Supporting Information

For

From Organic Wastes to Bioplastics: Feasibility of Non-Sterile Poly(3-hydroxybutyrate) Production by Zobellella denitrificans ZD1

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Supporting Information: 8 pages total, including Text, 6 Figures, and 5 Tables

Figure S1 The profiles of (A) pH, (B) glycerol, and (C) ammonia-nitrogen for each strain growing in mineral salts medium containing 5 g/L glycerol, 7.57 mM ammonium, and 0.05% of NaCl. Error bars represent standard deviation of duplicate measurements.

Figure S2 Growth curves of Burkholderia sp. with 5 g/L glycerol and different NaCl (0.05, 1, and 3%).

Figure S3 Growth curves of Zobellella denitrificans ZD1 under (A) different initial molar C/N ratios with initial pH = 7.3, (B) different initial pH conditions, and (C) different nitrogen sources.

Figure S4 SDS-PAGE analysis of EctABC expressed in strain ZD1 and in E. coli BL21. M: Mark12 Unstained Standard; 1: supernatant of cell extracts (at exponential phase) from sample of 0% NaCl with nitrate; 2: supernatant of cell extracts (at exponential phase) from sample of 1% NaCl with nitrate (sample from Exp. r); 3: supernatant of cell extracts (exponential phase) from sample of 3% NaCl with nitrate (sample from Exp. s); 4: supernatant of cell extracts (at stationary phase) from sample of 3% NaCl with nitrate (sample from Exp. s); 5: supernatant of cell extracts (at stationary phase) from sample of 0.5% NaCl with ammonium (sample from Exp. n); 6: supernatant of cell extracts (at stationary phase) from sample of 3% NaCl with ammonium (sample from Exp. o); 7: EctA from E. coli after 0.2 mM IPTG
induction; 8: EctB from *E. coli* after 0.2 mM IPTG induction; and 9: EctC from *E. coli* after 0.2 mM IPTG induction.

**Figure S5**  A contour graph simulates the combined effects of salinity and fatty acids on PHB production (g/L) by strain ZD1 from 18 sample sets.

**Figure S6**  Growth curves of strain ZD1 in batch reactors containing (A) sterile and non-sterile synthetic crude glycerol (SCG) with high salt content, 3% NaCl and (B) high strength synthetic wastewater (HSSW). Error bars represent range of duplicate measurements.

**Table S1**  Selection of *ectA*, *ectB*, and *ectC* from *Z. denitrificans* ZD1

**Table S2**  Primer sets used in this study. All primers were designed by primer design tool for In-fusion cloning.

**Table S3**  Ability of strains to grow on agar plates containing 5 g/L glycerol, 0.5 g/L (NH₄)₂HPO₄, mineral salts, noble agar, and varies NaCl content (0.05-12 %NaCl). The plus (+) and negative (−) signs indicate growth or no growth, respectively. * colonies showed up after 3 days of incubation. ** colonies showed up after 7 days of incubation.

**Table S4**  The lag phase time (λ), the maximum growth rate (νₘ), asymptote of maximum OD₆₀₀ (A), and coefficient of determination (RSQ) fitted in Gompertz model in different NaCl and fatty acid levels. Strain ZD1 was grown in ammonium mineral salts medium containing glycerol (5 g/L) with a molar C/N of 21.5 and 0.05% or 3% (w/w) of NaCl.

**Table S5**  Solubility of 1% (w/w) fatty acids in DI water vs. 3% of NaCl medium.
Supporting Text

SDS-PAGE Analysis

The ectoine synthesis genes (ectA, ectB, and ectC) in strain ZD1 were used to construct the recombinant plasmids. The gene information is described in Table S1, and the DNA of ZD1 was extracted using the FastDNA SPIN KIT (MP Biomedicals, Santa Ana, CA). The extracted DNA was used as a template for the PCR amplification, where the PCR primers targeting these genes are listed in Table S2. A 25 μL PCR reaction mixture, which contains 12.5 μL of 2X PCR Master Mix (Promega, Madison, WI), 1 μL of 100 ng/μL DNA, 1 μL of each primer (10 μM), and 9.5 μL of nuclease-free water, was used. The PCR products were purified by the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the restriction enzyme was double-digested using NdeI and NheI (New England Biolabs, Ipswich, MA). The 20-μL digestion mixture contained 1 μL of each restriction enzyme, 2 μL of CutSmart Buffer (New England Biolabs, Ipswich, MA), 5 μL of purified PCR products, and 11 μL of nuclease-free water. The digested products were repurified and cloned into pET11a plasmids between the NdeI-NheI cloning sites with T4 DNA ligase (New England Biolabs, Ipswich, MA). The 20-μL ligation mixture of ectA, ectB, and ectC comprised 3 μL of 50 ng/μL linearized pET11a vector, 2 μL of T4 DNA ligase buffer (10X), 3.5 μL of purified digested product, 10.5 μL of nuclease-free water, and 1 μL of T4 DNA ligase. Next, it was incubated at 23 °C for 2 h and inactivated at 65 °C for 10 min. After ligation, the constructed plasmids were transformed into Escherichia coli competent cells (NEB 5-alpha), screened on LB agar with 100-mg/L ampicillin as a selective marker, and then extracted using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). To verify the recombinant plasmid sequences, the plasmid was digested with restriction enzymes (EcoRV and BamH1 or NcoI and NdeI) at 37 °C for 1 h and then separated by gel electrophoresis. The verified plasmids were then transformed into E. coli BL21 (DE3) for protein expression.

An expression of the recombinant ectA, ectB, and ectC in each transformed E. coli BL21 (DE3) by induction with 0.2 mM IPTG as a positive control was confirmed by SDS-PAGE analysis. The E. coli BL21 with a recombinant plasmid and ZD1 cultures were concentrated (OD₆₀₀ = 16) in a phosphate-buffered saline (PBS, pH 7.0) and lyzed using FastPrep lysis beads and matrix (MP Biomedicals, Santa Ana, CA). A 15 μL supernatant of lysed cultures was mixed with 15 μL 2X SDS gel loading buffer (Sigma-Aldrich, St. Louis, MO) in a 1.5-mL microcentrifuge tube. After centrifuging at 13,000 rpm for 1 min, the samples were boiled for 10 min. SDS-PAGE was conducted on Novex™ wedge well 14% Tris-glycine gel (Invitrogen, Carlsbad, CA) in XCell SureLock™ Mini-Cell electrophoresis (Novel Experimental Technology, San Diego, CA). The minicell was filled with 1X Tris-Glycine (MP Biomedicals, Santa Ana, CA) running buffer. A 10 μL of the Mark12 Unstained Standard (Invitrogen, Carlsbad, CA) and 30 μL sample mixture (15 μL dye and 15 μL sample) were loaded on the gel. The electrophoresis was run at 90V for 2.5 hr. The gel was stained with Imperial™ protein stain for 1 hr, and then destained for 2 hrs with deionized water.
Figure S1. The profiles of (A) pH, (B) glycerol, and (C) ammonia-nitrogen for each strain growing in mineral salts medium containing initially 5 g/L glycerol, 7.57 mM ammonium, and 0.05% of NaCl. Error bars represent standard deviation of duplicate measurements.

Figure S2. Growth curves of Burkholderia sp. with 5 g/L glycerol and different NaCl (0.05, 1, and 3%).
**Figure S3.** Growth curves of *Zobellella denitrificans* ZD1 under (A) different initial molar C/N ratios with initial pH = 7.3, (B) different initial pH conditions, and (C) different nitrogen sources.

**Figure S4.** SDS-PAGE analysis of EctABC expressed in strain ZD1 and in *E. coli* BL21. M: Mark12 Unstained Standard; 1: supernatant of lysed cells (at exponential phase) from sample of 0% NaCl with nitrate; 2: supernatant of lysed cells (at exponential phase) from sample of 1% NaCl with nitrate (sample from Exp. r); 3: supernatant of lysed cell (exponential phase) from sample of 3% NaCl with nitrate (sample from Exp. s); 4: supernatant of lysed cells (at stationary phase) from sample of 3% NaCl with nitrate (sample from Exp. s); 5: supernatant of lysed cells (at stationary phase) from sample of 0.5% NaCl with ammonium (sample from Exp. n); 6: supernatant of lysed cells (at stationary phase) from sample of 3% NaCl with ammonium (sample from Exp. o); 7: EctA from *E. coli* after 0.2 mM IPTG induction; 8: EctB from *E. coli* after 0.2 mM IPTG induction; and 9: EctC from *E. coli* after 0.2 mM IPTG induction.
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Table S1. Selection of *ectA*, *ectB*, and *ectC* from *Z. denitrificans* ZD1

| Gene | Enzyme                               | Size (kB) | Size (AA) | MW (kDa) |
|------|---------------------------------------|-----------|-----------|----------|
| *ectA* | Diaminobutyrate acetyltransferase | 0.5       | 170       | 19       |
| *ectB* | Diaminobutyrate-2-oxoglutarate transaminase | 1.3       | 423       | 45       |
| *ectC* | L-ectoine synthase                   | 0.4       | 135       | 15       |

Table S2. Primer sets used in this study. All primers were designed by primer design tool for In-fusion cloning.

| Primer Name | Sequence (5’ -> 3’) | Primer Length (bp) | Melting Temperature (°C) |
|-------------|---------------------|--------------------|--------------------------|
| *ectA* _FW* | TAAGGACATATGATGGACACGGATACC ACACTAGAA | 36 | 61.9 |
| *ectA* _RV* | TTATTTGCTAGCTCAGGAGGGATGC | 29 | 65.1 |
| *ectB* _FW* | TAAGGCCATATGATGAGTATTTTTTGATG TAATGGAATCCAGTG | 43 | 61.6 |
| *ectB* _RV* | TTATTTGCTAGCTCAGGAGGGCTTGCCTC AGC | 31 | 64.6 |
| *ectC* _FW* | TAAGGACATATGATGATCGTACGTACTCTGCCGCC | 34 | 62.4 |
| *ectC* _RV* | TTATTTGCTAGCTCAGCCTCCGCCCTTCC | 31 | 64.4 |

Table S3. Ability of strains to grow on agar plates containing 5 g/L glycerol, 0.5 g/L (NH₄)₂HPO₄, mineral salts, noble agar, and varies NaCl content (0.05-12 %NaCl). The plus (+) and negative (−) signs indicate growth or no growth, respectively. * colonies showed up after 3 days of incubation. ** colonies showed up after 7 days of incubation.

| %NaCl | *Z. denitrificans* ZD1 | *Burkholderia sp.* | *P. oleovorans* | *A. lata* |
|-------|------------------------|-------------------|-----------------|-----------|
| 0.05  | +                      | +                 | +               | -         |
| 1     | +*                    | +**               | +**             | No data   |
| 3     | +                     | -                 | -               | No data   |
| 5     | +                     | -                 | -               | No data   |
| 12    | -                     | -                 | -               | No data   |
Table S4. The lag phase time (\(\lambda\)), the maximum growth rate (\(v_m\)), asymptote of maximum OD\(_{600}\) (A), and coefficient of determination (RSQ) fitted in Gompertz model in different NaCl and fatty acid levels. Strain ZD1 was grown in ammonium mineral salts medium containing glycerol (5 g/L) with a molar C/N of 21.5 and 0.05% or 3% (w/w) of NaCl.

| %NaCl | Fatty Acid Levels\(^b\) | Gompertz Model | DCW (g/L) | %PHB\(^e\) | Doubling time, \(t_d\) (h)\(^f\) |
|-------|--------------------------|-----------------|-----------|-------------|------------------|
| 0.05% | No fatty acids           | 7.33 0.23 2.64 0.999** 0.98 ± 0.15 84 ± 4.7 2 | | | |
|       | 0.5% S                   | 3.94 0.15 2.53 0.995* 1.02 ± 0.16 79 ± 7.3 6 | | | |
|       | 0.5% L                   | 6.03 0.22 2.34 0.999* 0.97 ± 0.14 83 ± 5.2 5.8 | | | |
|       | 1% S                     | 7.86 0.26 2.41 0.997* 0.83 ± 0.07 80 ± 4.5 4.6 | | | |
|       | 1% O                     | 22.59 0.87 1.94 0.966* 0.70 ± 0.03 71 ± 3.0 3 | | | |
|       | 1% L                     | 26.12 0.26 1.99 0.983* 0.68 ± 0.03 71 ± 6.9 5.1 | | | |
| 3%    | No fatty acids           | 15.52 0.50 5.06 0.997** 1.61 ± 0.02 51 ± 2.3 3.2 | | | |
|       | 1% S                     | 16.47 0.43 5.58 0.999** 1.88 ± 0.02 46 ± 3.4 6.8 | | | |
|       | 1% O                     | 17.17 0.48 5.40 0.996** 1.91 ± 0.01 55 ± 2.3 4.6 | | | |
|       | 1% L                     | 15.82 0.43 6.36 0.999** 1.97 ± 0.45 46 ± 4.2 3.4 | | | |
|       | 1% S and O\(^g\)         | 18.87 0.51 5.32 0.998** 2.08 ± 0.48 48 ± 2.7 7.2 | | | |
|       | 1% S, O, and L\(^g\)     | 7.06 0.19 5.35 0.997** 1.68 ± 0.72 39 ± 3.6 7.1 | | | |

\(^a\) %NaCl was calculated based on the weight of NaCl to the weight of water
\(^b\) The fatty acid level of stearate (S), oleate (O), and linoleate (L) is based on weight of fatty acid to the weight of glycerol.
\(^c\) The maximum OD\(_{600}\) obtained in this model is normalized with initial OD\(_{600}\) of each experiment as a baseline.
\(^d\) Adjusted RSQ \((R^2\text{ that considers degree of freedom})\) was applied to represent the goodness of fit between observed OD\(_{600}\) and model-predicted results. Asterisk mark represents the significance of the regression model based on an F-test (* p-value < 0.05 and ** p-value < 0.001).
\(^e\) The percent of PHB content in their DCW after reaching the stationary growth.
\(^f\) Represents the doubling time
\(^g\) The mixture of fatty acids contained 1% of each fatty acid in glycerol.

Table S5. The solubility of 1% (w/w) fatty acids in DI water vs. 3% of NaCl medium.

| Fatty Acid Levels | Theoretical COD (mg/L) | Actual COD (mg/L) | %Solubility |
|-------------------|------------------------|-------------------|-------------|
|                   |                        | DI Water | 3% NaCl     |             |
| 1% Stearate (S)   | 584                    | 476.3 ± 33.4 | 45.1 ± 4.5  | 10          |
| 1% Oleate (O)     | 578                    | 486.7 ± 1.8  | 248.3 ± 42.8| 51          |
| 1% Linoleate (L)  | 570                    | 313.7 ± 47.4 | 117.6 ± 29.3| 37.5        |
| 1%S+1%O+1%L       | 1732                   | 1337.7 ± 53.4 | 450.7 ± 4.5 | 33.7        |