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Characterization of Old Wine Yeasts Kept for Decades under a Zero-Emission Maintenance Regime

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Abstract: All laboratories dealing with microbes have to develop a strain maintenance regime. While lyophilization based on freeze-drying may be feasible for large stock centers, laboratories around the world rely on cryopreservation and freezing of stocks at −80 °C. Keeping stocks at these low temperatures requires investments of several thousand kW/h per year. We have kept yeast stocks for several decades at room temperature on agar slants in glass reagent tubes covered with vaspar and sealed with cotton plugs. They were part of the Geisenheim Yeast Breeding Center stock collection that was started in the 19th century, well before −80 °C refrigeration technology was invented. Of these stocks, 60 tubes were analyzed and around one-third of them could be regrown. The strains were typed by sequencing of rDNA PCR fragments. Based on BlastN analyses, twelve of the strains could be assigned to *Saccharomyces cerevisiae*, two to *S. kudriavzevii*, and the others to *Meyerozyma* and *Candida*. The strains were used in white wine fermentations and compared to standard wine yeasts Uvaferm/GHM (Geisenheim) and Lalvin EC1118. Even with added nitrogen, the strains exhibited diverse fermentation curves. Post-fermentation aroma analyses and the determination of residual sugar and organic acid concentrations indicated that some strains harbor interesting flavor characteristics, surpassing current standard yeast strains. Thus, old strain collections bear treasures for direct use either in wine fermentations or for incorporation in yeast breeding programs aimed at improving modern wine yeasts. Furthermore, this provides evidence that low-cost/long-term culture maintenance at zero-emission levels is feasible.

Keywords: strain collection; aroma profiling; gas chromatography; wine yeast; *Saccharomyces*; fermentation; volatile aroma compounds

1. Introduction

At the end of the 19th century, Emil Christian Hansen at the Carlsberg Laboratory in Copenhagen, Denmark, established the first pure culture lager yeast strain, Unterhefe No. 1 [1]. This strain then became known as *Saccharomyces carlsbergensis*. The finding that one yeast strain was sufficient to generate a fermented beverage of high quality started a new era and lead to new developments in the beer and dairy industry. It was soon recognized by Julius Wortmann at the Geisenheim Research Center in Germany that Hansen's findings were also applicable to wine making [2]. This started efforts in collecting wine yeast strains from different vineyards and wineries in the Rheingau area. These strains were characterized for their fermentation capacity and flavor attributes. At the "Geisenheimer

Hefe-Reinzuchtstation" (Geisenheim Yeast Breeding Center), founded in 1894, these strains were produced as liquid starter cultures and dispatched to the wineries upon request.

With the isolation of pure yeast cultures came the responsibility to maintain stocks of these cultures. Wine making requires yeast starter cultures only once a year just after the grape harvest. By contrast, yeasts for beer production are in constant use throughout the year. Even before the isolation of pure cultures, there was an interest in generating dry yeast cakes for longer term storage. The history of both the patents and literature in this field has been covered in depth by a recent excellent review [3]. A solid supply of dehydrated yeast became a necessity for long distance shipments, which came around 1940 when the Fleischman Co. produced active dry yeast. This yeast required 'reactivation', i.e., rehydration prior to use. In the 1970s, Lesaffre introduced an instant dry yeast which could be used directly without reactivation. In microbiological laboratories, however, bacterial and yeast cultures are nowadays generally preserved by storing at −80 °C which, since the 1970s, has become technically feasible on a larger scale [4].

Thus, for decades after the 1880s, yeast cultures had to be kept by other means. Two techniques used were water stocks and yeast slants covered with vaspar (a mix of paraffin and Vaseline) [5,6]. The method of storing yeasts in distilled water at room temperature, as proposed by Castellani, is not only a cheap way of preserving cultures but is also a very effective way of culturing a collection over many years without the need for constant propagation [7]. This method is particularly useful for yeasts [8]. Storage of fungal cells in distilled water can be extended for 20 years [9]. Therefore, it was stated that storing yeast cultures in distilled water may reach similar efficiencies as freezing at −80 °C [10]. The use of a paraffin or vaspar overlay is also a very cheap way of yeast culturing, although the viability may be reduced when compared to the other methods.

There are only a few long-term studies describing yeast viability, one of which used the traditional method of yeasts grown on slants and covered by paraffin oil and found cells to be viable after a seven-year incubation period [11]. At the Geisenheim Yeast Breeding Center, we have a large collection of wine yeasts and non-conventional yeasts dating back to the 1890s. Samples were routinely stored with a vaspar overlay. Of course, over time, the strain collection was transferred to either storage in liquid nitrogen or in freezers at −80 °C. Nevertheless, we still stored a few samples for over 30 years at room temperature in the old way. In this study, we examined 60 tubes containing these decade-old samples. The yeast were restreaked, and those strains that could be regrown were subjected to fermentation studies and volatile aroma analyses. Our results show that strain collections can safely be stored at room temperature. Such a strain maintenance regime could contribute to energy conservation and the reduction of CO_2 emissions.

2. Materials and Methods

2.1. Strains and Media

The yeast strains used in this study are shown in Table 1, including standard wine yeast strains used for comparison. Yeast strains were subcultured in YPD (1% yeast extract, 2% peptone, 2% glucose).

Table 1. Strains used in this study.

Original Label	Sequence-Based Assignment
Zell 1895	***Saccharomyces cerevisiae***
Valdepenas Criptana 1909	***Saccharomyces cerevisiae***
Brettanomyces claussenii IHG Berlin 1959	*Meyerozyma guilliermondii*
Ungstein 1892	***Saccharomyces cerevisiae***
Riesling Krim 1896	***Saccharomyces cerevisiae***
Olewig II 1896	*Meyerozyma guilliermondii*
Dürkheim 1892	*Meyerozyma guilliermondii*
Rüdesheimer Hinterhaus 1893	***Saccharomyces cerevisiae***
Alpiarca II 1896	***Saccharomyces cerevisiae***
Candida tropicalis	*Candida sanyaensis*
Heimersheimer Ruth 1895	***Saccharomyces cerevisiae***
Steinberg 1893	***Saccharomyces cerevisiae***
Rüdesheimer Berg	***Saccharomyces cerevisiae***
Würzburg (Stein)	*Saccharomyces kudriavzevii*
Winningen 1892	*Saccharomyces kudriavzevii*
Scy 1892	***Saccharomyces cerevisiae***
Bordeaux 1892	***Saccharomyces cerevisiae***
Geisenheimer (Mäuerchen) 1893	***Saccharomyces cerevisiae***

Bold: to indicate true *S. cerevisiae* strains. These strains will be important for winemaking. The others are non-conventional yeasts.

2.2. Molecular Analysis of Yeast Strains

Typing of the strains was determined by performing ITS-PCR (ITS, internal transcribed spacer) using standard ITS1F-fungal specific-(5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4-universal-(5′-TCCTCCGCTTATTGATATGC-3′) primers and sequencing of the PCR products as previously described [12]. Sequencing was conducted by Starseq, Mainz, Germany.

2.3. Fermentation Conditions

Lab-scale fermentations were carried out in duplicate with a standard pasteurized white wine must with a sugar concentration of 72 °Oechsle. The must was supplemented by the addition of Fermaid E (inactivated yeast product; according to the supplier's instructions; Lallemand, Vienna, Austria). Cells were inoculated at a density of OD_{600} = 0.5. The fermentation temperature was set to 18 °C and cultures were incubated with constant stirring at 300 rpm.

2.4. Analytical Methods

At the end of the fermentations, several compounds including fructose, glucose, ethanol, and organic acids were analyzed by high-performance liquid chromatography (HPLC) with an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany). Quantitative analyses were done as described in [13]. The HPLC equipment was equipped with a variable wavelength detector (UV/VIS) and a refractive index detector (RID). The column for separation is an Allure Organic Acids (Restek GmbH, Bad Homburg, Germany) with a 5 µm particle size, 60 A pore size and dimensions of 250 mm x i.d. 4.6 mm. A water-based solution of sulfuric acid (0.0139% *v/v*) and ethanol (0.5% *v/v*) was used as eluent. Gas chromatography was conducted using a GC 7890A (Agilent, Santa Clara, CA, USA), coupled with a MSD 5977B mass spectrometer (Agilent, Santa Clara, CA, USA). The determination of aroma

compounds followed the analytical approach described in Belda et al. [14] according to the method of Camara et al. [15].

3. Results

3.1. Regrowing of Dormant Strains Kept under Vaspar for Decades

At the Geisenheim Yeast Breeding Center (GYBC), we stored two racks containing 60 reagent tubes with yeast strains. The tubes contained slants on which yeast strains were spread and grown and then overlaid with vaspar (Figure 1). This was the standard procedure for maintaining strains at the GYBC. The collections were generated before the introduction of −80 °C refrigeration. These samples represent an even older stock as younger samples (20+ years of age) were kept in reagent tubes with screw cap closures. The apparent age of the old samples is, therefore, estimated to be over 30 years but less than 60 years, as one isolate carried a label with the year 1959 indicative of the year of isolation (Table 1).

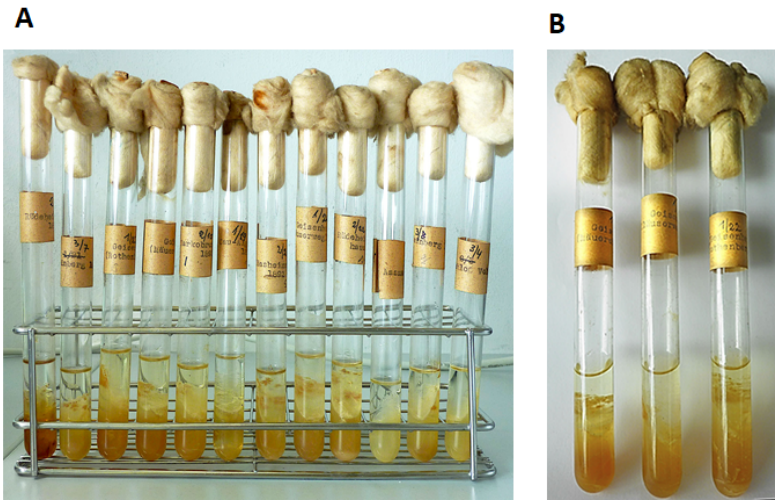


Figure 1. (**A**,**B**). Old samples from the Geisenheimer Yeast Breeding Center. Samples were generated as agar slants with yeasts grown and covered in vaspar. Tubes were plugged by a cotton ball. Samples were labeled according to the location of the isolate and the year of isolation (see Table 1).

We wanted to find out if these strains were still alive and, once propagated, what their fermentation behavior would be like. To this end, we either took samples with an inoculation loop and restreaked them on full medium YPD or inoculated them in liquid YPD. Astonishingly, about one-third (18 out of 60) of the strains could be regrown and cultivated under these conditions.

The strain labels often indicated the area where these strains had been isolated and did not necessarily identified the species. Thus, we went on to type the strains by PCR amplification of a region of the ribosomal DNA using a standard primer pair designed for fungal species (ITS1 and ITS4). These primers are located at the end of the 18S and start of the 28S rDNA and thus amplify the internal transcribed spacer (ITS) region including the 5.8S rDNA. This region is highly variable and allows strain determination to the species level. PCR products were sequenced, and the sequences compared to the NCBI non-redundant database using BlastN. The sequence comparisons indicated that most strains could indeed be assigned to *Saccharomyces cerevisiae*. Two of the strains were found to be *S. kudriavzevii* while three strains matched *Meyerozyma guilliermondii* and one strain could be assigned to a newly described species of *Candida sanyaensis* (Table 1).

3.2. Fermentation Performance

We went on to study the fermentation characteristics of the *Saccharomyces* strains. To this end, strains were used to ferment a standard white wine must of 72 °Oechsle to which additional amino

nitrogen was added via an inactivated yeast product (Fermaid E). Fermentations were carried out at 18 °C with stirring over a period of 12 days and fermentation rates were followed by daily measurements of CO_2 release. Strains were compared to the standard wine strain Lalvin EC1118 (Figure 2). It turned out that half of the strains generated a weight loss slightly larger than Lalvin EC1118, while the other half performed less well than Lalvin EC1118 in this respect. The largest weight loss was found with *S. cerevisiae* strain Steinberg 1893 and the *S. kudriavzevii* strain Würzburg (Stein). Lalvin EC1118 required a short lag phase of one day to enter alcoholic fermentation. Several of the tested yeast strains exhibited an extended lag phase of 3–4 days, particularly the strains that later showed the greatest weight loss and also Geisenheimer Mäuerchen, Winningen 1892, and Bordeaux 1892. Thus, even given the extended lag phase, these strains managed a complete fermentation within the 12-day fermentation window.

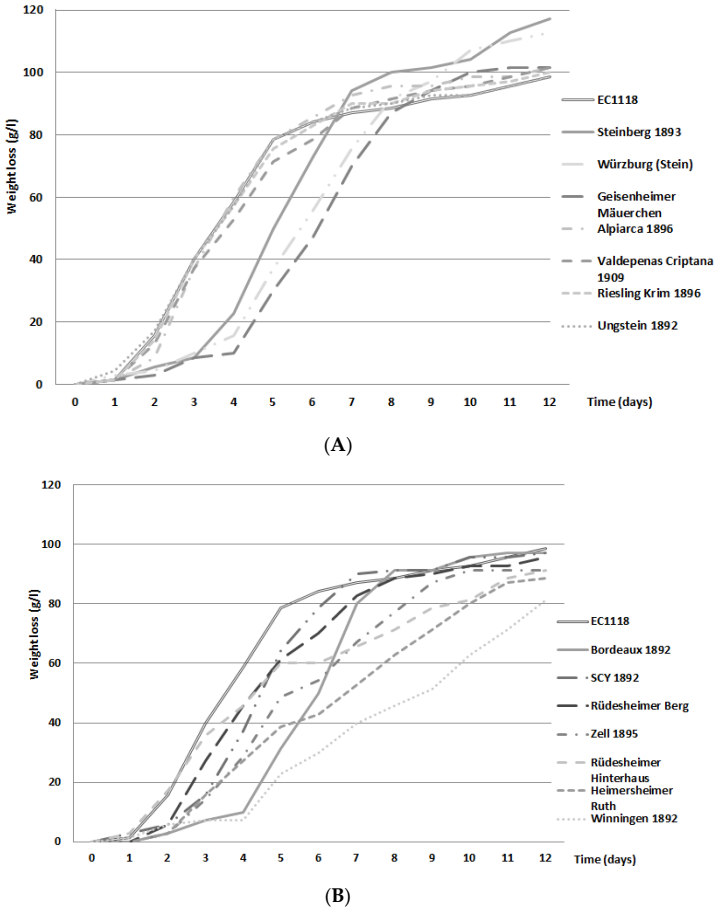


Figure 2. Fermentation curves based on CO_2 release/weight loss of *Saccharomyces* cultures derived from isolates of the old collection. Release of CO_2 was measured daily. (**A**) Strains are shown that released more CO_2 than the EC1118 control wine yeast strain. (**B**) Strains are shown that released less CO_2 than the EC1118 control wine yeast strain.

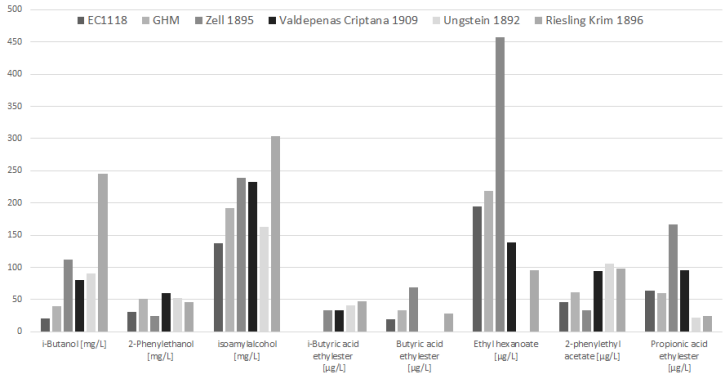
To analyze the fermented liquids in more detail, the residual sugars, organic acids, and final ethanol content were determined (Table 2). Most of the *Saccharomyces* strains reached complete fermentation with around 7% alcohol content. Two glucophilic strains, however, failed to utilize all of the fructose in the 12-day fermentation time. The *S. kudriavzevii* strains produced less alcohol than the *S. cerevisiae* strains. The two *S. cerevisiae* strains Rüdesheimer Hinterhaus 1893 and Heimersheimer Ruth, while using up glucose, did not utilize fructose completely during the 12-day fermentation. All strains showed a similar organic acid profile, with malate being the pronounced acid. Rüdesheimer Hinterhaus 1893, on the other hand, showed a surprising amount of shikimic acid (Table 2).

Table 2. HPLC analyses of residual sugars, organic acids, and total alcohol of *Saccharomyces* wine yeasts.

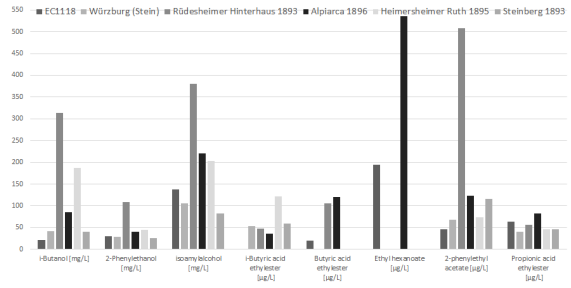
Strains	*Glucose*	*Fructose*	*Total Sugar*	*Malate*	*Shikimic Acid*	*Lactate*	*Acetate*	*Citric acid*	*Ethanol*	*Ethanol*
	[g/L]	**[g/L]**	**[g/L]**	**[g/L]**	**[mg/L]**	**[g/L]**	**[g/L]**	**[g/L]**	**[g/L]**	**[%]**
EC1118 wine yeast	<1	<1		4.6	38	0.2	0.1	0.2	60.7	7.7
GHM wine yeast	<1	<1		4.4	40	0.3	0.2	0.2	56.3	7.1
Saccharomyces cerevisiae										
Zell 1895	<1	<1		3.7	41	0.4	0.1	0.2	60.2	7.6
Valdepenas Criptana 1909	<1	<1		3.5	39	0.2	0.1	0.2	54.9	7.0
Ungstein 1892	<1	<1		2.9	28	0.2	0.2	0.2	56.6	7.2
Riesling Krim 1896	<1	<1		2.2	20	0.1	0.2	0.2	57.1	7.3
Rüdesheimer Hinterhaus 1893	<1	4.1	4.1	3.9	297	0.9	0.2	0.4	53.8	6.9
Alpiarca II 1896	<1	<1		4.1	38	0.2	0.1	0.2	56.8	7.2
Heimersheimer Ruth 1895	<1	6.9	6.9	3.4	34	0.3	0.2	0.2	56.4	7.2
Steinberg 1893	<1	<1		3.8	32	0.1	0.1	0.2	56.4	7.1
Rüdesheimer Berg	<1	<1		4.0	39	0.5	0.2	0.2	55.9	7.1
Scy 1892	<1	<1		3.6	31	0.1	0.2	0.2	58.7	7.4
Bordeaux 1892	<1	<1		2.9	29	0.2	0.1	0.2	56.9	7.2
Geisenheimer (Mäuerchen)	<1	<1		3.8	27	0.1	0.4	0.2	57.1	7.2
S. kudriavzevii										
Würzburg (Stein)	<1	<1		3.7	26	<0.1	0.2	0.2	54.2	6.9
Winningen 1892	2.1	19.2	20.8	4.1	37	0.5	0.2	0.2	49.7	6.3

3.3. Production of Aroma Compounds

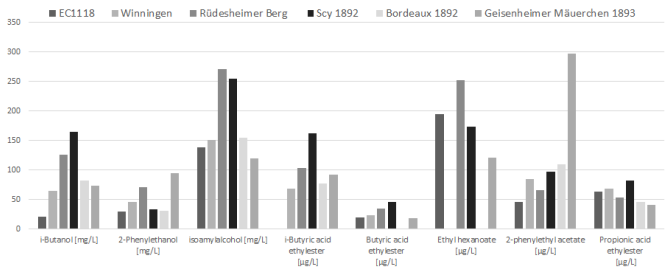
We routinely examined 28 aroma compounds, specifically alcohols and esters (Table S1). A comparison within strains using a selection of eight major compounds is shown in Figure 3 and Table 3. While all species produced a range of compounds, it was interesting to see that a major current wine production strain, EC1118, was actually not the highest producer of certain aroma compounds in our assay. The three strains that produced most fruity esters were Rüdesheimer Hinterhaus 1893, Alpiarca 1896, and Valdepenas Criptana 1909. Additionally, Rüdesheimer Hinterhaus 1893 championed the production of isoamyl acetate (acetic acid 3-methyl butyl ester) and 2-phenylethyl acetate (acetic acid 2-phenylethylester), which is the acetate ester of 2-phenylethanol. This is apparently a consequence of Ehrlich pathway output as regarding the production of alcohols, particularly i-butanol, isoamylalcohol (3-methyl-butanol), and 2-phenlyethanol, the strain that came on top for each of the compounds was also the Rüdesheimer Hinterhaus 1893 yeast (see Table S1).



(**A**)



(**B**)



(**C**)

Figure 3. (**A**–**C**). Bar charts with selected alcohol (in mg/L) and ester (in µg/L) aroma compounds of *Saccharomyces* strains compared to the EC1118 wine yeast. Flavor compounds were measured using gas chromatography at the end of fermentation. The full list of aroma compounds is shown in Table S1.

Table 3. Aroma compound generation determined at the end of fermentation.*

	i-Butanol [mg/L]	2-Phenyl-Ethanol [mg/L]	Isoamyl Alcohol [mg/L]	i-Butyric Acid Ethyl-Ester [µg/L]	Butyric Acid Ethylester [µg/L]	Hexanoic Acid Ethyl-Ester [µg/L]	2-Phenylethyl Acetate [µg/L]	Propionic Acid Ethyl-Ester [µg/L]
EC1118	21	30	138	nq	20	195	46	64
GHM	40	51	192	nq	34	218	62	60
Saccharomyces cerevisiae								
Zell 1895	112	24	238	34	69	**457+**	34	**167**
Valdepenas Criptana 1909	81	60	233	33	nq	138	94	**95**
Ungstein 1892	90	52	163	41	nq	nq	106	23
Riesling Krim 1896	**246**	46	303	48	28	96	98	25
Rüdesheimer Hinterhaus 1893	**314**	**108**	**380**	47	**106**	nq	**507**	57
Alpiarca 1896	85	41	220	35	**120**	**536**	123	82
Heimersheimer Ruth 1895	187	44	202	**122**	nq	nq	74	46
Steinberg 1893	40	25	83	59	nq	nq	116	46
Rüdesheimer Berg	126	72	**271**	104	35	252	67	54
Scy 1892	164	34	255	**162**	46	174	98	82
Bordeaux 1892	83	31	155	77	nq	nq	110	46
Geisenheimer Mäuerchen 1893	74	**95**	120	92	18	121	**297**	41
S. kudriavzevii								
Würzburg (Stein)	42	29	106	54	nq	nq	68	40
Winningen	65	46	151	68	23	nq	85	68

* nq = not quantified; + = pronounced values are shown in bold and highlighted in orange.

What was interesting to note is that some strains produced either high levels of hexanoic acid ethylester (ethyl hexanoate) or high levels of acetic acid phenylethylester, but not of both substances. This was observed when comparing Rüdesheimer Hinterhaus 1893 with Zell 1895 and Alpiarca 1896. The first was low on hexanoic acid ethylester but produced high levels of 2-phenylethyl acetate, while the latter strains produced high levels of hexanoic acid ethylester and much lower levels of 2-phenylethyl acetate (Table 3, Figure 3; see Section 4).

4. Discussion

Alcoholic beverage production has been carried out by spontaneous fermentation throughout the ages, and today, often still the preferred method of fermentation for some. In beer and baking enterprises, it was already realized in the middle ages that there are special properties in the slurry that leavens bread and makes beer ferment and foam. It was the traditional occupation of a 'Hefner' in Germany to maintain and provide sufficient supplies of this leavening activity [16]. Yet, as it was not clear what the causal activity in the slurry was, the German 'Reinheitsgebot' (purity law) from 1516 only stated that beer should be brewed using barley malt, hops, and water. With the work of Pasteur, published in his Etudes sur le vin and Etudes sur la biere, and the work of others, it became evident that yeast, *Saccharomyces cerevisiae*, was responsible for the observed fermenting power.

The pure yeast strain isolation procedure worked out by Hansen was transferred to the wine industry by the German Julius Wortmann, who founded the 'Geisenheim Yeast Breeding Center' in 1894 [1,2]. The isolation of pure yeast strains in Geisenheim had been initiated in the early 1890s and these strains are still preserved today. Today's strain maintenance relies on deep freezing of culture collections at −80 °C, while supplies for the industry are generally provided as instant dry yeasts [17,18].

Yet, at the Geisenheim Yeast Breeding Center, stocks were originally maintained as slants with a paraffin overlay. Cultures were stored either in this manner or, even simpler, in plain water [5,6]. A recent report analyzed long-term storage (12 years) of >1000 stocks and provided evidence that water storage yielded survival rates of 98.9%, closely resembling that of frozen stocks (99.5%), while survival rates under a mineral oil layer were a bit less with 88.2% [10]. Two racks of Geisenheim yeast stocks were kept over the years, more as display items than out of necessity. Such a long-term storage has not been previously reported. In fact, it is not entirely clear anymore when exactly these stocks were generated (besides the fact that they are very old and between 30 and 60 years of age). Collectively, these studies show, however, that yeast strain collections can be routinely kept under a zero-emission regime, which could be used as an incentive to reduce the energy-demanding storage of cultures at −80 °C.

The 'old' Geisenheim yeast strains can be both a heritage and a source of new strains for yeast breeding programs. As a heritage, they could be used to strengthen the local character of wines produced with them and thus contribute to the terroir of these wines [19–21]. To be useful as a breeding stock, these old strains need to be characterized in more detail, preferably including their genomes [22,23]. In our study, the yeast with the most pronounced flavor production capability of alcohols and esters was the *S. cerevisiae* strain Rüdesheimer Hinterhaus 1893 ('Hinterhaus' refers to backyard). It was apparent that this strain is a remarkable producer of acetate esters, but not so of medium chain fatty esters. The former are produced by the acetyl transferases Atf1 and Atf2, while the latter are generated by the acyl-coenzymeA:ethanol O-acyltransferases Eeb1 and Eht1 [24,25]. This suggests strong activity of the ATF alcohol acetyl-coA transferases in the Rüdesheimer Hinterhaus strain isolated already in 1893, with reduced formation of medium chain fatty acids. It will be interesting to explore these properties through a full-scale wine fermentation trial and use in-depth genomics and transcriptomics to generate molecular markers for yeast breeding.

The strive for more volatile aromas has spurred the search for alternative yeasts and renewed the interest in spontaneous fermentations [26]. Yet, due to the unpredictable and inconsistent outcomes of

spontaneous fermentations, the development of improved starter cultures or consortia may provide better alternatives [27,28].

5. Conclusions

In conclusion, our study of yeast isolates stored for a very long period opens several new research avenues in the use of these strains, either directly as starter cultures or as stocks for the Geisenheim Yeast Breeding Center; both will be interesting to exploit in the future. For general use, our data and previous work, e.g., on water cultures show that zero-emission strain-keeping of yeast cultures is feasible and should be more generally exploited.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/1/9/s1, Table S1. Full list of aroma compounds.

Author Contributions: Conceptualization, J.W., D.R., and C.v.W.; methodology, K.M., B.B., S.B., H.S.; validation, J.W., K.M., B.B., S.B., H.S.; formal analysis, J.W., K.M., S.B., H.S.; investigation, J.W., K.M., B.B., S.B., H.S.; resources, J.W.; D.R., C.v.W. data curation, J.W., K.M., S.B., H.S.; writing—original draft preparation, J.W.; writing—review and editing, J.W., D.R., C.v.W., K.M., B.B., S.B., H.S.; visualization, K.M. and J.W. supervision, J.W. and D.R.; project administration, J.W. and D.R.; funding acquisition, J.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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