Supplementary Materials

Optimized fermentation strategies for efficient bioethanol production

Kathiresan Subramanian[1*], Kagne Suresh[2]

[1] *Hospira Healthcare Pvt. Ltd, Chennai, Tamil Nadu 600017, India.*
[2]*Badrinarayan Barwale Mahavidhyalaya, Jalna, Maharashtra 431213, India*

Table S1 Various fermentation recipe combinations showing different substrate compositions and nutrient ratios.

Recipe 1	Recipe 2	Recipe 3	Recipe 4	Recipe 5
Biomass Concentration - 25g	Biomass Concentration - 35g	Biomass Concentration - 50g	Biomass Concentration - 100g	Biomass Concentration - 150g
Dextrose - 100mg	Dextrose - 300mg	Dextrose - 500mg	Dextrose - 700mg	Dextrose - 1g
Mannose - 100mg	Mannose - 250mg	Mannose - 350mg	Mannose - 500mg	Mannose - 1g
Glucose - 100mg	Glucose - 250mg	Glucose - 350mg	Glucose - 500mg	Glucose - 1g
Galactose - 100mg	Galactose - 250mg	Galactose - 350mg	Galactose - 500mg	Galactose - 1g
Xylose - 100mg	Xylose - 250mg	Xylose - 350mg	Xylose - 500mg	Xylose - 1g
Arabinose - 100mg	Arabinose - 250mg	Arabinose - 350mg	Arabinose - 500mg	Arabinose - 1g
Glycerol - 5g	Glycerol - 10g	Glycerol - 15g	Glycerol - 20g	Glycerol - 25g
Ammonium sulphate - 100mg	Ammonium sulphate - 250mg	Ammonium sulphate - 350mg	Ammonium sulphate - 700mg	Ammonium sulphate - 1g
Dipotassium phosphate - 250mg	Dipotassium phosphate - 500mg	Dipotassium phosphate - 750mg	Dipotassium phosphate - 3.5g	Dipotassium phosphate - 7g
Monopotassium phosphate - 100mg	Monopotassium phosphate - 250mg	Monopotassium phosphate - 500mg	Monopotassium phosphate - 1.5g	Monopotassium phosphate - 3g
Sodium citrate - 50mg	Sodium citrate - 150mg	Sodium citrate - 275mg	Sodium citrate - 500mg	Sodium citrate - 750mg
Magnesium sulphate - 10mg	Magnesium sulphate - 50mg	Magnesium sulphate - 100mg	Magnesium sulphate - 250mg	Magnesium sulphate - 500mg
Aspartic acid - 100mg	Aspartic acid - 300mg	Aspartic acid - 500mg	Aspartic acid - 700mg	Aspartic acid - 1g
Glutamic acid - 100mg	Glutamic acid - 300mg	Glutamic acid - 500mg	Glutamic acid - 700mg	Glutamic acid - 1g
Asparagine - 100mg	Asparagine - 300mg	Asparagine - 500mg	Asparagine - 700mg	Asparagine - 1g
Tryptophan - 100mg	Tryptophan - 300mg	Tryptophan - 500mg	Tryptophan - 700mg	Tryptophan - 1g
Isoleucine - 100mg	Isoleucine - 300mg	Isoleucine - 500mg	Isoleucine - 700mg	Isoleucine - 1g
Sodium Chloride -250mg	Sodium Chloride -500mg	Sodium Chloride -750mg	Sodium Chloride -1g	Sodium Chloride -2.5g
Temperature - 31.5	Temperature - 33.0	Temperature - 34.5	Temperature 35.5	Temperature 37
RPM - 35	RPM - 40	RPM - 45	RPM - 50	RPM - 60

Table S2 Effect of varying sugar concentrations in fermentation recipes.

Sugar conc. (v/v)	**Lag phase (h)**	**Diauxic phase (h)**	**Biomass produced (g/L)**	**Ethanol produced (g/L)**
30%	6	20	0.810	16.20
40%	12	36	1.020	18.15
50%	10	38	1.542	19.34
60%	13	37	1.821	17.30

Table S3 Effect of pH on ethanol production.

pH	Ethanol (g/L)	Xylose (g/L)	Theoretical yield	Observed yield
6.5	19.5	6.2	0.53	0.45

Table S4 Summary of fermentation run observations showing real-time monitoring of key byproducts and metabolites—ethanol, succinic acid, biomass, and residual sugar.

Run	Ethanol (g/L)	Succinic acid (g/L)	Biomass (g/L)	Residual sugar (g/L)
1	17.8	1.9	1.35	5.2
Duplicate	18.1	1.53	1.5	4.2

Table S5 Duration of fermentation and optical density (OD) measurements at 600 nm, indicating cell growth by monitoring the turbidity of the fermentation broth at specific time points to correlate with cell concentration.

Recipes	3h	6h	9h	121	l8h	24h	32h	48h	56h	64h
Recipe 1	0.21	0.31	0.42	0.53	0.67	1.56	1.33	1.05	1.00	0.92
Recipe 2	0.16	0.66	0.7l	0.75	0.81	1.33	1.4	0.94	0.94	0.83
Recipe 3	0.25	0.71	0.8	0.93	1.2	1.68	0.99	0.89	0.84	0.71
Recipe 4	0.32	0.78	0.9	1.26	1.03	1.72	1.31	1.10	0.93	0.91
Recipe 5	0.39	0.93	1.0	1.11	1.15	1.92	1.50	1.32	1.30	1.22

Table S6. Correlation of fermentation duration with sugar and xylose utilization, sugar consumption, and ethanol production, highlighting process control and optimization insights.

		Duration (Day)	Total Sugar utilized	Xylose utilized (%)	Ethanol yield (g/g sugar consumed)	Ethanol yield (g/g sugar available)	Ethanol Productivity	Succinic acid produced	Cell Mass (g/L)
Duration (Day)	Pearson Correlation	1	-0.348	-.502	-.613	-.693	-0.084	.477	.567
	Sig.(2-tailed)		0.095	0.012	0.001	0.000	0.697	0.019	0.004
	N	24	24	24	24	24	24	24	24
Total Sugar utilized	Pearson Correlation	-0.348	1	.743	-0.092	0.300	-.418	0.217	-.406
	Sig. (2-tailed)	0.095	1	0.000	0.668	0.154	0.042	0.309	0.049
	N	24	24	24	24	24	24	24	24
Xylose utilized	Pearson Correlation	-.502	.743	1	0.103	0.303	-0.329	0.033	- 0.396
	Sig. (2-tailed)	0.012	0.000	1	0.631	0.151	0.116	0.880	0.056
	N	24	24	24	24	24	24	24	24
Ethanol Yield (g/g sugar consumed)	Pearson Correlation	-.613	-0.092	0.103	1	.871	.641	-.637	- 0.002
	Sig. (2-tailed)	0.001	0.668	0.631	1	0.000	0.001	0.001	0.993
	N	24	24	24	24	24	24	24	24
Ethanol Yield (g/g sugar available)	Pearson Correlation	-.693**	0.3	0.303	.871**	1	.462*	-.516**	-0.133
	Sig. (2-tailed)	0	0.154	0.151	0	1	0.023	0.01	0.536
	N	24	24	24	24	24	24	24	24

Table S6 (contd.)

		Duration (Day)	Total Sugar utilized	Xylose utilized (%)	Ethanol yield (g/g sugar consumed)	Ethanol yield (g/g sugar available)	Ethanol Productivity	Succinic acid produced	Cell Mass (g/L)
Ethanol Productivity	Pearson Correlation	0.693	0.3	0.303	0.871	1	0.462	0.516	0.133
	Sig. (2-tailed)	0.697	0.042	0.116	0.001	0.023		0.084	0.007
	N	24	24	24	24	24	24	24	24
Succinic acid produced	Pearson Correlation	.477*	0.217	0.033	-.637**	-.516**	-0.36	1	0.194
	Sig. (2-tailed)	0.019	0.309	0.88	0.001	0.01	0.084	1	0.364
	N	24	24	24	24	24	24	24	24
Cell mass (g/L)	Pearson Correlation	.567**	-.406*	-0.396	-0.002	-0.133	.536**	0.194	1
	Sig. (2-tailed)	0.004	0.049	0.056	0.993	0.536	0.007	0.364	--
	N	24	24	24	24	24	24	24	24

Table S7 Significant correlation of key ethanol fermentation parameters, highlighting factors that may repress the uptake of other sugars through glycolysis.

Parameter	Significantly correlated
Duration	Xylose utilized, Ethanol yield consumed, Ethanol yields available, Succinic acid produced, Cell mass
Total sugar utilized	Xylose utilized, Ethanol productivity, Cell mass
Xylose utilized	Duration, Total sugar utilized
Ethanol yield consumed	Duration, Ethanol yield available, Ethanol productivity, Succinic acid produced
Ethanol yields available	Duration, Ethanol yield consumed, Ethanol productivity, Succinic acid produced
Ethanol productivity	Total sugar utilized, Ethanol yield consumed, Ethanol yields available, Cell mass
Succinic acid produced	Duration, Ethanol yield consumed, Ethanol yields available
Cell mass	Duration, Total sugar utilized, Ethanol productivity

Table S8 The effect of independent and dependent variables of ethanol production to determine B slope coefficients. The study enables to identify the variable(s) significantly affect the outcome of ethanol production. The signs of +/- indicates the direction of relationship reveals available sugar concentration versus utilization. A higher adjusted R^2 (AR^2) close to 1 indicates better fitting model, F- statistics (F) tests overall significance of regression model, R^2 (R-squared) indicates coefficient of determination within the predictors. R=0 predictor exhibits none of the variations, and R=1 predictor indicates all of the variations. t-statistics (t) tests significance of each predictor(s).

Predictor Variable	B (Slope)	B (Intercept)	t	R^2 (Level of prediction)	AR^2	F
Step 1				0.175	0.175	4.652
Total sugar utilized	-0.013	-0.418	-2.157			
Step 2				0.541	0.366	12.374
Total sugar utilized	-0.011	-0.362		-2.436		
Ethanol yield consumed	.2.484	0.608		4.0t94		

Table S9 Correlations and interrelationship between variables for ethanol fermentation.

Ethanol yield (g/g sugar available)	Pearson Correlation	-0.693**	0.3	0.303	0.871**	1	.462*	-0.516**	-0.133
	Sig. (2-tailed)	0	0.154	0.151	0		0.023	0.01	0.536
Ethanol productivity	Pearson Correlation	-0.084	-.418*	-0.329	0.641**	0.462*	1	-0.36	0.536**
	Sig. (2-tailed)	0.697	0.042	0.116	0.001	0.023		0.084	0.007
	N	24	24	24	24	24	24	24	24
Succinic acid produced	Pearson Correlation	0.477*	0.217	0.033	-0.637**	-0.516**	-0.36	1	0.194
	Sig. (2-tailed)	0.019	0.309	0.88	0.001	0.01	0.084		0.364
	N	24	24	24	24	24	24	24	24
Cell mass (g/L)	Pearson Correlation	0.567**	-0.406*	-0.396	-0.002	-0.133	0.536**	0.194	1
	Sig. (2-tailed)	0.004	0.049	0.056	0.993	0.536	0.007	0.364	--
	N	24	24	24	24	24	24	24	24

Table S10 Significant fermentation parameters control the fermentation by *Escherichia coli* strain.

Parameter	**Significantly correlated with**
Total sugar utilized	Xylose utilized, Ethanol yield consumed, Ethanol yields available, Succinic acid produced, Cell mass
Xylose utilized	Xylose utilized, Ethanol productivity, Cell mass
Ethanol yield consumed	Duration, Total sugar utilized
Ethanol yields available	Duration, Ethanol yield available, Ethanol productivity, Succinic acid produced
Ethanol productivity	Duration, Ethanol yield consumed, Ethanol productivity, Succinic acid produced
Succinic acid produced	Total sugar utilized, Ethanol yield consumed, Ethanol yields available, Cell mass
Cell mass	Duration, Ethanol yield consumed, Ethanol yields available

Table S11 Predictive steps for optimizing ethanol fermentation, guiding the experimental design to maximize ethanol yield by identifying key parameter interactions.

Step 3			0.558	0.017	8.429
Total sugar utilized	-0.016	-0.526	-2.214		
Ethanol yield consumed	0.900	0.220	0.478		
Ethanol welds available	1.580	0.428	0.888		
Step 4			0.732	0. 174	12.994
Total sugar utilized	-0.009	-0.292	-1.453		
Ethanol yield consumed	1.4 18	0.347	0.938		
Ethanol available	I .13 7	0.3 08	0.797		
Cell mass	0.356	0.459	3.5 14		

Table S12. Hierarchical regression of variance highlighting key predictors, prioritizing influential factors to improve experimental design and maximize ethanol production using *Escherichia coli*.

Hierarchical regression shows that 17.5% variance in ethanol productivity									
Ethanol yield (*gig* sugar available)	Pearson Correlation	-0.693**	0.3	0.303	0.871**	1	0.462*	-0.516**	-0.133
	Sig.(2-tailed)	0	0.154	0.151	0		0.023	0.01	0.536
Ethanol Productivity	Pearson Correlation	-0.084	-0.418*	-0.329	0.641**	0.462*	1	-0.36	0.536**
	Sig. (2-tailed)	0.697	0.042	0.116	0.001	0		0.084	0.007
	N	24	24	24	24	24	24	24	24
Succinic acid produced	Pearson Correlation	0.477*	0.217	0.033	-0.637**	-0.516**	-0.36	1	0.194
	Sig. (2-tailed)	0.019	0.309	0.88	0.001	0	0.084		0.364
	N	24	24	24	24	24	24	24	24
Cell mass (g/L)	Pearson Correlation	0.567**	-0.406*	-0.396	-0.002	-0	0.536**	0.194	1
	Sig. (2-tailed)	0.004	0.049	0.056	0.993	**1**	0.007	0.364	
	N	24	24	24	24	24	24	24	24

Table S13 Predictive steps for optimization of ethanol fermentation with significant correlated parameters.

Parameter	Significant correlations
Duration	Xylose utilized, Ethanol yield consumed, Ethanol yields available, Succinic acid produced, Cell mass
Total sugar utilized	Xylose utilized, Ethanol productivity, Cell mass
Xylose utilized	Duration, Total sugar utilized
Ethanol yield consumed	Duration, Ethanol yield available, Ethanol productivity, Succinic acid produced
Ethanol yields available	Duration, Ethanol yield consumed, Ethanol productivity, Succinic acid produced
Ethanol productivity	Total sugar utilized, Ethanol yield consumed, Ethanol yields available, Cell mass
Succinic acid produced	Duration, Ethanol yield consumed, Ethanol yields available
Cell mass	Duration, Total sugar utilized, Ethanol productivity