

Supplementary Materials for

Applying statistical design of experiments (DOE) to understanding the effect of growth media components on *Cupriavidus necator* H16 growth

Christopher C. Azubuike^{a,b}, Martin G. Edwards^a, Angharad M. R. Gatehouse^a, Thomas P. Howard^{a#}

^aSchool of Natural and Environmental Sciences, Faculty of Science, Agriculture and Engineering, Newcastle University, Newcastle-upon-Tyne, NE1 7RX, United Kingdom

^bDepartment of Microbiology, Faculty of Science, University of Port Harcourt, East-West Road, P.M.B. 5323, Choba, Port Harcourt, Rivers State, Nigeria

#corresponding author, Thomas P. Howard, email: Thomas.Howard@newcastle.ac.uk

Tel: +44(0)1208 48 54

Table S1 Previously described chemically defined media for the growth of *C. necator*.

Carbon	Phosphate buffer	Potassium	Magnesium	Calcium	Nitrogen	Trace elements	Reference
50 Fructose	1.5 Na ₂ HPO ₄ . 12H ₂ O, 0.25 KH ₂ PO ₄ ,		0.75 MgSO ₄ .7H ₂ O	0.015 CaCl ₂	2.39 (NH ₄) ₂ SO ₄ , 0.285 nitrilotriacetic acid, 28% 0.9 Fe(NH ₄) ₂ (citrate)	0.00045 H ₃ BO ₃ , 0.0003 CoCl ₂ , 0.00015 ZnSO ₄ .7H ₂ O, 0.000045 MnCl ₂ .4H ₂ O, 0.000045 NaMoO ₄ .2H ₂ O, 0.00003 NiCl ₂ .6H ₂ O and 0.000015CuSO ₄ . 5H ₂ O	(1)
0.5-1% Fructose	12 ml 1.1 M H ₃ PO ₄	0.45			0.1 or 0.01% NH ₄ Cl	24 mL/trace: 2.6 CaCl ₂ .2H ₂ O 0.1 MnSO ₄ .H ₂ O, 0.1 ZnSO ₄ .7H ₂ O, 0.02 CuSO ₄ .5H ₂ O, 0.015 FeSO ₄ .7H ₂ O	(2)
10-20 Fructose	1 KH ₂ PO ₄ ; 11.1 Na ₂ HPO ₄ . 12H ₂ O		0.2 MgSO ₄		3 (NH ₄) ₂ SO ₄	9.7 FeCl ₃ , 7.8 CaCl ₂ , 0.156 CuSO ₄ .5H ₂ O), 0.119 CoCl ₂ , 0.118 NiCl ₂ and 0.062 CrCl ₂	(3)
2% v/v biodiesel-driven glycerol, 2 % w/v glycerol bottom and 1% v/v free fatty acids	6.7 Na ₂ HPO ₄ . 2H ₂ O, 1.5 KH ₂ PO ₄ ,		0.2 MgSO ₄ .7H ₂ O	0.01 CaCl ₂ .2H ₂ O	1 (NH ₄) ₂ SO ₄ , 0.06 Fe(NH ₄) ₂ (citrate)	0.3 H ₃ BO ₃ , 0.2 CoCl ₂ , 0.1 ZnSO ₄ .7H ₂ O, 0.03 MnCl ₂ .4H ₂ O, 0.02 NaMoO ₄ .2H ₂ O, 0.02 NiCl ₂ .6H ₂ O and 0.01CuSO ₄ .5H ₂ O	(4)
20 Fructose	1.51 Na ₂ HPO ₄ , 2.65 KH ₂ PO ₄ ,		0.3 MgSO ₄ .7H ₂ O			1.97 FeCl ₃ , 9.7 CaCl ₂ .2H ₂ O, 0.156 CuSO ₄ .5H ₂ O, 0.184 CoCl ₂ .6H ₂ O, 0.118 NiCl ₂ .6H ₂ O and 0.062 CrCl ₃	(5)
^a 2% Fructose, 2% sodium gluconate, 1% palm oil or 0.5% Tween-60	4 NaH ₂ PO ₄ , 4.6 Na ₂ HPO ₄	0.45 K ₂ SO ₄	0.39 MgSO ₄	0.062 CaCl ₂	0.5 NH ₄ Cl	15 FeSO ₄ .7H ₂ O, 2.4 MnSO ₄ .H ₂ O, 2.4 ZnSO ₄ .7H ₂ O, 0.48 CuSO ₄ .5H ₂ O	(6, 7)
2-4 Fructose, 2 mL glycerol	1.5 KH ₂ PO ₄ , 9 Na ₂ HPO ₄		0.2 MgSO ₄ .7H ₂ O	0.01 CaCl ₂ .2H ₂ O	2 NH ₄ Cl	0.05 FeCl ₃ .6H ₂ O, 0.019-0.190 NiCl ₂ .6H ₂ O	(8)

All concentrations are g/L unless otherwise indicated. Starting medium for DOE investigation ^(a).

Table S2 Definitive screening design array. A definitive screening design (DSD1) was developed to assess the impact of 10 ingredients found within the chemically defined media.

Media	Fru.	NaH ₂ PO ₄	Na ₂ HPO ₄	K ₂ SO ₄	MgSO ₄	CaCl ₂	NH ₄ Cl	T.E.	A.A.	Vit.	OD1	OD2
1	20.25	0.1	0.1	0.01	0.01	0.01	0.01	0.01	0.05	0.01	1.06	1.37
2	20.25	6	6	4.8	2.8	0.8	4.8	4.8	19	4.8	1.14	1.62
3	0.5	3.05	0.1	4.8	0.01	0.01	0.01	4.8	19	4.8	0.52	0.47
4	40	3.05	6	0.01	2.8	0.8	4.8	0.01	0.05	0.01	0.09	0.78
5	0.5	6	3.05	0.01	2.8	0.01	0.01	0.01	19	4.8	0.77	0.71
6	40	0.1	3.05	4.8	0.01	0.8	4.8	4.8	0.05	0.01	-	-
7	40	6	0.1	2.41	2.8	0.01	4.8	4.8	19	0.01	0.93	0.69
8	0.5	0.1	6	2.41	0.01	0.8	0.01	0.01	0.05	4.8	0.11	0.28
9	0.5	6	0.1	4.8	1.41	0.01	4.8	0.01	0.05	0.01	0.80	0.64
10	40	0.1	6	0.01	1.41	0.8	0.01	4.8	19	4.8	1.62	1.48
11	0.5	6	6	0.01	2.8	0.41	0.01	4.8	0.05	0.01	0.29	0.32
12	40	0.1	0.1	4.8	0.01	0.41	4.8	0.01	19	4.8	1.86	1.41
13	0.5	6	6	4.8	0.01	0.8	2.41	0.01	19	0.01	0.41	0.55
14	40	0.1	0.1	0.01	2.8	0.01	2.41	4.8	0.05	4.8	-	0.01
15	0.5	0.1	6	4.8	2.8	0.01	4.8	2.41	0.05	4.8	0.17	0.43
16	40	6	0.1	0.01	0.01	0.8	0.01	2.41	19	0.01	1.69	1.67
17	0.5	0.1	0.1	4.8	2.8	0.8	0.01	4.8	9.53	0.01	0.17	0.28
18	40	6	6	0.01	0.01	0.01	4.8	0.01	9.53	4.8	-	0.19
19	0.5	0.1	0.1	0.01	2.8	0.8	4.8	0.01	19	2.41	0.48	0.70
20	40	6	6	4.8	0.01	0.01	0.01	4.8	0.05	2.41	-	0.04
21	20.25	3.05	3.05	2.41	1.41	0.41	2.41	2.41	9.53	2.41	1.76	1.70
22	0.5	0.1	6	0.01	0.01	0.01	4.8	4.8	19	0.01	0.86	0.71
23	0.5	6	0.1	0.01	0.01	0.8	4.8	4.8	0.05	4.8	0.10	0.19
24	40	6	0.1	4.8	2.8	0.8	0.01	0.01	0.05	4.8	1.64	1.56
25	40	0.1	6	4.8	2.8	0.01	0.01	0.01	19	0.01	0.20	0.23

A definitive screening design was developed to assess the impact of 10 ingredients found within the chemically defined media. All concentrations are in g/L except trace elements, amino acids and vitamins which are mL/L. Trace element working concentration contained (g/L): 15 FeSO₄·7H₂O, 2.4 MnSO₄·H₂O, 2.4 ZnSO₄·7H₂O, and 0.48 CuSO₄·5H₂O. A 100x stock amino acid mix contained (g/L): 12.9 arginine, and 10 each of histidine, leucine and methionine. A 1000x vitamin stock contained (g/L): 0.1 pyridoxine, 0.02 folic acid, 0.05 each of thiamine, riboflavin, niacin, pantothenic acid and nicotinamide. Abbreviations: Fru., fructose, T.E. trace element mixture; A.A., amino acid mixture; Vit., vitamin mixture. The DSD was performed in $n = 2$ biological replicates.

Table S3 Definitive Screening Design 2. A second definitive screening design (DSD2) was performed to assess the impact of the five ingredients identified as contributing significantly to *C. necator* growth.

Media	Fru.	NaH ₂ PO ₄	Na ₂ HPO ₄	K ₂ SO ₄	MgSO ₄	CaCl ₂	NH ₄ Cl	T.E.	A.A.	OD1	OD2
1	15	3.05	0.1	2.41	1.41	0.01	0.01	0.01	5	1.71	1.36
2	15	3.05	3.05	2.41	1.41	0.46	0.01	2.41	20	1.84	1.67
3	25	3.05	1.58	2.41	1.41	0.46	0.01	0.01	5	1.72	1.65
4	5	3.05	1.58	2.41	1.41	0.01	0.01	2.41	20	1.95	1.95
5	5	3.05	0.1	2.41	1.41	0.28	0.01	2.41	5	1.85	0.99
6	25	3.05	3.05	2.41	1.41	0.28	0.01	0.01	20	0.57	1.84
7	5	3.05	3.05	2.41	1.41	0.46	0.01	1.28	5	1.18	1.18
8	25	3.05	0.1	2.41	1.41	0.01	0.01	1.28	20	2.10	2.06
9	5	3.05	3.05	2.41	1.41	0.01	0.01	0.01	12.50	2.03	1.99
10	25	3.05	0.1	2.41	1.41	0.46	0.01	2.41	12.50	1.86	1.40
11	15	3.05	1.58	2.41	1.41	0.28	0.01	1.28	12.50	1.92	1.96
12	5	3.05	0.1	2.41	1.41	0.46	0.01	0.01	20	2.01	2.08
13	25	3.05	3.05	2.41	1.41	0.01	0.01	2.41	5	1.06	0.91

All concentrations are in g/L except trace elements and amino acids, which are mL/L. Trace element working concentration contained (g/L): 15 FeSO₄·7H₂O, 2.4 MnSO₄·H₂O, 2.4 ZnSO₄·7H₂O, and 0.48 CuSO₄·5H₂O. A 100x stock amino acid mix contained (g/L): 12.9 arginine, and 10 each of histidine, leucine and methionine. Abbreviations: Fru., fructose, T.E., trace element mixture; A.A., amino acid mixture. The DSD2 array was performed in duplicate.

Table S4 Definitive Screening Design 3. A final set of experiments were performed to re-assess the impact of components not deemed significant in the first DSD.

Media	Fru.	NaH ₂ PO ₄	Na ₂ HPO ₄	K ₂ SO ₄	MgSO ₄	CaCl ₂	NH ₄ Cl	T.E.	A.A.	OD1	OD2
1	5	0.1	0.1	0.01	0.01	0.46	4.8	1	10	0.13	0.17
2	5	0.1	0.1	1.21	1.41	0.46	0.01	1	10	1.79	1.74
3	5	0.1	0.1	2.41	0.01	0.46	0.01	1	10	1.64	1.71
4	5	0.1	0.1	0.01	1.41	0.46	2.41	1	10	0.95	0.57
5	5	3.05	0.1	1.21	0.01	0.46	4.8	1	10	1.85	1.76
6	5	3.05	0.1	0.01	0.71	0.46	0.01	1	10	1.86	1.65
7	5	3.05	0.1	2.41	1.41	0.46	0.01	1	10	1.89	1.88
8	5	1.56	0.1	0.01	0.01	0.46	0.01	1	10	1.76	1.92
9	5	3.05	0.1	0.01	1.41	0.46	4.8	1	10	0.84	0.79
10	5	0.1	0.1	2.41	0.71	0.46	4.8	1	10	0.66	0.73
11	5	1.56	0.1	2.41	1.41	0.46	4.8	1	10	1.51	1.55
12	5	3.05	0.1	2.41	0.01	0.46	2.41	1	10	1.95	1.89
13	5	1.56	0.1	1.21	0.71	0.46	2.41	1	10	1.49	1.76
14	20	0.1	0.1	0.01	0.01	0.46	4.8	1	10	0.15	0.81
15	20	0.1	0.1	1.21	1.41	0.46	0.01	1	10	1.86	2.09
16	20	0.1	0.1	2.41	0.01	0.46	0.01	1	10	2.09	1.92
17	20	0.1	0.1	0.01	1.41	0.46	2.41	1	10	1.66	0.95
18	20	3.05	0.1	1.21	0.01	0.46	4.8	1	10	1.79	1.52
19	20	3.05	0.1	0.01	0.71	0.46	0.01	1	10	1.71	1.90
20	20	3.05	0.1	2.41	1.41	0.46	0.01	1	10	2.05	1.73
21	20	1.56	0.1	0.01	0.01	0.46	0.01	1	10	1.81	1.85
22	20	3.05	0.1	0.01	1.41	0.46	4.8	1	10	1.08	0.98
23	20	0.1	0.1	2.41	0.71	0.46	4.8	1	10	1.17	0.96
24	20	1.56	0.1	2.41	1.41	0.46	4.8	1	10	1.65	1.48
25	20	3.05	0.1	2.41	0.01	0.46	2.41	1	10	1.74	1.80
26	20	1.56	0.1	1.21	0.71	0.46	2.41	1	10	1.65	1.39

All concentrations are in g/L except trace elements and amino acids, which are mL/L. Trace element working concentration contained (g/L): 15 FeSO₄·7H₂O, 2.4 MnSO₄·H₂O, 2.4 ZnSO₄·7H₂O, and 0.48 CuSO₄·5H₂O. A 100x stock amino acid mix contained (g/L): 12.9 arginine, and 10 each of histidine, leucine and methionine. Abbreviations: Fru., fructose, T.E., trace element mixture; A.A., amino acid mixture. The DSD array was performed in duplicate.

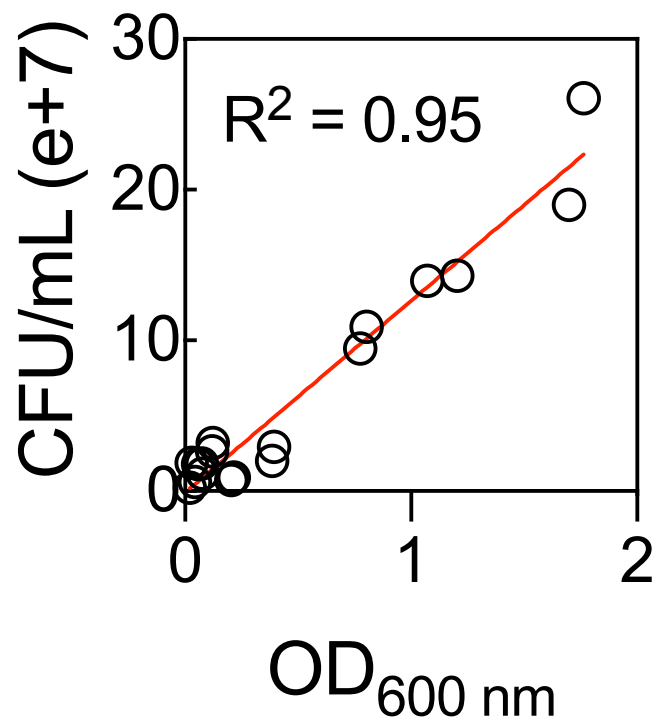


FIG S1 Correlation of colony forming unit per millilitre (CFU/mL) against optical density (OD_{600nm}). Data are for $n = 3$ biological replicates.

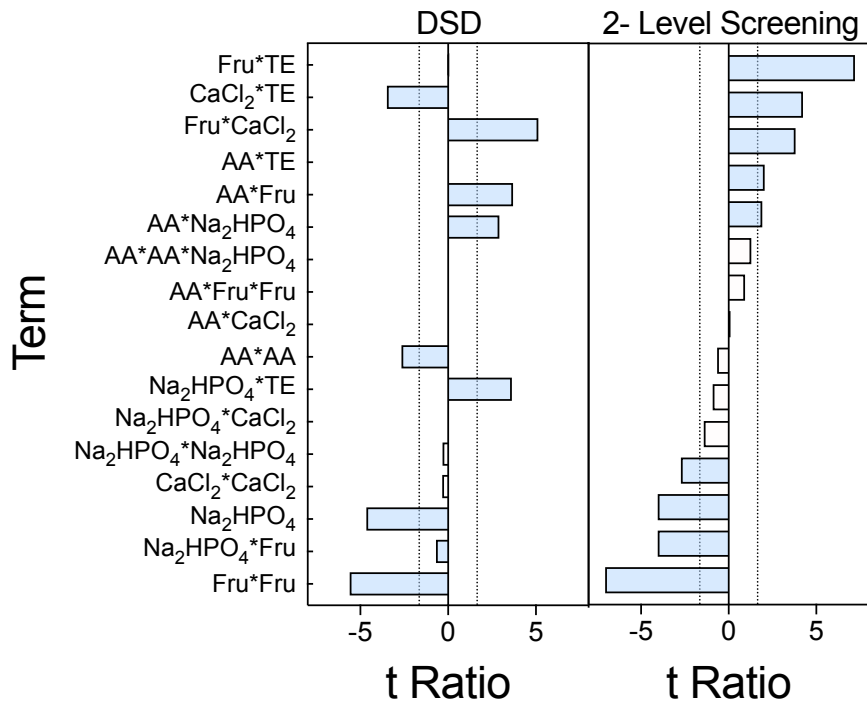


FIG S2 Two-way interaction between components. The comparative lengths of the t-ratios for factor interaction are shown. Bars extending to the right have a positive impact on growth at high concentration, those extending to the left have a negative impact on growth at high concentration. The broken vertical lines indicate threshold ($t < -1.65$ or > 1.65) level at 90% confidence level; terms (interaction) surpassing this threshold is deemed to have significant effect on the output, OD_{600nm} . Terms deemed significant for model projection are highlighted (blue). Analysis is based on two biological replicated arrays (Table S2).

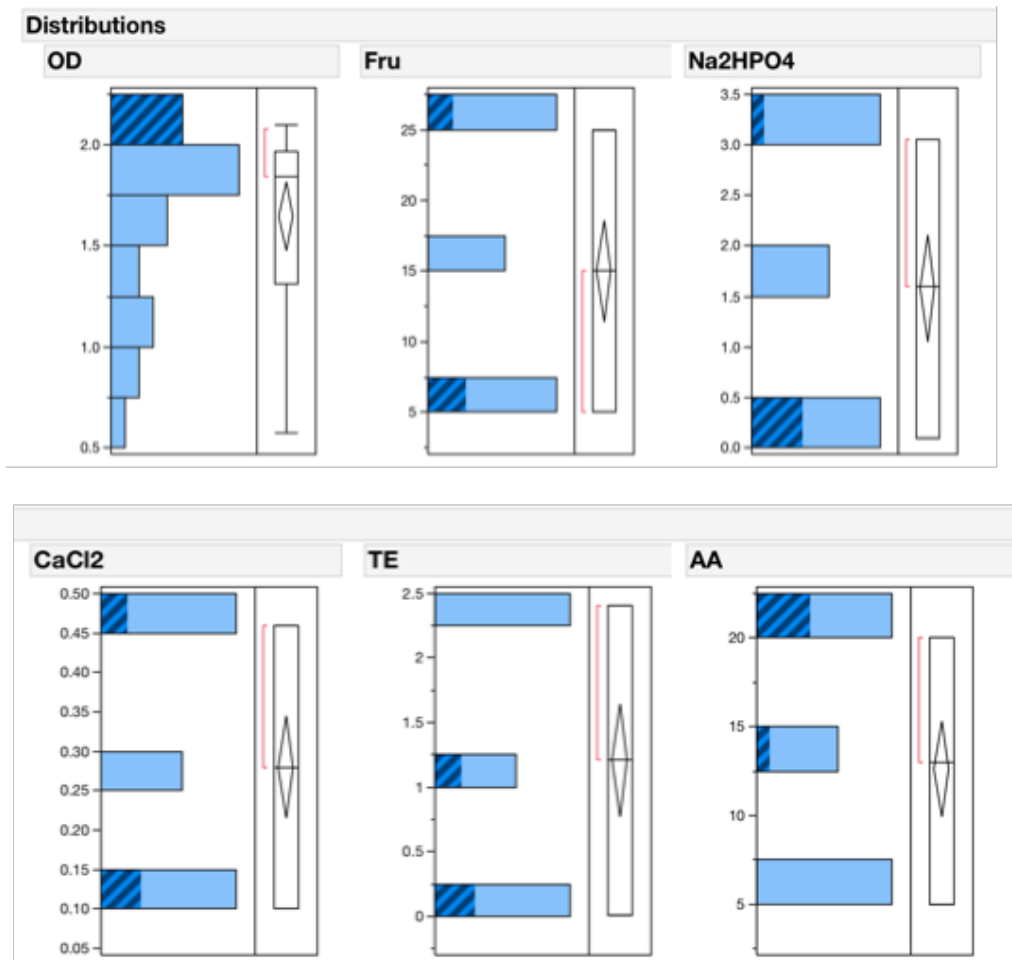


FIG S3 Distributions of data for Definitive Screening Design 2. Highlighted in blue crosshatch are the settings that resulted in the greatest OD_{600nm} at 72 h for DSD2. Fructose, Na₂HPO₄ and CaCl₂ may be at either the highest or lowest concentrations (g/L), whereas trace elements and amino acid concentrations are found at the lowest or highest concentrations (mL/L) respectively.

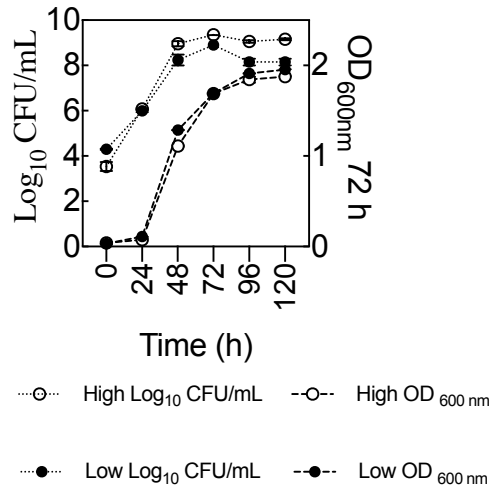


FIG S4 Validation of OD_{600nm} as surrogate for growth measurement. Viable cells (cfu/mL) of *C. necator* H16 correlated with optical density (OD_{600nm}) over prolonged cultivation. Experiment was performed at low and high concentration media formulations, guided by the growth model prediction (Fig. 7A). Cell were grown on defined media and plated on LB agar and incubated as appropriate for *C. necator*. Error bars are S.E. Mean, $n = 3$ biological replicates.

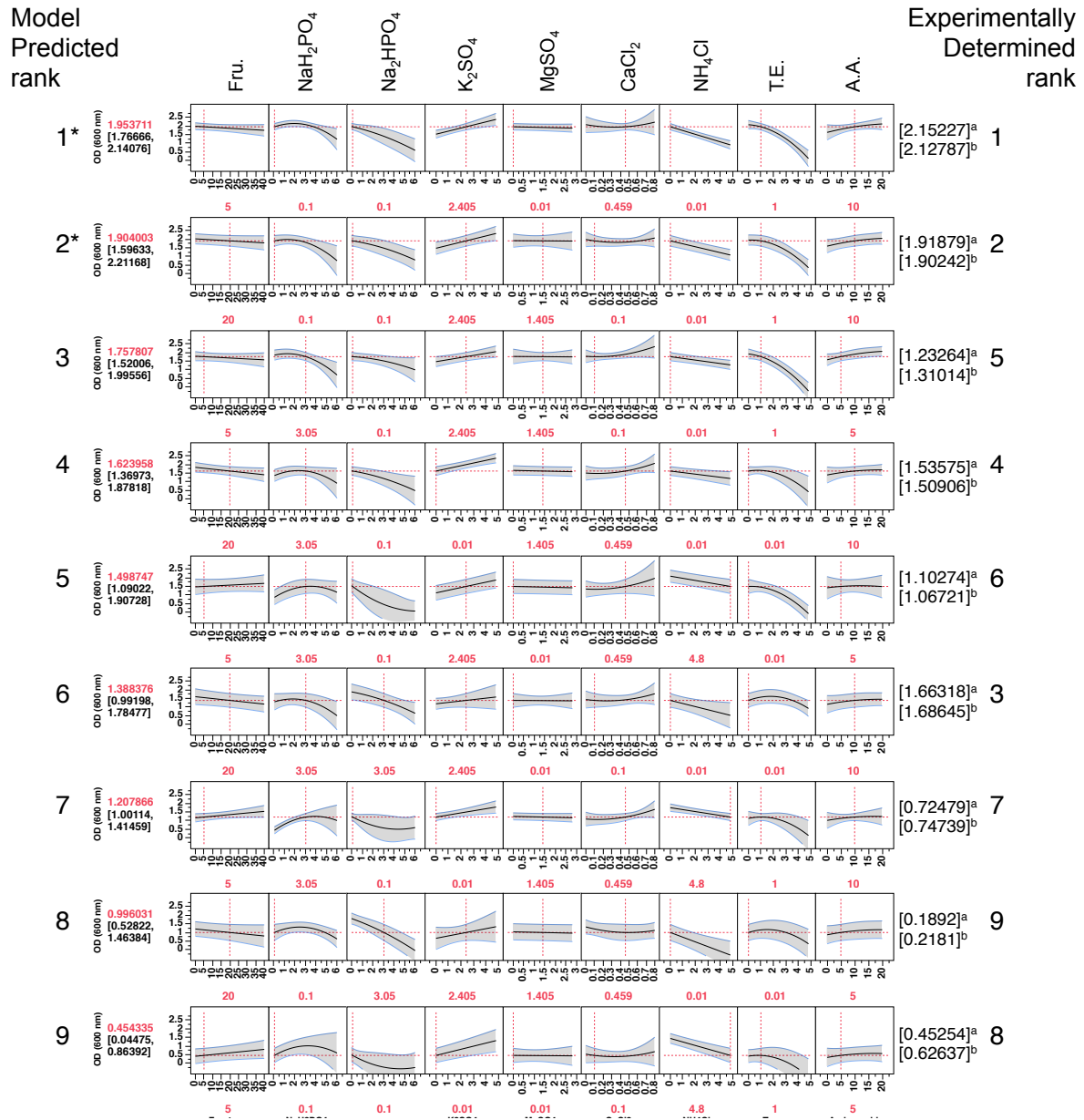


FIG S5 One hundred millilitre validation in shake flasks. Nine different formulations were assessed at 100 mL culture volumes. For each row, the model predicted rank and OD_{600nm} 72 h (values in brackets) are indicated on the left, media settings for each component are indicated in red underneath, the measured OD_{600nm} 72 h for two cultivation experiments (values in brackets) and experimentally determined rank are indicated on the right. Non-baffled flask cultivations (a); baffled flask cultivations (b); included in bioreactor cultivations and subsequently served as controls (*). All concentrations are in g/L except T.E. and A.A., which are in mL/L. Abbreviations: Fru., fructose; T.E., trace element mixture; A.A., amino acid mixture. The output is $n = 2$ biological replicated arrays of definitive screening designs (DSDs).

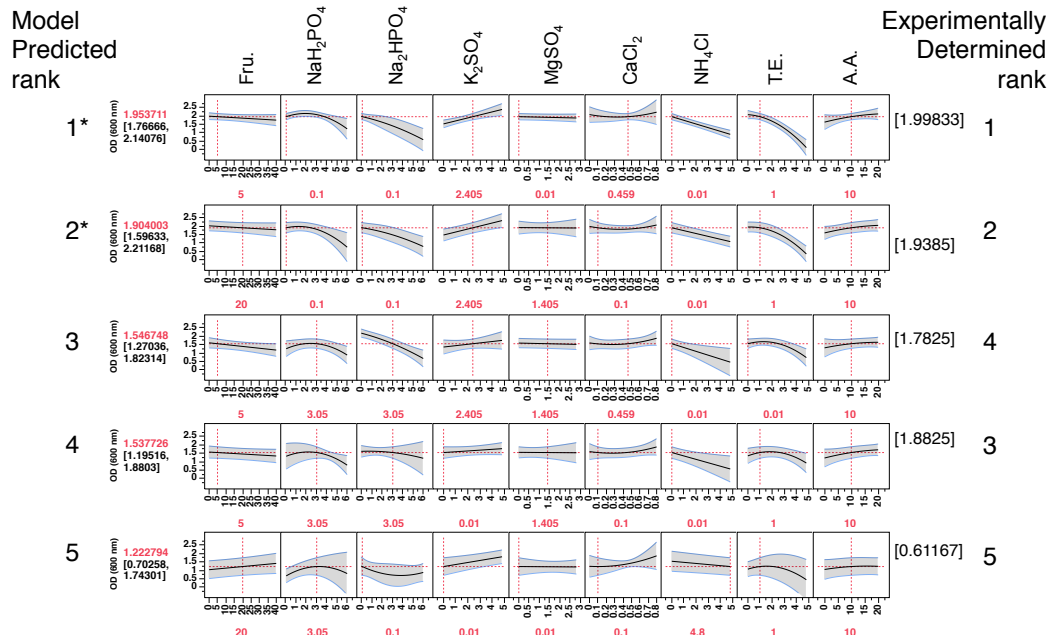


FIG S6 One litre (1 L) validation. Five different formulations were assessed at 1 L culture volumes. For each row, the model predicted rank and OD_{600nm} 72 h (values in brackets) are indicated on the left, media settings for each component are indicated in red underneath; the measured OD_{600nm} 72 h (values in brackets) and experimentally determined rank are indicated on the right. Controls (*). All concentrations are in g/L except T.E. and A.A., which are in mL/L. Abbreviations: Fru., fructose; T.E., trace element mixture; A.A., amino acid mixture.

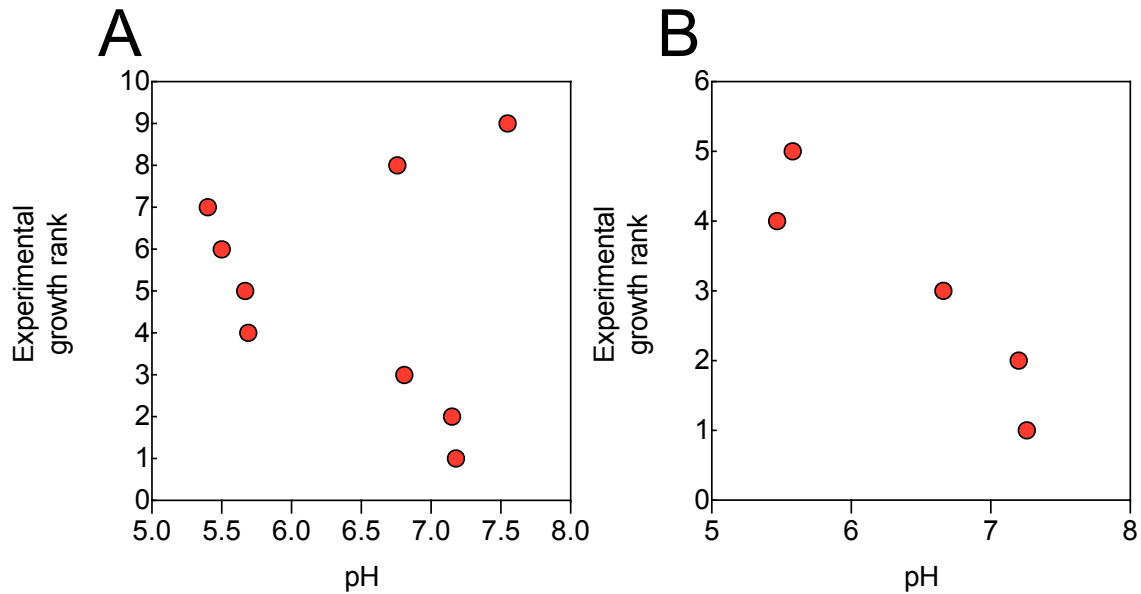


FIG S7 Effect of pH on growth. Spearman's correlation of experimentally determined growth ($OD_{600\text{ nm}}$) and pH after 72 h. **A.** Shake flask cultivation ($\rho = 0.23$). **B.** Bioreactor cultivation ($\rho = 0.52$).

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