Supplementary material:

S1. Media composition

S2. Box-Behnken DoE

S3. Microscopic analysis of cell size

S4. Flow cytometry analysis

S5. Evaluation of correlations between morphology descriptors and FT survivability

S1. Media composition

S1-1 – Cryopreservation medium

0.82 g K_2HPO_4, 0.18 g KH_2PO_4 ,0.59 g Na-citrate, 0.25 g MgSO4 x 7 H_2O, 172 ml glycerol (87%) 15% final concentration, Add dH_2O to 1000 mL

S1-2 – Homofermentative heterofermentative differential (HHD) agar

2.5 g Fructose, 2.5 g KH_2PO_4, 10.0 g Tryptone, 1.5 g Phytone Peptone, 3.0 g Casamino Acids, 10.0 g Yeast Extract, 1.0 g Tween 80, 20 mL Bromo Cresol Green dissolved in 0,01N NaOH (0.13 g in 40 mL NaOH), 20.0 g Agar, 1 L dH2O, pH correction at 50°C to the value of 6,85 + 0,05, using NaOH 35%. Sterilization by autoclavation at 121°C for 15 min.

Supplementary material S2. Box-Behnken DoE

Box Behnken method for designing the experiment at three levels generated 15 experiments (Table S2-1). Three of them were replicates of the middle point (0, 0, 0). For the other experiments one factor was kept constant at the middle level, (0) and the other two factors were either the higher or lower level, (1, -1), in total four different combinations.

Table S2-1. Box-Behnken design of experiments generated by MATLAB R2019a using the function *bbdesign* with three variables and three levels

Condition	Temperature (°C)	pH	$k_L a$ (h^{-1})
1	30	5	47.5
2	30	7	47.5
3	44	5	47.5
4	44	7	47.5
5	30	6	0
6	30	6	74.4
7	44	6	0
8	44	6	74.4
9	37	5	0
10	37	5	74.4
11	37	7	0
12	37	7	74.4
13	37	6	47.5
14	37	6	47.5
15	37	6	47.5

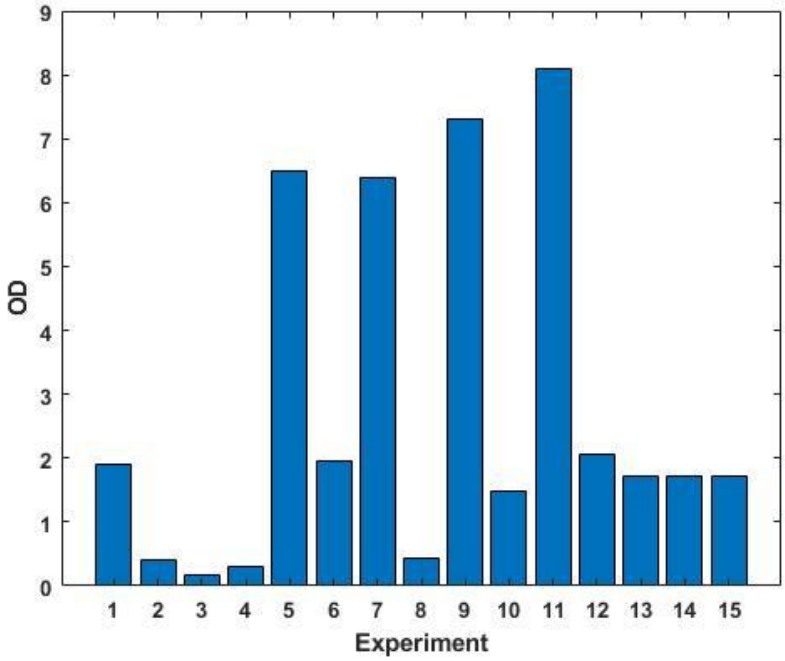

Figure S1-1. OD_{620} at 24h for conditions 1-15. Cells were cultivated for 24h in MRS under different conditions.

Supplementary material S3. Microscopic analysis of cell size

All the pictures were captured at 40X magnification with a phase contrast light microscope Leica DM750 (Leica Microsystems, Switzerland) integrated with phase turret condenser and phase contrast rings for each magnification. The pictures were captured with automatic exposure. Once the images were obtained, image processing was performed using free software package NIH ImageJ 1.52n. The first step is to take a picture of the scale was taken at 40X. This image is used for processing the length standard-length measuring unit such as µm. By using a straight-line tool, a known length of 100 µm was chosen. Then, a module named set scale was used where the conversion was made. Picture to be examined is loaded into the program. It is first converted to a 16-bit picture to distinguish more grey levels. Next step is to apply the Gaussian blur to reduce the background noise. Once the background noise is subdued, auto threshold is performed to highlight the cells from the dark background. The particles are analysed, and a summary table is obtained. Table consists of columns named Major which corresponds to the cell length and Minor which corresponds to the width.

Cell volume was calculated using length and width of the cell, assuming cells to have a cylinder shape. It was calculated by using equation 1, where w is the width and l is the length of the cell that was obtained from ImageJ 1.52n analysis.

$$V = \frac{\pi w^2 \left(\frac{l-w}{3}\right)}{4} \quad \text{..... Equation 1}$$

Table S3-1. Mean cell volume, and heterogeneity variables computed by microscopic analysis for conditions 1-15

Conditions	Mean cell volume (µm^3)	Skewness	CV	rCV
1	6.32	0.581	1.076	0.859
2	6.46	0.052	0.597	0.682
3	1.04	0.820	0.692	0.602
4	2.46	0.983	0.784	0.835
5	5.25	0.172	0.616	0.642
6	4.54	1.180	0.921	0.927
7	3.28	0.977	1.240	0.847
8	2.69	0.351	0.732	0.794
9	1.76	0.391	0.576	0.621
10	2.00	0.796	0.686	0.646
11	1.53	0.454	0.618	0.600
12	3.42	0.793	0.827	0.916
13	3.04	0.654	0.844	0.771
14	2.53	0.639	0.874	0.714
15	1.83	0.875	0.686	0.649

Supplementary material S4. Flow cytometry analysis

In this study, viability was studied using a mix of two dyes SYBR green and propidium iodide (PI). SYBR green binds to DNA of all the cells and PI is extensively used to evaluate cell membrane integrity (Bunthof et al., 2001).

Gating strategy to determine cell viability: FL1 vs FL3

First, the sample is cleaned by gating based on the scattering profiles (FSC and SSC) to remove the electronic noises. A log scale density plot of FL1-H vs FL3-H was obtained to visualize the fluorescence of the dyes. Live, damaged, and dead cells are gated in such a way that most of the population in all the samples are captured. Gating strategies and thresholds were kept the same for all the samples to attain comparable results.

Gating strategy and calculations of morphological descriptors

Mathematical functions such as Arithmetic mean, mode and median were used to evaluate average cell size based on the forward light scattering profile (Shapiro, 2000). Coefficient of variation (CV), Robust coefficient of variation (rCV) and skewness were evaluated for population heterogeneity. The FSC values required for the analysis were derived from the software FlowJo 10.6.

The skewness of the population distribution was performed by using mode and arithmetic mean. The mode of forward scatter was subtracted from the mean forward scatter resulting in positive value suggesting right skew or negative value suggesting negative skew (Heins et al., 2019).

Table S4-1. Morphological descriptors from FCM. Mean, skewness, CV, and rCV for FSC-H, and SSC-H, for conditions 1-15

Condition	Mean FSC-H	Mean SSC-H	Skewness FSC-H	CV FSC-H	rCV FSC-H	Skewness SSC-H	CV SSC-H	rCV SSC-H
1	4.988	4.196	0.163	1.853	1.800	0.444	2.076	2.097
2	5.085	4.195	0.203	1.846	1.827	0.444	2.100	2.149
3	4.794	3.953	-0.032	1.889	1.778	0.231	2.104	1.839
4	5.217	4.304	0.222	1.828	1.880	0.466	2.072	2.117
5	4.933	4.194	0.107	1.776	1.699	0.385	1.931	1.960
6	5.071	4.294	0.217	1.955	1.959	0.513	2.068	2.143
7	4.766	4.043	0.053	1.711	1.562	0.264	1.955	1.881
8	5.084	4.133	0.117	1.772	1.702	0.268	2.029	1.978
9	4.885	4.045	0.031	1.624	1.500	0.266	1.980	1.924
10	4.820	3.976	-0.063	1.897	1.839	0.225	2.124	1.799
11	4.854	4.037	0.028	1.683	1.529	0.259	2.255	1.894
12	5.060	4.157	0.121	1.819	1.723	0.405	2.041	2.093
13	4.795	4.018	-0.031	2.037	1.874	0.267	2.771	1.830
14	4.774	3.992	-0.052	2.029	1.866	0.269	2.710	1.802
15	4.778	3.988	-0.020	2.021	1.862	0.237	2.785	1.805

Table S4-2. Mean FSC, Mean SSC and Mean pulse width for cells cultivated at 30 ℃, 37 ℃ and 44 ℃.

Experiment	FSC mean	SSC mean	Mean pulse width
30 ℃	5.028	4.321	2.025
37 ℃	4.755	3.972	1.939
44 ℃	4.879	4.072	1.926
30 ℃ FD	5.026	3.842	1.994
37℃ FD	4.827	4.008	1.903
44℃ FD	4.815	4.066	1.863

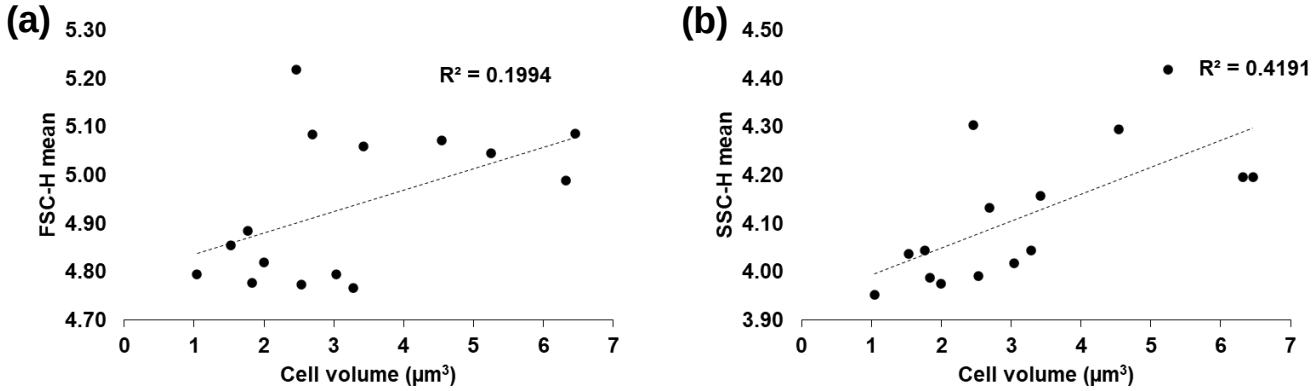

Figure S4-1. Correlations between Cell volume and (a) FSC-H mean, and (b) SSC-H mean. FSC-H mean obtained the poorest correlation with R^2 value of 0.1994, and mean SSC-H mean with R^2 value of 0.4191, respectively.

Gating strategy for subpopulations based on pulse-width

Heterogeneity was also assessed using pulse width descriptor. Peaks were gated using the create gates on peaks function. Five gates were found, and the gating was kept the same for all the samples to attain comparable results.

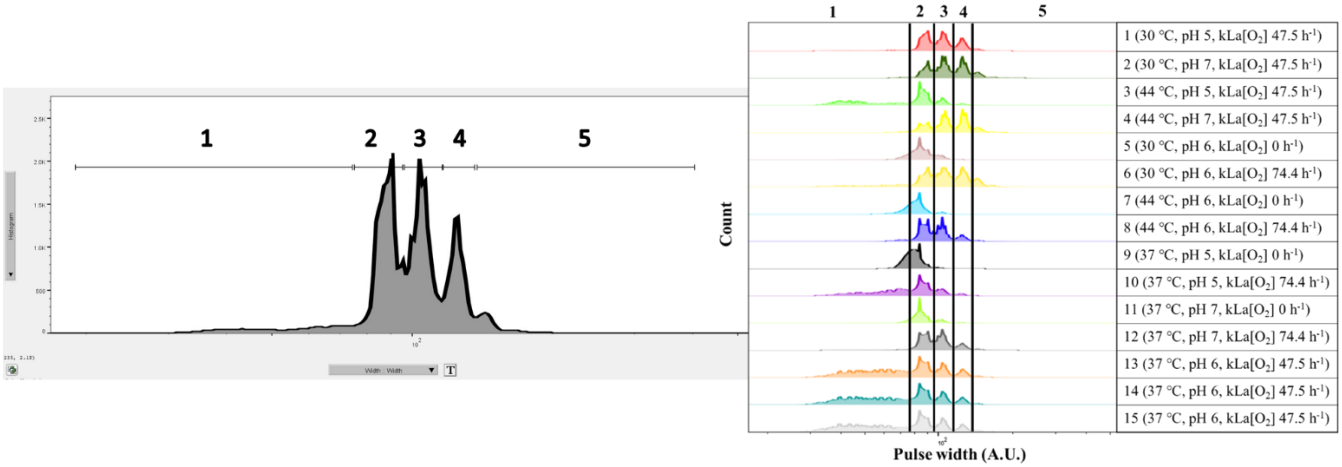

Figure S4-2. Gating performed based on peaks using FlowJo software on pulse width parameter. Histogram displaying gating performed for 15 conditions for total cells. Black line separates gates from each other.

Populations were divided based on pulse width, and tabulated. Also, individual populations such as live and dead cells were subjected to the same gating strategy.

Table S4-3. Population distribution based on pulse width gating for conditions 1-15

condition	Total cells (%)					Dead cells (%)					Live cells (%)				
	Gate 1	Gate 2	Gate 3	Gate 4	Gate 5	Gate 1	Gate 2	Gate 3	Gate 4	Gate 5	Gate 1	Gate 2	Gate 3	Gate 4	Gate 5
1	7.55	36.2	31.2	18.4	4.54	87.9	10.1	0.77	0.03	0.05	0.32	38.4	34	20.2	4.86
2	2.7	23.4	32.1	26.7	12.7	53.6	33.6	7.12	1.35	0.39	1.37	23.1	32.8	27.5	12.9
3	42.10	40.6	11.90	3.35	0.90	98.0	1.49	0.13	0.03	0.01	1.87	69.20	20.40	5.59	1.43
4	3.13	22.8	31.9	29.8	10.2	74.7	20.1	2.36	0.32	0.18	0.81	22.2	32.9	31.4	10.7
5	12.36	42.4	24.35	13.5	4.56	74.7	19.8	2.61	0.93	0.43	8.57	45.15	25.65	13.53	4.04
6	14.6	24.7	26.7	20.7	10.6	76	17.1	4.01	0.79	0.17	0.63	26	32	25.7	12.9
7	21.2	67.3	6.37	0.54	0.39	92.6	5.71	0.16	0.04	0.08	13	75	6.65	0.47	0.36
8	5.77	44.8	32.8	10.9	3.25	66.4	27.3	1.98	0.26	0.16	0.3	46.5	35.9	11.8	3.28
9	24.6	65.7	2.68	0.6	0.65	83.6	12.2	0.59	0.36	0.2	22.7	67.7	2.62	0.56	0.62
10	44.1	36.8	12.5	3.61	0.8	81.4	14.6	1.49	0.29	0.2	1.72	62.2	25.1	7.27	1.14
11	8.58	73.8	11.8	2.28	1.19	88.6	8.94	0.53	0.13	0.05	3.96	77.7	12.4	2.36	1.2
12	5.94	42	34.1	11.6	3.97	80.8	15	1.54	0.36	0.16	0.53	43.9	36.6	12.4	4.12
13	48	26.6	13.9	7.59	2.3	91.1	7.06	0.67	0.15	0.04	3.23	45.8	28.1	15.9	4.65
14	50.8	26.5	12.9	6.79	1.55	94.2	4.69	0.37	0.08	0.01	3.75	49.3	27	14.6	3.21
15	50	26.4	13.3	6.92	1.86	94.9	3.98	0.42	0.06	0.02	3.53	48.5	27.3	14.6	3.76

Table S4-4. Population distribution based on pulse width gating for cells cultivated at 30 ℃, 37 ℃ and 44 ℃.

condition	Total cells (%)					Dead cells (%)					Live cells (%)				
	Gate 1	Gate 2	Gate 3	Gate 4	Gate 5	Gate 1	Gate 2	Gate 3	Gate 4	Gate 5	Gate 1	Gate 2	Gate 3	Gate 4	Gate 5

30 ℃	12.36	42.4	24.35	13.5	4.56	74.7	19.8	2.61	0.93	0.43	8.57	45.15	25.65	13.53	4.04
37 ℃	13.32	71.9	9.62	2.20	1.15	72.8	23.4	1.02	0.29	0.27	9.49	75.33	10.09	2.24	1.08
44 ℃	26.7	54.1	14.5	1.82	0.39	70.6	24	1.16	0.22	0.12	0.72	73.2	21.8	2.61	0.35
30 ℃ FD	10.9	35	30.7	15.3	4.9	58.8	31.5	4.72	0.99	0.51	0.44	31.9	39.9	20.3	4.21
37 ℃ FD	36.7	50.3	4.68	2.46	2.94	88	7.06	1.53	1.15	0.74	9.77	77.4	5.22	1.61	2.38
44 ℃ FD	64.9	26.5	3.4	1.09	0.68	71.7	21.5	1.91	0.92	0.52	0.48	76.5	20.3	0.95	0.075

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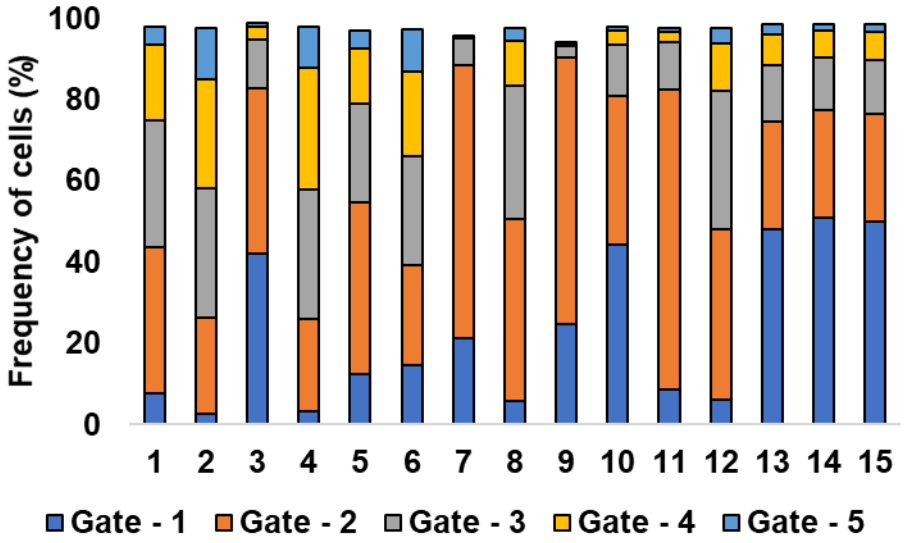
(a)



(b)

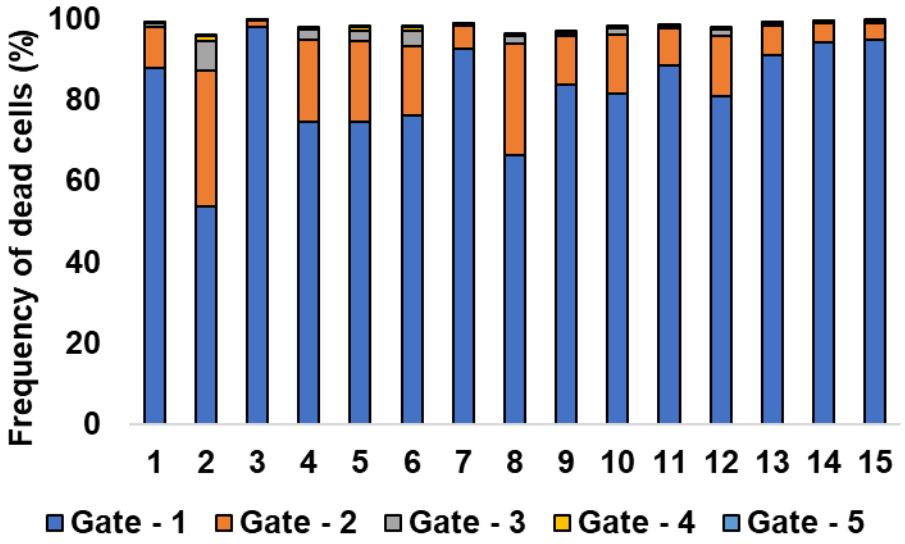


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Figure S4-3. Population distribution based on pulse width peaks for conditions 1-15. (a) Total cells, (b) Dead cells.

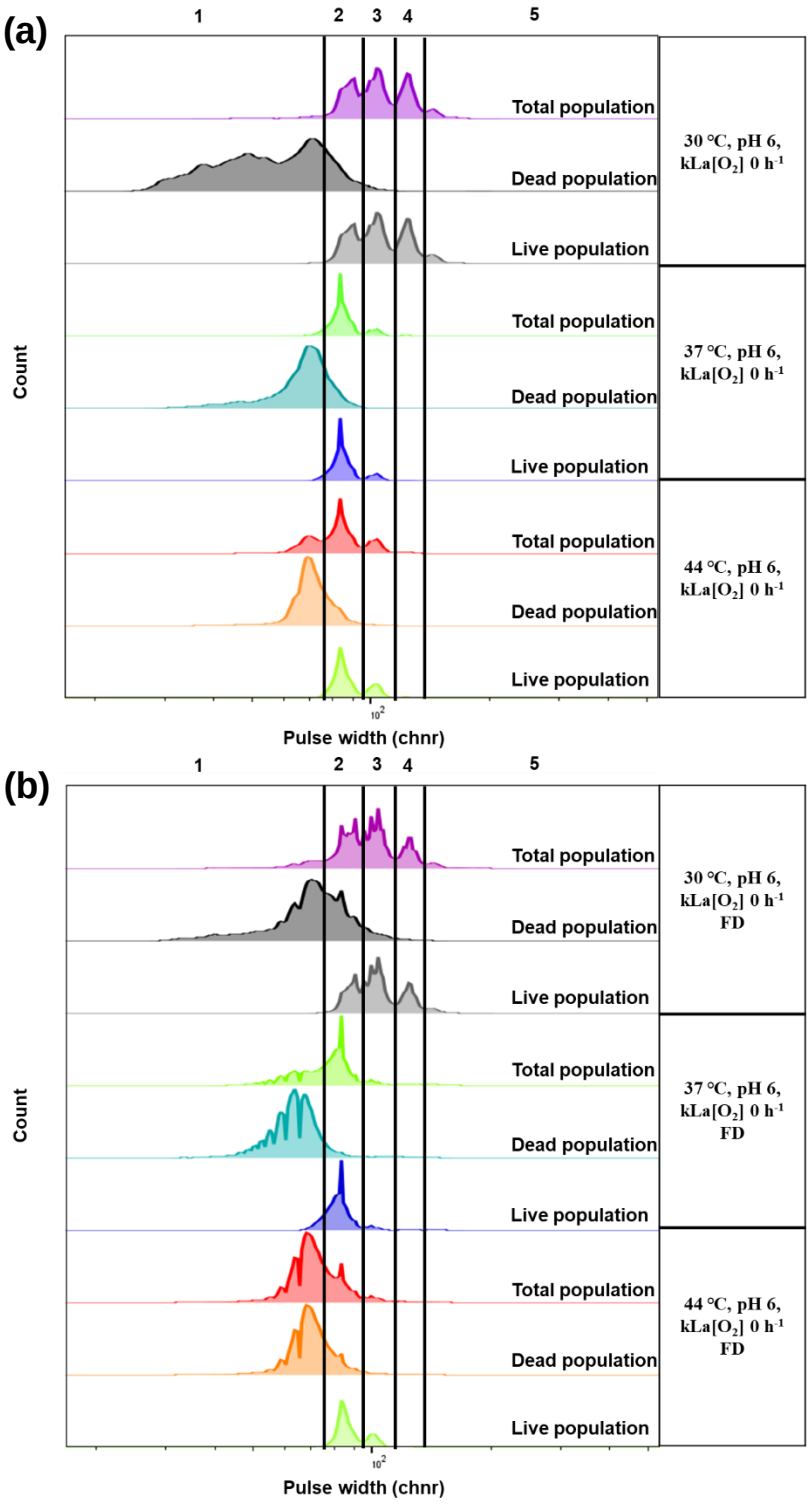


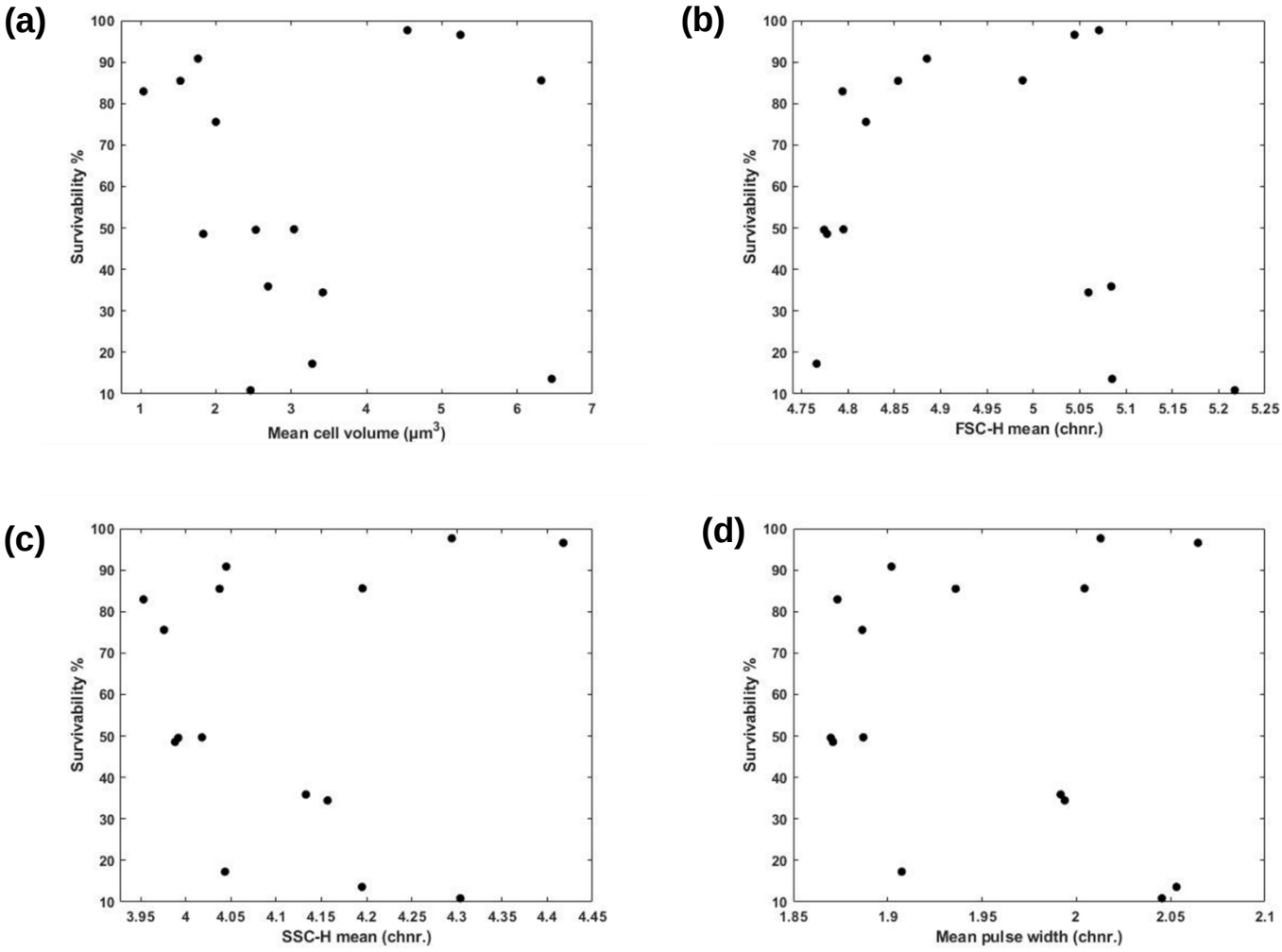
Figure S4-4. Comparison of difference in pulse width peaks for cells cultivated at 30 °C, 37 °C and 44 °C. (a) at harvest, (b) after FD. Line represents the separation between each gate.

Table S4-5. Three-way ANOVA for impact of temperature, pH and oxygen on heterogeneity variables

Source	Skewness-FSC	CV- FSC	rCV-FSC	Skewness – Microscope	CV – Microscope	rCV – Microscope
	p-value	p-value	p-value	p-value	p-value	p-value
T	0.0067	0.0094	0.0038	0.1745	0.3069	0.2848
pH	0.0151	0.0035	0.0074	0.4719	0.3028	0.3045
Oxy	0.0806	0.0009	0.0004	0.1787	0.8803	0.1492
T*pH	0.0561	0.0197	0.004	0.1439	0.1542	0.1862
T*Oxy	0.1052	0.0184	0.0134	0.0486	0.0928	0.1995
Oxy *pH	0.0283	0.0139	0.0074	0.8274	0.6723	0.1408
T*Oxy *pH	-	-	-	-	-	-

T- Temperature, Oxy- oxygen concentration

Supplementary material S5. Evaluation of correlations between survivability and cell morphology descriptors



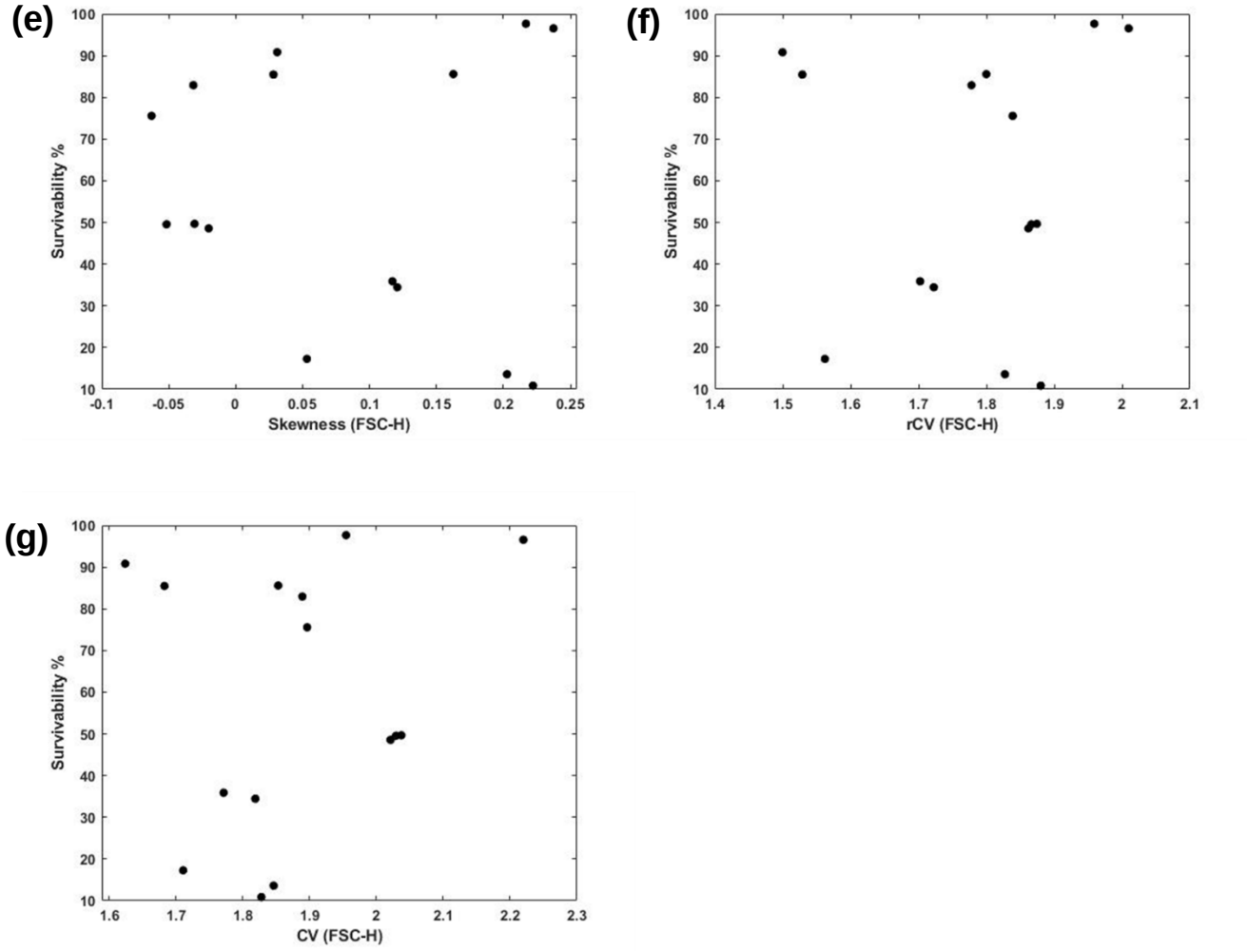


Figure S5. Morphology descriptors as a function of FT survivability for conditions 1-15 (a) Mean cell volume obtained from microscopic analysis, (b) Mean FSC, (c) Mean SSC, (d) Mean pulse width, (e) Skewness – FSC-H, (f) rCV – FSC-H, (g) CV – FSC-H.