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GROWTH TEMPERATURES AND ELECTROPHORETIC KARYOTYPING AS TOOLS FOR PRACTICAL DISCRIMINATION OF *SACCHAROMYCES BAYANUS* AND *SACCHAROMYCES CEREVISIAE*

MUNEKAZU KISHIMOTO* AND SHOJI GOTO[1]

Technology Department, Sapporo Wines Ltd., Higashiyamanashi-gun, Yamanashi 409–13, Japan
[1] *The Institute of Enology and Viticulture, Yamanashi University, Kofu 400, Japan*

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Growth temperatures, fermentation characteristics and electrophoretic karyotype of sixteen strains of *Saccharomyces bayanus* and nine strains of *Saccharomyces cerevisiae* were examined. Growth temperatures of 1 and 2°C accompanied by 35°C clearly distinguished these two species, and also fermentation characteristics, such as fermentation velocity at a low temperature (7°C) and ethanol yield for fermentation at an intermediate temperature (28°C), supported this distinction. Additionally, in pulsed-field gel electrophoresis under the conditions for separating large DNA molecules, specific chromosomal bands were observed in each of the two species. From these results, it was concluded that growth temperatures and electrophoretic karyotyping were convenient tools for practical discrimination of the two species.

Recently, it was recognized that *Saccharomyces* sensu stricto yeasts, which were grouped by Yarrow (*20*) under *Saccharomyces cerevisiae*, can be separated on the basis of DNA similarity into four species: *S. cerevisiae*, *S. bayanus*, *S. pastorianus* and *S. paradoxus* (*13–15*). This separation is also supported by the fact that interspecific hybrids of *S. cerevisiae*, *S. bayanus*, and *S. paradoxus* produce non-viable ascospores (*9*). Additionally, Yamada et al. (*18*) reported that identification based on utilization of sugars did not correspond to the classification based on DNA similarity. Therefore, it is necessary to establish practical methods to identify the industrial yeast *S. cerevisiae* and its related yeasts more efficiently.

We previously reported that the seven strains of cryophilic wine yeasts, which were classified as *S. bayanus* on the basis DNA similarity in photobiotin microplate hybridization, exhibit the following growth and fermentation characteristics: non-

* Address reprint requests to: Dr. Munekazu Kishimoto, Technology Department, Sapporo Wines Ltd., 577 Watazuka, Katsunuma, Higashiyamanashi-gun, Yamanashi 409–13, Japan.

growth above 35°C, 2–3 times faster fermentation velocity at an early stage than that of mesophilic wine yeasts *S. cerevisiae* at a low temperature (7°C), and premature cessation of fermentation and a reduction in the yield of ethanol at intermediate temperatures (22–30°C) (*5–8*).

In this study, growth temperatures, fermentation characteristics and electrophoretic karyotyping of sixteen strains of *S. bayanus* and nine strains of *S. cerevisiae* were examined to evaluate the possibility of utilization as tools for practical discrimination of these two species. We also re-identified twelve strains of wine, sherry, sake and brewer's yeasts on the basis of growth temperatures, fermentation characteristics and electrophoretic karyotyping.

MATERIALS AND METHODS

Strains. The sixteen strains of *Saccharomyces bayanus* and nine strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. All of these strains were classified on the basis of DNA similarity by the photobiotin microplate hybridization method (*3, 8, 18, 19*). Twelve strains of wine, sherry, sake and brewer's yeasts (Table 3) were used for re-identification by growth temperatures, fermentation characteristics and electrophoretic karyotyping.

Growth test. For the growth tests, approximately 1×10^3 cells from each seed culture were spread on a YM (glucose 1%, polypeptone 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 2%) plate, and then cultivated at both 1 and 2°C for four weeks or at 35°C for a week in a forced convection system low-temperature incubator LTI-600D (Tokyo Rikakikai, Tokyo, Japan).

Fermentation test. To estimate the fermentation velocity and ethanol yield, fermentation tests were carried out in 200 ml of PYG-F (glucose 18%, polypeptone 0.75%, yeast extract 0.45%, pH 4.5) liquid medium according to a previous report (*6*). Fermentation velocity was expressed as the weight of CO_2 evolved, and ethanol yield at the end of fermentation was measured by gas chromatography.

Pulsed-field gel electrophoresis. The Crossfield system AE-6800 (Atto, Tokyo, Japan) was employed to separate the chromosomal DNA. Sample plugs of chromosomal DNA were prepared by the method of Carle and Olson (*2*) from yeast cells grown in 5 ml of YPD (glucose 2%, polypeptone 2%, yeast extract 1%) liquid medium. Electrophoresis was carried out at 180 V for 20 h with a switching interval of 70 s, and then at 120 V for 20 h with a switching interval of 250 s to separate the large DNA molecules (*8*).

RESULTS AND DISCUSSION

Growth temperatures and fermentation characteristics

Growth temperatures and fermentation characteristics of the sixteen strains of *S. bayanus* were compared with the nine strains of *S. cerevisiae.* The results are shown in Table 2. All sixteen strains of *S. bayanus* were able to grow at 1 and 2°C,

Table 1. Strains used in this study.

Strain No.[a]	Labeled name	DNA similarity		G+C mol%
		S. cerevisiae IFO 10217	*S. bayanus* IFO 1127	
Saccharomyces bayanus				
IFO 1127[b]	*S. bayanus*[T]	39	100	39.0
RIFY 1071 (YM-27)[b]	*S. uvarum*	38	82	39.2
RIFY 1112 (YM-82)[b]	*S. uvarum*	43	88	39.3
RIFY 1113 (YM-83)[b]	*S. uvarum*	42	98	39.4
RIFY 1114 (YM-84)[b]	*S. uvarum*	29	86	39.0
RIFY 1218 (YM-126)[b]	*S. uvarum*	32	82	38.9
RIFY 7143 (NZ-681)[b]	*S. chevalieri*	30	80	39.4
IAM 12242 (YM-94)[b]	*S. uvarum*	31	79	39.5
IFO 0213[c]	*S. cerevisiae*	38	84	40.0
IFO 0251[c]	*S. cerevisiae*	43	91	40.2
IFO 0573[c]	*S. uvarum*	31	85	40.1
IFO 0676[d]	*S. pastorianus*	45	101	ND[f]
IFO 1048[c]	*S. heterogenicus*	48	86	38.1
IFO 1344[d]	*S. bayanus*	33	97	ND
IFO 1948[c]	*S. pastorianus*	39	97	39.0
IFO 2031[e]	*S. bayanus*	26	94	39.9
Saccharomyces cerevisiae				
IFO 10217[b]	*S. cerevisiae*[T]	100	41	37.1
RIFY 1001 (W3)[b]	*S. cerevisiae*	81	37	36.7
IAM 4274 (OC-2)[b]	*S. cerevisiae*	95	41	36.4
IAM 4206 (YM-95)[b]	*S. carlsbergensis*	79	35	37.9
IFO 0290[c]	*S. uvarum*	82	42	38.1
IFO 0291[c]	*S. uvarum*	81	38	38.1
IFO 1661[c]	*S. cerevisiae*	91	42	37.7
IFO 1662[c]	*S. cerevisiae*	128	39	37.4
IFO 2359[e]	*S. cerevisiae*	85	28	37.0

[a] RIFY, The Institute of Enology and Viticulture, Yamanashi University, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Osaka, Japan.

[b,c,d,e] DNA similarity value by photobiotin microplate hybridization method and G+C content of DNA by HPLC analysis of the strains are cited from Refs. *8*, *19*, *3* and *18*), respectively.

[f] Not determinded.

[T], type strain.

but were unable to grow at 35°C. The maximum growth temperature for each was 34°C or lower. However, all of the nine strains of *S. cerevisiae* were unable to grow at 1 and 2°C, but were able to grow at 35°C and often up to 40–42°C. In fermentation at 7°C, the CO_2 evolution velocities of *S. bayanus* ranged from 4.0 to 5.8 g during the first 20-day period, and these values were 1.5–4.5 times higher than those of *S. cerevisiae.* On the other hand, the ethanol yields of *S. bayanus* in fermentation at 28°C were reduced to 3.9–6.5%. In contrast, all *S. cerevisiae* but the type strain IFO 10217 showed high yields of ethanol. From these results, it was suggested that *S. bayanus* was a species suitable for growth and fermentation at low

Table 2. Growth temperatures and fermentation characteristics of *Saccharomyces bayanus* and *Saccharomyces cerevisiae*.

Strain No.	Growth[a]			Fermentation velocity at 7°C[b] (CO_2 g/200 ml for 0–20 days)	EtOH yield[b] (v/v %) at	
	1°C	2°C	35°C		7°C	28°C
S. bayanus						
IFO 1127[T]	+	+	−	4.9	10.3	5.1
RIFY 1071	+	+	−[c]	4.2[c]	10.3[c]	4.4[c]
RIFY 1112	+	+	−[c]	4.1[c]	10.2[c]	5.9[c]
RIFY 1113	+	+	−[c]	4.0[c]	10.2[c]	5.7[c]
RIFY 1114	+	+	−[c]	5.8[c]	10.3[c]	3.9[c]
RIFY 1218	+	+	−[c]	4.7[c]	10.3[c]	6.5[c]
RIFY 7143	+	+	−[c]	5.6[c]	10.3[c]	4.5[c]
IAM 12242	+	+	−[c]	4.6[c]	10.3[c]	5.3[c]
IFO 0213	+	+	−	4.8[d]	10.4[d]	4.9[d]
IFO 0251	+	+	−	4.8[d]	10.4[d]	5.2[d]
IFO 0573	+	+	−	5.4[d]	10.4[d]	4.7[d]
IFO 0676	+	+	−	4.5[d]	10.4[d]	4.7[d]
IFO 1048	+	+	−	4.6[d]	10.2[d]	4.9[d]
IFO 1344	+	+	−	5.6[d]	10.3[d]	5.3[d]
IFO 1948	+	+	−	5.0[d]	10.2[d]	4.0[d]
IFO 2031	+	+	−	4.7[d]	10.4[d]	4.9[d]
S. cerevisiae						
IFO 10217[T]	−	−	+	1.5	ND[e]	7.8
RIFY 1001	−	−	+[c]	2.4[c]	10.2[c]	9.8[c]
IAM 4274	−	−	+[c]	2.2[c]	10.1[c]	10.1[c]
IAM 4206	−	−	+	1.7	10.1	9.7
IFO 0290	−	−	+	2.6	10.2	10.4
IFO 0291	−	−	+	1.3	ND	10.2
IFO 1661	−	−	+	2.4	10.2	10.2
IFO 1662	−	−	+	1.8	ND	10.1
IFO 2359	−	−	+	2.4	10.1	10.3

[a] Growth ability on YM plate at the indicated temperatures. Symbols: +, growth (more than one hundred colonies were detected on the plate); −, non-growth (no colonies were detected on the plate).

[b] Fermentation was carried out in 200 ml of PYG-F liquid medium. Fermentation velocity is expressed as the weight of CO_2 evolved and ethanol yield was measured at the end of fermentation.

[c,d] The data of growth at 35°C, fermentation velocity and ethanol yield are cited from Refs. 5 and 8), respectively.

[e] ND, not determined because it took more than 130 days to finish fermentation.

[T], type strain.

temperatures, and these growth temperatures and fermentation characteristics were considered important to distinguish between these two species. Vaughan-Martini and Martini (*16*) examined growth at 34–37°C, growth without a vitamin supplement (vitamin-free medium), the presence of an active transport mechanism for fructose and assimilation of D-mannitol of the four species *S. cerevisiae*, *S. bayanus*, *S. pastorianus* and *S. paradoxus*; they recognized *S. bayanus* was the species which was unable to grow above 35°C, was able to grow without vitamin supplement and

possessed an active transport mechanism for fructose. The non-growth above 35°C of *S. bayanus* determined in this study was in agreement with their assertion. Additionally, growth at 1 and 2°C accompanied by non-growth at 35°C ensured distinction by growth temperature, and also fermentation characteristics such as fermentation velocity at low temperatures and ethanol yield at intermediate temperatures supported this distinction. However, a few strains of *S. bayanus* tested in this study were unable to grow without a vitamin supplement (data not shown), and this result did not agree with the finding of Vaughan-Martini and Martini (*16*).

Electrophoretic karyotypes

We previously reported that the cryophilic wine yeast *S. bayanus* and the mesophilic wine yeast *S. cerevisiae* possess specific chromosomal bands which correspond to the respective type strains of the species (Fig. 1) (*8*). In this study, electrophoretic karyotypes of *S. bayanus* were compared with those of *S. cerevisiae.* The results are shown in Fig. 2. The electrophoretic karyotypes of *S. bayanus* were similar to the type strain of *S. bayanus* IFO 1127, although some differences in numbers and mobilities of chromosomal bands were observed among the species. Especially, two specific chromosomal bands, **a** (between chromosome IV and XV, VII of *S. cerevisiae* YNN 295) and **b** (between chromosome XV, VII and XVI of *S. cerevisiae* YNN 295), indicated by arrows, were observed in all of *S. bayanus*, and it was considered that the electrophoretic karyotype of *S. bayanus* was character-

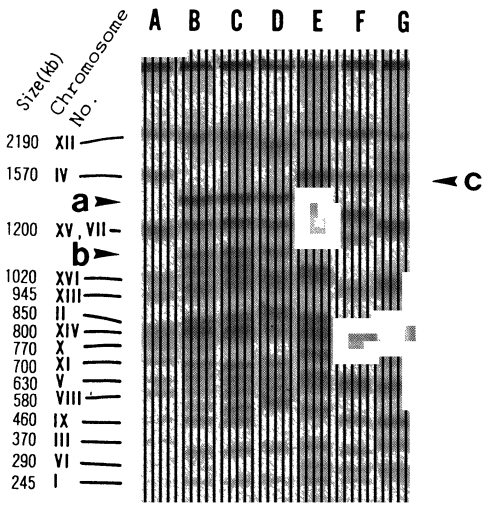

Fig. 1. Electrophoretic karyotypes of *Saccharomyces bayanus* and *Saccharomyces cerevisiae.*

A laboratory haploid strain *S. cerevisiae* YNN 295 was used as a size marker. Lane A, YNN 295; lane B, IFO 1127 (type strain of *S. bayanus*); lane C, RIFY 1114; lane D, RIFY 1218; lane E, IFO 10217 (type strain of *S. cerevisiae*); lane F, IAM 4274; lane G, RIFY 1001.

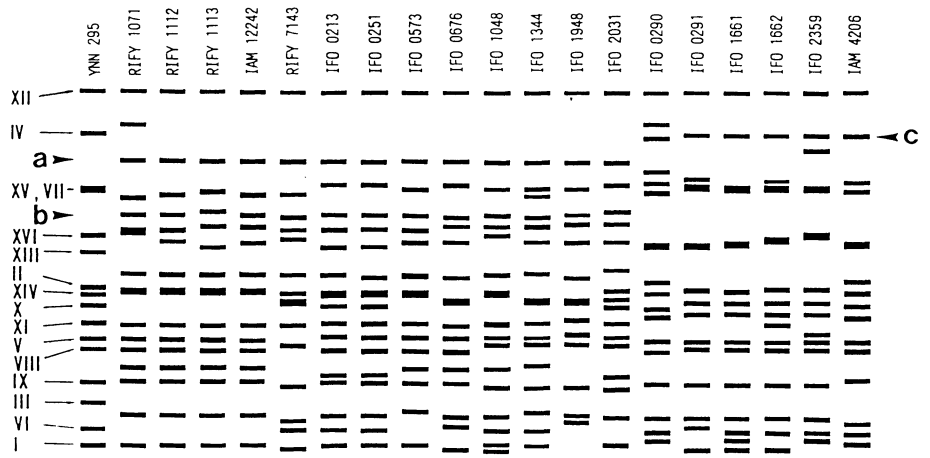

Fig. 2. Illustration of electrophoretic karyotypes of *Saccharomyces bayanus* and *Saccharomyces cerevisiae.*

ized by the presence of both of these chromosomal bands. On the other hand, a chromosomal band of **c**, which corresponds to chromosome IV of *S. cerevisiae* YNN 295, was observed in all of *S. cerevisiae.* This chromosomal band was not observed in any of *S. bayanus.*

Vaughan-Martini et al. (*17*) suggested the usefulness of electrophoretic karyotyping as a tool for classification of the genus *Saccharomyces.* Naumov et al. (*10–12*) reported that *S. bayanus* displayed specific chromosome patterns which could be distinguished from those of *S. cerevisiae* and *S. paradoxus.* Yamada et al. (*19*) also showed the presence of specific chromosomal bands for *S. bayanus*, and described that electrophoretic karyotyping makes it possible to distinguish *S. bayanus* and *S. cerevisiae.* Our results supported their assertion. However, it is not clear whether the specific chromosome patterns or the specific chromosomal bands for *S. bayanus* correspond to our own because of differences in the electrophoresis apparatus and conditions.

From our results, it was concluded that *S. bayanus* and *S. cerevisiae* which were classified on the basis of DNA similarity could be reliably distinguished by a combination of growth temperatures, fermentation characteristics and electrophoretic karyotyping.

Re-identification of industrial strains

Twelve strains of wine, sherry, sake and brewer's yeasts were re-identified by growth temperatures, fermentation characteristics, and electrophoretic karyotype. The results are summarized in Table 3. All of the wine, sherry and sake yeasts were unable to grow at 1 and 2°C, but were able to grow at 35°C. In fermentation at 7°C, their CO_2 evolution velocity ranged from 1.2 to 2.6 g during the first 20-day period. Additionally, the ethanol yield for fermentation at 28°C was 9.8–10.4%.

Table 3. Growth temperatures, fermentation characteristics and electrophoretic karyotype of wine, sherry, sake and brewer's yeasts.

Strain No.[a]	Re-identified as	Labeled name	Growth[b]			Fermentation velocity at 7°C[c] (CO_2 g/200 ml for 0–20 days)	EtOH yield at 28°C[c] (v/v %)	Chromosomal band in electrophoresis[d]
			1°C	2°C	35°C			
Wine yeast								
RIFY 1032 (AWRI 2A70)	*S. cerevisiae*	*S. cerevisiae*	−	−	+	1.8	10.1	c
RIFY 1069 (AWRI 4A797)	*S. cerevisiae*	*S. chevalieri*	−	−	+	1.9	10.4	c
RIFY 1054 (ENSA, Montpellier S-5)	*S. cerevisiae*	*S. bayanus*	−	−	+	1.7	10.1	c
RIFY 1043 (FAG, Montrachet 1107)	*S. cerevisiae*	*S. cerevisiae*	−	−	+	2.3	10.3	c
RIFY 1045 (FAG, Wadenswil 27)	*S. cerevisiae*	*S. cerevisiae*	−	−	+	2.8	10.3	c
Sherry yeast								
RIFY 3010 (Jerez No. 5)	*S. cerevisiae*	*S. bayanus*	−	−	+	1.4	10.2	c
RIFY 3011 (Xerez No. 2)	*S. cerevisiae*	*S. bayanus*	−	−	+	1.2	10.3	c
RIFY 3012 (SJ-75)	*S. cerevisiae*	*S. bayanus*	−	−	+	1.4	9.8	c
Sake yeast								
Kyokai, No. 7	*S. cerevisiae*	*S. cerevisiae*	−	−	+	2.4	10.3	c
Kyokai, No. 9	*S. cerevisiae*	*S. cerevisiae*	−	−	+	2.2	10.3	c
Brewer's yeast								
Sapporo, YB12-5[e]	Hybrid[f]	*S. carlsbergensis*	−	−	−	3.9	8.1	a, b, c
Sapporo, YB15-1[e]	Hybrid[f]	*S. carlsbergensis*	−	−	−	3.0	8.1	a, b, c

[a] RIFY, The Institute of Enology and Viticulture, Yamanashi University, Japan; AWRI, Australian Wine Research Institute, Glen Osmond, Australia; ENSA, Ecole Nationale Superieure Agronomique, Montpellier, France; FAG, Institut für Mikrobiologie unt Biochemie, Forschungsanstalt Geisenheim, Germany; Kyokai, Nippon Jōzōkyōkai (Brewing Society of Japan), Tokyo, Japan; Sapporo, Sapporo Breweries Culture Collection, Sapporo Breweries Ltd., Yaizu, Japan.

[b,c] See footnote in Table 2.

[d] Existence of specific chromosomal bands indicated in Fig. 1.

[e] The data of fementation velocity, ethanol yield at 28°C and chromosomal bands in electrophoresis of the brewer's yeasts are cited from Ref. *8*).

[f] Natural hybrid of *S. bayanus* and *S. cerevisiae* (*8*).

These growth temperatures and fermentation characteristics were in agreement with those of *S. cerevisiae* shown in Table 2. In the electrophoresis, the chromosomal band **c**, which was specific for *S. cerevisiae*, was observed in all of the yeasts. From these results, the five strains of wine yeasts, the three strains of sherry yeasts and the two strains of sake yeasts were re-identified as *S. cerevisiae*. Banno and Kaneko (*1*), and Naumov (*9*) recognized that an interspecific hybrid of *S. bayanus* and *S. cerevisiae* formed non-viable ascospores. We also suggested that classification based on the genetic hybridization analysis corresponded to that based on DNA similarity by the photobiotin microplate-hybridization method (*8*). Therefore, wine yeast strain RIFY 1069, a strain with a high frequency of sporulation and high ascospore viability, was conjugated with an arginine-requiring mutant of *S. bayanus* RIFY 1114 (*4*), but their hybrid formed non-viable ascospores (data not shown). This result confirmed identification based on growth temperatures, fermentation characteristics and electrophoretic karyotype. The brewer's yeasts YB 12-5 and YB 15-1 were considered natural hybrids of *S. bayanus* and *S. cerevisiae* from their fermentation characteristics, electrophoretic karyotype and DNA similarity (*8*). Non-growth at temperatures of 1, 2 and 35°C supported this consideration.

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