Microbial production of lactic acid: the latest development

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Abstract
Lactic acid is an important platform chemical for producing polylactic acid (PLA) and other value-added products. It is naturally produced by a wide spectrum of microbes including bacteria, yeast and filamentous fungi. In general, bacteria ferment C5 and C6 sugars to lactic acid by either homo- or hetero-fermentative mode. Xylose isomerase, phosphoketolase, transaldolase, L- and D-lactate dehydrogenases are the key enzymes that affect the ways of lactic acid production. Metabolic engineering of microbial strains are usually needed to produce lactic acid from unconventional carbon sources. Production of D-LA has attracted much attention due to the demand for producing thermostable PLA, but large scale production of D-LA has not yet been commercialized. Thermophilic Bacillus coagulans strains are able to produce L-lactic acid from lignocellulose sugars homo-fermentatively under non-sterilized conditions, but the lack of genetic tools for metabolically engineering them severely affects their development for industrial applications. Pre-treatment of agriculture biomass to obtain fermentable sugars is a pre-requisite for utilization of the huge amounts of agricultural biomass to produce lactic acid. The major challenge is to obtain quality sugars of high concentrations in a cost effective-way. To avoid or minimize the use of neutralizing agents during fermentation, genetically engineering the strains to make them resist acidic environment and produce lactic acid at low pH would be very helpful for reducing the production cost of lactic acid.

Keywords
Application, fermentation, lactic acid, metabolic engineering, microorganism, product separation, renewable resource

Introduction
Traditionally, petroleum derivatives were used for the production of plastics. The rapid depletion of fossil fuels and the concerns for the environment have motivated people to develop clean and green approaches to produce recyclable and biodegradable plastics using renewable biomass as feed stocks (Abdel-Rahman et al., 2013; Okano et al., 2010). Optically pure L- and D-lactic acids (LA) are the precursors for producing biodegradable polylactic acid (PLA), which is the major component of bioplastic polymers. Historically, LA was first discovered by Carl Wilhelm Scheele from sour milk in 1790 and the industrial production of LA by microbial fermentation was developed by a French scientist Frémy in 1811. Traditionally, LA is used in the food industry (processing meat, preservative and salad dressing), pharmaceutical industry (pH regulator, metal sequestration, chiral intermediate and as a natural component of drugs), detergent industry (detergent agent) and dairy industry (improving performances of farm animals; http://www.lactic-acid.com).

From 2010 to 2016, the global demand for LA and PLA were expected to increase from 482.7 to 1076.9 kilo tons (annual growth rate 14.2%) and from 248.8 to 870.8 kilo tons (annual growth rate 20.8%), respectively. Nature Works LLC (140 000 tons, USA), Purac (100 000 tons, Netherlands), Pyramid Bioplastics Guben GmBH (60 000 tons, Germany), Archer Daniels Midland Company (USA) and Henan Jindan (China) are the leading companies for LA and PLA production (Sauer et al., 2008; http://www.marketsandmarkets.com/Market-Reports/polylacticacid-387.html).

Along with the increasing demand, the increasing price of LA is one of the major concerns for making commercially viable PLA. To meet the requirements of PLA as per the demand, the production cost of LA should be less than US$0.8 per kg and its selling price should go to half its current market price (Okano et al., 2010; Taskila & Ojamo, 2013). Currently, LA is produced from glucose derived from starchy materials, which not only contributes to a significant proportion of LA production costs but also competes with food and feed supply. Therefore, the use of inexpensive, abundant and renewable lignocellulosic biomass as an alternative carbon source has attracted much attention in recent years. This review considers the latest progress regarding the isolation and genetic engineering of microbes for LA production, structural biology of key enzymes involved in LA metabolic pathway, raw materials especially lignocellulose and key ingredients for LA fermentation, product separation, purification and applications of LA based on recent 5 years’ publication and patents.
Lactic acid is produced by a wide spectrum of natural microbes. Isolation of various LA producing microbes is an important way to obtain excellent LA producers. These new isolates have been characterized for their ability to utilize various carbohydrates (C5 and C6 sugars) and yield LA (Abdel-Rahman et al., 2013; Ye et al., 2013a). The LA producing microbes are usually isolated from soil, fruits, milk products and the feces of animals. Some thermophilic lactic acid bacteria that are able to digest C5 sugars have been isolated from soils (Ye et al., 2013c). Soil samples were enriched by growing overnight at 50°C in the enrichment media containing xylose as the sole carbon source. The cultures were diluted and plated onto agar plates containing Bromocresol Purple. The bacteria that are able to produce organic acids cause a color change from green to yellow around the colonies. The disadvantage of this screening method is that the color change is not only specific for LA but also for other acids (Ye et al., 2013c). A similar approach was applied to isolate lactic acid bacteria from food samples using the Bromocresol Purple (Mohd Adnan & Tan, 2007). Similarly, a native strain able to convert glyceral to LA was isolated from soil by enriching the soil flora at 37°C in the enrichment medium containing glyceral. After enrichment, the sample was diluted and plated over the enrichment medium plates containing CaCO₃. When glyceral was consumed to acids, the acids reacted with CaCO₃ forming halos around the colonies. Colonies with bigger size and larger transparent zones were selected for verification of LA production (Hong et al., 2009). The isolated colonies were further grown in shaking flasks to confirm the production of LA by HPLC. A method specifically designed for high throughput screening of lactic acid bacteria has not yet been available.

Native lactic acid bacteria, that were isolated from diverse natural sources, are listed in Supplemental Table S1. The bacteria that can meet industrial requirements such as fast growing, high cell density in minimal media, ability to metabolize all sugars and resistance to high concentrations of sugars and LA (>200 g l⁻¹) are industrially attractive. Supplemental Table S2 lists the patented lactic acid bacteria. Although a large number of lactic acid bacteria have been isolated, only a few are able to meet the strict industrial requirements. Therefore, further improvement in lactic acid bacteria by whole genome mutagenesis or metabolic engineering is often needed for industrial applications.

Microbial production of lactic acid
Lactic acid was chemically produced using lead as the catalyst from the plant biomass fraction containing crystalline cellulose and water at a yield of 60% (Wang et al., 2013), giving a racemic mixture containing both D- and L-isomers (Gao et al., 2011). LA production by microbial fermentation harbors multiple advantages over the chemical routes, such as the availability of a broad range of low-cost substrates and low energy consumption due to moderate temperatures employed (Gao et al., 2011; Okano et al., 2010). In nature, LA is produced by a wide spectrum of microorganisms including bacteria, fungi, cyanobacteria and algae.

Lactic acid production by bacteria
Lactic acid is produced by many genera of bacteria as either primary or secondary fermentation products. Among these genera, the term lactic acid bacteria is reserved specifically for the genera in the order Lactobacillales, which include Lactobacillus, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Tetragenococcus, Vagococcus, Leuconostoc, Oenococcus, Weissella, Streptococcus and Lactococcus. Among them, Lactobacilli and cocci have been predominantly used in food industries to preserve food and inhibit microbial contamination. Due to their traditional use in the food industry and their non-pathogenic character, lactic acid bacteria have been given a unique Generally Recognized As Safe (GRAS) status (Abdel-Rahman et al., 2013; Reddy et al., 2008). Lactic acid bacteria are Gram negative, non-sporulating, devoid of cytochromes and produce LA as the major product during carbohydrate fermentation and they have cocci or bacilli shapes. Due to their deficiency in cytochromes, they lack an electron transport chain and rely on the fermentative mode for respiration. Due to the low energy yield in the fermentative mode of respiration, the growth of lactic acid bacteria is relatively slow when compared to LA producing Bacilli (Reddy et al., 2008). In industrial fermentation processes, the slow-growing lactic acid bacteria take a long time to reach high cell densities. Consequently, completion of fermentable sugar uptake requires a longer time affecting the productivity (Abdel-Rahman et al., 2013).

Lactic acid bacteria are grouped into homo- and hetero-fermentative strains. Homo-fermentative lactic acid bacteria contain the gene expressing the aldolase enzyme with LA being the major product. They convert one molecule of glucose to two molecules of LA generating two molecules of ATP (Figure 1). Hetero-fermentative lactic acid bacteria use the phosphoketolase pathway to convert one molecule of xylose to one molecule of LA and one molecule of ethanol or acetic acid (Figure 1). They convert one molecule of glucose to one molecule of LA, one molecule of acetic acid or ethanol, one molecule of CO₂ and generates one molecule of ATP. Homo-fermentative strains that produce optically pure L (+) or D (-) LA are industrially attractive compared to hetero-fermentative strains due to the higher LA yield and easier downstream processing (Abdel-Rahman et al., 2013; Reddy et al., 2008; Teusink et al., 2011). Lactobacillus manihotivorans LMG 18010, L. lactis and S. bovis 148 produce L-LA, L. plantarum A6 produce racemic D-, L-LA and L. delbrueckii is the only known D-LA producer (Okano et al., 2010).

Co-cultivation of lactic acid bacterial strains was conducted to obtain high cell density to increase the LA productivity from Cassava bagasse hydrolysate. L. paracasei subsp. paracasei and a fast-growing mutant of L. delbrueckii subsp. delbrueckii were grown separately to high cell densities (10⁹ CFU/ml) and mixed in a 1:1 ratio as a starter culture for inoculation. Maximum LA (>38 g/l) was achieved from 40 g/l fermentable sugars within 12 h with a productivity of 3.2 g/l/h (2.5 g/l dry cell mass). Application of polyurethane cubes (PUC) immobilized with co-cultures took 24 h to
reach the same LA concentration, when the cubes were further coated with calcium alginate it took 36 h for the maximum yield. PUC immobilized and calcium alginate coated cubes decreased the productivity but had the advantages of easier recycling for multiple rounds in LA fermentation (John & Madhavan Nampoothiri, 2011).

Lactic acid bacteria natively lack various biosynthetic pathways, so they are fastidious microbes and require expensive yeast extract and peptone as the nitrogen sources. Their optimal growth conditions are pH 3.5–10 and 5–45 °C. Since they grow at lower temperatures, sterilization of the culture media is required before starting the fermentation. These two factors significantly affect the production cost of LA (Abdel-Rahman et al., 2013; Okano et al., 2010).

It is worth mentioning that some lactic acid bacteria are able to directly utilize starch materials as the substrates as they can secrete amylases into the medium (Reddy et al., 2008). However, their optimal pH and temperature are 6.5 and 30 °C (Shibata et al., 2007), respectively, which is unfavorable for the starch hydrolysis catalyzed by amylases which are most active at higher temperatures (>50°C). Therefore, screening thermophilic amylase-secreting lactic acid bacteria is needed to develop more efficient fermentation processes for direct conversion of starch to LA.

Bacilli are Gram negative, sporulating, motile and facultative anaerobic bacteria. The optimal temperature for their growth is 50 °C. In comparison to the conventional lactic acid bacteria, Bacilli have multiple advantages in terms of reducing the production cost of LA, including: (1) they can grow in simpler mineral media with inexpensive nitrogen sources such as corn steep liquor or (NH₄)₂SO₄ (Wang et al., 2011a); (2) they ferment at 50 °C so sterilization of the media before the fermentation is not necessary; (3) they can metabolize both pentose and hexose sugars giving an opportunity to utilize all sugars from lignocellulose (Ye et al., 2013a; Zhou et al., 2013). These Bacilli utilize xylose via pentose phosphate pathway to produce LA (Figure 2) converting three molecules of xylose to five molecules of LA (Patel et al., 2006); and (4) Bacillus sp has been accredited by both European Food Safety Authority (EFSA) and FDA to the Qualified presumption of safety (QPS) list and GRAS status for applications in livestock production (EFSA Panel, 2008). Surprisingly, all Bacillus sp. that have been reported produce only L-LA (Supplemental Table S3).

Corynebacterium glutamicum has been used for production of amino acids by fermentation. C. glutamicum is a Gram positive, non-sporulating, non-motile saprophytic microorganism (Leuchtenberger et al., 2005). It produces organic acids under oxygen-limiting conditions (Kawaguchi et al., 2008). C. vitaeruminis MTCC 5488 produced 38.5 g/l LA in fed-batch fermentation (Shukla et al., 2012). A mutant of C. glutamicum, F172-8, that is deficient of H⁺-ATPase, produced 24.6 g/l LA (Sekine et al., 2001). Engineering the metabolic pathways for fermenting xylose and arabinose in oxygen-deprivation conditions produced a mixture of organic acids including LA, succinic acid and acetic acid (Kawaguchi et al., 2006, 2008). A Δ L-LDH knockout strain of C. glutamicum over-expressing E. coli or L. delbrueckii D-LDH gene produced 120 g/L D-LA at an optical purity of 99.9% in mineral salt medium in 30 h (Okino et al., 2008).
Metabolically engineering *C. glutamicum* strains to minimize or eliminate the production of succinic acid and acetic acid is worth investigating to favor their industrial application for LA production. The LA produced by *C. glutamicum* strains can be utilized for bio-medical applications (Zahoor et al., 2012).

*Escherichia coli* can natively produce LA by metabolizing both pentose and hexose sugars. It requires simpler LB broth for growth when compared to the conventional lactic acid bacteria. Transformation of plasmids and gene knock-outs are simpler compared to those for lactic acid bacteria and *Bacillus* sp. The major drawback is the carbon flux distribution leading to the production of a mixture of acids (D-LA, acetic acid, succinic acid, formic acid) and ethanol (Okano et al., 2010).

D-Lactic acid or L-LA production using recombinant *E. coli* was first reported in 1999. A double knock out mutant of *E. coli* deficient of phosphoenolpyruvate carboxylase gene (*pca*), stops the metabolic flux towards acetate) and pyruvate-formate lyase gene (*ppc*, stops the metabolic flux towards succinate) was grown to high biomass concentrations in aerobic phase and shifted to anaerobic phase for D-LA fermentation, producing 62.2 g/l of D-LA in 60 h. The double knock out mutant lacking *pca* and native D-LDH genes was transformed with L-LDH gene from *Lactobacillus casei*, generating a mutant able to produce 45 g/l L-LA from glucose in 67 h (Chang et al., 1999).

*Escherichia coli* strains have been metabolically engineered to produce platform strains to produce D-LA on glycerol. The homo-fermentative route of D-LA production was constructed by over-expressing the pathways involved in the conversion of glycerol to glycolytic intermediates [glycerol kinase-glycerol-3-phosphate dehydrogenase (*GlpK-GlpD*)]. To inhibit the flux distribution from pyruvate to competing byproducts (ethanol, acetate, formate and lactate to pyruvate), the genes encoding the relevant enzymes [pyruvate-formate lyase (*pflB*), fumarate reductase (*frdA*), phosphate acetyltransferase (*pta*), alcohol/acetaldehyde dehydrogenase (*adhE*) and native D-lactate dehydrogenase (*ldh*)] were knocked out, resulting in two platform strains, LA01 and LA02. LA01 is a double mutant (*ΔpflBΔfrdA*) strain with its succinate formation being inhibited, giving a D-LA at 12.4 g/l and a yield of 0.69 g lactate/g glycerol. LA02 is a triple mutant (*ΔptaΔadhEΔfrdA*) strain giving a D-LA at 10.9 g/l with minimized formation of ethanol and succinic acid. The LA02 strain that expresses the enzymes involved in conversion of glycerol to glycolytic intermediates with its native D-LDH being knocked out to avoid the reversion of lactate to pyruvate produced 32.3 g/l D-LA from 39.5 g/l glycerol in 60 h with a yield of 0.83 g lactate/g of glycerol (Mazumdar et al., 2010).

These platform strains (LA01 and LA02) were further engineered by (1) replacing native D-lactate specific dehydrogenase with *Streptococcus bovis* L-LDH, (2) deleting methylglyoxal bypass pathways to avoid the synthesis of a racemic mixture of D- and L-lactates and accumulation of toxic methylglyoxal and (3) blocking the native aerobic L-LDH to prevent the undesired utilization of L-lactate. The engineered strain produced 50 g/l of L-LA from 56 g/l crude glycerol in 60 h with a yield of 99.9% and chemical (97%) purities (Mazumdar et al., 2013).

Ethanologenic *E. coli* mutant, SZ470 (*ΔfrdB CDCΔldhA ΔackA ΔpflB ΔpdxR::pflBp6-acEF-lpd ΔmgsA*) (Wang et al., 2011b), was further engineered to produce a homo-fermentative L-LA producing strain on xylose, converting 1.2 mole of xylose to 2 mole of L-LA. The native alcohol
dehydrogenase gene (adhE) was deleted and integrated with the L-LDH gene from *Pediococcus acidilactici*. The resulting strain, WL203, was metabolically evolved for anaerobic growth in the screw-cap tubes containing xylose. Multiple rounds of sub-cultivation evolved strain WL204 with improved anaerobic cell growth. WL204 strain produced 62 g/l L-LA (99.5% optical purity) from 70 g/l xylose at 1.63 g/l/h with a yield of 97% based on metabolized xylose (Zhao et al., 2013).

**Lactic acid production by filamentous fungi**

Lactic acid is also produced by filamentous fungi, particularly *Rhizopus* sp. *R. oryzae* can produce L-LA, fumaric acid and ethanol on different carbon sources with LA being the major product (Meussen et al., 2012). *R. oryzae* 365 produced 65–67% d-LA based on glucose consumed (Ward et al., 1936). The highest LA yield reported for *Rhizopus* was 0.88 g/g with ethanol being the main byproduct (Zhou et al., 1999). To further decrease the ethanol yield during LA fermentation, alcohol dehydrogenase (ADH) inhibitors were added to the fermentation broth and 2,2,2-trifluoroethanol was found to be the most effective ADH activity inhibitor, which gave the highest lactate yield of 0.47 g/g glucose at the lowest concentration of 0.01 mM, corresponding to a 38% increase in lactate yield compared to the control (0.34 g/g glucose) (Thitiprasert et al., 2011). Insights into the genomics of *R. oryzae* NRRL 395 indicated the presence of two NAD+-dependent LDH genes A and B. *LDH-A* is activated in the presence of fermentable sugars such as D-glucose and D-xylose. *LDH-B* is activated in the presence of non-fermentable carbon sources such as ethanol, glycerol and lactate (Skory, 2000). Metabolic engineering of the strain by deleting the alcohol dehydrogenase and malate dehydrogenase genes moved the metabolic flux to LA formation. *R. oryzae* NRRL 395 produced 140 g/l LA on potato hydrolysate (Liu et al., 2008) and 48 g/l LA from 75 g/l glycerol enriched with 25 g/l Lucerne green juice and inorganic nutrients (Vodnar et al., 2013). However, a drawback of filamentous fungi is their fast growth in media containing nitrogen sources producing predominantly chitin instead of LA. Therefore, utilizing fungal pellets instead of spores, growing fungal biomass in the presence of nitrogen and using a high content of carbon sources and low content of nitrogen sources have been proposed to improve their LA production (Liu et al., 2008).

There is no information on the direct production of LA by other fungi. The fungus, *Aspergillus niger*, was used together with *Lactobacillus* sp. for simultaneous saccharification and fermentation of artichoke to LA, where the fungus provided the enzymes required for the de-polymerization of carbohydrate polymers to fermentable sugars for use by the bacterium (Ge et al., 2009).

**Lactic acid production by yeasts**

Yeasts do not have the native pathway to produce LA. The first paper on metabolic engineering of a yeast strain to produce LA was published in 1994 (Sauer et al., 2010). *Candida sonorensis* is a methylotrophic yeast that can convert glucose and pentose sugars including xylose and arabinose to ethanol. It can resist acidic pH and has simple growth requirements. *L-LDH* genes from *L. helveticus*, *B. megaterium* and *R. oryzae* were heterologously expressed in it to construct mutants for LA production. The mutants obtained produced both LA and ethanol. To eliminate ethanol production, two pyruvate decarboxylase genes (*PDC* 1 and 2) were deleted, which did not affect cell growth. The PDC-knocked out strain, harboring the *L. helveticus* LDH gene, produced 92 g/l LA at a yield of 0.94 g/g glucose, free of ethanol in minimal medium containing 5 g/l cell dry weight (Ilmeén et al., 2013).

*Candida boidinii* is a native Crabtree-negative methylotrophic haploid yeast. To minimize its ethanol production, its *PDC* 1 gene was knocked out, leading to 17% less ethanol production compared to wild-type strain. Bovine *L-LDH* gene was integrated under the PDC1 promoter for heterologous expression. In a 5 L fermentation, the LA production reached 85.9 g/l at a productivity of 1.79 g/l/h (Osawa et al., 2009).

*Pichia stipitis* can metabolize C5 and C6 sugars from lignocellulose hydrolysates to produce ethanol. Metabolic engineering of the strain by deleting the alcohol dehydrogenase-1 (*ADH 1*) and heterologous expressing *L. helveticus* *L-LDH* under the ADH1 promoter was conducted. This mutant can metabolize glucose, xylose, producing 58 and 41 g/l of LA from 100 of xylose and 94 g/l glucose, respectively. In the presence of both sugars, xylose and glucose were consumed simultaneously. The LA was accompanied by the production of ethanol, which decreased to 15–30% in the xylose medium and 70–80% in the glucose medium compared to the wild-type strain (Ilmeén et al., 2007).

The *S. cerevisiae* PDC1 gene on chromosome XII was substituted with *L-LDH* genes from bovine and *Bifidobacterium longum*. Expression of the *LDH* genes was under the control of the PDC1 promoter. The strain containing bovine *LDH* gene produced higher amount of LA than the strain expressing *B. longum* *L-LDH*, the strain containing bovine *LDH* gene converted 62.2% of glucose to LA (55.6 g/l) and ethanol (16.9 g/l) when CaCO₃ was used as the neutralizing agent and produced 50.2 g/l LA and 16.7 g/l ethanol under non-neutralizing conditions. The yeast strain expressing *B. longum* *L-LDH* produced 25.7 g/l LA and 31.1 g/l ethanol under neutralizing conditions (Ishida et al., 2005).

The β-glucosidase from *Aspergillus aculeatus* was expressed in a LA-producing *S. cerevisiae* strain by a cell surface display technique to produce LA from cellulose. The highest enzyme activity was obtained from the strain PB2 bearing two copies of bgg1 gene under the control of the PDC1 promoter. LA fermentation with strain PB2 under microaerophilic condition in a non-selective enriched medium containing 98 g/l cellulose or 100 g/l glucose gave a maximum rate of l-lactate production of 2.8 g/l/h on cellulose and 3.0 g/l/h on glucose (Tokuhiro et al., 2008).

**Raw materials for lactic acid production**

Lactic acid is traditionally produced from glucose, lactose, dairy plant wastes (skim milk, whey, paneer whey), starch (potato, cassava, wheat, rice and sorghum), molasses and glycerol from biodiesel industry (Hofvendahl & Hahn-Hägerdal, 2000). However, the use of traditional raw materials...
competes with the supply of foods and feeds increasing the production cost. Therefore, producing LA using less expensive carbon sources has now become a focus of LA production.

**Lactic acid production from dairy plant wastes**

Dairy plant wastes have been used for LA production by *Lactobacilli* for a long history. *Lactobacilli* are fastidious microbes requiring complex macro and micro nutrients. Due to the poor nutritional status of dairy plant wastes such as whey, which is the waste product produced during the cheese production and contains salts, lactose and small amounts of proteins, enrichment of whey with nitrogen sources such as yeast extract, peptone, soy flour or corn steep liquor significantly improved LA production (Abdel-Rahman et al., 2013; Hofvendahl & Hahn-Hägerdal, 2000).

**Lactic acid production from starch**

Cassava starch is the most commonly used substrate for LA production. Starch is a carbohydrate polymer and cannot be directly utilized by most microbes for metabolism. To release fermentable sugars (glucose) from starch, the addition of amylases is usually a must. *L. plantarum* MTCC 1407 is an amyloytic lactic acid bacterium that produces α-amylase. Semi-solid state fermentation of cassava fibrous residue (CFR) to LA was conducted by using this bacterium for at pH 6.5 and 35 °C for 120 h, giving a maximal starch to LA conversion of 63.3%. The organism produced 29.86 g of L-LA from 60 g of starch present in CFR (Ray et al., 2009).

Fermentation of Maltodextrin DE 11–14 (95% dry solids, 382 g) and liquefied starch DE 9–11 (35% dry solids, 1.11) to LA using *B. coagulans* was conducted at 56 °C, pH 5.6 for 40 h with added Novozyme amylases (AMG E) in two rounds, giving 180–200 g/l of LA with an optical purity of 99.5% after 40 h of simultaneous saccharification and fermentation (Otto, 2003).

**Lactic acid production from algal biomass**

Algal biomass is rich in carbohydrates and proteins which could be a good source for producing LA. Lack of lignin in algal biomass is an additional advantage over plant biomass. The fresh water algae *Hydrodictyon reticulum* contains 47.5% of polysaccharides, which were converted to glucose and mannose as fermentable sugars predominantly after saccharification. Simultaneous saccharification and co-fermentation (SSCF) using *Lactobacillus paracasei* LA104 produced 37.1 g/l of LA with a productivity of 1.03 g/l/h (Nguyen et al., 2012a) from 80 g of this algae. The SSCF using *L. coryniformis* subsp. torquens produced 36.6 g/l of D-LA in 36 h (Nguyen et al., 2012b). *Nannochloropsis salina* is an oleaginous microalga with its dry biomass containing 40% of lipids, 20% of carbohydrates and 40% of proteins. The algal biomass was pre-treated with dilute H2SO4 (5%) at 121 °C for 1 h followed by treatment with hexane to remove lipids. The lipid-free residue was neutralized and concentrated to 64.3% of sugars (glucose and xylose) followed by inoculation of *L. pentosus* to ferment under anaerobic conditions at 30 °C, 150 rpm for 48 h yielding 10.1 g/l of LA at a substrate conversion of 92.8% (Talukder et al., 2012).

**Lactic acid production from lignocellulose**

Plant biomass is the most abundant renewable resource on earth. Plant cell wall contains primarily cellulose, hemicellulose and lignin. Cellulose is the homopolymer of glucose existing in crystalline form and is resistant to depolymerization (Juturu & Wu, 2014). Hemicellulose is amorphous heteropolymer composing of C5 and C6 sugars with xylan being the major component existing as the backbone. Depolymerization of the carbohydrate polymers to release fermentable sugars is a pre-requisite for microbial fermentation (Juturu & Wu, 2013). Pre-treatment of biomass can be conducted by either chemical or enzymatic routes. Enzymatic depolymerization is conducted under milder conditions and does not generate degradation products, while chemical hydrolysis is performed at high temperatures (≥100 °C) and generate degradation products such as furfural, 5-hydroxy methyl furfural (HMF) and aromatics, which are toxic and inhibit microbial fermentation of the produced sugars. Therefore, detoxification is needed to remove the degradation products from the hydrolysate.

Biological detoxification of furfural and HMF from lignocellulose hydrolysate is commercially attractive due to its low energy consumption. The cells of *Enterobacter* sp. FD8 were added to the acid hydrolysate of oil palm empty fruit bunch (EFB), giving the furfural and HMF detoxification rates at 0.54 and 0.12 g/l/h, respectively, which are the highest bio-detoxification rates of furfural and HMF ever reported. The total sugar loss was less than 5%. The bacterial cells could be recycled and reused for at least five times without losing their detoxification capability. When used for fermentation by *L. pentosus*, the biologically detoxified EFB hydrolysate gave higher LA productivity (1.7-fold), titer (1.5-fold) and yield (1.8-fold) than the un-detoxified hydrolysate (Zhang et al., 2013).

*Bacillus coagulans* JI12 was used to produce L-LA from whole EFB hydrolysate at 50 °C. EFB was pre-treated with dilute H2SO4 (2% w/v) and H3PO4 (0.8% w/v) to obtain hemicellulose sugars and cellulose–lignin complex in the hydrolysate, into which was added CTec2 cellulase (25 FPU per gram of cellulose) to commence simultaneous detoxification, saccharification and co-fermentation at pH 5.5 and 50 °C. After 24 h, 80.6 g/l of LA was obtained at a productivity of 3.4 g/l/h and a yield of 0.49 g LA per g of EFB consumed. *B. coagulans* JI12 was tolerant to both furfural (4 g/l) and acetate (20 g/l). Neither pre-detoxification nor separation of fermentable sugars from lignin was needed before the fermentation (Ye et al., 2014).

Overlimbed EFB acid hydrolysate containing 63.1 g/l of total sugars along with inhibitors (acetic acid 19.17 g/l, furfural 0.41 g/l and HMF 0.01 g/l) was used by *B. coagulans* JI12 to produce L-LA at pH 6 and 50 °C, giving 59.2 g/l of L-LA at a yield of 97% and productivity of 6.2 g/l/h (Ye et al., 2013a).

Corn cob molasses is a major waste in the xylitol production industry. It contains high amounts of fermentable sugars including glucose (9% w/v), xylose (45% w/v) and arabinose
Batch fermentation of molasses by Bacillus sp. XZL9 under static conditions at 30°C, pH 5.6–6.0 gave 80.8 g/l of LA at a yield of 0.98 g/g. Fed-batch fermentation increased to LA concentration to 115.2 g/l at a productivity of 0.80 g/l/h (Wang et al., 2010).

Raw sugar beet juice with a Brix of at least 60 was used for LA production in 3000 l starting volume in a 5000 l fermenter. The fermentation medium contained 2000 l of water, 1000 l of beet juice (70°Brix), 18.16 kg of nutrients and 500 l of inoculum. The fermentation was carried out at 54°C giving a L-LA yield of 95% with an optical purity of 99% (Visser et al., 2012).

Unpolished rice from agging paddy was used as major nutrients to produce D-LA. The unpollished rice saccharificate, wheat bran powder and yeast extract were employed as carbon source, nitrogen source and growth factors, respectively. When the fermentation was carried out under optimal conditions, the D-LA yield reached 731.50 g/kg unpollished rice with a productivity of 1.50 g/l/h (Yin et al., 1997).

Lactic acid production from glycerol

Glycerol is a byproduct of the biodiesel industry, which yields at about 10% biodiesel. The bio-diesel production has increased from 3.2 million tons in 2005 to nearly 5.71 million tons in 2007 in the EU and from 25 million gallons in 2004 to 450 million gallons in 2007 in the US. This crude glycerol can be used as an inexpensive carbon source for microbial LA production (Hong et al., 2009; Johnson & Taconi, 2007).

A native E. coli strain AC-521 was isolated from soil in China which can produce LA from glycerol as a carbon source at optimal conditions of 42°C, pH 6.5 with a K_{dG} of 0.85 min^{-1}. After 88 h of fed batch fermentation in 5 l fermenter, the LA concentration achieved 85.8 g/l at a productivity of 0.97 g/l/h and a yield of 0.9 mol/mol glycerol (Hong et al., 2009).

Nitrogen sources for lactic acid fermentation

Nitrogen is the building block for living cells and is essential for the synthesis of amino acids, purines, pyrimidines, some carbohydrates and lipids, co-factors and other nitrogen-containing substances. In general, yeast extract and peptone are used as nitrogen sources. Yeast extract is rich in vitamin B and is known to enhance LA production. An economic analysis of LA production at the industrial scale shows that yeast extract accounts for 38% of the medium cost (Tejayadi & Cheryan, 1995). A large number of inexpensive renewable nitrogenous materials such as industrial by-products and agricultural wastes were tested for LA fermentation. Altaf et al. (2005, 2007) found that the flour of red lentils along with yeast cells produced 92% (w/w) of L-LA from starch. Wheat contains gluten which is rich in protein but lacks the micronutrients present in yeast extract. Using gluten as the major nitrogen source and a small amount of yeast extract as the micronutrient source, the LA fermentation by Lactobacillus sp. MKT-LC878 was conducted with a productivity of 2.31 g/l/h (Hetenyi et al., 2008). Corn steep liquor that contains soluble proteins, amino acids, vitamin B and other nutrients has also been used as an organic nitrogen source for LA fermentation by L. rhamnosus CGMCC 1466 producing 113 g/l LA under optimal conditions (Yu et al., 2008a). The inorganic nitrogen source (NH_{4})_{2}SO_{4} was used for LA fermentation by Rhizopus at up to 4 g/l (Zhang et al., 2007).

Neutralizing agents used in lactic acid fermentation

Neutralizing agents to adjust the broth pH are employed in LA fermentation. In general, NH_{3}, NH_{4}OH, KOH, NaOH, NaHCO_{3}, Ca(OH)_{2} and CaCO_{3} are routinely used as neutralizing agents (Yen et al., 2010; Zhao et al., 2012). Among them, Ca(OH)_{2} and CaCO_{3} have the advantage that they precipitate LA as calcium lactate reducing the toxic effects of LA on the growth and morphology of microbial cells (Yen et al., 2010). The disadvantages of using calcium substances include that they are slow and mild neutralizers with a limited capacity to regulate pH (Yen et al., 2010). The release of LA from its calcium salt requires acid (H_{2}SO_{4}) treatment generating calcium sulphate (gypsum) as a byproduct, which is of limited commercial value and its disposal is a large environmental problem (Pal et al., 2009).

Ammonia and its hydroxide are most commonly used as neutralizing agents after calcium substances. The advantages of using ammonia are (1) NH_{4}OH can regulate pH easily, (2) it is beneficial to cell growth as an additional nitrogen source, (3) (NH_{4})_{2}SO_{4} obtained after acid hydrolysis can be used as a fertilizer with good market value. The disadvantages of using ammonia substances as neutralizing agents are their toxicity to microbial growth and the osmotic stress on the microbial cells caused by ammonium lactate in the late phase decreasing the LA titer. In addition, separation of dissolved ammonium lactate from the fermentation broth is cumbersome. Similar to ammonia, sodium has the same inhibitory effect on LA production when used as the neutralizing agent (Tian et al., 2014; Wang et al., 2014; Zhao et al., 2012).

Recently, we found that the LA production might be relevant to the ionic strength (I) of the fermentation broth. The fermentation using Bacillus coagulans WCP-10-4 stopped at I = 1.1 in both cases using Ca(OH)_{2} and NH_{4}OH as the neutralizing agents (unpublished data).

Development of mutant strains resistant to acid pH

Although typical commercial Lactobacillus fermentations were conducted at a minimal pH of 5.0–5.5, fermentation at or below the pKa of LA (~3.8) is more commercially beneficial and thus desirable. At this low pH, a substantial proportion of the product is in the free acid form and can be purified by direct organic extraction of the fermentation broth. At higher pH, LA exists in lactate form and requires extensive purification generating considerable waste. Engineering the strains to increase the growth and LA production at low pH will decrease waste formation and reduce LA production cost. The pH 3.8 is a critical threshold for LA fermenting microorganisms as it is the pKa of LA. Free LA is believed to be the ultimate cause of Lactobacillus growth inhibition at low pH presumably because it can diffuse back into the cell, short-circuiting proton export and inhibiting enzymes in the cell (John et al., 2010; Patnaik et al., 2002). A wild-type of Lactobacillus strain grows at pH 5.5–6.0. Acid tolerance of
this strain was improved by genome shuffling. Two variants of the *Lactobacillus* population were developed by two different methods. The first set of *Lactobacillus* mutants was adapted to pH 4 (Pop adap) and screening the mutants in low pH plates (Pop NTG). Genome shuffling between the two populations was achieved by recursive protoplast fusion. After five rounds of recursive protoplast fusion, a mutant was obtained which resisted pH 4 and produced >5 g/l LA. The increase in LA production was three times greater compared to the wild-type strain (John et al., 2010; Patnaik et al., 2002).

*Lactobacillus pentosus* ATCC 8041 wild-type strain grows at pH 6. Acid tolerance of *L. pentosus* was significantly improved by error-prone PCR of the whole genome using random primers and Taq DNA polymerase. The amplified DNA fragments with random mutations were transformed back into the parental strain and mutants with improved acid tolerance were screened on agar plates with low pH. After one round of mutation, a mutant (MT3) was screened, which completely consumed 20 g/l of glucose to LA at a yield of 95% in MRS medium, pH 3.8 within 36 h, whereas no growth or LA production was observed for the wild-type strain under the same conditions. The acid tolerance of mutant MT3 remained genetically stable after sub-culturing for 25 cycles (Ye et al., 2013b).

The pH tolerance and LA productivity *L. rhamnosus* ATCC 11443 were improved by genome shuffling using protoplast fusion. Mutants with minute improvements were generated separately using UV irradiation and NTG. These mutants were subjected to recursive protoplast fusion for genome shuffling. After three rounds of protoplast fusion, four strains that could resist pH 3.6 were obtained. These strains showed 3.1-fold increase in LA production and 2.6-fold improvement in cell growth at pH 3.8, respectively. The maximal LA productivity was 5.77 g/l/h when fermented with 10% glucose in neutralizing conditions using CaCO₃, which was 26.5% higher than that of the wild-type strain (John et al., 2010; Wang et al., 2007).

*Lactobacillus rhamnosus* ATCC 11443 was improved for its ability to resist high concentrations of glucose by genome shuffling using protoplast fusion. The starting populations required for the genome shuffling were created separately by UV irradiation and NTG. The mutant strains generated were subjected to repetitive protoplast fusion to bring genome shuffling. The colonies with improved ability to withstand high concentrations of glucose were screened on agar plates with high concentrations of glucose enriched with 2% CaCO₃. After two rounds of protoplast fusion, strains, with improved resistance to high concentrations of glucose and producing high amounts of LA, were obtained and tested in 16 l fermentor with 10% glucose in neutralizing conditions using CaCO₃, pH 3.8 within 36 h, whereas no growth or LA production was observed for the wild-type strain under the same conditions. The acid tolerance of mutant MT3 remained genetically stable after sub-culturing for 25 cycles (Ye et al., 2013b).

Applications of LA can be broadly divided into food and non-food industries. In the food industry, LA is used in a wide range of applications such as bakery products, beverages, meat products, confectionery, dairy products, salads, dressings, instant meals, etc. Lactic acid present in the food products usually serves as a pH regulator or a preservative. It is also used as a flavoring agent. In addition, lactic acid bacteria can be used as probiotics. In non-food industries, the applications include pharmaceutical, biomaterials, detergents and animal feed industries. The recent reports on applications of LA and lactic acid bacteria in various fields are summarized below.

1. Polyactic acid (PLA) is an eco-friendly plastic with excellent biocompatibility and processability. It has been used in biomedical applications such as medical implants in the shapes of rod, plate, fiber and beads for bone and tissue engineering (Rasal et al., 2010).

2. Poly lactic-glycolic acid polymer (PLGA) is considered as one of the best biomaterials for drug delivery with respect to design and performances. Depending on the time frame required for the release of drug, tailor-made PLGA is available for short term (1 month) and long term (up to 6 months) release. Depending on the needs and types of materials to be encapsulated (drugs, peptides and proteins), PLGA can be molded into nano/microspheres, hollow fibers and millimeter size implants that can be delivered into human body by diverse routes (Behera, 2013; Makadia & Siegel, 2011).

3. In general, lactic acid bacteria are considered as safe having Qualified Presumption of Safety-EU (QPS) and GRAS status and play an essential role in food preservation. Lactic acid bacteria protect foods mainly by their ability to produce antifungal compounds such as carboxylic acids, fatty acids, ethanol, carbon dioxide, hydrogen peroxide and bacteriocins. These lactic acid bacteria can also positively contribute to the flavor, texture and nutritional values of food products. *L. plantarum* FST 1.7 was used for preservation of bread, *L. fermentum* Te007, *L. pentosus* G004, *L. paracasei* D5 and *P. pentosaceus* Te010 were used in the preservation of dairy products, fresh fruits and vegetables (Pawlowska et al., 2012).

4. Lactic acid bacteria secrete exopolysaccharides (EPS), which are considered to play a vital role in protection against desiccation, toxic compounds, bacteriophages and osmotic stress, and to permit adhesion to solid surfaces and biofilm formation. Apart from these, they have antitumor, antulcer, immunomodulating and cholesterol-lowering activities which have resulted in their applications in food industry. In addition, bacterial EPSs are resistant to human gastrointestinal juices selectively enhancing the colonization of human beneficiary bacteria
in the colon whose function resembles probiotics that help in digesting polysaccharides present in the human diet such as fructooligosaccharides, galactooligosaccharides and inulin, which are not digested by the native human digestive system (Hongpattarakere et al., 2012).

(5) Sporulated Lactobacilli, Lactococcus, Pediococcus, Bacillus coagulans (7.5 × 10⁷ cfu/g) were mixed with hard fat (hydrophobic carrier), polyacrylic acid (super-absorbent) and zeolite (odor absorbent) for the application in baby diapers, sanitary napkins and panty liners. These bacteria are the normal microbial flora of the vagina and their applications in the sanitary napkins will be beneficial in women to avoid occurrence of vaginal infections (Di et al., 2001; Jiffer, 2010).

Concluding remarks

Lactic acid is a bulk chemical with a market demand of 500 000 ton and current production of 300 000–400 000 ton per year (www.nnfcc.co.uk). The US is the largest consumer of LA followed by Western Europe and China (http://www.ihb.com). The increasing demand for LA makes it uneconomical to produce LA from the traditional raw materials such as starch and glucose, as it will severely affect the global supply of foods and feeds. According to United Nations Environment Programs (UNEPs), 5 billion metric tons of plant biomass waste is generated annually from agriculture. This is equivalent to about 1.2 billion tons of oil, i.e. 25% of the current global oil production (http://www.unep.org/gpwwm/FocalAreas/WasteAgriculturalBiomass/tabid/56456/Default.aspx). Pre-treatment of agriculture biomass in order to obtain fermentable sugars (>100 g l⁻¹) is a pre-requisite for utilization of the huge amount of agricultural biomass to produce LA by fermentation. The major challenge lies in obtaining high concentrations of sugars and LA titers for industrial applications.

Development of a cost-effective method for the separation of LA from the fermentation broth is also essential. During the pre-treatment of plant biomass, inhibitors (furfural, hydroxyl methyl furfural and phenolic wastes from lignin) are also produced along with fermentable sugars. Cost-effective removal of these inhibitors before or after the fermentation is challenging but worth investigating.

Bacillus coagulans strains have emerged as commercially promising strains for producing LA from lignocellulose due to their ability to utilize both C5 and C6 sugars, but the lack of native D-LA producing strains and difficulty in genetic manipulation of these native strains severely hampers their industrial applications. Developing novel genetic tools for metabolically engineering these strains is a priority of research and development.

For traditional LA fermentation, Ca(OH)₂ is often used as the neutralizing agent. However, the recovery of LA from calcium lactate generates a lot of gypsum which is almost useless. To avoid or minimize the use of neutralizing agents, genetically engineering the strains to make them resist acidic environment and produce LA at low pH would be very useful and thus in high demand.

Declaration of interest

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Supplementary material available online.
Supplemental Figures S1–S4 and Tables S1–S5.