Supplementary Information

Enrichment D-lactic acid from organic wastes catalyzed by zero-valent iron: an approach for sustainable lactate isomerization

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6 Tables & 17 Figures

Materials and Methods

WAS and Food Waste. Waste Sludge was collected from a municipal sewage treatment plant in Songjiang district Shanghai, and settled for 1 d to decant the supernatant before use. The concentrated sludge was characterized (averaged data plus standard deviation of triplicate tests): pH 6.3 ± 0.3, TSS 13.66 ± 0.19 g/L, VSS 12.58 ± 0.18 g/L, TCOD 15.98 ± 4.14 g/L and SCOD 0.05 ± 0.01 g/L. Food waste from dinning canteen of Donghua University was smashed to slurry state according to previous method [1]. The characteristic of food waste was as follow: TSS 210.47 ± 21.91 g/L, VSS 207.09 ± 23.04 g/L, TCOD 304.20 ± 25.67 g/L and SCOD 65.19 ± 15.67 g/L. WAS (0.55 L) was mixed with food waste (0.15 L) to make a total of 0.9 L fermentative substrate (diluted by 0.2 L tap water).

Miseq Pyrosequencing Method. Immediately after sampling, 2 mL of mixture was centrifuged at 10,000×g for 5 min and the supernatant was decanted. The pellets were washed twice with 1.5 mL of PBS (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4 \cdot 7H_2O$, and 0.14 mM KH_2PO_4) and centrifuged again under the same condition. The supernatants were carefully decanted, and the pellets were resuspended in 5 mL extracted buffer (100 mMTris·Cl, 100 mMEDTA-Na_2, 200 mM NaCl, 2% CTAB, pH 8.0) followed by incubation at 37 ^oC for 45 min, and then 750 μL of 20% sodium dodecyl sulfate (SDS) were added followed by incubation at 65 ^oC for 1 h in water bath. After centrifugation at 12,000×g for 10 min, 1 mL of the supernatant was carefully collected, and 1 mL of phenol-chloroform-isoamyl alcohol (25:24:1) was used to extract twice. Subsequently, 1 mL of 0.3 M sodium acetate (NaAC) and 2 mL of 100% ethanol were added to form precipitate for 1 h at ambient temperature, which was then centrifuged (12,000×g for 20 min) at 4 ^oC. Pellets with extracted DNA was washed twice with 70% ethanol and dried before being eluted in

50 μL of nuclease-free water. In order to conduct the pyrosequencing, the extracted DNA was amplified by PCR using the primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') for the V1-V3 region. The 20.0 mL PCR mixture contained 4 mL of 5× FastPfu Buffer, 2.0 mL of 2.5 mM dNTPs, 0.4 mL of each primer, 10.0 ng template of DNA and 0.4 mL FastPfu Polymerase (TransStart FastPfu DNA Polymerase,TransGen). The amplification program was processed according to the modified procedural: starting with an initial denaturation step at 94 ºC for 2 min, 25 cycles of denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 30 s, followed by a final extension at 72 ºC for 5 min (Ma et al., 2013). Pyrosequencing was conducted via Illumina MiSeq sequencing system (Illumina, USA) in Sangon Co., Ltd. in Shanghai. Based on these clusters, Chao 1 and ACE richness, Shannon diversity and Good's coverage were all calculated at a dissimilarity level of 3% for each sample. Raw sequences have been deposited in the NCBI Short Read Archive (SRA) database under the accession number SRR3723632.

Analytical Methods. In order to see the morphology of the fermentative matter, iron should be removed from the solid phase as followed: sludge samples were centrifuged at 8000 g for 10 min. Then, the sludge pellet was re-suspended by tap water and stirred completely. A bulk magnet was dropped into the beaker to suck the residual iron from the sludge, and took out to clear up the iron on the magnet surface. The procedural was repeated five times to guaranteed the solid sample was free of iron. Before substrate mixture observation by scanning electron microscopy using Fei Quanta 200 electron microscope, the samples were washed with PBS and fixed with 2.5% glutaraldehyde (pH 7.2-7.4) overnight at 4 ^{o}C, then washed with PBS for 2 h. Samples were fixed for 1.5 h by 1% osmic acid, then washed with double distilled water for 2 h. To obtain

cleaved preparations of the granules, the fixed samples were quick-frozen in liquid nitrogen and cleaved with a rotary microtome. Then samples were dehydrated by successively passaging through 25, 50, 75, 80, 90, 95, and 100% ethanol followed by drying with a critical drier and platinizing with an ion coater.

X-ray photoelectron spectroscopy (XPS) allows us to access the local environment of atoms and their oxidation states, and thus in current study it will allow us to investigate the iron state in substrate with or without Fe dosing. X-ray photoelectron spectroscopy was conducted using Escalab 250Xi with Al Kα radiation as the exciting source.

To analyze VFA, which included acetic, propionic, n-butyric, isobutyric, n-valeric, and iso-valeric acids, the filtrate sample (through 0.45 μm –pore-size filter) was collected in a 1.5 mL gas chromatography (GC) vial, and 3% H_3PO_4 was added to adjust the pH to around 3.0. Agilent GC 7820 with flame ionization detector and equipped with a DB-WAX column (30 m×1.0 mm×0.53 mm) was applied to analyze the composition of VFA. Nitrogen was the carrier gas and the flux was 50 mL/min. The injection port and the detector were maintained at 200 and 220 ^{o}C, respectively. The oven of GC was programmed to begin at 80 ^{o}C and to remain there for 1 min, then to increase at a rate of 40 ^{o}C/min to 110 ^{o}C, and to hold for 1 min, then further to increase at a rate of 10 ^{o}C/min to 170 ^{o}C, which was hold for 1min, and to boost at 40 ^{o}C/min to final 230 ^{o}C for an additional 2 min.

Molecular weight (M_w) distributions of the liquid phase were determined by gel-filtration chromatography (GFC) analyzer (Shimadzu Co., Japan), which was equipped with the gel column (TSK G4000SW type Tosoh Co., Japan) and a refractive index detector (RID-10A) in the present study. The M_w was calculated with class VP 5.03 software (Shimadzu Co., Japan). The flow

rate of the mobile phase (Milli-Q water) was set as 0.5 mL/min. The samples were diluted properly and filtered through a 0.45 μm hydrophilic filtration membrane before being assayed.

Fluorescence spectroscopy analysis of protein was conducted using excitation emission matrix (EEM) fluorescence spectroscopy with a luminescence spectrometry (Fluoromax-4 Spectrofluorometer, HORIBA Scientific, France). Prior to the EEM test, the sample was filtered through a 0.45 mm hydrophilic filtration membrane and diluted to an approximate concentration. To get the fluorescence of EEM, the excitation and emission wavelengths were gradually increased from 300 to 600 nm at 2 nm increments and 400 to 700 nm at a 2 nm step, respectively. The analyses of α-glucosidase and protease were according to the reported method[2].

Table S1. Statistical Analysis Results of the Influences of Iron Dosing (Fe-I, Fe-IV, and Fe-VII) on the Concentration of Lactic Acid Compared to Blank Tests (B-I, B-II, B-III)

Items	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$
Fe-I *vs* B-I	49.353	7.708	0.0022
Fe-IV *vs* B-II	12.526	7.708	0.0241
Fe-VII *vs* B-III	110.044	7.708	0.0005

Table S2. Statistical Analysis of the Stabilization of D-lactic Acid After 4 d of Fermentation from R-15, R-30, and R-60

Items	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$
R-15	2.928	4.066	0.099
R-30	0.182	3.478	0.942
R-60	0.069	3.478	0.990

Table S3. Statistical Analysis of the VFA Production Obtained at 4 d at Different ZVI Dosage Compared to Blank.

Items	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$
Fe-15 *vs* Blank	0.004	7.708	0.955
Fe-30 *vs* Blank	0.359	7.708	0.581
Fe-60 *vs* Blank	6.016	7.708	0.071
Fe-150 *vs* Blank	3.438	7.708	0.137
Fe-500 *vs* Blank	0.1×10^{-4}	7.708	0.997
Fe-900 *vs* Blank	7.043	7.708	0.056

Table S4. Summary of Pyrosequencing Data for Blank, R-60 and R-900 (α = 0.03).

Samples	Reads	OTUs	ACE[a]	Chao1[a]	Shannon[a]	Simpson	Coverage
Blank	13073	328	765	568	3.37	0.079	0.983
R-60	10385	1017	3836	2164	4.52	0.039	0.939
R-900	10074	537	1571	1169	3.98	0.052	0.971

[a] ACE and Chao richness estimator: the total number of OTUs estimated by infinite sampling. A higher number indicates higher richness. Shannon diversity index: an index to characterize species diversity. A higher value represents more diversity.

Table S5. Lactic Acid Producing from *B. cepacia and B. japonicum*. [a]

Serum bottles [b]	D-lactic acid (g/L)	L-lactic acid (g/L)	OA (%)
B-*c1*	0.44	1.22	<0
B-*c2*	2.33	0.88	47.7
B-*cj1*	dl[c]	<0.01	<0
B-*cj2*			

[a] data was collected after 2 d of fermentation.

[b] *B. cepacia* was added to serum bottles B-*c1* and B-*c2*, and *B. japonicum* was to B-*j1* and B-*j2*. While, B-*c1* and B-*j1* were at anaerobic condition, and B-*c2* and B-*j2* were at facultative condition

[c] symbol of "dl" represents concentration of lactic acid was under detected limit.

Table S6. Acetic Acid Production for Fermentation with Addition of Ferrous L-lactate in Fe(II)-0.4 and Fe(II)-4.5. Unit: g/L.

Time (d)	0	2	4
	Acetic acid	Acetic acid	Acetic acid
Fe(II)-0.4	dl	1.0	1.2
Fe(II)-4.5	dl	1.6	1.8

Symbol: dl means measured under detected limit (<0.001g/L).

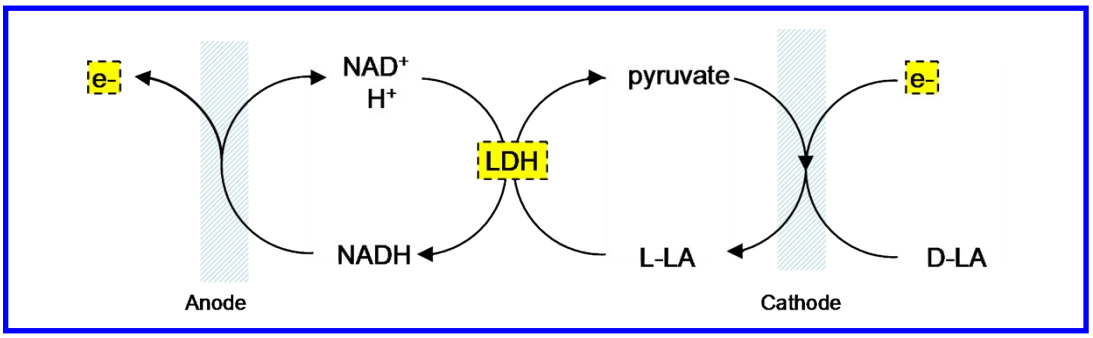

Fig. S1 - Illustration of the electrochemical reaction to convert L-lactate to D-lactate via electrochemical oxidation of NADH, and electrochemical reduction of pyruvate by L-lactate dehydrogenase (L-LDH, E.C. l. l. l .27.). D-LA represent to D-lactate, L-LA represents to L-lactate. [3]

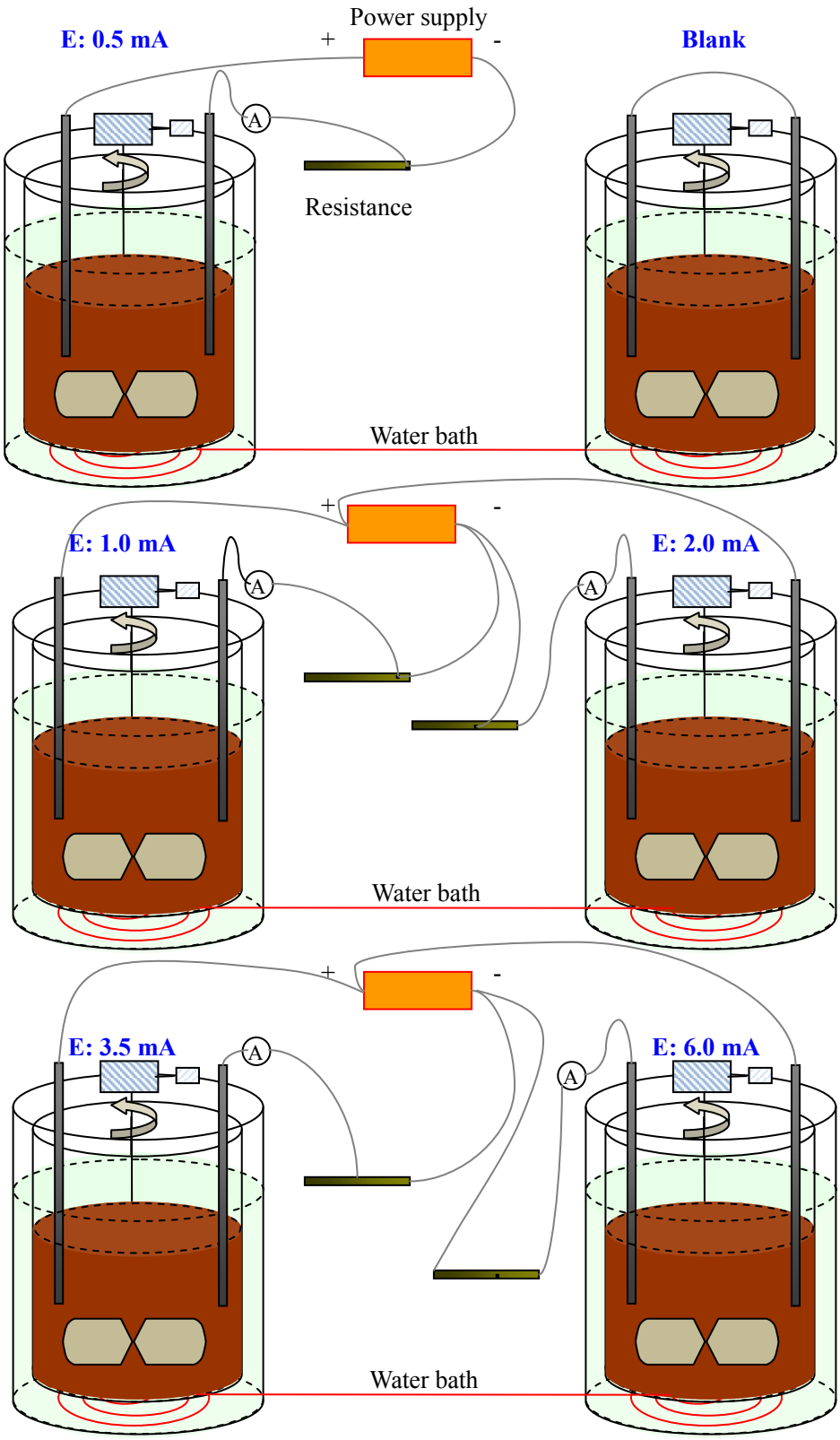

Fig. S2 - Overview of bioelectrochemical fermentation system for lactate isomerization test.

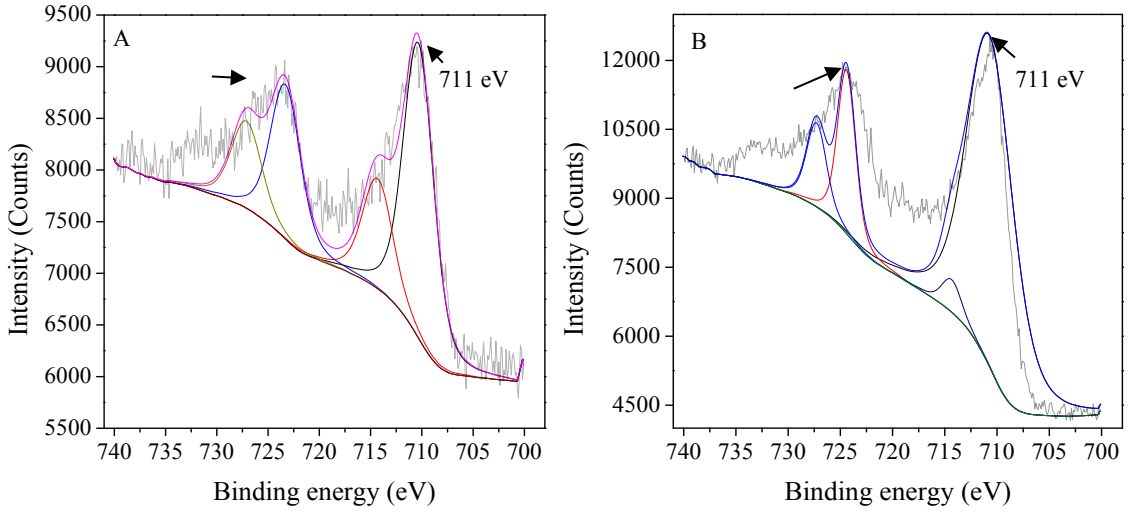

Fig. S3 - X-ray photoelectron of Fe $2p_{3/2}$ spectra from solid phase of Blank (A) and R-60 (B) after fermentation 6 d. Binding energy of Fe(III) presents a major contribution near 711 eV due to that sludge derived from WWTP had been conditioned by $FeCl_3$. Iron incorporation in samples of R-60 was negligible compared to Blank.

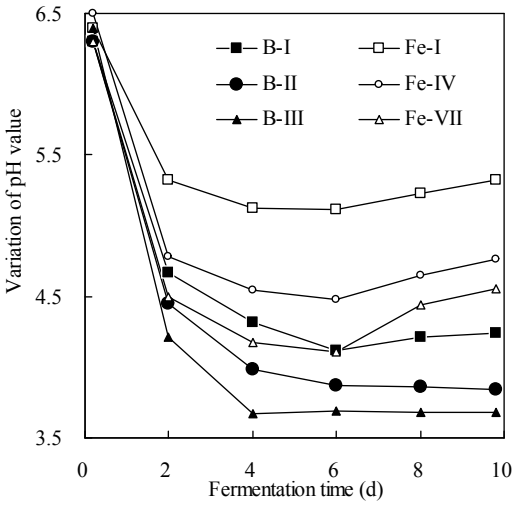

Fig. S4 - Variation of pH value during fermentation from reactors without pH control.

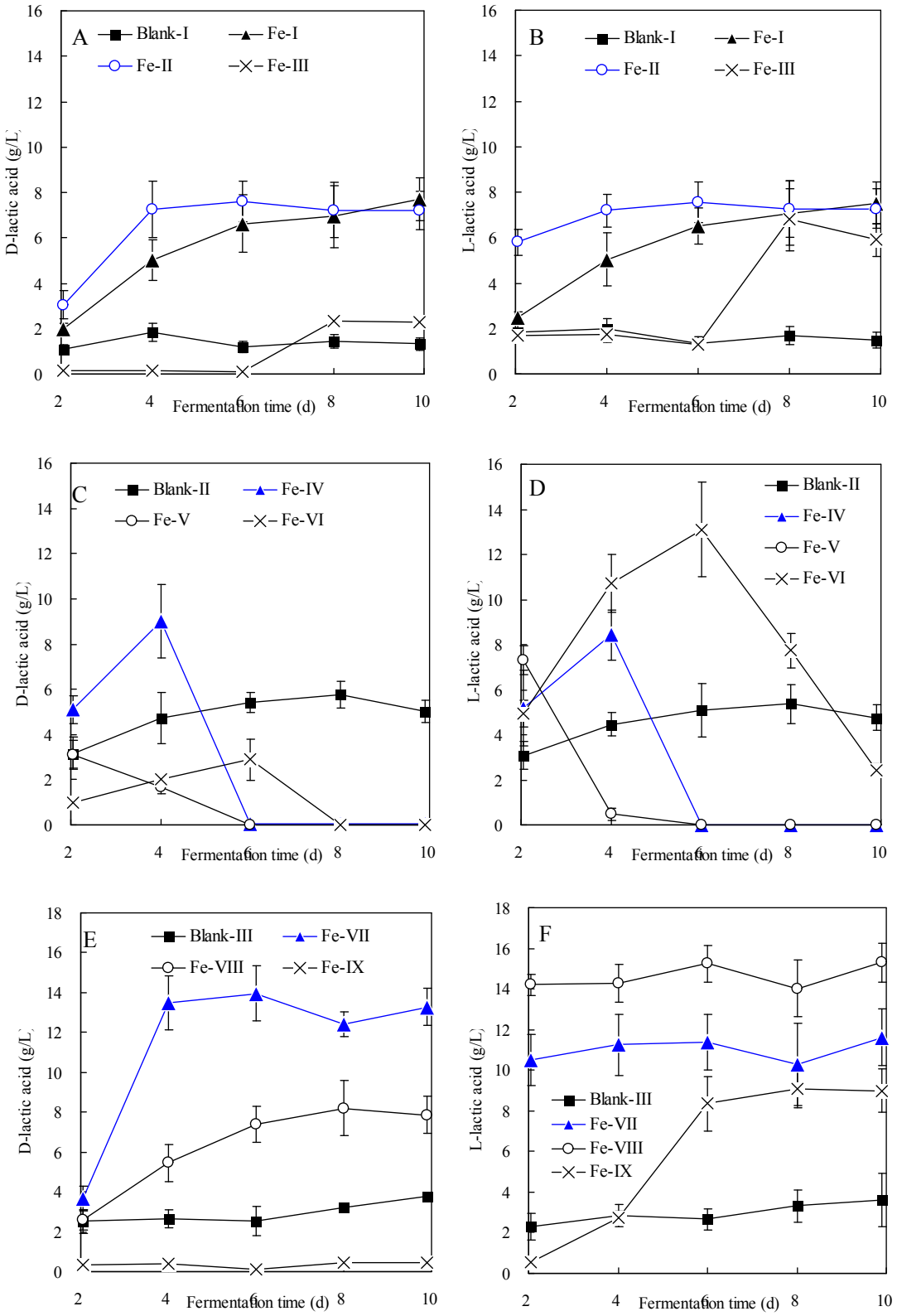

Fig. S5 - Time curves of accumulative lactic acid from different fermentation parameters. A, B: ambient temperature; C, D: mesophilic condition; E, F: thermophilic condition. Blue ones represented the corresponding optimal fermentation parameters.

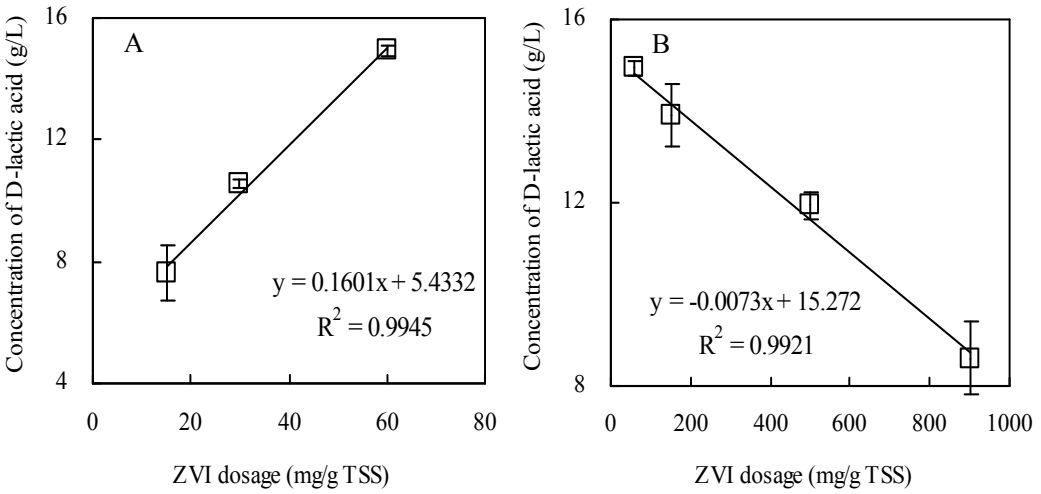

Fig. S6 - Linear regression of the maximal concentration of D-lactic acid and ZVI dosage from interval A: ZVI dosage 20-60 mg/g TSS, interval B: dosage 60-900 mg/g TSS.

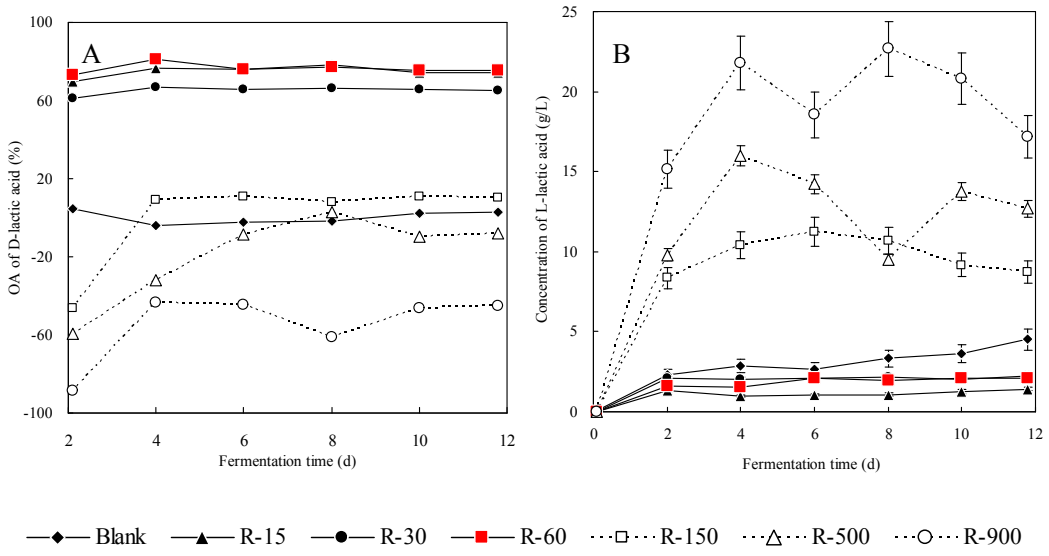

Fig. S7 - Effect of ZVI dosage on OA of D-lactic acid (A) and L-lactic acid production (B) during the whole fermentation.

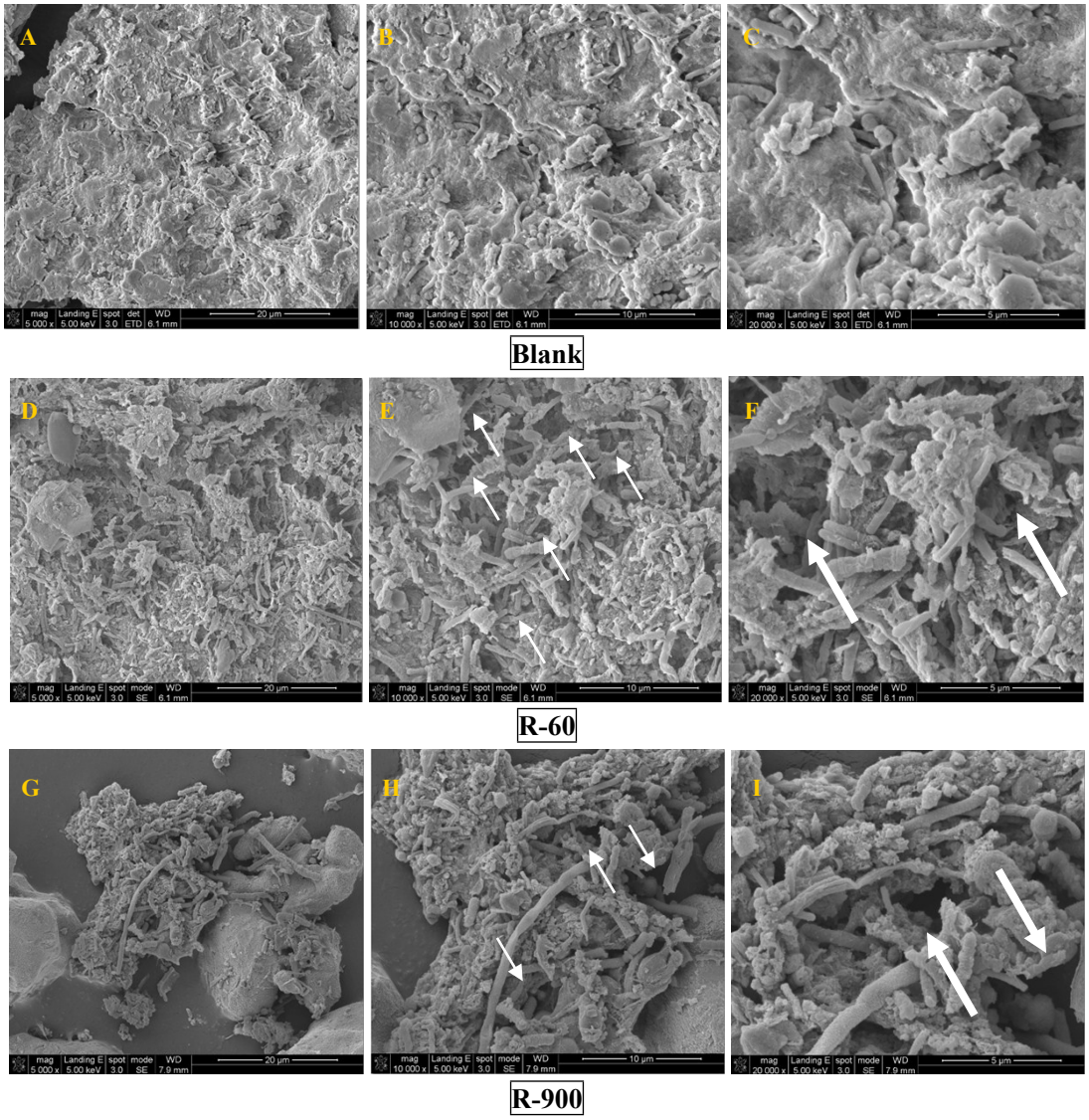

Fig. S8 - SEM of fermentation solid sample from Blank (A-C), R-60 (D-F) and R-900 (G-I).

White arrows indicated fine porous in the surface.

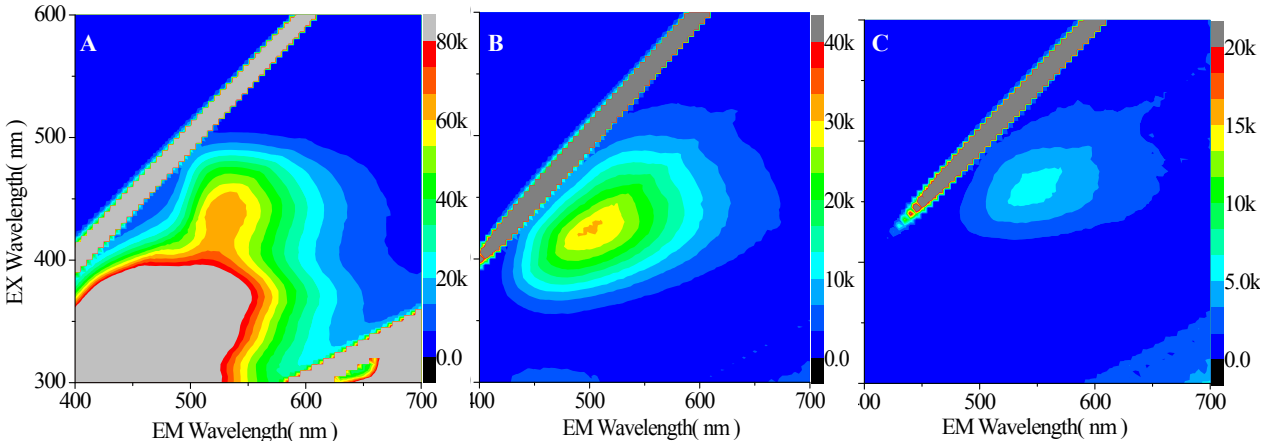

Fig. S9 - Fluorescence spectra of soluble protein-like substrate at 4 d fermentation from Blank (A), R-60 (B) and R-900 (C). For clear comparison, color legends were shrunk properly.

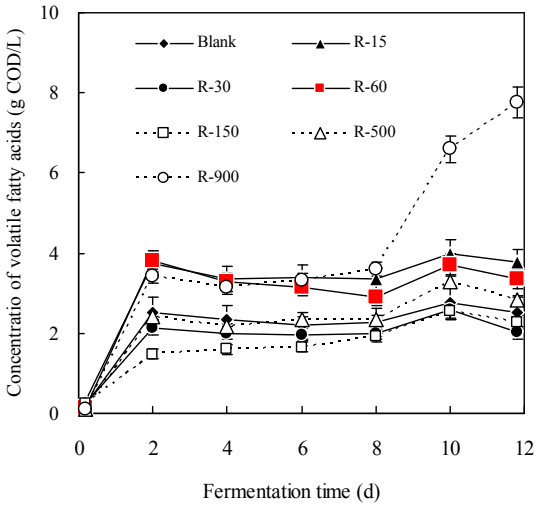

Fig. S10 - Effect of ZVI dosage on VFA generation during the entire fermentation.

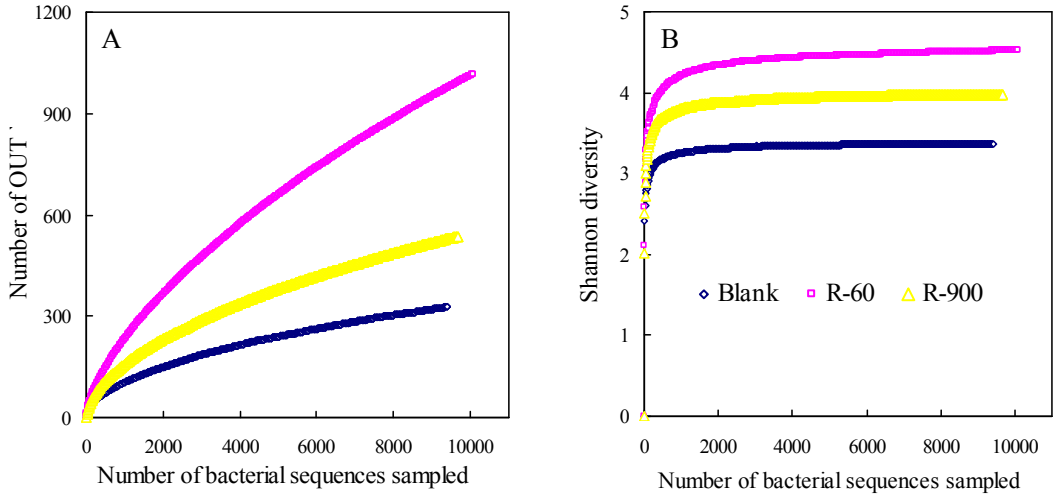

Fig. S11 - Rarefaction curves (A) and shannon diversity (B) of bacterial sequences from Blank, Fe-60 and Fe-900. The OTUs were defined by clustering sequences at the dissimilarity levels of 3%, respectively.

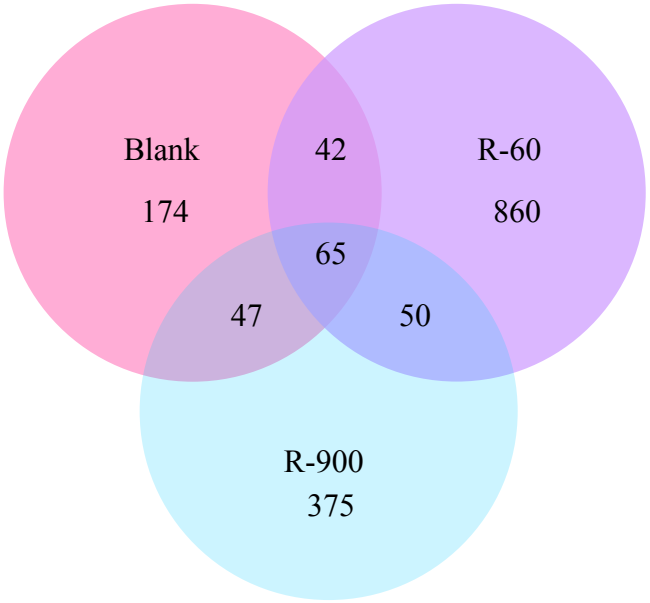

Fig. S12 - Venn diagram at distance 3% level from Blank, R-60 and R-900. The number of species is 328 in Blank, 1017 in R-60, and 537 in R-120. The total richness of all the groups is 1613. The number of species shared between groups is 107 (Blank and R-60), 112 (Blank and R-900), 115 (R-60 and R-900), and 65 (total shared)

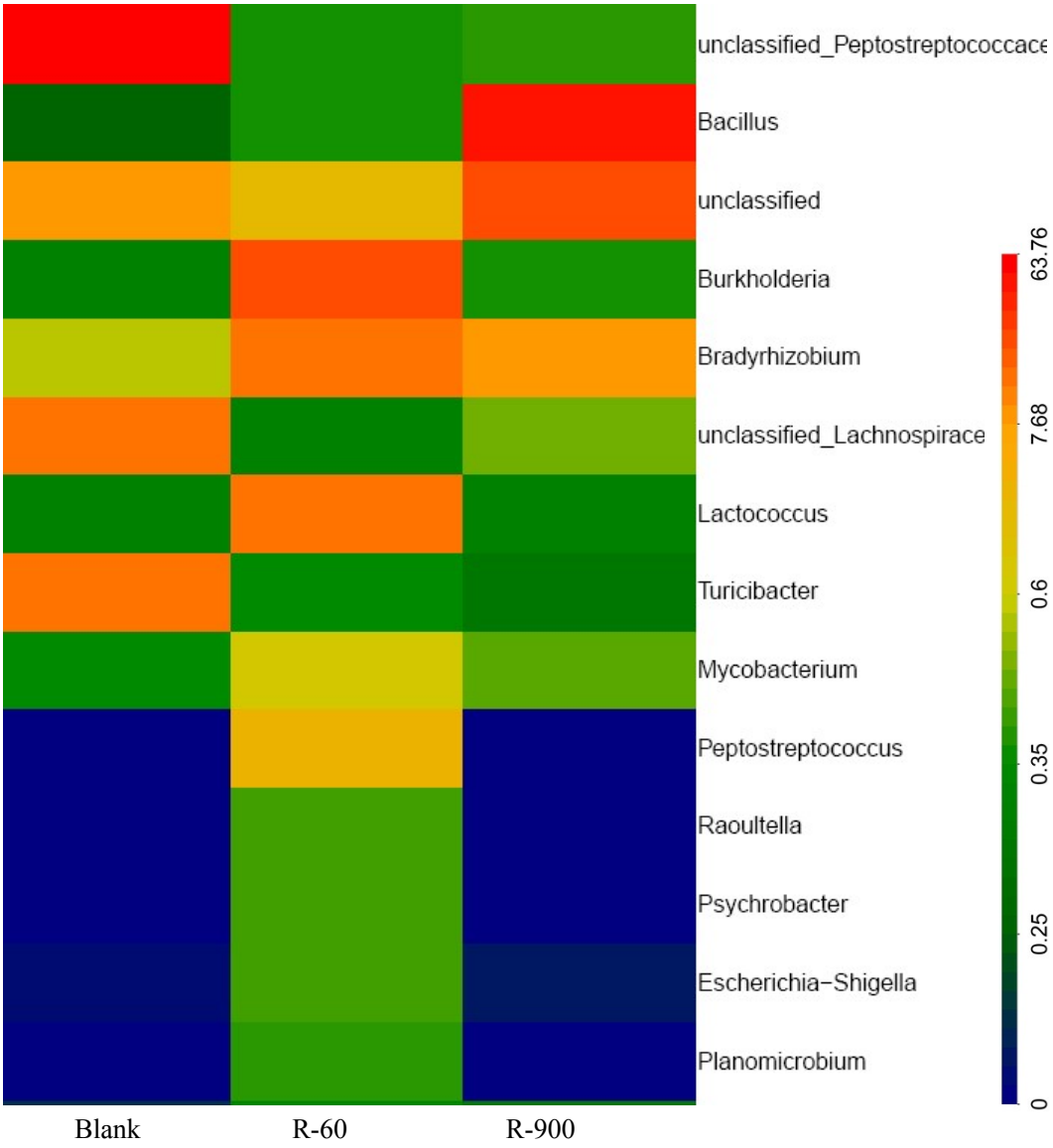

Fig. S13 - Heatmap abundance annotated at genus level from Blank, R-60 and R-900. Map of tree on the top represents the similarity of the samples in genus level.

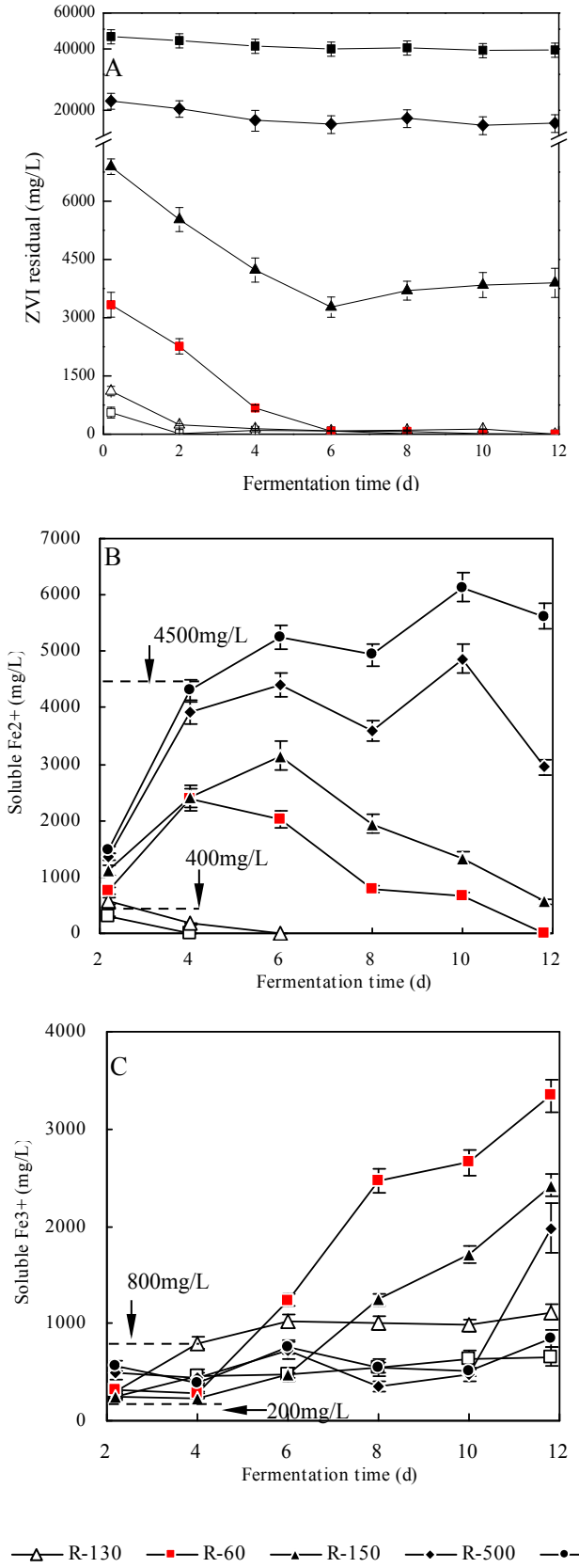

Fig. S14 - Iron variation during fermentation: A: rZVI, B: Fe(II), C: Fe(III). Fe(II) and Fe(III) were 0.4 - 4.5 g/L and 0.2 to 0.8 g/L during lactic acid producing stage (Day 4)

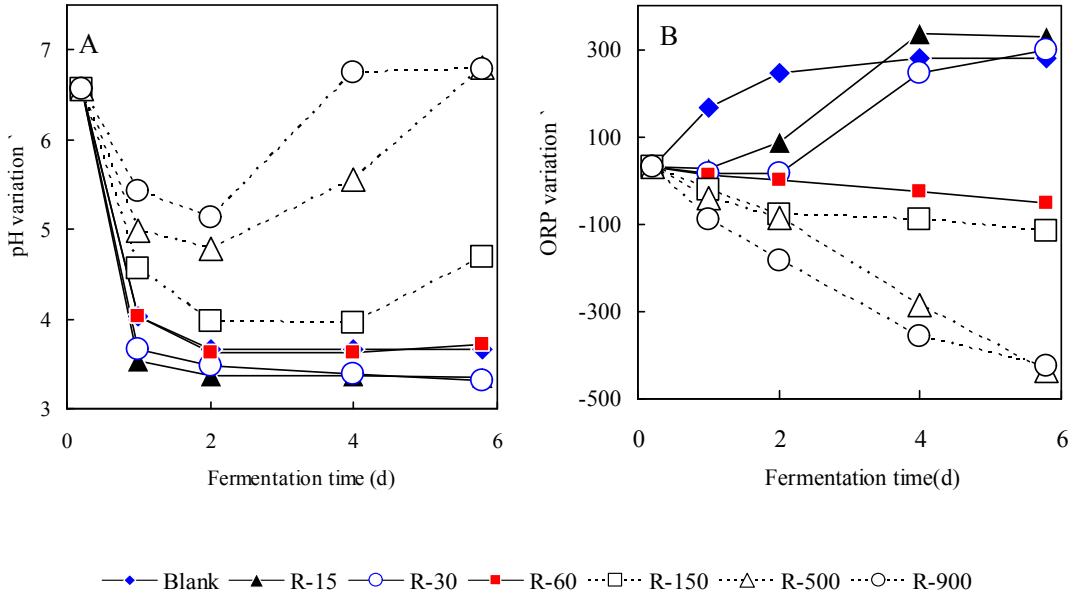

Fig. S15 - Variations of pH and ORP during the fermentation time when most of lactic acid was produced.

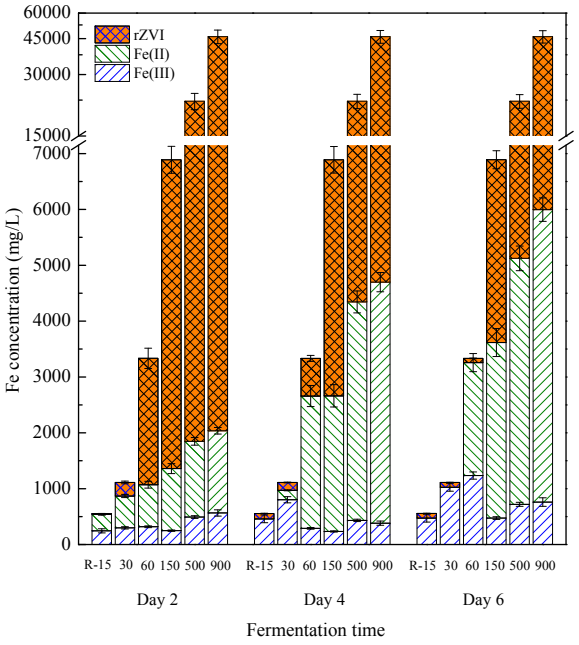

Fig. S16 - Influence of ZVI dosage on concentration of different valent iron during fermentation of Day 2 to 6. rZVI represents residual ZVI in the liquid phase which calculated according to *eq 2*.

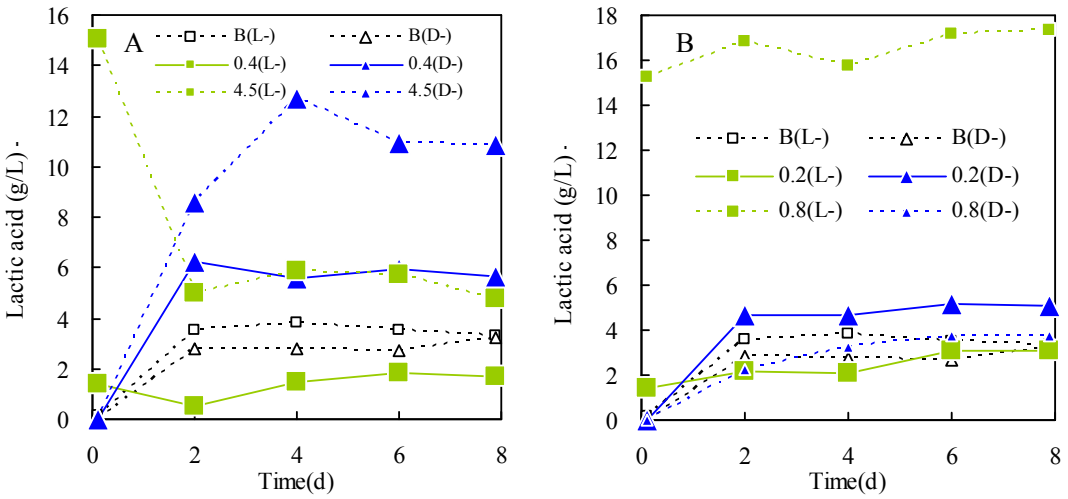

Fig. S17 - Effect of ferrous (A) and ferric (B) on isomerization of L-lactate to D-lactate. Fe(II)-0.4 and Fe(III)-0.2 were initially introduced 1.4 g/L L-lactate; Fe(II)-4.5 and Fe(III)-0.8 were initially introduced 15 g/L L-lactate.

Reference

1. Li, X.; Chen, Y.; Zhao, S.; Chen, H.; Zheng, X.; Luo, J.; Liu, Y., Efficient production of optically pure L-lactic acid from food waste at ambient temperature by regulating key enzyme activity. *Water Res* **2015,** *70*, (1), 148-157.

2. Goel, R.; Mino, T.; Satoh, H.; Matsuo, T., Enzyme activities under anaerobic and aerobic conditions inactivated sludge sequencing batch reactor. *Water Res* **1998,** *32*, (7), 2081-2088.

3. Biade, A. E.; Bourdillon, C.; Laval, J. M.; Mairesse, G.; Moiroux, J., Complete conversion of L-lactate into D-lactate. A generic approach involving enzymic catalysis, electrochemical oxidation of NADH and electrochemical reduction of pyruvate. *Journal of the American Chemical Society* **1992,** *114*, (3), 893-897.