Producing Non-Native Ethanol as a Major Product from Syngas Fermentation of an Engineered *Eubacterium limosum* KCTC13263BP

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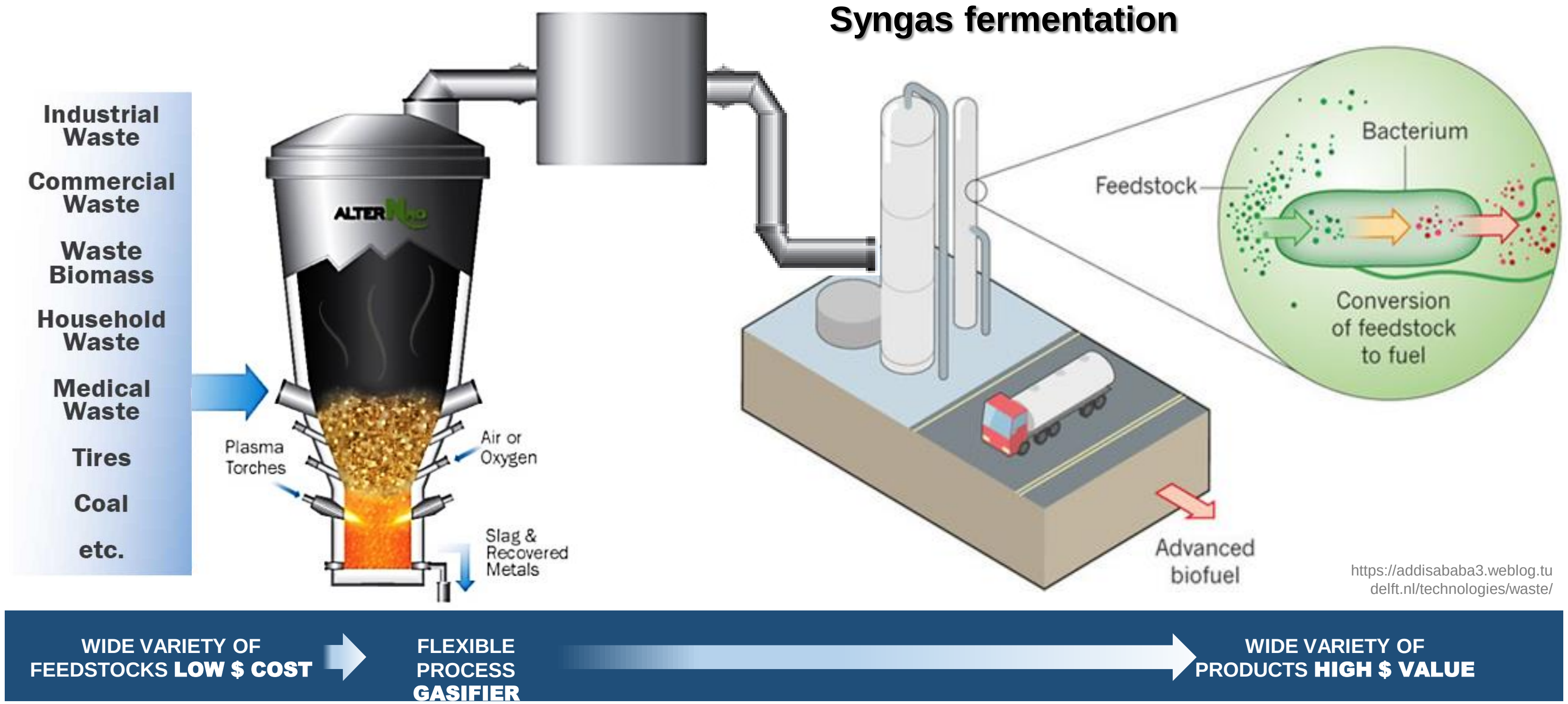
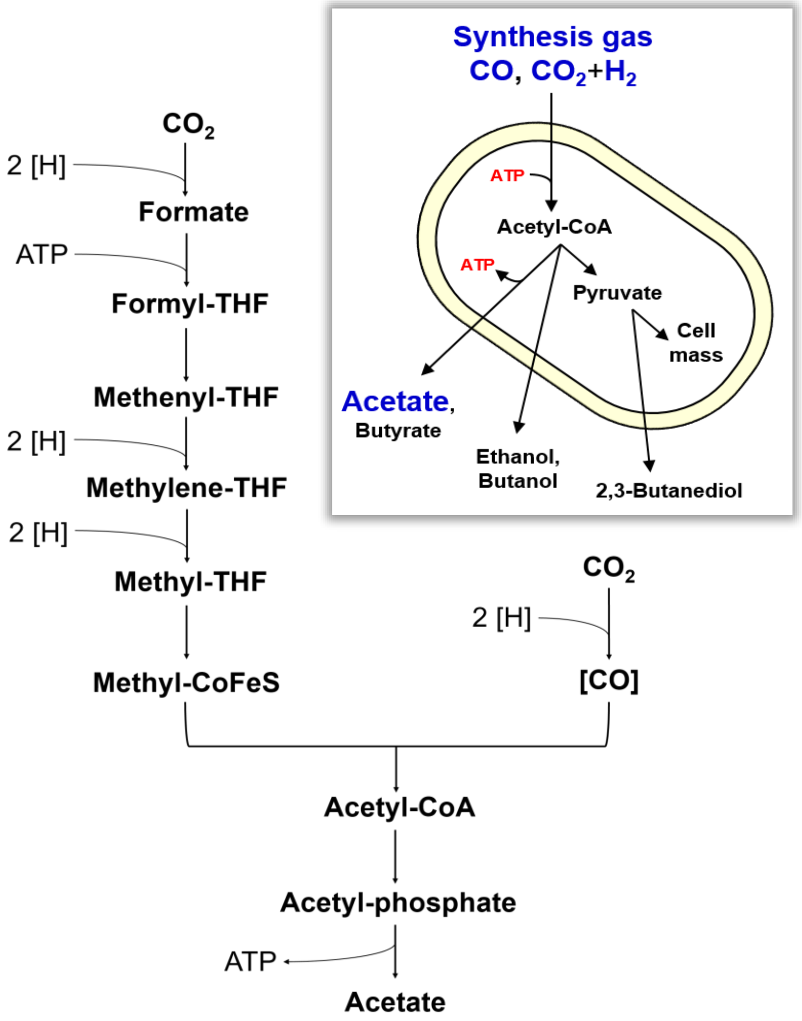
Syngas fermentation and acetogens



(Homo)Acetogens



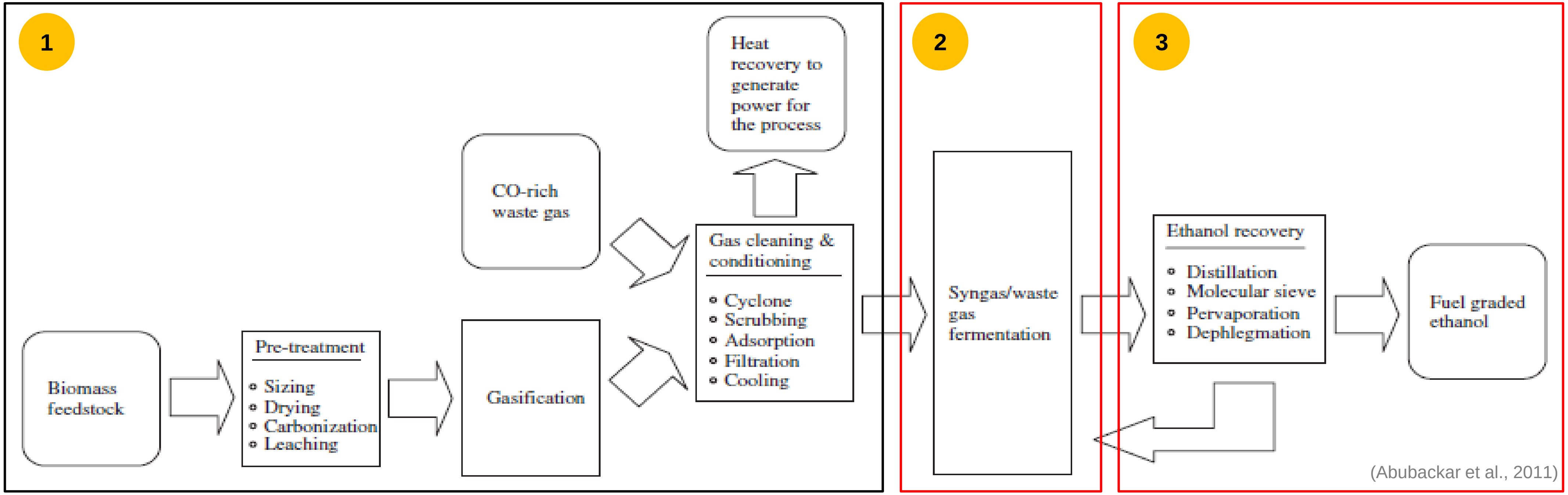
$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$

Wood-Ljungdahl pathway (WLP)

- Syngas fermentation is a microbioal process that converts syngas (CO_2, H_2, CO) to biofuel or biochemical using acetogens as a biocatalyst.
- Acetogens are group of bacteria which are capable of chemolithoautotrophic growth using CO_2.
- Acetogens use Wood-Ljungdahl pathway to synthesis acetyl-CoA from CO_2 + H_2 or CO for an energy conservation and a carbon fixation.

Syngas bioconversion and industrial strain development

Syngas bioconversion process



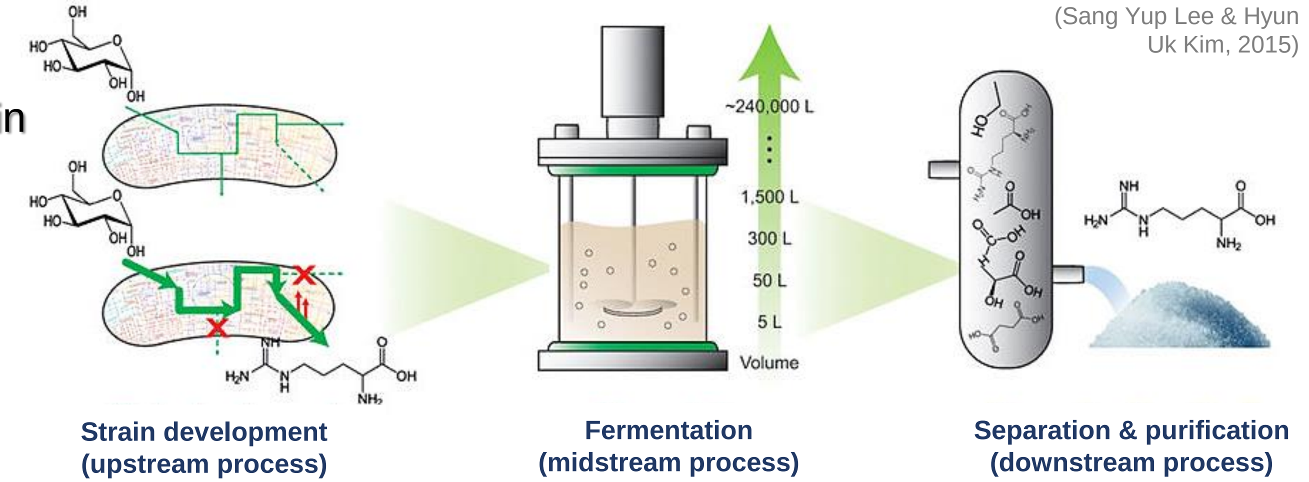
1 Preparation of syngas

2 Acetogenic fermentation
- Microbial resources
- Reactor configuration

3 Product purification

Industrial strain development

(Sang Yup Lee & Hyun Uk Kim, 2015)



Eubacterium limosum KCTC13263BP (formely, *E. limosum* KIST612)

- Isolated in anaerobic digestor (early 1990)
- Obligate anaerobic acetogen
- Autotrophic/heterotrophic growth is possible
- Secured genetic data base → complete genome sequence, gene annotation

Organism	Products
Acetobacterium woodii	Acetate
Clostridium aceticum	Acetate
Clostridium carboxidivorans	Acetate, ethanol, butyrate, butanol
Clostridium ljungdahlii	Acetate, ethanol
Eubacterium limosum	Acetate, butyrate
Morella thermoacetica	Acetate

- Fast growth rate (μ = 0.17-0.25 h^{-1}) under 1 atm of CO partial pressure
- High organic acid (acetate, butyrate) production rate from CO with high threshold on CO substrate inhibition
- Production diversity as compared with other (homo) acetogens
- Bioenergetics on syngas metabolism

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Formulation of Defined Media for Carbon Monoxide Fermentation by *Eubacterium limosum* KIST612 and the Growth Characteristics of the Bacterium

IN-SEOP CHANG,[1,2] BYUNG-HONG KIM,[1]* DO-HEE KIM,[1] ROBERT W. LOVITT,[2] AND HA-CHIN SUNG[3]

JOURNAL OF BACTERIOLOGY, Jan. 2011, p. 307–308
0021-9193/11/$12.00 doi:10.1128/JB.01217-10

Complete Genome Sequence of a Carbon Monoxide-Utilizing Acetogen, *Eubacterium limosum* KIST612▽

Hanseong Roh,[1] Hyeok-Jin Ko,[1] Daehee Kim,[2] Dong Geon Choi,[2] Shinyoung Park,[2] Sujin Kim,[1] In Seop Chang,[2] and In-Geol Choi[1]*

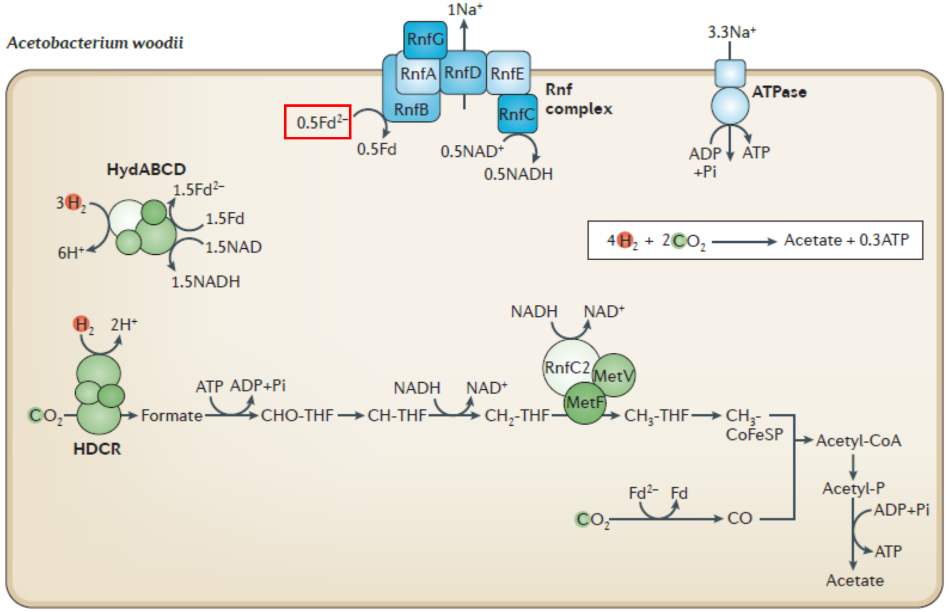
AEM
Journals.ASM.org

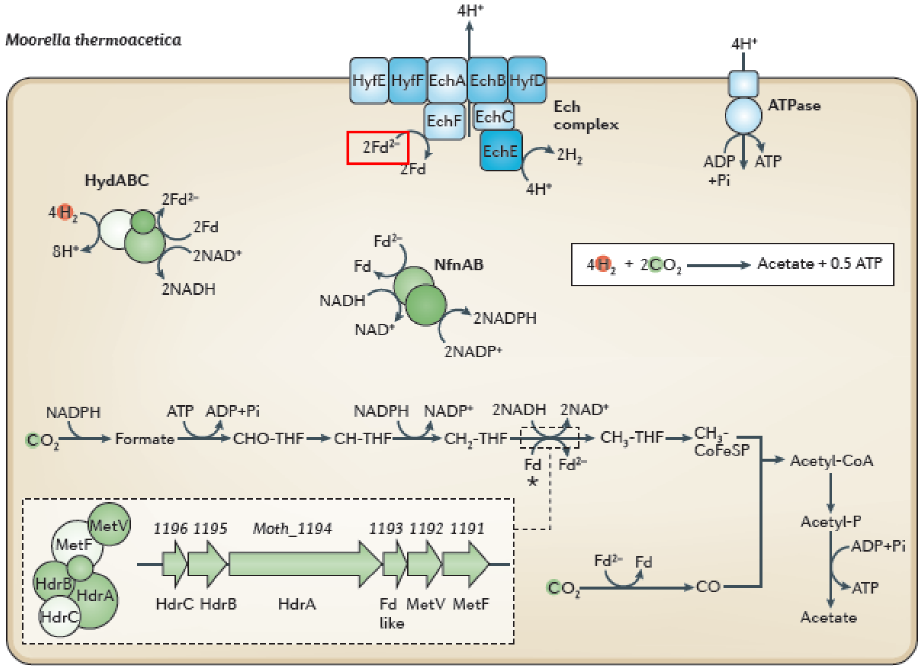
Energy Conservation Model Based on Genomic and Experimental Analyses of a Carbon Monoxide-Utilizing, Butyrate-Forming Acetogen, *Eubacterium limosum* KIST612

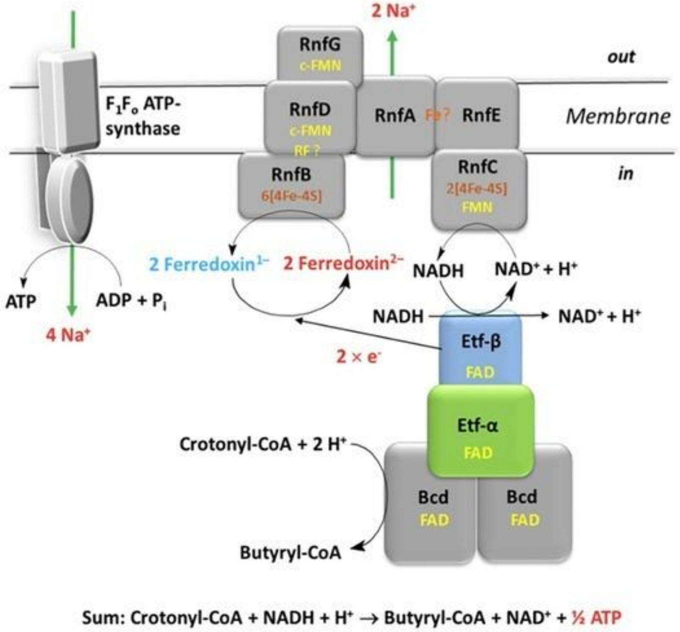
Jiyeong Jeong,[a] Johannes Bertsch,[b] Verena Hess,[b] Sunju Choi,[a] In-Geol Choi,[c] In Seop Chang,[a] Volker Müller[b]

A promising biocatalyst for application in the syngas industry

Bioenergetics of acetogens







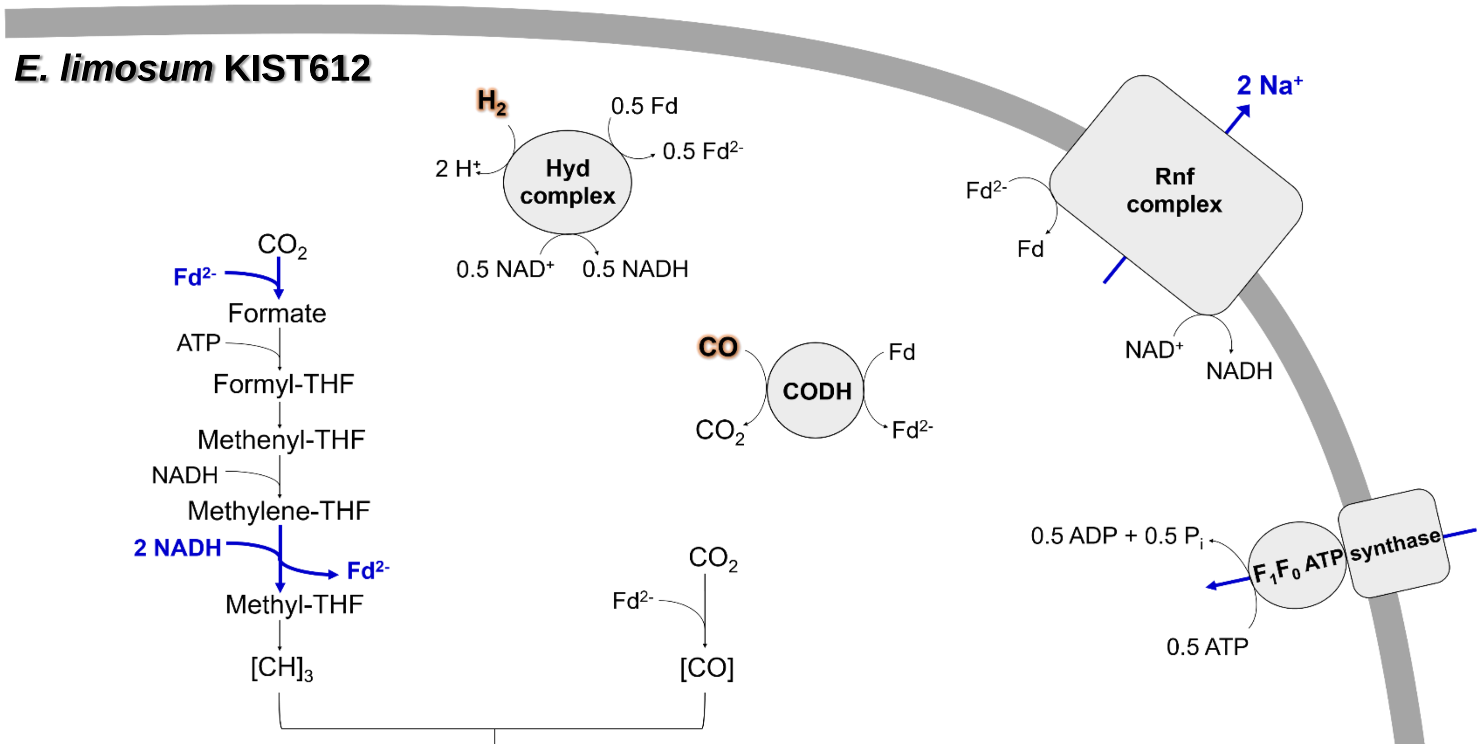
(Chowdhury et al. 2016)

The mechanism of SLP coupled with chemiosmotic energy conservation is highly strain-specific

< Major point of view>

- The enzymes related to the redox reaction of **H^+/H_2**, **Fd/Fd^{2-}**, and **$NAD(P)^+/NAD(P)H$**
- Transmembrane ion pump; **Rnf**(ferredoxin: NAD^+ oxidoreductase) or **Ech**(energy-conserving hydrogenase)
- Ion (**Na^+** or **H^+**) translocating **ATP synthase**

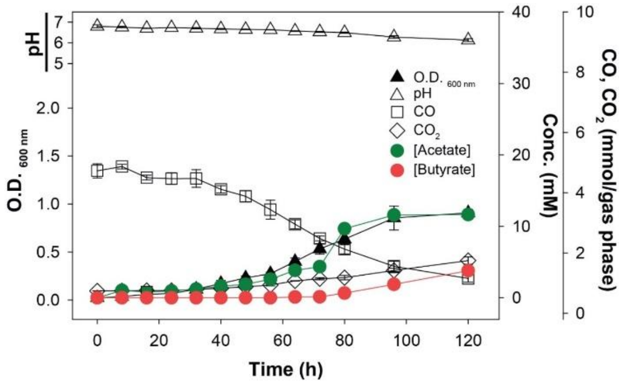
Bioenergetic Benefit: Factor affecting cell growth and metabolites formation



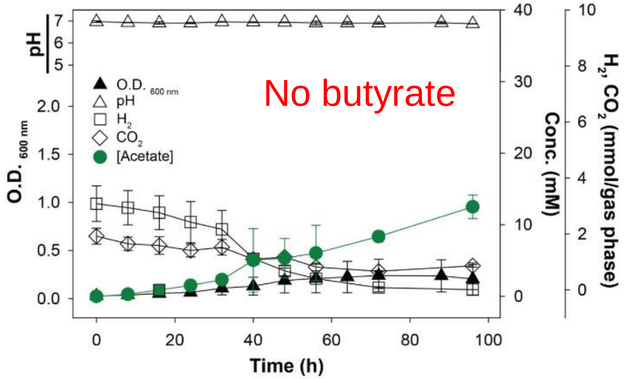
4CO + $2H_2O$ + 1.5ADP + 1.5P_i → **Acetate** + $2CO_2$ + H^+ + 1.5ATP	**0.375 ATP/CO**
10CO + $4H_2O$ + 3.5ADP + 3.5P_i → **Butyrate** + $6CO_2$ + H^+ + 3.5ATP	**0.35 ATP/CO**
$4H_2$ + $2CO_2$ + 0.5ADP + 0.5P_i → **Acetate** + H^+ + 0.5ATP	**0.125 ATP/H_2**
$10H_2$ + $4CO_2$ + ADP + P_i → **Butyrate** + H^+ + $6H_2O$ + ATP	**0.1 ATP/H_2**

- **CO** is more **energetically favorable electron source** than H_2 in *E. limosum* KIST612 strain
- The **energetics reason** according to the type of electron source **affects** whether the **butyrate** was produced or not.

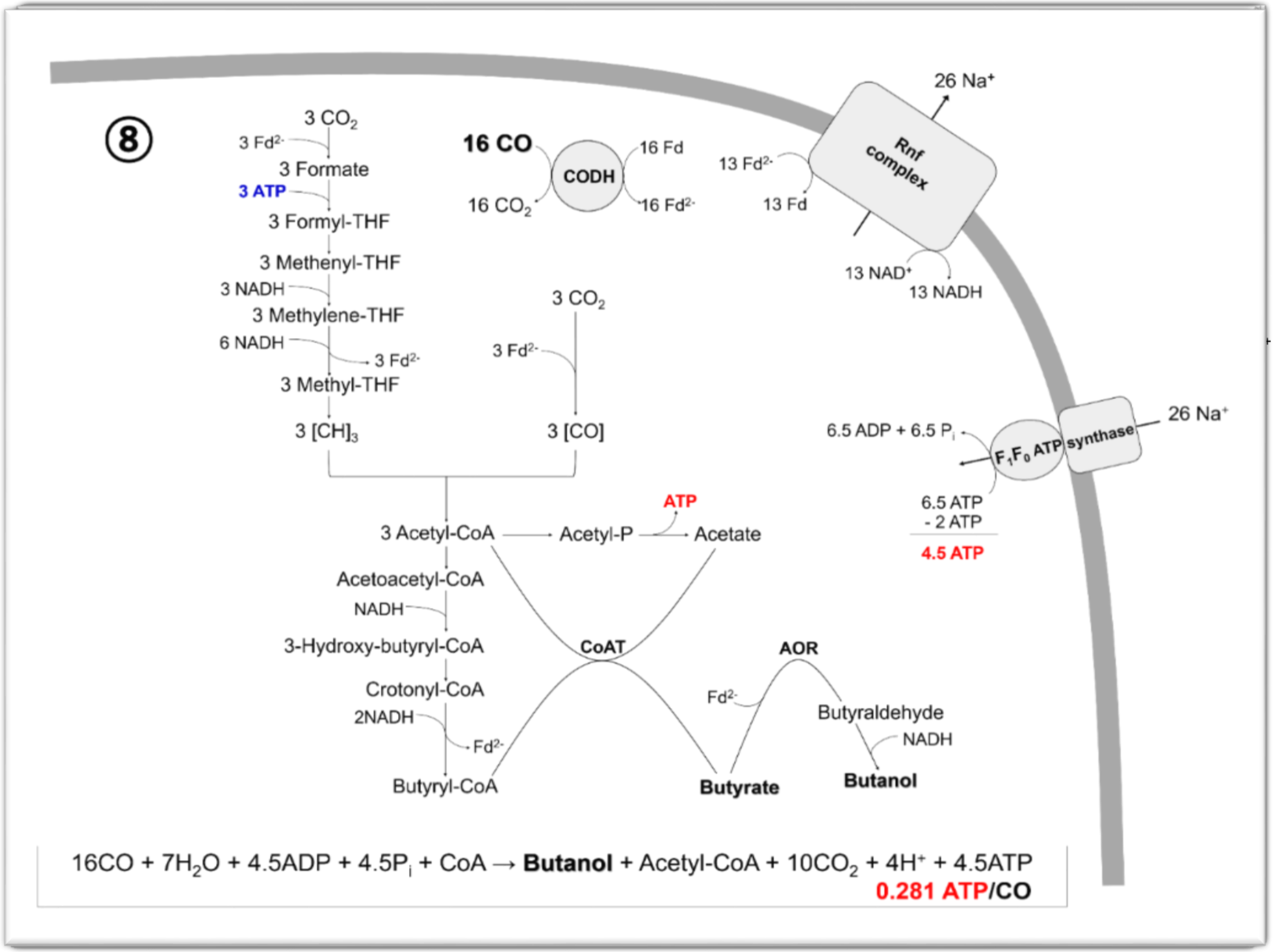
CO condition



H_2/CO_2 condition



Metabolites Available from CO & Bioenergetics Differences



① Acetate :	0.375 ATP/CO
② Ethanol :	0.25 ATP/CO
③ Ethanol(*via* AOR) :	0.333 ATP/CO
④ Butyrate :	0.35 ATP/CO
⑤ Butyrate(*via* CoAT) :	0.286 ATP/CO
⑥ Butanol :	0.292 ATP/CO
⑦ Butanol(*via* AOR) :	0.333 ATP/CO
⑧ Butanol(via CoAT, AOR) :	0.281 ATP/CO

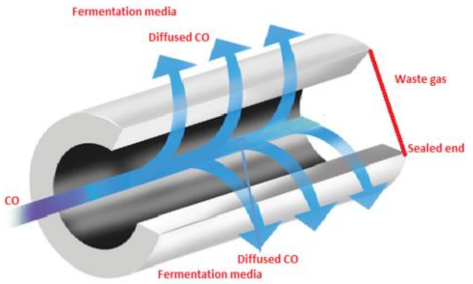
①>④>③=⑦>⑥>⑤>⑧>②

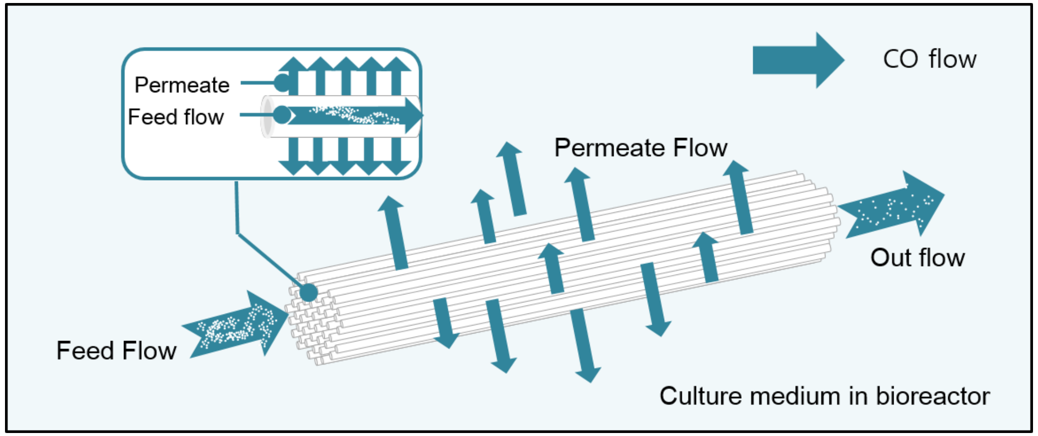
- Acetate production has the highest ATP yield (energetically most preferred).
- Especially, the pathway *via* AOR has high ATP yield to produce alcohol by assimilation of acid product.

Bioreactor configuration and operation

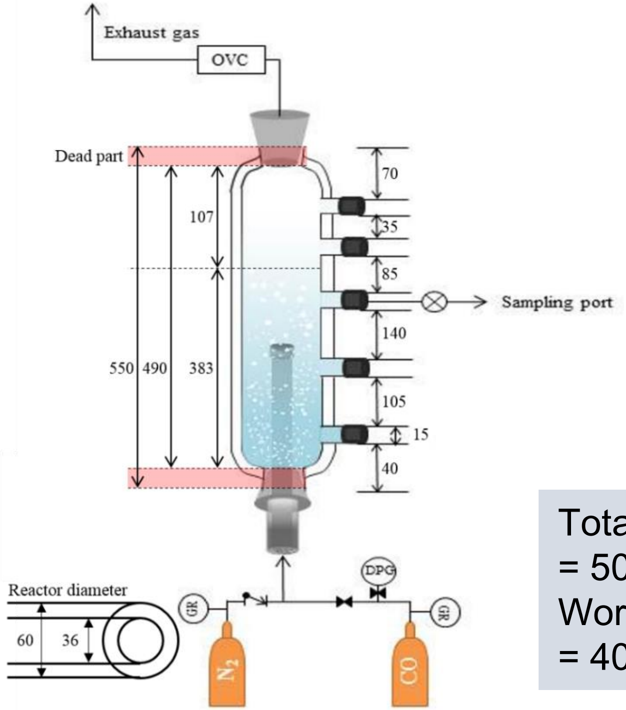
Hollow fiber membrane bioreactor

Membrane characteristics				Configuration	A_s/V_L (m^{-1})	Pressure through lumen (kPa)/Water recirculation rate (mL min^{-1})	Mixing reservoir (rpm)	k_La (h^{-1})	Reference
Membrane Material	Water interaction	Pore size (μm)	ID/OD (μm)						
PVDF	Hydro phobic (on CO)	0.1	700/1200	Stand alone[1] (inside out)	62.5	37.23/not used	Not used	155.2	Yasin et al. (2014)
PP	Hydro phobic (on CO)	0.04	220/300	External[2] (inside out)	175	103.4/1000	200	1096.2	Shen et al. (2014b)
PVDF	Hydro phobic (on CO)	0.2	800/1400	Internal[3] (inside out)	2250	203/1500	Not used	1.36^	Zhao et al. (2013)
PS	Hydrophilic (on O_2)	n/a	500/660	External (inside out)	4366	1-2SLPM*/80	Not used	55	Orgill et al. (2013)
PES	Hydrophilic (on O_2)	n/a	1100/1300	External (inside out)	2271	1-2SLPM*/80	Not used	23	Orgill et al. (2013)
PDMS	Hydrophobic (on O_2)	Non-porous	200/300	External (inside out)	10,000	1-2SLPM*/400	Not used	1062.0	Orgill et al. (2013)
PP	Hydro Phobic (on CO)	0.2	376/426	External (inside out)	56	114.5/670	90	385.01	Lee et al. (2012b)
CHF	Hydro Phobic (on CO)	n/a	200/240	External (outside in)	200	206.8/1500	Not used	946.0	Munasinghe and Khanal (2012)
CHF	Hydro Phobic (on CO)	n/a	n/a	Internal (inside out)	200	241/500	Not used	1.08	Munasinghe and Khanal (2010b)



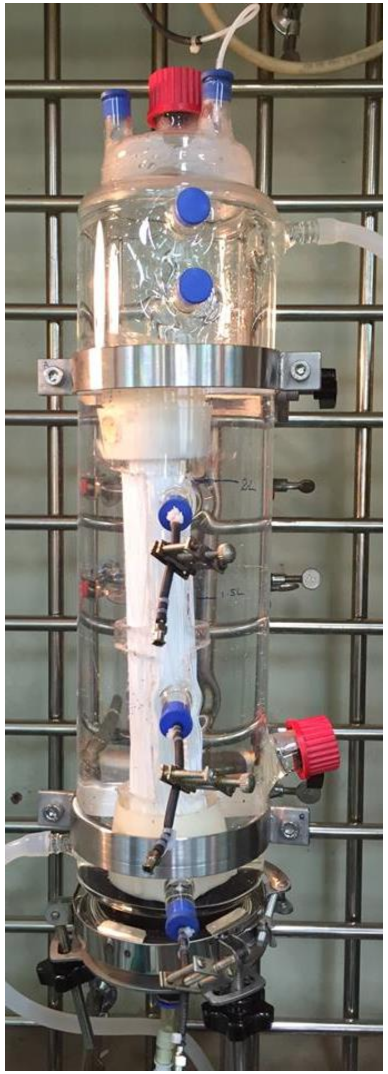


Hollow fiber membrane module (Master thesis book, Yeseul Jeong, 2014)



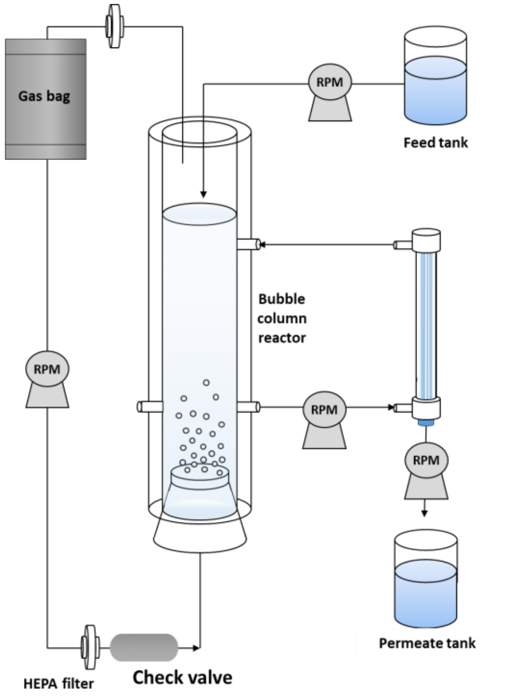
Schematic of reactor setup used to measure k_La.
Reactor dimensions are not to the scale. GR (gas regulator), DPG (digital pressure gauge), OVC (overhead vapours collector)

Total volume (V_T) = 500 mL
Working volume (V_W) = 400 mL



GAS-LIFT REACTOR

Reactor operation: Continuous CO fermentation by AdhE2 transformants of KCTC13263BP

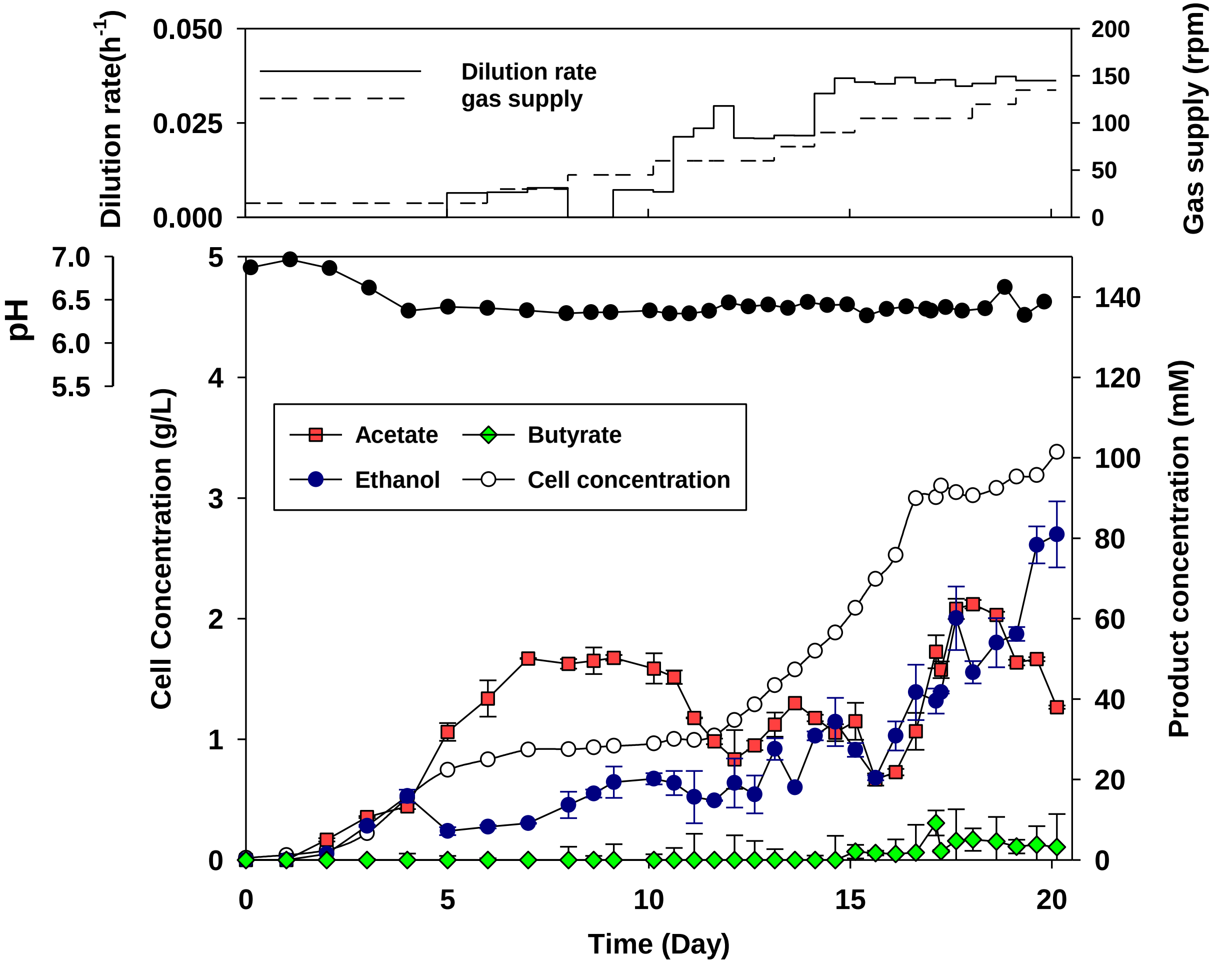


Culture condition for *E. limosum* KCTC13263BP

CBBM	Amount
Basal medium	50ml/L
Trace solution	10ml/L
Yeast extract	2g/L
L-cysteine	0.5g/L
Rezazurin	0.1ml/L
Bicarbonate	2.1g/L
pH	7.0
Vitamin	1 %
P-source (Sodium phosphate buffer)	1 %
Na_2S	Added during the operation for Batch mode

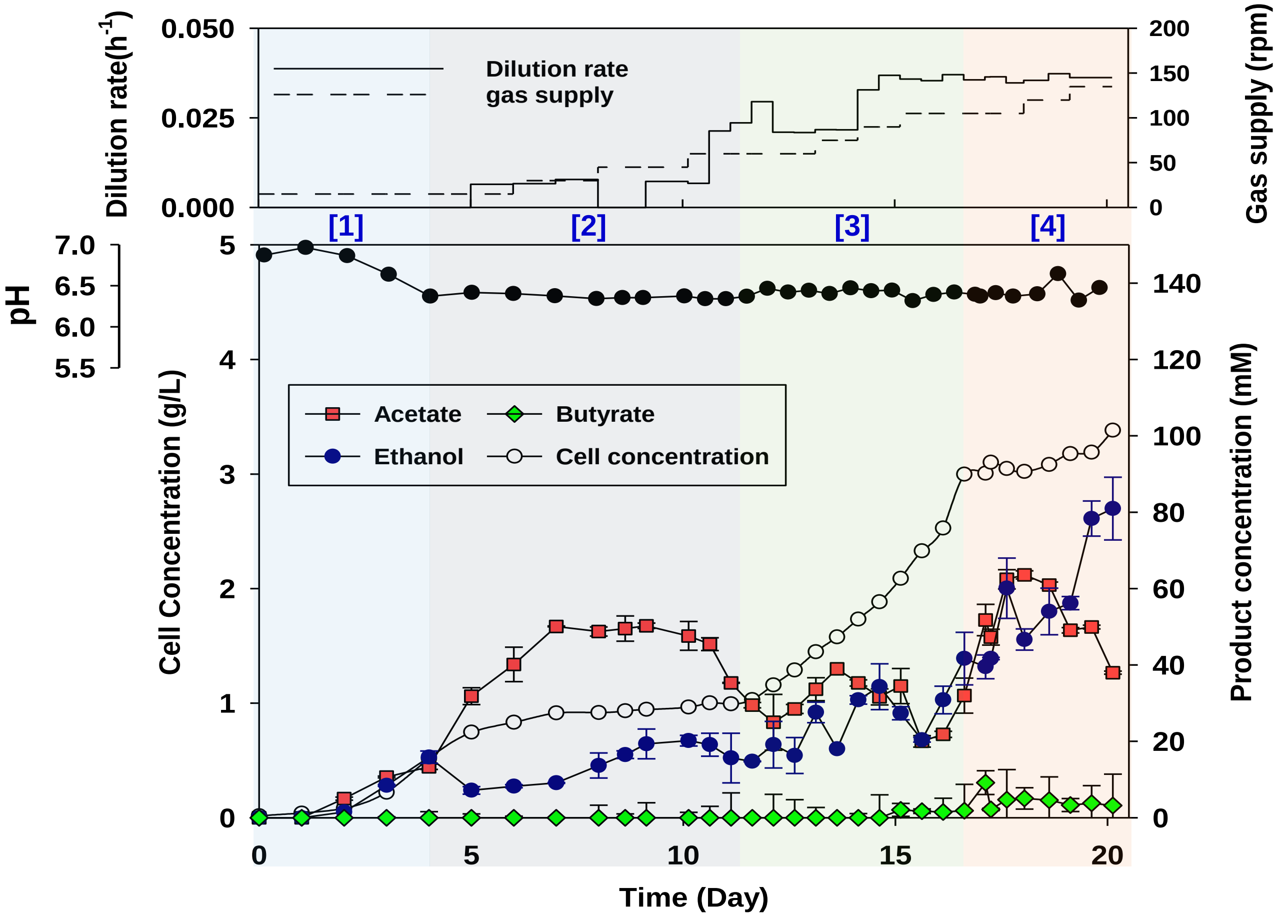
Specification

Reactor volume : 0.5 L
Working volume : 0.4 L
Gas supply : polymeric Spurger
Gas : 8:2 mixture at 1atm (Injected with gasbag)
Temperature : 37℃



Acetate (mM)	Ethanol (mM)
37.9	80.9

Reactor operation; Continuous CO fermentation by AdhE2 transformants of KCTC13263BP



[1] Batch mode operation, inoculation stage

- Initial operation : Batch mode (5days)
- pH decrease to 6.5 and monitoring products

[2] Convert to the continuous mode

- Convert to continuous mode, maintain pH 6.5
- Dilution rate (D) : 0.007 h^{-1}
- Mass transfer (kLa) : 15 rpm to 60 rpm
- Concentration of ethanol increased with maintain biomass

[3] Biomass increasing stage

- **Increase** Dilution rate (D) : 0.02 to 0.03 h^{-1}
- **Increase** Mass transfer (kLa) : increased to 105 rpm
- Cell concentration increased : 1.0 g/L to 2.9 g/L
- Product concentration decreased by increasing dilution rate

[4] Ethanol production stage

- Cell concentration maintained
- Acetate concentration decrease, ethanol concentration increased

BCR operation results

Biomass [g/L]	**Max. μ (/hr)**	**Batch mode (hr)**
3.4	0.041	96
Acetic acid [mM]	**Ethanol [mM]**	**Continuous mode (hr)**
37.9	**80.9**	**390**

- ➢ During continuous mode, the production rate of acetate is faster than the re-assimilation rate of acetate to ethanol due to the continuous growth of cells.
- ➢ In the stagnation period of cell growth, acetate assimilatory ethanol production was observed.

Take-Home Message

[Specific Features in Elm]

- Acetate is #1 metabolite but others could be naturally/artificially available from CO oxidation **(TRUE)**
- Butyrate is naturally producible **(TRUE)**
- Na+ dependent Rnf system: Fd2- for Na+ translocation and ATP synthesis (Like *A. woodii*) **(Confirmed)**
- Existed Fd dependent Acetate OxidoReductase (AOR) **(function?)**
- ……

[In this study]

- One of Two routes for ethanol production from acetyl-CoA: Acetate-assimilatory ethanol production.
- Aldehyde Fd oxidoreductase (AOR) is a key enzyme for Acetate-assimilatory ethanol production
- AdhE not Adh for Acetate-assimilatory ethanol production in Elm transformant.
- AdhE transformant showed no butyrate formation (could be competitive product to consume reducing power)
- Acetate-assimilatory ethanol production only showed in CO (autotrophic) fed condition (could be energetics issue?)
- Adh transformant not showed production of ethanol (could be due to type of reducing power, NADPH?)
- High production of ethanol (as major product on CO condition): Homoethanogenesis
- Acetate and ethanol titers: depending on the difference of acetate production rate and reassimilation rate of acetate to ethanol

Meet Scientists and Engineers for detail info

[On 21 Jan]

Board No. 16 “Heterologous Expression of Alcohol Dehydrogenase Enables Concentrated Production of Non-Native Ethanol from Carbon Monoxide in Acetogen, *Eubacterium limosum* KCTC13263BP”

Board No. 17 “Development of Optimised CRISPR/Cas9 System for Acetogenic Bacterium, *Eubacterium limosum* KIST612”

Board No. 19 “Simultaneous Gas- and Cell-Recycled Continuous Carbon Monoxide Fermentation Under Open-, Closed- and Mixed Circuit to Boost-up Biomass and Product Titer Using Acetogen Strain, *Eubacterium limosum* KIST612”

[On 23 Jan]

9:40-10:00 “Microbioprocessing: Optimum Reactor Design for Carbon Monoxide Fermentation”

By Dr Muhammad YASIN

External collaborators

Prof. In Geol Choi
at Korea Univ.
(Genomics & Transcriptomics)

Prof. Lee Krumholz
at Oklahoma Univ, USA
(Acetogens Physiology)

Prof. Dr. Volker Müller
at Goethe-University Frankfurt
(Electron bifurcation)

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