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Biotechnological production of succinic acid: current state and perspectives

Ke-Ke Cheng, Xue-Bing Zhao, Jing Zeng and Jian-An Zhang, Tsinghua University, Beijing, P.R. China

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Abstract: Succinic acid has multiple practical applications (e.g. synthesis of 1,4-butanediol, tetrahydrofuran, gamma-butyrolactone, and as a monomer of some biodegradable polymers). Bio-based succinic acid is a potential substitute for current petrochemical production. Facing a shortage of crude oil supply and sharply rising oil prices, biological production of succinic acid from abundant and available biomass has become a topic of worldwide interest. Although great progress has been made in recent decades, much needs to be developed further in order to achieve economic viability. This paper reviews developments in technology and updates research progress of bio-succinate production, including pathways, micro-organisms, culture conditions, as well as integrated production with other high-value-added products. Finally, strategies are proposed for successful commercialization of fermentative succinic acid production. © 2012 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: bioconversion; biomass; fermentation; succinic acid

Introduction

Succinic acid (IUPAC name: butanedioic acid) is a colorless crystal soluble in water, ethanol, and acetone. It is also known as amber acid since it was first obtained by distilling amber in 1550. Succinic acid has a wide range of industrial applications, such as being used as a chemical intermediate for the production of lacquers and perfume esters as well as a flavor, bacteriostatic, or neutralizing agent in the food industry. Furthermore, succinic acid also has a special chemical market for producing coatings, surfactants, dyes, detergents, green solvents, biodegradable plastics, and ingredients stimulating animal and plant growth. Based upon the structure of linear and saturated dicarboxylic acid, succinic acid can be readily converted to other bulk chemicals, such as 1, 4-butanediol, gamma-butyrolactone, tetrahydrofuran, adipic acid, n-methylpyrrolidone or linear aliphatic esters.[1-4] With various environmental implications, the demand for succinic acid is expected to increase significantly. For example, a new biodegradable polymer, poly(1,3-propylene succinate), can be derived by thermal polycondensation of succinic acid with 1,3-propanediol.[5] Succinic acid can be converted to 1,4-butanediol,[6-8] which can be further used for the production of biodegradable poly(butylene succinate) (PBS) with excellent thermal and mechanical properties as well

Correspondence to: Jian-An Zhang, Institute of Nuclear and New Energy Technology, Tsinghua University, Beijing 100084, P.R. China.
E-mail: zhangja@tsinghua.edu.cn

as thermoplastic processability.[9] While the current global succinic acid production is 30 000 to 50 000 tons per year with the market price of US$2400–3000 per ton, the market is expected to reach 100 000 tons per year by 2015.[1,3,10]

Succinic acid can be produced via chemical routes, which mainly include paraffin oxidation,[11] catalytic hydrogenation, or electroreduction of maleic acid or maleic anhydride.[12,13] In the paraffin oxidation process, a calcium or manganese catalyst is employed and several kinds of dicarboxylic acids are obtained at the same time. Succinic acid is then recovered and purified by distillation, crystallization, and drying. However, the yield and purity of succinic acid obtained by this process are relatively poor.[11] Industrialized as early as the 1930s, the hydrogenation process is one of the mature technologies for chemical production of succinic acid. The reaction can be conducted homogeneously or heterogeneously by careful selection of the catalyst. While high yield, purity, and selectivity of succinic acid can be obtained, the operation of this process is complicated, expensive, and might have environmental implications.[13]

Recent developments in the production of succinic acid have been focused on biotechnological alternatives, in particular microbial transformation based upon the use of renewable biomass as feedstock.[14-17] Note that the fact that CO_2 is assimilated during succinic acid fermentation can be considered as an environmental advantage.

At present, the biotechnological production of succinic acid is still at demonstration scale, but significant progress is expected in light of major business joint ventures and research and development activities. For example, BioAmber, a US company dedicated to the production of bio-based succinic acid, has constructed a demonstration plant in Pomacle, France, with a capacity of 2000 tons per year. In parallel, BioAmber developed a turn-key technological package available for licensing in 2011. DSM and Roquette are currently building a large-scale plant with a capacity of 10 000 tons per year and will begin commercial production in 2012. Myriant, a successor to BioEnergy International, was recently awarded US$50 million by the US Department of Energy to construct a succinic acid plant with an initial capacity of 13 500 tons per year in Louisiana, USA, which is expected to be running in 2012. In addition, BASF, Purac, Mitsubishi Chemicals, and PTT have their individual plans for commercial bio-based production of succinic acid.[8]

Recognizing the importance of the biotechnological production of succinic acid, herein we intend to review relevant synthetic pathways, micro-organism producers, cultivation technologies, as well as propose effective strategies toward bioprocess commercialization through the integrated production of succinic acid with other high-value-added products.

Metabolic pathway

Being a key intermediate of the tricarboxylic acid (TCA) cycle, succinic acid can also act as a fermentation end-product for a selection of bacterial strains when glucose or glycerol is used as a carbon source with the fixation of carbon dioxide. There are three routes that can form succinate: the reductive branch of the TCA cycle, also known as the fermentative pathway (which is primarily active under fully anaerobic conditions), the oxidative branch of the TCA cycle (which is primarily active under aerobic conditions), and the glyoxylate pathway, which is essentially active under aerobic conditions upon adaptation to growth on acetate.

Depending on the stoichiometric assimilation of carbon dioxide and hydrogen, the overall yield can vary significantly: [4,10,18]

$$C_6H_{12}O_6 + CO_2 \longrightarrow HOOCCH_2CH_2COOH + CH_3COOH + HCOOH$$

$$7C_6H_{12}O_6 + 6CO_2 \longrightarrow 12\ HOOCCH_2CH_2COOH + 6\ H_2O$$

$$C_6H_{12}O_6 + 2CO_2 + 2H_2 \longrightarrow 2HOOCCH_2CH_2COOH + 2H_2O$$

Under anaerobic conditions, succinate derived from phosphoenolpyruvate (PEP), via several intermediate compounds of the TCA cycle, including oxaloacetate (OAA), malate, and fumarate, is recognized as the primary pathway. Depending on the micro-organism and cultivation condition, other metabolites, such as ethanol, acetate, lactate, and formate, can be synthesized when pyruvate is further oxidized.[19-20] The fermentative pathway converts oxaloacetate to malate, fumarate, and then succinate and this pathway requires 2 moles of NADH per mole of succinate produced. One major obstacle to high succinate yield through the fermentative pathway is due to NADH limitation. This is because 1 mole glucose can provide only 2 moles of NADH through the glycolytic pathway; however, the formation of 1 mole succinate through

the native fermentative pathway requires 2 moles NADH. Therefore, the maximum molar theoretical yield of succinate from glucose is limited to 1 mol mol^{-1} glucose assuming that all the carbon flux will go through the native succinate fermentative pathway. In the bacterium *Actinobacillus succinogenes*, glucose is metabolized to PEP by glycolysis and the oxidative pentose phosphate pathway.[20,21] To further synthesize succinate from PEP, four key enzymes, including phosphoenolpyruvate carboxykinase, malate dehydrogenase, fumarase and fumarate reductase are required (Fig. 1).

In *E. coli*, succinate is a metabolite that is formed under both anaerobic and aerobic conditions. Under aerobic conditions, acetyl-CoA generated from pyruvate mainly enters the TCA for energy and cell intermediates production. Succinate is formed by succinyl-CoA synthetase and subsequently converted to fumarate by succinate dehydrogenase. As a result, succinate does not accumulate in wild-type *E. coli* cultures under aerobic condition. To realize succinate aerobically accumulation, inactivation of sdhA gene to block the conversion of succinate to fumarate in TCA cycle is necessary.[22]

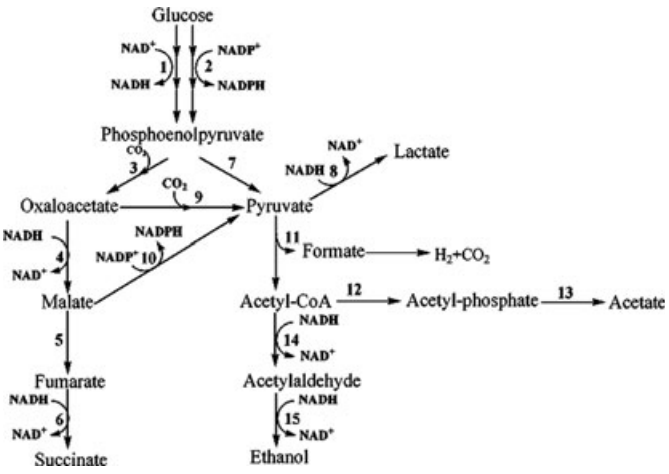

Figure 1. Succinic acid production pathway in *A. succinogenes* (simplified, based on van der Werf *et al*.[20]). (1) Embden–Meyerhof pathway enzymes; (2) pentose phosphate pathway enzymes; (3) phosphoenolpyruvate carboxykinase; (4) malate dehydrogenase; (5) fumarase; (6) fumarate reductase; (7) pyruvate kinase; (8) lactate dehydrogenase; (9) oxaloacetate decarboxylase; (10) malic enzyme; (11) pyruvate–formate lyase; (12) acetate kinase; (13) phosphotransacetylase; (14) acetaldehyde dehydrogenase; (15) alcohol dehydrogenase.

During anaerobic culture, *E. coli* undergoes mixed-acid fermentation that yields acetate, ethanol, formate, and lactate as its major fermentation products with only a small amount of succinate formed. The primary pathway for succinate synthesis occurs by carboxylation of PEP to oxaloacetate using phosphoenolpyruvate carboxylase, which is irreversible (Fig. 2). Different from *A. succinogenes*, conversion of PEP to oxaloacetate using phosphoenolpyruvate carboxykinase is reversible and usually active during gluconeogenesis. Another potential biosynthetic route for succinate in *E. coli* is through the glyoxylate pathway, which is active under aerobic conditions. The glyoxylate pathway converts 2 mol acetyl CoA and 1mol OAA to 1mol succinate and 1mol malate, which can be further converted to succinate using only 1mol NADH. The *IclR* transcriptional repressor (encoded by the gene *iclR*), regulates the expression of the *aceBAK* operon involved in the induction of the glyoxylate pathway upon growth on acetate under aerobic conditions. By deleting the *iclR*, the glyoxylate pathway will be activated in anaerobic condition, which will benefit to achieve higher succinate yield.

The yield of succinate from sugars or other carbon sources is strongly decided by available NADH and ATP produced in the C3 route which results in by-product accumulation.[23,24] In view of the high cost of product recovery, a high final product concentration with high yield is desirable. To achieve this, the formation of acetate, lactate, formate, and ethanol should be controlled at the minimum levels.[25,26] The metabolic pathway indicates that by-product formation is the result of carbon distribution among phosphoenolpyruvate–pyruvate node. The flux toward pyruvate should be controlled so that it allows necessary pyruvate as a biosynthetic precursor. Metabolic engineering can be very helpful in manipulating the pathways so that by-product pathways will be controlled or even eliminated. Changing the activity of enzymes around PEP, pyruvate and OAA would also entail flux redistribution, which could bring improvement in the succinate synthesis.

Micro-organisms

Natural succinate producing strain

Many natural bacterial species have the pathway for convert PEP to succinate. Experimental data infer the presence of the succinic acid pathway (in whole or in part) in the following species: *Actinobacillus succinogenes*,[27-28] *Anaerobiospirillum*

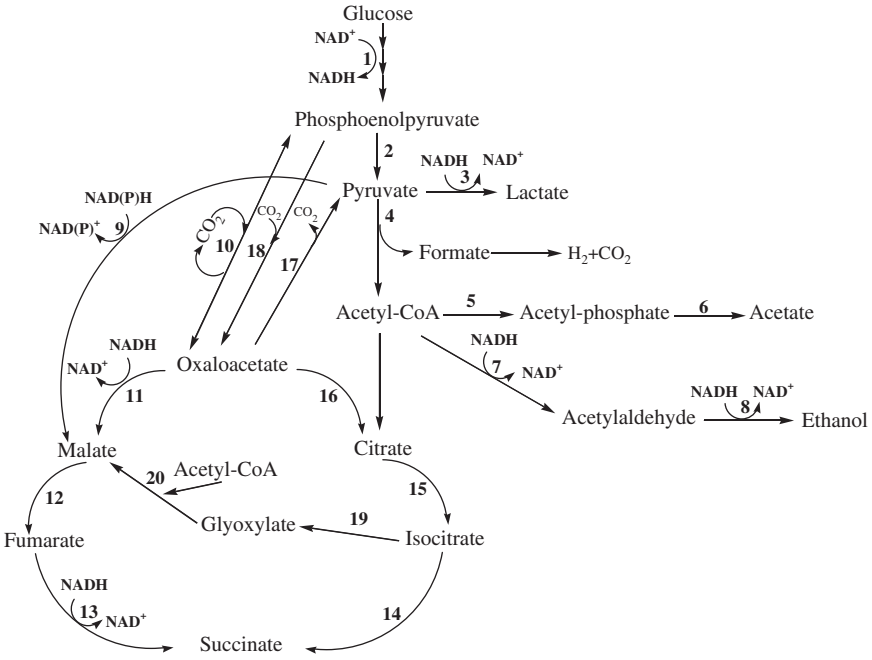

Figure 2. Succinic acid production pathway in *E. coli* (simplified, based on Jantama *et al.*).[17] (1) Embden–Meyerhof pathway enzymes; (2) pyruvate kinase; (3) lactate dehydrogenase; (4) pyruvate–formate lyase; (5) phospho-transacetylase; (6) acetate kinase; (7) acetaldehyde dehydrogenase; (8) alcohol dehydrogenase; (9) malic enzyme; (10) phosphoenolpyruvate carboxylase; (11) malate dehydrogenase; (12) fumarase; (13) fumarate reductase; (14) isocitrate lyase; (15) aconitase; (16) citrate synthase; (17) oxaloacetate decarboxylase; (18) PEP carboxylase; (19) isocitrate lyase; (20) malate synthase.

succiniciproducens,[29] *Bacteroides amylophilus*,[30] *Bacteroides fragilis*,[31-33] *Bacteroides succinogenes*,[34,35] *Clostridium thermosuccinogenes*,[36] *Cytophaga succinicans*,[37] *Escherichia coli*.,[18,38] *Fibrobacter succinogenes*,[39] *Klebsiella pneumoniae*,[40] *Klebsiella oxytoca*,[41] *Mannheimia succiniciproducens*,[42] *Paecilomyces varioti*,[43] *Penicillium simplicissimum*,[44] *Ruminococcus flavefaciens*,[45] and *Succinivibrio dextrinosolvens*.[46] It is worth noting that not all species produce succinate as an end-product and sometimes succinate as an intermediate can be further converted to another product, such as propionate.[31] Some publications report yeast can synthesize succinate. However, the productivity is very poor. [47-49]

Currently, high efficient producers of succinic acid are *Actinobacillus succinogenes*,[50-55] *Anaerobiospirillum succiniciproducens*,[56-59] and *Mannheimia succiniciproducens*.[60-62] *Corynebacterium glutamicum* has been recognized recently to be a very important organism for succinate prodction.[63-65]

Genetically modified strain

Although *A. succinogenes*, *A. succiniciproducens*, *M. succiniciproducens* are well known succinate producers, not many reports have been published on metabolic engineering of these species.[61,66] Due to a rich set of genetic tools available and the fact of fast cell growth and simple culture medium, *E. coli* is one of the most wholly studied systems for succinate production. Strategies in metabolic engineering of *E. coli* can be classified as four main methods: improvement of substrate transportation, enhancement of pathways directly involved in the succinate production, deletion of pathways involved in by-product accumulation, and their combinations.[67-76] These four methods have been studied in many reports and some high efficient succinic acid producers have been constructed.[77-84] Succinic acid production using different bacteria species is compared in Table 1.

Table 1. Microbial succinic acid production using different bacteria species.

Strains	Microbial succinate production					References
	Substrates	Methods	Concentration ($g\ l^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	
		A. succinogenes				
FZ53	Glucose	Anaerobic, batch	105.8	1.36	0.83	27
FZ6	Corn fiber hydrolyzate	Anaerobic, batch	70.6	0.7	0.88	27
130Z	Sake lees	Anaerobic, batch	48	0.94	0.75	85
130Z	Whey	Anaerobic, batch	21.5	0.44	0.57	86
130Z	Glucose	Anaerobic, batch	67.2	0.8	0.7	87
130Z	Glucose	Anaerobic, batch	45.8	1.55	0.83	87
CGMCC 2650	Corn straw	Anaerobic, batch	15.8	0.62	1.23	55
CGMCC 1593	Cane molasses	Anaerobic, batch	50.6	0.84	0.8	88
CGMCC1593	Corn stover hydrolyzate	Anaerobic, fed-batch	53.2	1.21	0.83	89
CGMCC 1593	Glucose	Anaerobic, batch	60.2	1.3	0.75	90
CGMCC 1716	Corn fiber hydrolyzate	Anaerobic, batch	35.4	0.98	0.73	91
CIP 106512	Sugarcane bagasse hydrolyzate	Anaerobic, batch	22.5	1.01	0.43	92
		A. succiniciproducens				
ATCC53488	Glucose	Anaerobic, continuous culture with membrane for cell recycling	83	10.4	0.88	51
ATCC53488	Glucose	Anaerobic, batch	34.4	1.8	0.86	57
ATCC53488	Glucose	Anaerobic, batch	32.2	1.2	0.99	59
ATCC53488	Glucose	Anaerobic, batch	50.3	2.1	0.9	93
ATCC53488	Glucose	Anaerobic, continuous culture	39.1	2.03	0.85	94
ATCC29305	Whey	Anaerobic, fed-batch	34.7	1.02	0.91	95
ATCC29305	Whey	Anaerobic, continuous culture	19.8	3	0.64	95
ATCC29305	Glucose	Anaerobic, continuous culture with membrane for cell recycling	14.3	3.3	0.71	58
ATCC29305	Glycerol	Anaerobic, batch	19	0.15	1.6	96
ATCC29305	Glucose/Glycerol	Anaerobic, batch	29.6	1.35	0.97	96
ATCC29305	Wood hydrolyzate	Anaerobic, batch	24	0.74	0.88	97
ATCC29305	Galactose	Anaerobic, batch	15.3	1.46	0.87	97
		M. succiniciproducens				
MBEL55E	Glucose	Anaerobic, batch	14	1.87	0.70	42
MBEL55E	Whey	Anaerobic, batch	13.4	1.18	0.71	60
MBEL55E	Whey	Anaerobic, continuous culture	6.4	3.9	0.69	60
MBEL55E	Glucose	Anaerobic, batch	10.5	1.75	0.59	99
MBEL55E	Wood hydrolyzate	Anaerobic, batch	11.7	1.17	0.56	100
MBEL55E	Wood hydrolyzate	Anaerobic, continuous culture	8.2	3.19	0.55	100
LPK7	Glucose	Anaerobic, fed-batch	52.4	1.75	0.76	61
		E. coli				
NZN111	Glucose	Anaerobic, batch	12.8	0.29	0.64	101
NZN111	Glucose	Dual phase aeration, Fed-batch	28	0.7	0.7	102
AFP111	Glucose	Dual phase aeration, batch	51	0.52	0.54	67

Table 1. Continued.

Strains	Microbial succinate production					References
	Substrates	Methods	Concentration ($g\ l^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	
AFP111	Glucose	Dual phase aeration, batch	101	1.18	0.78	103
AFP111-*pyc*	Glucose	Dual phase aeration, batch	99.2	1.31	1.1	104
AFP184	Glucose	Dual phase aeration, batch	38	1.27	0.8	105
AFP184	Fructose	Dual phase aeration, batch	30	1.01	0.7	105
AFP184	Xylose	Dual phase aeration, batch	23	0.78	0.5	105
AFP184	Glucose	Dual phase aeration, fed-batch	77	0.71	0.75	76
AFP184	Softwood dilute acid hydrolyzate	Dual phase aeration, batch	42.2	0.78	0.72	103
KJ060	Glucose	Anaerobic, batch	86.5	0.9	0.93	18
KJ060	Glucose	Anaerobic, batch	73.4	0.61	1.06	18
KJ073	Glucose	Anaerobic, batch	78.8	0.82	0.79	18
KJ122	Glucose	Anaerobic, batch	83	0.88	0.9	107
SBS550MG	Glucose	Anaerobic, Fed-batch	40	0.42	1.06	108
SBS550MGpHL314	Glucose	Anaerobic, Fed-batch	40	0.42	1.1	17
HL51276k- pepc	Glucose	Aerobic, batch	8.3	0.16	0.72	109
HL27659k-pepc	Glucose	Aerobic, Fed-batch	58.3	0.72	0.62	109
JCL1208	Glucose	Anaerobic, batch	10.7	0.59	0.29	110
W3110	Sucrose	Dual phase aeration, batch	24	0.81	1.2	70
W3110	Cane molasses	Dual phase aeration, batch	26	0.87	0.52	111
SD121	Corn stalk enzymatic hydrolyzate	Dual phase aeration, batch	57.8	0.8	0.87	112
		C. glutamicum				
R	Glucose	Micro-aerobic, fed-batch with membrane for cell recycling	23	3.83	0.19	64
R $^{\Delta}$*ldh*A-pCRA717	Glucose	Micro-aerobic, fed-batch with membrane for cell recycling	146	3.17	0.92	65
		B. fragilis				
MTCC1045	Glucose	Anaerobic, batch	12.5	0.42	0.62	32
MTCC1045	Glucose	Anaerobic, batch	20	0.83	0.57	33
		P. ruminocola				
ATCC 19188	Glucose	Anaerobic, batch	18.9	0.52	–	87
		F. succinogenes				
S85	Wheat straw	Anaerobic, batch	1.55	0.022	0.05	113
S85	Orange peel	Anaerobic, batch	1.75	0.025	0.044	113
		S. cerevisiae				
AH22ura3 $^{\Delta}$*sdh2*$^{\Delta}$*sdh1*$^{\Delta}$*idh1*$^{\Delta}$*idp1*	Glucose	Aerobic, batch	3.62	0.022	0.072	49

Currently, major research results are obtained using prepared medium and pure sugar as feedstock. Succinate productions are mostly carried out in batch fermentations, while higher concentration and yield are obtained in fed-batch or continuous culture systems. The highest productivity, 10.4 $g\ l^{-1}\ h^{-1}$ was obtained in a continuous culture of *A. succiniciproducens* with

integrated membrane for cell recycling at a dilution rate of 0.98 h^{-1}. Succinate concentration up to 146 g l^{-1} was obtained in a cell recycling fed-batch culture of *C. glutamicum*. In an *E. coli* dual phase (aerobic/anaerobic) batch fermentation using sucrose as substrate the highest described yield of 1.2 g g^{-1} sucrose was obtained. Using lactose as inducer, *E. coli* SD111 also produces succinate with a high yield of 1.1 g g^{-1} glucose in aerobic/anaerobic combined fed-batch fermentations.[114]

Culture conditions

In addition to essential nutrient components for sustaining bacterial cell growth and physiological maintenance, other substrates, including major carbonaceous feedstock, carbon dioxide, and hydrogen, as well as culture pH are also critical for economic production of succinate. Using a selection of bacterial strains, these technical issues and general cultivation strategies are reviewed.

Substrate

Feedstock represents the major cost for most bioprocesses for the production of bulk chemicals. Therefore, identifying inexpensive feedstock for the production of succinate becomes critical to making the bioprocess economical. Among various feedstock options, waste by-products (such as glycerol from biodiesel production) and waste biomass (such as corn stover and corncob) appear to be promising based upon many successful attempts.

A selection of substrates has been used for growing *Actinobacillus succinogenes* to produce succinate, include sake lees,[85] cane molasses,[88] corn stover,[89,115,116] corn fiber,[91,117] wheat flour,[118] corncob hydrolysates,[119] corn stalk and cotton stalk,[55] sugarcane bagasse hemicellulose hydrolyzate,[92] and whey.[86] Cane molasses, pre-treated by sulfuric acid, were consumed as carbon resource for succinate synthesis using *A. succinogenes* CGMCC1593. In anaerobic bottles fermentation, 50.6 g l^{-1} succinate with a productivity of 0.84 g l^{-1} h^{-1} was obtained, and the sugar conversion ratio was about 95.6%. During the fed-batch fermentation with external CO_2 aeration and controlled pH, higher succinate concentration (55.2 g l^{-1}) and productivity (1.15 g l^{-1} h^{-1}) were reached.[88] Succinate was synthesized by *A. succinogenes* 130 Z from cheese whey. At pH 6.8, inoculum size of 5%, 0.5 vvm CO_2 aeration and 200 rpm, initial 50 g l^{-1} cheese whey, 21.5 g l^{-1} succinate with a yield of 0.57 g g^{-1} whey and productivity of 0.44 g l^{-1} h^{-1} were obtained.[86] Corn stover as a substrate was used for succinate fermentation by *A. succinogenes* CGMCC1593.[89] The raw material was pre-treated by diluted alkaline before simultaneous saccharification and fermentation so that part of the lignin could be removed. Cellulose and hemicellolose were further hydrolyzed by cellulase and cellobiase, and used as a medium in succinate production. Corn stalk after steam explosion and then NaOH-H_2O_2 pre-treatment were hydrolyzed by enzyme, obtaining a glucose, xylose, cellubiose medium for succinate production. Using this substrate, *A. succinogenes* CGMCC2650 can produce 15.8 g l^{-1} succinate. The authors observed that glucose and xylose were consumed simultaneously, while cellubiose was not used until glucose and xylose were exhausted.[55]

A. succiniciproducens can produce succinate from whey,[95] glycerol,[96] pre-treated wood hydrolyzate,[97] and galatose.[98] In the latter study, the authors examined succinate production using galactose, galactose/glucose, or galactose/lactose medium, respectively. *Anaerobiospirillum succiniciproducens* ATCC 29305 can consume galactose as the sole substrate at a rate of 2.4 g g dry cell $weight^{-1}$ h^{-1} and synthesis succinate in a yield of 0.87 g g^{-1}. When glucose and galactose coexisted, *A. succiniciproducens* consumed two kinds of sugars synchronously. Further, when a lactose and galactose mixture was used as substrate, galactose consumption was not affected by lactose. The authors concluded that the productivity and economy of biological succinate production could be improved by co-fermentation from galactose and other sugars. Succinate production from whey, whose main compositions are lactose, protein, and lactic acid, was investigated in batch, continuous, and fed-batch cultures using *A. succiniciproducens* ATCC 29305. Using a CO_2 limited medium (1g l^{-1} $MgCO_3$), only 48% of lactose was metabolized and lactic acid accumulated as a major product. Using a high CO_2 level medium (35 g l^{-1} $MgCO_3$), more than 90% of the lactose was metabolized and succinate accumulated as major product. The highest succinate productivity of 3 g l^{-1} h^{-1} reached in continuous culture and the yield was 0.64 g g^{-1}. Higher product concentration and yield (34.7 g l^{-1} and 0.906 g g^{-1}) were obtained in fed-batch cultures.[95] Lee *et al.*[96] found that *A. succiniciproducens* ATCC 29305 can metabo-

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lize glycerol as a carbon source to accumulate succinate and glycerol consumption depending on the yeast extract concentration in the medium. When glycerol was used as the only carbon source in a medium added with yeast extract, the highest 19 g l^{-1} succinate was obtained. When a glycerol-glucose mixture was used as co-substrate, 29.6 g l^{-1} succinate was produced. The authors concluded that succinate production from glycerol has some advantages over glucose, such as increased succinate yield and decreased acetate formation, which benefit downstream processes because acetate is not easy to separate from succinate.

M. succiniciproducens produced succinate when grown on whey[60] and wood hydrolyzate.[101] In the latter study, oak wood cellulosic residues after steam explosion pre-treatment were hydrolyzed by cellulase, obtaining a glucose/xylose mixture. This mixture was treated with sodium hydroxide before sterilization for reducing inhibitors. *M. succiniciproducens* MBEL55E metabolized xylose/glucose co-substrate in the wood hydrolyzate-based medium for succinate production. In batch fermentations, 11.7 g l^{-1} succinate was obtained, giving a succinate yield of 0.56 g g^{-1} sugar and a productivity of 1.17 g l^{-1} h^{-1}; while the continuous fermentations at a dilution rate of 0.4 h^{-1} obtained similar succinate yield (0.55 g g^{-1}) but higher productivity of 3.19 g l^{-1} h^{-1}. Using the same strain, another substrate, whey and corn steep liquor, were investigated for succinate production in batch and continuous fermentation. In batch fermentations, 13.4 g l^{-1} succinate was obtained, giving a succinate yield of 0.71 g g^{-1} lactose and a productivity of 1.18 g l^{-1} h^{-1}. In the continuous fermentations succinate yield is in the range of 0.63–0.69 g g^{-1} lactose. The highest productivity of 3.9 g l^{-1} h^{-1} was obtained at a dilution rate of 0.6 h^{-1}.[60]

Succinate production using corn stalk enzymatic hydrolyzate by recombinant strain *E. coli* SD121 was studied by Wang *et al*.[112] In this case, a two-stage aeration strategy was used. The aerobic culture was carried out for the first 12 h with a reducing sugar concentration about 44 g l^{-1} and the cell growth entered into the middle exponential phase with a dry cell weight of about 7.6 g l^{-1}. Then the anaerobic culture was triggered by CO_2 aeration. The final concentration of succinate was 57.81 g l^{-1}. The overall productivity and yield of succinate in the whole anaerobic stage were 0.96 g l^{-1} h^{-1} and 0.87 g g^{-1} total sugar, respectively. *E. coli* SBS550MG bearing both the pHL413 plasmid, which contains *L. lactis* pycA gene, and the pUR400 plasmid, which contains the *scrK*, *Y*, *A*, *B*, and *R* genes for sucrose uptake and catalyzation was tested using fructose, sucrose, a mixture of glucose and fructose, a mixture of glucose, fructose and sucrose, and sucrose hydrolysis solution medium for succinate production. Compared to the culture grown on fructose or sucrose alone, co-utilization of glucose with fructose and sucrose increased in succinate productivity. When SBS550MG pHL413 pUR400 was cultured using a glucose–fructose–sucrose mixture medium, glucose was utilized preferentially to sucrose and sucrose was utilized preferentially to fructose.[120]

Sugars from waste biomass hydrolyzates (especially those derived from the hemicellulose fraction) have been used for succinate fermentation. However, the cellulosic feedstock often contains inhibitors which negatively affect the fermentation efficiency and succinate yield. These inhibitors might include weak acids, furans, and phenolic compounds, and can be partly removed by detoxification process, such as treatment with overliming or activated carbon adsorption. However, the fermentation parameters for the treated hydrolyzate are still lower than those obtained with a synthetic medium. This shows that there were some leftover toxic components in the treated hydrolyzate that negatively affected the fermentation performance. To further improve the fermentation efficiency, proper culture conditions and detoxification techniques should be developed to alleviate the inhibitions.

CO_2 supply

CO_2 supply is an important variable in succinate fermentation. Not only can external CO_2 gas supply but also some carbonates in the medium can be considered as a source of CO_2. As a consequence, for a high succinate yield, CO_2 or some carbonates are needed. When CO_2 or carbonates dissolve in water, dissolved CO_2 or carbonates react with water producing HCO_3^- and CO_3^-. The equilibrium among CO_2, HCO_3^- and CO_3^- are decided by pH in medium.

The effects of carbon dioxide levels on the glucose fermentation and cells grown at pH 6.2 were studied by Samuelov *et al*.[121] In this study, CO_2 source was provided by $MgCO_3$. When the molar ratio between CO_2-HCO_3^- and glucose was 0.5–1.0, about 15% of the available carbon (glucose plus CO_2-HCO_3^-) was converted to biomass. Sixty-five percent of the carbon was converted to succinate. When the molar

ratio between CO_2-HCO_3^- and glucose was 0.065, only 8% of the carbon was converted into biomass. Approximately 50% of the carbon was fermented into lactate, and 30% was converted into succinate. Under low CO_2-HCO_3^- concentrations, the ATP yield was 0.75 mol mol^{-1} glucose, whereas under high CO_2-HCO_3^- concentrations, the ATP yield was 2.55 and 2.47, respectively. The ATP yield were significantly increased under sufficient CO_2-HCO_3^- conditions, suggesting that there is a critical value of CO_2 in *A. succiniciproducens* above which succinate production improves significantly. The authors concluded that lactate production in *A. succiniciproducens* was controlled by high pH and succinate production was controlled by CO_2 availability. At pH 6.2 and sufficient CO_2-HCO_3^- conditions, succinate accumulates as a major product. PEP carboxykinase activity is high, but lactate dehydrogenase and ethanol dehydrogenase activity are lacking; whereas at pH 7.2 and insufficient CO_2-HCO_3^- conditions, PEP carboxykinase activity is lower and lactate dehydrogenase and alcohol dehydrogenase activity are detected.

The effect of CO_2 aeration at different pH on cell growth and succinate formation were investigated by Lee *et al*.[122] using *A. succiniciproducens* in pH-controlled batch fermentations at 0.25 vvm CO_2 flow. Inhibited cell growth by CO_2 aeration was observed at pH 6.2 and 6.5. At pH 6.2, there is a constant succinate yield of 0.82–0.83 g g^{-1} glucose with or without CO_2 supply. However, succinate yield increased from 0.84 g g^{-1} without CO_2 supply to 0.88 g g^{-1} with CO_2 supply at pH 6.5. At pH 7.2, both dry cell weight and succinate yield decreased sharply. They concluded that different succinate yields were due to different CO_2 solubilities at different pH. It is reasonable to explain succinate yield increase with CO_2 aeration at pH 6.5. However, it can't explain why succinate yield keeps the same at pH 6.2 with or without CO_2 supply. Different to succinate yield, biomass was adversely affected by CO_2 aeration. These phenomena hint that CO_2 has selective inhibition on metabolism by affecting intracellular enzymatic activities. This hypothesis was proved by the phenomena that some enzymes, particularly, involved in carboxylation or decarboxiation reactions can be affected by CO_2.

The effect of the CO_2 availability on biomass formation and succinate fermentation under various CO_2 partial pressures (calculated by a model developed by the authors) were investigated in batch culture using *M. succiniciproducens* MBEL55E.[123] Biomass formation was strongly inhibited at low CO_2 availability. Only weak biomass formation was found during the first 0–4 h under dissolved CO_2 concentration of 8.74 mM. Biomass formation and succinate production enhanced in proportion as CO_2 availability improved. For further enhancing CO_2 availability in the medium, batch cultures were fulfilled with varied concentrations of $NaCO_3$, $MgCO_3$, or $CaCO_3$ implement as an additional CO_2 source. When 119 mM of $NaHCO_3$ or $MgCO_3$ (both corresponding 141 mM dissolved CO_2 concentration) was added, biomass formation and succinate fermentation were further increased. Compared with the yields of biomass and succinate at dissolved CO_2 concentration of 8.74 mM, the yields increased at dissolved CO_2 concentration of 141 mM by 49% and 52%, respectively. However, biomass formation and succinate production were inhibited to some degree in the media with 238 mM of $NaHCO_3$ and $MgCO_3$ (corresponding to dissolved CO_2 concentration of 260 mM and 163 mM, respectively). The addition of $CaCO_3$ strongly suppressed biomass formation because the maximum specific growth rate and the maximum dry cell weight were even less than those at dissolved CO_2 concentrations of 8.74 mM.

These results tell us that CO_2 availability has a great impact on biomass and product formations. The pH is also a key factor because it affects the solubility of CO_2 in the medium, and as a consequence influences the availability of CO_2 for micro-organisms. Because different micro-organisms can tolerate different CO_2 levels during the fermentation, the best CO_2 concentration should be obtained on an individual basis for each micro-organism and medium used.

H_2

In addition to CO_2, H_2 as a potential electron donor can affect cellular metabolism. Lee *et al*.[122] found that H_2/CO_2 mixed aeration improved *A. succiniciproducens* biomass formation and succinate fermentation and that the ratio of H_2/CO_2 was optimized. Compared with 100% CO_2 flow, the succinate yield and productivity using 5% H_2 and 95% CO_2 increased by 5.8% (0.86 *vs* 0.91 g g^{-1}) and 80% (1 *vs* 1.8 g $l^{-1}h^{-1}$), respectively. They concluded that the enhancement was due to the decreased cellular redox potential and the improved NADPH recycles. Therefore, the outside supplement of H_2 seems to have two major functions: (i) to decrease intracellular redox potential which will enhance

biomass formation and (ii) to promote glucose bioconversion to succinate due to the incorporation of electrons derived from H_2. Similar phenomena were observed by Van der Werf *et al*;[21] succinate production by *Actinobacillus* sp. 130Z was enhanced when 100% H_2 was aerated. Compared with pure CO_2 flow, acetate formation declined by 6% when external supply of 5% H_2 was added. They deduced that succinate is a highly reduced fermentative product using four electrons per molecule formed, and therefore the supplementation of H_2 may lead to the accumulation of a greater amount of more reduced fermentative product succinate, rather than acetate.

The supply of other electron donors, such as electrically reduced neutral red, also led to the obvious improvement of succinate fermentation.[66] These conclusions are in accordance with that the use of more reduced substrates such as mannitol and arabitol which led to considerable enhancement in the succinate yield compared with glucose (increase by 21.3% and 37.3%, respectively), while the use of more oxided sugars such as gluconate which resulted in decrease in the succinate yield.[21,124]

pH

The pH is a key parameter in the bioconversion process because both intracellular enzymatic activities and cellular maintenance are strictly pH dependent. Generally, the optimal pH for bacterial culture is about 6–7, among which the optimum biomass yield will be obtained. Most succinate production by bioconversion is accompanied by accumulation of other organic acids, such as acetate and lactate. Thus, during succinate fermentation, if no pH buffer is included, the medium will acidulate gradually. The undissociated acids, which will be harmful for biomass formation and substrate consumption, will increase. Due to fermentative product inhibitions, the metabolism will end.

With uncontrolled pH from an initial pH of 7.0, *Actinobacillus* sp. 130Z produced acetate, formate, ethanol, and succinate as the major products, with a gradual pH decline to 5.2, at which biomass formation ended. The optimal pH for biomass formation was found at 7.0. The concentrations of product accumulated were almost the same in the scope of pH 6.0–7.4.[21] Similar results were found in *A. succinogenes* CGMCC1593; it did not propagate and did not accumulate succinate at pH below 5.5. The pH for the highest succinate yield was found at pH 6.7. The molar ratios of succinate/acetate in pH 6.0–7.2 were stable though the concentrations were different.[90] The effects of some neutralizers (including $MgCO_3$, $CaCO_3$, Na_2CO_3, NaOH and NH_4OH) were investigated for pH buffer in succinate fermentations using *A. succinogenes* CGMCC1593. The authors observed that there was no succinate or other organic acid accumulation if NH_4OH was used as a neutralization buffer, and the strains could not propagate due to the toxicity NH_4OH. When Na_2CO_3 or NaOH was used, cells flocculated and lumped after 16 h, and biomass dropped sharply. However, cell growth was normal during the whole culture process with $MgCO_3$ as pH neutralizer.

According to Nghiem *et al*., [59] the optimum pH value for succinate production by *A. succiniciproducens* ATCC 53438 is the pH 6. At pH both above and below 6.0, the rate of cell growth was significantly decreased. At pH 5.0, cell didn't grow at all. The consumption of glucose follows the same pattern. A range of pH 6.2–6.5 was thought to be fit for succinate fermentation using *A. succiniciproducens* ATCC 29305.[122] However, in this case, succinate formation was slightly higher at higher pH (35 g l^{-1} at pH 6.5 compared to 33.2 g l^{-1} at pH 6.3), although the productivity (2.01 g $l^{-1}h^{-1}$ at pH 6.2 compared to 1.92 g $l^{-1}h^{-1}$ at pH 6.5) was higher at lower pH.

It was found that *Enterococcus flavescens* accumulated succinate in range of pH 4.0–9.0. The maximum productivity of 0.92 g l^{-1} h^{-1} was reached at pH 6.5, above which there was a decrease in the succinate concentration.[125] The enzymes involved in succinate formation, such as phosphoenol pyruvate carboxylase (PPC), phosphoenol pyruvate carboxykinase (PPCK), and malate dehydrogenase, showed maximum activities at pH 6.5. Similarly it was found that *M. succiniciproducens* MBEL 55E showed the same cell growth at a pH of 6.0–7.5 and accumulated succinate, acetate and formate at a constant ratio of 2:1:1.[43]

Yuzbashev *et al*.[126] developed a yeast *Yarrowia lipolytica* Y-3314 in which the gene coding of one of the succinate dehydrogenase subunits was deleted and could use glycerol as a substrate to produce succinate even at pH below 3.5. When $CaCO_3$ was used for buffer during fermentation in shaking flasks, the average succinate concentration was 45.5 g l^{-1}. When no buffer was used, the pH dropped below 3.5 after 72 h of fermentation. Nevertheless, 17 g l^{-1} succinate accumulation was observed in the cell stationary phase.

Integrated production with other high-value-added product

NADH and ATP have to be yielded for biomass formation and succinate production by oxidation of glucose or another substrate to some products, such as acetate and lactate. Many of these undesired products are low-value-added. Genetic engineering approaches and biorefining strategies provide highly effective methods for producing desired and high-value-added compounds. Because the major object is to produce economic and green biological succinate, a type of fermentation that produces two commercial interests might be considered for a promising biological production process.

Using a low level of alkali metals medium (total salts 4.2 g l^{-1}), *E. coli* KJ073(*ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*, *poxB*) produces 78.9 g l^{-1} succinate and 15.8 g l^{-1} malic acid in a batch fermentation with initial 100 g l^{-1} glucose, strain KJ071 (*ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*), can produce 33.1 g l^{-1} succinate and 69.2 g l^{-1} malic acid with total molar yields (succinate+malate) of 2.2 per mole of glucose metabolized. This study could have the advantages of flexible production using different microorganisms (KJ073 or KJ071) and producing different compounds (succinate or malic acid) depending on the market situation.[18]

An isoamyl acetate production pathway was designed in *E. coli* by expression pyruvate carboxylase and alcohol acetyltransferase for accumulation isoamyl acetate and succinate with high yields of both high value products.[127] Since the two products have different volatility, they can be easily purified. Using 20 g l^{-1} glucose and 0.8 g l^{-1} isoamyl alcohol, *E. coli* SBS990MG(pHL413, pKmAT) produced 1.22 g l^{-1} isoamyl acetate and 5.37 g l^{-1} succinate. In this process, succinate formation was used to keep the reasonable balance between NADH and NAD^+ to maximize isoamyl acetate yield.

A genetically modified *E. coli* QZ1112 was constructed by inactivation of sdhA, Pta, and PoxB gene and expressing phbCAB genes from *Ralstonia eutropha* to accumulate extra-cellular succinate and intra-cellular polyhydroxybutyrate at the same time in aerobic culture.[22] In a batch culture using 45 g l^{-1} glucose, *E. coli* QZ1112 can accumulate 24.6 g l^{-1} succinate and 4.95 g l^{-1} polyhydroxybutyrate, which occupied 41.3% of dry cell weight. During polyhydroxybutyrate formation, a great deal of NADPH was used, which would promote the tricarboxylic acid cycle and benefit succinate production. Further, polyhydroxybutyrate formation reduced pyruvate and acetate secretion and had a positive effect on the cell growth. Therefore, integrated production of succinate and polyhydroxybutyrate made the carbon flux more balanced. Based on *E. coli* LR1110, an *E. coli* KNSP1 strain was developed by deletions of ptsG, sdhA and pta genes and overexpression of phaC1 from *Pseudomonas aeruginosa*. Using a pulse glycerol feeding strategy in aerobic cultivation, *E. coli* KNSP1 can produce 21.07 g l^{-1} succinate and 0.54 g l^{-1} PHA (occupied 41.3 % of dry cell weight) from glycerol and fatty acid mixture. The generated PHA composed 58.7 mol% 3-hydroxyoctanoate and 41.3 mol% 3-hydroxydecanoate. This strain would be useful for utilization of by-product glycerol and fatty acid from the biodiesel production process.[128]

Glycerol metabolism in *Klebsiella pneumoniae* includes two routes: the reductive route and the oxidative route. In the reductive route, glycerol is first converted to 3-hydroxypropanal by glycerol dehydratase. 3-Hydroxypropanal is then converted to 1,3-propanediol by 1,3-propanediol oxidoreductase. In the oxidative route, some by-products, such as 2,3-butanediol, acetate, lactate, succinate, and ethanol, are produced. *K. pneumoniae* HR526, a new screened organism, exhibited high productivity but excrete lactate in the late-exponential phase as the main by-product. When *K. pneumoniae* LDH526 was developed with D-lactate pathway deletion, intra-cellular NADH/NAD^+ increased significantly. Due to more NADH formation in the late-exponential phase, cell growth in fed-batch fermentation by *K. pneumoniae* LDH526 was quicker and biomass increased by about 6% at the stationary phase. 1,3-propanediol concentration increased from 95.4 to 102.1 g l^{-1}. Succinate formation, as an alterative NADH consumption route, was also enhanced and its concentration increased from 9.2 to 13.8 g l^{-1}.[129]

A biorefining strategy based on solid state fermentation using wheat as feedstock for succinate production was developed by Du *et al*.[118] At first, wheat was fractionatingly treated to bran, gluten, and gluten-free flour by size reduction and extraction. The bran was used for *Aspergillus awamori* glucoamylase preparation and *Aspergillus oryzae* protease preparation by solid state fermentation, respectively. The prepared raw enzymes were separately utilized

to hydrolyze flour and gluten to produce over 140 g l^{-1} of glucose solution and a more than 3.5 g l^{-1} of amino nitrogen solution. A mixed medium consisting of these two solutions contained all the necessary nutrients required for succinate production. In a fermentation using *A. succinogenes* ATCC 55618 from the mixed medium and addition of $MgCO_3$, around 64 g l^{-1} succinate was obtained. These results demonstrate that wheat can be efficiently utilized for the production of succinate and other high-value-added products by biorefining strategies.

Conclusions and perspectives

Succinate has been included in the US Department of Energy's Top Value Added Chemicals from Biomass based on its potential to become an important building block for deriving both commodity and specialty chemicals.[130] Three important process parameters decide the economical viability of a bioprocess: yield, concentration, and productivity. The yield relates more to the variable cost of raw feedstock and will be of increasing importance with increasing sugar prices. Productivity and concentration relate more to the fixed cost and total investment. Low rates imply larger energy and labor cost; low concentrations will result in larger energy input for product recovery. Compared with petrochemical-derived succinate, biological succinate production is still not economically competitive, because of its major drawbacks: high cost of the feedstocks, low product concentration in the fermentation broth, the co-production of low-value acid by-products, and difficult product recovery. To increase the competitiveness of the biological production of succinate, future work should be addressed as follows: increasing succinate concentration and yield through metabolic engineering; introducing and optimizing the succinate synthetic pathway in the species with high succinate tolerance; overcoming the substrate repression effect and utilizing low-cost non-food-based feedstocks; and integrating production of succinate with other high-value-added products. Lignocellulose is the most abundant renewable resource on Earth. Succinate production from lignocellulose is promising using a consolidated bioprocessing system in which cellulase and hemicellulase production, substrate hydrolysis, and succinate fermentation are combined or partially combined by a succinate-producing organism that expresses a cellulolytic or a cellulolytic enzyme gene or engineering cellulolytic organism with succinate production properties. By studying metabolic regulation and metabolic engineering bacteria, combined with physical and chemical pre-treatment technology, it is expected to design new integrated biorefinery technology to transform lignocellulosic biomass into succinate successfully.

Recovery and purification of succinate represents a technological challenge and an economical obstacle for an efficient microbial production on a large scale. All methods used at present showed unsatisfied product yield and purity. For further development, traditional separation techniques should to be improved and coupled with upstream technology. For example, by target deletion of acid by-products using genetic modification to lower or eliminate acid by-products' accumulation, the selectivity of succinate in reactive extraction over other acid by-products will be improved. Since product inhibition is found in succinic acid fermentation,[131] another approach worth considering and an attractive method for increasing productivity of the process is fermentation combined with *in situ* product removal. Such an approach, if precisely optimized, could result in a highly efficient bioprocess. Furthermore, a novel separation method using an emulsion liquid membrane, which shows high selectivity in the separation of acetate from succinate, deserves attention in the future.[132]

Biological succinate can decrease the use of non-renewable resource and reduce greenhouse gas emissions. Currently, the demand for succinate has been entering a time of rapid increase, bringing both profits and a driving force. We believe that through extensive industry-university-research co-operation, a sustainable and economically viable process for biosuccinate production can be established in the near future.

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Keke Cheng

Dr Keke Cheng received his PhD from the Department of Chemical Engineering, Tsinghua University in 2005. He has worked at the Institute of Nuclear and New Energy Technology, Tsinghua University, as an Assistant Professor since April 2005. His specialized fields are biorefinery, biofuel and fermentation technology.

Xuebing Zhao

Dr Zhao obtained his PhD in 2009 and continued his post-doctoral work at the Department of Chemical Engineering, Tsinghua University. He became an Assistant Professor in the same department in 2011. His current research topics are the biorefining of lignocellulosic biomass to produce biofuels and chemicals.

Jing Zeng

Jing Zeng received her Master's degree from the Department of Chemical Engineering, Tsinghua University in 2006. She has worked at the Department of Chemical Engineering, Tsinghua University, as a research assistant since April 2006. Her specialized field is biofuel policy.

Jian-An Zhang

Dr Jian-An Zhang is Associate Professor at the Institute of Nuclear and New Energy Technology in Tsinghua University. His main research interests are bioenergy (bioethanol, biodiesel, biobutanol), biochemical engineering and the utilization of lignocellulose biomass.

