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An Assessment of Serial Co-cultivation Approach for Generating Novel Zymomonas Mobilis Strains

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Abstract

Objective

The alphaproteobacterium *Zymomonas mobilis* is an efficient ethanol producer.Utilizing its distinctive physiological features, *Z. mobilis*-based biorefinery shows a great potential for an industrial biofuel production at large scale.Serial co-cultivation based adaptation that promotes species-interaction has been an emerging approach to improve or rewire metabolic features in industrially useful microorganisms by inducing frequent mutations. We applied this method to assess if adaptation to long term co-culture improves or rewire the desirable physiological features of *Z. mobilis*.

Results

We have performed serial co-culture of *Z. mobilis* mixed with the baker yeast *Saccharomyces cerevisiae*. We observed filamentation of *Z. mobilis* cell in the co-culture, indicating that *Z. mobilis* cells were stressed due to the presence of competitor and that there appeared to be a selective pressure. After 50 times of serial transfers, we characterized the generated *Z. mobilis* strains. The analysis showed that long term co-culture did not drive significant changes in growth or excreted metabolites profile of generated strains. In line with this, whole genome sequencing of the generated *Z. mobilis* strains revealed only minor genetic variations from parental strain. The result indicates that co-culture method should be carefully optimized for *Z. mobilis* strain improvement.

Introduction

The alphaproteobacterium *Z. mobilis* is the best bacterial ethanol producer endowed with unique physiological features [1]. Therefore, *Z. mobilis* based-biorefinery is a promising biofuel production system at an industrial scale [2]. Despite of its efficient ethanol production capacity, there are remaining challenges in employing *Z. mobilis* as biocatalyst. For example, *Z. mobilis* consumes limited range of feedstock as substrate [2]. It is also known to be sensitive to certain abiotic stress [2, 3]. Yet, recent advances in *Z. mobilis* metabolic engineering has been overcoming these drawbacks and advancing its potential use for attaining environmentally-friendly biorefinery [2, 3].

In addition to ethanol production, rewiring metabolic pathways to produce other useful compounds has been lately explored in *Z. mobilis* [3–7]. This is to utilize and exploit its intrinsic capacity of fast catabolism that comes with small biomass accumulation [1]. Considering that its prolific potential is expanding, novel approach to engineer or generate desirable *Z. mobilis* strains should be assessed.

Recently, co-culture based adaptive evolution, mixing several species in same culture to stimulate inter-species interaction, has been shown as a novel approach for improving physiological feature of industrially beneficial microorganisms by inducing frequent mutations [8]. For example, serial co-culture of *Candida glabrata* and *Pichia kudriavzevii* significantly influenced growth and fermentation profile of co-evolved strains [9]. The evolved strains conferred altered chemical complexity in produced wine [9].

Zhou et al. showed that long term bacterial-yeast competition induced chromosomal arrangements in the yeast, rendering stress-tolerance, altered metabolism and other physiological features in the yeast *Lachancea kluyveri* [10]. In addition to promoting mutation, co-culture has been also shown to promote production of particular metabolites in *Streptomyces* species, which otherwise not expressed in pure monoculture [11, 12]. Thus, inter-species interaction stimulates expression of cryptic genes. [11–13]. Such a response might also bias mutational events if the cryptic genes were continuously expressed during serial co-culture.

In addition to gaining potentially desirable traits, another advantage of the serial co-culture based adaptation method is to shed light on understanding of basic ecological aspect of species interaction [14, 15]. Given that *Z. mobilis* ecology and its natural habitat is yet rather enigmatic [16], the approach should be worth being examined in this regard as well.

In the present work, we adopted serial co-culture of *Z. mobilis* mixed with baker yeast *Saccharomyces cerevisiae* for strain generation. The aim of study was to see if *Z. mobilis* changes or rewire its ethanologenic feature through competition and interaction with *S. cerevisiae* over serial co-cultivation.

Methods

Cultivation

Z. mobilis Zm6 and *S. cerevisiae* strain CEN.PK113-7D strain were used for adaptive evolution by co-culture. Cells were grown in growth medium containing glucose (20 g/L or 100 g/L), yeast extract (5 g/L), NH_4SO_4 (1 g/L), KH_2PO_4 (1 g/L) and MgSO (0.5 g/L). The growth medium was flushed by nitrogen gas prior to cultivation. The co-culture was grown at 30 °C in a tightly capped test tube with shaking at 200 rpm throughout the study.

For serial co-culture, 10 µL of Zm6 overnight anaerobic culture and *S. cerevisiae* anaerobic culture were used as starters. The mixed cultures typically spent all nutrients within a day. The fully grown cultures (10 µL) were transferred to fresh identical medium next day and the co-culture continued. After the transfer was repeated for 5 times, we observed that *Z. mobilis* competes out in the co-culture under the condition. To maintain balanced co-culture, we inoculated additional *S. cerevisiae* overnight culture 40 µL, in addition to 10 µL of previous co-culture, upon each transfer from 6th round. This gave a good balance for continuing co-culture.

For *Escherichia. coli* co-culture with *Z. mobilis*, *E. coli* strain K12 grown in LB medium under aerobic condition was used as starter. We started co-culture with mixing 10 µL of fully grown monocultures of *Z. mobilis* and *E. coli*. During the experiments, we learned that inoculating 10 µL of fully grown co-culture of previous round with 20 µL of fully grown *E. coli* monoculture upon a transfer gave a good balance for continuation of co-cultures, which we performed for all transfer. All lines were replicated for whole passages.

After the serial co-culture of 50 transfers, cells were streaked out on the solid identical medium. Isolated pure strains are designated as follows, Zs100: *Z. mobilis* Zm6 derived strain obtained from last round of *Z. mobilis* vs *S. cerevisiae* serial co-culture supplemented with glucose 100 g/L. Zs100R is obtained from a parallel replicate of *Z. mobilis* vs *S. cerevisiae* glucose 100 g/L. Zs20: Zm6 derived strain obtained from the last round of serial co-culture with *S. cerevisiae* supplemented with 20 g/L glucose, and Zs20R was obtained from a last culture of parallel run of Zs20R. Similarly, Ze20 designates *Z. mobilis* strain obtained from last round of *Z. mobilis* vs *E. coli* co-culture with 20 g/L glucose, and Ze20R as a parallel replicate.

Characterization of growth and ethanol production

Growth profiles of all strains were analyzed using flat bottom 96-well microplate in plate-reader spark 20M (Tecan) by measuring its absorbance at 600 nm. Overnight anaerobic monoculture was used as an inoculum. Temperature control was set at 30 °C. Three technical replicates were repeated for each time points. The used medium in plate reader was same as for co-culture, with supplement of glucose 20 g/L. We also measured growth profiles in test tubes under anaerobic condition using spectrophotometer (VWR), showing similar trends from microplate experiments.

Acetate, lactate, ethanol and glucose in the overnight monoculture of generated *Z. mobilis* strains were measured using Waters 2695e Alliance HPLC (Waters) with Hi-plex column (300 × 7.7 mm, Agilent). The spent medium of anaerobic overnight culture in the test tube was analyzed for HPLC analysis. The collected supernatant was filtered through 0.2 µm Supor® polyether sulfone membrane (PALL) before the analysis. HPLC analysis was run under the condition; 0.05 M sulfuric acid as mobile phase at a flow rate of 0.8 mL/minute. External standard curve was used for converting obtained peaks to concentration of analytes.

Microscopy

Growing sample was directly mounted on Phosphate-buffered saline (PBS)-agarose pad before imaging. Zeiss Axio Imager Z2 microscope (ZEISS) equipped with camera Axiocam MR R3 (ZEISS) was used for capturing phase contrast images. Software ZEN 3.1 (ZEISS) was used for image analysis.

Whole genome sequencing

Total DNA was extracted by combining a lysozyme treatment [17] and D-neasy blood tissue kit (Qiagen). Whole genome of the co-cultured *Z. mobilis* strains was sequenced by GATC re-sequencing service (INVIEW Genome sequencing). The reference sequence was obtained from NCBI [18].

Results

In order to generate novel *Z. mobilis* strains, we assessed the co-culturing based adaptive evolution. We chose serial transfer instead of continuous chemostat co-culture, for the simplicity of method, and baker yeast *S. cerevisiae* as a competitor since it also produces ethanol and likely share an ecological niche with *Z. mobilis*. During the pilot experiment, we observed that many *Z. mobilis* cells showed abnormal cell

shape in the co-culture with *S. cerevisiae* supplemented with 100 g/L glucose. (Fig. 1). Most of *Z. mobilis* cells formed filamentous structures which can be up to 10 times longer than regular cell (Fig. 1, shown as C in the lower-right panel). Some of the *Z. mobilis* cells were burst or exhibiting a membrane protrusion from cell (Fig. 1, shown as A in lower-right panel) and several cells were found dead as shown by lighter phase contrast (Fig. 1B). Dead *S. cerevisiae* cells were also observed (Fig. 1, shown as D in lower-right panel). Bacterial filamentation is often an adaption to stress [19] Thus, we found the observed phenomenon as a good indication that the Zm6 cells were interacting and competing with *S. cerevisiae*. This prompted us to expect that frequent mutations should arise in the *Z. mobilis* cells to gain advantages for the competition during serial co-culture.

We then performed serial transfer of each mixed culture for 50 passages. For co-culture with *S. cerevisiae*, we made two lines of culture supplemented with 20 g/L or 100 g/L glucose. For *E. coli* co-culture, we performed cultivation only with 20 g/L glucose. All cultures were run with parallel replicate, resulting in 6 lines of serial co-cultures.

After 50 transfers of serial co-cultures, we characterized growth profile and ethanol production by monoculture of obtained *Z. mobilis* strains from last batch of long-term co-cultures. It was to be noted that the elongated morphology observed during serial co-culture was not retained in *Z. mobilis* cells grown in monoculture, suggesting that filamentation was due to stress. The HPLC and growth curve data showed that excreted production and growth profiles by obtained strains were nearly identical to those by parental strain (Fig. 2). This was somewhat contrary to what we anticipated, since Zs100 cells underwent long cultivation under constant stress, which could have influenced its metabolism or growth profile for better survival. This result is in s sharp contrast to our previous experience with *Z. mobilis* adaptive evolution against abiotic stress, in which 10–20 passages of serial transfer drove strains to gain adapted phenotype [20]. The obtained result here showed that the competition with yeast in the co-culture did not influence *Z. mobilis* ethanologenic physiology.

Next, to see if there are mutations not reflected in the measured phenotypes, we sequenced whole genome of the obtained *Z. mobilis* strains. The analysis revealed that several mutations in the ORF regions of the generated strains, comparing to the reference genome [18]. Yet, it was not clear how the mutations could relate to advantage in the species interaction. Interestingly, *Z. mobilis* strain Zs100R did not show any mutation in the ORF regions, despite of long-term constant pressure from *S. cerevisiae*. This suggests that genomic stability was very high in *Z. mobilis* during the serial co-culture.

Discussion

Despite that co-culture based adaptive evolution has been powerful tool for several bacterial and yeast species, we recommend that the method should be carefully fine-tuned for *Z. mobilis*. Our data suggest that serial co-culture method utilizing species-interaction was not optimally done to drive mutation in our experimental setting, or, *Z. mobilis* genomic stability was very stable during species interaction. *Z. mobilis* was previously shown to possess polyploidy [21, 22], and that could render genetic stability during stress

response by frequent homologous recombination. Interestingly, recent report suggests that *Z. mobilis* is not much involved in pair-wise cross feeding with other bacteria [23], implying that *Z. mobilis* does not cooperate with other microbes for its survival.

Table 1. A table of list of mutations found in generated *Z. mobilis* strains. NA stands for not available.

Strain	Position	Referencee	Mutation	Gene	Annotation
Zs100	87016	GA	G	ZZ6_0076	hypothetical protein
Zs100R	NA		NA		
Zs20	NA		NA		
Zs20R	114095	C	T	ZZ6_0103	hypothetical protein
Ze20	1195056	C	T	ZZ6_1040	TonB-dependent receptor
Ze20R	1185646	A	AGGCTCAG	ZZ6_1032	heptose-I-phosphate ethanolaminephosphotransferase

Limitation

Apart from growth curves and excreted metabolites profiles, there might be hidden phenotypes in generated strains that we did not detect.

Abbreviations

HPLC
high-pressure liquid chromatography
OD
Optical Density
ORF
Open Reading Frame

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets and strains generated from the present work are available from the corresponding author upon on a reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KF conceptualized and designed the study, conducted the whole experiments, interpreted and analyzed the data, and wrote and edited the draft manuscript. PB supervised the study and experiments, interpreted and analyzed the data, and edited the draft manuscript. All authors read and approved the final manuscript.

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References

1. Kalnenieks U: **Physiology of Zymomonas mobilis**: **Some Unanswered Questions**. In: *Advances in Microbial Physiology.* Edited by Poole RK, vol. 51: Academic Press; 2006: 73–117.
2. He MX, Wu B, Qin H, Ruan ZY, Tan FR, Wang JL, Shui ZX, Dai LC, Zhu QL, Pan K, et al. Zymomonas mobilis: a novel platform for future biorefineries. Biotechnol Biofuels. 2014;7(1):101.
3. Wang X, He Q, Yang Y, Wang J, Haning K, Hu Y, Wu B, He M, Zhang Y, Bao J, et al. Advances and prospects in metabolic engineering of Zymomonas mobilis. Metab Eng. 2018;50:57–73.
4. Yang S, Mohagheghi A, Franden MA, Chou Y-C, Chen X, Dowe N, Himmel ME, Zhang M: **Metabolic engineering of Zymomonas mobilis for 2,3-butanediol production from lignocellulosic biomass sugars**. *Biotechnology for Biofuels* 2016, **9**(1):189.
5. Qiu M, Shen W, Yan X, He Q, Cai D, Chen S, Wei H, Knoshaug EP, Zhang M, Himmel ME, et al. Metabolic engineering of Zymomonas mobilis for anaerobic isobutanol production. Biotechnol Biofuels. 2020;13(1):15.
6. Kalnenieks U, Balodite E, Strähler S, Strazdina I, Rex J, Pentjuss A, Fuchino K, Bruheim P, Rutkis R, Pappas KM, et al: **Improvement of Acetaldehyde Production in Zymomonas mobilis by Engineering**

of Its Aerobic Metabolism. *Frontiers in Microbiology* 2019, 10(2533).

7. Yang S, Fei Q, Zhang Y, Contreras LM, Utturkar SM, Brown SD, Himmel ME, Zhang M. Zymomonas mobilis as a model system for production of biofuels and biochemicals. Microb Biotechnol. 2016;9(6):699–717.
8. Swamy KBS, Zhou N. Experimental evolution: its principles and applications in developing stress-tolerant yeasts. Appl Microbiol Biotechnol. 2019;103(5):2067–77.
9. Morrison-Whittle P, Lee SA, Fedrizzi B, Goddard MR. **Co-evolution as Tool for Diversifying Flavor and Aroma Profiles of Wines**. *Frontiers in Microbiology* 2018, 9(910).
10. Zhou N, Bottagisi S, Katz M, Schacherer J, Friedrich A, Gojkovic Z, Swamy KBS, Knecht W, Compagno C, Piškur J. **Yeast–bacteria competition induced new metabolic traits through large-scale genomic rearrangements in Lachancea kluyveri**. *FEMS Yeast Research* 2017, 17(6).
11. Pishchany G, Mevers E, Ndousse-Fetter S, Horvath DJ, Paludo CR, Silva-Junior EA, Koren S, Skaar EP, Clardy J, Kolter R: **Amycomicin is a potent and specific antibiotic discovered with a targeted interaction screen**. *Proceedings of the National Academy of Sciences* 2018, **115**(40):10124.
12. Traxler MF, Kolter R. Natural products in soil microbe interactions and evolution. Nat Prod Rep. 2015;32(7):956–70.
13. Okada BK, Seyedsayamdost MR. Antibiotic dialogues: induction of silent biosynthetic gene clusters by exogenous small molecules. FEMS MicroBiol Rev. 2017;41(1):19–33.
14. Conacher CG, Rossouw D, Bauer FFB. **Peer pressure: evolutionary responses to biotic pressures in wine yeasts**. *FEMS Yeast Research* 2019, 19(7).
15. Lenski RE. Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. The ISME Journal. 2017;11(10):2181–94.
16. Weir PM. The ecology of Zymomonas: a review. Folia Microbiol. 2016;61(5):385–92.
17. Gill C, van de Wijgert JHHM, Blow F, Darby AC. Evaluation of Lysis Methods for the Extraction of Bacterial DNA for Analysis of the Vaginal Microbiota. PLOS ONE. 2016;11(9):e0163148.
18. Desiniotis A, Kouvelis VN, Davenport K, Bruce D, Detter C, Tapia R, Han C, Goodwin LA, Woyke T, Kyrpides NC, et al. Complete Genome Sequence of the Ethanol-Producing Zymomonas mobilis subsp. mobilis Centrotype ATCC 29191. J Bacteriol. 2012;194(21):5966.
19. Rizzo MG, De Plano LM, Franco D. **Regulation of filamentation by bacteria and its impact on the productivity of compounds in biotechnological processes**. *Applied Microbiology and Biotechnology* 2020.
20. Fuchino K, Bruheim P. **Increased salt tolerance in Zymomonas mobilis strain generated by adaptative evolution**. *Researchsquare (preprint)* 2020.
21. Skerker JM, Leon D, Price MN, Mar JS, Tarjan DR, Wetmore KM, Deutschbauer AM, Baumohl JK, Bauer S, Ibáñez AB, et al. Dissecting a complex chemical stress: chemogenomic profiling of plant hydrolysates. Mol Syst Biol. 2013;9(1):674.

22. Brenac L, Baidoo EEK, Keasling JD, Budin I. Distinct functional roles for hopanoid composition in the chemical tolerance of Zymomonas mobilis. Mol Microbiol. 2019;112(5):1564–75.
23. Pacheco AR, Moel M, Segrè D. Costless metabolic secretions as drivers of interspecies interactions in microbial ecosystems. Nat Commun. 2019;10(1):103.

Figures

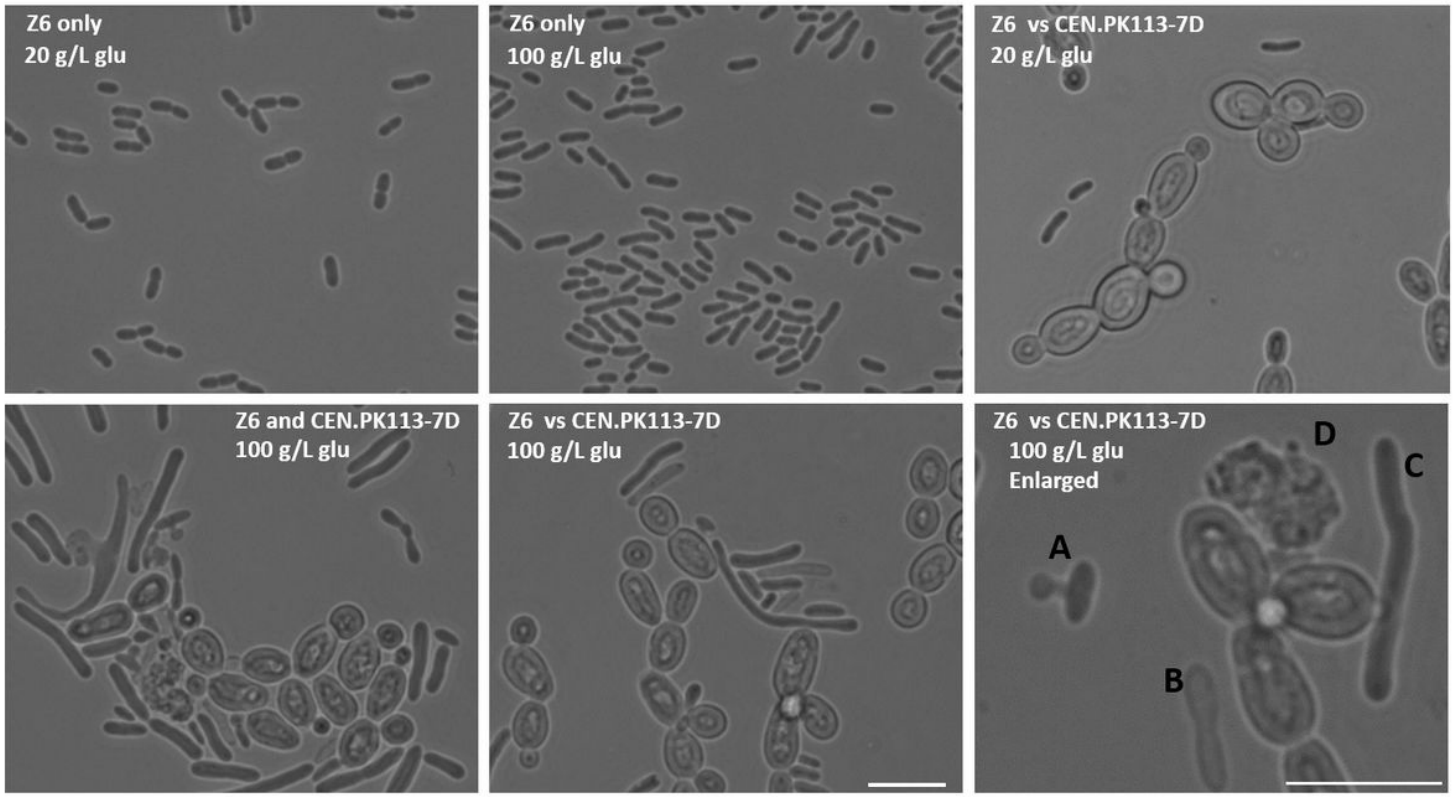

Figure 1

Phase contrast images of Z. mobilis monoculture and co-culture with S. cerevisiae. Used strains and conditions are as shown in white texts in the images. Abbreviation glu stands for glucose in the medium. It is to be noted that Z. mobilis strain in the co-culture vs yeast with 100 g/L glucose formed elongated filamentous structure, while other conditions did not induce such drastic morphological changes in Z. mobilis cells. Right-bottom panel is an enlarged image with scale bar 10 μm, while all other panel sizes are corresponding with the scale bar 10 μm, found in bottom-middle panel. Black A in the right-bottom panel indicates bursting cell with membrane protrusion from cytoplasm. B; Lysed cell exhibiting lighter phase contrast than that of live cells. C; Elongated Z. mobilis cell. D; Dead yeast cell.

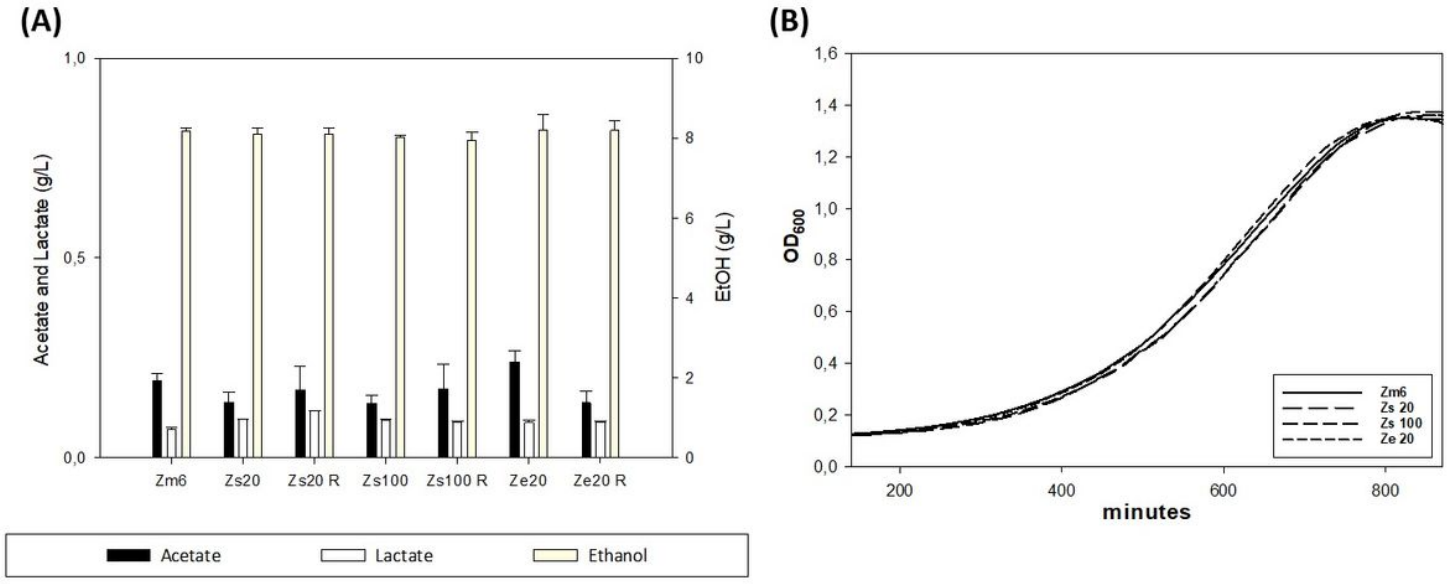

Figure 2

Characterization of strains that generated from serial co-culture-based laboratory adaptation.(A) Total production of acetate, lactate and ethanol from the overnight monoculture of the generated Z. mobilis strains and parental strain Zm6. (B) Growth profiles of monoculture Z. mobilis strain obtained in this study and parental strain Zm6. Strain information can be found in method section. Note that there was no significant difference in ethanol production and growth profiles between strains. Error bars; standard deviations from 3 independent measurements.