POLYTECHNIQUE MONTRÉAL

LE GÉNIE EN PREMIÈRE CLASSE

Canada Research Chair on the Development of Metabolic Engineering Tools

Acetone-Butanol-Ethanol (ABE) Fermentation by *Clostridium* Species Study

Xinhe Zhao (Ph.D candidate)

Supervised by: Prof. Mario Jolicoeur

Table of Content

1. Background

OIL CRISIS

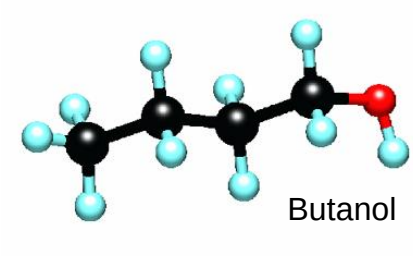
Abundant and economical energy is the life blood of modern civilizations.

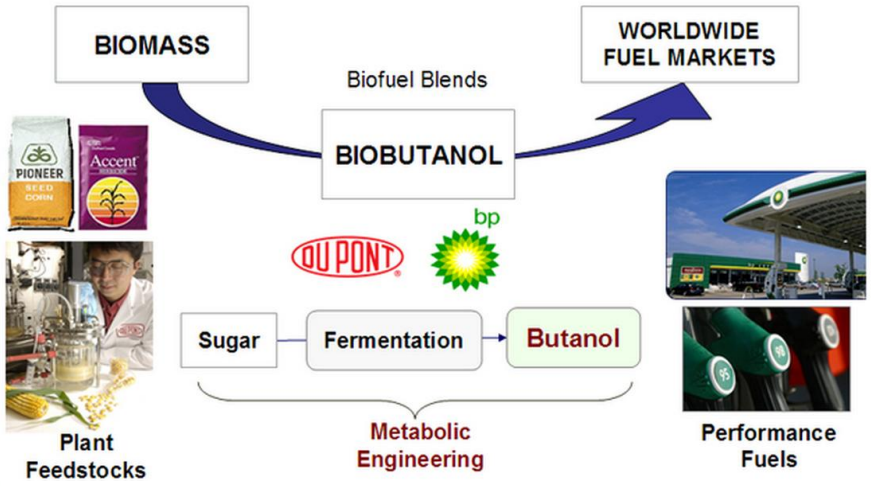
The coming era of limited and expensive energy will be very difficult for everyone

”Clearly, we live in the age of oil, but the age of oil is drawing to a close”.

If oil production remains constant until it's gone, there is enough to last **42** years.

1. Background





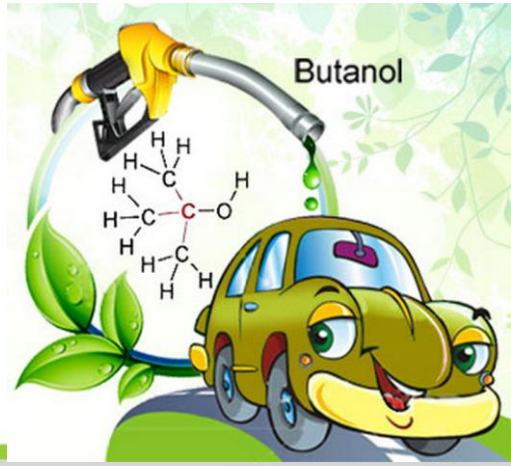
non-corrosive and can be **shipped via pipeline** rather than in rail tanks and tank trucks.

Can be **blended** up to 40% with diesel.

Lower emissions: reduces hydrocarbon emissions by 95%; carbon monoxide to 0.1%; and oxides of nitrogen by 37%.

Can be made from a **wide variety of non-food feedstocks** such as wood and forest residues…….

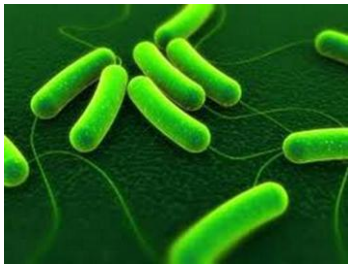
Fuel Type	Density (kg/m3)	Specific energy (kJ/g)	Energy content (MJ/L)
n-butanol	810	36	29.2
Ethanol	794	26.5	23.5
Gasoline	740	44	32.6



1. Background

Chaim Weizmann

1916



Clostridium acetobutylicum

"Weizmann Organism"

ATCC 824

In ABE fermentation, acetone, ethanol and butanol are produced from glucose or other carbon resource using strains of *Clostridia*, which are strictly anaerobic bacteria.

The current international price of biobutanol is about $4 per gallon and the worldwide market is about 10-12 billion pounds per year and with 3% growth [a].

As far back as 2007, the United States have passed the new energy bill (H.R.6) which defined butanol as "advanced biofuels". U.S Navy also cooperated to Cobalt Technologies company develop biobutanol as military jet fuel recently . (www.cobalttech.com)

a: Ranjan, A. and V.S. Moholkar, *Biobutanol: science, engineering, and economics*. International Journal of Energy Research, 2012. **36**(3): p. 277-323

2. Cells view – strain statistics

Strain	Butanol yield (g/L)		T (℃)
	glucose as substrate	other substrates	
C. acetobutylicum ATCC 824	16.9	11-17	37
C. beijerinckii NCIMB 8052	10.7	8-12.3	35-37
C. saccharoperbutylacetonicum N1-4 (ATCC 13564)	20.1	10-20	30
C. beijerinckii BA101	17,19	13-17	37
C. beijerinckii P260	-	14	35
C. beijerinckii ATCC 10132	20	-	37
C. beijerinckii DSM 6423	11.2	-	37
C. beijerinckii mutant RT66	-	9.3	35
C. beijerinckii ATCC 55025 （derived from ATCC 4259）	-	8.8	37
C. acetobutylicum DSM 792	-	9.5-11	37
C. acetobutylicum B3 (CGMCC 5234)	-	15.4	37
C. acetobutylicum JB200 (derived from ATCC 55025)	19	-	37
C. acetobutylicum mutant NT642 （D64）	-	15.4	37
C. acetobutylicum BKM19(derived from PJC4BK)	17.6g/L	-	37
C. acetobutylicum EA 2018 (CCTCC M94061)	-	13	37
C. tyrobutyricum ATCC 25755	20.5	16	37
C. saccharobutylicum DSM 13864	-	13.4	35
C. pasteurianum CH4	13.3		37
C. pasteurianum MBEL_GLY2(derived from *C. pasteurianum* ATCC 6103)	-	10.8	37

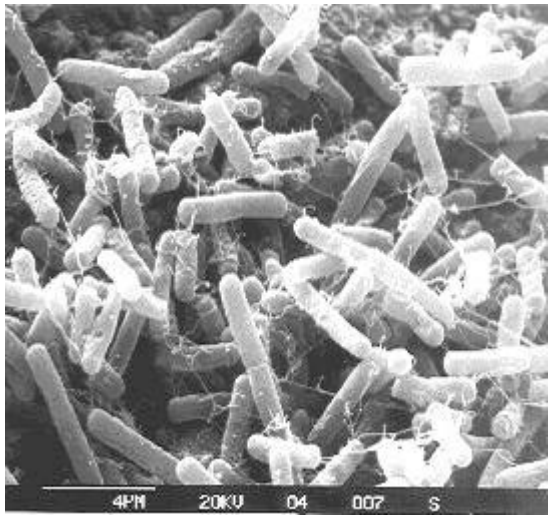
C. acetobutylicum
C. beijerinckii
C. saccharobutylicum
C. saccharoperbutylacetonicum

E. coli

Saccharomyces cerevisiae

In summary, *Clostridium* is still the most dominant species for biobutanol production.

2. Cells view



https://www.youtube.com/watch?v=9QSIWObVya4

https://www.youtube.com/watch?v=EWdKuhCmEnI

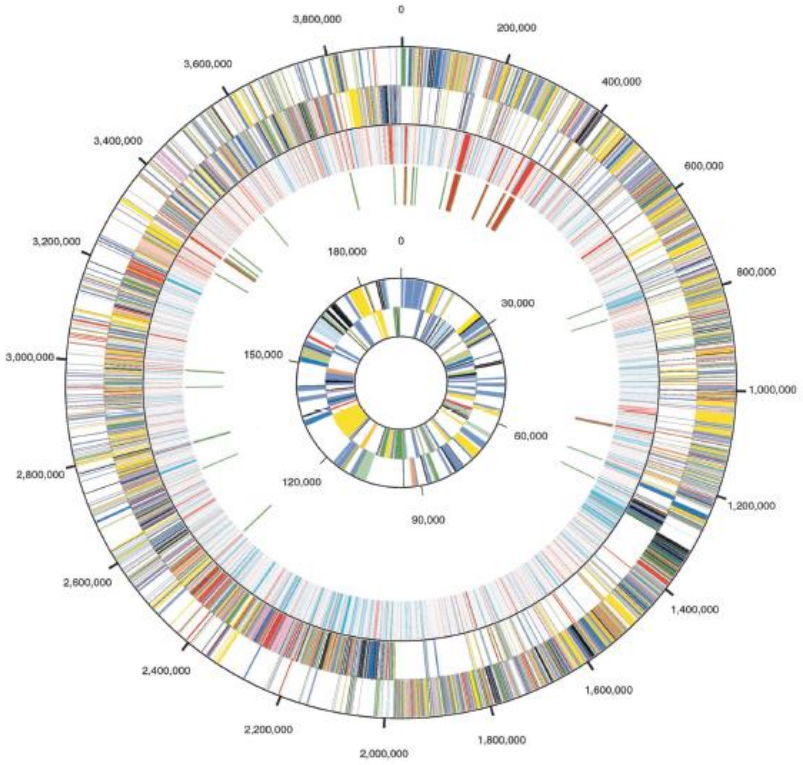
2. Cells view- Genome Sequence

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

In 2001

Genome Sequence and Comparative Analysis of the Solvent-Producing Bacterium *Clostridium acetobutylicum*

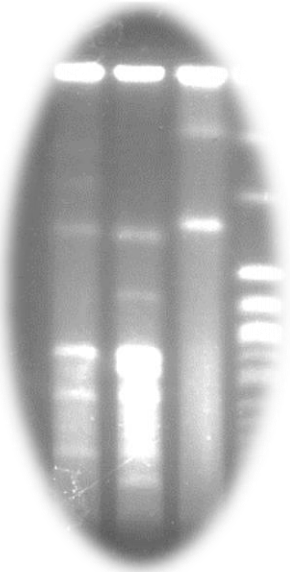
Jörk Nölling[1], Gary Breton[1], Marina V. Omelchenko[2], Kira S. Makarova[2,3], Qiandong Zeng[1], Rene Gibson[1], Hong Mei Lee JoAnn Dubois[1], Dayong Qiu[1], Joseph Hitti[1], GTC Sequencing Cente Production Finishing, and Bioinformatics Teams[1 †], Yuri I. Wolf[3], Roman L. Tatusov[3], Fabrice Sabathe[4], Lynn Doucette-Stamm[1], Philippe Soucaille[4], Michael J. Daly[2], George N. Bennett[5], Eugene V. Koonin[3], and Douglas R. Smith[1,*]



2. Cells view- Genetic technology

Genetic modification of *Clostridium* is widely used by inserting some heterologous genes or overexpressing or knocking out/down some relative endogenous genes to improve the butanol production

Example: The acetoacetate decarboxylase gene (adc), which has been proved being responsible for acetone production in hyper-butanol producing industrial stain.

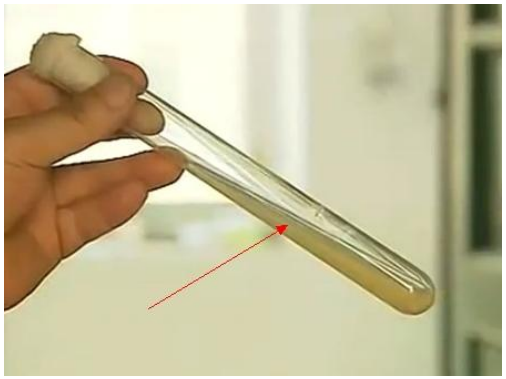


2. Cells view- Genetic technology

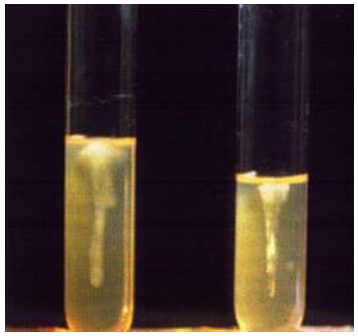
The list of key enzymes of the butanol synthetic pathway in *C. acetobutylicum*

Pathway	Key enzyme	ab.	Gene	EC no.	Mr (kDa)
Lactate synthetic pathway	Phosphate acetyltransferase	PTA	pta	2.3.1.8	36.2
	Acetate kinase	AK	ack	2.7.2.1	44.3
Butyrate synthetic pathway	Phosphate butyryltransferase	PTB	ptb	2.3.1.19	264
	Butyrate kinase	BK	buk	2.7.2.7	85
Butanol synthetic pathway	Acetyl-CoA acetyltransferase	THL	thiL	2.3.1.9	41
	β-hydroxybutyryl-CoA dehydrogenase	BHBD	hbd	1.1.1.35	30.5
	Enoyl-CoA hydratase (crotonase)	CRT	crt	4.2.1.17	158
	Butyryl-CoA dehydrogenase	BCD	bcdetfBetfA	1.3.99.2	33
	Butyraldehyde dehydrogenase	BAD	aad	1.2.1.57	56
	Butanol dehydrogenase	BDH	bdh AB	1.1.1.1	42
Acetone synthetic pathway	Acetoacetate decarboxylase	AADC	adc	4.1.1.4	28
	CoA-transferase	CoAT	CtfA/B	2.8.3.9	93
Ethanol synthetic pathway	Acetaldehyde dehydrogenase	ALDH	aad	1.2.1.10	96
	NAD(P)H alcohol dehydrogenase	ADH	adh	1.1.1.2	44

3. Culture experience – cell storage



4℃， 3-6 month



4℃， 1-3 month

Half-solid medium

-80℃， 1-2 years

Glycerin or dimethyl sulfoxide

5-15 years

Skimmed milk powder

1-10 years

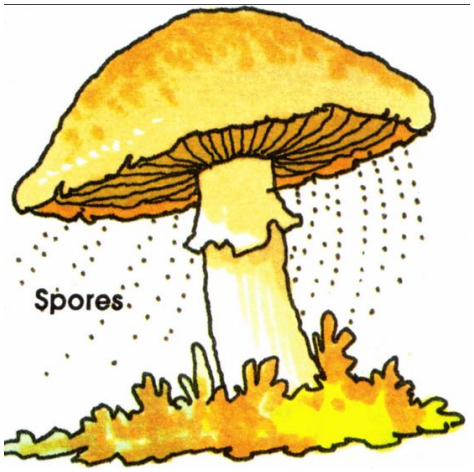
Sand with medium

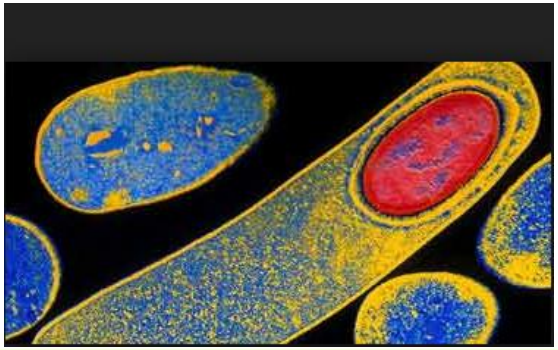
-196℃， 1-2 years

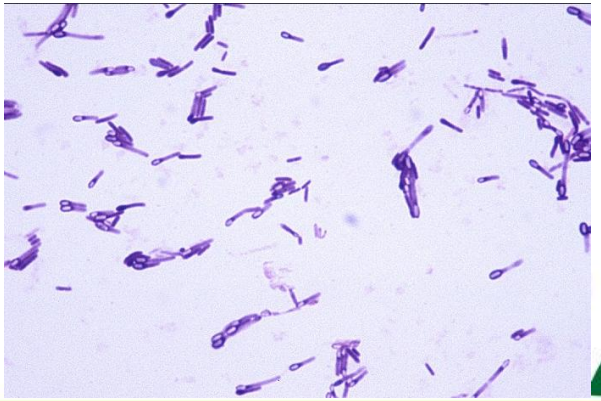
Glycerin or dimethyl sulfoxide

Discussion 1 :

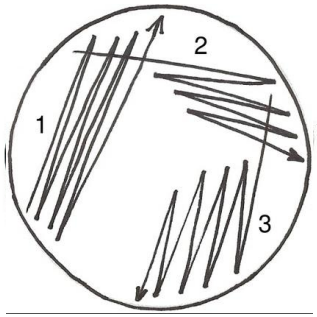
Describe multiplicative process and mechanism of *Clostridium* species in different environments.

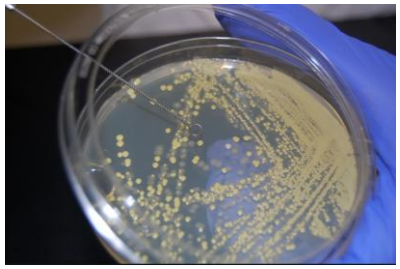


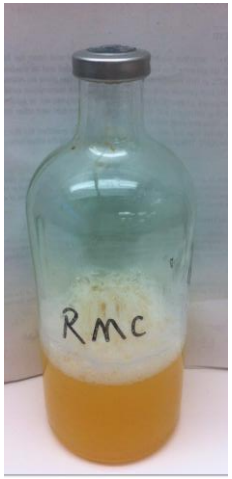




3. Culture experience – scale up







3. Culture experience –industrialization & scale up

The scale up process of cell /bioreactor platform includes a lot of influencing factors.

1. First of all, the design of the bioreactor when scaling up is very important**. H/D ratio** of bioreactor is one key element of fermentation tank design, when the height of the liquid increase, the oxygen pressure in the liquid will increase, so the oxygen transfer coefficient $K_L a$ will increase accordingly. So, keeping an appropriate H/D ratio is very important.

ÉCOLE POLYTECHNIQUE MONTRÉAL

Cours GCH8650

GÉNIE BIOCHIMIQUE

La problématique cellulaire

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Professeur titulaire

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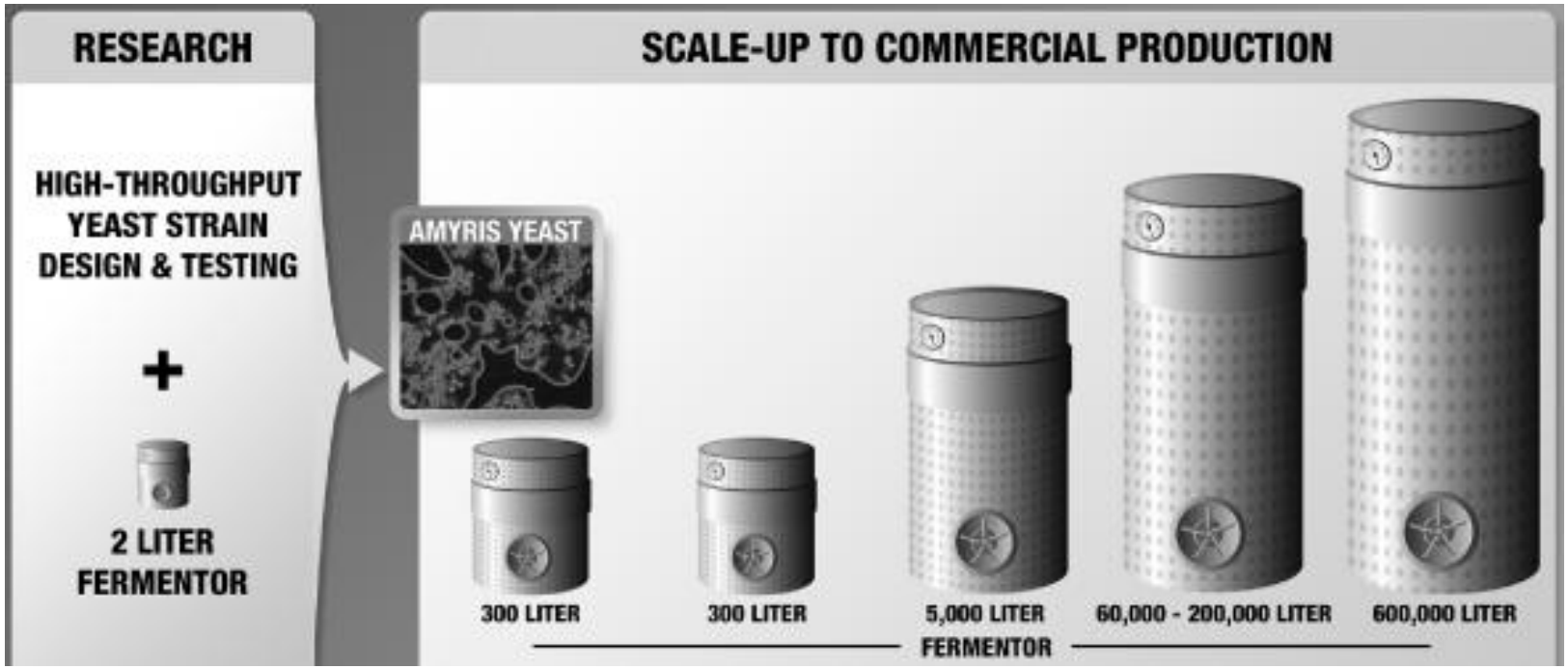
bureau JAB-3069

3. Culture experience –industrialization & scale up

2. The **pressure**, **temperature**, **oxygen absorption** et. al. We should also consider the **economy of energy consumption, environment protection** and **human resources** in scale up.



3. Culture experience -industrialization

Cathay biotechnology company was claimed the world's largest bio-butanol producer based on active production capacity, 100,000 ton/year in 2011 (reported from consulting firm CMAI in 2011, Chemical Market Associates, Inc.).

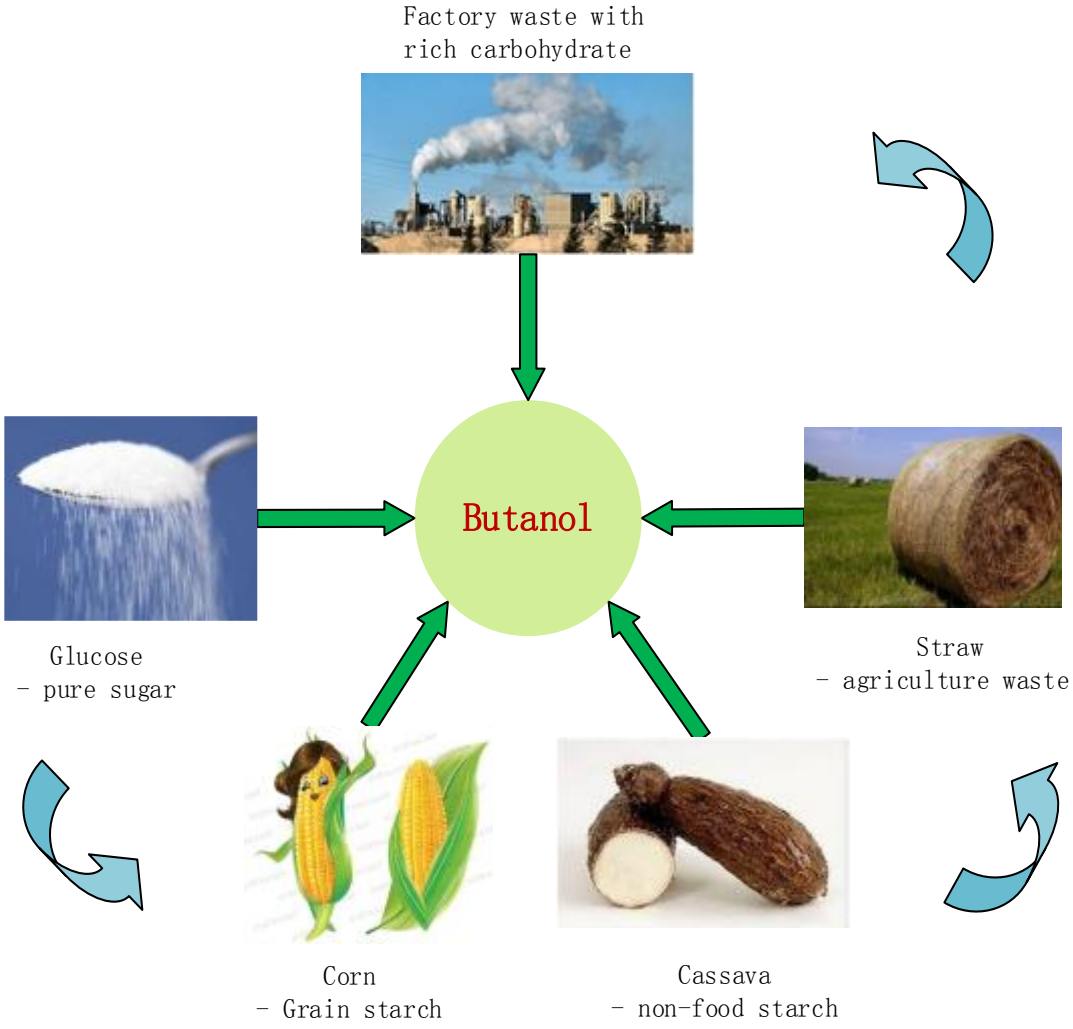
3. Culture experience -industrialization

3. Culture experience -industrialization

Cathay Industrial Biotech Ltd.

Demonstration factory location of Tianguan Group Co., Ltd.

3. Culture experience- raw materials



3. Culture experience

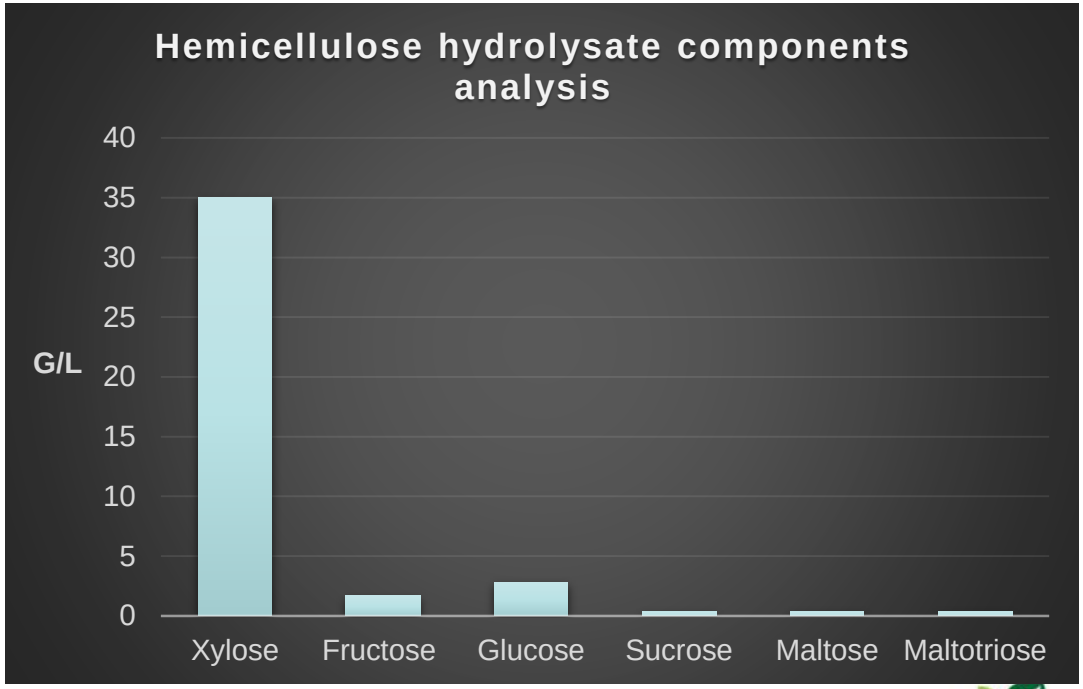
Substrate	Hydrolysis method	Strain used	Yield (g/g)/ Productivity (g/L h)	Total ABE (g/L)
Wheat straw	H_2SO_4 +enzyme	*C. beijerinckii* P260	0.60/0.42	25
Wheat straw	H_2SO_4 + enzyme	*C. beijerinckii* P260	0.41/0.31	21.42
Corn fiber	H_2SO_4	*C. beijerinckii* BA101	0.39/0.10	9.3
Palm oil mill effluent + sago starch	Enzyme	*C. saccharoperbutylace-tonicum* N1-4	0.40/0.10	14.38
Dried distillers' grains and soluble (DDGS)	Ammonium fiber expansion + enzyme	*C. beijerinckii* BA101	0.34/0.14	10.4
Rice bran and defatted rice bran	HCl + enzyme	*C. beijerinckii* NCIMB 8052	0.31/0.26	16.42
Barley straw	H_2SO_4 + enzyme	*C. beijerinckii* P260	0.43/0.39	26.64
Corn stover	H_2SO_4 + enzyme	*C. beijerinckii* P260	0.44/0.31	26.27
Switchgrass	H_2SO_4 + enzyme	*C. beijerinckii* P260	0.39/0.17	14.61
Wheat bran	H_2SO_4	*C. beijerinckii* ATCC 55025	0.32/0.16	11.8
SO_2–ethanol–water (SEW) spent liquor	SO_2–ethanol–water	*C. acetobutylicum* DSM 792	0.20/0.09	8.79
Sugar maple wood	Hot water extraction + sulfuric acid	*C. acetobutylicum* ATCC 824	0.22/0.15	11
Rice straw	H_2SO_4 + enzyme	*C. acetobutylicum* MTCC 481	1.04[a]/0.017	3
Cassava baggase	Enzyme	*C. acetobutylicum JB200*	0.39/0.62	33.87
Maize stalk juice	–	*C. beijerinckii* NCIMB 8052	0.27[a]/0.30	11.5

3. Culture experience

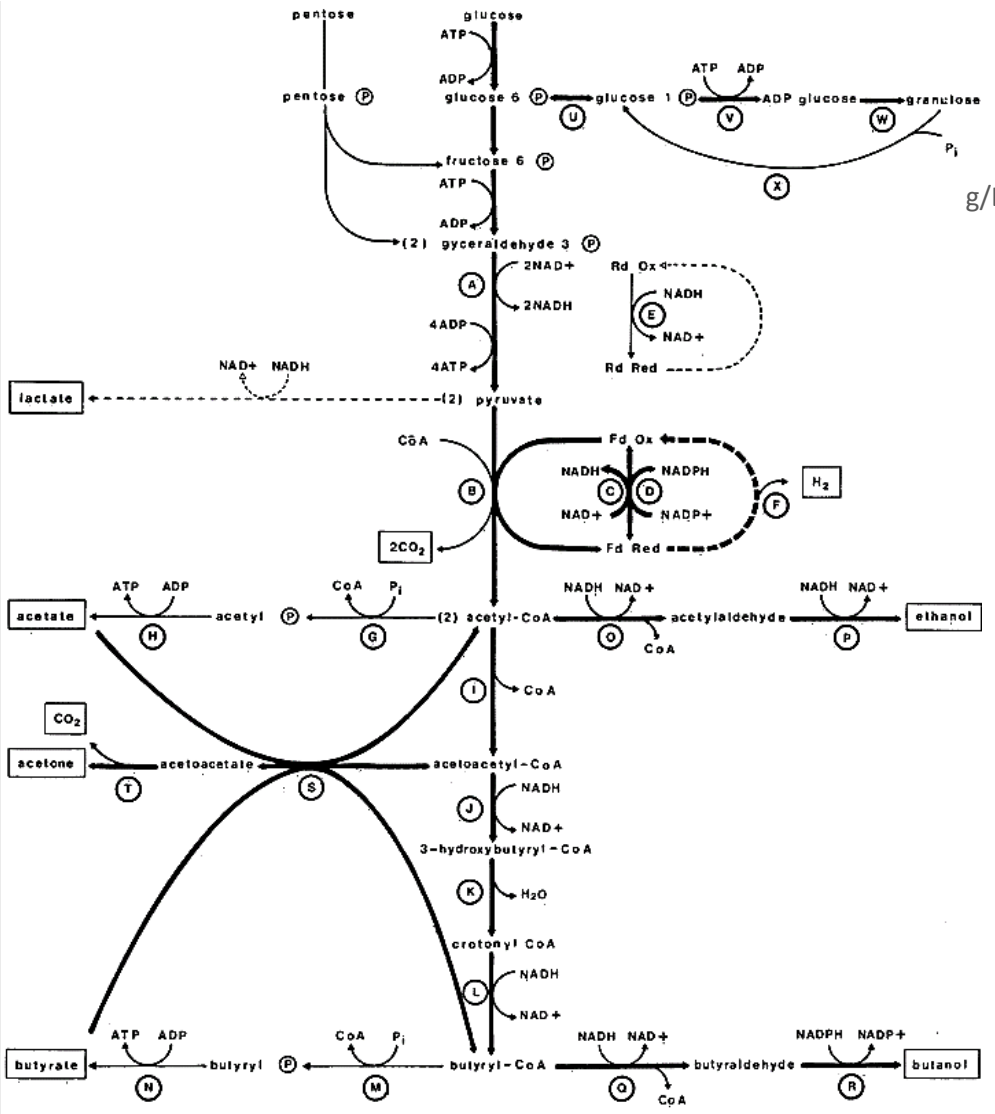
Paper waste water
Black liquor with abundant hemicellulose

→ Hydrolysate → Xylose with other sugars → Fermentation for Butanol

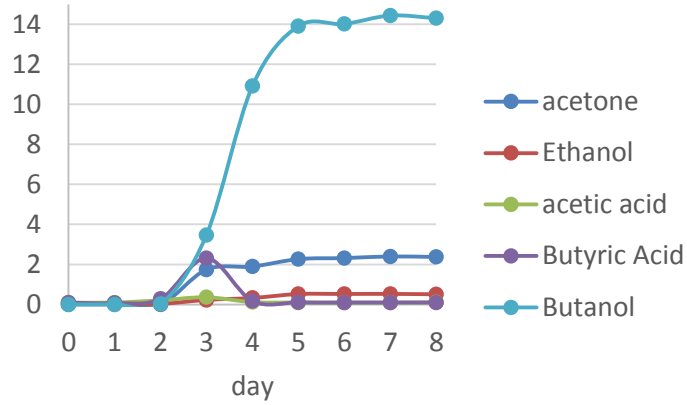
Low cost and be good for environment



4. Metabolism analysis



***C. acetobutylicum* ATCC 824 fermentation**



It is considered as two parts:

Acetogenesis stage

Solventogenesis stage

The central pathway of *Clostridium*

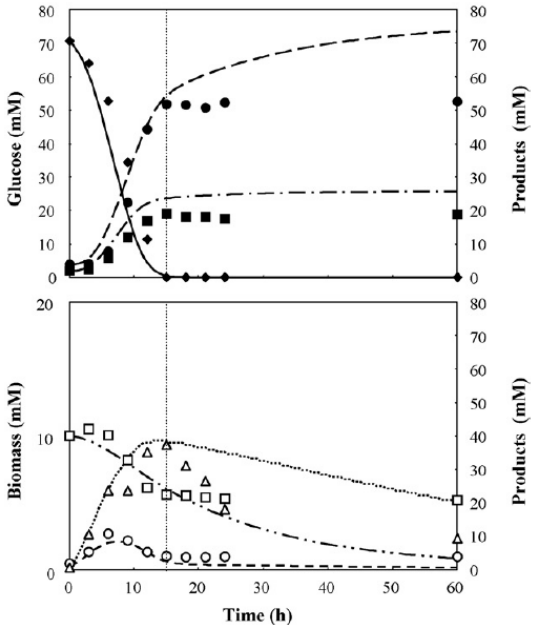
DAVID T. JONES, D.R.W.: 'Acetone-Butanol Fermentation Revisited', American Society for Microbiology, 1986, pp. 484-524

4. Metabolism analysis- Modeling

In 1984, Papoutsakis wrote a stoichiometric balance equation with carbon, hydrogen, oxygen elemental compositions.

Metabolic Flux Analysis (MFA) with stoichiometric and static state studying

In 2007, Shinto *et al* established a dynamic base on central metabolic pathway.



Didn't consider ATP and NADP metabolism

Only simulated substrates, biomass and products, but didn't include any intracellular metabolites

Just considered acetyl-CoA contributed to biomass

Parameters identification used one-at-a-time method.

Shinto, H., Tashiro, Y., Yamashita, M., Kobayashi, G., Sekiguchi, T., Hanai, T., Kuriya, Y., Okamoto, M., and Sonomoto, K.: 'Kinetic modeling and sensitivity analysis of acetone-butanol-ethanol production', Journal of biotechnology, 2007, 131, (1), pp. 45-56

5. Problems identification

- **Solvent toxicity** limited ABE fermentation development.

- **High cost** of raw materials limited ABE industrialization, finding one cost-effective resource become significant.

6. My work in the lab

1. Objectives
2. Culture calibration
3. Pathway development
4. Modeling
5. Conclusion and future works

6.1 Objectives of my project

- Culture calibration and culture process optimization
- Kinetic metabolic mathematical modeling
- Cell engineering for production improvement
- Raw materials hydrolysate inhibition study

6.1 Culture calibration

Strain: ***Clostridium acetobutylicum*** **ATCC824**

Culture conditions: **37℃, statistic culture, strict anaerobic culture**

Medium:

Seed medium: Modified Reinforced Clostridial broth (**MRC**) medium (ATCC Medium No. 2107)

Production medium: Modified Clostridia Growth Medium (**CGM**) (xylose instead of glucose)

Analysis equipment

Instruments	Info.	Comments
GC-FID	Perkin Elmer	Clarus 480
LC/MS/MS	Agilent	HPLC 1290; Mass sp. 6460A; Trip Quadrupole
LC/MS	Waters	MS: ZQ; HPLC:1250
IC	Dionex	HPLC IP20； Conductivity detector
NMR	Agilent	400MHz; 5mm direct probe, 5mm 2D probe, 10mm BB probe, 16mm BB probe.

Choi, S.J., Lee, J., Jang, Y.S., Park, J.H., Lee, S.Y., and Kim, I.H.: 'Effects of nutritional enrichment on the production of acetone-butanol-ethanol (ABE) by Clostridium acetobutylicum', Journal of microbiology, 2012, 50, (6), pp. 1063-1066

Modified Reinforced Clostridial broth (**MRC**) medium

ATCC Medium: 2107 Modified Reinforced Clostridial Agar/Broth (pre-reduced)

Agar Medium

Reinforced Clostridial Medium (BD 218081).....38 g
Agar.....14.5 g
DI Water.....1000 ml

Combine ingredients and boil to dissolve agar. Dispense and autoclave at 121ºC. If making plates, autoclave at 121ºC, let cool to 55ºC and dispense.

Broth Medium

Peptone.....10.0 g
Beef Extract.....10.0 g
Yeast Extract.....3.0 g
Dextrose.....5.0 g
NaCl.....5.0 g
Soluble Starch.....1.0 g
L-Cysteine HCl.....0.5 g
Sodium Acetate.....3.0 g
Resazurin (0.025%).....4 ml
DI Water.....1000 ml

Combine ingredients and dissolve. Adjust pH to 6.8. Dispense and autoclave at 121ºC.

Modified Clostridia Growth Medium (**CGM**)

The Journal of Microbiology (2012) Vol. 50, No. 6, pp. 1063–1066
DOI 10.1007/s12275-012-2373-1

NOTE

Effects of Nutritional Enrichment on the Production of Acetone-Butanol-Ethanol (ABE) by *Clostridium acetobutylicum*

chamber (Forma Scientific, USA) filled with 4% H_2 balanced with N_2 gas. The clostridial growth medium (CGM) containing 0.75 g K_2HPO_4, 0.75 g KH_2PO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.017 g $MnSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$, 1 g NaCl, and 2 g L-asparagine, 0.004 g *p*-aminobenzoic acid, 30 mmol $CH_3COONa \cdot 3H_2O$, and 5 g yeast extract (all per L) was used for test tube, flask and bioreactor experiments throughout this study. Glucose or xylose 80g/L

Choi, S.J., Lee, J., Jang, Y.S., Park, J.H., Lee, S.Y., and Kim, I.H.: 'Effects of nutritional enrichment on the production of acetone-butanol-ethanol (ABE) by Clostridium acetobutylicum', Journal of microbiology, 2012, 50, (6), pp. 1063-1066

Discussion 2:

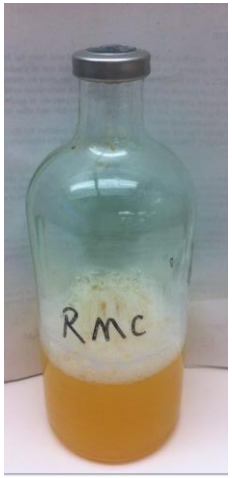
Describe functions and reactive process of each component in MRC medium, and compare differenc between MRC and CGM medium from culture objective.

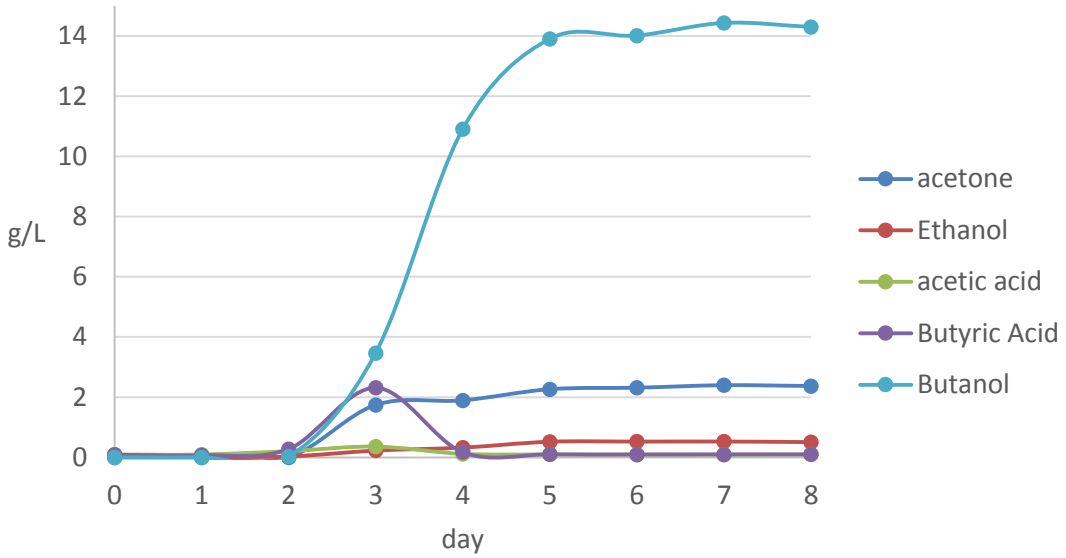
Broth Medium

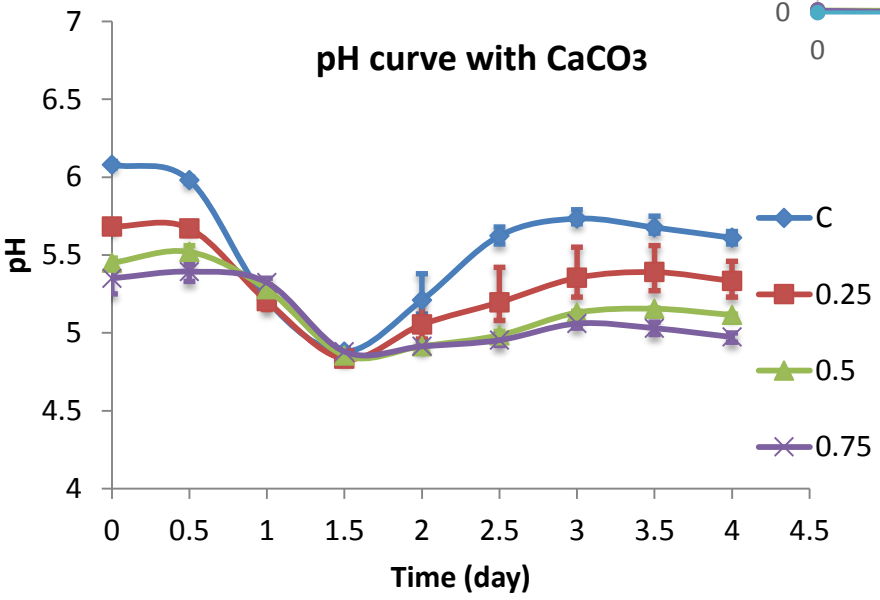
Peptone	10.0 g
Beef Extract	10.0 g
Yeast Extract	3.0 g
Dextrose	5.0 g
NaCl	5.0 g
Soluble Starch	1.0 g
L-Cysteine HCl	0.5 g
Sodium Acetate	3.0 g
Resazurin (0.025%)	4 ml
DI Water	1000 ml

chamber (Forma Scientific, USA) filled with 4% H_2 balanced with N_2 gas. The clostridial growth medium (CGM) containing 0.75 g K_2HPO_4, 0.75 g KH_2PO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.017 g $MnSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$, 1 g NaCl, and 2 g L-asparagine, 0.004 g *p*-aminobenzoic acid, 30 mmol $CH_3COONa \cdot 3H_2O$, and 5 g yeast extract (all per L) was used for test tube, flask and bioreactor experiments throughout this study. Glucose or xylose 80g/L

Preliminary results



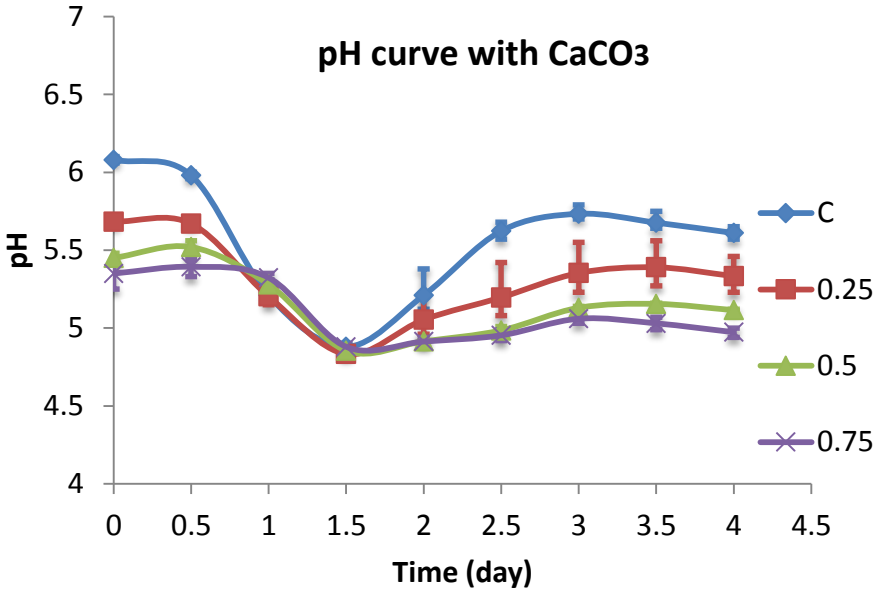




The phenomenon known as **"acid crash"** is an occasional feature of batch fermentations which are performed without any pH control.

Discussion 3:

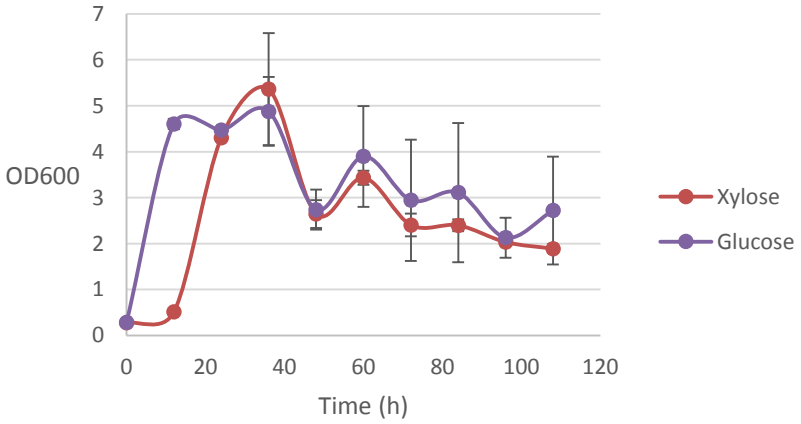
Talk about the functions and reactive principle of $CaCO_3$ during fermentation process. (it should include Ca iron and carbonate)



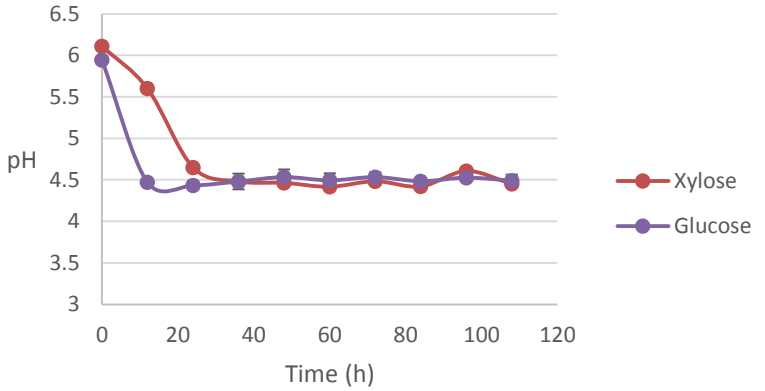
Preliminary results

Comparison of glucose and xylose as carbon source

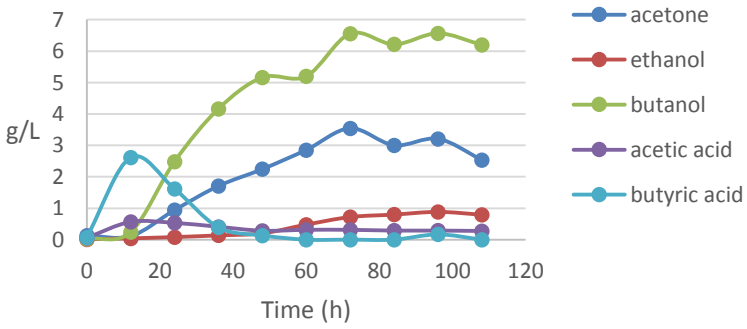
Clostridium acetobutylicum biomass by glucose and xylose as substrate



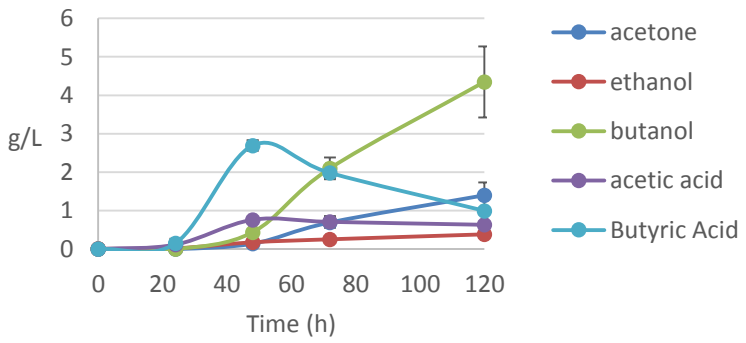
pH change utilizing glucose and xylose as substrate



Glucose fermentation by *Clostridium acetobutylicum*

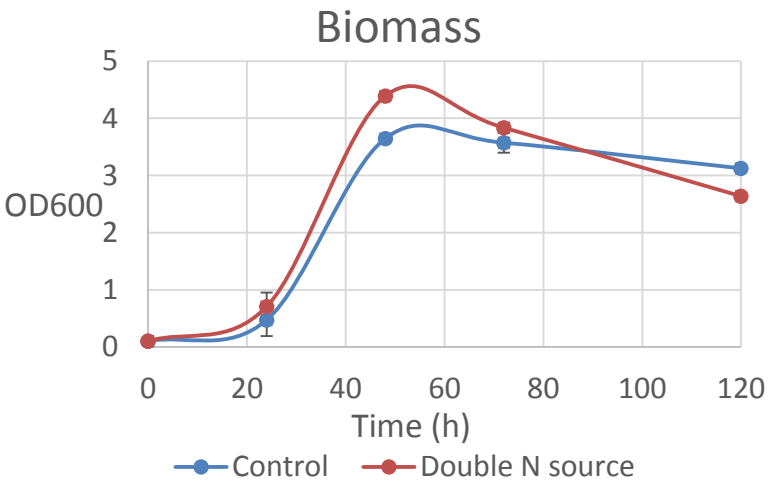


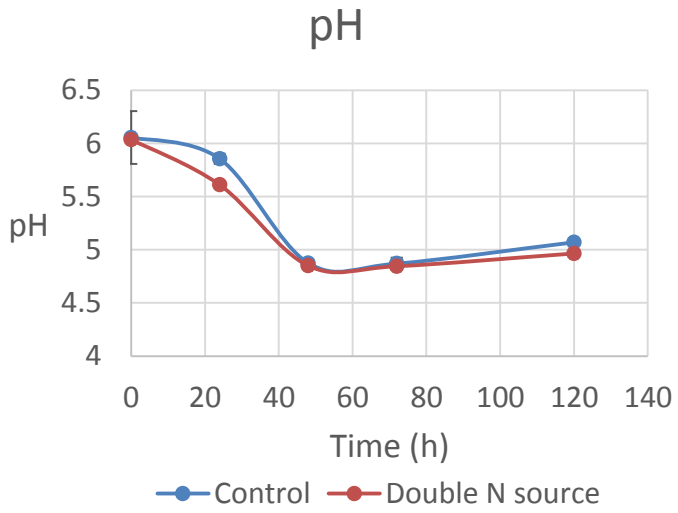
Xylose fermentation by *Clostridium acetobutylicum*

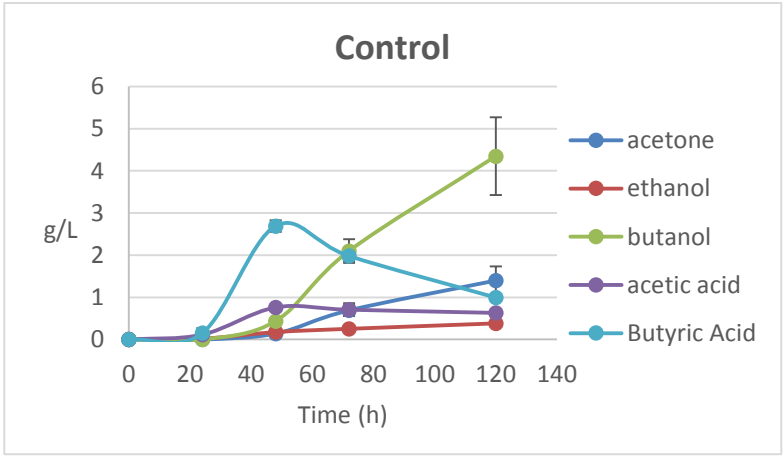


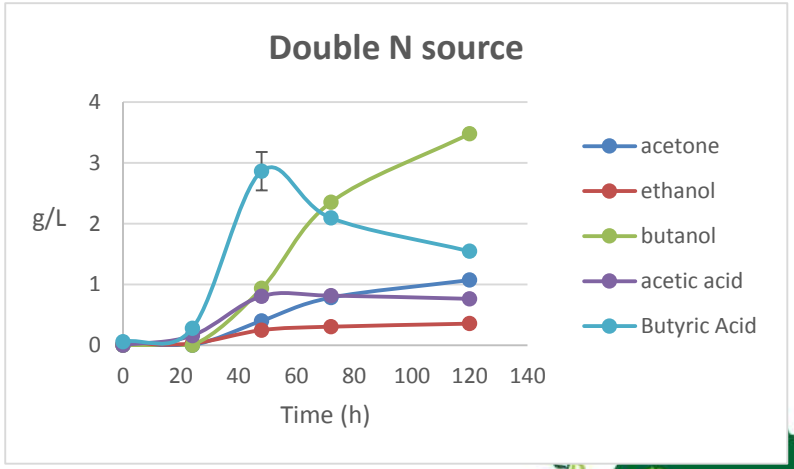
Preliminary results

Comparison of different N content – xylose as carbon source

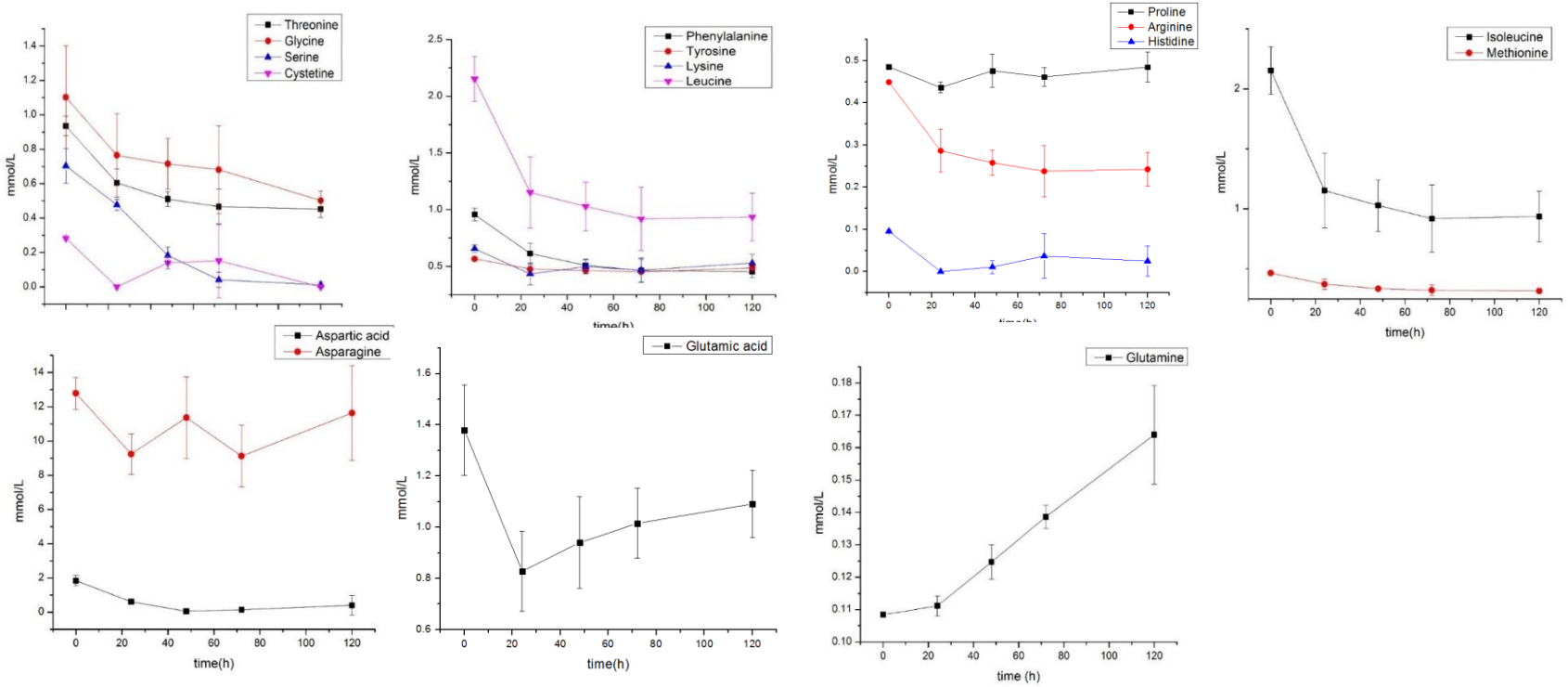






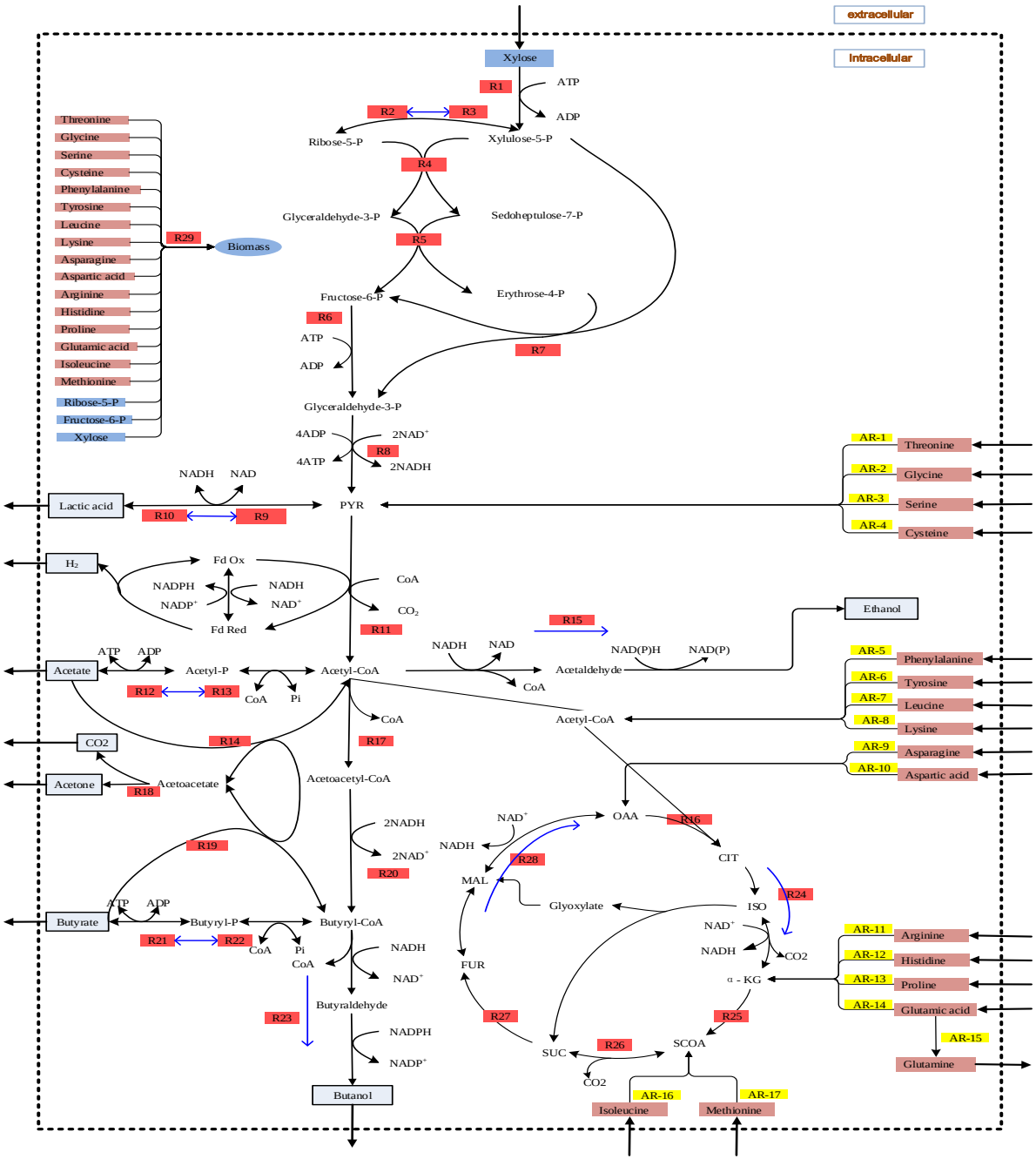


6.2 Pathway development- Amino acid consumption



Group types	Amino acids
Pyruvate group	threonine, glycine, serine, cysteine
Acetyl-CoA group	Phenylalanine, tyrosine, leucine, lysine
Oxaloacetate group	asparagine, aspartic acid
α- ketoglutarate group	arginine, histidine, proline, glutamic acid
Succinyl-CoA group	isoleucine, methionine
Product group	glutamine

Classify of amino acids



Improved metabolic pathway of *Clostridium* species (developed nitrogen source consumption pathway)

6.3 modeling- processing

Hypothesis:

- Just considered **carbon metabolism**, but no energy and other resource metabolism (e.g. nitrogen source).
- Just considered **central pathway**, ignore little flow rate branch.
- For small molecule products (e.g. ABE), using **extracellular** contents instead of intracellular.
- Assumed xylose, acetyl-CoA and ribose-5-phosphate contribute to **biomass**.

Modeling- theory

Multi-Michaelis-Menten equation

$$V = V_{\max}\left[\prod_{i=1}^{N_s}\frac{C_{S_i}}{K_{mSi}+C_{S_i}}\right]$$

V: reaction rate of each intracellular biochemical reaction;
$V_{\max}$: constant of the maximum reaction rate;
S_i: substrate of each reaction;
N: the number of substrates;
C: concentration of each substrate;
Km: constant of the substrate concentration at reaction rate is half of $V_{\max}$.

$$V = V_{\max}\left[\prod_{i=1}^{N_s}\frac{C_{S_i}}{K_{mSi}\times\left(1+\frac{C_{butanol}}{b}\right)+C_{S_i}\times\left(1+\frac{C_{butanol}}{b}\right)}\right]$$

Inhibition term

$C_{butanol}$: concentration of butanol;

b: inhibition constant

Mass balance

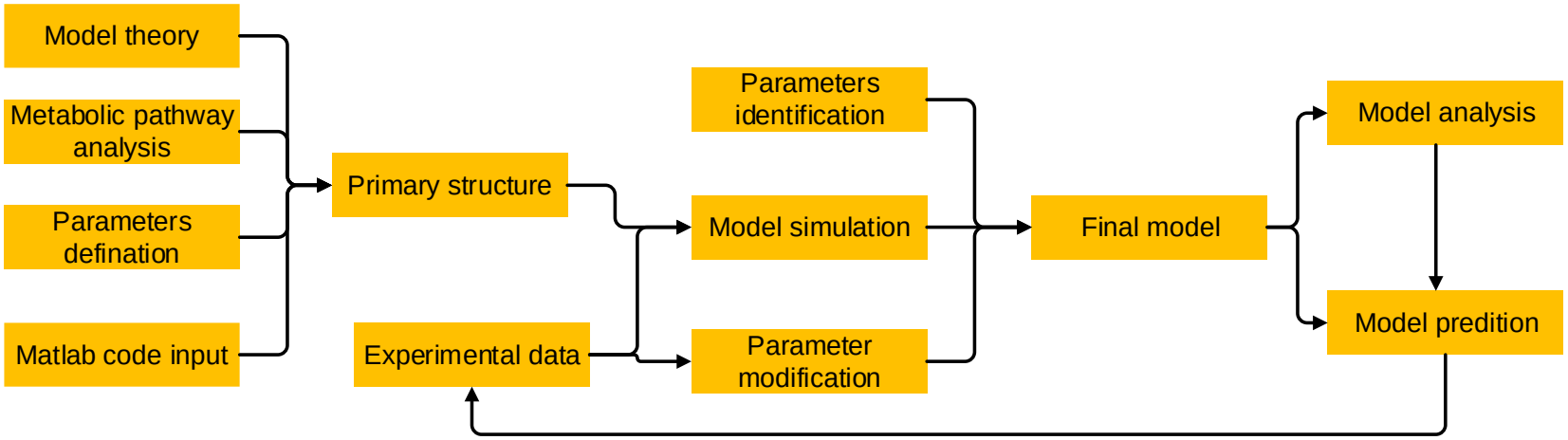
$$\frac{d[S]}{dt} = \sum_{i=1}^{M} V_{input_i} - \sum_{j=1}^{N} V_{output_j} - \mathrm{a}\times V_{growth} - [S]\times V_{growth}$$

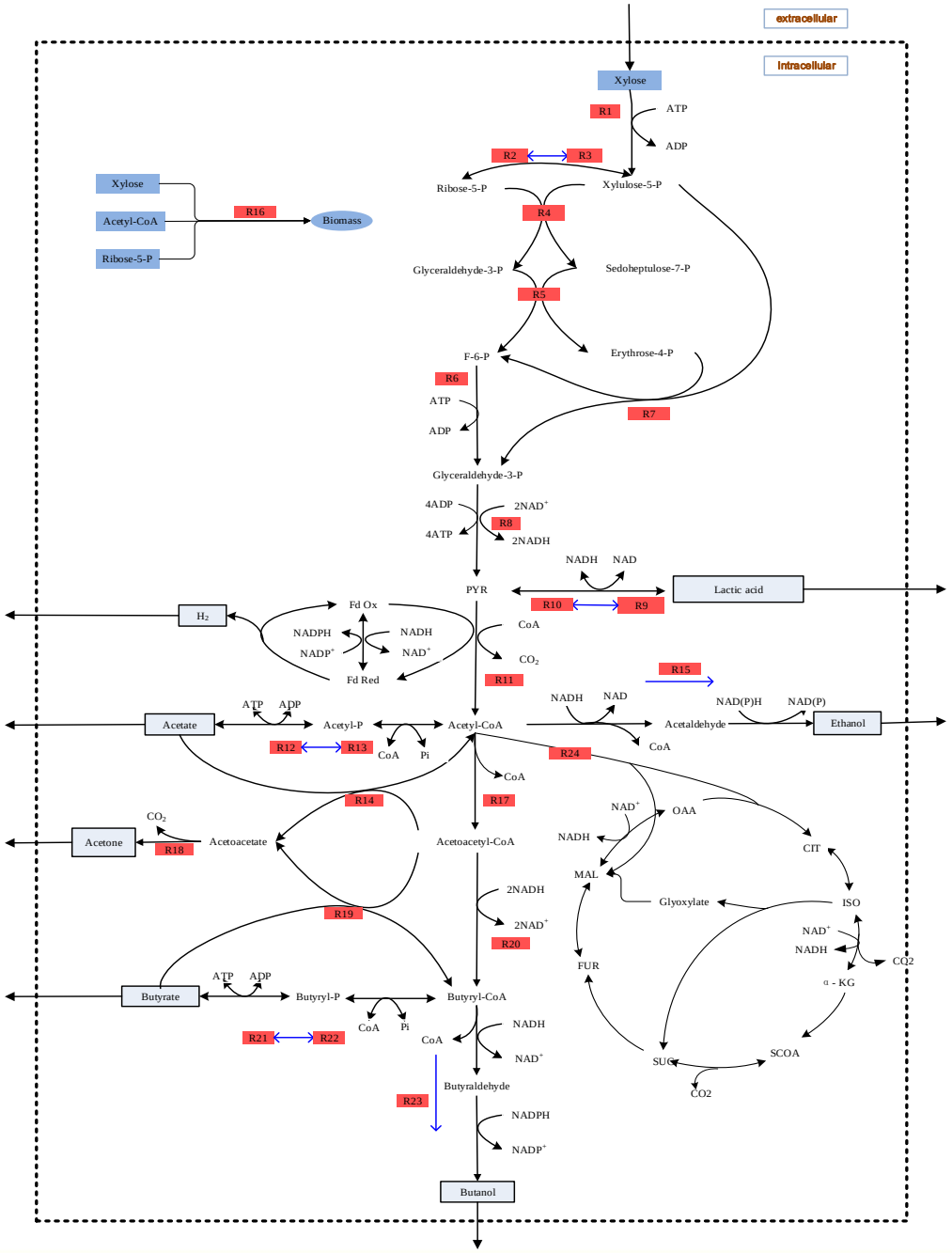
Cell growing consumption

Cell dilution

[S]: concentration of metabolite in each node.
M: the number of input flux;
N: the number of output flux;
a: constant of distribution to cell growth;

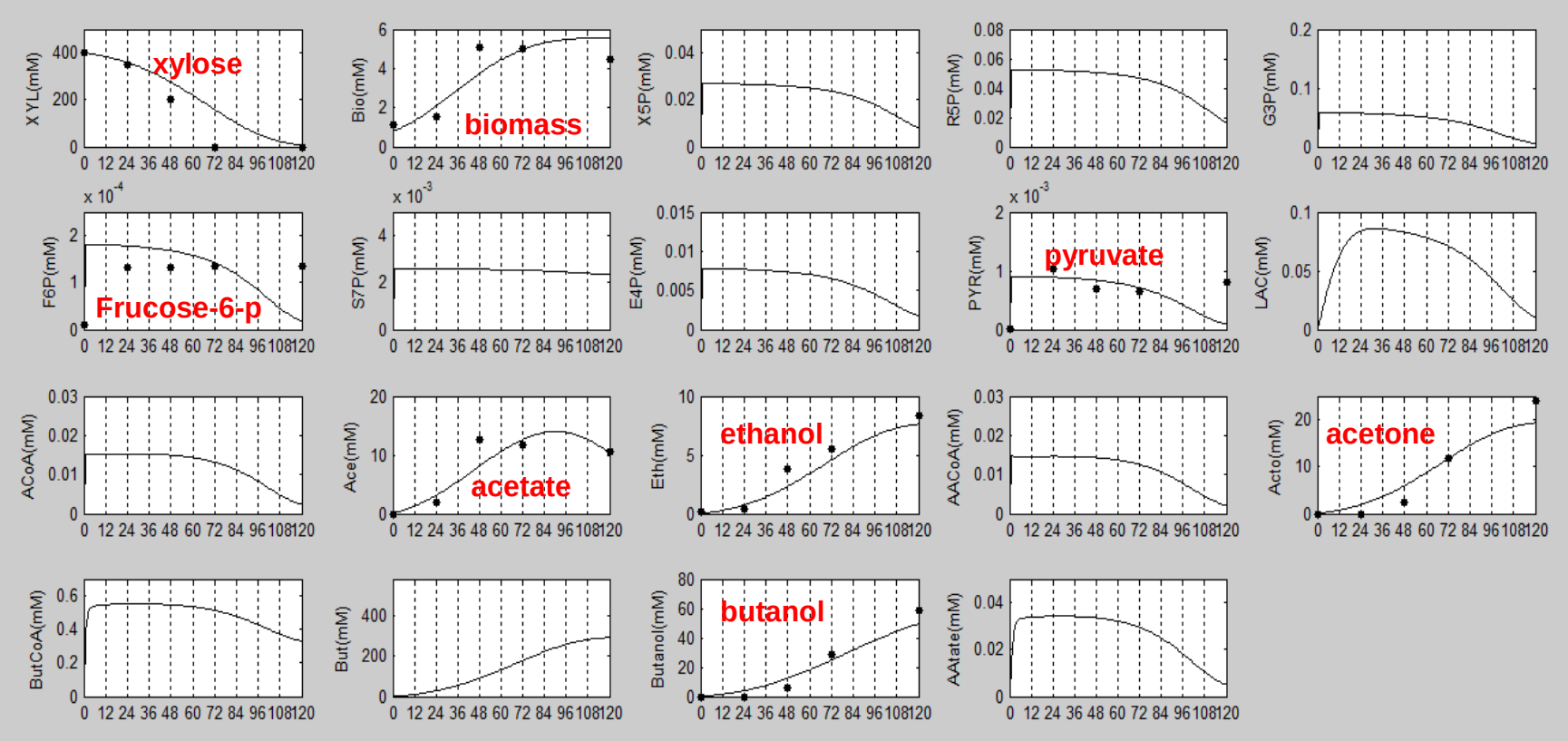
Modeling- process





Metabolic map structure for modeling

6.3 modeling- Simulation result in Matlab



7. Conclusion and future works

7.1 Conclusion- Current and on-going works

1. Compared with general **glucose** ABE fermentation and **xylose** fermentation. Based on xylose fermentation, different content of **nitrogen** resource was investigated. **Calcium carbonate** was used in batch culture as a buffer.

2. Based on existing central pathway, one **main structure of model** has been established which including 24 reactions ad 18 metabolites. Depending on experimental data, parts of **parameters** were modified.

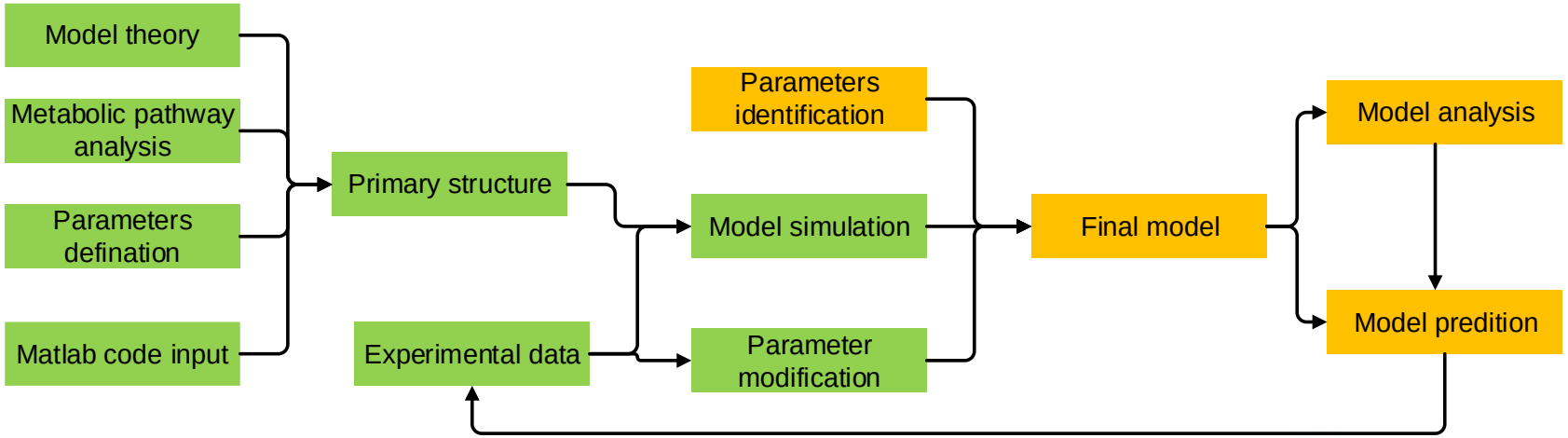
3. During batch culture, 20 kinds of **amino acids** metabolism was studied and their variation trends were tracked along with culture period. According to their metabolic nodes entering metabolism, 6 groups were defined.

7. Conclusion and future works

Future works

1. Model development

- Depending improved metabolic pathway, adding more notes and mass balance in model structure.
- Parameters identification, goodness fitting simulation study.
- Model analysis and model prediction study.



7. Conclusion and future works

2. Cell engineering

- Depending on genetic technology and model prediction, modify key point in metabolic pathway.

3. Biological NMR (Nuclear Magnetic Resonance)

- Depending on the information of P^{31} metabolism aim to understand energy metabolism *in vivo* situation

4. Raw materials study

- Different hydrolysis byproducts inhibition will be studied with copying into standard xylose fermentation.
- Inhibition mechanisms and metabolic influence will be studied through intracellular and extracellular analysis, which include nucleotides, sugar phosphates, amino acid and ABE.

Lab teaching plan:

Anaerobic culture tools:

Special tools are as follows:
Screwed glass tubes (Chemglass, CLS-4208-01 Glass 15 ml): tube culture for seed growth;
Serum Bottle (Wheaton, 223952 Serum Bottle 500 ml): for production culture;
Rubber snap-on style stopper (Wheaton, W224100-342 30 mm) and Center vial seal (IV packs, 30 mm): be matched to serum bottle;
Vial Crimper (IV packs, 30mm All Aluminum Seals): be used to seal serum bottle;
Anaerobic jar (Fisher, OXAG0025A, 2.5L), ANAEROGEN (Fisher, 2.5 L consume oxygen for anaerobic environment) and Anaerobic indicator (Fisher, REZAS) will be used for generating an anaerobic environment.

Medium: MRC, CGM, Cooked meat medium, Half-solid medium

O_2 removing operation: N_2 flow, positive press, anaerobic jar application.

Take sample: syringe using, high press by gas production, inoculation.

Cell watching: Microscope application.

Acknowledgement

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NSERC
CRSNG

Fonds de recherche sur la nature et les technologies
Québec

Canada Research Chair on the Development of Metabolic Engineering Tools


Thank you! Merci!
“You must be the change you wish to see in the world.”
- Mahatma Gandhi

Discussion 4 :

Describe the difference between anaerobic and aerobic cell culture.