

Supplemental material

Fig. S1. Temperature tolerance assay of parental strains and hybrids. All strains were spotted onto YPD 2% medium and incubated at different temperatures to assay their growth. Hybrids of the aromatic parents Y354 and Y397 were generated using mass mating of cells, whereas the other hybrids result from mass mating of spore cultures of Y181 and Y184 with Y115 and Y927 (H38: Y115-S1xY354-S6, H39: Y115-S2xY354-S6, H40: H28-S1xY397-S2, H41: H28-S2xY397-S2, H42: Y115-S3xY397-S2, H43: Y115-S4xY397-S2). All strains were tested in duplicate, of which only one representative is shown. Strains selected for field trials are highlighted in orange.

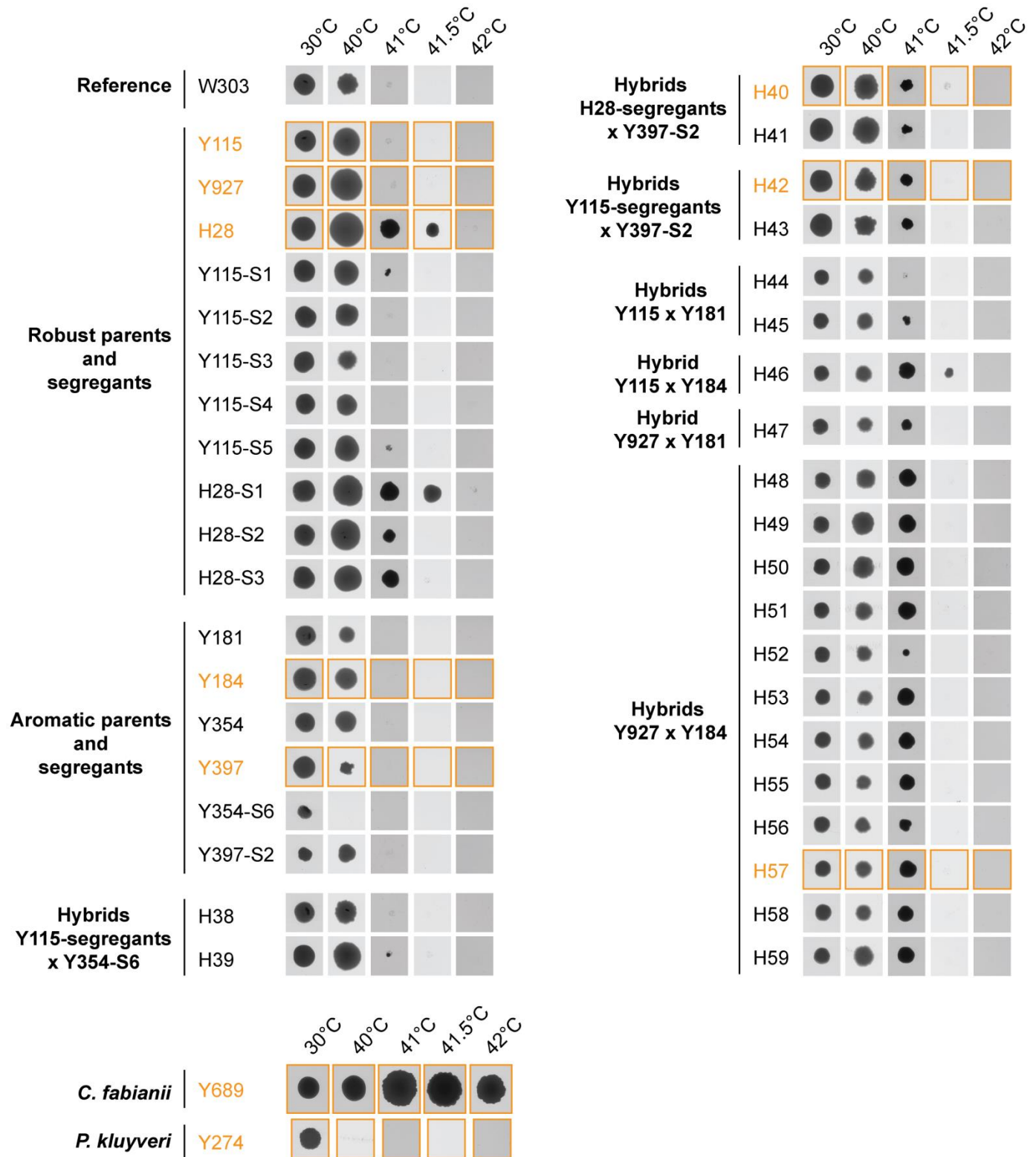


Fig. S2. PCR-based fingerprinting (“interdelta analysis”) distinguishes between inbreds and outcrossed hybrids generated using mass mating of spore cultures. The 90 strains selected after mass mating the robust parents Y115 and Y927 with the aromatic parents Y181 and Y184 were identified as 16 Y115 inbreds, 58 Y927 inbreds and 16 outcrossed hybrids (H44-H59: two Y115xY181, one Y115xY184, one Y927x181, and twelve Y927xY184). Diploids resulting from mating two cells of opposite mating types of the same parent, either germinating spores or haploid segregants, were classified as inbreds. A representative selection of these different groups is shown as an illustration. Strains selected for pilot-scale fermentations are indicated in orange.

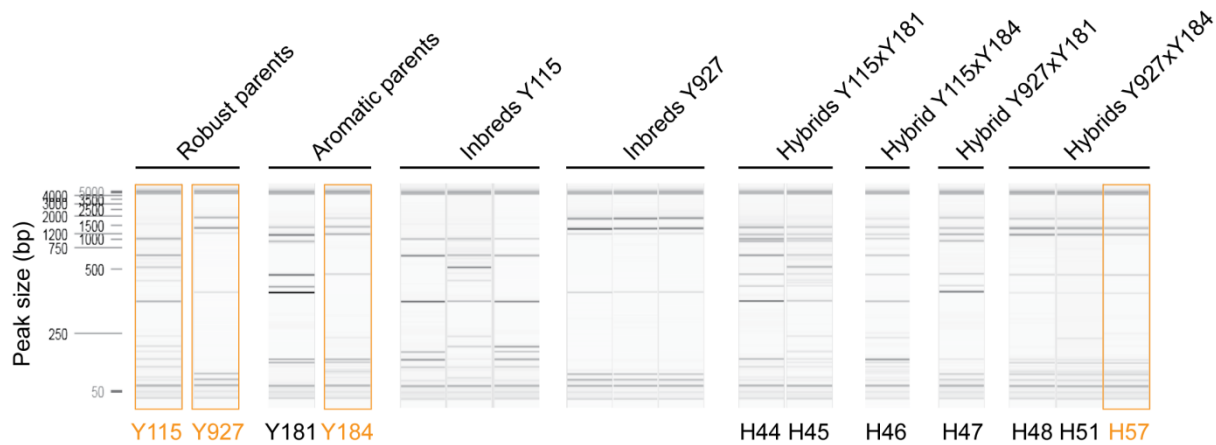


Fig. S3. Yeast population dynamics of inoculated fermentations in Malaysia. All yeasts were tested in two independent fermentation assays, graphs displayed here are the “B”-duplicates from the inoculated fermentations shown in Fig. 3. Temperature (right Y-axis) is indicated as a dashed black line. The dashed arrows indicate when the yeast cell count rises to or drops below the detection limit. Note that the scale for the total yeast count (left Y-axis) is logarithmic, whereas individual yeast species in the population at a given time point (indicated by the colors) are presented as the relative fraction of the total population. Absolute concentrations for individual species are given in Table S3. Percentages of identity are reported when <100% identity with type strain sequences (see Materials and Methods). Cocoa beans were turned 48 h of fermentation (solid vertical arrows). *Cyb.*: *Cyberlindnera*, *C.*: *Candida*, *H.*: *Hanseniaspora*, *P.*: *Pichia*, *S.*: *Saccharomyces*.

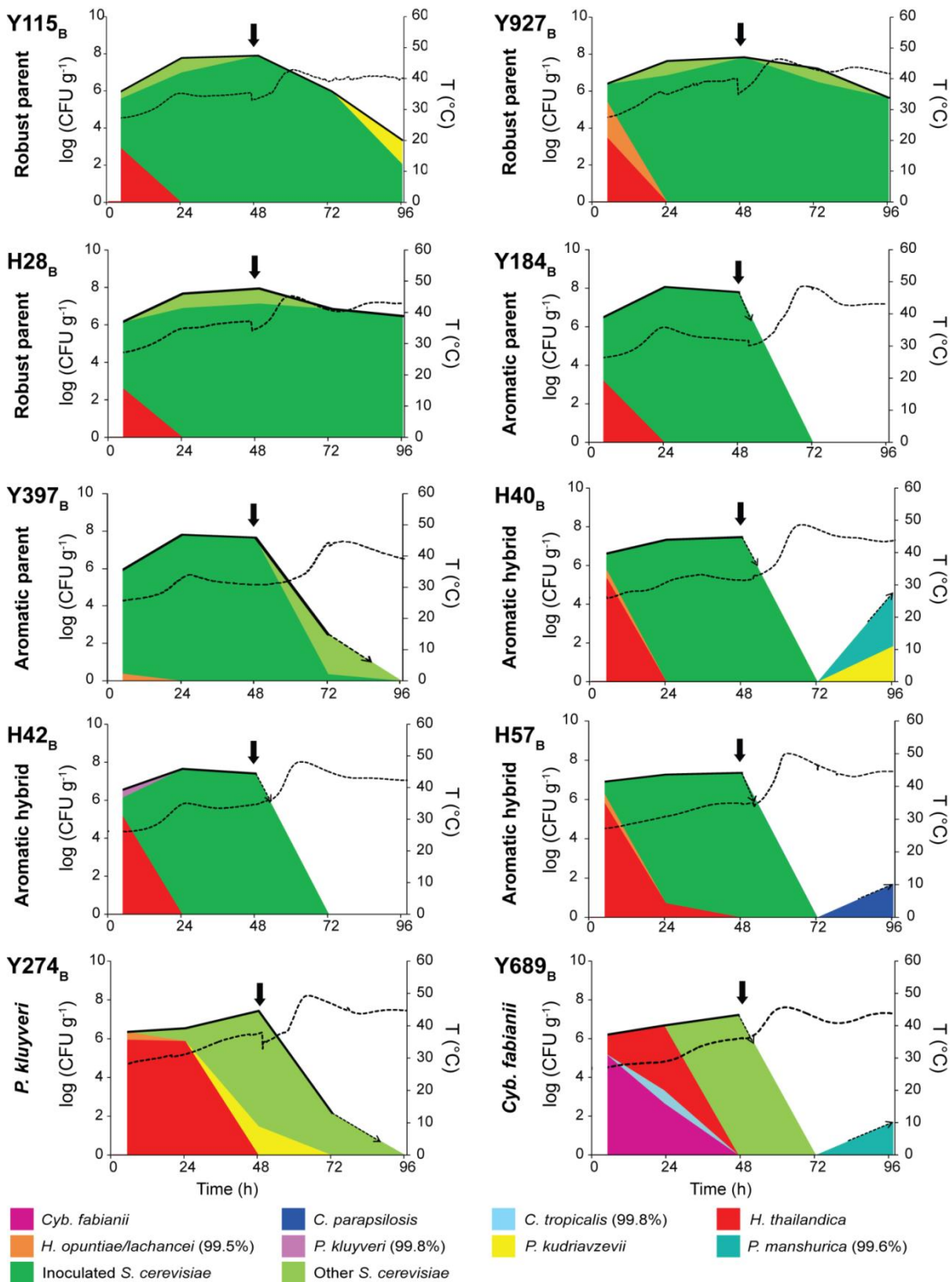


Fig. S4. Retention of acetate and ethyl esters during chocolate production from cocoa liquor. Values are represented as the averages of ten yeast inoculated fermentations and one spontaneous control \pm SD.

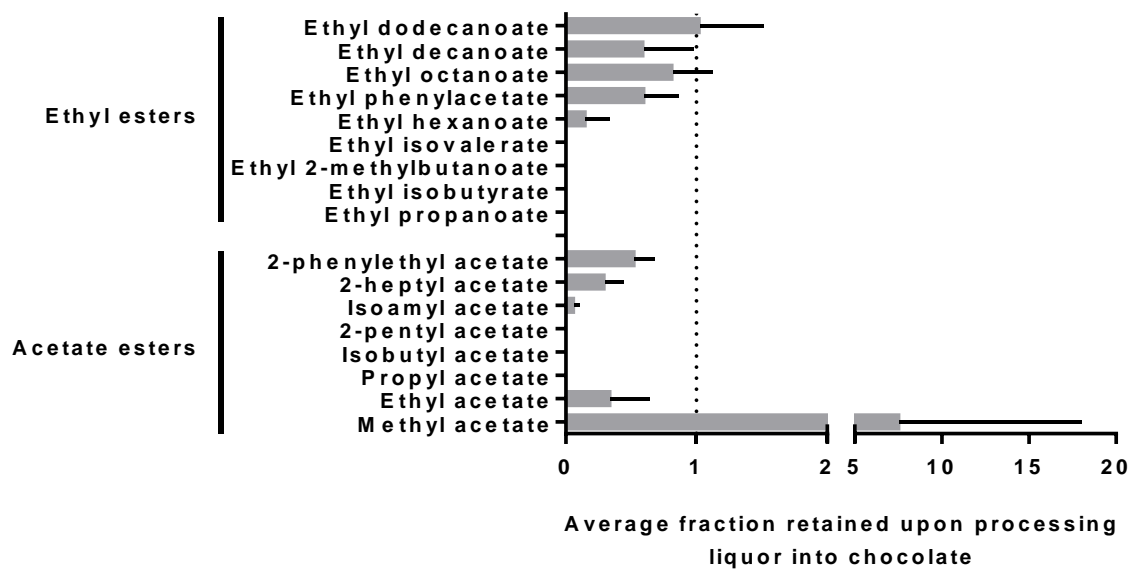


Table S1. Overview of hybrids between aromatic and robust parents generated in this study.

Hybrid	Robust parent	Aromatic parent	Diploids after mating (%)
H38	Y354-S6 (α)	Y115-S1 (a)	NA
H39	Y354-S6 (α)	Y115-S2 (a)	50
H40	Y397-S2 (a)	H28-S1 (α)	4
H41	Y397-S2 (a)	H28-S2 (α)	14
H42	Y397-S2 (a)	Y115-S3 (α)	10
H43	Y397-S2 (a)	Y115-S4 (α)	14
H44	Y115	Y181	NA
H45	Y115	Y181	NA
H46	Y115	Y184	NA
H47	Y927	Y181	NA
H48	Y927	Y184	NA
H49	Y927	Y184	NA
H50	Y927	Y184	NA
H51	Y927	Y184	NA
H52	Y927	Y184	NA
H53	Y927	Y184	NA
H54	Y927	Y184	NA
H55	Y927	Y184	NA
H56	Y927	Y184	NA
H57	Y927	Y184	NA
H58	Y927	Y184	NA
H59	Y927	Y184	NA

In case the hybrids were developed using mass mating of cells (H38-H43), the code and mating type of the haploid segregant (S) that served as parental strain is indicated.

Table S2. Overview of pH and temperature in spontaneous and inoculated fermentations

	T (°C)				pH			
	T _i	T _f	T _{min}	T _{max}	pH _i	pH _f	pH _{min}	pH _{max}
S _A	25.9	43.9	25.7	49.3	3.8	4.1	3.6	4.1
S _B	25.6	42.3	25.5	45.8	3.8	4.3	3.7	4.3
H28 _A	27.3	41.4	27.2	46.1	3.8	5.4	3.8	5.5
H28 _B	27.2	42.9	27.1	45.3	3.9	4.0	3.9	4.8
H40 _A	26.2	43.6	25.8	49.3	3.7	4.1	3.6	4.4
H40 _B	26.2	42.3	25.9	48.6	3.7	4.3	3.6	4.6
H42 _A	25.8	43.9	26.2	48.4	3.6	4.4	3.7	4.2
H42 _B	26.0	44.0	26.1	48.1	3.6	4.4	3.7	4.4
H57 _A	26.9	44.4	26.8	50.0	3.6	3.2	2.9	4.1
H57 _B	27.2	44.5	27.1	50.1	3.7	4.1	3.7	4.4
Y115 _A	27.6	41.7	27.5	45.9	3.8	4.3	3.7	4.7
Y115 _B	27.3	40.1	27.3	43.0	3.7	6.0	3.7	6.1
Y184 _A	26.7	43.1	26.7	46.9	3.5	4.3	3.5	4.7
Y184 _B	26.5	43.0	26.4	48.6	3.5	4.4	3.5	4.4
Y397 _A	26.5	43.1	26.4	49.1	3.7	4.2	3.6	4.2
Y397 _B	25.8	39.1	25.7	44.7	3.5	4.2	3.5	4.5
Y927 _A	30.0	41.2	27.3	45.6	3.7	4.7	3.6	4.7
Y927 _B	27.5	41.7	27.5	46.4	3.6	4.7	3.6	4.7
Y274 _A	27.5	44.1	27.5	51.5	3.6	4.2	3.6	4.2
Y274 _B	27.0	44.7	27.0	49.3	3.5	3.8	3.5	4.3
Y689 _A	26.6	43.3	26.5	45.8	3.5	4.1	3.5	4.2
Y689 _B	26.6	43.9	26.6	45.7	3.7	4.1	3.7	4.3

Different parameters are listed for each fermentation: pH_i = initial pH, pH_f = final pH, pH_{min} = minimal pH, pH_{max} = maximal pH, T_i = initial temperature, T_f = final temperature, T_{min} = minimal temperature, T_{max} = maximal temperature.

Two spontaneous fermentations (S_A and S_B) and 20 inoculated fermentations, 10 yeast strains tested in duplicate, indicated by the subscripts A and B.

Table S3. Population sizes of the inoculated and most prevalent yeast species during spontaneous and inoculated cocoa pulp fermentations.

	Time (h)	log (CFU g ⁻¹)																					
		H28 _A	H28 _B	H40 _A	H40 _B	H42 _A	H42 _B	H57 _A	H57 _B	S _A	S _B	Y115		Y184		Y274		Y397		Y689		Y927	
												A	B	A	B	A	B	A	B	A	B	A	B
<i>H. thailandica</i>	4	5.81	5.78	6.17	6.53	5.77	6.43	6.06	6.84	5.18	5.84	5.36	5.65	5.88	6.18	6.05	6.32	5.70	-	5.95	5.38	5.85	6.12
	24	-	-	-	-	-	-	6.70	6.27	6.11	5.52	-	-	-	-	7.04	6.49	6.64	-	6.31	6.38	7.20	-
	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. cerevisiae</i> (inoculated)	4	5.81	5.89	6.00	5.71	5.55	5.62	5.81	5.73	-	-	5.83	5.59	5.94	6.18	-	-	5.76	5.86	-	-	5.85	5.47
	24	7.77	7.63	7.69	7.33	7.48	7.65	7.65	7.23	-	-	7.59	7.73	7.71	8.07	-	-	7.19	7.81	-	-	7.57	7.58
	48	7.48	7.89	7.68	7.47	-	7.41	7.26	7.36	-	-	7.92	7.90	7.72	7.79	-	-	6.51	7.66	-	-	7.71	7.83
	72	6.41	6.85	-	-	-	-	-	-	-	-	5.71	5.98	-	-	-	-	-	1.63	-	-	6.59	7.17
	96	5.34	6.47	-	-	-	-	-	-	-	-	3.32	3.14	-	-	-	-	-	-	-	-	7.07	5.64
<i>S. cerevisiae</i> (other)	4	5.11	-	-	-	4.60	-	-	-	4.63	-	-	4.75	-	-	-	-	-	-	-	-	-	-
	24	-	6.67	-	-	-	-	-	-	5.72	5.82	6.63	6.78	-	-	-	5.54	6.34	-	-	-	-	6.63
	48	-	6.94	-	-	-	-	-	-	7.14	7.25	7.55	-	-	-	7.32	7.33	-	-	6.90	7.22	-	-
	72	-	-	-	-	-	-	-	-	-	-	5.35	-	2.80	-	-	1.70	-	2.41	-	-	5.64	6.22
	96	4.19	-	-	-	-	-	-	-	1.90	2.54	-	-	-	-	3.54	-	-	-	2.65	-	-	-
<i>P. kluyveri</i>	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.27	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cyb. fabianii</i>	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.25	6.12	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.49	6.28	-	-
	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The relative populations sizes of the different yeast species were calculated based on a representative number of yeast isolates taken per time point (see Materials and Methods for details). Multiplication by the total yeast population size led to the absolute population sizes, displayed as log(CFU g⁻¹). Interdelta analysis was used to differentiate between the inoculated and other *S. cerevisiae* strains. S: spontaneous fermentation, Y274: *P. kluyveri*, Y689: *Cyb. fabianii*, all other strains are *S. cerevisiae*. *H.*: *Hanseniaspora*, *S.*: *Saccharomyces*, *P.*: *Pichia*, *Cyb.*: *Cyberlindnera*.

Table S4. Overview of all detected lactic and acetic acid bacterial species in the different cocoa pulp fermentations in Malaysia.

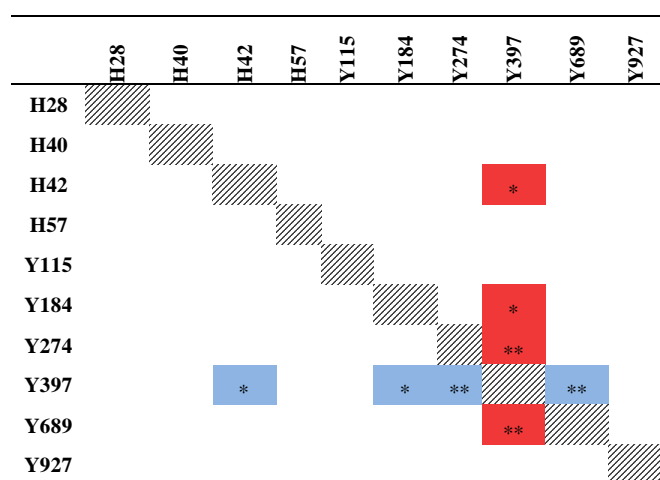
	N° isolates	Time point (h)				
		4	24	48	72	96
Lactic acid bacteria						
Inoculated						
<i>Lactobacillus fermentum</i>	38	x	x	x	x	x
<i>Leuconostoc pseudomesenteroides</i> (99.6%)	34	x	x	x	x	x
<i>Lactobacillus plantarum/paraplantarum/pentosus/fabifermentans</i> (99.6%)	33	x	x	x	x	x
<i>Fructobacillus pseudoficulneus</i>	28	x	x	x		
<i>Lactobacillus brevis</i> (99.6%)	9		x	x	x	
<i>Lactobacillus amylovorus/ultunensis</i>	7	x	x			x
<i>Fructobacillus tropaeoli</i>	4			x	x	
<i>Lactobacillus mali/aquaticus/hordei/uvarum</i> (99.6%)	2			x	x	
<i>Lactobacillus rhamnosus</i>	1	x				
<i>Weissella fabaria/fabalis/ghanensis/beninensis</i>	1	x				
<i>Lactobacillus casei/paracasei</i>	1					x
Spontaneous						
<i>Lactobacillus fermentum</i>	17	x	x	x	x	x
<i>Lactobacillus plantarum/paraplantarum/pentosus/fabifermentans</i> (99.6%)	4	x	x	x		
<i>Lactobacillus brevis</i> (99.6%)	3		x	x		
<i>Leuconostoc pseudomesenteroides</i> (99.6%)	2		x			
<i>Fructobacillus tropaeoli</i>	1			x		
Acetic acid bacteria						
Inoculated						
<i>Acetobacter pasteurianus</i>	175	x	x	x	x	x
<i>Acetobacter tropicalis/sicerae</i> (99.8%)	24	x	x	x		x
<i>Acetobacter ghanensis</i>	4			x		
Spontaneous						
<i>Acetobacter pasteurianus</i>	22	x	x	x	x	x
<i>Acetobacter tropicalis/sicerae</i> (99.8%)	5	x	x	x		

All fermentations were performed in October-November 2013 in Malaysia. Data is collected from 20 inoculated and 2 spontaneous fermentations. Bacterial sequences were compared to type strain sequences using nucleotide-nucleotide BLAST (nblast) and the EzTaxon database (1), percentages of identity are reported when <100% (see Materials and Methods).

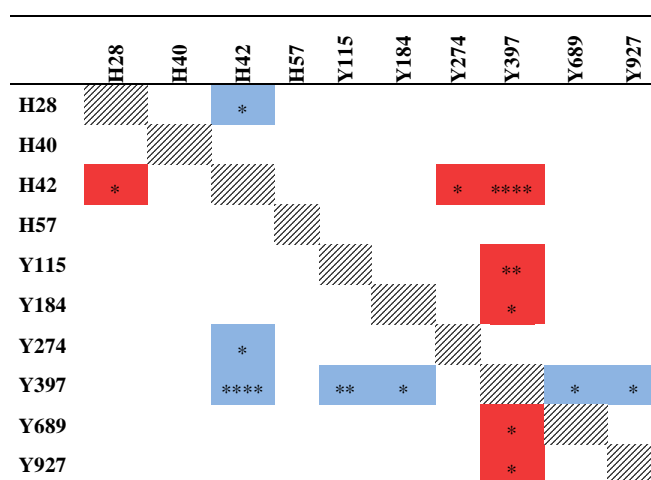
Table S5. Sensory analysis by an expert panel of chocolate from inoculated fermentations.

	Bitter	Cocoa	Floral	Fruity	Roasted	Sour	Vegetal	Yellow fruits
H28	1.5±0.3	3.1±0.8	0.3±0.4	0.6±0.2	2.5±0.3	1.6±0.6	0.3±0.2	0.4±0.5
H40	1.6±0.5	3.3±0.8	0.4±0.6	1.8±0.7	2.4±0.8	1.6±0.5	0.8±0.4	0.7±0.7
H42	1.9±0.7	3.8±0.4	0.1±0.4	0.8±0.3	2.9±0.7	1.1±0.4	0.3±0.3	0.4±0.7
H57	1.7±0.6	3.2±0.4	0.7±1.3	1.0±0.4	2.0±0.9	1.6±0.5	1.0±0.6	0.3±0.7
Y115	1.5±0.0	3.5±0.0	1.0±0.0	1.5±0.0	2.5±0.0	1.5±0.0	1.0±0.0	1.0±0.0
Y184	1.9±1.0	3.4±0.4	0.1±0.2	1.6±0.5	2.2±1.1	2.0±0.5	0.3±0.2	0.4±0.6
Y274	2.0±0.8	3.1±0.5	0.6±0.9	1.1±0.4	2.1±0.6	1.6±0.7	1.1±0.5	0.3±0.5
Y397	1.2±0.6	2.7±0.9	0.4±0.5	0.5±0.3	1.7±0.8	1.1±0.4	0.8±0.3	0.4±0.6
Y689	2.0±0.5	3.4±0.4	0.7±0.7	0.7±0.4	2.4±0.6	1.4±0.7	1.2±0.4	0.2±0.4
Y927	1.6±0.3	3.4±0.3	0.3±0.6	0.9±0.3	2.7±0.4	1.7±0.3	0.5±0.3	0.6±0.5

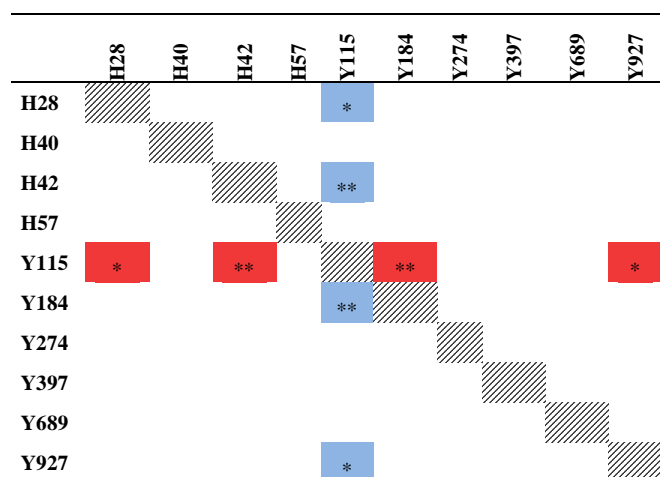
Bitter



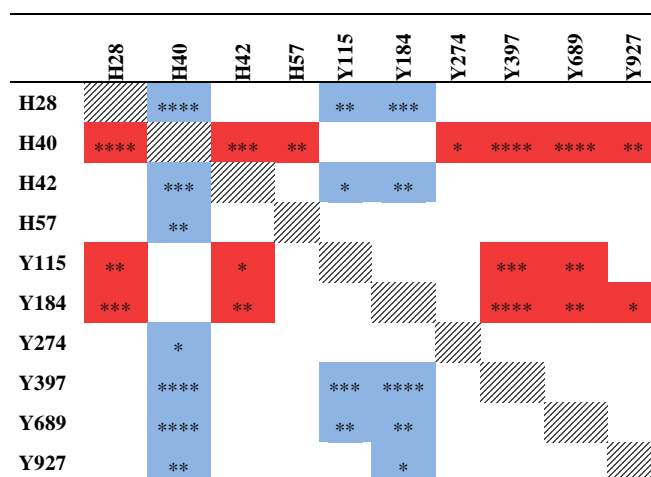
Cocoa



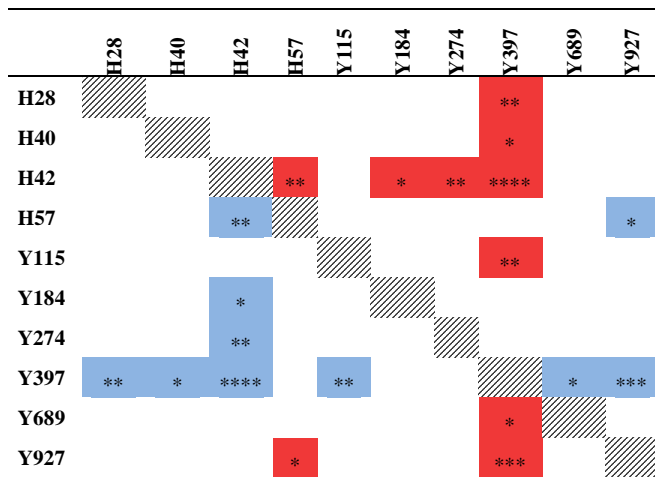
Floral



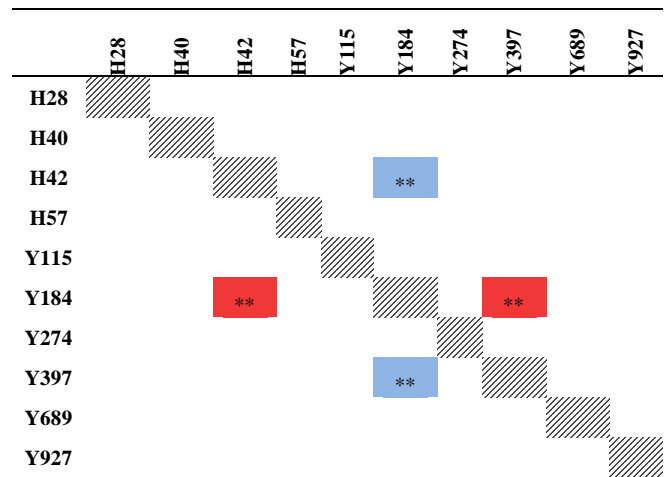
Fruity



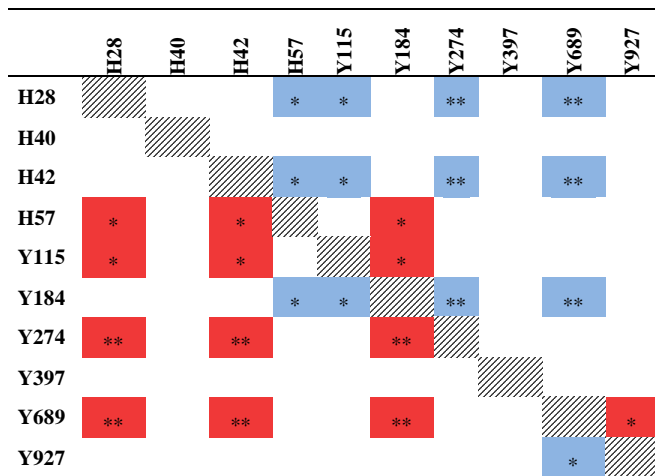
Roasted



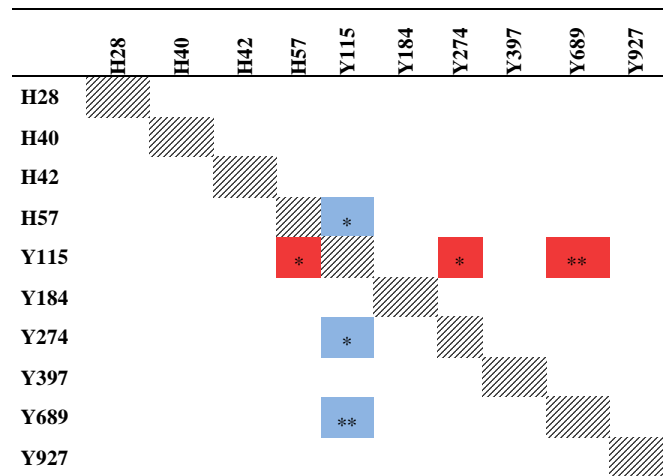
Sour



Vegetal



Yellow fruits



All inoculated yeasts are *Saccharomyces cerevisiae* strains, except for Y274 (*Pichia kluyveri*) and Y689 (*Cyberlindnera fabianii*). The expert panel consisted of nine experienced panelists, who were asked to score all categories from 0 to 5, half points were also allowed. Values represent average concentrations \pm SD. All pair-wise significant differences are shown calculated using Tukey's multiple comparisons test, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$. A red color indicates that the strain displayed left had a significantly higher score, whereas a blue color indicates that the strain displayed on top scored significantly higher.

References

1. **Kim O-S, Cho Y-J, Lee K, Yoon S-H, Kim M, Na H, Park S-C, Jeon YS, Lee J-H, Yi H, Won S, Chun J.** 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**:716–21.