***In silico* Analysis of Synthetic Multispecies Biofilms for Cellobiose-to-Isobutanol Conversion Reveals Design Principles for Stable and Productive Communities**

**Table S1. Genome-scale metabolic properties of each strain model**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Strain** | **Number of genes** | **Number of metabolites** | **Number of reactions** | **ATP Maintenance (mmol/gDW/h)** | **Reference** |
| *R. champanellensis* | 486 | 862 | 893 | 5 | [www.vmh.life](http://www.vmh.life) |
| *C. lentocellum* | 777 | 1086 | 1239 | 10 | [www.vmh.life](http://www.vmh.life) |
| *E. coli*-ib | 1261 | 1672 | 2387 | 8.39 | [1-3] |
| *E. coli*-ac | 1261 | 1668 | 2382 | 8.39 | [1, 3, 4] |
| *G. metallireducens* | 987 | 1109 | 1285 | 0.81 | [5] |

**Table S2. Nutrient requirements for each modeled strain**

|  |  |  |  |
| --- | --- | --- | --- |
| **Strain** | **Consumed nutrients** | **Amino acid status** | **Oxygen status** |
| *R. champanellensis* | cellobiose, glucose, alanine, cysteine, isoleucine, leucine, methionine and valine | essential | anaerobe |
| *C. lentocellum* | cellobiose, glucose, alanine, cysteine and glutamine | essential | anaerobe |
| *E. coli-ib* | glucose, oxygen, alanine, cysteine, glutamine isoleucine, leucine, methionine and valine | not essential | aerobe |
| *E. coli-ac* | acetate, oxygen, alanine, cysteine, glutamine, isoleucine, leucine, methionine and valine | not essential | aerobe |
| *G. metallireducens* | acetate, formate, ethanol, fe(III), isoleucine, leucine and valine | not essential | anaerobe |

We considered biofilm thicknesses of 200-300 m based on experimental observations [4] and cellobiose supply concentrations in range as 20-40 mmol/L. The glucose and oxygen diffusion coefficients within biofilms were obtained our previous work [3] based on experimental observations. Complete sets of parameter values are provided in Table S3 and S4.

**Table S3. Nominal biofilm model parameter values**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Symbol** | **Description** | | **Value** | **Source** |
| *L* | Biofilm thickness | | 250 m | Specified |
| *Xb,i* | Bulk planktonic biomass concentrations | All strains | 0 g/L | Specified |
| *Cb, O2*(0) | Supplied O2 concentration at top of biofilm | | 0.21 mmol/L | [6] |
| *Cb,Clb*(*L*) | Supplied nutrient concentration at the bottom of biofilm | Cellobiose | 30 mmol/L | Adapted from [4] |
| *Cb,Fe(III)*(*L*) | Iron | 5 mmol/L | [3] |
| *Cb,AA*(*L*) | Total amino acids | 4 mmol/L | Adapted from [7] |
| *Di* | Biomass diffusion coefficient | All strains | 10-9 cm2/s | [3] |
| *kd* | Cell death rate | *Rc*, *Cl*, *Gm* | 0.03() hr- | Specified |
| *Ec*-ib, *Ec*-ac | 0.03 hr-1 |
| *Dk* | Diffusion coefficients of metabolites | Cellobiose | 5.88x10-7 cm2/s | Adapted from [8] |
| Glucose | 8.04x10-7 cm2/s | Adapted from [8] |
| Alanine | 10.9x10-7 cm2/s | Adapted from [8] |
| Cysteine | 9.36x10-7 cm2/s | Adapted from [8] |
| Glutamine | 8.52x10-7 cm2/s | Adapted from [8] |
| Isoleucine | 9x10-7 cm2/s | Adapted from [8] |
| Leucine | 8.76x10-7 cm2/s | Adapted from [8] |
| Methionine | 8.44x10-7 cm2/s | Adapted from [8] |
| Valine | 9.96x10-7 cm2/s | Adapted from [8] |
| Acetate | 2.78x10-6 cm2/s | Adapted from [8] |
| O2 | 2.90x10-5 cm2/s | Adapted from [8] |
| Iron | 4.5x10-6 cm2/s | [3] |
| Ethanol | 4.14x10-6 cm2/s | Adapted from [8] |
| Formate | 4.23x10-6 cm2/s | Adapted from [8] |
| Isobutanol | 3.27x10-6 cm2/s | Adapted from [8] |
| *,* | Mass transfer coefficients of metabolites | Glucose | 5x10-5 cm/s | [3] |
| Cellobiose, Alanine, Cysteine, Glutamine, Isoleucine, Leucine, Methionine, Valine | 5x10-5 cm/s | Specified |
| O2, Iron | 1 cm/s | [3] |
| Acetate, Ethanol, Formate, Isobutanol | 2x10-6 cm/s | Specified |
| *Xi*(*z*,0) | Initial biomass concentrations | All strains | 1 g/L | [3] |
| *v1j,max* | *Rc* maximum uptake rates for metabolites | Glucose | 10 mmol/gDW/h | [9] |
| Cellobiose | 5 mmol/gDW/h | Half of glucose |
| Alanine, Cysteine, Isoleucine, Leucine, Methionine, Valine | 1 mmol/gDW/h | Adapted from [7, 9] |
| Acetate, O2,Ethanol, Formate, Isobutanol | 0 mmol/gDW/h | Specified |
| *V2j,max* | *Cl* maximum uptake rates for metabolites | Glucose | 10 mmol/gDW/h | [9] |
| Cellobiose | 5 mmol/gDW/h | Half of glucose |
| Cysteine, Glutamine, Alanine | 1 mmol/gDW/h | Adapted from [7, 9] |
| Acetate, O2,Ethanol, Formate, Isobutanol | 0 mmol/gDW/h | Specified |
| *v3j,max* | *Ec-ib* maximum uptake rates | Glucose | 10 mmol/gDW/h | [9] |
| Alanine, Cysteine, Glutamine, Isoleucine, Leucine, Methionine, Valine | 1 mmol/gDW/h | Adapted from [7, 9] |
| O2 | 7.5 mmol/gDW/h | [3] |
| Cellobiose, Acetate, Ethanol, Formate, Isobutanol | 0 mmol/gDW/h | Specified |
| *v4j,max* | *Ec-ac* maximum uptake rates | Acetate | 10 mmol/gDW/h | [3] |
| Alanine, Cysteine, Glutamine, Isoleucine, Leucine, Methionine, Valine | 1 mmol/gDW/h | Adapted from [7, 9] |
| O2 | 8.5 mmol/gDW/h | [3] |
| Cellobiose, Glucose Ethanol, Formate, Isobutanol | 0 mmol/gDW/h | Specified |
| *v5j,max* | *G. metallireducens* maximum uptake rates | Acetate | 20 mmol/gDW/h | [10] |
| Isoleucine, Leucine, Valine | 1 mmol/gDW/h | Adapted from [7, 9] |
| Iron | 100 mmol/gDW/h | [10] |
| Formate | 10 mmol/gDW/h | [5] |
| Ethanol | 10 mmol/gDW/h | Specified |
| Cellobiose, Glucose, O2, Isobutanol | 0 mmol/gDW/h | Specified |
| *Kij* | Michaelis-Menten constants for uptake of metabolites | Glucose | 0.5 mmol/L | [9] |
| All other metabolites | 0.5 mmol/L | Adapted from [9] |
| O2 | 0.003 mmol/L | [9] |
| *C1k,max* | *R. champanellensis* uptake inhibition by metabolites | Acetate | 80 mmol/L | [4] |
| O2 | 0.125 mmol/L | Specified |
| Ethanol | 600 mmol/L | [11] |
| Formate | 400 mmol/L | [12] |
| Isobutanol | 135 mmol/L | [13] |
| All other metabolites | 106 mmol/L | Specified |
| *C2k,max* | *C. lentocellum* uptake inhibition by metabolites | Acetate | 80 mmol/L | [4] |
| O2 | 0.125 mmol/L | Specified |
| Ethanol | 600 mmol/L | [11] |
| Formate | 400 mmol/L | [12] |
| Isobutanol | 135 mmol/L | [13] |
| All other metabolites | 106 mmol/L | Specified |
| *C3k,max* | *E. coli-ib* uptake inhibition by metabolited | Acetate | 80 mmol/L | Specified |
| Ethanol | 600 mmol/L | [11] |
| Formate | 400 mmol/L | [12] |
| Isobutanol | 135 mmol/L | [13] |
| All other metabolites | 106 mmol/L | Specified |
| *C4k,max* | *E. coli-ac* uptake inhibition by metabolites | Ethanol | 600 mmol/L | [11] |
| Formate | 400 mmol/L | [12] |
| Isobutanol | 135 mmol/L | [13] |
| All other metabolites | 106 mmol/L | Specified |
| *C5k,max* | *G. metallireducens* uptake inhibition by metabolites | O2 | 0.125 mmol/L | Specified |
| All other metabolites | 106 mmol/L | Specified |

**Table S4. Enzyme synthesis and cellobiose degradation model parameter values**

|  |  |  |  |
| --- | --- | --- | --- |
| **Symbol** | **Description** | **Value** | **Source** |
|  | Basal enzyme synthesis rate | 0.05 | Specified |
|  | Maximum rate of induced synthesis | 0.005 hr-1 | [14] |
|  | Saturation constant for induction | 0.02 g/L | [14] |
|  | Inhibition constant of glucose on induced beta glucosidase synthesis | 0.07 g/L | [15] |
|  | Enzyme degradation rate constant | 0.3 hr-1 | Specified |
| Kp | Enzyme penalty constant | 0.1 | Specified |

We performed biofilm simulations with DFBAlab [16] that uses lexicographic optimization to overcome the problem of possible alternative optima in the LP problems, achieving unique exchange fluxes and establishing a well-defined dynamic model. Following our previous research [3, 7], we specified the lexicographic optimization objectives as shown in Table S4-S7 for the four system designs. The four triculture biofilm designs were numbered follows,

1. *R. champanellensis (Rc)*, *E. coli-ib (Ec*-ib*)* and *E. coli-ac (Ec*-ac*)*
2. *Rc, Ec*-ib and *G. metallireducens (Gm)*
3. *C. lentocellum (Cl), Ec*-ib and *Ec*-ac
4. *Cl, Ec*-iband *Gm*

**Table S5. Lexicographic optimization objectives for community system design 1.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Rank** | **Flux** | ***R. champanellensis*** | | ***E. coli*-ib** | | ***E.coli*-ac** | |
| **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** |
| 1 | Growth rate |  | Maximize |  | Maximize |  | Maximize |
| 2 | Acetate | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 3 | Ethanol | secretion | Maximize | secretion | Maximize | NA | Maximize |
| 4 | Formate | secretion | Maximize | secretion | Maximize | NA | Maximize |
| 5 | Isobutanol | NA | Maximize | secretion | Maximize | NA | Maximize |
| 6 | Cellobiose | uptake | Maximize | NA | Maximize | NA | Maximize |
| 7 | Glucose | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 8 | Alanine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 9 | Cysteine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 10 | Isoleucine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 11 | Leucine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 2 | Methionine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 13 | Valine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 14 | Oxygen | NA | Maximize | uptake | Maximize | uptake | Maximize |

**Table S6. Lexicographic optimization objectives for community system design 2.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Rank** | **Flux** | ***R. champanellensis*** | | ***E. coli*-ib** | | ***G. metallireducens*** | |
| **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** |
| 1 | Growth rate |  | Maximize |  | Maximize |  | Maximize |
| 2 | Acetate | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 3 | Ethanol | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 4 | Formate | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 5 | Isobutanol | NA | Maximize | secretion | Maximize | NA | Maximize |
| 6 | Cellobiose | uptake | Maximize | NA | Maximize | NA | Maximize |
| 7 | Glucose | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 8 | Alanine | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 9 | Cysteine | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 10 | Isoleucine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 11 | Leucine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 12 | Methionine | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 13 | Valine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 14 | Oxygen | NA | Maximize | uptake | Maximize | NA | Maximize |
| 15 | Iron | NA | Maximize | NA | Maximize | uptake | maximize |

**Table S7. Lexicographic optimization objectives for community system design 3.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Rank** | **Flux** | ***C. lentocellum*** | | ***E. coli*-ib** | | ***E.coli-ac*** | |
| **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** |
| 1 | Growth rate |  | Maximize |  | Maximize |  | Maximize |
| 2 | Acetate | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 3 | Ethanol | secretion | Maximize | secretion | Maximize | NA | Maximize |
| 4 | Formate | secretion | Maximize | secretion | Maximize | NA | Maximize |
| 5 | Isobutanol | NA | Maximize | secretion | Maximize | NA | Maximize |
| 6 | Cellobiose | uptake | Maximize | NA | Maximize | NA | Maximize |
| 7 | Glucose | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 8 | Cysteine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 9 | Glutamine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 10 | Alanine | uptake | Maximize | uptake | Maximize | uptake | Maximize |
| 11 | Oxygen | NA | Maximize | uptake | Maximize | uptake | Maximize |

**Table S8. Lexicographic optimization objectives for community system design 4.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Rank** | **Flux** | ***C. lentocellum*** | | ***E. coli*-ib** | | ***G. metallireducens*** | |
| **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** | **Uptake/**  **secretion** | **Direction** |
| 1 | Growth rate |  | Maximize |  | Maximize |  | Maximize |
| 2 | Acetate | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 3 | Ethanol | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 4 | Formate | secretion | Maximize | secretion | Maximize | uptake | Maximize |
| 5 | Isobutanol | NA | Maximize | secretion | Maximize | NA | Maximize |
| 6 | Cellobiose | uptake | Maximize | NA | Maximize | NA | Maximize |
| 7 | Glucose | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 8 | Cysteine | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 9 | Glutamine | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 10 | Alanine | uptake | Maximize | uptake | Maximize | NA | Maximize |
| 11 | Oxygen | NA | Maximize | uptake | Maximize | NA | Maximize |
| 12 | Iron | NA | Maximize | NA | Maximize | uptake | maximize |

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**List of Supplemental Figures**

1. Steady-state exchange flux profiles for coculture design 1 consisting of *Rc* and *Ec*-ib. (a) *Rc* and *Ec*-ib effective growth rates. (b) *Rc* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Rc* amino acid uptake rates. (e) *Ec*-ib amino acid uptake rates. (f) *Rc* and *Ec*-ib oxygen uptake rates. (g) *Rc* byproduct secretion rates. (h) *Ec*-ib byproduct secretion rates. (i) *Rc* and *Ec*-ib isobutanol secretion rates.
2. Steady-state exchange flux profiles for triculture design 1 consisting of *Rc*, *Ec*-ib and *Ec*-ac. (a) *Rc*, *Ec*-ib and *Ec*-ac effective growth rates. (b) *Rc* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Ec*-ib and *Ec*-ac oxygen uptake rates. (e) *Rc* byproduct secretion rates. (f) *Ec*-ib byproduct secretion rates. (g) *Ec*-ac byproduct uptake rates. (h) *Rc*, *Ec*-ib and *Ec*-ac isobutanol secretion rates.
3. Steady-state exchange flux profiles for triculture design 2 consisting of *Rc*, *Ec*-ib and *Gm*. (a) *Rc*, *Ec*-ib and *Gm* effective growth rates. (b) *Rc* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Ec*-ib oxygen and *Gm* iron uptake rates. (e) *Rc* byproduct secretion rates. (f) *Ec*-ib byproduct secretion rates. (g) *Gm* byproduct uptake rates. (h) *Rc*, *Ec*-ib and *Gm* isobutanol secretion rates.
4. Steady-state exchange flux profiles for coculture design 3 consisting of *Cl* and *Ec*-ib. (a) *Cl* and *Ec*-ib effective growth rates. (b) *Cl* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Cl* amino acid uptake rates. (e) *Ec*-ib amino acid uptake rates. (f) *Cl* and *Ec*-ib oxygen uptake rates. (g) *Cl* byproduct secretion rates. (h) *Ec*-ib byproduct secretion rates. (i) *Cl* and *Ec*-ib isobutanol secretion rates.
5. Steady-state exchange flux profiles for triculture design 3 consisting of *Cl*, *Ec*-ib and *Ec*-ac. (a) *Cl*, *Ec*-ib and *Ec*-ac effective growth rates. (b) *Cl* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Ec*-ib and *Ec*-ac oxygen uptake rates. (e) *Cl* byproduct secretion rates. (f) *Ec*-ib byproduct secretion rates. (g) *Ec*-ac byproduct uptake rates. (h) *Cl*, *Ec*-ib and *Ec*-ac isobutanol secretion rates.
6. Steady-state exchange flux profiles for triculture design 4 consisting of *Cl*, *Ec*-ib and *Gm*. (a) *Cl*, *Ec*-ib and *Gm* effective growth rates. (b) *Cl* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Ec*-ib oxygen and *Gm* iron uptake rates. (e) *Cl* byproduct secretion rates. (f) *Ec*-ib byproduct secretion rates. (g) *Gm* byproduct uptake rates. (h) *Cl*, *Ec*-ib and *Gm* isobutanol secretion rates.
7. Steady-state concentrations average across the biofilm for nine combinations of the supplied cellobiose concentration (Clb) and biofilm length for triculture design 3 consisting of *Cl*, *Ec*-ib and *Ec*-ac. (a) *Cl* biomass. (b) *Ec*-ib biomass. (c) *Ec*-ac biomass. (d) cellobiose. (e) glucose. (f) acetate. (g) ethanol. (h) formate. (i) isobutanol.
8. Steady-state concentrations average across the biofilm for nine combinations of the supplied cellobiose concentration (Clb) and biofilm length for triculture design 2 consisting of *Rc*, *Ec*-ib and *Gm*. (a) *Rc* biomass. (b) *Ec*-ib biomass. (c) *Gm* biomass. (d) cellobiose. (e) glucose. (f) acetate. (g) ethanol. (h) formate. (i) isobutanol.
9. Steady-state concentrations average across the biofilm for nine combinations of the supplied cellobiose concentration (Clb) and biofilm length for triculture design 1 consisting of *Rc*, *Ec*-ib and *Ec*-ac. (a) *Rc* biomass. (b) *Ec*-ib biomass. (c) *Ec*-ac biomass. (d) cellobiose. (e) glucose. (f) acetate. (g) ethanol. (h) formate. (i) isobutanol.
10. Steady-state concentrations profiles biofilm for five species design with *Rc, Cl, Ec*-ib, *Gm* and *Ec*-ac (a) *Rc* biomass. (b) *Cl* biomass. (c) *Ec*-ib biomass. (d) Gm biomass (e) *Ec*-ac biomass. (f) beta glucosidase (g) cellobiose and glucose. (h) oxygen and iron. (i) byproducts (acetate, ethanol and formate). (j) isobutanol.
11. Steady-state exchange flux profiles biofilm for five species design with *Rc, Cl, Ec*-ib, *Gm* and *Ec*-ac (a) *Rc, Ec*-ib and *Gm* effective growth rates. (b) *Rc* cellobiose and glucose uptake rates. (c) *Ec*-ib glucose and cellobiose uptake rates. (d) *Ec*-ib oxygen and *Gm* iron uptake rates. (e) *Rc* byproduct secretion rates. (f) *Ec*-ib byproduct secretion rates. (g) *Gm* byproduct uptake rates. (h) *Rc*, *Ec*-ib and *Gm* isobutanol secretion rates.