ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Microbial Granulation for Lactic Acid Production

Dong-Hoon Kim,[1] Mo-Kwon Lee,[2] Yuhoon Hwang,[3,4] Wan-Taek Im,[5] Yeo-Myeong Yun,[4] Chul Park,[6] Mi-Sun Kim[2,7]

[1]Department of Civil Engineering, Inha University, 100 Inharo, Nam-gu, Incheon 402-751, Republic of Korea

[2]Biomass and Waste Energy Laboratory, Korea Institute of Energy Research, 152 Gajeong-ro, Yuseong-gu, Daejeon 305-343, Republic of Korea

[3]Department of Environmental Engineering, Technical University of Denmark, Miljøvej, Bygning 113, DK-2800 Kgs. Lyngby, Denmark

[4]Department of Civil and Environmental Engineering, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea

[5]Department of Biotechnology, Hankyoung National Univeristy, 327 Chungang-no Anseong-si, Kyonggi-do 456-749, Republic of Korea

[6]Department of Civil and Environmental Engineering, University of Massachusetts Amherst, 130 Natural Resources Road, Amherst, Massachusetts 01003

[7]Division of Renewable Energy Engineering, University of Science and Technology, 217 Gajeong-ro, Yuseong-gu, Daejeon 305-350, Republic of Korea; telephone: +82-42-860-3707; fax: +82-42-860-3134; e-mail: bmmskim@kier.re.kr

ABSTRACT: This work investigated the formation of microbial granules to boost the productivity of lactic acid (LA). The flocculated form of LA-producing microbial consortium, dominated by *Lactobacillus* sp. (91.5% of total sequence), was initially obtained in a continuous stirred-tank reactor (CSTR), which was fed with 2% glucose and operated at a hydraulic retention time (HRT) of 12 h and pH 5.0 ± 0.1 under a thermophilic condition (50°C). The mixed liquor in the CSTR was then transferred to an up-flow anaerobic sludge blanket reactor (UASB). The fermentation performance and granulation process were monitored with a gradual decrease of HRT from 8.0 to 0.17 h, corresponding to an increase in the substrate loading from 60 to 2,880 g glucose $L^{-1}d^{-1}$. As the operation continued, the accumulation of biomass in the UASB was clearly observed, which changed from flocculent to granular form with decrease in HRT. Up to the HRT decrease to 0.5 h, the LA concentration was maintained at 19–20 g L^{-1} with over 90% of substrate removal efficiency. However, further decrease of HRT resulted in a decrease of LA concentration with increase in residual glucose. Nevertheless, the volumetric LA productivity continuously increased, reaching 67 g L-fermenter$^{-1}h^{-1}$ at HRT 0.17 h. The size of LA-producing granules and hydrophobicity gradually increased with decrease in HRT, reaching 6.0 mm and 60%, respectively. These biogranules were also found to have high settling velocities and low porosities, ranging 2.69–4.73 cm s^{-1} and 0.39–0.92, respectively.

Biotechnol. Bioeng. 2016;113: 101–111.

KEYWORDS: lactic acid; microbial granules; up-flow anaerobic sludge blanket reactor; volumetric productivity; pyrosequencing; settling experiments

Corresponding author: M.-S. Kim
Contract grant sponsor: The Ministry of Education, Science and Technology
Contract grant number: 2011-0031955
Contract grant sponsor: The Korea Institute of Energy Technology Evaluation and Planning (KETEP)
Contract grant sponsor: The Ministry of Trade, Industry & Energy, Republic of Korea
Contract grant number: 20132020000170
Received 23 July 2014; Revision received 24 December 2014; Accepted 5 January 2015
Accepted manuscript online 28 April 2015;
Article first published online 5 May 2015 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.25540/abstract).
DOI 10.1002/bit.25540

Introduction

For the development of sustainable industrial society and effective management of greenhouse gas emissions, an alternative supply of materials and chemicals is considered as critical as that of energy and fuels (Ragauskas et al., 2006). In fact, most industrial chemicals are derived from fossil fuels, and therefore different sustainable production patterns are urgently needed. The US Department of Energy (DOE) identified several chemicals that can be produced by microbial processes, and among them, organic acids including lactic acid (LA) constitute a key group (FitzPatrick et al., 2010; Sauer et al., 2008).

Lactic acid (LA, 2-hydroxypropanoic acid, CH_3-CH(OH)-COOH) is a natural organic acid with a long history of application in food

and non-food industries, including the cosmetic and pharmaceutical industries, and for the production of oxygenated chemicals, plant growth regulators, and special chemical intermediates (Abdel-Rahman et al., 2011). Currently, there is an increased demand for LA as a feedstock for the production of biopolymers and chemicals such as polylactide, acetaldehyde, acrylic acid, and 2,3-pentadione (Dusselier et al., 2013). The annual production of LA in 2012 was 260,000 tons and its production is expected to grow to 600,000 metric tons by the year 2020.

Over 90% of the current commercial production of LA is carried out by microbial fermentation, specifically using pure cultures in a batch manner. This type of fermentation has advantages, in that it reduces the risk of contamination, and obtains high LA yield and concentration (Hofvendahl and Hahn-Hägerdal, 2000). However, it suffers from the high cost of sterilization, and low volumetric productivity due to the long fermentation time required and end-product inhibition. From a practical point of view, a continuous process using mixed cultures is therefore vital, since (i) it is free from sterilization; (ii) it can tolerate the complexity and variability of substrates; and (iii) it can achieve high productivity under high cell concentration (Kleerebezem and van Loosdrecht, 2007). Although LA produced by mixed cultures may be unfit for food and cosmetic purposes, it can be used as a platform chemical making pyruvic acid, acrylic acid, propanediol, ester, and other substances (Gao et al., 2011).

Various techniques have been employed in continuous LA production to separate cell retention from hydraulic retention. Examples include the use of immobilizing matrix, a centrifugal system, membrane filtration units, or other external sources of materials (Chang et al., 1994). Instead of using extra equipment, microbial granulation can be an alternative method to attain high LA productivity. The granules are discrete well-defined cell aggregates formed by cell-to-cell attraction, which usually occurs in up-flow type reactors (Hulshoff et al., 2004). Compared to conventional microbial flocs, granules have a regular, dense, and strong structure with excellent settleability, enabling high cell retention and withstand high organic loading (Liu et al., 2009). Until the twentieth century, the main research on microbial granules was focused on producing methane along with wastewater treatment, but, today it has expanded to various microbial processes, such as aerobic wastewater treatment, anaerobic ammonium oxidation, sulfate reduction, and hydrogen production (Cui and Kim, 2013; Gonzalez-Gil et al., 2012; Li and Yu, 2011; Tang et al., 2014).

This paper presents the formation of LA-producing granules in an up-flow anaerobic sludge blanket reactor (UASB), which enabled us to achieve the highest volumetric LA productivity ever reported. To obtain the microbial consortium effectively producing LA, anaerobic mixed cultures were initially inoculated in a continuous stirred-tank reactor (CSTR) operated at a hydraulic retention time (HRT) of 12 h under thermophilic condition (50°C). The change in microbial community in the CSTR with time was analyzed using a pyrosequencing tool. After seeding the microbial consortium to the UASB, a substrate loading rate (SLR, g glucose $L^{-1}d^{-1}$) was gradually increased by shortening the HRT from 8.0 to 0.17 h. The granulation process was monitored by measuring the size of microbial aggregates and hydrophobicity. In addition, images were taken with a scanning electron microscope (SEM) to investigate the surface characteristics of the granules. Finally, information about the physicochemical characteristics of LA-producing granules was obtained by carrying out settling experiments.

Materials and Methods

Inoculum and Substrate

The inoculum for a CSTR was taken from an anaerobic digester in a local wastewater treatment plant. The pH, alkalinity, and volatile suspended solids (VSS) concentration of the sludge were 7.7, 2.5 g $CaCO_3$ L^{-1}, and 5.3 g L^{-1}, respectively.

Glucose of 20 g L^{-1} was used as a substrate. Concentrations of NH_4Cl, KH_2PO_4, and $FeCl_2.4H_2O$ were added to yield a C:N:P:Fe ratio of 100:5:1:0.33. The feed also contained the following nutrients (in mg L^{-1}): yeast extract, 3,000; $NaHCO_3$, 1,000; $MgCl_2.6H_2O$, 100; $CaCl_2.2H_2O$, 75; $Na_2MoO_4.4H_2O$, 0.01; H_3BO_3, 0.05; $MnCl_2.4H_2O$, 0.5; $ZnCl_2$, 0.05; $CuCl_2$, 0.03; $NiCl_2.6H_2O$, 0.05; $CoCl_2.2H_2O$, 0.5; Na_2SeO_3, 0.05 (Kim et al., 2012). Substrate and nutrients were not sterilized.

Reactor Operation

In this study, a CSTR with a working volume of 2.0 L (120 mm ID) was used. After being seeded with anaerobic digester sludge equivalent to 30% of the total effective volume, the reactor was purged with N_2 gas for 5 min to provide anaerobic conditions. The reactor was mixed by mechanical stirring at 100 rpm and pH was maintained at 5.0 ± 0.1 using a pH sensor, pH controller, and 2 N KOH solution. According to our previous work, temperature was controlled using a water bath circulator and a built-in water jacket at 50°C (Kim et al., 2012). At first, the reactor was operated by batch mode for one day, and then SLR was maintained at 40 g glucose $L^{-1}d^{-1}$, corresponding to HRT 12 h.

After 5 days of operation, 1 L of mixed liquor in the CSTR was transferred to the UASB (working volume 2.9 L, Fig. 1) as seed biomass. As there was no mechanical mixing, the pH inside the UASB was not uniform, and, accordingly, controlling pH was difficult. Therefore, in order to provide buffer capacity, $NaHCO_3$ (5.0 g L^{-1}) was added to the medium. The SLR was gradually increased from 60 to 2,880 g glucose $L^{-1}d^{-1}$ by shortening the HRT from 8.0 to 0.17 h. The interval of SLR increase was set at 1.5 times of the previous SLR. At each phase, the reactor was operated for at least 5 days, more than 10 times of HRT, in order to establish steady-state conditions, judging from the metabolic products.

Microbial Community Analysis

Sampling, DNA Extraction and PCR

To analyze the structure of bacterial communities in the CSTR, 0.5 g of mixed liquor was used for DNA extraction using the Fast DNA Spin Kit for soil (QBIOgene, Carlsbad, CA) following the manufacturer's instructions. The DNA obtained was confirmed by electrophoresis on 0.8% agarose-gel (Mupid, Japan). Partial sequences of the 16 S rRNA gene including the variable V1–V3 region (corresponding to the 9–536 regions in *E. coli*) were amplified from the obtained DNA using two primers: Bac9f (5′- GAGTTTGATCMTGGCTCAG-3′) (Weisburg et al., 1991) and Bac536r (5′- WTTACCGCGGCTGCTGG -3′) (Muyzer et al., 1993). On both primers, barcode sequences were attached as a unique tag for sample identification when multiple samples were

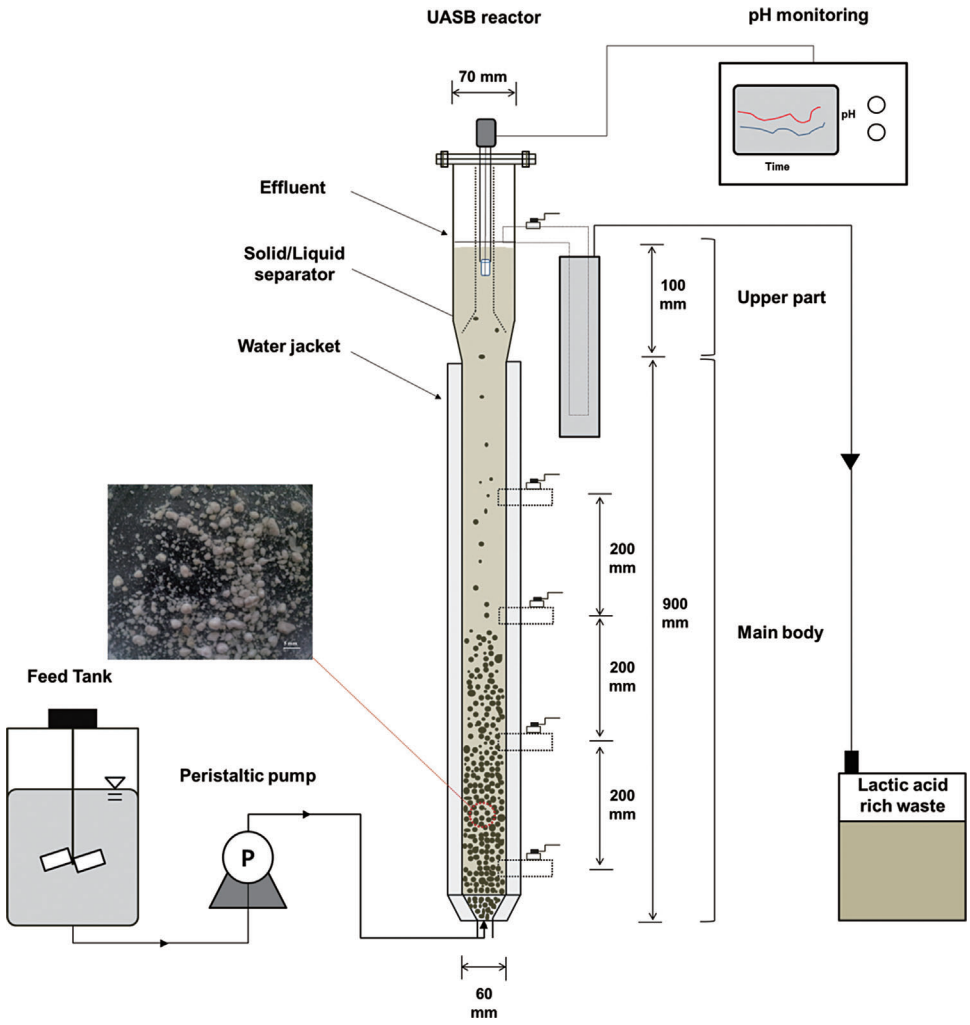

Figure 1. A schematic of lactic acid producing up-flow anaerobic sludge blanket reactor.

analyzed in parallel on one 454 picotiter plate. All PCR amplification were performed in 40 μL volumes containing 40 ng of template DNA, 1 × f-Taq Buffer (Solgent Co., Ltd, South Korea), 200 μM dNTP, 40 pmol of each primer, and 2 units of Taq polymerase (Solgent Co., Ltd, South Korea) using a MJ Mini personal thermalcycler (Bio-rad laboratories, Inc. Hercules, CA, USA). The PCR conditions were as follows: 94°C for 1 min; 30 cycles of denaturation (94°C; 1 min), annealing (58°C; 45 s), and extension (72°C; 1 min); followed by the final elongation (72°C; 7 min). The PCR products were examined by electrophoresis in a 0.8% (w/v) agarose gel, stained with ethidium bromide, in TAE buffer, and purified using a PCR purification kit (Solgent Co., Ltd, Korea) according to the manufacturer's instructions.

Pyrosequencing and Data Analysis

The 7 PCR products were mixed together and transferred to the Macrogen Co. Ltd. (Seoul, Korea) for pyrosequencing using the 454 GS-FLX sequencer (Roche diagnostics Korea Co., Ltd, Seoul, Korea) utilizing the Titanium Sequencing Kit (Roche) to generate 400-bp sequence reads. The pyrosequencing methodology has been described in a previous review (Armougom and Raoult, 2009). All of the raw sequence data were sorted based on sample-specific barcode tags and primer and tag sequences were trimmed from sorted sequences. Raw sequences were processed through the Ribosomal Database Project II pyrosequencing pipeline (http://wildpigeon.cme.msu.edu/pryo/index.j) (Cole et al., 2009). First, ambiguous and short sequences with a length less than 300 nucleotides were removed. Second, qualified sequences were clustered into OTUs defined by a 3% distance level using a complete-linkage clustering. Third, these were assigned to phyla using the RDP-II classifier at a 50% confidence threshold (Wang et al., 2007). The sequences were clustered based on the similarities of 97%, 95%, and 90% using the CDHIT program (Li and Godzik, 2006). OTU-based diversity analyses and diversity indexes calculation were performed using mother package (Schloss et al., 2009).

Physicochemical Characteristics Analysis

Settling Experiment

The settling experiment was performed to obtain the physicochemical characteristics of the LA-producing granule as described in a previous study (Cho et al., 2013). The experimental apparatus consisted of a transparent acrylic column and collection unit. The collection unit consisted of a bottom well and filtration unit with a Buchner funnel and vacuum pump.

Each granule was transferred into the top of the settling column, and the settling velocity at a point of 15–25 cm from the top of the column was measured. After a settling experiment, the size and dry weight of granules were determined. The size (projection diameter) was determined by calculating the diameter of a circle which had the same projection area as the granule. A total of 40 granules obtained at the end of the operation were used for the settling experiment.

Calculated Granule Properties

Several physicochemical properties of the granules were calculated based on the Stokes' law for a porous but impermeable microbial aggregate. The settling velocity of a microbial aggregate predicted from Stokes' law (U_s) can be derived according to Equation (1).

$$U_s = \left[\frac{8gf}{\pi}\left(\frac{1}{\rho_l}-\frac{1}{\rho_c}\right)\frac{W_d}{C_d d^2}\right]^{1/2} \quad (1)$$

where g is the gravitational constant (cm s^{-2}), ρ_c is the density of a microbial aggregate (g cm^{-3}), ρ_l is the density of the liquid (g cm^{-3}), C_d is the empirical drag coefficient, W_d is the dry weight of the granule, and f is a ratio factor between dry mass and wet mass. The C_d is adjusted for higher Reynolds numbers ($Re > 1$) and it can be obtained from Equation (2) and (3).

$$C_d = \frac{24}{Re}+\frac{6}{1+\sqrt{Re}}+0.4 \quad (2)$$

$$Re = \rho_l U d/\mu \quad (3)$$

where U is the actual settling velocity (cm s^{-1}) and μ is the fluid viscosity (g $cm^{-1}s^{-1}$).

The two parameters for the general characteristics of cells, ρ_c and f, were determined separately. The ρ_c was determined using a series of sugar solution according to the method described by(Zheng et al. 2005). The ρ_c and f were found to be 1.06 g cm^{-3} and 3.48, respectively.

The permeable structure of a granule can be characterized by a fractal dimension and permeability. The fractal dimension can be found from the slope of a log-log plot of the dry mass and size of the granule (Equation 4).

$$W_c = fW_d \sim d^D \quad (4)$$

where D is the fractal dimension and W_c is the wet mass of granule

Highly porous and fractal structure of the granule may permit a significant intra-granule flow, resulting in a reduced drag coefficient and thus increased settling velocity, as compared to what can be predicted by Stokes' law. The predicted settling velocity from Equation (1), $U_{S,}$ can be compared with the actual settling velocity, U. The ratio of U/U_S will be then:

$$\Gamma = \frac{U}{U_s} = \frac{\xi}{\xi - \tanh(\xi)} + \frac{3}{2\xi^2} \quad (5)$$

where the dimensionless permeability factor, ξ, is a function of the size and the hydraulic permeability of the granule, k, is described as Equation (6).

$$\xi = d/2k^{1/2} \quad (6)$$

The internal permeation of the granule maybe more directly indicated by its fluid collection efficiency, e_f, which is defined as the ratio of the interior flow passing through the granule to the flow approaching it (Equation 7).

$$e_f = \frac{9U_s}{2\xi^2 U} \quad (7)$$

Analytical Methods

Organic acids including volatile fatty acids (VFAs, C_2–C_6) and lactate were analyzed by a high performance liquid chromatograph (HPLC) (Finnigan Spectra SYSTEM LC, Thermo Electron Co.) with an ultraviolet (210 nm) detector (UV1000, Thermo Electron) and an 100 × 7.8 mm Fast Acid Analysis column (Bio-Rad Lab.) using 0.005 M H_2SO_4 as mobile phase. The liquid samples were pretreated with a 0.45 μm membrane filter before injection to both HPLCs. VSS and alkalinity were measured according to Standard Methods (APHA, 1998). The remained glucose in the broth was measured using the Dubois' method (Dubois et al., 1956).

The size of flocs was analyzed with the free UTHSCSA Image Tool program, a program developed at the University of Texas Health Science Center at San Antonio, Texas. The sample of sludge (0.2 mL) was spread over a petri dish and fixed within a transparent 25 g-gelatin L^{-1} gelatin solution (5 mL). After the gelatin solidified, the sample dishes were placed over the scanner surface. Once eight-bit greyscale images had been obtained, they were analyzed. The software provided the information of area, particle number, diameter, and other characteristics of the particles in the digital image. The microstructures of granules were investigated by SEM (LEO 1455VP) equipped with a secondary electron and quadrant back-scattering detector (QBSD). Cell hydrophobicity was measured by conventional microbial attached to hydrocarbons (MATH) method using n-hexadecane as the hydrocarbon and phosphate urea magnesium sulfate buffer as the water phase (Rosenberg et al., 1980). To disperse the cells in granules, grinding and low sonication (50 W for 2 min with 5 s pulse and 5 s interval) were applied (Guo et al., 2011). The extraction of EPS from granules was followed the procedure described by(Brown and Lester, 1980).

Results and Discussion

Microbial Community Change in CSTR

As the CSTR operation started, an LA concentration gradually increased, reaching 18 g L^{-1} (90% of input glucose) by the 5th day of operation (Fig. S1), which indicated that there was a microbial shift favorable for LA production under the operating condition. A biomass concentration gradually decreased and reached to 1.2 g VSS L^{-1}, accounting for 8.5% of input glucose on chemical oxygen demand (COD) basis. The COD of biomass was calculated by assuming a composition of $C_5H_7O_2N$, resulting in a COD of 1.42 g COD VSS^{-1} (Kim et al., 2014). The total output COD (produced LA + biomass + residual glucose) was 105–110% of input glucose, probably due to the additional supply of 0.3% yeast extract. Assuming that yeast extract contains 20% ash and corresponds to 1.42 g COD/g ($yeast\ extract_{volatile}$), a COD balance reached to 90–95% (total input COD = 23.4 g/L). The produced LA was mostly (>95%) l−(+) form, as shown in other studies (Kwon et al., 2001; Lu et al., 2012).

To study the bacterial community structure of the LA-producing CSTR, the collected mixed liquor samples were subjected to pyrosequencing analysis. A total of 68,530 reads were obtained from a single lane of an 8-lane picotiter plate on a Genome Sequencer FLX titanium system. The type of samples and the number of reads, good reads, average length, and operational taxonomic units (OTUs) from this pyrosequencing work are listed in Table SI.

Figure 2 shows the distribution of sequence at the genus level in each sample. After only one day of CSTR operation, the genus *Lactobacillus* jumped from 0.1% to 49.4%, occupying the predominant composition, which later increased up to 91.5% by the 5th day of operation. Meanwhile, the genus *Clostridium*, *Anaerobacter*, *Sarcina*, and *Pelobacter* gradually decreased and completely disappeared after 5 days of CSTR operation. Instead, *Leuconostoc*, which has similar LA productivity with *Lactobacillus* emerged after 5 days of CSTR operation, constituting around 7% of total sequences (Dartois et al., 1995).

To examine the diversity of genus *Lactobacillus* at the species level, a representative of retrieved sequences was analyzed in the EzTaxon server (Chun et al., 2007). Among the genus *Lactobacillus*, only limited kinds of taxonomical groups related to *Lactobacillus delbrueckii* were observed throughout the operation (Fig. 3). The majority sequence (Group I) belonging to the genus *Lactobacillus* showed a sequence similarity of 99–100% to that of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T which is the same as *L. delbrueckii* subsp. *delbrueckii* ATCC 9649^T and *L. delbrueckii* subsp. *indicus* NCC725^T. As shown in Table I, subgroup I in *L. delbrueckii* was found after the batch operation (Day 0) and developed their realm continuously as CSTR operation continued. Subgroup II, III, and IV accounted for a smaller composition, which fluctuated with time. It was impressive to observe that only 0.1% of the *L. delbrueckii*-related group was observed among the total 1,385 sequences after batch operation. However, their abundance reached 85% of total sequences after five days of CSTR operation, suggesting that although the initial population of effective microbial consortium producing LA was very low in the inoculum, environmental conditions prevalent in the CSTR was suitable for making them dominate so quickly.

Fermentation Performance in UASB

The daily performance of the UASB including LA concentration, residual substrate concentration, and LA productivity at various

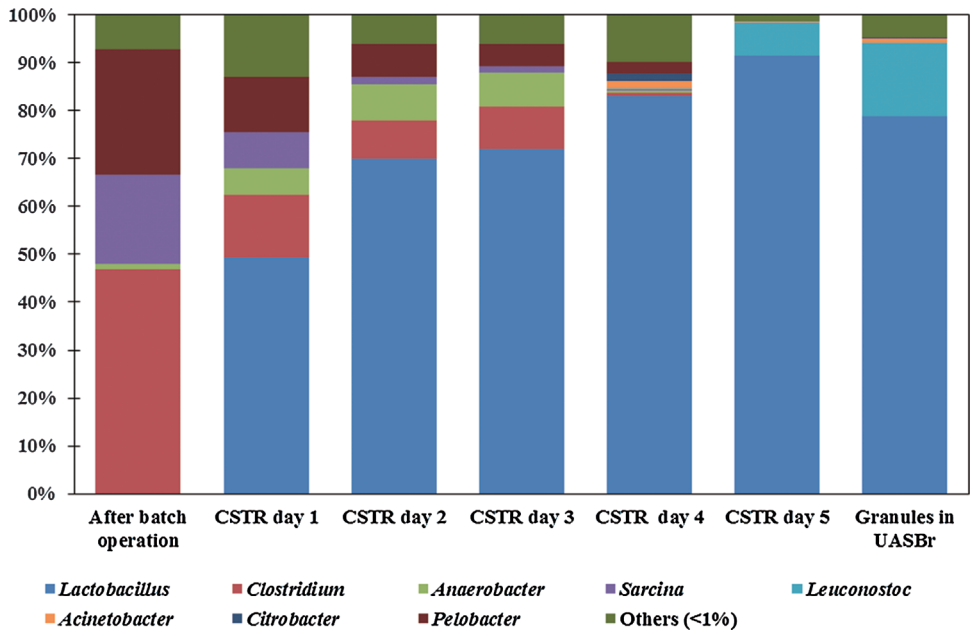

Figure 2. Taxonomic compositions of bacterial communities at genus level for the sequence retrieved from each sample.

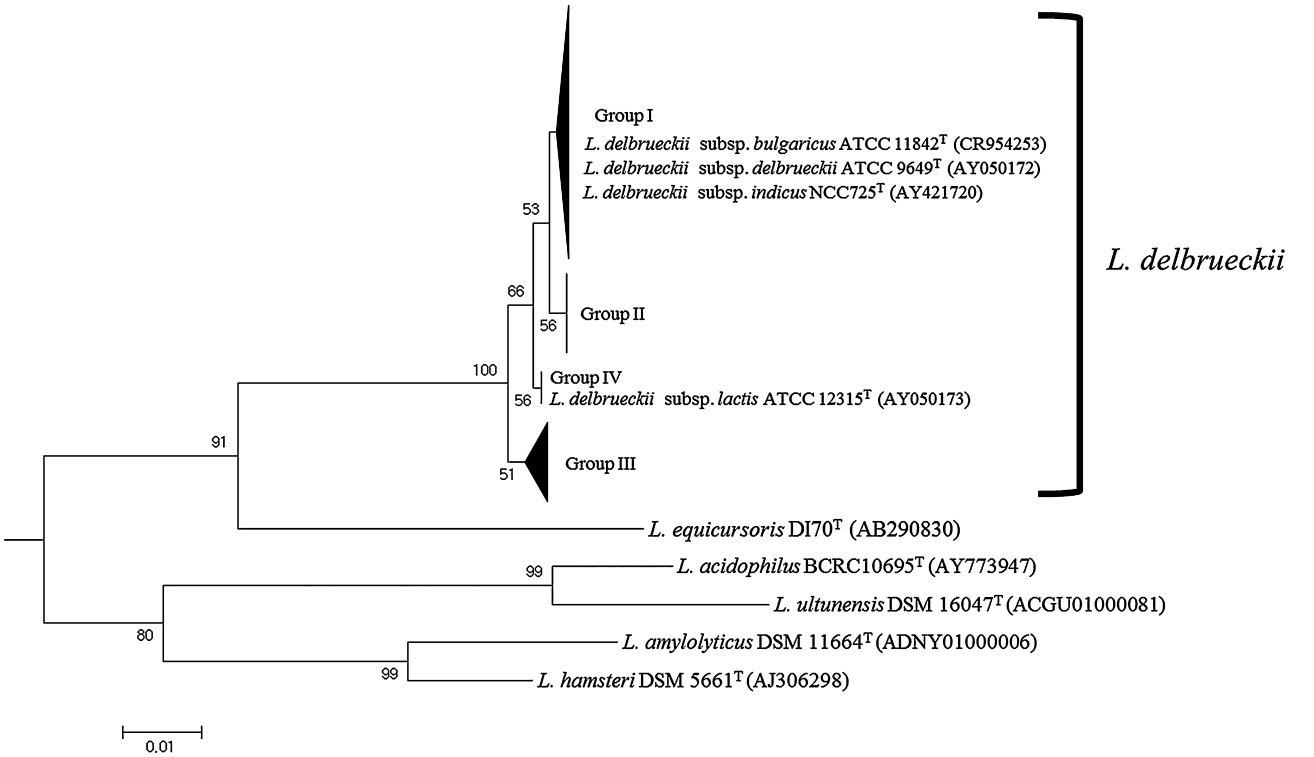

Figure 3. Phylogenetic tree of the representative sequences that belong to the genus *Lactobacillus* and their abundances during the operation of lactic acid producing bioreactor. (Scale bar of tree indicates the 0.01 nucleotide substitutions per site. Abbreviations for genus names: *L, Lactobacillus*).

HRTs is shown in Figure 4. After transferring 1 L of mixed liquor from the CSTR to the UASB, an LA concentration gradually increased and reached 20–21 g L^{-1} with over 95% of substrate removal within 5 days, suggesting a successful start-up of UASB. It seemed that addition of yeast extract by 0.3% in the feed contributed to yield over 100% of input glucose (>20 g L^{-1}) (Altaf et al., 2006; Yilmaz et al., 2010). The sum of soluble portion (produced LA + residual glucose) in the effluent was around 90% of total input COD (23.4 g COD/L), indicating that 10% COD was used for cell synthesis. The production of other organic acids such as acetate, butyrate, and propionate was negligible (<0.01 mM). The LA concentration was maintained up to HRT 3.5 h, but a sudden decrease of LA concentration with the increase of residual substrate concentration was observed after decreasing HRT to 2.0 h. However, it was soon recovered and showed a stable performance. During this fluctuation period, pH suddenly increased from 4.0–4.5 to 5.0–5.5, but it soon returned with process recovery. Indeed, this kind of phenomenon was observed every time when HRT was further reduced, which might be related to the lack of biomass to treat an increased load of substrate (Lu et al., 2012). As operation went on, however, it was possible to see the accumulation of biomass from the lower part of the main body of the UASB, which could handle higher substrate loading. From HRT 1.0 h, the biomass concentration in the blanket zone of UASB ranged 40–60 g dry cell weight

Table I. The subgroup ratio in *L. delbrueckii* and their abundances during the operation of lactic acid producing bioreactor (unit, %).

		CSTR					
	After batch operation (Day 0)	Day 1	Day 2	Day 3	Day 4	Day 5	Granules in UASB
Subgroup I[a]	42.3	61.5	69.0	76.3	79.5	74.5	81.8
Subgroup II[a]	—	8.8	11.4	8.2	3.3	—	—
Subgroup III[a]	—	2.3	7.0	—	6.5	1.9	7.8
Subgroup IV[a]	—	—	4.6	5.5	2.6	16.2	—
L. delbrueckii group in Lactobacillus[b]	42.3	72.6	92.0	90.1	92.0	92.5	89.6
L. delbrueckii group in total sequences[c]	0.1	35.9	64.3	65.0	77.9	85.0	70.6

[a]Subgroups were divided by DNA G+C contents in a L. delbrueckii group.
[b]Relative abundance of a L. delbrueckiigroup in total Lactobacillus sequences.
[c]Relative abundance of a L. delbrueckiigroup in total microbial sequences.

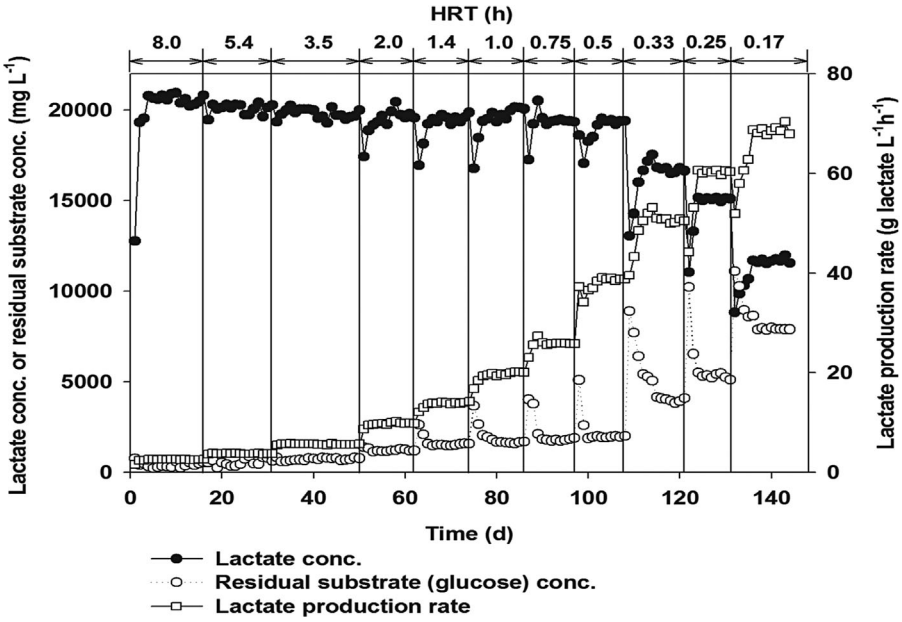

Figure 4. Daily lactic acid fermentation performance at various hydraulic retention times.

(dcw) L^{-1} (70–110 as optical density). Up to HRT 0.5 h, the accumulated biomass, formed as granules, could successfully produce LA, ranging 19–20 g L^{-1}.

When HRT was shortened to 0.33 h, there was a drastic decrease of LA concentration to 13 g L^{-1}, accompanied by a drop of substrate removal around 60%. Although LA concentration increased again and showed a stable performance within 5 days, it was still 20% lower than that in the previous operation. As HRT was further shortened, an LA concentration kept decreasing. Under steady-state condition with operation at HRT 0.17 h, LA concentration and substrate removal were 11.5 g L^{-1} and 60%, respectively. However, the volumetric LA productivity continuously increased, reaching 67 g $L^{-1}h^{-1}$, which, to our best knowledge, was the highest value reported ever. Previously, LA productivities of 22–57 g $L^{-1}h^{-1}$ were obtained using a membrane-coupled reactor (Kwon et al., 2001; Schepers et al., 2006; Tejayadi and Cheryan, 1995; Xu et al., 2006). The microbial granule system for LA production carries significant advantage over the membrane bioreactor such as being free from technical challenges associated with membrane fouling. Actually, the continuous operation lasted less than 5 days in the previous works while the UASB in the current study showed a stable performance for 10 days even at HRT 0.17 h. The future work would be focused on making LA-producing granules by using cheap feedstock including molasses, potato waste, and corn hydrolysate.

Granulation Process and SEM Image Analysis

To elucidate the granulation process in the UASB, samples were prepared to get microscopic images and to measure the mean size and hydrophobicity of granules. One day before changing HRT, 100 mL of mixed liquor were taken from the two different ports (5 and 25 cm from the bottom) and used for these analyses. Since there was not much biomass accumulated at HRT 8 h, sampling and analysis were done from HRT 5.4 h.

The LA-producing granules had creamy color, probably due to the suppression of sulfate reducing bacteria at pH lower than 5.0 (Fig. 5; Mu and Yu, 2006). Actually, the change of color was obviously seen in the CSTR operation, which became white and yellow as operation continued. From the microscopic images, it was easily visible that bigger size granules were more dominant at HRT 1.0 and 0.5 h, compared to HRT 5.4 and 2.0 h. The reactor hydrodynamics is known to have a significant role in the granulation process, which is, in general, facilitated under high up-flow velocity conditions (Arcand et al., 1994; Pan et al., 2004; Verawaty et al., 2013). The up-flow velocity applied in this study increased from 0.13 to 6.0 m h^{-1} with a decrease of HRT from 8.0 to 0.17 h.

Microbial granules are formed by dynamic processes involving microbial attachment, detachment and growth, and reach a certain stable size, which is influenced by various parameters including up-flow velocity (Verawaty et al., 2013). As HRT decreased, the size of granules gradually increased, reaching 6.0 mm at HRT 0.17–0.25 h (Fig. 6). In particular, there was a drastic increase of size at HRT 1.0 h, which was consistent with the biomass movement behavior in the UASB. It seemed that a transition occurred from a fixed-bed regime to fluidized-bed regime at HRT 1.0 h. At HRT $\leq$1 h, it was easy to see a lot of movement of granules, up and down, in the UASB (Fig. S2). This process can lead to washout of non-granulated biomass and to enhance mass transfer to the core of granules, resulting in an increase of granule size.

Like the size of granules, hydrophobicity also increased with decreased HRT. It reached around 60% of hydrophobicity at HRT

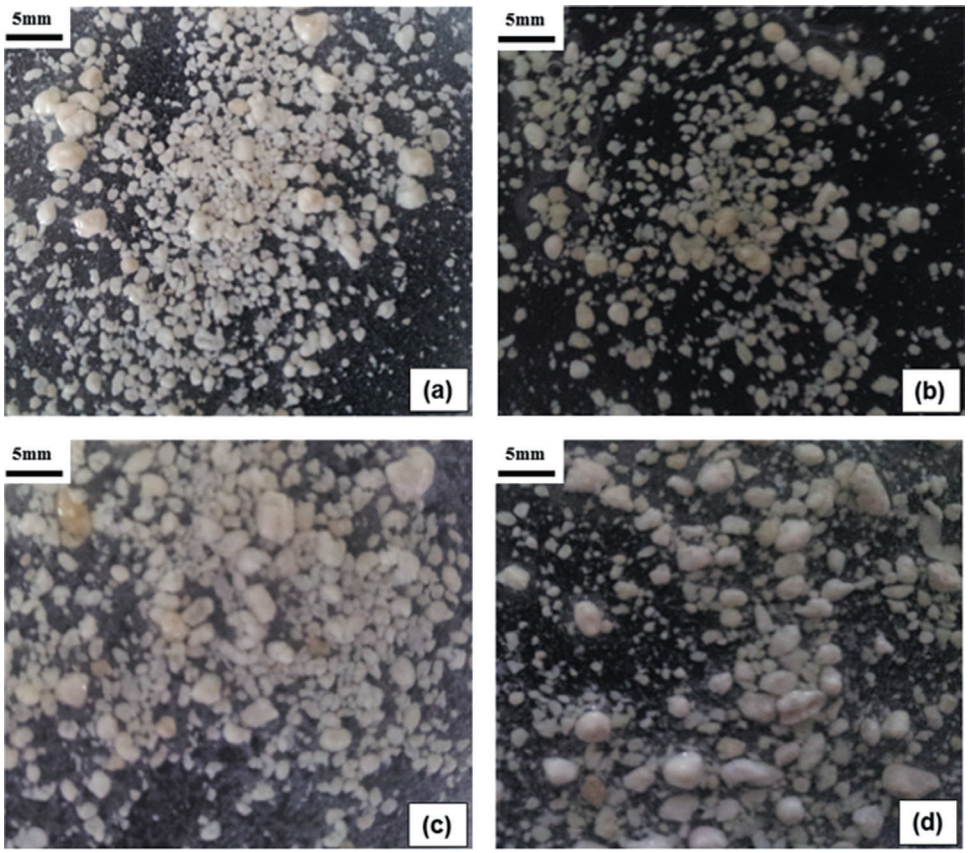

Figure 5. Microscopic images of lactic acid producing granules (a–d), applied hydraulic retention times are 5.4, 2.0, 1.0, and 0.5 h, respectively.)

≤1 h, which was in the similar range of anaerobic methanogenic granules and aerobic sludge granules, determined by MATH method (Pan et al., 2004; Liu and Tay, 2004). MATH method can be easily applied to flocculated cells and mixed culture, in which "%" indicates the percentage of cells adhering to hexadecane after partitioning for 15 min. It was reported that an adequate HRT or hydraulic selection pressure can compel the microorganisms to modify their surface properties and increase their cell hydrophobicity (Pan et al., 2004; Mahoney et al., 1987). Moreover, high hydrophobic force causes a decrease in the excess Gibbs energy of the surface and facilitates cell-to-cell interactions, resulting in a compact and strong structure of granules (Liu et al., 2004).

The SEM images of granules obtained at HRT 1 h are shown in Figure 5. At macro-scale, the surface of granules was smooth (Fig. 7a). However, cracks became visible as magnification increased, which might be related with mass transfer and the release of gaseous products from microbial metabolic processes within the granules (Fig. 7b and c). These kinds of cavities also have been observed in other kinds of biogranules (Zhang et al., 2008). The granules comprised uniform rod bacteria (Fig. 7d), presumably rod bacterium *Lactobacillus*, which is consistent with the results of pyrosequencing analysis. Although, there was a slight increase of *Leuconostoc* population in the UASB operation, *Lactobicillus* sp. were the dominant species, occupying about 80% of total bacterial composition (Fig. 2).

Physicochemical Characteristics of Granules

At the end of operation, 40 granules were taken from the reactor and a settling experiment was carried out to measure their physicochemical characteristics. The granule size varied from 3.4 to 9.7 mm, with a dry mass ranging from 2.6 to 25.9 mg (Fig. 8a). Based on the slope of logarithmic relationship between the dry mass and size, the fractal dimension of the granules was calculated to be $D = 2.00$. This fractal dimension was within the range that is expected for biological aggregates. Li and Ganczarczyk (1989) reported a relatively wide range of fractal dimensions, 1.4–2.85, for particle aggregates generated in water and wastewater treatment processes.

The granule became more porous as its size increased, with porosity ranging from 0.39 to 0.92 (Fig. 8b). It seemed that the granules have lower porosity compared with conventional aerobic granules or anaerobic methanogenic granules which normally have porosity greater than 0.96 or in a range of 0.64–0.90, respectively (Li and Yuan, 2002). The LA-producing granules were much denser than other bio-aggregates, as demonstrated by their lower porosity.

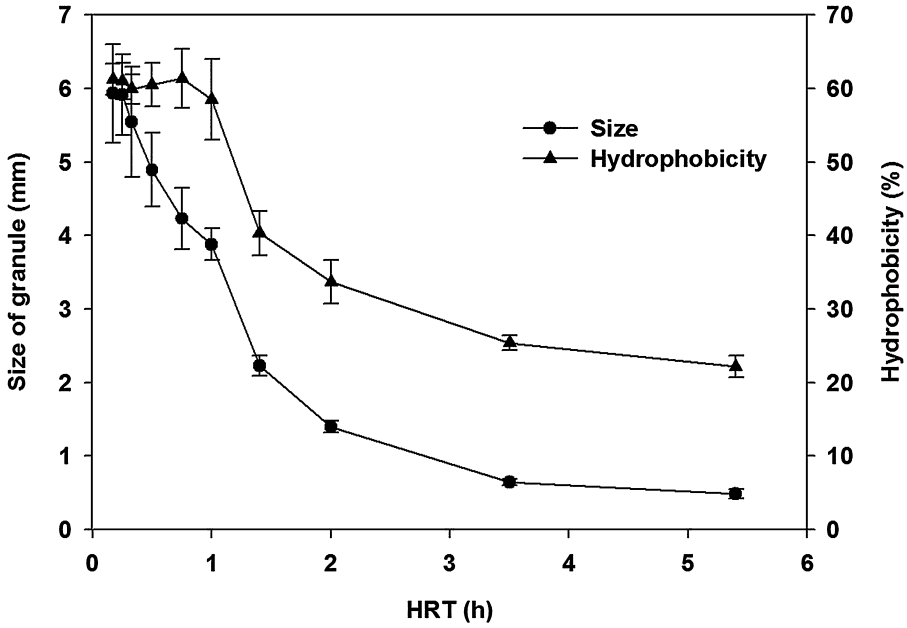

Figure 6. Hydrophobicity and mean size of granules adapted at different HRTs.

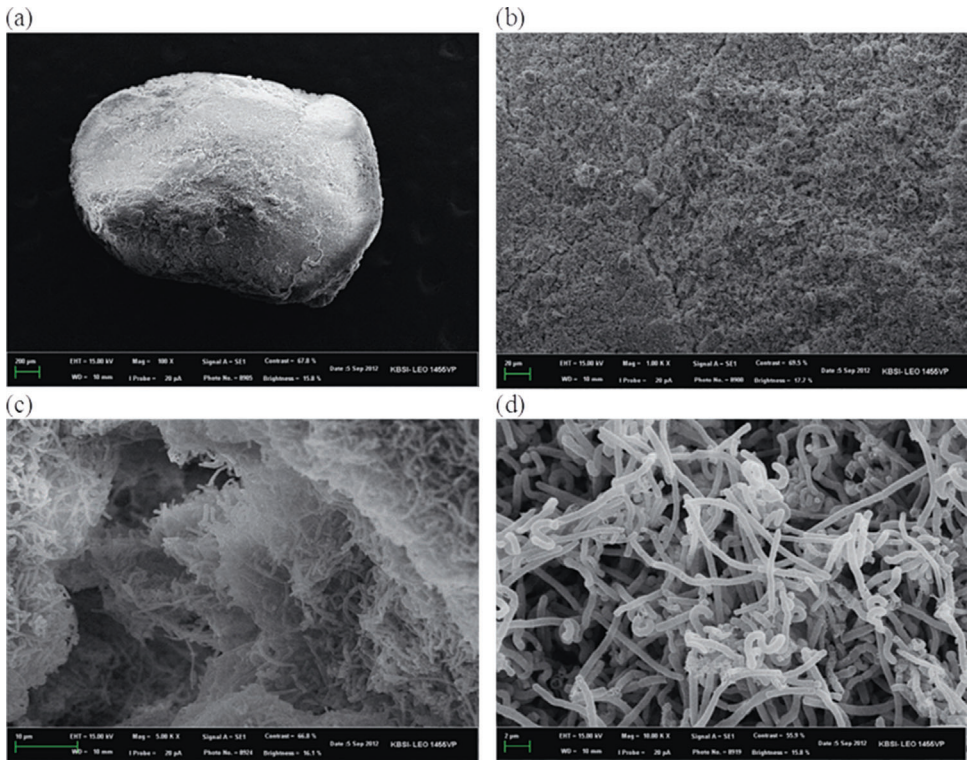

Figure 7. SEM images of lactic acid producing granules obtained at HRT 1 h: (a) 100× magnification; (b) 1,000× magnification; (c) 5,000× magnification; and (d) 10,000× magnification.

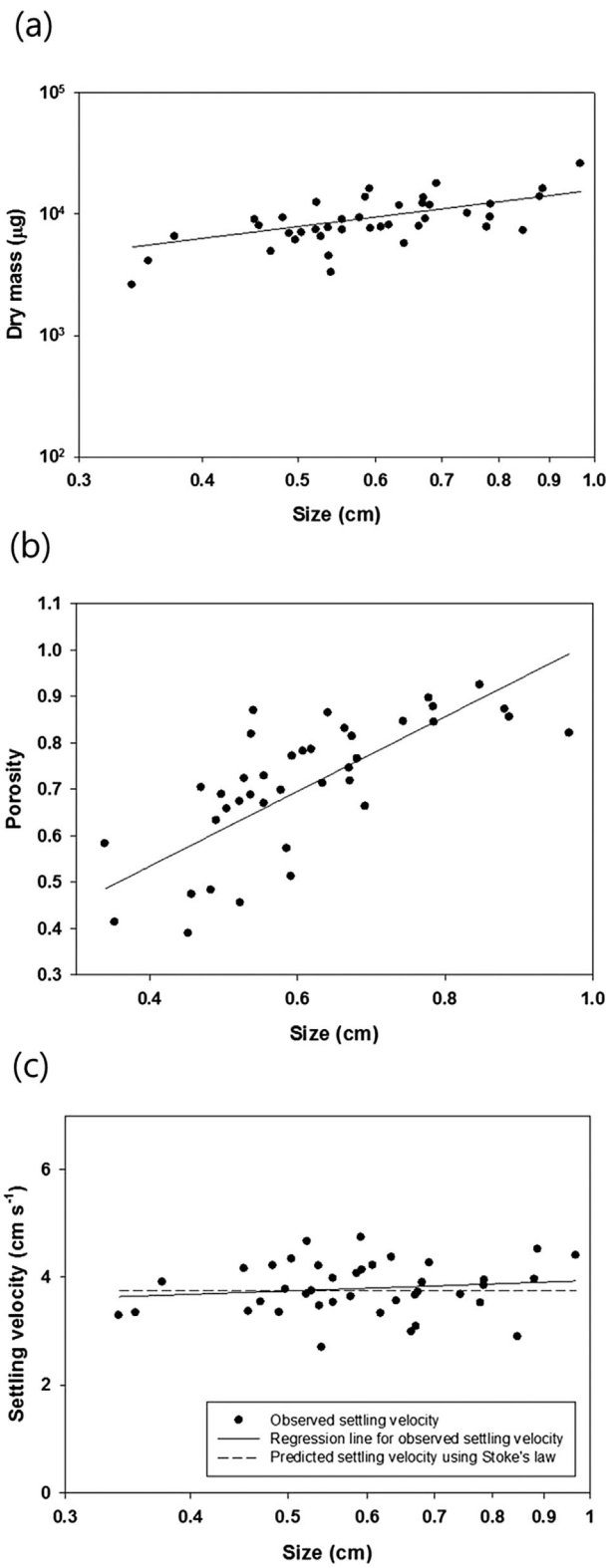

Figure 8. Physicochemical characteristics of lactic acid producing granules ((a) size vs. dry mass; (b) size vs. porosity; and (c) size vs. settling velocity).

This might be linked to the fact that LA production from glucose does not involve gaseous matters ($C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$) while anaerobic methanogenic granules produce CH_4 and CO_2 and aerobic granules require O_2 as an electron acceptor.

The settling velocities of granules varied from 2.69 to 4.73 cm s^{-1} with an average of 3.79 cm s^{-1} (Fig. 6c), which were higher than those for aerobic granules (0.17–3.21 cm s^{-1}), anaerobic H_2-producing granules (0.89–2.08), and anaerobic mathanogenic granules (0.37–6.60 cm s^{-1}). The high settling velocity in this study enabled successful operation of the UASB under very short HRT conditions. The corresponding Reynolds numbers were 231.2 ± 67.3, which were greater than unity. Therefore, the application of Equation (2) for calculation of C_d (empirical drag coefficient) was found to be suitable. The observed settling velocities were in good agreement with the predictions for porous but impermeable objects from Stokes' law.

The dimensionless ratio of the observed to predicted velocities, Γ, is 1.03 ± 0.14. This value close to unity suggests that the internal permeation of the granule was not sufficient to affect their settling behavior. The impermeable nature of the granule could also be explained by a permeability and fluid collection efficiency. The permeability and fluid collection efficiency were calculated to be 0.016 ± 0.0017 and 0.0487 ± 0.021 cm^2, respectively. In general, these granules had much lower permeability compared with those previously reported for aerobic and anaerobic granules, which ranged 0.14–0.19 (Cho et al., 2013; Johnson et al., 1996; Li and Yuan, 2002; Mu et al., 2006).

The observation of this study was consistent with the argument that although microbial granules are highly porous and fractal, the internal permeation of the granules did not appear to have much hydrodynamic significance. Highly permeable inorganic aggregates have been found to have 4–8 times faster settling velocity than was predicted by Stokes' law, but the granules in this study had settling velocities that generally agreed with those predicted by Stokes' law for porous but impermeable spheres. This agreement suggests that there was little convective flow through the granule interior, probably due to pore clogging by large amounts of EPS generated by LA-producing granules. The high concentration of extracted EPS from the granule (145 ± 16 mg carbohydrate g VSS^{-1} and 68 ± 4 mg protein g VSS^{-1}) is consistent with the explanation of low permeability of this granular biomass.

This work was supported by the Intelligent Synthetic Biology Center of Global Frontier Project funded by the Ministry of Education, Science and Technology (2011-0031955), and by the Energy Efficiency & Resources Core Technology Program of the Korea Institute of Energy Technology Evaluation and Planning (KETEP), granted financial resource from the Ministry of Trade, Industry & Energy, Republic of Korea (20132020000170).

References

Abdel-Rahman MA, Tashiro Y, Sonomoto K. 2011. Lactic acid production from lignocelluloses-derived sugars using lactic acid bacteria: Overview and limits. J Biotechnol 156(4):286–301.

Altaf M, Naveena BJ, Venkateshwar M, Kumar EV, Reddy G. 2006. Single step fermentation of starch to L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using inexpensive nitrogen source to replace peptone and yeast extract - Optimization by RSM. Process Biochem 41(2):465–472.

American Public Health Association (APHA) 1998. Standard Methods for the examination of Water and Wastewater. 20th ed Washington, DC: APHA, ASCE, WEF.
Arcand Y, Guitot SR, Desrochers M, Chavarie C. 1994. Impact of the reactor hydrodynamics and organic loading on the size and activity of anaerobic granules. Chem Eng J Biochem Eng J 56(1):23–35.
Armougom F, Raoult D. 2009. Exploring microbial diversity using 16S rRNA high-throughput methods. J Comput Sci Syst Biol 2: 74–92.
Brown MJ, Lester JN. 1980. Comparison of bacterial extracellular polymer extraction methods. Appl Environ Microbiol 40(2):179–185.
Chang HN, Yoo IK, Kim BS. 1994. High density cell culture by membrane-based cell recycle. Biotechnol Adv 12(3):467–487.
Cho SK, Hwang YH, Kim DH, Jeong IS, Shin HS, Oh SE. 2013. Low strength ultrasonication positively affects the methanogenic granules toward higher AD performance. Part I: Physico-chemical characteristics. Bioresource Technol 136: 66–72.
Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 57(Pt 10):2259–2261.
Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37: D141–D145.
Cui F, Kim M. 2013. Use of Steay-stage biofilm model to characterize aerobic granular sludge. Environ Sci Technol 47(21):12291–12296.
Dartois V, Phalip V, Schmitt P, Divies C. 1995. Purification, properties and DNA sequence of the D-lactate dehydrogenase from *Leuconostoc mesenteroides* sub sp. *cremoris*. Res Microbiol 146(4):291–302.
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colormetric method for determination of sugars and related substances. Anal Chem 28(3):350–356.
Dusselier M, Wouwe PV, Dewaele A, Makshina E, Sels BF. 2013. Lactic acid as a platform chemical in the biobased economy: the role of chemocatalysis. Energ Environ Sci 6: 1415–1442.
FitzPatrick M, Champagne P, Cunningham MF, Whitney RA. 2010. A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Bioresource Technol 101: 8915–8922.
Gao C, Ma C, Xu P. 2011. Biotechnological routes based on lactic acid production from biomass. Biotechnol Adv 29: 930–939.
Gonzalez-Gil G, Lopes SIC, Saikaly PE, Lens PNL. 2012. Leaching and accumulation of trace elements in sulfate reducing granular sludge under concomitant thermophilic and low pH conditions. Bioresource Technol 126: 238–246.
Guo F, Zhang SH, Yu X, Wei B. 2011. Variations of both bacterial community and extracellular polymers: The inducements of increase of cell hydrophobicity from biofloc to aerobic granule sludge. Bioresource Technol 102(11):6421–6428.
Hofvendahl K, Hahn-Hägerdal B. 2000. Factors affecting the fermentative lactic acid production from renewable resources. Enzyme Microb Technol 26(2–4):87–107.
Hulshoff Pol, Castro LW, Lopes SI, Lettinga G, Lens PNL. 2004. Anaerobic sludge granulation. Wat Res 38(6):1376–1389.
Johnson CP, Li X, Logan BE. 1996. Settling velocities of fractal aggregates. Environ Sci Technol 30(6):1911–1918.
Kim DH, Lee JH, Kang S, Hallenbeck PC, Kim EJ, Lee JK, Kim MS. 2014. Enhanced photo-fermentative H_2 production using *Rhodobacter sphaeroides* by ethanol addition and analysis of soluble microbial products. Biotechnol Biofuels 7(79):1–10.
Kim DH, Lim WT, Lee MK, Kim MS. 2012. Effect of temperature on continuous fermentative lactic acid (LA) production and bacterial community, and development of LA-producing UASB reactor. Bioresource Technol 119: 355–361.
Kleerebezem R, van Loosdrecht MC. 2007. Mixed culture biotechnology for bioenergy production. Curr Opin Biotechnol 18(3):207–212.
Kwon S, Yoo IK, Lee WG, Chang HN, Chang YK. 2001. High-rate continuous production of lactic acid by *Lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor. Biotechnol Bioeng 73(1):25–34.
Li DH, Ganczarczyk JJ. 1989. Fractal geometry of particle aggregates generated in water and wastewater treatment processes. Environ Sci Technol 23(11):1385–1389.
Li XY, Yuan Y. 2002. Settling velocities and permeabilities of microbial aggregates. Wat Res 36(12):3110–3120.
Liu XW, Sheng GP, Yu HQ. 2009. Physicochemical characteristics of microbial granules. Biotechnol Adv 27(6):1061–1070.
Liu Y, Tay JH. 2004. State of the art of biogranulation technology for wastewater treatment. Biotechnol Adv 22(7):533–563.
Liu Y, Yang SF, Tay JH, Liu QS, Qin L, Li Y. 2004. Cell hydrophobicity is a triggering force of biogranulation. Enzyme Microb Technol 34(5):371–379.
Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22: 1658–1659.
Li WW, Yu HQ. 2011. Physicochemical characteristics of anaerobic H_2-producing granular sludge. Bioresource Technol 102(18):8653–8660.
Lu Z, Wei M, Yu L. 2012. Enhancement of pilot-scale production of L(+)-lactic acid by fermentation coupled with separation using membrane bioreactor. Process Biochem 47(3):410–415.
Mahoney EM, Varangu LK, Cairns WL, Kosaric N, Murray RGE. 1987. The effect of calcium on microbial aggregation during UASB reactor start-up. Water Sci Technol 19(1–2):249–260.
Mu Y, Yu HQ. 2006. Biological hydrogen production in a UASB reactor with granules. I: Physicochemical characteristics of hydrogen-producing granules. Biotechnol Bioeng 94(5):980–987.
Mu Y, Yu HQ, Wang G. 2006. Permeabilities of anaerobic CH_4-producing granules. Wat Res 40(9):1811–1815.
Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695–700.
Pan S, Tay JH, He YX, Tay STL. 2004. The effect of hydraulic retention time on the stability of aerobically grown microbial granules. Lett Appl Microbiol 38(2):158–163.
Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ, Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T. 2006. The path forward for biofuels and biomaterials. Science 311: 484–489.
Rosenberg M, Gutnick D, Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9(1):29–33.
Sauer M, Porro D, Mattanovich D, Branduardi P. 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol 26: 100–108.
Schepers AW, Thibault J, Lacroix C. 2006. Continuous lactic acid production in whey permeate/yeast extract medium with immobilized Lactobacillus helveticus in a two-stage process: Model and experiments. Enzyme Microb Tech 38(3–4):324–337.
Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.
Tang CJ, Zheng P, Ding S, Lu HF. 2014. Enhanced nitrogen removal from ammonium-rich wastewater containing high organic contents by coupling with novel high-rate ANAMMOX granules addition. Chem Eng J 240: 454–461.
Tejayadi S, Cheryan M. 1995. Lactic acid from cheese whey permeate. Productivity and economics of a continuous membrane bioreactor. Appl Microbiol Biotechnol 43(2):242–248.
Verawaty M, Tait S, Pijuan M, Yuan Z, Bond PL. 2013. Breakage and growth towards a stable aerobic granule size during the treatment of wastewater. Wat Res 47(14):5338–5349.
Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73: 5261–5267.
Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.
Xu G, Chu J, Wang Y, Zhuang Y, Zhang S, Peng H. 2006. Development of a continuous cell-recycle fermentation system for production of lactic acid by *Lactobacillus paracasei*. Process Biochem 41(12):2458–2463.
Yilmaz LS, Kontur WS, Sanders AP, Sohmen U, Donohue TJ, Noguera DR. 2010. Electron partitioning during light- and nutrient-powered hydrogen production by *Rhodobacter sphaeroides*. Bioenergy Res 3(1):55–66.
Zhang ZP, Adav SS, Show KY, Tay JH, Liang DT, Lee DJ, Su A. 2008. Characteristics of rapidly formed hydrogen producing granules and biofilms. Biotechnol Bioeng 101(5):926–936.
Zheng YM, Yu HQ, Sheng GP. 2005. Physical and chemical characteristics of granular activated sludge from a sequencing batch airlift reactor. Process Biochem 40: 645–650.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.