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D-Fructose Assimilation and Fermentation by Yeasts Belonging to Saccharomycetes: Rediscovery of Universal Phenotypes and Elucidation of Fructophilic Behaviors in *Ambrosiozyma platypodis* and *Cyberlindnera americana*

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Abstract: The purpose of this study was to investigate the ability of ascomycetous yeasts to assimilate/ferment D-fructose. This ability of the vast majority of yeasts has long been neglected since the standardization of the methodology around 1950, wherein fructose was excluded from the standard set of physiological properties for characterizing yeast species, despite the ubiquitous presence of fructose in the natural environment. In this study, we examined 388 strains of yeast, mainly belonging to the Saccharomycetes (Saccharomycotina, Ascomycota), to determine whether they can assimilate/ferment D-fructose. Conventional methods, using liquid medium containing yeast nitrogen base +0.5% (*w/v*) of D-fructose solution for assimilation and yeast extract-peptone +2% (*w/v*) fructose solution with an inverted Durham tube for fermentation, were used. All strains examined (n = 388, 100%) assimilated D-fructose, whereas 302 (77.8%) of them fermented D-fructose. In addition, almost all strains capable of fermenting D-glucose could also ferment D-fructose. These results strongly suggest that the ability to assimilate/ferment D-fructose is a universal phenotype among yeasts in the Saccharomycetes. Furthermore, the fructophilic behavior of *Ambrosiozyma platypodis* JCM 1843 and *Cyberlindnera americana* JCM 3592 was characterized by sugar consumption profiles during fermentation.

Keywords: physiology; physiological characterization; ascomycetous yeasts; fructophily; Kluyver rule

1. Introduction

Physiological tests have long been utilized to characterize yeast species with poor morphological traits. As is similar to the situations in taxonomic studies for the majority of bacteria, physiological properties have been a major feature in distinguishing and identifying yeast species until the era of molecular phylogeny. Although the importance of molecular phylogeny is widely accepted in the field of the systematics of microbes, physiological characterization has been an important aspect of new taxon descriptions. A set of common sugars, alcohols, sugar alcohols, and organic acids has been routinely used for assimilation tests of carbon compounds for yeasts. The fundamental methodology of carbon assimilation test for yeasts was published more than 70 years ago [1] and then standardized in the monograph "*The Yeasts, a Taxonomic Study*" [2]. In the latest version of the monograph, "*The Yeasts, a Taxonomic Study, 5th edition*", published in 2011 [3], assimilation of 36 carbon compounds was routinely profiled for almost all ascomycetous yeast species. However, D-fructose was still not included.

Fructose is a ketonic C6-monosaccharide naturally found in many plants. For instance, grapes are a rich source of sugars, containing equal amounts of glucose and fructose, and their total hexose content typically ranges from 160 to 300 g·L^{-1} [4]. The ability of *Saccharomyces cerevisiae* to ferment fructose was well documented in the early 20th century [5]. The presence of fructose in the natural environment, such as in fruits, and its importance as a carbon source have been highlighted in some previous studies, particularly in the field of food microbiology. For instance, fructose serves as the carbon source metabolized by yeasts during grape spoilage [6,7] or wine fermentation [8]. The fructophilic yeasts *Zygosaccharomyces rouxii* and *Z. bailii* are involved in canned fruit or fruit juice spoilage [9]. The characteristic fructophilic behavior of *Zygosaccharomyces* species is associated with the presence of the fructose facilitator *Zygosaccharomyces* genes, which encode hexose transporters [10]. Despite its ubiquitous presence in the natural environment and despite knowledge of its potential utility as a substrate for fermentation, fructose has not been included in the standard set of assimilation of carbon compounds since the first publication of the monograph, "*The Yeasts, a Taxonomic Study*". Consequently, there is still limited data available on the ability of yeasts to assimilate/ferment fructose.

According to Wickerham and Burton (1948) [1] and Miller and Phaff (1958) [11], carbon assimilation tests were perhaps first applied to yeasts by Beijerinck in 1889 [12]. The methods were reexamined by Wickerham and Burton 1948 [1], and then well-establish in the monograph "*The Yeasts, a Taxonomic Study*" [2], which has long been accepted as the gold standard of characterization for yeasts. Wickerham and Burton 1948 [1] mentioned—"Up to the present time the carbon sources used in assimilation tests in the major attempts at yeast classification have been limited to glucose, fructose, mannose, . . . " However, fructose was not included in the standard set of carbon assimilation tests in "*The Yeasts, a Taxonomic Study*" (1952) [2]. This monograph mentioned that tests with fructose had been omitted because, during many years, experiences have taught us that the rule, first formulated by Kluyver, according to which a yeast able to ferment glucose can also ferment fructose and mannose, holds good without a single exception [2] (p. 22); perhaps the "Kluyver" mentioned herein would designate the literature of Kluyver (1912) [13]. Due to this exclusion of fructose from the standard set, studies on fructose fermentation/assimilation have been intermittent since then. In 1985, Konno et al. examined the so-called Kluyver rule using over 200 yeast type strains and reported the results very briefly on half of one page [14]. The authors mentioned that the "Kluyver rule" was generally true, with a special emphasis on *Torulopsis halophila* (current name: *Wickerhamiella versatilis*; the strain used in the study was not specified) that was negative for fructose fermentation, despite being positive for glucose fermentation. It is very disappointing that the materials and methods were not described in detail [14]. Therefore, very little detailed information is available about the previous examination of the "Kluyver rule".

In the course of phenotypic quality control of yeast strains in our culture collection at Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Research Center (RIKEN BRC-JCM), we confirmed that many strains actually had the ability to ferment fructose. This led to the survey of the capability of a wide variety of yeasts in the Saccharomycetes (Saccharomycotina, Ascomycota) to assimilate/ferment fructose, namely reexamination of the "Kluyver rule" by using JCM strains. Thus, the purpose of this study was to identify the range of yeast species that are capable of utilizing fructose. We also reexamined their ability to assimilate/ferment sucrose. Sucrose is a common disaccharide that can be hydrolyzed by invertase to glucose and fructose [15]. The ability of glucose and sucrose assimilation or fermentation has been examined in almost all known ascomycetous yeast species. Specifically, in the present study, the generality of fructose assimilation or fermentation was evaluated by comparing their positive percentages.

As will be presented hereafter in this paper, universal phenotypes of "fructose assimilation" and "fructose fermentation" of yeasts in the Saccharomycetes (Saccharomycotina, Ascomycota) were rediscovered in this study. The reasons underlying the lack of information about the phenotypes have also been discussed. In addition, fructose and glucose consumption in the fermentation liquid media by two yeast strains, *Ambrosiozyma platypodis* JCM 1843 and *Cyberlindnera americana* JCM 3592, was monitored as they exhibited specific fructophilic behaviors. Please note that the term "fructose" in the present paper always indicates D-fructose.

2. Materials and Methods

2.1. Yeast Strains

Yeast strains used in this study were obtained from RIKEN BRC-JCM. The strains examined for the assimilation/fermentation tests are listed in Table 1. Strain information, including the voucher numbers, isolation source, and GenBank accession numbers of the reference nucleotide sequence, is described in the online strain catalog of RIKEN BRC-JCM (https://jcm.brc.riken.jp/en/catalogue_e; 16 March 2021). Most of them belonged to the Saccharomycetes (Saccharomycotina, Ascomycota). A few species in the genus *Schizosaccharomyces* and *Saitoella complicata* in Taphrinomycotina and *Trichosporiella flavificans* in Pezizomycotina were also employed. The yeast strains were incubated at 25 °C for preculitivation, assimilation, and fermentation, with the exception of *Cyberlindnera rhizosphaerae* JCM 16499 (8 °C), *Debaryomyces coudertii* JCM 2387 (15 °C), *Kazachstania telluris* JCM 5298 (37 °C), and *Wickerhamomyces patagonicus* JCM 16381 (15 °C).

Table 1. Fructose assimilation and fermentation profiles of Saccharomycetes yeasts.

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Saccharomycetes, Saccharomycotina Saccharomycetales Cephaloascaceae							
Cephaloascus fragrans	7613	+	+	-	-	- [1]	- [1]
Debaryomycetaceae							
Candida aaseri	1689	+	+	+/s	-	-	-
Candida albicans	1542	+ST	+ST	+ST	+ST	+ST	-
Candida atlantica	9548	+ST	+ST	+ST	w/-	w/-	-
Candida atmosphaerica	9549	+ST	+ST	+ST	+	+	-
Candida boleticola	1500	+	+	-	+ST	+ST	-
Candida buinensis	9453	+ST	+ST	+ST	+ST	+ST	-
Candida conglobata	2373	+	+	-	+ST	+ST	-
Candida dendronema	1803	+	+	+	+ST	+ST	-
Candida diddensiae	9598	+	+	+	+	+	-
Candida fluviatilis	9552	+	+	+	+	+	-
Candida friedrichii	9553	+	+	+	+	+	-
Candida glaebosa	1590	+ST	+ST	+	-	- [1]	- [1]
Candida insectamans	9611	+	+	-	-	-	-
Candida insectorum	9457	+	+	+	+	+	-
Candida lyxosophila	7532	+	+	+	+ST	+	-
Candida maltosa	1504	+ST	+ST	+	+ST	+ST	+ST
Candida multigemmis	9559	+	+	+	+	+	-
Candida oleophila	1620	+	+	+	+ST	+ST	-
Candida palmioleophila	5218	+	+	+	-	- [1]	- [1]
Candida membranifaciens	9450	+ST	+ST	+ST	+	+	+
Candida naeodendra	1509	+	+	+	+ST	+ST	-
Candida neustonensis	14892	+ST	+ST	+ST	+	+	+/s
Candida parapsilosis	1612	+ST	+	+	+ST	+ST	-
Candida pseudoglaebosa	2168	+	+	+	+	+	-
Candida saitoana	1438	+ST	+	+	-	-	-
Candida santamariae	1816	+	+	-	+ST	+ST	-
Candida schatavii	1778	+ST	+ST	-	s	s	-
Candida sojae	1644	+	+	+	+ST	+ST	+
Candida tammaniensis	10730	+	+	+	+ST	+ST	-
Candida thasaenensis	17817	+	+	+	+ST	+ST	+
Candida tropicalis	1541	+ST	+ST	+ST	+ST	+ST	+ST
Candida trypodendroni	10731	+	+	+	+	+	-
Candida psychrophila	2388	+	+	-	-	- [1]	- [1]
Candida viswanathii	9567	+	+	+	+ST	+ST	-
Candida xestobii	9569	+	+	+	-	-	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Candida zeylanoides	1627	+	+	-	-	-	-
Danielozyma ontarioensis	10729	+	+	+	+	+	+
Debaryomyces coudertii	2387	+	+	-	+	+	-
Debaryomyces hansenii	1990	+ST	+ST	+ST	+/s	+/s	+/s
Debaryomyces maramus	1528	+	+	+	w/-	w/-	w/-
Debaryomyces nepalensis	2095	+ST	+ST	+ST	+	+	+
Debaryomyces prosopidis	9913	+	+	+	+/s	s/w	s/w
Debaryomyces udenii	7855	+ST	+ST	+ST	+/d	+	d/w
Kurtzmaniella fragi	1791	+ST	+ST	+ST	+ST	+ST	-
Kurtzmaniella natalensis	1445	+	+	+	+ST	+ST	-
Kurtzmaniella quercitrusa	9832	+	+	+	+ST	+ST	-
Lodderomyces elongisporus	1781	+ST	+ST	+ST	+	+	-
Meyerozyma guilliermondii	10735	+ST	+ST	+ST	+	+	+
Millerozyma acaciae	10732	+ST	+ST	-	+ST	+ST	-
Millerozyma farinosa	10734	+ST	+ST	-	+ST	+ST	-
Millerozyma koratensis	12576	+ST	+ST	+ST	+	+	+
Priceomyces carsonii	8121	+ST	+ST	+	-	- [1]	- [1]
Priceomyces castillae	10733	+	+ST	-	-	-	-
Priceomyces fermenticarens	9589	+ST	+ST	-	-	-	-
Priceomyces haplophilus	1635	+ST	+ST	-	-	- [1]	- [1]
Priceomyces medius	10737	+	+	-	-	-	-
Priceomyces melissophilus	1707	+ST	+ST	+ST	-	- [1]	- [1]
Scheffersomyces coipomensis	8916	+ST	+ST	+	+ST	+ST	-
Scheffersomyces ergatensis	9599	+ST	+ST	+	+ST	+	-
Scheffersomyces insectosa	9842	+	+	+	+ST	+ST	-
Scheffersomyces lignosum	9837	+ST	+	+	+ST	+ST	-
Scheffersomyces segobiensis	10740	+	+	+	+ST	+ST	-
Scheffersomyces shehatae	9840	+	+	s	+ST	+ST	-
Scheffersomyces spartiniae	10741	+	+	+	+ST	+ST	-
Scheffersomyces stipitis	10742	+	+	+	+ST	+ST	-
Schwanniomyces capriottii	6177	+ST	+ST	+ST	+ST	+ST	+ST
Schwanniomyces etchellsii	3656	+ST	+ST	+ST	+ST	+ST	-
Schwanniomyces occidentalis var. *occidentalis*	8123	+ST	+ST	+ST	+ST	+	+
Schwanniomyces occidentalis var. *persoonii*	8127	+	+	+	+	+	+
Schwanniomyces polymorphus var. *africanus*	7443	+ST	+ST	+ST	+ST	+ST	+ST
Schwanniomyces polymorphus var. *polymorphus*	3647	+ST	+ST	+ST	+ST	+ST	+
Schwanniomyces pseudopolymorphus	3652	+ST	+ST	+ST	+ST	+ST	+ST
Schwanniomyces vanrijiae	3657	+ST	+ST	+ST	w	w	w
Schwanniomyces yamadae	6191	+	+	+	+ST	+ST	-
Wickerhamia fluorescens	1821	+	+	+	+ST	+ST	+
Yamadazyma akitaensis	10738	+ST	+ST	+ST	+	+	-
Yamadazyma kitorensis	31005	+ST	+ST	s	+	+	-
Yamadazyma mexicana	1835	+ST	+ST	+ST	+	+	-
Yamadazyma nakazawae	7529	+ST	+ST	+ST	+ST	+ST	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Yamadazyma philogaea	10739	+	+	+	+	+	-
Yamadazyma scolyti	3654	+	+	+	+	+	-
Yamadazyma takamatsuzukensis	15410	+	+	+	+	+	-
Yamadazyma tenuis	9827	+ST	+	+	+	+	-
Yamadazyma triangularis	9449	+	+	+	w/-	w/-	-
Yamadazyma tumulicola	15403	+ST	+ST	+	+	+	-
Dipodascaceae							
Dipodascus aggregatus	31687	+	+	-	-	-	-
Dipodascus australiensis	31688	+	+	-	-	-	-
Dipodascus eriense	3912	+	+	-	+/s	w/-	-
Dipodascus fermentans	2468	+	+	-	+ST	+ST	-
Dipodascus ingens	9471	+	+	-	-	-	-
Dipodascus ovetensis	3706	+	+	-	-	-	-
Dipodascus reessii	1943	+	+	-	-	-[1]	-[1]
Dipodascus tetrasperma	6361	+ST	+ST	-	+ST	+ST	-
Geotrichum rectangulatum	1750	+ST	+	-	+ST	+ST	-
Lipomycetaceae							
Babjevia anomala	5988	+	+	-	-	-[1]	-[1]
Lipomyces kononenkoae	5989	+	+	+	-	-	-
Lipomyces lipofer	3769	+	+	+	-	-	-
Lipomyces smithiae	8928	+	+	+	-	-[1]	-[1]
Lipomyces spencermartinsiae	5990	+	+	+	-	-	-
Lipomyces starkeyi	5995	+	+	+	-	-	-
Lipomyces suomiensis	7660	+	+	-	-	-[1]	-[1]
Lipomyces tetrasporus	6000	+	+	+	-	-[1]	-[1]
Myxozyma geophila	5220	+	+	-	-	-[1]	-[1]
Myxozyma kluyveri	7661	+	+	w	-	-[1]	-[1]
Myxozyma lipomycoides	5198	+	+	-	-	-[1]	-[1]
Myxozyma melibiosi	5194	+	+	-	-	-[1]	-[1]
Myxozyma mucilagina	1834	+	+	+	-	-[1]	-[1]
Myxozyma neglecta	5197	+	+	-	-	-[1]	-[1]
Myxozyma udenii	8927	+	+	+	-	-[1]	-[1]
Metschnikowiaceae							
Aciculoconidium aculeatum	13354	+	+	+	s	s	-
Clavispora fructus	1513	+	+	-	+ST	+ST	-
Clavispora lusitaniae	7533	+	+	+	+ST	+ST	-
Candida akabanensis	9115	+ST	+ST	+ST	+ST	+ST	+ST
Candida auris	15448	+ST	+ST	+ST	+	+	+
Candida haemulonii	3762	+ST	+ST	+ST	+ST	+ST	+
Candida intermedia	1607	+ST	+	+	+ST	+ST	+ST
Candida melibiosica	9558	+	+	+	+ST	+ST	-
Candida mogii	1611	+	+	+	+ST	+ST	+ST
Candida pseudointermedia	1592	+ST	+ST	+ST	+ST	+ST	+ST
Candida fukazawae	1641	+	+	+	+ST	+ST	-
Candida fungicola	10142	+ST	+ST	+ST	-	-	-
Candida mesenterica	2368	+	+	+	-	-	-
Candida musae	1598	+	+	+	+ST	+ST	-
Candida oregonensis	1811	+ST	+ST	+	+ST	+ST	-
Candida pseudohaemulonii	12453	+ST	+ST	+ST	+ST	+	+
Candida tsuchiyae	1638	+ST	+ST	+ST	+ST	+ST	+
Hyphopichia burtonii	3708	+	+	+	+	+	+
Hyphopichia fennica	9849	+	+	+	+ST	+ST	+
Hyphopichia gotoi	10145	+	+	+	+ST	+ST	+ST
Hyphopichia homilentoma	1507	+ST	+	+	+ST	+ST	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Hyphopichia khmerensis	13262	+ST	+ST	+ST	+	+	+
Hyphopichia pseudoburtonii	16346	+	+	+	+ST	+ST	+
Hyphopichia rhagii	9839	+ST	+ST	+ST	+ST	+ST	+ST
Metschnikowia agaves	31832	+	+	+	+/s	+/s	-
Metschnikowia kofuensis	12563	+	+	+/s	+	+	-
Metschnikowia lunata	1798	+	+	+	+ST	+ST	-
Metschnikowia reukaufii	7534	+	+	+	+	+	-
Metschnikowia torresii	1845	+ST	+ST	-	+ST	+ST	-
Metschnikowia viticola	12561	+	+	+	+	+	-
Phaffomycetaceae							
Komagataella pastoris	3650	+ST	+ST	-	+ST	+ST	-
Phaffomyces opuntiae	1836	+	+	-	-	- [1]	- [1]
Phaffomyces thermotolerans	1837	+	+	-	-	- [1]	- [1]
Pichiaceae							
Candida ethanolica	9588	+	+	-	s/-	s/-	-
Candida inconspicua	9555	+	+	-	+	+	-
Candida pseudolambica	9830	+ST	+ST	-	+ST	+ST	-
Candida rugopelliculosa	1593	+	+	-	+ST	+ST	-
Candida silvatica	9828	+	+	-	-	-	-
Dekkera anomala	31686	+ST	+ST	+	+ST	+ST	+
Dekkera bruxellensis	11407	+	+	+	+ST	+ST	+ST
Kregervanrija fluxuum	3646	+	+	-	+/s	+/s	-
Pichia cactophila	1830	+	+	-	+/s	+/w	-
Pichia exigua	1829	+	+	-	w	w	-
Pichia heedii	1833	+	+	-	+/w	+/w	-
Pichia kluyveri var. *kluyveri*	11403	+	+	-	+ST	+ST	-
Pichia membranifaciens	1442	+	+	-	w	w	-
Pichia myanmarensis	12922	+ST	+ST	+ST	+ST	+ST	+
Pichia nakasei	1699	+ST	+ST	-	+ST	+ST	-
Pichia occidentalis	1711	+ST	+	-	+ST	+ST	-
Pichia rarassimilans	14993	+	+	-	s	s	-
Pichia terricola	1709	+ST	+	-	+	+	-
Saturnispora ahearnii	10726	+	+ST	-	+ST	+ST	-
Saturnispora besseyi	1706	+ST	+	-	+ST	+ST	-
Saturnispora dispora	1795	+	+	-	+ST	+ST	-
Saturnispora diversa	1848	+	+	-	+ST	+ST	-
Saturnispora saitoi	1793	+ST	+ST	-	+ST	+ST	-
Saturnispora silvae	6352	+ST	+	-	+/s	+/s	-
Saturnispora zaruensis	1515	+ST	+	-	+ST	+ST	-
Saccharomycetaceae							
Candida castellii	9550	+	+	-	+ST	+ST	-
Candida glabrata	3761	+	+	-	+ST	+ST	-
Issatchenkia orientalis	1710	+ST	+ST	-	+ST	+ST	-
Kazachstania aerobia	31691	+	+	-	+ST	+ST	-
Kazachstania bulderi	31689	+	+	+	+ST	+ST	+
Kazachstania exigua	1790	+	+	+	+ST	+ST	+
Kazachstania humilis	9852	+	+	-	+ST	+ST	-
Kazachstania servazzii	5179	+	+	-	+ST	+ST	-
Kazachstania telluris	5298	+	+	-	+ST	+/w	-
Kazachstania transvaalensis	5178	+	+	-	+ST	+ST	-
Kazachstania unispora	5180	+	+	-	+ST	+ST	-
Kluyveromyces marxianus	9556	+	+	+	+ST	+ST	+ST
Kluyveromyces nonfermentans	10232	+	+	-	-	-	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Lachancea kluyveri	7257	+	+	+	+ST	+ST	+ST
Lachancea thermotolerans	19085	+	+	+	+ST	+ST	+
Lachancea waltii	10745	+	+	+	+ST	+ST	+ST
Saccharomyces bayanus	7258	+	+	+	+ST	+ST	+ST
Saccharomyces cerevisiae	7255	+	+	+	+ST	+ST	+ST
Saccharomyces pastorianus	7256	+	+	+	+ST	+ST	+ST
Tetrapisispora arboricola	10813	+	+	-	+ST	+ST	-
Tetrapisispora iriomotensis	10810	+	+	-	+ST	+ST	-
Tetrapisispora namnaoensis	12664	+	+	-	+ST	+ST	-
Tetrapisispora nanseiensis	10811	+	+	-	+ST	+ST	-
Torulaspora delbrueckii	31684	+	+	-	+ST	+ST	-
Torulaspora pretoriensis	3662	+	+	+	+ST	+ST	+
Zygosaccharomyces rouxii	7619	+	+	w/-	+ST	+ST	s
Zygosaccharomyces rouxii	22060	+	+	-	+ST	+ST	-
Zygosaccharomyces siamensis	16825	+	+	-	+ST	+ST	s/w
Zygotorulaspora mrakii	1800	+	+	+	+ST	+ST	+
Saccharomycodaceae							
Hanseniaspora opuntiae	31690	+	+	-	+ST	+ST	-
Saccharomycopsidaceae							
Candida fragicola	1589	+	+	-	+ST	+ST	-
Saccharomycopsis capsularis	7619	+	+	w/-	+ST	+ST	s
Saccharomycopsis crataegensis	1700	+	+	-	+	+	-
Saccharomycopsis fibuligera	7609	+	+	+	+ST	+ST	+
Saccharomycopsis javanensis	3707	+	+	-	-	- [1]	- [1]
Saccharomycopsis malanga	7620	+	+	-	+/s	+	-
Saccharomycopsis selenospora	7616	+	+	-	-	-	-
Saccharomycopsis synnaedendra	7607	+	+	-	-	- [1]	- [1]
Saccharomycopsis vini	7623	+	+	+	+	+	+
Trichomonascaceae							
Blastobotrys adeninivorans	8914	+ST	+ST	+ST	+ST	+	+/s
Blastobotrys arbuscula	2926	+	+	-	+ST	+	-
Blastobotrys aristata	2929	+	+	s	+ST	+ST	-
Blastobotrys capitulata	2934	+	+	-	+ST	+ST	-
Blastobotrys chiropterorum	9597	+	+	+	-	-	-
Blastobotrys elegans	2931	+	+	-	+	+/w	-
Blastobotrys gigas	2927	+	+	-	+ST	+	-
Blastobotrys nivea	2933	+	+	s	+	+	-
Blastobotrys parvus	9487	+	+	s	-	-	-
Blastobotrys proliferans	2928	+	+	+	+	+	-
Blastobotrys terrestris	8913	+	+	+	-	-	-
Candida santjacobensis	8924	+	+	+	+	+	-
Groenewaldozyma auringiensis	9593	+	+	-	+ST	+	-
Groenewaldozyma salmanticensis	8896	+	+	+	+ST	+ST	+ST
Middelhovenomyces petrohuensis	8922	+	+	+	-	-	-
Middelhovenomyces tepae	10265	+	+	s	w	-	-
Sugiyamaella castrensis	9585	+	+	+	w/-	-	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Sugiyamaella paludigena	9614	+	+	+	-	-	-
Sugiyamaella valdiviana	9565	+	+	+	+/w	w/-	-
Trichomonascus ciferrii	7621	+	+	+	-	-	-
Wickerhamiella azyma	1691	+	+	+	-	-	-
Wickerhamiella domercqiae	9478	+	+	+/w	-	- [1]	- [1]
Wickerhamiella galacta	8257	+	+	-	-	-	-
Wickerhamiella hasegawae	12559	+	+	-	+	+/w	-
Wickerhamiella kazuoi	12558	+	+	-	-	-	-
Wickerhamiella pararugosa	1512	+	+	-	-	- [1]	- [1]
Wickerhamiella sorbophila	1514	+	+	-	-	-	-
Wickerhamiella spandovensis	9562	+	+	+	+ST	w	w
Wickerhamiella vanderwaltii	9615	+	+	-	-	-	-
Wickerhamiella versatilis	8065	+	+	+	+ST	+ST	+ST
Zygoascus biomembranicola	31007	+	+	-	+ST	+	-
Wickerhamomycetaceae							
Barnettozyma salicaria	3653	+	+	-	-	-	- [1]
Barnettozyma wickerhamii	21961	+ST	+ST	+ST	+	+	-
Candida berthetii	9594	+	+	-	+	+	-
Candida danieliae	17247	+ST	+ST	+	+ST	+	-
Candida dendrica	9605	+	+	-	+	+/s	-
Candida easanensis	12476	+	+	+/s	+	+	-
Candida eppingiae	17241	+ST	+ST	+ST	+ST	+ST	-
Candida freyschussii	9850	+	+	+/s	+	+	-
Candida maritima	9612	+	+	+	+	+	+
Candida montana	2323	+	+	-	-	- [1]	- [1]
Candida nakhonratchasimensis	12474	+	+	+	+ST	+ST	+ST
Candida norvegica	8897	+	+	-	-	-	-
Candida pattaniensis	12475	+ST	+ST	+ST	+ST	+ST	+
Candida pseudoflosculorum	17242	+ST	+ST	+ST	+ST	+ST	+ST
Candida quercuum	1587	+ST	+	+	+/w	+	-
Candida robnettiae	17243	+ST	+ST	+ST	+ST	+ST	-
Candida silvicultrix	9831	+	+	+	+ST	+ST	+ST
Candida solani	2339	+	+	+	+ST	+ST	-
Candida vartiovaarae	3759	+	+	+	+ST	+ST	+
Cyberlindnera americana	3592	+	+	+	-	+/s	-
Cyberlindnera americana	3593	+	+	+	w	s	-
Cyberlindnera amylophila	1702	+	+	+	+ST	+ST	-
Cyberlindnera bimundalis	3591	+	+	+	+/s	+/s	-
Cyberlindnera fabianii	3601	+ST	+	+	+ST	+ST	+ST
Cyberlindnera jadinii	3617	+	+	+	+ST	+ST	+ST
Cyberlindnera japonica	11402	+	+	+	s	s/w	-
Cyberlindnera mississippiensis	1703	+ST	+	+	+ST	+ST	-
Cyberlindnera mrakii	3614	+	+	-	+ST	+ST	-
Cyberlindnera petersonii	3619	+	+	+	+	+	+
Cyberlindnera rhizosphaerae	16499	+	+	s	+	+	+
Cyberlindnera rhodanensis	3649	+ST	+ST	+ST	+ST	+ST	-
Cyberlindnera samutprakarnensis	17816	+	+	+	+ST	+ST	+ST
Cyberlindnera subsufficiens	3625	+	+	+	+	+	+
Starmera amethionina	1831	+	+	-	s	s	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Starmera pachycereana	1832	+	+	-	-	-	-
Starmera quercuum	3659	+	+	-	+	+	-
Starmera stellimalicola	3546	+	+	-	+ST	+ST	-
Wickerhamomyces anomalus	3585	+ST	+ST	+ST	+ST	+ST	+ST
Wickerhamomyces bisporus	3590	+	+	+	w	w	-
Wickerhamomyces bovis	3640	+ST	+ST	+ST	+ST	+ST	-
Wickerhamomyces canadensis	3597	+ST	+ST	+ST	-	- [1]	- [1]
Wickerhamomyces chaumierensis	17246	+ST	+ST	+ST	+ST	+ST	-
Wickerhamomyces ciferrii	3599	+ST	+ST	+ST	+	+ST	+
Wickerhamomyces mucosus	6814	+	+	+	+ST	+ST	-
Wickerhamomyces patagonicus	16381	+	+	-	-	-	-
Wickerhamomyces pijperi	11406	+	+	-	+ST	+ST	-
Wickerhamomyces silvicola	3627	+ST	+ST	+	+ST	+ST	-
Wickerhamomyces subpelliculosus	3631	+ST	+ST	+	+ST	+ST	+ST
Wickerhamomyces sydowiorum	9455	+ST	+ST	+ST	+	+ST	+
Saccharomycetales *incertae sedis*							
Ambrosiozyma cicatricosa	7598	+ST	+	+	+/s	+/s	+/s
Ambrosiozyma kamigamensis	14990	+	+	+/s	+	+	-
Ambrosiozyma kashinagicola	15019	+	+	-	+	+	-
Ambrosiozyma llanquihuensis	8918	+	+	-	+	+	-
Ambrosiozyma monospora	7599	+	+	+	+ST	+ST	+/w
Ambrosiozyma neoplatypodis	14992	+	+	-	+	+	-
Ambrosiozyma oregonensis	1797	+ST	+	+	+	+	-
Ambrosiozyma philentoma	7600	+	+	+	+/d	+	d/w
Ambrosiozyma platypodis	1843	+	+	+	w	+	-
Ambrosiozyma platypodis	1796	+	+	+	+/s	+	-
Ambrosiozyma pseudovanderkliftii	15025	+	+	s	+	+	-
Ambrosiozyma vanderkliftii	15029	+ST	+ST	+	+ST	+ST	w
Babjeviella inositovora	10736	+	+	+	-	-	-
Candida arabinofermentans	10727	+	+	-	+	+	-
Candida blankii	8259	+	+	+	+/s	w	w
Candida boidinii	9604	+	+	-	+ST	+ST	-
Candida chilensis	1693	+	+	+	+	+	-
Candida cylindracea	9586	+	+	-	+	+	-
Candida digboiensis	12330	+	+	+	-	-	-
Candida entomophila	9607	+	+	+	+ST	+ST	+ST
Candida incommunis	8258	+	+	+	+	+	-
Candida insectalens	9610	+	+	-	-	-	-
Candida krabiensis	12266	+	+	-	-	-	-
Candida maris	9853	+	+	-	-	-	-
Candida methanosorbosa	9620	+	+	-	+	+	-
Candida nanaspora	9590	+	+	-	+ST	+ST	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Candida nemodendra	9855	+	+	-	s/-	w/-	-
Candida nitratophila	9856	+	+	-	+	+	-
Candida ovalis	9444	+	+	-	+ST	+ST	-
Candida pini	9826	+	+/s	-	+/s	+/s	-
Candida sake	2951	+ST	+	+	+ST	+ST	-
Candida savonica	9561	+	+	-	+	+	-
Candida sequanensis	9841	+	+	-	+ST	+ST	-
Candida silvanorum	1804	+	+	+	+ST	+ST	-
Candida sithepensis	12265	+	+	-	+ST	+ST	-
Candida sonorensis	1827	+	+	-	+ST	+ST	-
Candida sophiae-reginae	8925	+ST	+ST	+	+ST	+ST	-
Candida sorboxylosa	1536	+ST	+ST	-	+	+	-
Candida succiphila	9445	+	+	-	+ST	+ST	-
Citeromyces matritensis	2333	+	+	+	+ST	+ST	+ST
Citeromyces siamensis	11522	+	+	+	+ST	+ST	+ST
Diutina catenulata	1604	+ST	+	-	+	+	-
Diutina rugosa	1619	+ST	+	-	-	- [1]	- [1]
Kuraishia capsulata	1991	+	+	-	+	+	-
Nadsonia commutata	10138	+	+	-	-	- [1]	- [1]
Nadsonia fulvescens var. *fulvescens*	9992	+	+	-	+ST	+ST	-
Nadsonia starkeyi-henricii	11408	+	+	-	-	-	-
Nakazawaea anatomiae	9547	+	+	-	+	+	-
Nakazawaea holstii	3608	+	+	+/s	+ST	+ST	-
Nakazawaea ishiwadae	9451	+	+	+	+ST	+ST	-
Nakazawaea peltata	9829	+	+	+	+ST	+ST	-
Nakazawaea populi	9833	+	+	s	+ST	+ST	-
Nakazawaea wickerhamii	9568	+	+	-	+ST	+ST	-
Ogataea angusta	3635	+	+	+	+ST	+ST	-
Ogataea glucozyma	3607	+	+	-	+ST	+ST	-
Ogataea henricii	3611	+	+	-	-	- [1]	- [1]
Ogataea kodamae	11404	+	+	-	+/s	+/s	-
Ogataea methanolica	10240	+	+	-	+ST	+ST	-
Ogataea methylivora	22142	+	+	+	+	+	-
Ogataea minuta	3622	+	+	-	+	+ST	-
Ogataea naganishii	22078	+	+	+	+	+	-
Ogataea nonfermentans	3615	+	+	-	-	-	- [1]
Ogataea philodendri	22070	+	+	-	-	-	-
Ogataea pignaliae	9836	+	+	-	+ST	+ST	-
Ogataea pini	3655	+	+	-	s/-	s/-	-
Ogataea salicorniae	10744	+	+	-	+ST	+ST	-
Ogataea siamensis	12264	+	+	+	+/s	+/s	-
Ogataea thermomethanolica	12984	+	+	+	+	+	-
Ogataea trehalophila	3651	+ST	+ST	-	+ST	+ST	-
Pachysolen tannophilus	31685	+	+	-	+ST	+ST	-
Peterozyma toletana	3658	+ST	+	+	+	+	-
Saprochaete japonica	2451	+ST	+ST	-	+ST	+ST	-
Sporopachydermia cereana	9480	+	+	-	-	-	-
Sporopachydermia lactativora	9485	+	+	-	-	- [1]	- [1]
Sporopachydermia quercuum	9486	+	+	-	+	-	-
Starmerella apicola	9592	+	+	+	+	+	+
Starmerella apis	8256	+ST	+ST	+	-	w	-
Starmerella bombi	9595	+	+	+	+ST	+ST	+
Starmerella bombicola	9596	+	+	+	+ST	+ST	+ST
Starmerella etchellsii	8066	+	+	-	+	s/w	-

Table 1. *Cont.*

Species	JCM no.	Assimilation			Fermentation		
		Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
Starmerella floricola	9439	+	+	+	+ST	+ST	+ST
Starmerella geochares	9851	+	+	+	+	+	+/s
Starmerella gropengiesseri	8255	+	+	+	+	+	w
Starmerella lactis-condensi	9472	+	+	+	+ST	+ST	+ST
Starmerella magnoliae	1446	+	+	+	+	+	+
Starmerella stellata	9476	+	+	+	+ST	+ST	+ST
Starmerella vaccinii	9446	+	+	+	+	+	+
Suhomyces tanzawaensis	1648	+	+	+	s/w	+/w	-
Teunomyces kruisii	1779	+	+	+	+ST	+ST	-
Trigonopsis cantarellii	8260	+	+	-	+ST	+ST	-
Trigonopsis variabilis	1823	+	+	-	-	- [1]	- [1]
Trigonopsis vinaria	1813	+	+	-	-	- [1]	- [1]
Yarrowia deformans	1694	+	+	-	-	- [1]	- [1]
Yarrowia keelungensis	14894	+ST	+ST	-	-	-	-
Yarrowia lipolytica	2320	+ST	+ST	-	-	- [1]	- [1]
Yarrowia yakushimensis	12782	+ST	+ST	-	-	-	-
Taphrinomycotina							
Saitoella complicata	7358	+	+	+	-	-	-
Schizosaccharomyces japonicus	8264	+	+	+	+ST	+ST	+ST
Schizosaccharomyces octosporus	8261	+	+	w	+	+	w
Schizosaccharomyces pombe	8274	+	+	+	+ST	+ST	+ST
Pezizomycotina							
Trichosporiella flavificans	1506	+	+	-	+ST	+ST	-

[1] Fermentation test examined once; +ST, strongly positive; +, positive; d, delayed positive; s, slowly positive; w, weakly positive; -, negative; a diagonal line "/" indicates "or".

2.2. Assimilation of Fructose

The assimilation of fructose was examined using the conventional method for yeast identification [3]. Experiments on fructose and sucrose assimilation were performed twice independently using commercially available highly pure reagents obtained from the two different suppliers. Briefly, an aqueous stock solution containing 6.7% (*w/v*) yeast nitrogen base (YNB, Difco Labs, Thermo Fisher Scientific, Waltham, MA, USA, 239210) and 5% (*w/v*) fructose (Nacalai Tesque, Inc., Kyoto, Japan, GR grade, cat. 16315-55; FUJIFILM Wako Chemical Corporation, Miyazaki, Japan, GR grade, cat. 147-02765) was filter-sterilized, and 0.2 mL of the sterilized stock solution was mixed with 1.8 mL of sterile distilled water in a sterile glass test tube to prepare a working liquid medium containing 0.67% (*w/v*) YNB and 0.5% (*w/v*) fructose. Glucose (Nacalai Tesque, Inc., GR grade, cat. 16806-25) or sucrose (Nacalai Tesque, Inc., GR grade, cat. 30404-45; Kanto Chemical Co.,INC., Tokyo, Japan, GR grade, cat. 37000-01) were also employed instead of fructose in the above-mentioned assimilation medium as a reference. A plain YNB solution was used as a negative control. Yeast culture was prepared on YM agar (2.1% (*w/v*) of YM broth (Difco Labs., Thermo Fisher Scientific, 271120) plus 2% (*w/v*) agar (Nacalai Tesque, Inc., cat. 01028-85)) 2–7 days before inoculation and a vigorously grown culture was inoculated into the liquid media. Growth was visually monitored and scored weekly for up to four weeks. Growth was measured according to the above-mentioned monograph with some modifications [3]. Briefly, the degree of growth in the liquid medium was observed by the naked eye after shaking the test tube to disperse the yeast cells. The test tube was placed on a white file card, on which 0.75 mm thick black lines were drawn at intervals of approximately 5 mm. The results were scored as 3+ when the lines were completely obscured, 2+ when the lines appeared as diffused bands, 1+ when the lines were distinguishable but had blurred edges, and negative when the lines were distinct with sharp edges. The results were as follows: Strongly positive (3+ reading developed within 1 week), positive (2+ or 3+ reading developed within 2 weeks), slowly positive (2+ or 3+ reading developed slowly over a period exceeding two weeks), delayed positive (2+ or 3+ reading developed rapidly but after two weeks), weakly positive (1+ reading developed), and negative (little (less than 1+ reading) or no growth).

2.3. Fermentation of Fructose

Fermentation of fructose was also examined by the conventional method for yeast identification [3]. Experiments on fructose and sucrose fermentation were performed twice independently using commercially available highly pure reagents obtained from the two different suppliers. Briefly, 4.5 mL of sterile fermentation basal medium containing 0.45% (*w/v*) bacto yeast extract (Difco Labs., Thermo Fisher Scientific, 212750), 0.75% (*w/v*) bacto peptone (Difco Labs., 211677), and ~50 ppm bromothymol blue (Sigma-Aldrich, St. Louis, MO, USA, B8630) was prepared in a glass test tube with a small, inverted Durham tube inside. An aqueous stock solution of 20% (*w/v*) fructose (Nacalai Tesque, Inc., GR grade, cat. 16315-55; FUJIFILM Wako Chemical Corporation, GR grade, cat. 147-02765) was filter-sterilized, and 0.5 mL of the sterilized stock solution was added to the fermentation basal liquid medium to obtain a final concentration of 2% (*w/v*) fructose. Glucose (Nacalai Tesque, Inc., GR grade, cat. 16806-25) or sucrose (Nacalai Tesque, Inc., GR grade, cat. 30404-45; Kanto Chemical Co.,INC., GR grade, cat. 37000-01) were also employed instead of fructose in the above-mentioned fermentation medium as a reference. Yeast culture was prepared in the same manner as for assimilation tests, and a vigorously grown culture was heavily inoculated into the liquid media. Filling with gas in the inverted tube (Supplementary Figure S1) was visually monitored and scored about every second day up to one week and then at two and three weeks after inoculation. The results were scored according to the above-mentioned monograph with some modifications as follows [3]: Strongly positive (the Durham tube rapidly filled with gas within three days), positive (more than half of the Durham tube filled with gas within seven days), slowly positive (more than half of the Durham tube filled with gas after more than seven days), delayed positive (more than half of the Durham tube rapidly filled with gas, but only after more than seven days), weakly positive (less than half of the Durham tube filled with gas), or negative (no gas accumulation observed in the Durham tube).

2.4. Sugar Consumption during Fermentation

Sugar consumption by *A. platypodis* JCM 1843, *C. americana* JCM 3592, and *S. cerevisiae* JCM 7255 in the fermentation liquid media was monitored, as the former two strains exhibited apparent fructophilic behaviors during fermentation (see Section 3.2.).

Fermentation liquid media were prepared in the same manner as described in Section 2.3 with the following modifications. The total amount of medium was 7 mL in a glass test tube to allow a series of liquid medium sampling, and bromothymol blue was not added to the media to avoid interference with absorbance at 340 nm in the subsequent measurement using a spectrophotometer. Three kinds of fermentation media were prepared: (i) 2% (*w/v*) fructose, (ii) 2% (*w/v*) glucose, and (iii) 2% (*w/v*) fructose plus 2% (*w/v*) glucose (final concentrations in the media). To prepare the fructose–glucose mixed medium (iii), an aqueous stock solution of 20% (*w/v*) fructose (Nacalai Tesque, Inc.) plus 20% (*w/v*) glucose (Nacalai Tesque, Inc.) was filter-sterilized, and then 0.7 mL of the sterilized stock solution was added to 6.3 mL of the fermentation basal liquid medium.

Strains JCM 1843, JCM 3592, and JCM 7255 were cultured on YM agar at 25 °C for 2–3 days, and the freshly prepared culture was incubated in the basal fermentation medium at 25 °C for 2 days. The three fermentation media (i), (ii), and (iii) were inoculated with the culture and incubated at 25 °C without shaking. Inoculation was done in quadruplicates. The fermentation medium was sampled after gentle mixing by pipetting at approximately 12 h intervals for JCM 7255 and approximately 12–48 h intervals for JCM 1843 and JCM

3592. The sampled media were centrifuged to remove cells, and the supernatant was heated at 90 °C for 10 min to deactivate enzymes and then stored at −20 °C for measuring the fructose and glucose concentrations.

The concentration of fructose and glucose in the fermentation media was measured and calculated using an enzymatic test kit D-glucose/D-fructose (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany, cat. 10 139 106 035) following the manufacturer's instructions with some modifications. The absorbance of the solution in a 96-well microplate was measured at 340 nm using a spectrophotometer Multiskan SkyHigh (Thermo Fisher Scientific).

3. Results

3.1. Assimilation of Fructose

All 388 strains tested had the ability to assimilate fructose as well as glucose, utilizing fructose as the sole carbon source (Table 1). Sucrose was assimilated by fewer yeast strains than those capable of assimilating fructose; 229 (59.0%) out of the 388 strains assimilated sucrose (including strains of positive reaction delayed, slowly, and weakly positive).

3.2. Fermentation of Fructose

Three hundred and two (77.8%) out of the 388 strains had the ability to ferment glucose, and most of these strains could also ferment fructose (Table 1), with the exception of *Sporopachydermia quercuum* JCM 9486, which fermented glucose but not fructose. In contrast, *Ambrosiozyma platypodis* JCM 1843 showed a stronger and quicker positive reaction to fructose than to glucose. A preference for fructose was also observed; *Cyberlindnera americana* JCM 3592 fermented fructose well, but not glucose. Thus, 302 (77.8%) of the 388 strains had the ability to ferment fructose. These observations of JCM 9486, JCM 1843, and JCM 3592 were reproduced in three independent trials (Supplementary Table S1).

Similar to the results of assimilation, the number of strains capable of fermenting sucrose (99 strains) was much lower than that of strains capable of fermenting glucose/fructose. In addition, all the strains fermenting sucrose were capable of fermenting both glucose and fructose.

3.3. Sugar Consumption during Fermentation

Sugar consumption by *A. platypodis* JCM 1843 and *C. americana* JCM 3592 in the fermentation liquid media was monitored using only 2% fructose or 2% glucose (sugar solo fermentation), or both 2% fructose and 2% glucose (sugar duo fermentation). Figure 1 shows the time-course consumption profiles of fructose and glucose, where the amount of sugars at 0-h (sampled immediately after inoculation) was set as 100%.

In *A. platypodis* JCM 1843 and *C. americana* JCM 3592, the sugar consumption profiles were similar to each other in both sugar solo fermentation and sugar duo fermentation. Fructose was more rapidly consumed than glucose in sugar solo fermentation. On the contrary, fructose consumption was substantially slower in sugar duo fermentation than in sugar solo fermentation; instead, glucose consumption was observed before fructose consumption.

Saccharomyces cerevisiae JCM 7255 rapidly consumed both fructose and glucose, which were almost used up at 36 h in both sugar solo/duo fermentation. Glucose consumption was always more rapid than fructose consumption. Fructose consumption in sugar duo fermentation was apparently less rapid than in sugar solo fermentation.

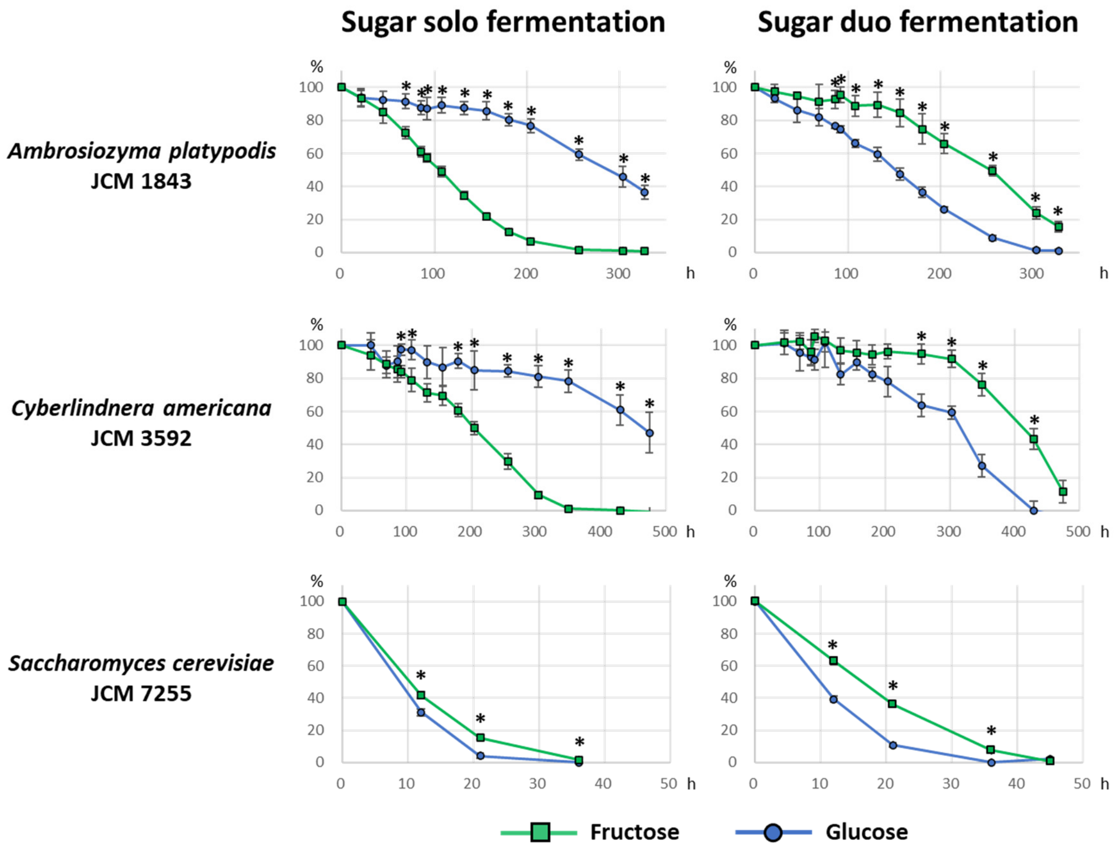

Figure 1. Sugar consumption profiles in the fermentation liquid media. The green square and blue circle indicate the percentage of fructose and glucose concentrations, respectively, compared with the initial amount of each sugar at 0 h. The left three graphs show sugar consumption by yeasts incubated in only 2% fructose or 2% glucose in the fermentation media. The right three graphs show sugar consumption by yeasts incubated in the fermentation medium containing both 2% fructose and 2% glucose. The bar on the symbols indicates standard deviation. Asterisk (*) indicates a significant difference between fructose and glucose percentages (Welch's *t*-test, $p < 0.01$).

4. Discussion

The results of this study are very simple; all the yeast strains tested could assimilate glucose, and glucose fermenters were fructose fermenters, with a few exceptions. This strongly suggests that the utility of fructose is universal among yeasts in the Saccharomycetes. Positive reactions in assimilation and fermentation of fructose should be regarded as universal phenotypes rediscovered by this survey. We employed approximately 380 species of yeasts belonging to the Saccharomycetes. This accounts for almost one-third of the described ascomycetous yeast species. As we used a taxonomically wide variety of yeasts, there is no doubt about the generality of the positive reactions in the fructose assimilation/fermentation tests, at least for the Saccharomycetes. Thus, the "Kluyver rule" was confirmed on the whole.

We searched the capability of assimilation/fermentation of other common sugars by ascomycetous yeasts, based on the data in the monograph "*The Yeasts, a Taxonomic Study, 5th edition*" [3] (Table 2). As shown in Table 2, the percentage of assimilating/fermenting sucrose was 59.0%/25.5% in this study, whereas it was 60.7%/24.2% in the monograph, suggesting that the selection of yeast species employed in this study was unbiased. Judging from the higher positive percentages for both assimilation and fermentation of fructose compared to those of the other sugars, it is safe to say that fructose is an easy-to-use carbon source for the yeasts.

Table 2. Percentage of yeast species in the Ascomycota capable of assimilating/fermenting common sugars.

Sugars	This Study		*The Yeasts* *	
	Assimilation	**Fermentation**	**Assimilation**	**Fermentation**
Glucose	100% (388/388)	77.8% (302/388)	100% (827/827)	72.8% (602/827)
Fructose	100% (388/388)	77.8% (302/388)	nd	nd
Sucrose	59.0% (229/388)	25.5% (99/388)	60.7% (502/827)	24.2% (200/827)
Galactose	nt	nt	65.4% (541/827)	30.6% (253/827)
Trehalose	nt	nt	70.0% (579/827)	30.1% (249/827)
Maltose	nt	nt	56.8% (470/827)	18.3% (151/827)
Raffinose	nt	nt	28.5% (236/827)	13.8% (114/827)

* Data collected from "*The Yeasts, a Taxonomic Study, 5th edition*" [3]; "v (variable)" counted as positive; the percentages were calculated on the number of species basis. nt, not tested; nd, no data.

The ability to ferment fructose by brewer's yeast was well-known as early as in the first half of the 20th century, particularly in the context of "selective fermentation" observed in a mixture of glucose and fructose ([5,16] and the literature cited therein). However, yeast taxonomists have not paid serious attention to fructose. To the best of our knowledge, no recent work, except one, has employed fructose to characterize new yeast species [17]. In a recent publication, fructose was used to prepare an enrichment medium for the isolation of highly osmotolerant yeasts from natural substrates, as its solubility is much higher than that of glucose; unfortunately, assimilation/fermentation of fructose was not determined in the characterization of new species [18].

Why have such simple phenotypes largely neglected until now? The reasons would be: (1) fructose was not selected in the standard set of physiological characterization throughout the monograph "*The Yeasts, a Taxonomic Study*"; (2) physiological profile has been used mostly just as a key for yeast taxonomy; thus, fructose has been out of focus even though it occurs abundantly in the natural environment, such as in honey and fruits. Probably due to such a historical background, most of the recent researchers excluded physiological tests of fructose from the description of new yeast species, likely without paying attention to the "Kluyver rule".

In the present work, some strains exhibited fructophily during fermentation, preferring fructose, as a substrate for fermentation, to glucose. As stated in the results section, *A. platypodis* JCM 1843 and *C. americana* JCM 3592 appeared to be fructophilic in the regular fermentation test (Table 1). Furthermore, JCM 1843 and JCM 3592 demonstrated a fructophilic behavior, as determined by sugar consumption profiles in sugar solo fermentation, and this is contradictory to the pattern of sugar consumption by *S. cerevisiae* JCM 7255, which always preferred glucose to fructose (Figure 1). Initially, we hypothesized that JCM 1843 and JCM 3592 might exhibit a fructophilic behavior even in sugar duo fermentation (mixed fermentation), similar to *Zygosaccharomyces* species [19]. However, to our surprise, fructose consumption appeared to be suppressed in sugar duo fermentation (Figure 1). It is remarkable that glucose fermentation by JCM 1843 and JCM 3592 seemed to be activated by the presence of fructose in the medium. To the best of our knowledge, this is a new "irregular" pattern of sugar consumption profile. Further molecular biological investigations are required to clarify the mechanism underlying this phenomenon.

In contrast to *A. platypodis* and *C. americana*, *S. quercuum* JCM 9486 exclusively prefers glucose over fructose, and this is similar to the case reported previously for *W. versatilis* [14]. The reason for this exception, however, remains unknown. These specific preferences of sugar may be related to their lifestyles in the natural environment, which is a fascinating research theme from the viewpoint of yeast ecology. For instance, *A. platypodis* and *C. americana* likely inhabit a fructose-rich environment, and therefore, possess potent fructose transporter(s). Furthermore, the fructophilic behavior in *A. platypodis* and *C. americana* would be adaptive to such an environment.

Zygosaccharomyces rouxii and *Z. bailii* were reported to be fructophilic [19], although a clear fructophilic reaction was not observed in our simple experiments. In a previous study, *Z. bailii* was found to first ferment fructose and then glucose in a medium containing both glucose and fructose [19]. The experimental conditions in the present study differed from those in the previous one. As the fermentation test was performed using either glucose or fructose separately in the present study, the priority of sugar utilization remained unknown. Additional yeast strains exhibiting a fructophilic phenotype may be found if fermentation tests using a medium containing both glucose and fructose are performed. Later, fructose transporters in the plasma membrane of the *Zygosaccharomyces* yeasts were studied with molecular biological interests in their fructophilic behavior [20–22]. In addition, the mechanism of fructose fermentation has been well investigated on a molecular basis in the wine yeast *S. cerevisiae* [4]. *S. cerevisiae* contains at least 20 transporters associated with hexose uptake [23]. Glucose uptake is facilitated by hexose transporters [24]. Following its uptake into the cell cytoplasm, glucose is phosphorylated to glucose-6-phosphate, subsequently isomerized to fructose-6-phosphate, and finally metabolized through the glycolytic pathway [25]. Fructose is transported by the hexose transporter (HXT) family of proteins [26] and directly phosphorylated to fructose-6-phosphate by hexokinases, such as Hxk1 and Hxk2 [27]. Our data indicate that most of the yeasts belonging to the Saccharomycetes would exhibit fructose transporters and express specific hexokinases that metabolize fructose to fructose-6-phosphate. Indeed, a novel proton-coupled fructose transporter, Frt1, has been identified in *Kluyveromyces lactis* [28]. The mechanism of fructose uptake and its subsequent metabolism would be further investigated from the viewpoint of molecular biology using a wider variety of ascomycetous yeast species. Novel fructose transporters may be identified by exploring *FRT1* gene analogs using the draft genome sequences of ascomycetous yeasts.

In this study, we aimed to survey a wide variety of yeast species belonging to Saccharomycetes; thus, a single strain of each species was tested for its ability to assimilate and ferment fructose, except *A. platypodis* (JCM 1843 and JCM 1796) and *C. americana* (JCM 3592 and JCM 3593). Although the fructophilic behavior was less apparent in JCM 1796 and JCM 3593 than in JCM 1843 and JCM 3592, respectively (Table 1), both *A. platypodis* and *C. americana* preferably fermented fructose. Additional reference strains should be surveyed for fructose assimilation and fermentation, particularly for *A. platypodis*, *C. americana*, *S. quercuum*, and *W. versatilis,* to conclude the exceptions are species-specific. In addition, we should examine the assimilation/fermentation profiles of basidiomycetous yeasts in future studies to determine whether fructose assimilation/fermentation is a universal phenotype in yeasts irrespective of their taxonomic position.

Lastly, it is suggested that tests for fructose should be resurrected in the standard set of physiological characterization for yeasts in the Saccharomycotina subphylum in order not to miss the special characteristics of yeasts.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9040758/s1, Figure S1: Gas filling in a Durham tube in the fermentation liquid medium, Table S1: (a) Growth in the assimilation media containing each sugar, (b) Filling of gas in Durham tube in the fermentation media containing each sugar.

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