United Nation Environmental Program
Regional Workshop on
Waste Agricultural Biomass

March 2-5, 2010, Osaka, Japan

RITE
Research Institute of Innovative Technology for the Earth

Technology and Innovation for Production of Cellulosic Biofuels

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Molecular Microbiology and Biotechnology Group
Research Institute of Innovative Technology for the Earth (RITE)

Today's outline

- RITE: Who we are
- Biofuel: current status
- RITE Bioprocess
  - Features
  - Biofuels
  - Biochemicals

Research Institute of Innovative Technology for the Earth (RITE)

Established: 1990

Status: Non-profit organization under the Ministry of Economy, Trade and Industry

Annual budget: JPY 5.1 billion ($ 51 million)

Mission:

- **Development of environmental technologies against global warming problems**

Main research fields:

- **Biorefinery**
- **CO_2 geological sequestration**

Biorefinery group

Production of biofuels / chemicals from biomass

Research fields

Biorefinery: Production of energy and chemicals from Biomass

Research projects

Chemicals

- Succinic acid
- L-Lactate, D-Lactate
- Propanol (Raw material for propylene)
- Ethanol (Raw material for ethylene)
- Amino acid

Biofuels

- Ethanol
- Propanol
- Butanol

Energy

- H_2

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Biofuels: Current situation

- Sustainability?

  Food-Fuel issue, environmental effect…

- Solution: Cellulosic biofuels

  - Non-food resources

  - Countermeasures against global warming problems

Challenge: Development of a cost-effective production process

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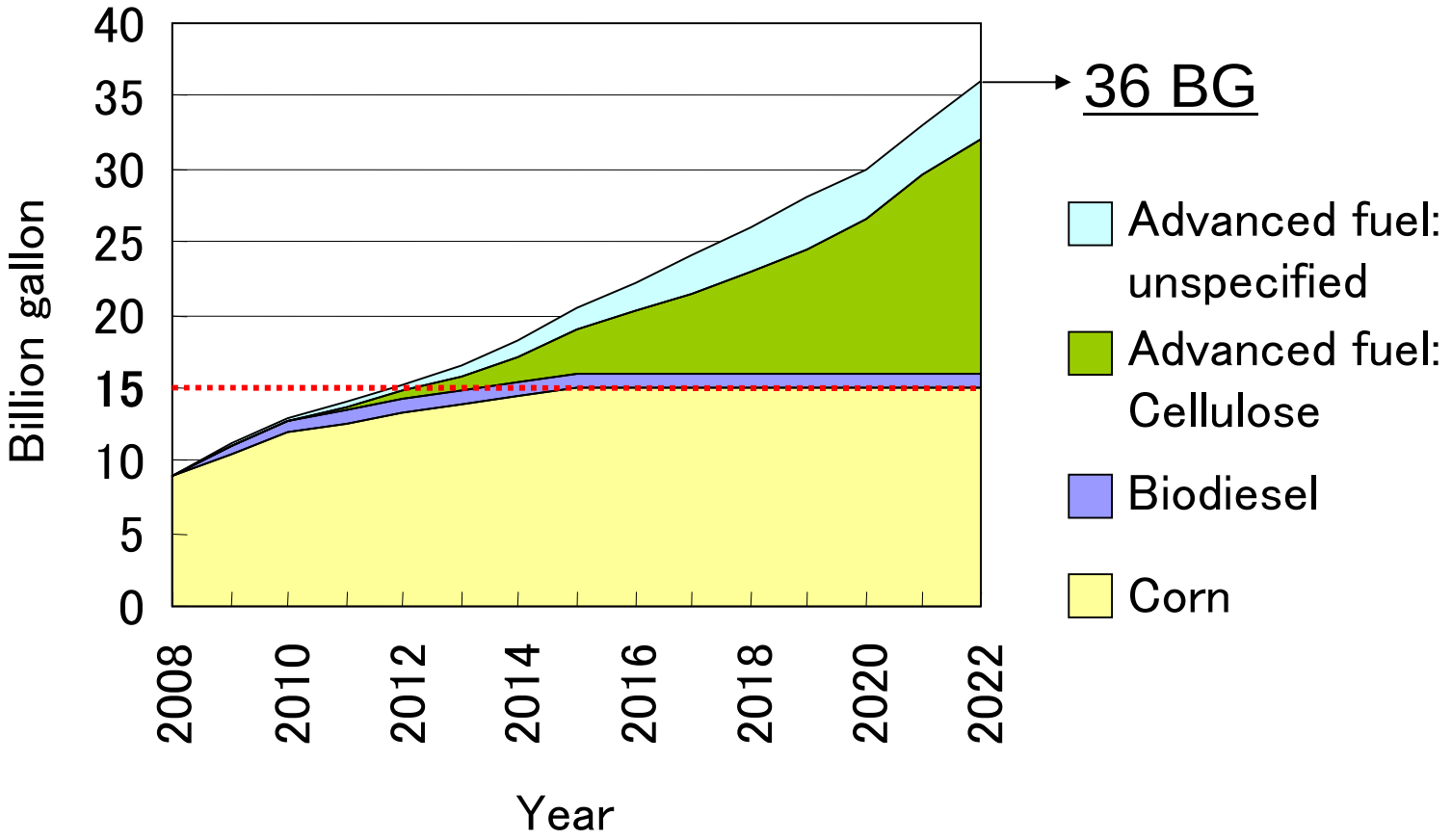
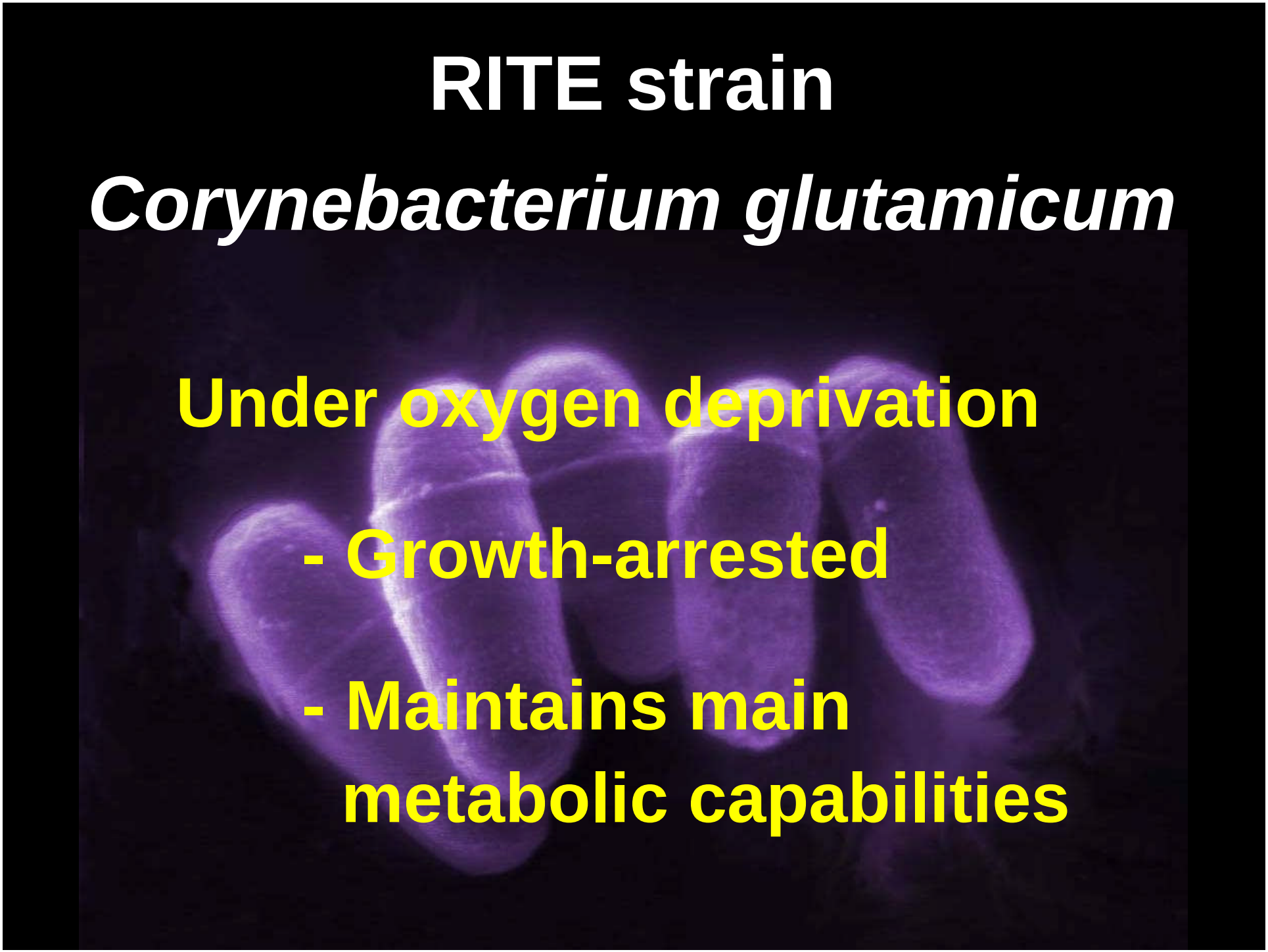
Cellulosic biofuel in USA



Source: EPA Renewable Fuel Standard2

A novel and highly efficient bioprocess

Growth-Arrested Bioprocess (RITE bioprocess)

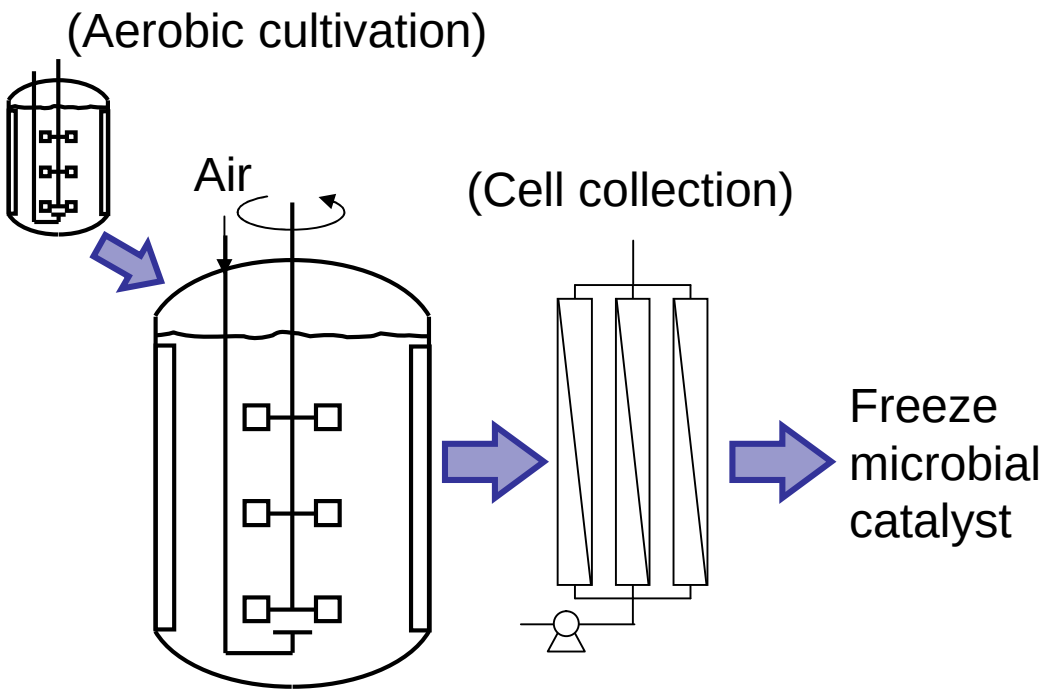

RITE strain
Corynebacterium glutamicum
Under oxygen deprivation
- Growth-arrested
- Maintains main metabolic capabilities

RITE bioprocess

JP-Patent 3869788

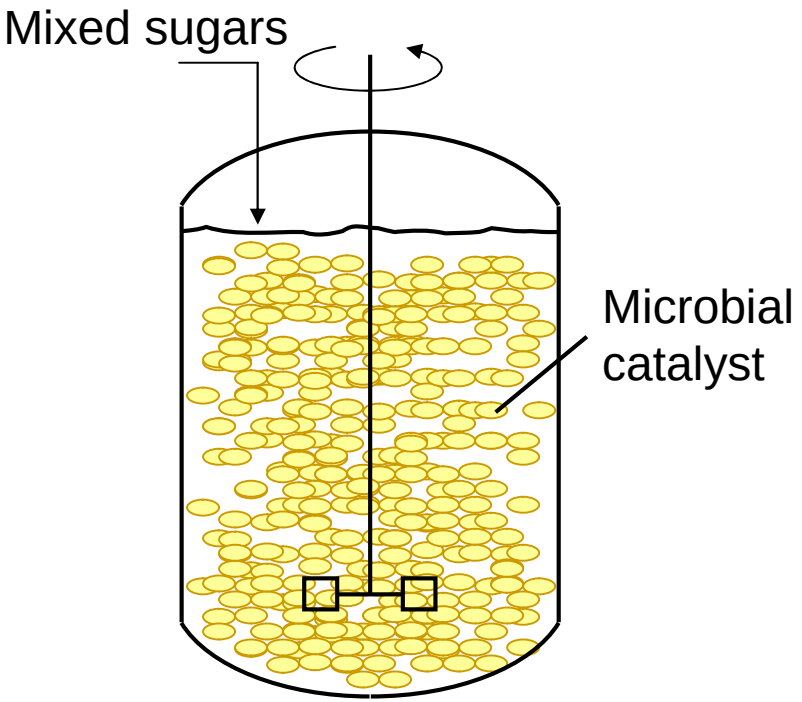
INDIA 209524

Microbial catalyst preparation



Growth by cell division

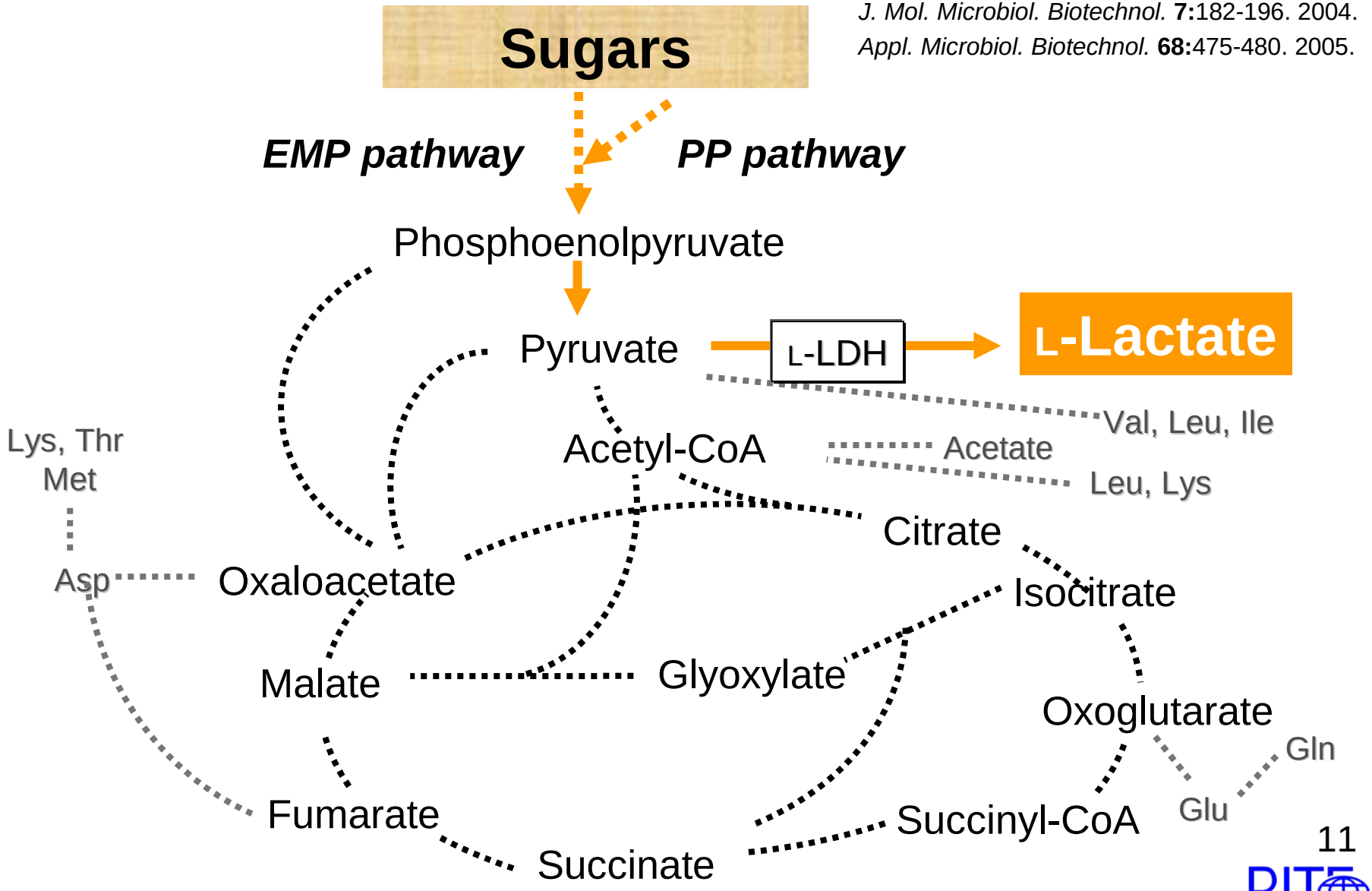
Bioconversion



Growth-arrested cells

Metabolic pathways of *C. glutamicum* under oxygen deprivation (without CO_2)

J. Mol. Microbiol. Biotechnol. **7:**182-196. 2004.
Appl. Microbiol. Biotechnol. **68:**475-480. 2005.



Metabolic pathways of *C. glutamicum* under oxygen deprivation (with CO_2)

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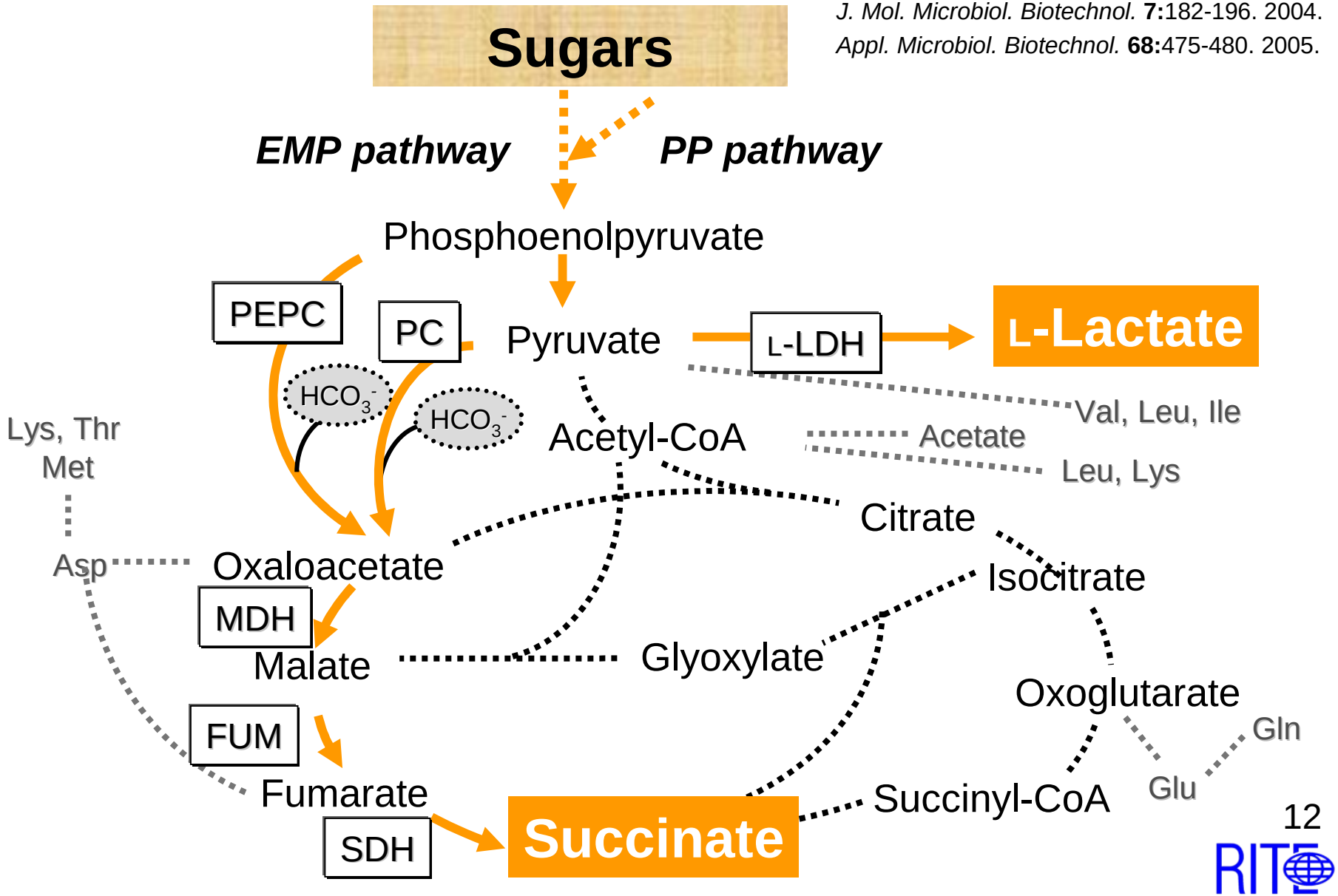
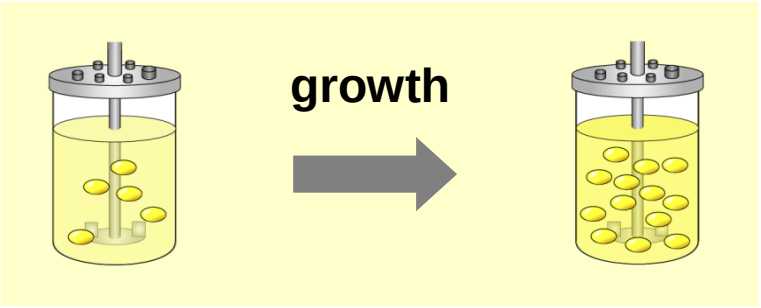


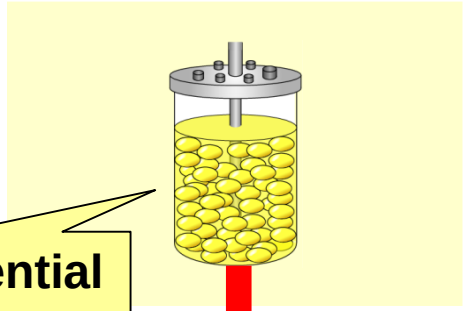
Analysis of metabolic shift under oxygen deprivation

Aerobic cultivation
(Cell propagation)



harvest and wash

Oxygen-deprived condition

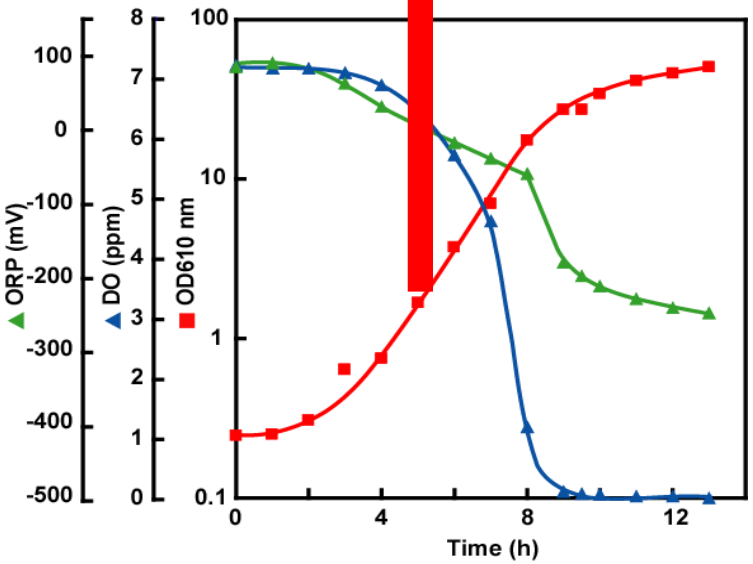


Redox potential
- 450mV

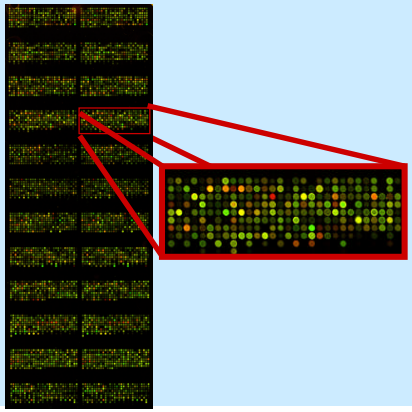
Mid-exponential phase

Sampling

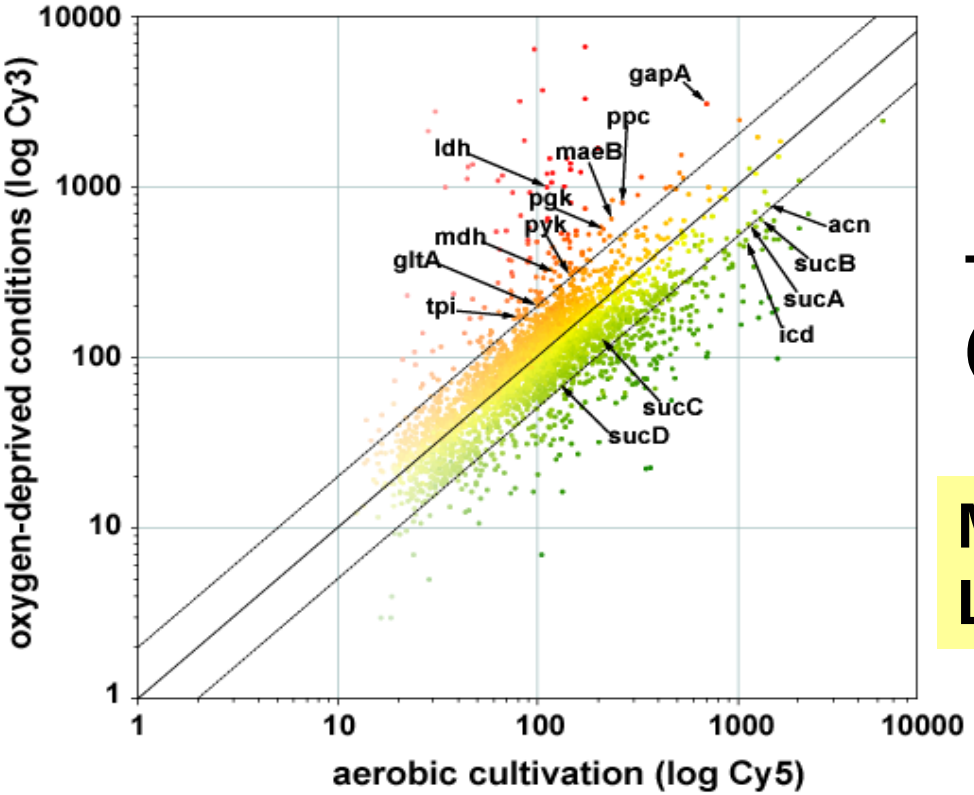
Sampling



Transcriptome (DNA microarray) analysis
Mid-exponential-phase cells vs
Oxygen-deprived cells



Gene expression analysis



- **Entire gene (3080 genes)**

The ratios of mRNA levels
(oxygen-deprived conditions /aerobic cultivation)

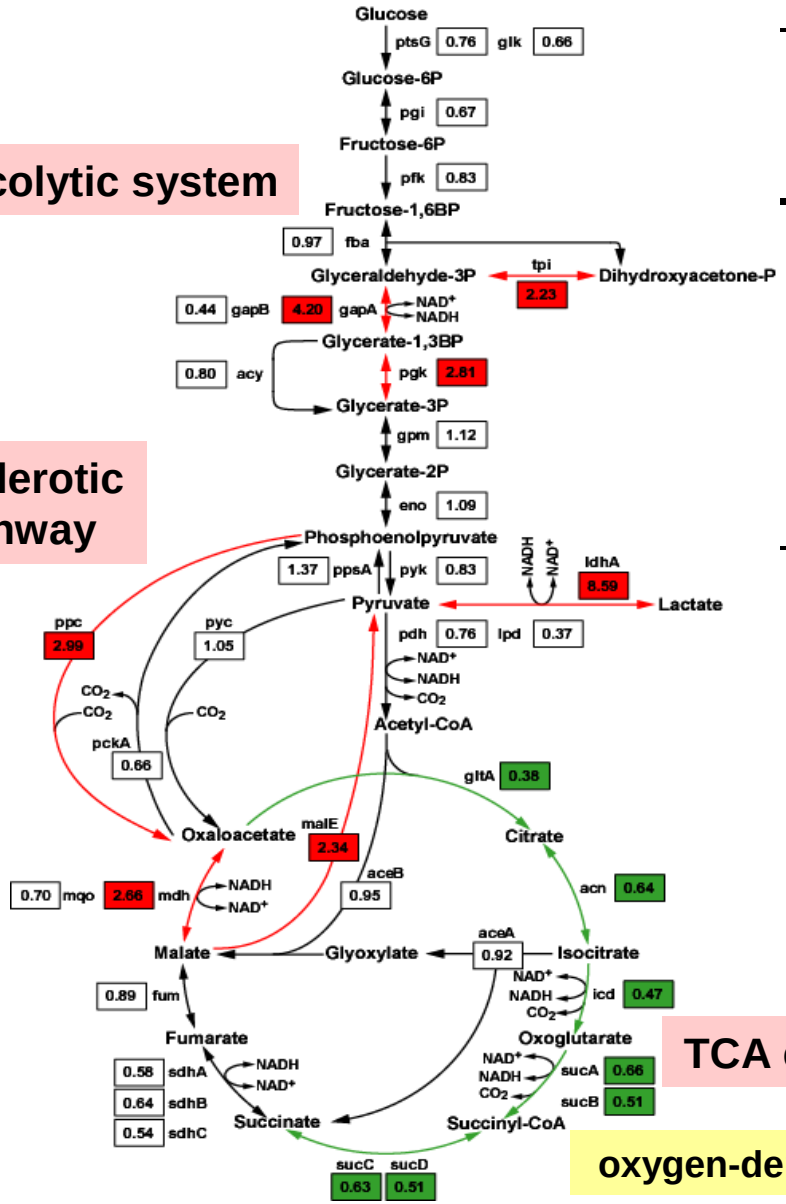
More than 2-fold	**161 genes**
Less than 1/2-fold	**221 genes**

A gene expression profile is different greatly between aerobic and oxygen-deprived conditions

Expression analysis of glucose metabolism

glycolytic system

anaplerotic pathway



TCA cycle

Enzyme	Relative enzyme activities: oxygen-deprived conditions / aerobic
GAPDH	5.3
PGK	10.5
TPI	19.1
PEPC	4.5
LDH	14
MDH	25.8

Genes encoding several key enzymes involved in the glycolytic and organic acid production pathways were significantly up-regulated under growth-arrested bioprocess.

Microbiology. **153:**2491-2504. 2007.

oxygen-deprived conditions / aerobic cultivation

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Application of “RITE bioprocess”

For the production of biofuels

- Ethanol
- Propanol
- Butanol

Biofuels: Current situation

- Sustainability?

  Biofuels “pros & cons”

- Cellulosic biofuels:

  - Non-food resources
  - Countermeasures against global warming problems

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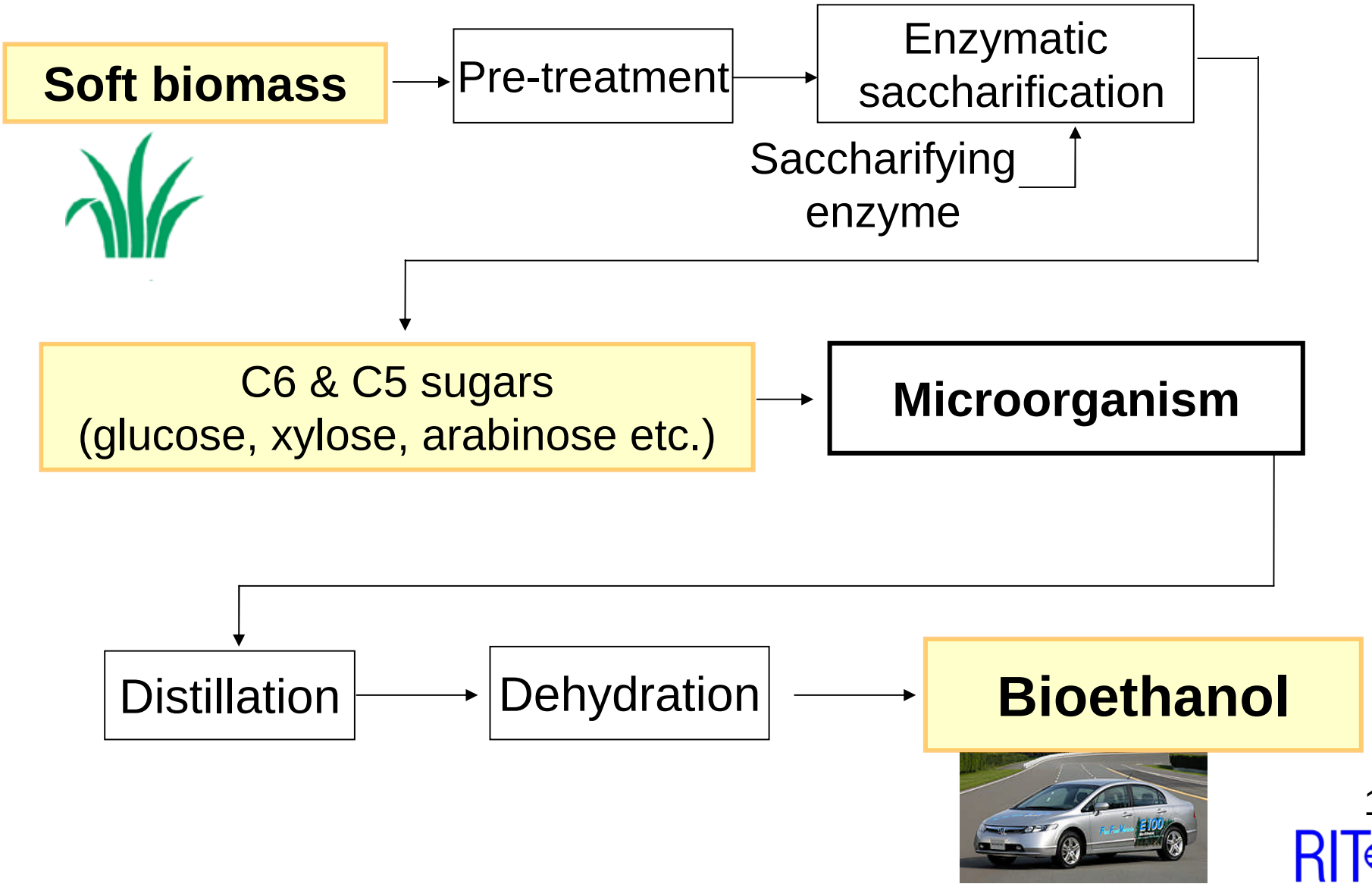
Ethanol production from soft biomass



Ethanol production

Important traits for industrialization

- High productivity
- Simultaneous utilization of C_6 & C_5 sugars
- Tolerance to “fermentation inhibitors”

Dien BS *et al*. *Appl Microbiol Biotechnol* (2003) 63: 258-266

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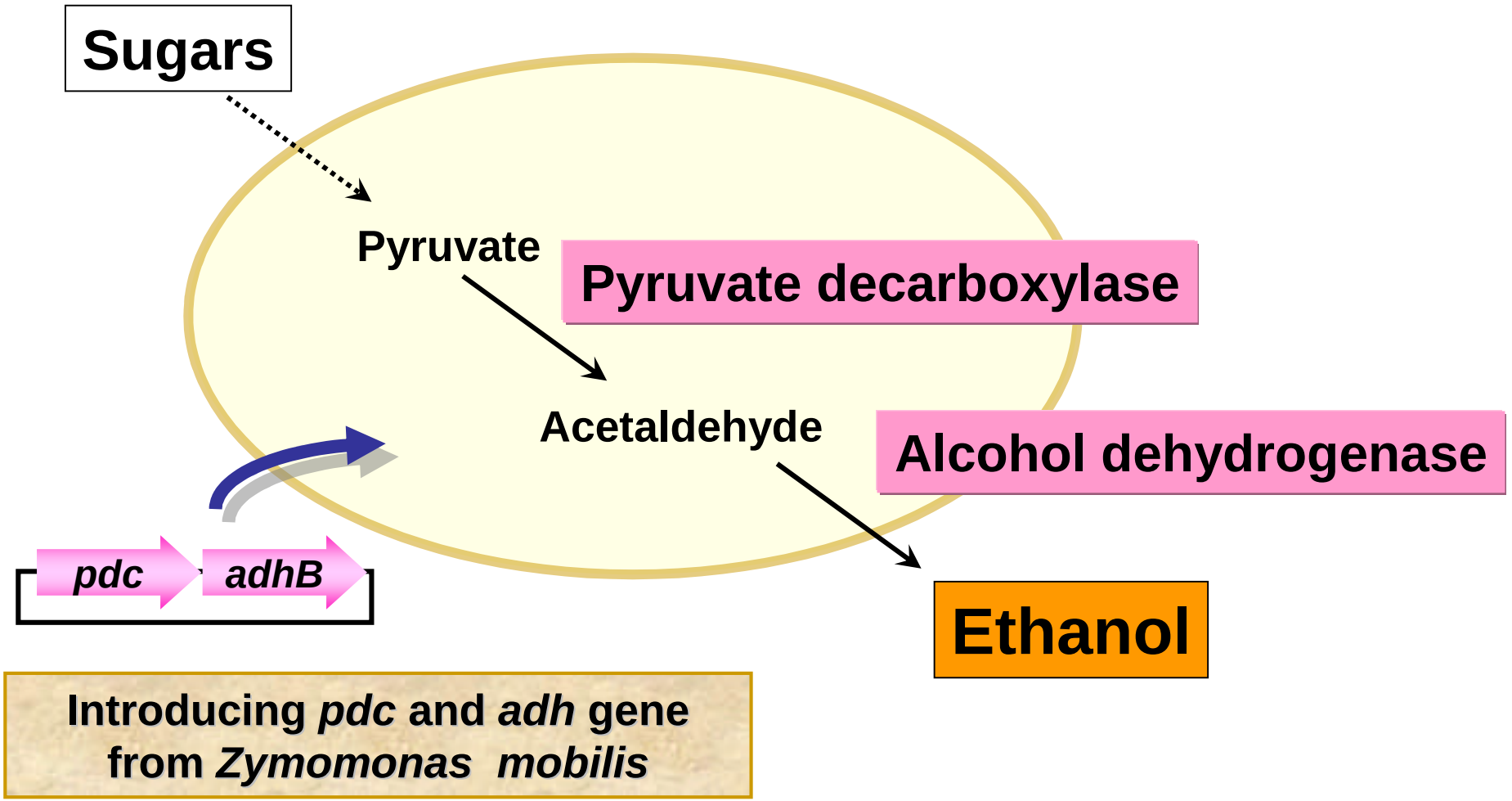
Development of the ethanol producing strain



J. Mol. Microbiol. Biotechnol. **8:** 243-254. 2004.

Ethanol production

Important traits for industrialization

- High productivity
- **Simultaneous utilization of C_6 & C_5 sugars**
- Tolerance to “fermentation inhibitors”

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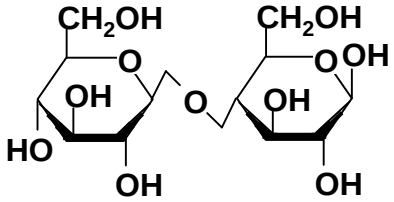
Introducing ability to utilize sugars derived from biomass

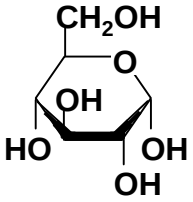
Cellulose

Cellobiose (C_6-C_6)



Adaptive mutant for cellobiose uptake ability 1)

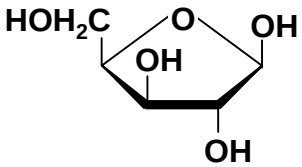
Glucose (C_6)



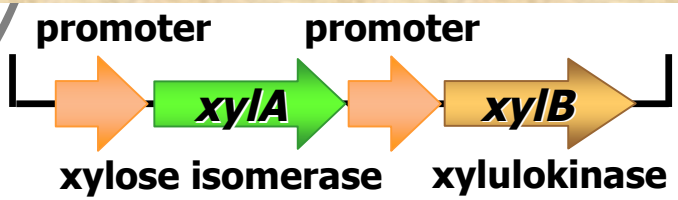
RITE strain

Hemicellulose

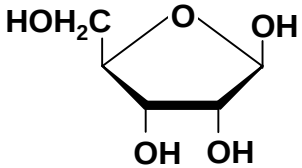
Xylose (C_5)



Chromosomal integration for xylose metabolic ability 2)



Arabinose (C_5)

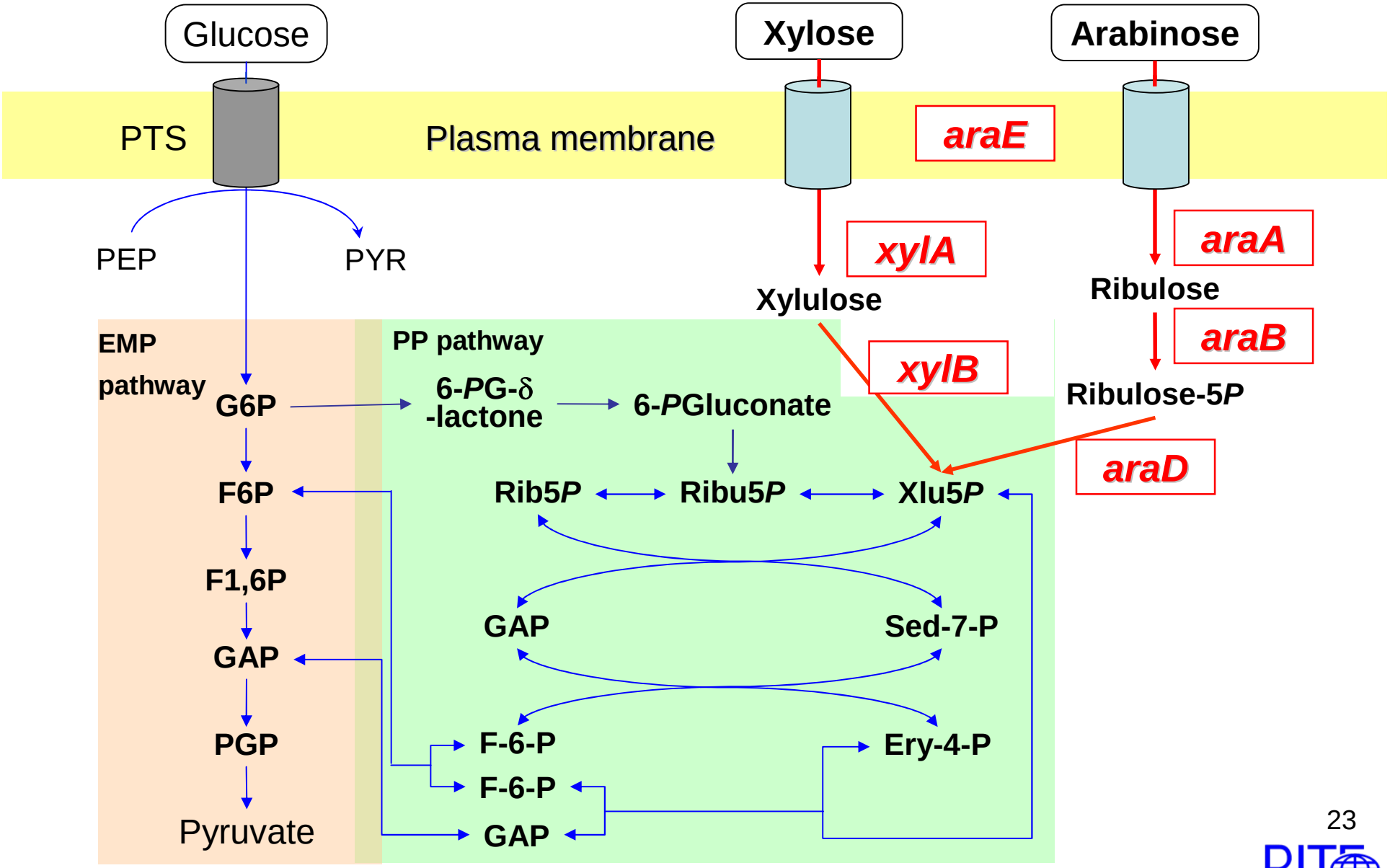


Chromosomal integration for arabinose metabolic ability 3)

L-arabinose isomerase
L-ribulokinase L-ribulose-5-P-4-epimerase

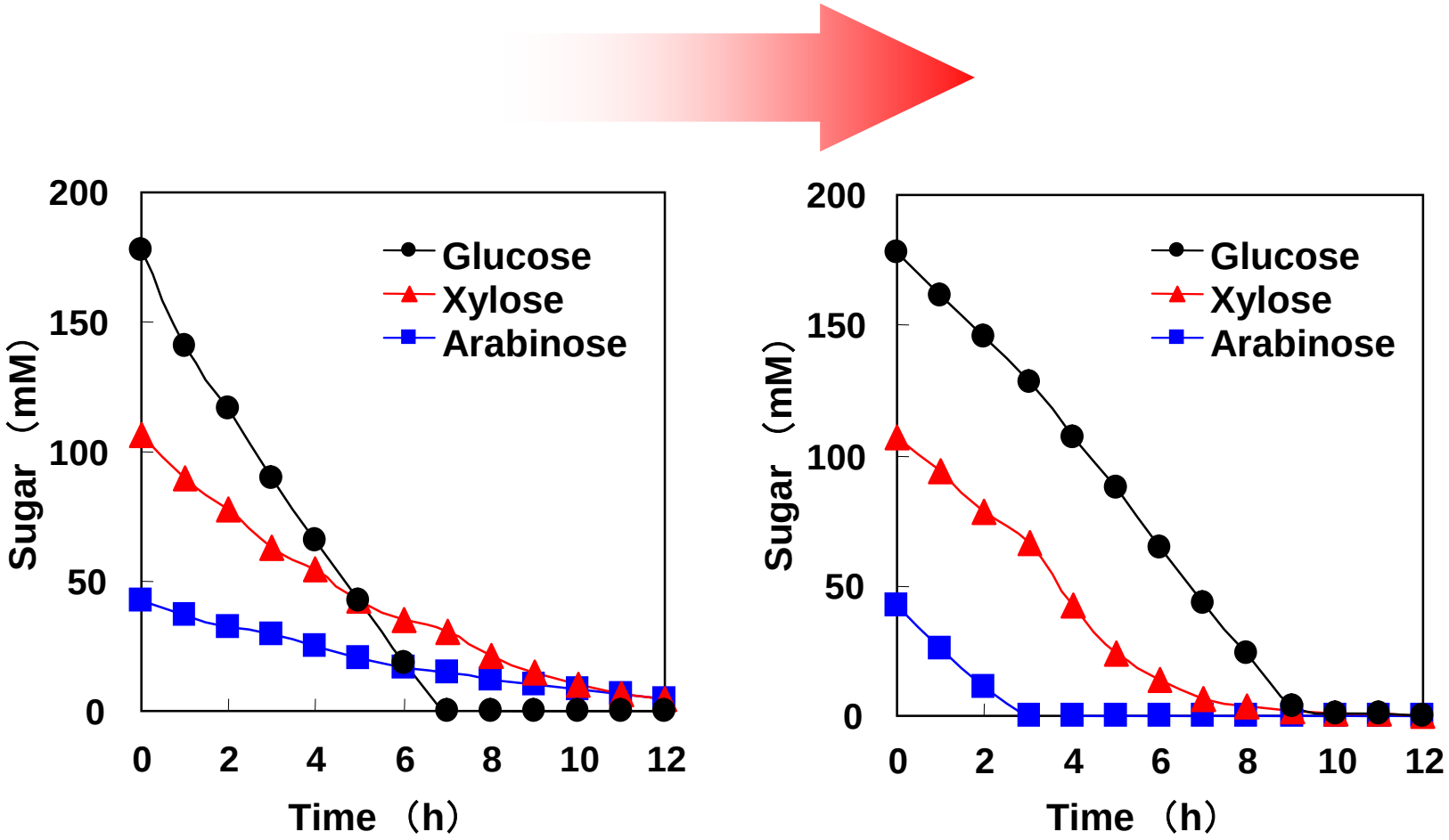
1) *Microbiology* **149:**1569-80. 2003. 2) *Appl. Environ. Microbiol.* **72:**3418-28. 2006. 3) *Appl. Microbiol. Biotechnol.* **77:**1053-62. 2008.

Engineering of xylose and arabinose metabolic pathways



Simultaneous Utilization of Mixed Sugar

Introduction of a pentose transporter



Appl. Microbiol. Biotechnol. (2009) 85: 105-115

Ethanol production

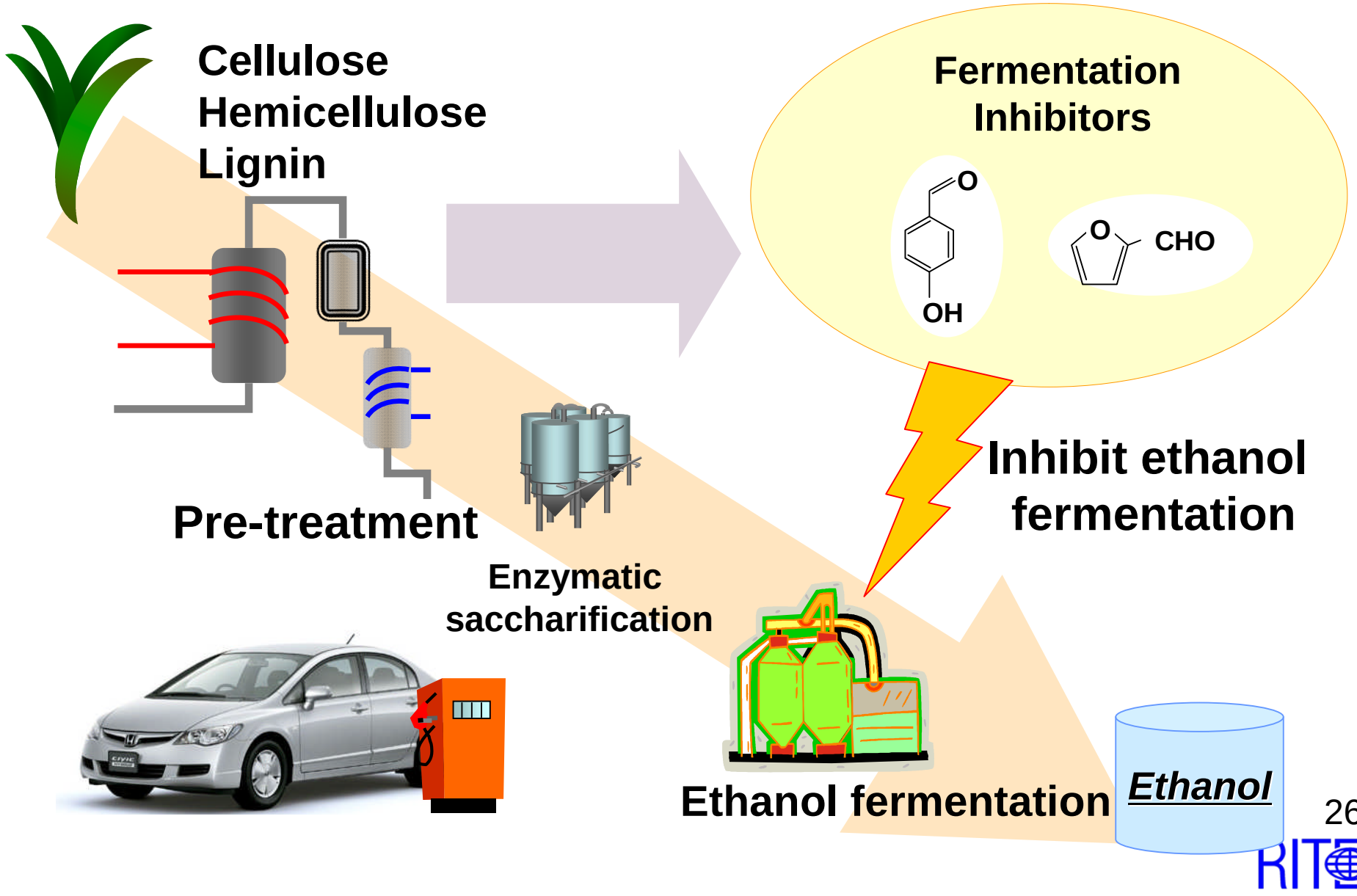
Important traits for industrialization

- High productivity
- Simultaneous utilization of C_6 & C_5 sugars
- **Tolerance to “fermentation inhibitors”**

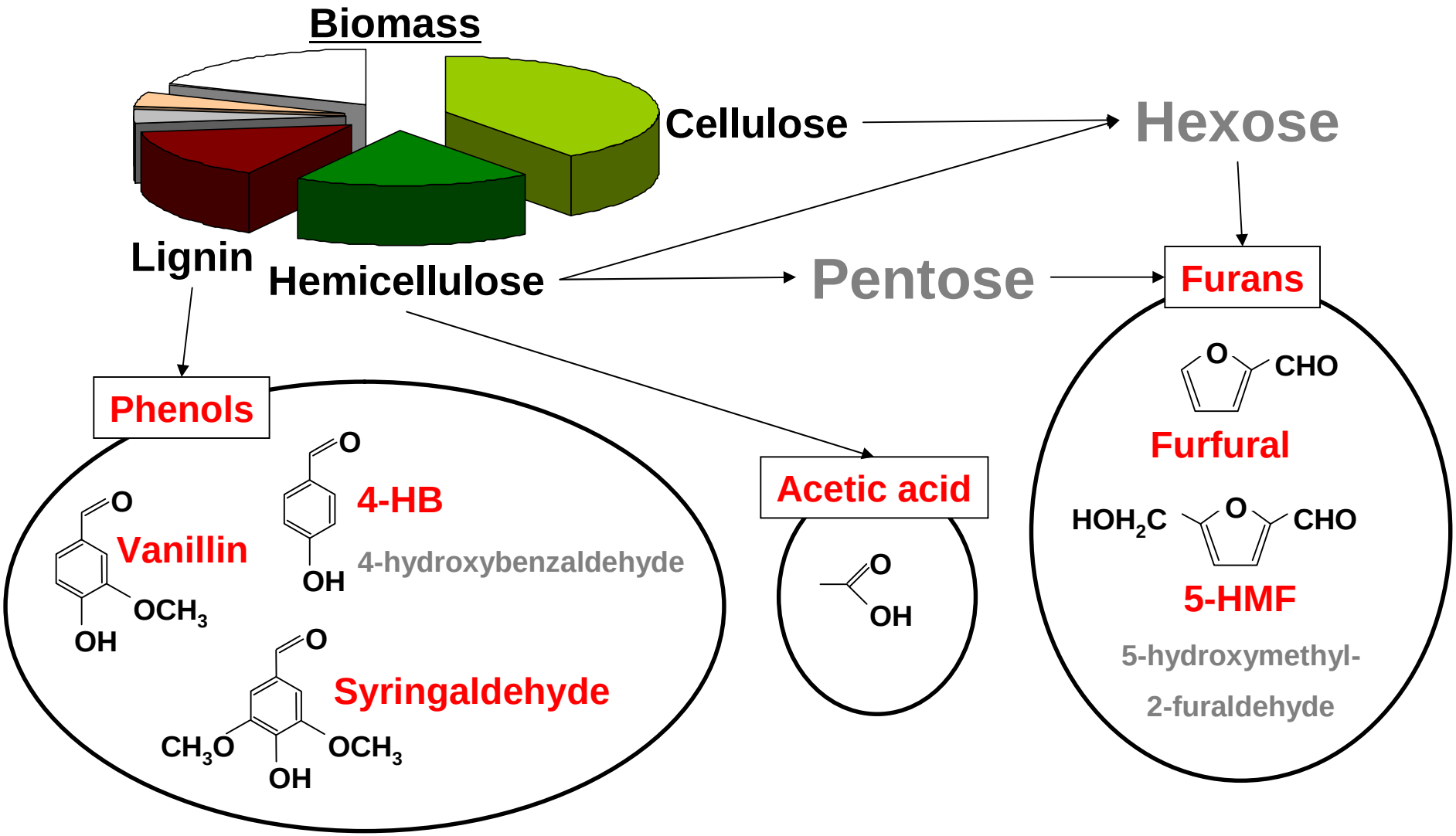
Dien BS *et al*. *Appl Microbiol Biotechnol* (2003) 63: 258-266

RITE

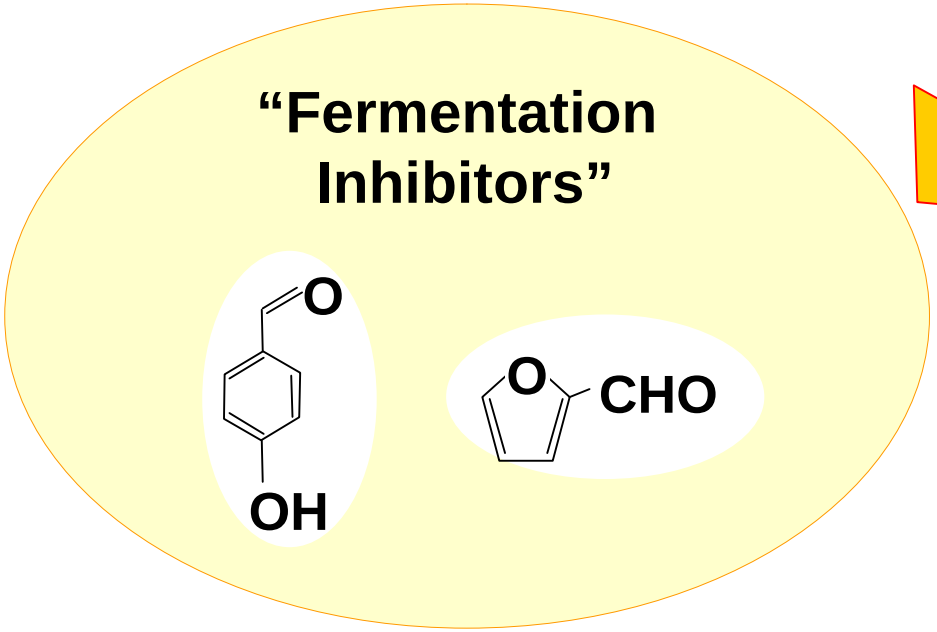
What is “fermentation inhibitors”?

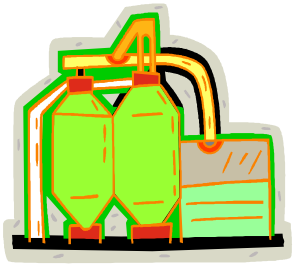


Major “fermentation inhibitors”



Inhibition mechanism





Ethanol fermentation

Growth Inhibition

No inhibition to the ethanol producing metabolic pathway!

Effect of lignocellulose-derived inhibitors on growth and ethanol production by growth-arrested *Corynebacterium glutamicum* R. *Appl. Environ. Microbiol.* **74:**754-760. 2007.

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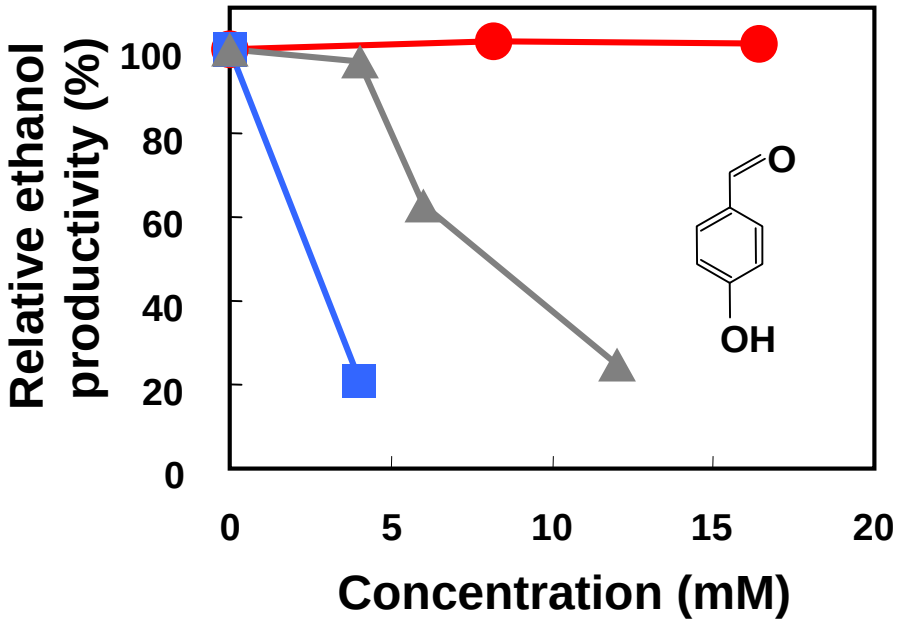
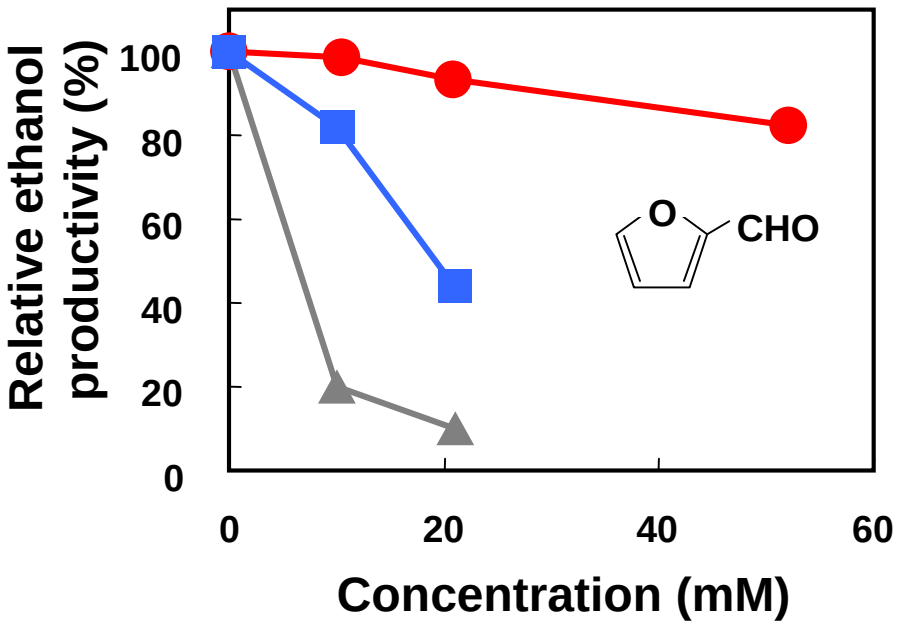
Tolerance to the inhibitors

●RITE Bio-Process ■*Z. mobilis* ▲*S. cerevisiae*

4-HB



Furfural



Biobutanol as “fuel”

- Diesel additive
- High energy density
- Low water solubility

Expected use as;

- Fuel for diesel engines
- Aviation fuel
- Ethanol – Butanol: Mixed-use complementally

(Synergistic effects)

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Trends in Biobutanol R&D

- Will improve ABE fermentation
- Create novel producing microorganisms

<u>Prediction of practical application</u>

Fundamental research (3-5 years)

+

Industrialization research (2 years)

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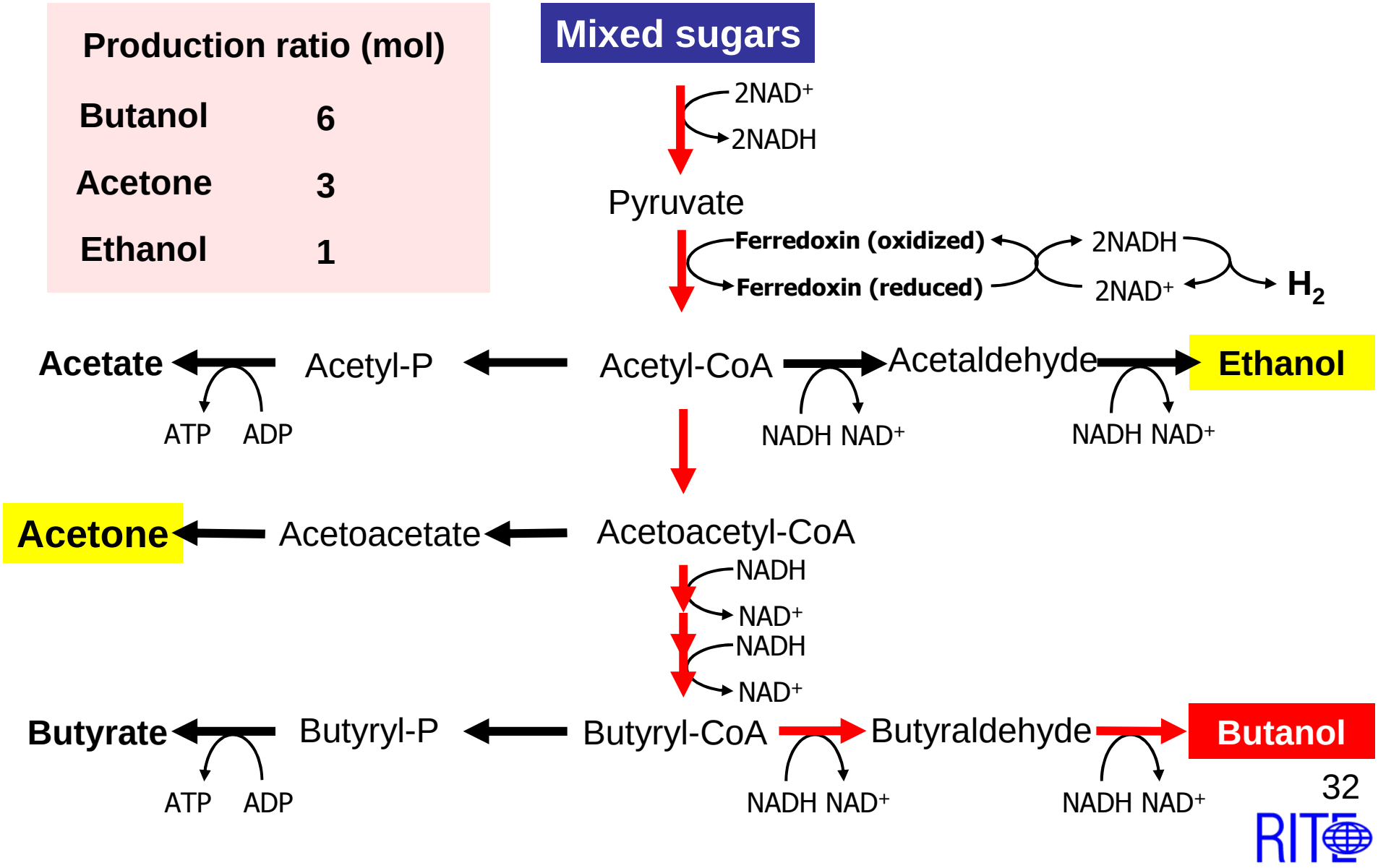
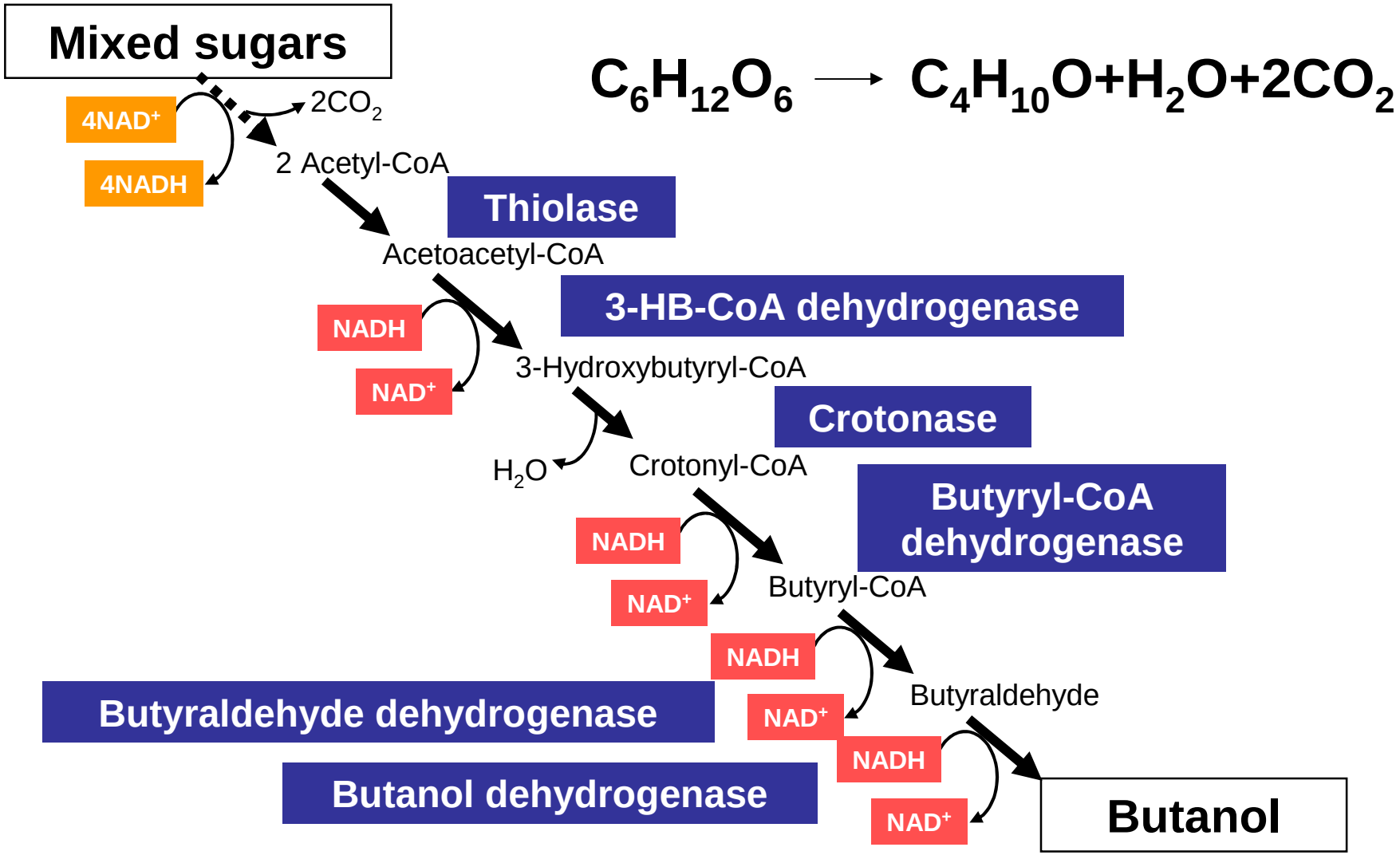
Clostridial ABE fermentation pathway

Production ratio (mol)	
Butanol	6
Acetone	3
Ethanol	1



Butanol production by recombinant *E.coli*.



Appl. Microbiol. Biotechnol. **77:**1305-1316.

Application of “RITE bioprocess” for the production of biochemicals

Examples;

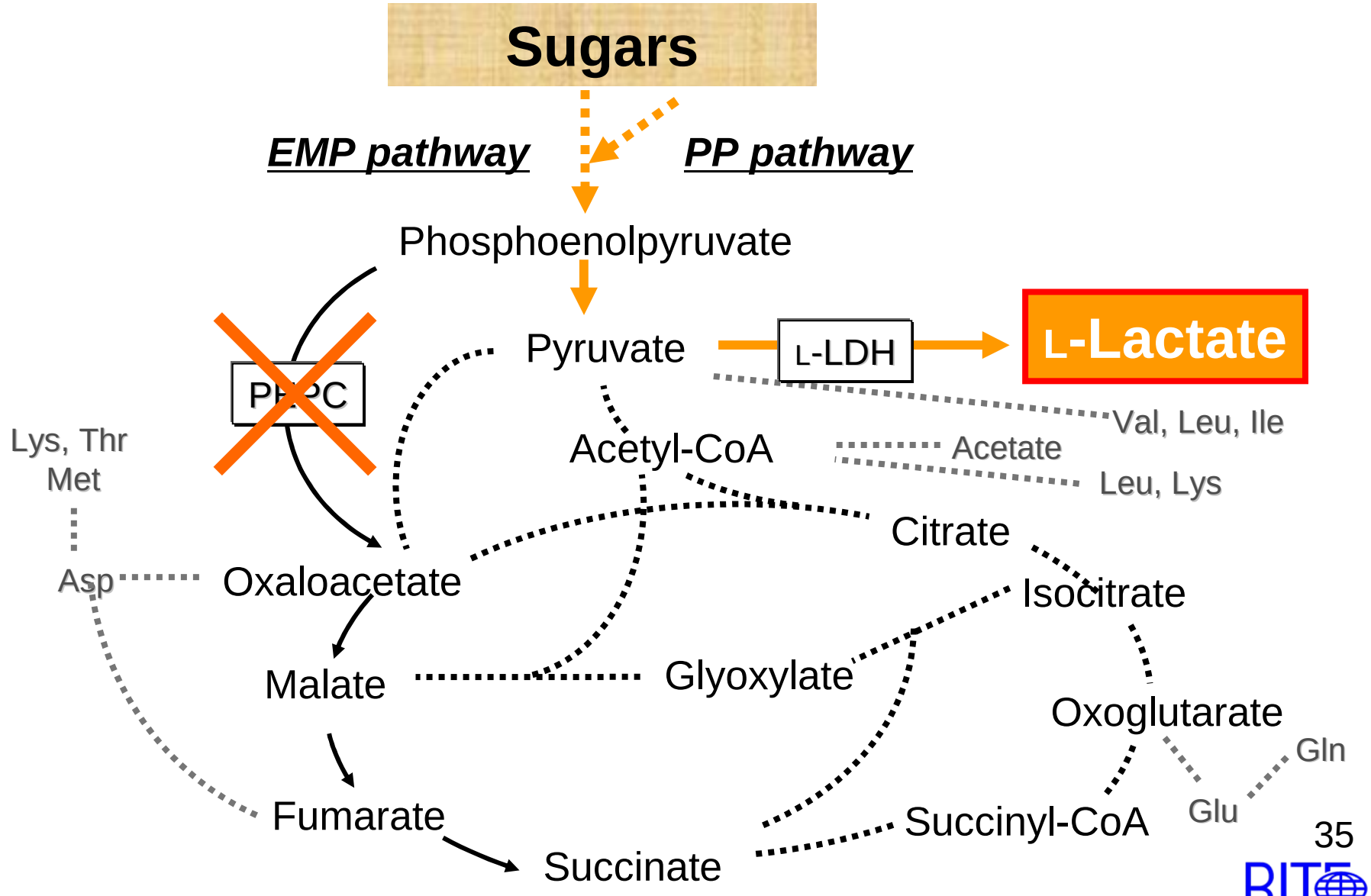
L-Lactate

D-Lactate

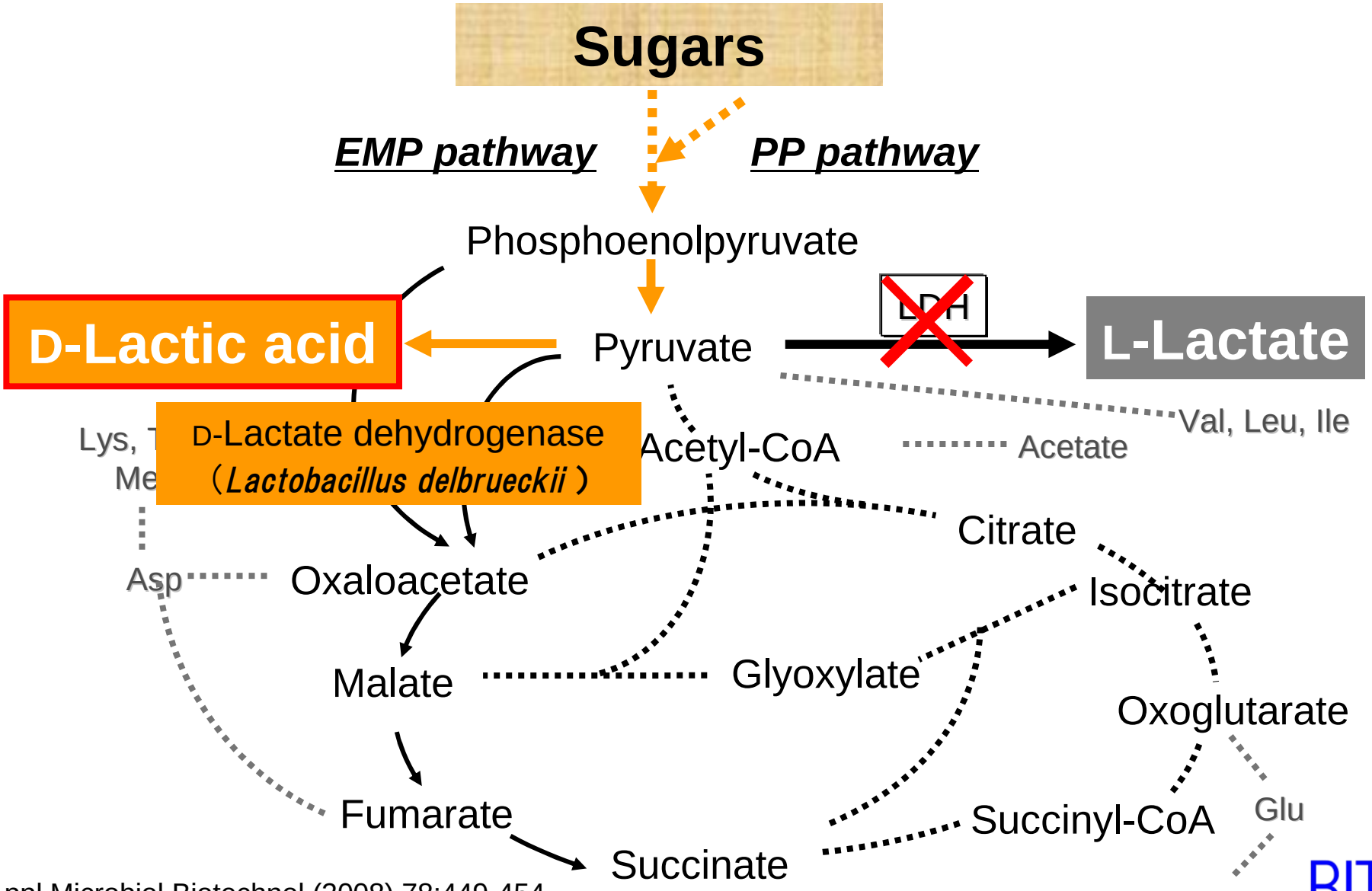
Succinate

Amino acid (L-Alanine)

Metabolic engineering for L-Lactate production



Metabolic engineering for D-Lactate production

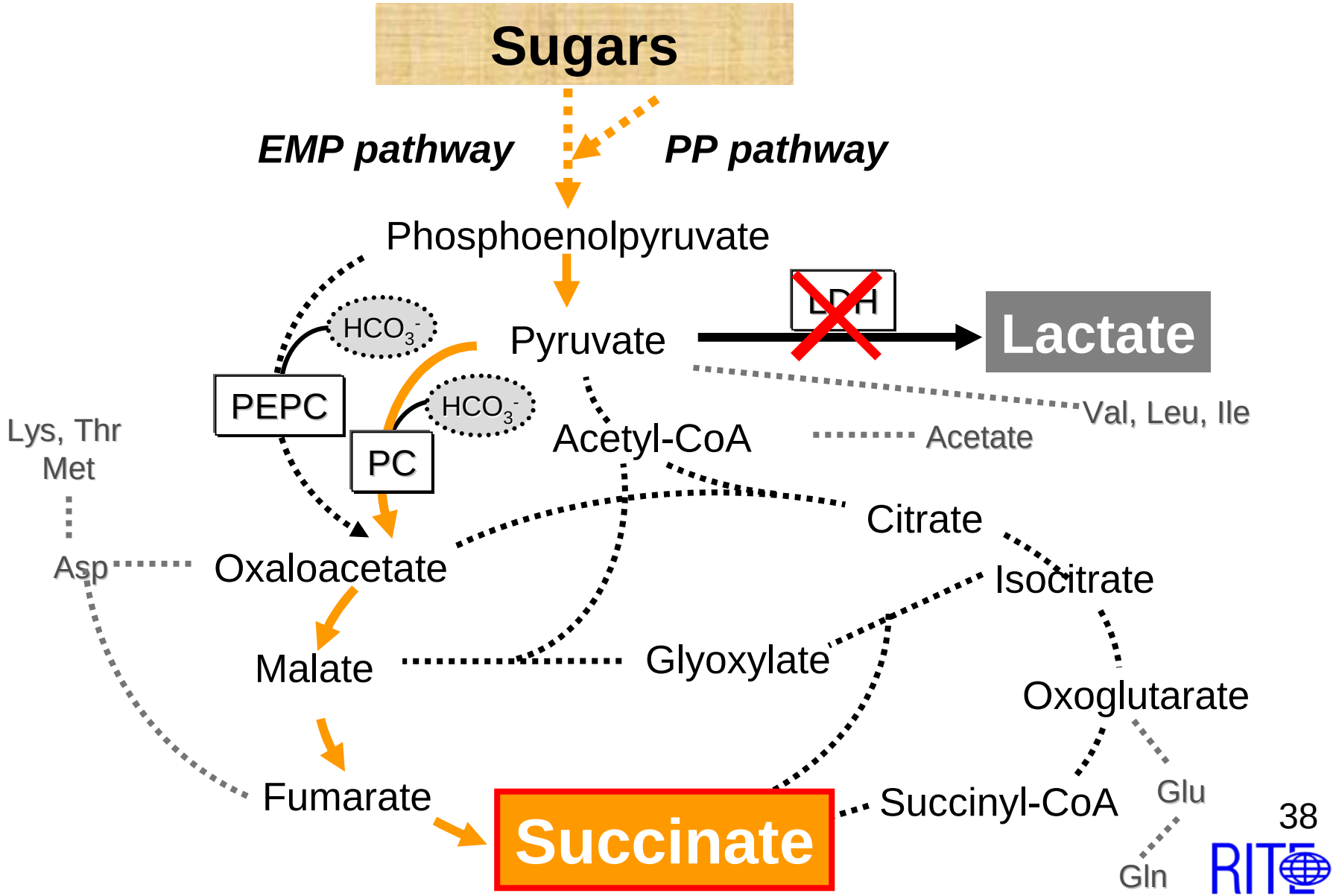


Appl Microbiol Biotechnol (2008) 78:449-454

Comparison of D-lactate production

Microorganism	Titer (g/l)	Productivity (g/l/h)	Reference
Lactobacillus delbrueckii	63	1.3	*Macromolecul Biosci.* 4: 1021-1027. (2004)
E.coli RR1	62	2.1	*Appl Environ Microbiol.* 65: 1384-1389. (1999)
E. coli W3110 SZ63	49	0.5	*Appl Environ Microbiol.* 69: 399-407. (2003)
E. coli MT-10934/pGlyldhA	65	1.3	JP 2005-102625 (2005)
Saccharomyces cerevisiae OC2	62	1.4	*J Biosci Bioeng.* 101: 172-177. (2006)
E. coli SZ194	92	2.1	*Biotechnol lett.* 28: 663-670. (2006)
RITE bioprocess	110	40.0	*Appl Microbiol Biotechnol.* 78: 449–454. (2008)

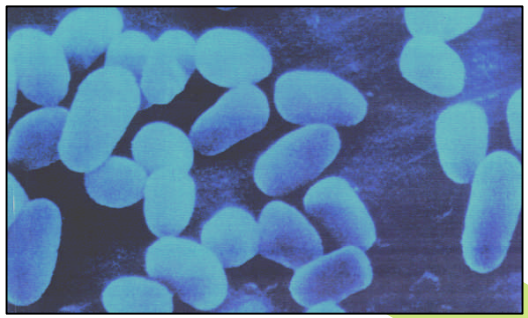
Metabolic engineering for succinic acid production



Comparison of succinate production

Microorganism	Titer (g/l)	Productivity (g/l/h)	Reference
A. Succiniciproducens	50	2.1	United States Patent 5143834 (1992)
A. Succiniciproducens	84	10.4	*Biotechnol Bioeng.* 99:129-135. (2008)
A. succinogens FZ53	106	1.4	United States Patent 5573931 (1996)
E. coli NZN111	28	0.7	*Appl Environ Microbiol.* 73:7837-7843. (2007)
E. coli AFP111/pTrc99A-*pyc*	99	1.3	*J Ind Microbiol Biotechnol.* 28:325-332. (2002)
RITE bioprocess (case 1)	146	3.2	*Appl Microbiol Biotechnol.* 81:459-464. (2008)
RITE bioprocess (case 2)	83	11.8	*Appl Microbiol Biotechnol.* 81:459-464. (2008)

Amino acids production by RITE bioprocess



Metabolic engineering
of *C. glutamicum* R

RITE bioprocess

- High productivity
- No energy loss for growth
- Simple system

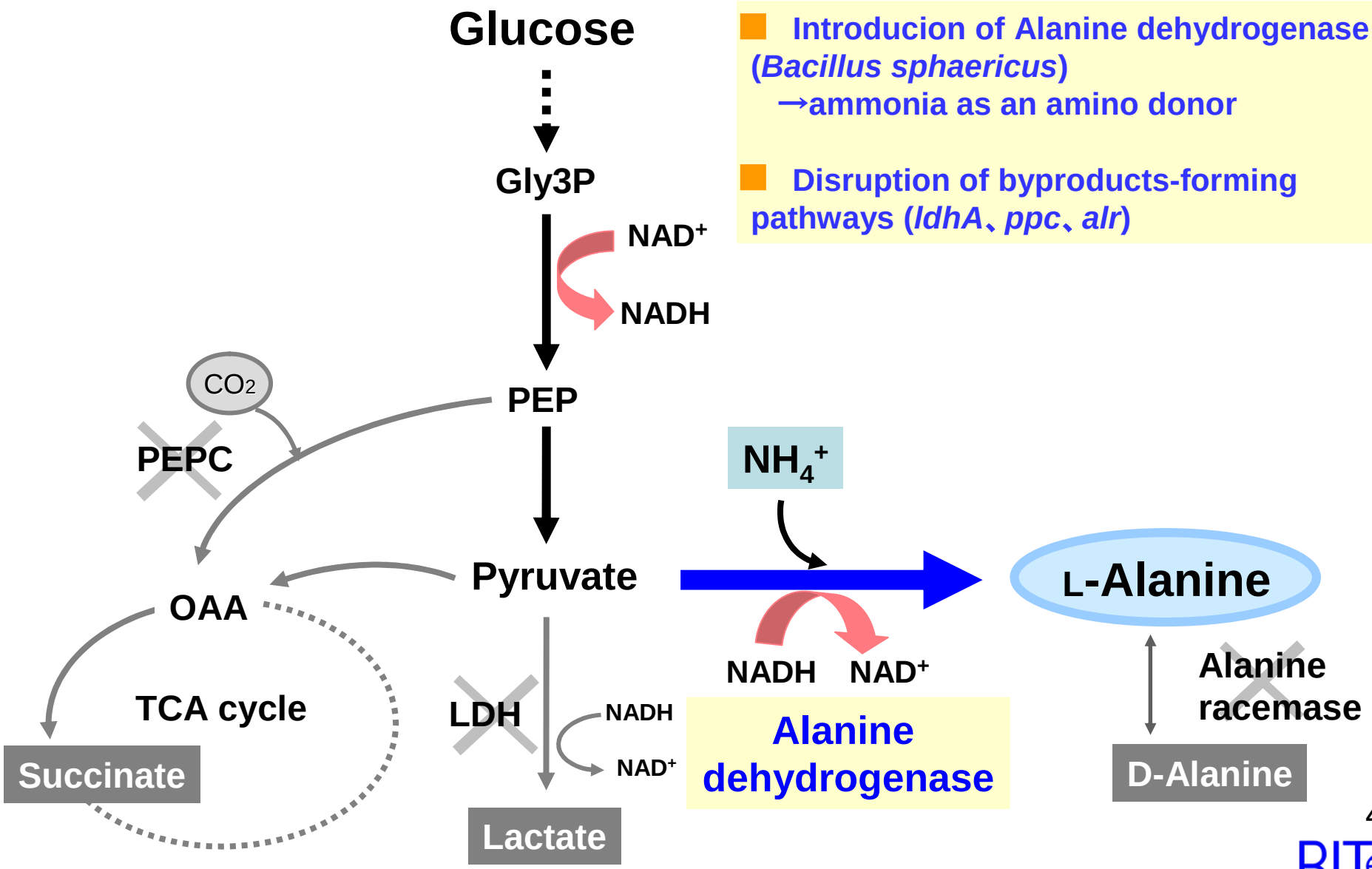
No growth

No aeration

Amino acids

L-Alanine: First trial of amino acid production by RITE bioprocess

Metabolic engineering for L-Alanine production



- Introducion of Alanine dehydrogenase (*Bacillus sphaericus*)
  →ammonia as an amino donor
- Disruption of byproducts-forming pathways (*ldhA*、*ppc*、*alr*)

Summary

- We have developed a novel process “RITE bioprocess” where we can utilize *C. glutamicum* cells like a catalyst.
- RITE bioprocess shows high productivity in bioethanol and biochemicals production.
- Genetically-engineered *C. glutamicum* consumes mixed sugar simultaneously.
- RITE-bioprocess is tolerant to fermentation inhibitors.
- We continue to improve productivity of butanol.

Host vector system

- *Agric. Biol. Chem*. **54:**443-447. 1990.
- *J. Industrial. Microbiol.* **5:**159-165. 1990.
- *Appl. Environ. Microbiol.* **57:**759-764. 1991.
- *Res. Microbiol.* **144:**181-185. 1993.
- *Biosci. Biotech. Biochem.* **57:**2036-2038. 1993.
- *Appl. Microbiol. Biotechnol.* **81:**1107-1115. 2009.

Gene expression system

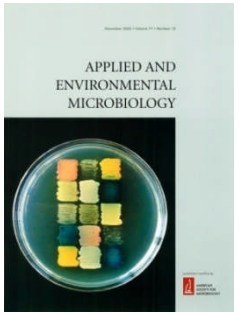
- *FEMS Microbiol. Lett.* **131:**121-126. 1995.
- *Appl. Microbiol. Biotechnol.* **82:**491-500. 2009.

Physiology of corynebacteria

- *DNA seq.* **11:**383-394. 2000.
- *Biochem. Biophys. Res. Commun.* **289:**1307-1313. 2001.
- *J. Biosci. Bioeng.* **92:**502-517. 2001.(Review)
- *Mol. Gen. Genomics.* **271:**729-741. 2004.
- *J. Mol. Microbiol. Biotechnol.* **8:**91-103. 2004.
- *Microbiology* **153:**1042-1058. 2007.
- *Appl. Microbiol. Biotechnol.* **75:**889-897. 2007.
- *Microbiology* **153:**2190-2202. 2007.
- *Appl. Microbiol. Biotechnol.* **76:**1347-1356. 2007.
- *Microbiology* **154:**264-274. 2008.
- *Mol. Microbiol.* **67:**597-608. 2008.
- *J. Mol. Microbiol. Biotechnol.* **15:**264-276. 2008.
- *Appl. Microbiol. Biotechnol.* **78:**309-318. 2008.
- *J. Bacteriol.* **190:**3264-3273. 2008.
- *Appl. Environ. Microbiol.* **74:**5290-5296. 2008.
- *Microbiology* **154:**3073-3083. 2008.
- *Appl. Microbiol. Biotechnol.* **81:**291-301. 2008.
- *J. Bacteriol.* **191:**968-977. 2009.
- *Microbiology* **155:**741-750. 2009.
- *Appl. Microbiol. Biotechnol.* **83:**315-357. 2009.
- *J. Bacteriol.* **191:**2964-2972. 2009.
- *Appl. Environ. Microbiol.* **75:**3419-3429. 2009.
- *Appl. Environ. Microbiol.* **75:**3461-3468. 2009.
- *J. Bacteriol.* **191:**4251-4258. 2009.
- *J. Biol. Chem.* **284:**16736-16742. 2009.
- *Microbiology* **155:**3652-3660. 2009.

***C. glutamicum* R**

The cover of MM



The cover of AEM

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Thank you for your attention

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