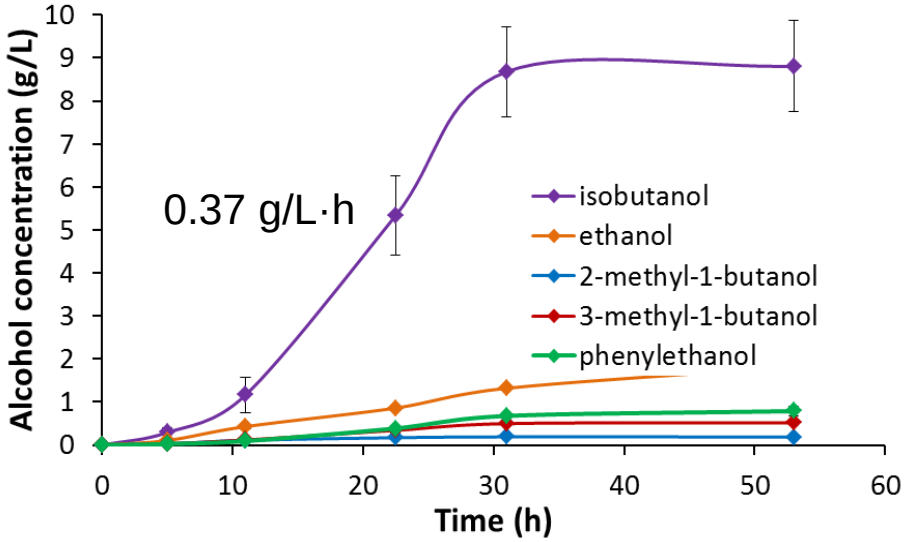
# Alcohols production from glucose and xylose by *E. coli* BLF2

- The uptake of xylose was slow until glucose was completely metabolized, which is the result of carbon catabolite repression.
- Up to 12 g/L total fusel alcohols were produced.
- Isobutanol comprised ~80% of the alcohol mixture.

Glucose and xylose consumption in sugar mixture




Fusel alcohols production from sugar mixture

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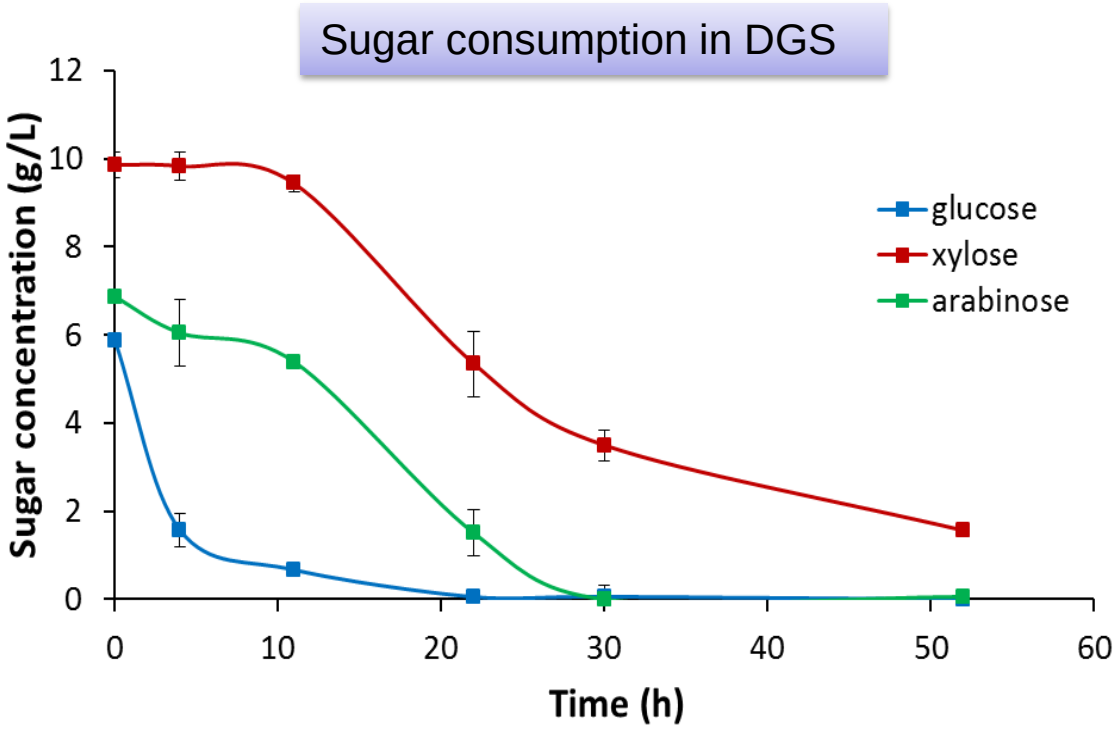
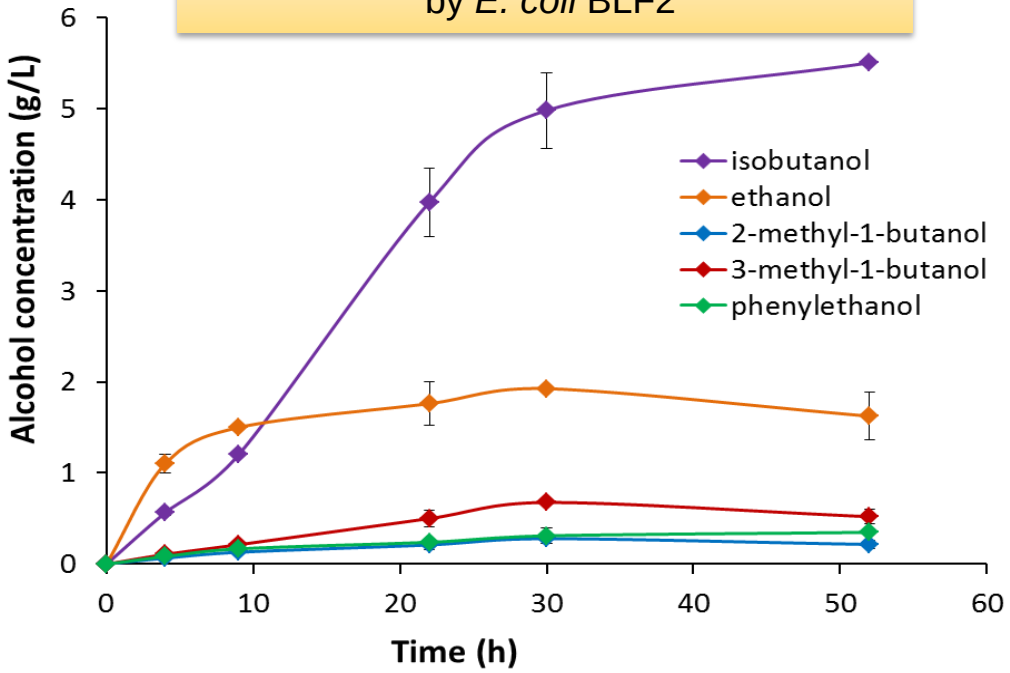
# DGS pretreatment and monoculture fermentation by *E. coli* BLF2

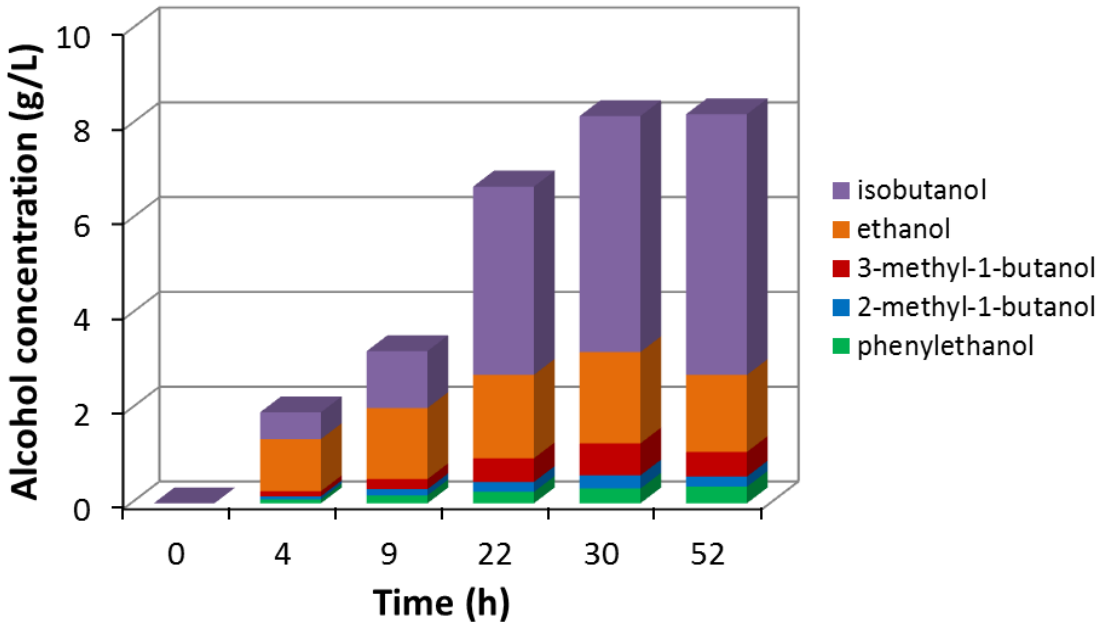
- DGS samples (35% solids) were from Aemetis, Inc., a bioethanol company in CA.
- Pretreatment of DGS with 8.5% solid loading: dilute acid (4% $H_2SO_4$, incubated in 90˚C water bath for 5 hrs) & enzymatic (1.5 mg/ml, 37˚C for 48 hrs).
- DGS fermentation by *E. coli* BLF2
  - Glucose was preferentially utilized.
  - Arabinose and xylose were utilized when glucose's concentration was low.
  - Incomplete utilization of xylose with a 84% conversion.



## Fusel alcohols production from DGS by *E. coli* BLF2

- ~ 8.2 g/L total fusel alcohols were produced at 52 hrs.
- Isobutanol comprised 67%.

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# Integrated sugar & protein fermentation of DGS hydrolysates




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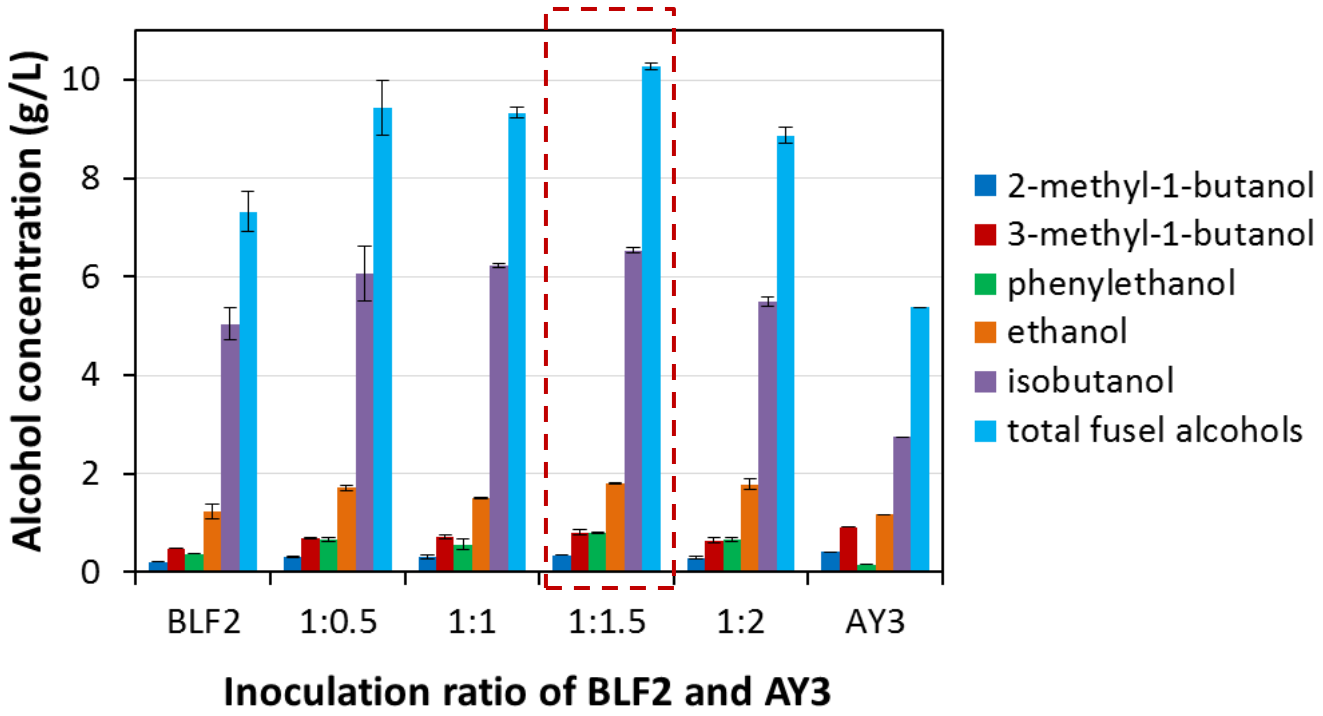
# Bioconversion of DGS hydrolysates by the *E. coli* co-culture

- The consortium with the 1:1 inoculation ratio of *E. coli* BLF2 and AY3 achieved the highest fuel yield.
- Up to 10.3 g/L of total fusel alcohols were produced, including 63% isobutanol.

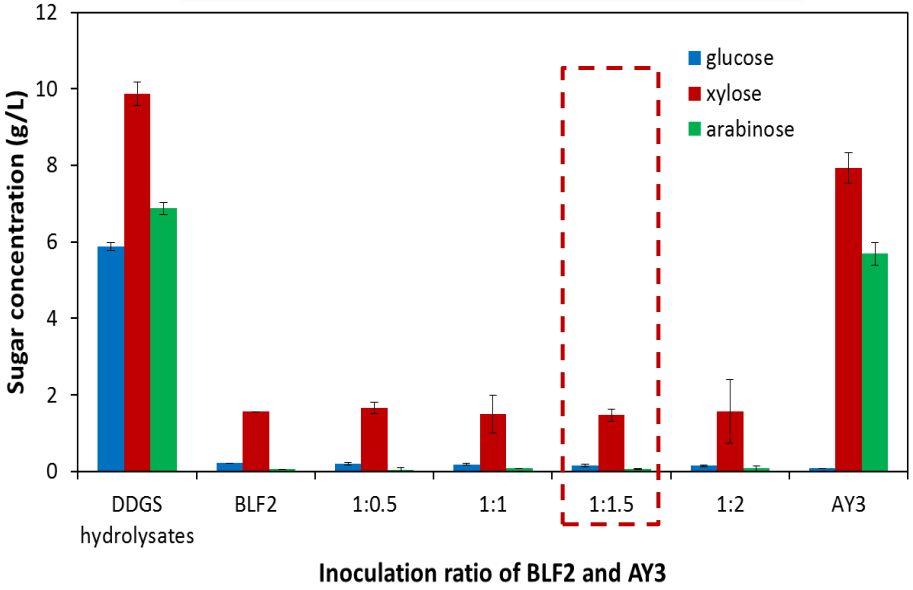
Alcohols production from DGS by the *E. coli* co-culture



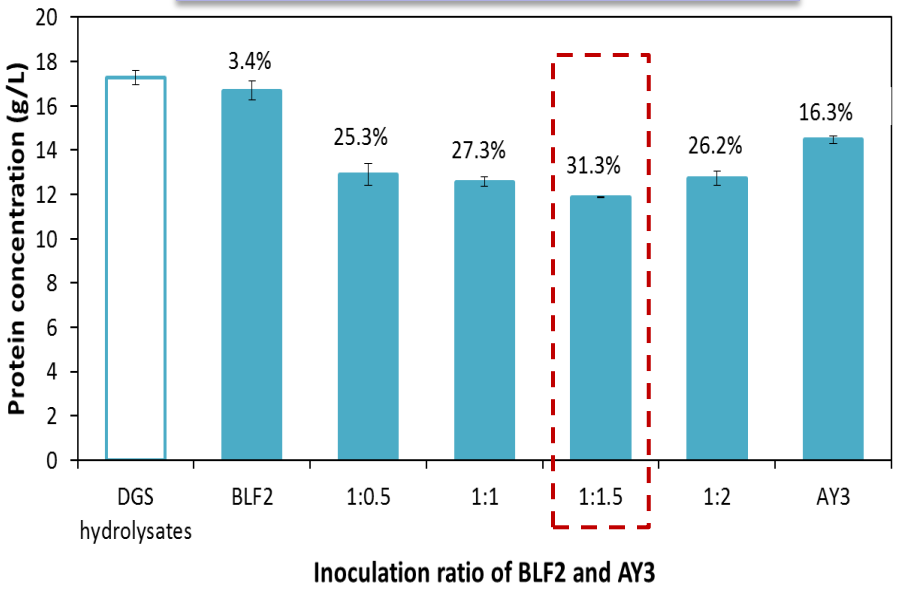
# Bioconversion of DGS hydrolysates by *E. coli* co-culture

- The consortium with the 1:1.5 inoculation ratio utilized glucose and arabinose completely and about 85% of the xylose in the DGS hydrolysates.
- ~31% of the total DGS proteins were converted.

Sugar consumption

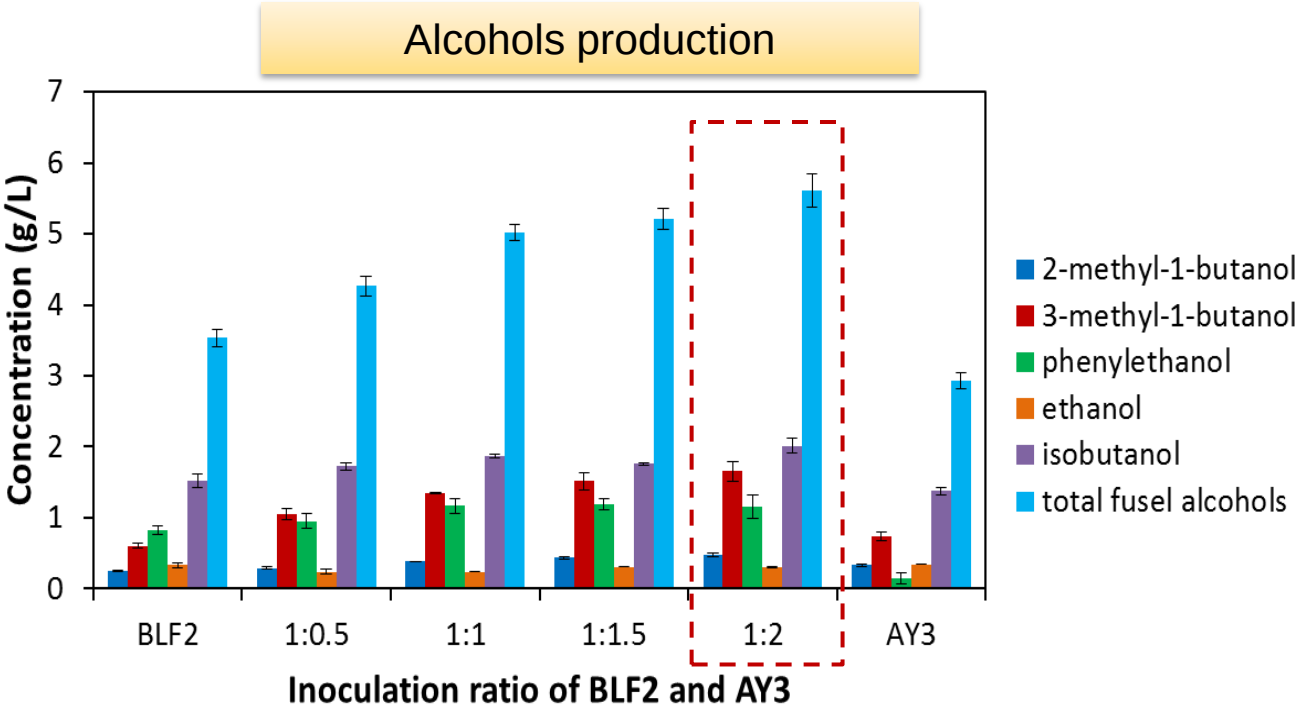



Protein conversion



# Bioconversion of algal hydrolysates by *E. coli* co-culture

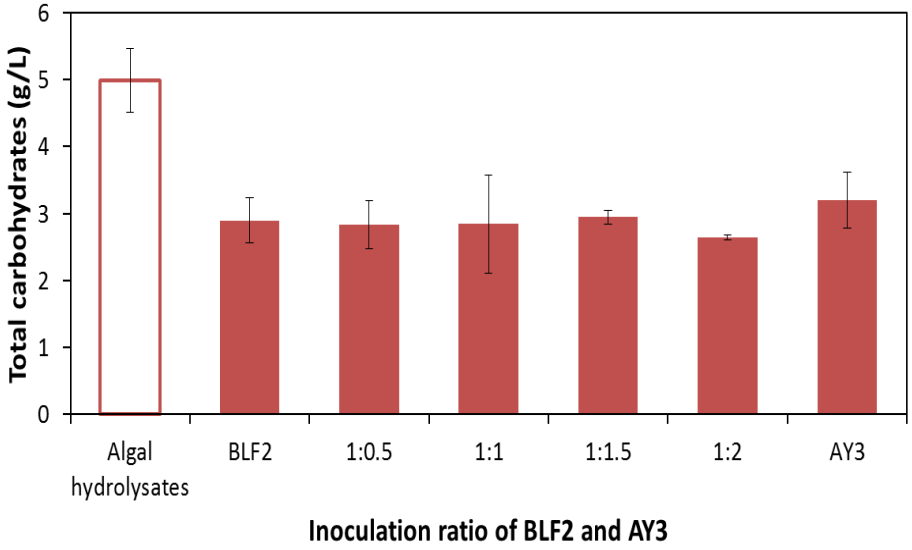
- Algae *Reed Nanochloropsis* were pretreated by 10% dilute acid & enzymatic (2 g/L, 55˚C for 48 hr).
- The consortium with the 1:2 inoculation ratio of *E. coli* BLF2 and AY3 achieved the highest fuel yield.
- Up to 5.6 g/L of total fusel alcohols were produced with isobutanol and isopentanol as the major products.



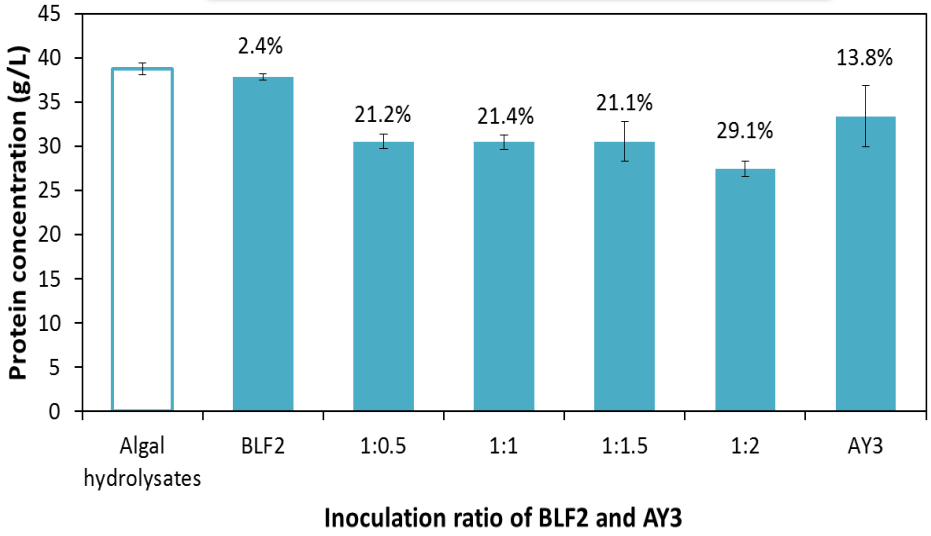
# Bioconversion of algal hydrolysates by *E. coli* co-culture

- Up to 47% of total carbohydrates were converted.
- 21-29% of the total proteins were converted by the *E. coli* consortia.

Carbohydrate consumption




Protein conversion



# Conclusions

- We demonstrated “one-pot” bioconversion of the protein and carbohydrate fractions of a DGS hydrolysate into higher fusel alcohols through development of a microbial consortium incorporating two engineered *E. coli* strains
- Th d an glu
- Th nd AY g/L
- Th also useful for other sugar and protein rich hydrolysates such as algae biomass.
- The mixed fusel alcohol has potential applications as a fuel additive in gasoline, diesel, jet fuel, heating oil or as a neat fuel of itself.

**Poster: ENFL 183**

Title: Production, blending, and upgrading of advanced renewable fuels for the co-optimization of fuels and engines

Monday, 2:00 pm - 4:00 pm
Halls B/C - Moscone Center

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# Acknowledgements


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AEMETIS

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# Thank you !

# Any questions?

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# Future work

- Optimization of the DGS fermentation system.
- Scale up the DGS fermentation in 1-L fermenter.
- Improve fusel alcohol production by doing further strain engineering, e.g. deleting the completing pathways for the byproducts.

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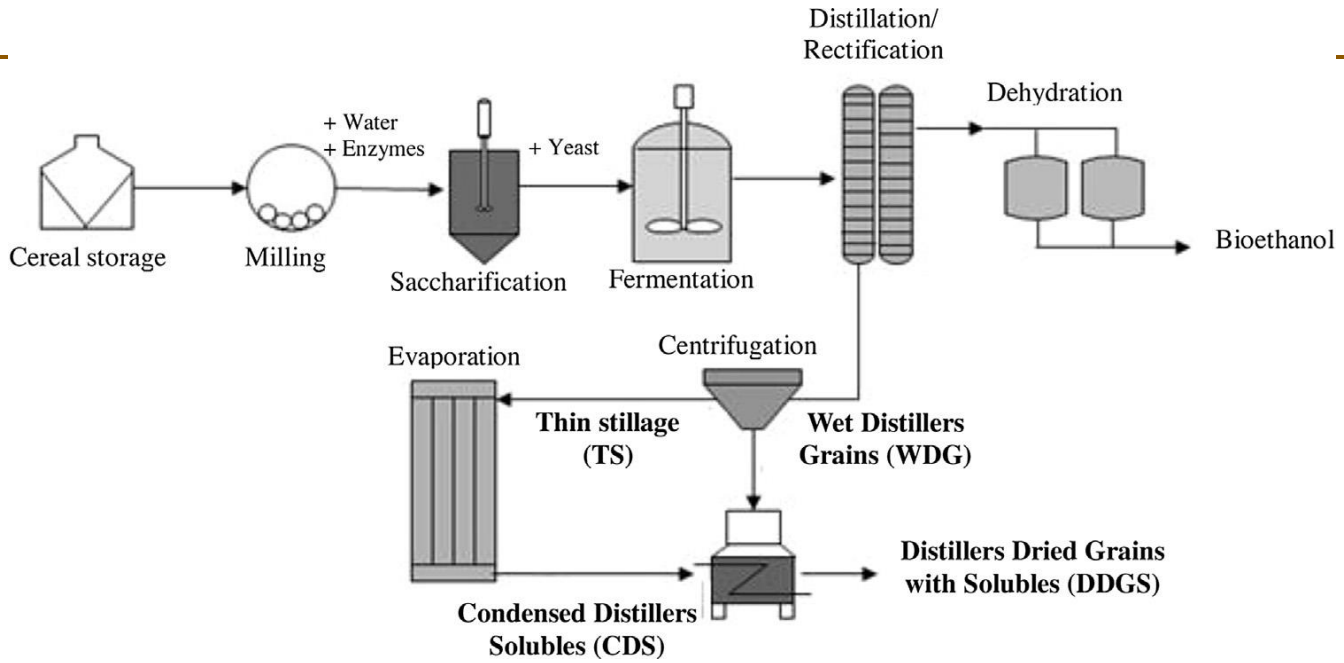
# Dry-mill bioethanol production process and by-product production streams




(A. Chatzifragkou et al. *Process Biochemistry*. 2015, 50: 2194–2207)

- The whole grain is milled and liquefied → amylolytic enzymes convert starch into fermentable glucose→ fermentation to ethanol and $CO_2$ by yeast
- Ethanol is distilled and dehydrated, whereas the non-volatile components are centrifuged to produce a liquid fraction (thin stillage, TS) and a solid fraction (wet distillers' grains, WDG)
- 15% or more of the thin stillage is used as backset for the liquefaction of the ground grain and the rest is concentrated into condensed distiller soluble (CDS)
- CDS is mixed with WDG and drum dried at high temperatures to produce the final DDGS.
- The utilization of a bushel of corn (56 pounds) results in 2.8 gallon of ethanol and 18 pounds of DGS.

# Higher carbon fusel alcohols

- Advantages: lower hygroscopicity, vapor pressure and corrosivity, allowing safer handling and more efficient use than ethanol.
- Higher chain alcohols possess elevated energy densities.
- Full compatibility with existing engines and pipelines.

| Fuel | Gasoline | n-Butanol | Isobutanol | Ethanol | Methanol |
|---|---|---|---|---|---|
| Energy density (MJ/L) | 32 | 29 | 29 | 19.6 | 16 |
| Vapor pressure (kPa) at 20 ° C | 0.7-207 | 0.53 | 1.17 | 7.58 | 12.8 |
| Vapor pressure of mixture with gasoline (kPa) | 53.8-103.4 | 44.1 | 46.9 | 138 | 800 |
| Hygroscopicity | Low | Low | Low | High | High |
| Compatibility with existing infrastructure | Yes | Yes | Yes | No | No |

(Source: BiofuelsDigest, 2016/09/27)

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# Engineering carbohydrate fermentation strain

- Strain selection: *E. coli* ATCC11303 (Luria strain B)
  - able to metabolize glucose as well as xylose which offers an opportunity to convert both hexose and pentose fractions of biomass.

| Sugar | Plasmid | Final $OD_{550}$ of *E. coli* ATCC strain: | | | | | |
|---|---|---|---|---|---|---|---|
| | | 8677 | 8739 | 9637 | 11303 | 11775 | 14948 |
| Glucose | None | 4.0 | 3.7 | 6.1 | 6.0 | 4.7 | 5.6 |
| | pLOI297 | 10.0 | 10.5 | 10.5 | 10.0 | 9.5 | —[a] |
| | pLOI308-11 | 9.8 | 9.5 | 11.4 | 11.2 | — | 9.3 |
| Lactose | None | 4.3 | 3.8 | 7.5 | 6.0 | 4.5 | 6.1 |
| | pLOI297 | 13.0 | 6.8 | 11.6 | 10.8 | 7.6 | — |
| | pLOI308-11 | 10.0 | 10.0 | 11.5 | 11.0 | — | 7.3 |
| Xylose | None | 4.1 | 3.7 | 7.7 | 7.3 | 4.9 | 5.9 |
| | pLOI297 | 8.1 | 10.6 | 10.8 | 10.6 | 4.7 | — |
| | pLOI308-11 | 10.0 | 6.8 | 11.4 | 8.5 | — | 11.4 |

[a] —, No data available.

( Alterthum et al. *Appl Environ Microbiol*. 1989, 55(8): 1943-1948 )

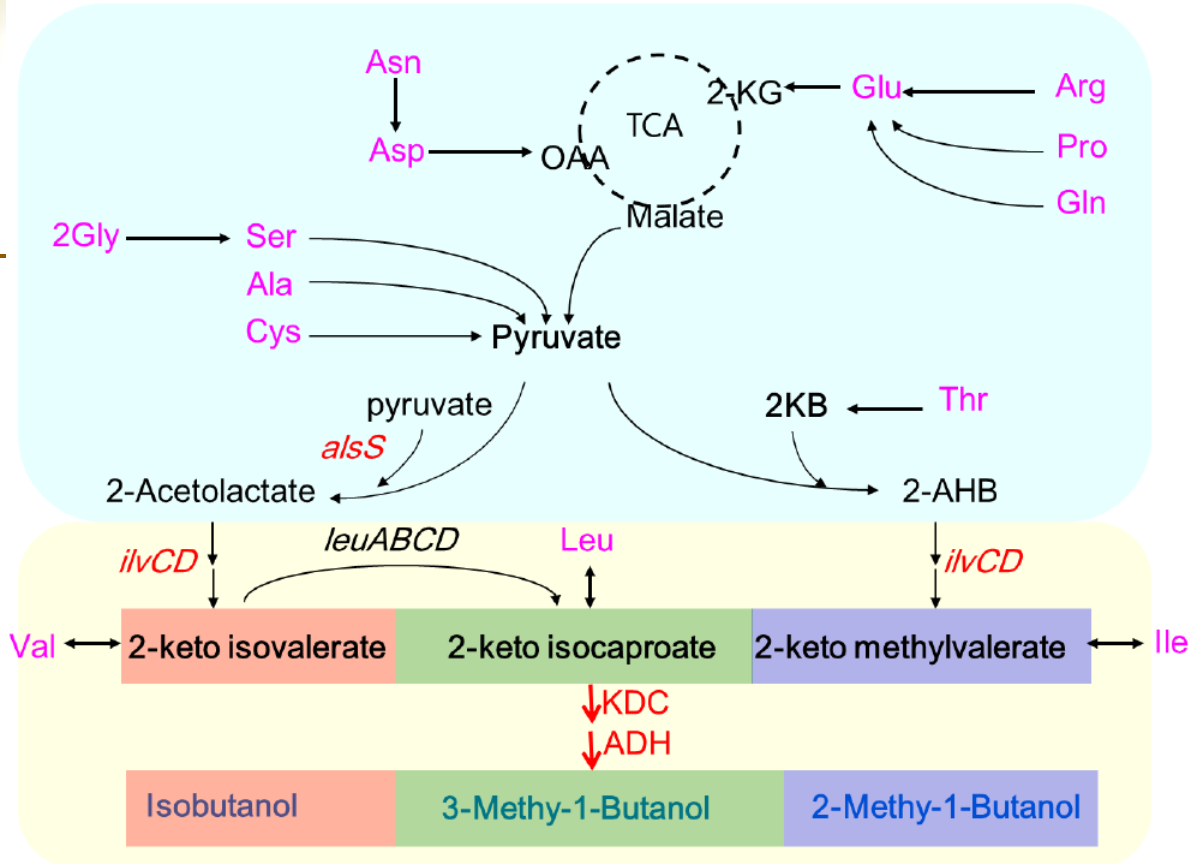
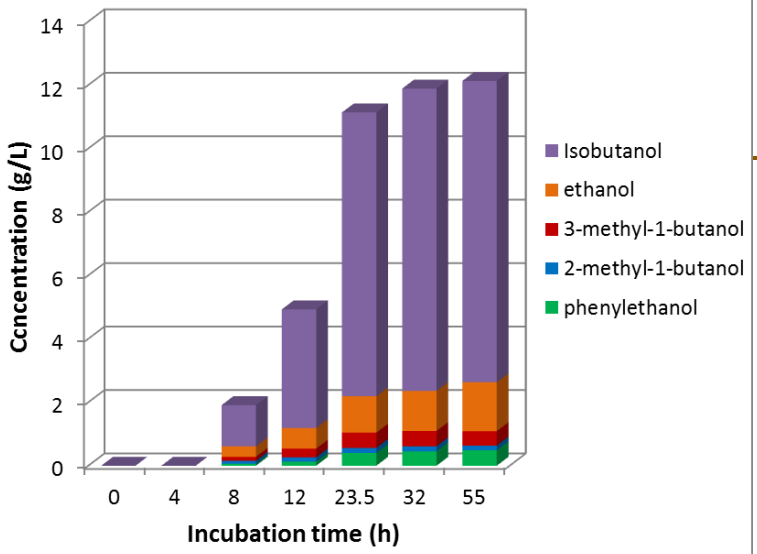
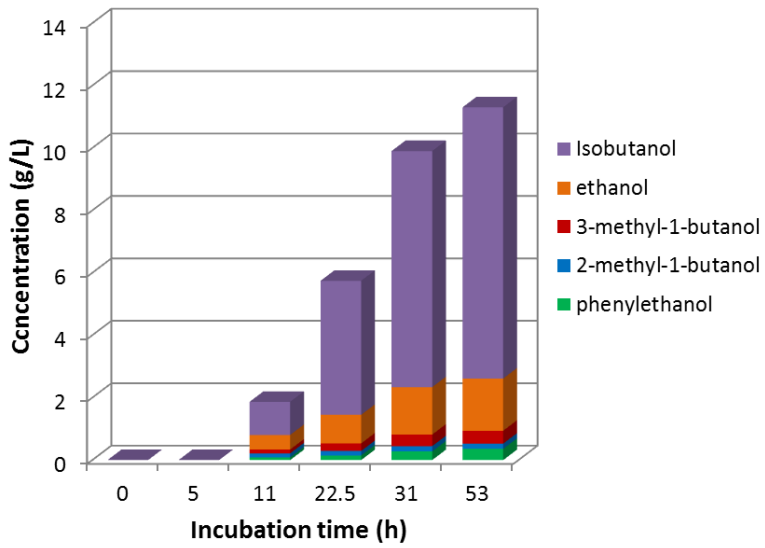



Figure S2. The metabolic networks for the biofuel production from Amino acids. Amino acids could be deaminated to 2-keto acids or TCA intermediates, which can be directed to pyruvate. Pyruvate can be extended to longer keto acids by acetohydroxy acid synthase (e.g. AlsS) or isopropylmalate synthase chain elongation pathways (LeuABCD). The keto acids could be converted to aldehydes by broad substrate-range 2-keto acid decarboxylase (KDCs), and then to alcohols by alcohol dehydrogenases (ADHs).

## Glucose as carbon source

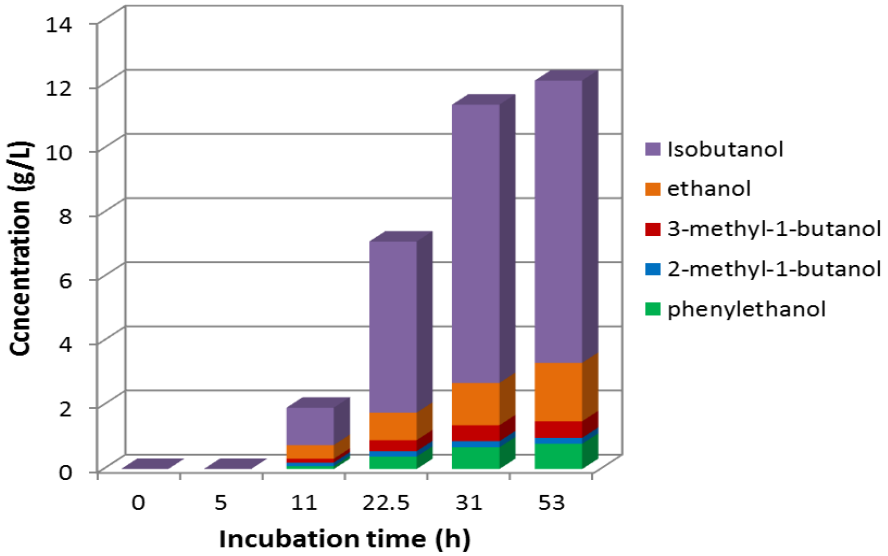



## Xylose as carbon source



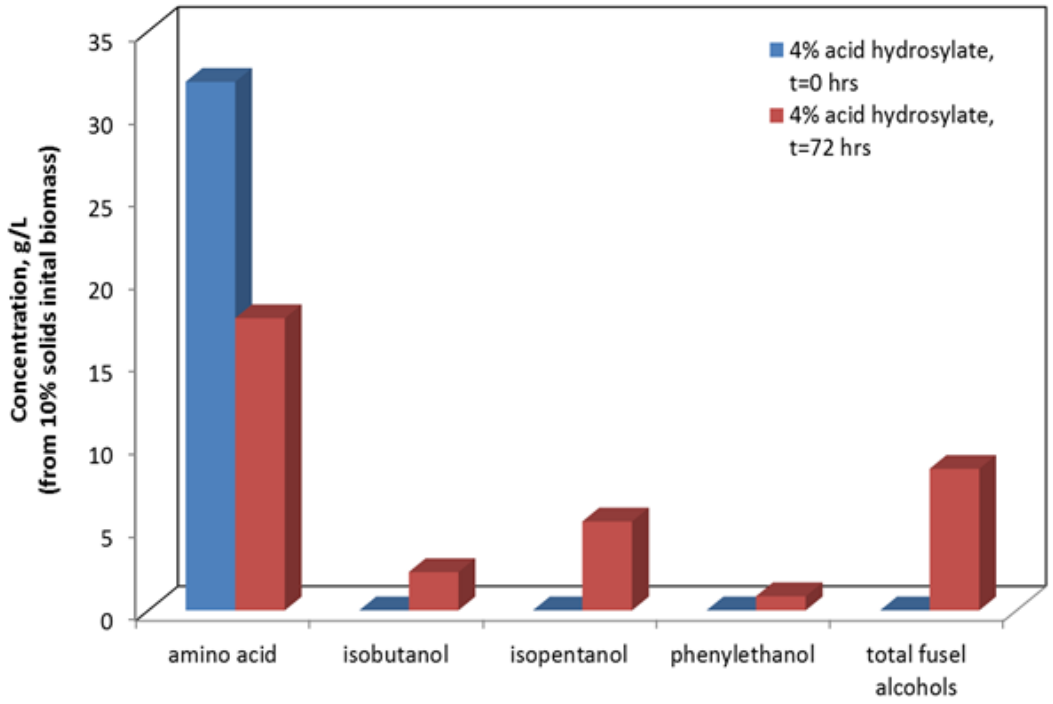

- Up to 12 g/L total fusel alcohols were produced.
- Isobutanol comprised ~80%.

## Glucose and xylose as carbon source



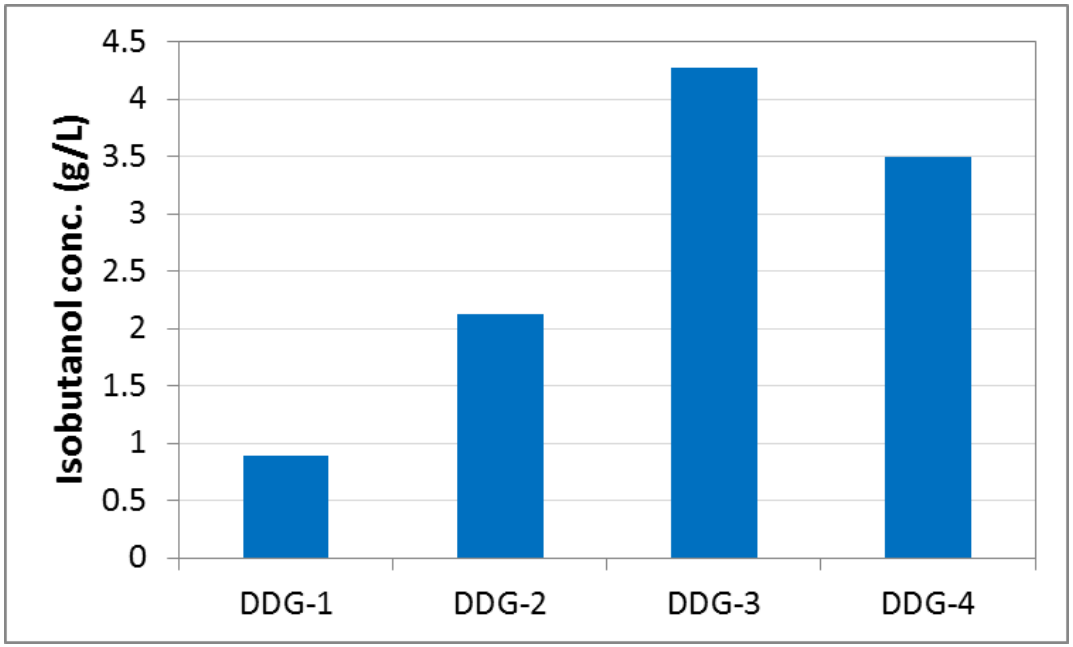
# DGS mono-culture fermentation by *E. coli* AY3

- AY3 converted about 46% protein in DGS hydrolysates .
- Produced a total of 7 g/L fusel alcohols where isopentanol (2-methyl-1-butanol and 3-methly-1-butanol) are the major products.



# The effect of DGS pretreatment on carbohydrate fermentation

- Best pretreatment condition: 4% $H_2SO_4$, incubate at 90° C for 5h in water bath.




| | |
|---|---|
| DDG-1 | 4% $H_2SO_4$, 121 ° C for 1h (autoclave) |
| DDG-2 | 4% $H_2SO_4$, 90° C for 1h (autoclave) |
| DDG-3 | 4% $H_2SO_4$, 90° C for 5h (water bath) |
| DDG-4 | 10% $H_2SO_4$, 90° C for 5h (water bath) |