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Resurrection of the lager strain *Saccharomyces pastorianus* TUM 35

Saccharomyces pastorianus lager yeast strains are some of the most important industrially used microbes used in fermentations. Lager beer types dominate the market with over 90 % of the market share. Although some popular and widespread lager strains, such as the most used strain _Saccharomyces pastorianus_ TUM 34/70, are well characterized, little or nothing is known about old and seldom used lager strains from long-standing strain collections. Only two _Saccharomyces pastorianus_ lager strain subgroups are known to date, 'Frohberg' and 'Saaz'. Most industrial, modern, high-performance lager strains belong to the 'Frohberg' group. In this study our group reactivated a freeze-dried stock of a yeast culture (carrier matrix unknown, probably dry milk powder) of the historic strain TUM 35. The strain was presumed to have been lost over time. Fortunately, the freeze-dried stock was found in a forgotten box in a storage room (together with other historic strains) at the Research Center Weihenstephan for Brewing and Food Quality. TUM 35 grew after two weeks of applying a tailored reactivation protocol in liquid wort. This paper presents research on the history of the strain TUM 35. Its journey could be traced back from Freising-Weihenstephan to Nuremberg and to its origin Coburg in upper Franconia. Its history also revealed why this formerly very successful old yeast strain disappeared completely in the mid to late 1950s. We also confirmed the species using specific qPCR systems with marker DNA-regions for _S. pastorianus_ identification. PCR-capillary electrophoresis of the IGS2-314 rDNA fragment showed the close relation to the strain TUM 34/70 but also the subtle differences in the DNA-fingerprint pattern. Phenotypic experiments and beer fermentation trials at volumes of 30 L could prove that TUM 35 performed like a typical Frohberg-type lager strain. It produced a straight, neutral and soft aroma profile in the final beer with a high degree of fermentation. Results of fermentation by-product analysis and other main beer parameters of the beer produced with TUM 35 lie within the specifications and reference values for Frohberg-type lager beers. In contrast to most other lager beer strains, TUM 35 produced no sulfuric aromas that could be sensed by the tasting panel in the final beer. This study is a first approach to improve the understanding of old lager beer yeast strains and also opens up opportunities for breweries to use forgotten old strains for standard or historic lager beer production.

Descriptors: yeast, *Saccharomyces pastorianus*, TUM 35, lager beer, fermentation, history, identification, differentiation

1 Introduction

The most important yeast species for fermentation technology belong to the *Saccharomyces* genus [1-4]. Presently the genus includes eight natural species (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, *S. jurei*, *S. eubayanus* and *S. uvarum*), together with two artificial and hybrid species exclusively associated with human-made fermentative environments (*S. pastorianus* and *S. bayanus*) [5, 6]. *Libkind* et al. published that the yeast strains of the species *Saccharomyces pastorianus* used in lager beer production are genetic hybrids of *Saccharomyces cerevisiae* and the Patagonian wild yeast *Saccharomyces eubayanus* [7]. Older literature (pre-1987) refers to lager yeasts as *S. carlsbergensis* instead of *S. pastorianus*. Although the two names refer to the same group of yeasts, two distinct type strains have to be considered. CBS 1538 is the type strain of *S. pastorianus* and CBS 1513 is the type strain of *S. carlsbergensis*. As explained above, the nomenclatural change concerned the belated recognition that *S. pastorianus* was an older name than *S. carlsbergensis*. This hybrid species is by far the most relevant brewing yeast, since lager beer accounts for 94 % of the world market [8]. However, *S. pastorianus* has never been isolated outside the brewing environment; therefore, it appears to be an exclusively domesticated species.

Although the precise origin of lager yeasts is unknown, it is logical to assume that their emergence is intimately related with the origin and development of lager-brewing in the 15th century in Bavaria [9,

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10]. *S. pastorianus* was described by *Hansen* in 1904, however, the hybrid nature of lager yeasts was first hypothesized much later in the 1980s [11-13, 6]. Later *S. euybayanus* was also discovered in North America, Asia and New Zealand [14-16]. *Dunn* and *Sherlock* postulated that at least two hybridization events took place and that all *Saccharomyces pastorianus* lager strains consist of at least two types [17]. Other studies also confirmed the existence of two different lager yeast groups [18, 1, 19, 20]. The *S. pastorianus* strains were divided into the Saaz and Frohberg groups [18, 6, 20]. *Gallone* et al. described different hypothetical evolutionary models that could have led to the lager groups Saaz and Frohberg [21].

The terms 'Frohberg' and 'Saaz' were coined at the VLB at Berlin by *Delbrück* and *Lindner* at the turn of the 19th century, describing two pure type strains with a characteristic fermentation behavior. They had been isolated from bottom-fermenting 'Stellhefen' (mixed brewing yeast populations) obtained from Frohberg's brewery at Grimma (Saxony) and the Saaz brewhouse (Bohemia) [10, 22]. The most famous Saaz-type yeast strains are Carlsberg 'Unterhefe Nr. 1' (CBS 1513) and 'Unterhefe Nr. 2' (CBS 1503) isolated by Emil Christian Hansen whereas the most famous member of the Frohberg group is *Saccharomyces pastorianus* TUM 34/70 [1, 6, 20]. Industrial strains exhibiting strong fermentation performance belong to the Frohberg group [18, 1]. Besides distinct genetic compositions, the two lineages of lager yeasts have different flavor and fermentation profiles. Saaz strains grow better at low temperatures (10 °C), have a relatively poor fermentation performance compared with Frohberg strains at 22 °C, do not utilize maltotriose, and produce lower amounts of esters [18, 20]. *Saccharomyces pastorianus* lager yeast strains are bottom-fermenting, while *Saccharomyces cerevisiae* brewing yeast strains are top-fermenting. Both differ significantly from one another with regard to numerous characteristics. The former are able to ferment at lower temperatures, flocculate well during primary fermentation and are harvested from the bottom of a fermentation tank, e.g. cylindroconical tank (CCT). The latter cease to function or they ferment very slowly at low temperatures (6 – 10 °C). They ferment most readily at temperatures between 15 and 25 °C, depending on the strain and type of wort. Cell growth is more vigorous among top-fermenting brewing yeast strains, and the cells do not flocculate as rapidly compared with bottom-fermenting yeast strains [1, 6]. Therefore, while both groups evidence their alloploid hybrid nature by combining the cryotolerant phenotype of *S. eubayanus* with the good fermentation performance of *S. cerevisiae*, detectable differences both in cryotolerance and in fermentation have been linked to the proportional amount of *S. eubayanus* DNA retained in their respective genomes. Moreover, the different phenotypes offer an explanation for the dominance of Frohberg strains in modern industrial brewing [6].

A brewing yeast strain should be taxonomically classified by means of molecular biological methods at the species and strain level. Its propagation and fermentation performance, as well as its aroma profile, should also be characterized [1]. TUM 34/70 is one of the most abundant lager yeast strains in the brewing industry, and is a strain with which many other lager strains are compared regarding fermentation performance and the pure flavor of lager beers produced with it [1]. The genome of TUM 34/70 was also the first of the bottom-fermenting strains to be sequenced and published [23]. A study conducted by *Mueller-Auffermann* used the characteristics of TUM 34/70 as a reference for developing a method to rapidly compare the performance of lager yeast strains [24]. Studies that compare a broader set of lager strains phenotypically and in terms of fermentation and technological characteristics in brewing are rare. A study by *Donhauser* et al. also compared a broad set of lager strains on a 20-L scale pilot fermentation set-up [25, 26]. This group confirmed that TUM 34/70 is one of the best-performing strains in terms of fermentation degree and beer maturation. The fermentation by-products were within the desired range and the sensorial impression scored highly. These findings confirmed those of Prof. Narziß in 1956, TUM 34 (the ancestor of TUM 34/70) being the most appropriate lager strain for lager beer production within a strain set of seven lager strains and one lager strain yeast mixture [27, 28].

Before *S. pastorianus* TUM 34/70 started its triumphal march after the dissertation and publication of *Narziß*, the yeast strain TUM 35 was one of the most widespread lager yeast strains in Franconia (Northern Bavaria). The aim of this study was to characterize the recultivated or 'resurrected' lager strain TUM 35. In this study we gathered historical data on why this formerly widespread industrial lager strain completely disappeared. A lot of the historic facts related to strain TUM 35 are based on personal correspondence and records belonging to Prof. Ludwig Narziß, who is one of the authors of this study. It was not included in the strain set of Narziß's studies in the 1960s whereas *Wagner* used that strain 2002 in a comparative lager strain study [29]. In 2002, Wagner presented at the first Weihenstephaner Hefesymposium (yeast symposium) that TUM 35 was the 16th most appropriate flocculating lager strain whereas TUM 34/70 was the most appropriate flocculating lager strain with regard to various technological and sensorial evaluation parameters. In this context TUM 35 was actively used in Wagner's studies but it was not actively sold or promoted during that time and only used for research purposes. Before and after these studies the lager strain TUM 35 was neither used for industrial applications nor for scientific studies. This paper describes why this strain was of great importance after the Second World War, that it had extraordinary aroma profile properties in the past, and why it disappeared completely for industrial-scale fermentations. Following Wagner's study in 2002 the strain was completely forgotten and was neither present as an active slant agar culture nor as a cryo stock culture at – 80 °C. The strain was lost. Fortunately, our group found a freeze-dried stock on milk-powder (composition unknown) in HPLC flasks. These were found in a forgotten box in a storage room also containing other yeast strain stocks at our institute. We reactivated the yeast from the dried pellet in liquid wort according to a tailored protocol that is described in the paper. The aim of the study was to characterize the strain TUM 35 genetically, to evaluate its fermentation characteristics in a 30-L brewing trial and the quality of the final beer. Additionally, the strain history and route should be revealed.

2 Materials and methods

2.1 Yeast strains

Two lager yeast strains (*Saccharomyces pastorianus* TUM 34/70 and TUM 35) and one German wheat beer strain (*Saccharomyces*

Table 1 Brewing yeast strains, their industrial application and their original conservation stock type

Yeast strains (TUM identifier)	Industrial application	Conservation stock type
Saccharomyces pastorianus TUM 35	Lager beer production, bottom-fermenting flocculant yeast strain	Freeze-dried culture on milk powder in sealed HPLC glass flasks
Saccharomyces pastorianus TUM 34/70	Lager beer production, bottom-fermenting flocculant yeast strain	Slant agar
Saccharomyces cerevisiae TUM 68	German wheat beer production, top-fermenting yeast strain	Slant agar

cerevisiae TUM 68) were obtained from the Yeast Center division of the Research Center Weihenstephan for Brewing and Food Quality (Table 1). The origin and the historic importance of the yeast strain TUM 35 are described in detail in the next chapter 'reactivation of a freeze-dried culture' and in the first chapter of the results section 'historic information research'.

2.2 Reactivation of a freeze dried yeast stock

An old *S. pastorianus* TUM 35 conservation stock in an HPLC glass flask (age unknown) was found in 2018 at the Research Center Weihenstephan in a forgotten box in a storage room containing approx. 20 different old freeze-dried stock cultures. Historic strains were stored in a box in the same order and with the same strain number descriptions as written on the list shown in figure 2 (results, see page 72). Figure 2 only shows the top section of the historic list. As the order and numbers are identical for all strains we assume that the HPLC vial with the number 35 found in 2018 is really the historic strain TUM 35. Until then, *S. pastorianus* TUM 35 had seemed to be lost. Our group reactivated the freeze-dried culture of TUM 35 according to the steps shown in figure 1.

The sealed HPLC flask was opened aseptically and the pellet was placed in 50 mL sterile liquid wort medium in a 100 mL glass flask with sterile cotton plug. The flask was incubated at 27 °C for 14 days under aerobic conditions and was shaken continuously at 80 rpm (Wiseshake, Witeg GmbH, Wertheim, Germany). A loop of the yeast suspension was streaked on wort slant agar and incubated aerobically at 27 °C for 5 days. The slant agar was stored at 4 °C and used as new stock culture for further studies.

2.3 Genetic identification and strain determination

2.3.1 DNA extraction

To isolate the DNA from each investigated yeast isolate, cultures were taken from wort agar slants using a sterile inoculation loop, transferred to a 1.5 mL micro centrifuge tube, and mixed with an aliquot of 200 µL InstaGeneTM Matrix solution (Biorad, Munich, Germany). Each tube was vortexed for ten seconds and incubated at 56 °C for 30 minutes, followed by another ten seconds of vortexing and incubation at 96 °C for eight minutes. The incubation steps occurred in a Thermomix 5436 (Eppendorf, Hamburg, Germany). After incubation, the tubes were centrifuged at 13,000 × g for two minutes and then a 100 µL aliquot of the supernatant containing the DNA was transferred to a new 1.5 mL micro centrifuge tube [30-32]. The DNA concentration was adjusted to 25 ng/µL after being measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

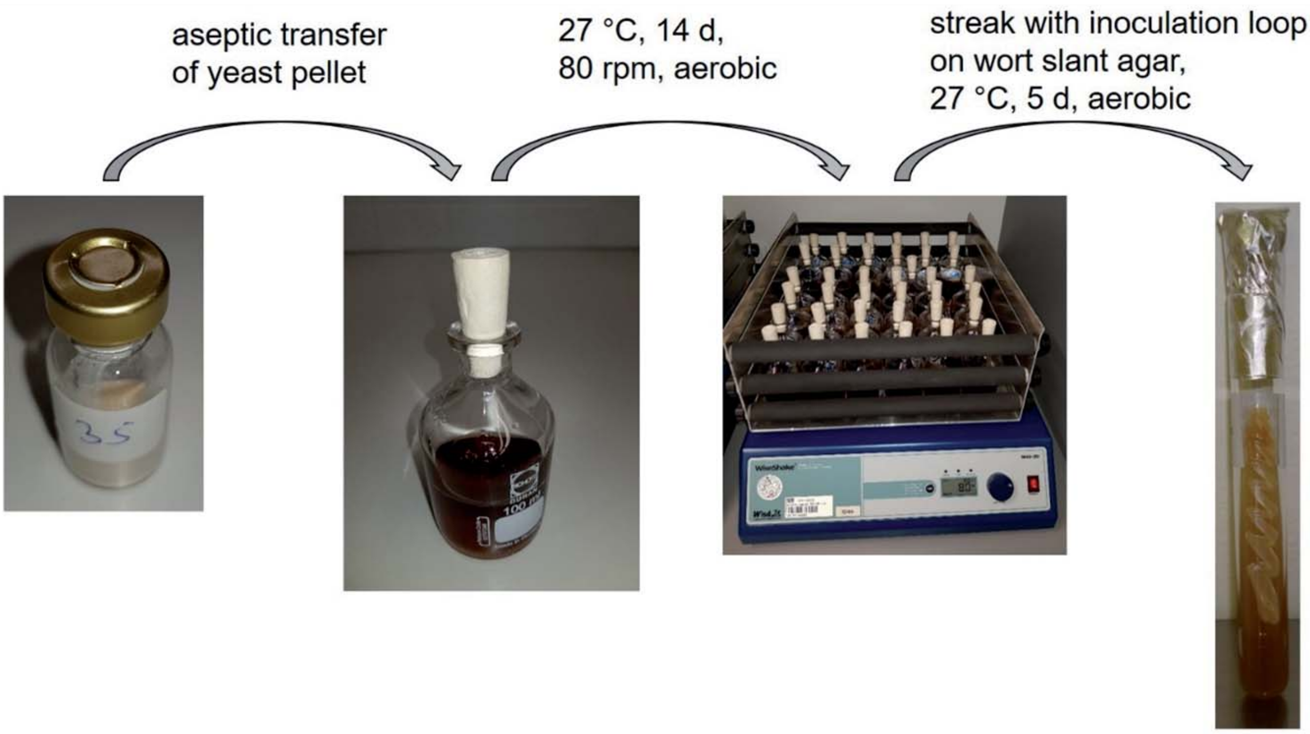

Fig. 1 Reactivation of freeze-dried culture of Saccharomyces pastorianus TUM 35 in aerobic shaking culture in sterile wort with subsequent streak on wort agar

Stamm	Auftraggeber bzw. Herkunft	Ursprung	Vergärung
7		Rosenbr.Pößneck	
26		Lütschena	mittel
34 Rz70	Staatsbr. Wstephan	Nürnberg, Stamm Reif	hoch
34 Rz78	Staatsbr. W`stephan	Nürnberg, Stamm Reif	hoch
35		Nürnberg, Stamm Coburg	

Fig. 2 Section of historic TUM strain list (in German) that documents the origin of strain TUM 35 and TUM 34/70

2.3.2 Quantitative polymerase chain reaction (q-PCR)

RT-PCR (Light Cycler® 480 II. Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used to taxonomically classify the isolates. The applied primer and TaqMan® probe sequences and RT-PCR procedure followed that of *Hutzler* and publications of our group [30, 31, 1, 33, 32, 6]. The RT-PCR systems Sc-GRC3, Sce, TF-COXII, Sbp, Seub, BF-300, BF-LRE1, Sdia are described as compatible and were performed with 10 µL 2x Mastermix (Light Cycler® 480 Probe Master, Roche, Germany), 1.4 µL DNA-free water, 0.8 µL (400 nM) of each primer (Biomers, Ulm, Germany), 0.4 µL (200 nM) probe (Biomers, Ulm, Germany; MGB probe from ThermoFisher scientific, Applied Biosystems®, USA). The yeast strains *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 were used as a positive and negative control according to the RT-PCR system tested.

2.3.3 DNA fingerprinting (PCR-amplicon-capillary electrophoresis of the IGS2-314 fragment)

In order to determine if isolates represented different or similar strains, genetic fingerprints were generated using the IGS2-314 method [30, 31]. The IGS2 is a spacer region within of the ribosomal cluster. To a partial sequence of the intergenic spacer 2 (IGS2-314) the specific primers IGS2-314f (5'-CGGGTAACCCAGTTCCTCACT-3') and IGS2-314r (5'-GTAGCATATATTTCTTGTGTGAGAAAGGT-3') (Biomers GmbH, Ulm, Germany) [34, 30, 31] were used at a concentration of 600 nM as described by Hutzler 2010 [30, 31].

PCR was performed with 22.5 µL RedTaq Mastermix (2x) (Genaxxon, Ulm, Germany) and 2.5 µL template DNA with a total reaction volume of 25 µL. The Mastermix contained 12.5 µL buffer solution (RedTaq Mastermix), 7.0 µL DNA-free PCR water and 1.5 µL of each primer (Biomers, Munich, Germany). Cycling parameters are described by Hutzler [30, 31]. PCR was performed using a SensoQuest LabCycler48s (SensoQuest GmbH, Gottingen, Germany). Amplified fragments were analyzed using a capillary electrophoresis system (Agilent DNA 1000 kit) following the manufacturer's recommendations (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA).The results of the PCR-product separation (DNA-Fingerprint) using the capillary electrophoresis were imaged as a gel image and eletropherograms using the Bioanalyzer 2100 software.

2.4 Brewing trials

2.4.1 Wort

For the fermentation trial, industrial hot wort (IBU 31) for the beer type Weihenstephaner Original (a Bavarian Helles Lager beer type with 21 IBU, used hop variety Hallertauer Perle) was sampled aseptically from the State Brewery Weihenstephan in 20 L Cornelius KEGs and 30 L was aseptically transferred to pilot fermentation tanks. When wort reached pitching temperature after cooling, the wort was pitched as described below. The original gravity and pH value of the wort are shown in table 3 (results section, see page 73)

2.4.2 Propagation

In order to propagate yeasts, isolates were inoculated from agar slants (yeast pure culture) into 2 × 60 mL of sterile wort medium in 2 × 100 mL Erlenmeyer flasks and incubated for 72 h at ambient temperature (20 °C) and pressure, and agitated at 80 rpm using a WiseShake 207 orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany). After incubation, the 2 × 60 mL were transferred to 2 × 2 L of sterile wort medium in 2 × 5 L glass flasks closed with sterile cotton plug and further propagated at the same conditions for an additional 72 hours. After allowing six hours for sedimentation, the supernatant was decanted and the sediments of both 5 L flasks were combined in a new sterile 5 L flask. The yeast cell concentration was determined using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area per square of 0.00025 m^2 (Brand GmbH&Co.KG, Wertheim, Germany). The volume of the yeast suspension was adjusted to pitch the 30 L of wort at a starting yeast cell concentration of 15 × 10^6 cells per mL in the pitched wort.

Table 2 Qualitative results of the qPCR systems used for the investigated isolates to differentiate Saccharomyces sensu stricto species; positive (+), negative (–)

Species	Yeast isolates / reference strains	qPCR System: Sc-GRC3	Sce	TF-COXII	Sbp	Seub	BF-LRE1	BF-300	Sdia
S. cerevisiae	TUM 68	+	+	+	–	–	–	–	–
S. pastorianus	TUM 35	+	+	–	+	+	+	+	–
	TUM 34/70	+	+	–	+	+	+	+	–

Table 3 **Chemical analyses of the beer produced with TUM 35**

Parameters	Units	Wort	Final beer
Original gravity	°P	12.2	12.2
Final gravity	°P	potentially 1.3*	1.6
Attenuation	%	potentially 89.0*	87.4
pH value	-	5.45	4.63

*determined with an excess amount of the yeast strain TUM 34/70 at 20 °C

2.4.3 Fermentation

Pilot-scale brewing trials were performed with volumes of 30 L using stainless steel fermentation tanks (Gresser C., Regensburg, Germany) with a total volume of 60 L. Brewing trials were evaluated by pitching the calculated volume of yeast suspension to start the 30 L scale fermentation with 15×10^6 cells per mL. The wort was aerated to obtain an oxygen concentration of 8 mg/L. Specific gravity was measured daily as cells in suspension. Yeast isolates were added at an inoculation rate of 15 million cells/g of homogeneous mixed wort. Primary fermentation was pitched at 10 °C and maintained at 11 °C. After 13 days cooling down to 1 °C started. Lager time was 14 days at 1 °C. A temperature of 11 °C was maintained until day 13 and diacetyl reduction and therefore maturation was almost complete at day 13. The specific gravity and pH of samples were determined from the filtered fermentation samples using a DMA 35N (Anton-Paar GmbH, Graz, Austria) for specific gravity and a pH3210 (WZW, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) for pH measurement. The samples were filtered using a Whatman® folded filter paper with a diameter of 320 mm (GE Healthcare Europe GmbH, Freiburg, Germany).

2.5 Analytical methods

After 14 days lagering, the finished beers were analyzed for physical and chemical attributes, which included the following parameters: ethanol, pH, specific gravity, degree of attenuation, free vicinal diketones and the concentration of fermentation by-products. Ethanol, pH, specific gravity, and degree of attenuation were measured using an Anton Paar DMA 5000 Density Meter with Alcolyzer Plus measuring module, pH measuring module, and Xsample 122 sample changer (Anton-Paar GmbH, Ostfildern, Germany). Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, USA) with a headspace unit and Elite 5 60 m 1.5DF column using a 2,3-hexanedione internal standard. The final concentrations of fermentation by-products (e.g. acetaldehyde, ethyl acetate, n-propanol, i-butanol, isoamyl acetate, amyl alcohols, diacetyl, 2,3-pentanedione) were measured according to MEBAK II (3.2.21) methods using a gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 µm column (Perkin-Elmer, USA). Fatty acids and fatty acid esters were determined by gas chromatography with a flame ionization detector (GC-FID) Clarus 500 (Perkin-Elmer, USA) according to the following temperature protocol (1 min, 60 °C; 3 min, 22 °C (5 °C/min); 8 min, 240 °C (20°C/min)), detector temperature 250 °C, injector temperature 200 °C, column: 50 m × 0.32 mm, Phenomenex FFAP, 0.25 µm, carrier gas helium 5.0 ECD-quality, 20 mL/min, 2 bar.

2.5.1 Phenolic off-flavor test (POF-test)

TUM yeast culture isolates were taken from wort agar slopes and spread on a YM-agar plate containing one of the precursor substances: ferulic acid, cinnamic acid and coumaric acid. After three days of incubation at 24 °C, the three single agar plates per yeast isolate were evaluated by sniffing (blind, 8 testers that were trained on the target substances 4-VG, 4-VS, 4-VP) to detect any of the following aromas: ferulic acid becomes 4-vinylguajacol (4-VG, clove-like), cinnamic acid becomes 4-vinylstyrene (4-VS, styrofoam-like) and coumaric acid becomes 4-vinylphenol (4-VP, medicinal-like). *S. cerevisiae* LeoBavaricus - TUM 68® and *S. pastorianus* Frisinga - TUM 34/70® were used as a positive and a negative control, respectively [30, 31]. A YM-media was made for the YM-agar plates by adding distilled water to 3.0 g malt extract, 3.0 g yeast extract, 5.0 g peptone, 11.0 g glucose monohydrate and 20.0 g agar to 1000 mL and autoclaved. After autoclaving, an aliquot of the following stock solutions was added to the YM-media at 45–50 °C under sterile conditions. For the stock solution of coumaric acid, 100 mg of the instant was dissolved in 10 mL of 96 % [v/v] ethanol. The stock solution of ferulic and cinnamic acid was made by dissolving 1 g in 20 mL of 96 % [v/v] ethanol. 10 mL coumaric acid, 2 mL ferulic acid or 2 mL cinnamic acid stock solution was added for 1000 mL YM-media.

2.6 Sensory evaluation

The beer produced by TUM 35 was judged by 10 trained panelists with long-standing experience in the sensorial evaluation of beer. The beer was judged according to the DLG-scheme (Deutsche Landwirtschafts-Gesellschaft). In this scheme, the flavor, taste, quality of the bitterness, mouthfeel and carbonization of the beer are judged and each awarded 1–5 points, one being the lowest (negative), and five being the highest (positive) score. A further descriptive sensory evaluation of the beer was performed.

3 Results and discussion

3.1 Historic information research

Most of the historic facts in this chapter are based on information and personal correspondence provided by Prof. Ludwig Narziß who is also a co-author of the present study. Due to the Second World War (1939–1945) and the resulting shortage of malt and hops, German breweries were forced to produce beer referred to as “Dünnbier” (translation: thin beer) up until 1948. A maximum original gravity of 1.7 °P was allowed for beer production. In these times, there was a variety of different bottom-fermenting yeast strains used in Bavarian breweries. Breweries around Nuremberg and Augsburg were supplied with yeast by the Hasen-Bräu Augsburg brewery. Shortly before 1955, the yeast strain of the Hasen-Bräu was deposited by the Reif brewery Nuremberg (Brauhaus Nürnberg) at the TU München as the strain TUM 34. This yeast strain is an ancestor of today’s most commonly used bottom-fermenting yeast strain worldwide – Frisinga - TUM 34/70 (progenies of the strain in other collections are denominated with the identifiers 34/70, W 34/70 or Weihenstephan 34/70).

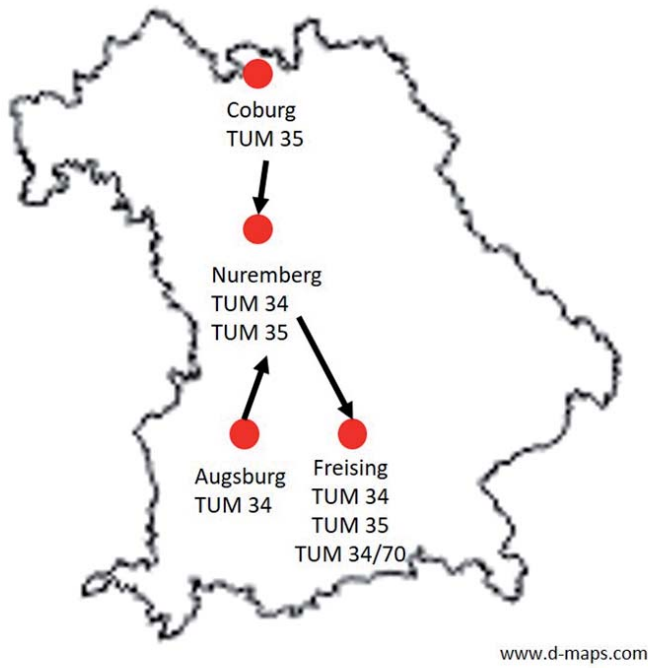

Fig. 3 Origin and traffic of yeast strains TUM 34 and TUM 35 illustrated in a profile map of Bavaria (free profile map of www.d-maps.com)

Shortly after the Second World War however, another yeast strain called "C" was common in many breweries around Franconia (northern Bavaria). The name came from the city name Coburg and the brewery Hofbrauhaus Coburg, where it originally came from (Fig. 2 & 3). The strain we found marked with the number 35 on the HPLC vial with the freeze-dried culture was in a box with other freeze-dried cultures. The number order was completely congruent with the order of the numbers in the historic list (Fig. 2). This is strong evidence that it must be TUM 35. It is not known where it came from before the time in Coburg. This strain was first deposited at an early research center for brewing called the Nürnberger Versuchsstation für Bierbrauerei der Landesgewerbeanstalt (LGA). In 1948 the German economy recovered, as did the breweries and the malt and hops market. From that point, beer with an original gravity of 8 °P could be brewed. The beers produced at that time with the yeast strain C had a mild, balanced flavor with fine notes of hops according to Prof. Ludwig Narziß. However, the yeast strain C was again deposited by brew master Carl Hager from the Brauhaus Nürnberg before 1952 at the TU München and called strain TUM 35. Figure 2 proves that strain 35 derives from Coburg and was isolated at Nuremberg. Strain 34/70 also originated from the Brauhaus Nürnberg (brewery Reif) and Professor Narziß Ludwig knows from his master brewer colleague Carl Hager that the strain TUM 34 originally came from Hasen-Bräu, Augsburg.

In 1952, bad weather conditions forced the barley to ripen prematurely. As a result, malt produced from this barley was low in amino acids and soluble protein. Furthermore, the final attenuation of wort produced from this malt was analyzed at 76 % and lower. This exceptional composition of the produced worts had a strong impact on the bottom-fermenting yeast strains used everywhere. Once fermenting, the yeasts did not flocculate well and stayed in suspension much longer than usual. Even though varying strains were used in different breweries, they all faced similar problems related to poorly flocculating yeasts. The strain TUM 35 had the worst flocculation of all the strains and caused the greatest problems during production. It fermented until the determined final attenuation but it was very difficult to crop enough yeast biomass for further pitching, even with enforced cooling. In 1953 there was a lot of rainfall, which again led to a bad barley harvest and the brewer's yeast strains behaved in the opposite manner. The yeast cells flocculated early, which is now known as PYF (premature yeast flocculation). Again, TUM 35 was one of the worst-fermenting yeast strains and was therefore no longer used by any of the breweries. All the breweries switched to a new yeast strain called TUM 44, which was much more robust against changes in raw materials. However, the flavor was not comparable to the mild balanced aroma profile of beer produced using the strain TUM 35. Since this time, the yeast strain TUM 35, also just called "C", was lost and not used in any brewery. The flavor issues that a lot of the breweries experienced with TUM 44 meant the start of TUM 34's success story, which continues today with the yeast strain Frisinga TUM 34/70. The only time TUM 35 was used again, was in strain comparison studies in 1987 and 2002 by Donhauser et al. and Wagner but no further industrial application followed [25, 26, 29]. The strain was forgotten again and no active or cryo culture was deposited at the Research Center Weihenstephan.

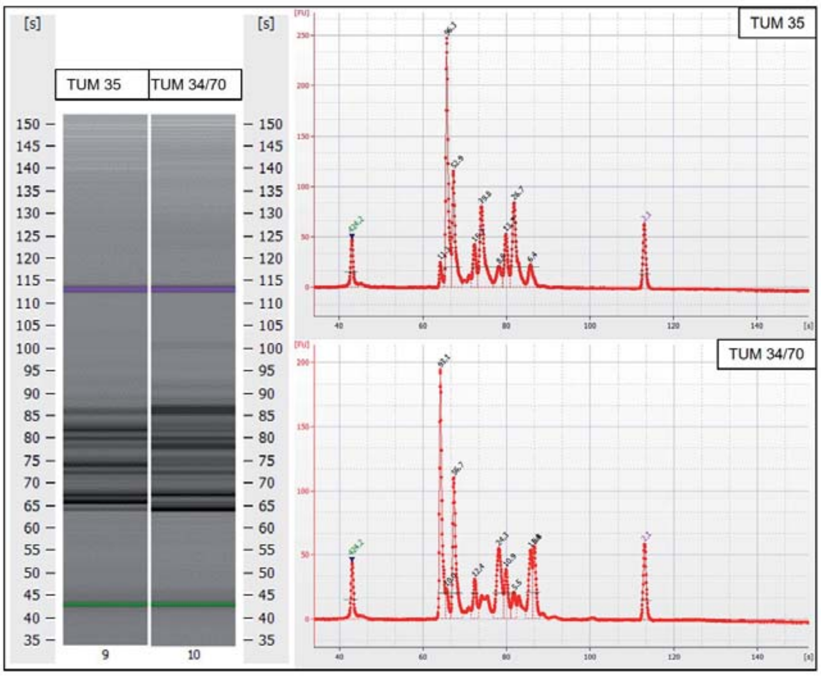

Fig. 4 DNA Fingerprint of PCR amplicons of the IGS2-314 rDNA fragments of the lager brewing yeast strains *S. pastorianus* TUM 35 and TUM 34/70 (left: Bioanalyzer gel image; right electropherogram images)

Table 4 POF results of the investigated yeast strains Saccharomyces pastorianus TUM 35 (sample) and TUM 34/70 (negative control) and Saccharomyces cerevisiae TUM 68 (positive control)

Product/Precursor	TUM 35	TUM 34/70	TUM 68
4-vinylguajacol/ferulic acid	–	–	+
4-vinylphenol/coumaric acid	–	–	+
4-vinylstyrene/cinnamic acid	–	–	+

3.2 Genetic analyses

The qPCR analysis results of various published marker DNA-sequences revealed that the DNA of the strain *S. pastorianus* TUM 35 has a typical marker DNA-sequence pattern like the *S. pastorianus* strain TUM 34/70, which is like a type-strain for the Frohberg lager yeast subgroup (Table 2). The marker gene pattern differs from the pattern of the top-fermenting wheat beer strain *S. cerevisiae* TUM 68.

Quantitative PCR analyses confirmed that the strain TUM 35 belongs to the *Saccharomyces pastorianus* species. A genetic strain comparison using the capillary electrophoresis of PCR amplicons of the IGS2-314 rDNA fragments revealed that TUM 34/70 and TUM 35 are very similar in terms of these highly discriminating regions. There are some quantitative differences for some specific DNA amplicons and there are bands between 80 and 90 s runtime that can be used for qualitative differentiation. Further investigations such as the whole-genome sequencing (WGS) of TUM 35 and subsequent comparison with other lager strains would reveal the detailed phylogenetic relation of TUM 35 within the *Saccharomyces pastorianus* lager clade. Some researchers conducted detailed WGS comparisons for *Saccharomyces cerevisiae* beer strains. *Saccharomyces pastorianus* strains were not the focus of their studies [35, 36, 6]. Probably such WGS genetic comparisons for hybrid species such as *Saccharomyces pastorianus* will follow soon.

3.3 Aroma profiling and brewing trials

The applied yeast strain TUM 35 fermented well at 11 °C, resulting in a final gravity of 1.6 °P (original gravity 12.2 °P) and a final attenuation of 87.4 % after 9 days (Table 3). The previously determined final attenuation of 89 % using the yeast strain TUM 34/70 at 20 °C was almost achieved (MEBAK method), showing a good fermentation performance. After achieving the final gravity of 1.6 °P and no change in gravity for three following days, the beer was cooled down for maturation and stored at 0 °C for 14 days.

The fermentation kinetics of the main fermentation are shown in figure 5. The fermentation kinetics are comparable to the pilot fermentation kinetics of other studies applying other lager yeast strains [25, 26, 33, 24, 29].

3.4 Phenolic off-flavor

Table 4 shows the results of the POF-tests evaluated by sniffing. As shown in table 4, only the wheat beer strain *Saccharomyces cerevisiae* TUM 68 (positive control) is capable of building phenolic off-flavors. For strain TUM 68, all three corresponding POF-flavors were detected by sniffing. The lager yeast strains *Saccharomyces pastorianus* TUM 34/70 (negative control) and TUM 35 are POF negative. These two yeast strains cannot decarboxylate any of the precursor acids. Therefore the PAD or/and FDC activity might be inactive or blocked [37, 38].

3.5 Flavor compounds and fermentation by-products

The results of the measurements of the fatty acids, esters, higher alcohols and fermentation by-products of the beer fermented with TUM 35 presented in tables 5 and 6 show typical values for a

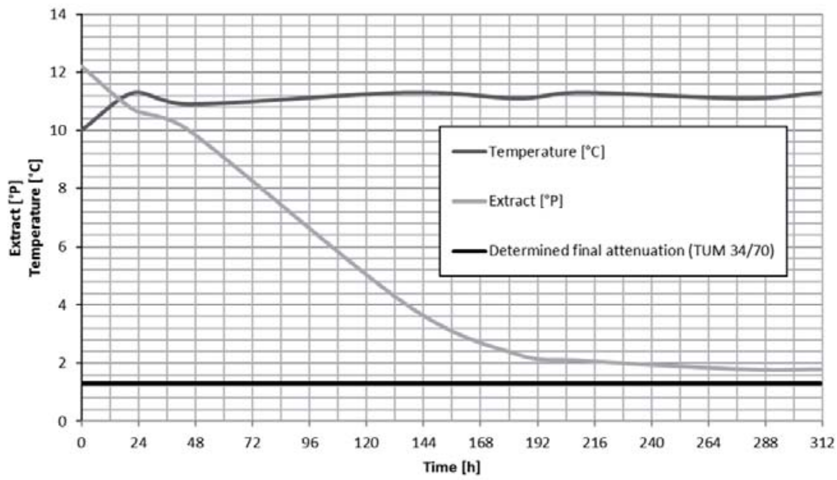

Fig. 5 ***S. pastorianus*** **TUM 35 fermentation profile of a lager beer wort with an original gravity of 12.2 °P at a main fermentation temperature of 11 °C**

Table 5 Fatty acids, fatty acid esters and acetate esters detected in the beer sample fermented with TUM 35

Fatty acids	Content [mg/L]	Fatty acid esters	Content [mg/L]	Acetate esters	Content [mg/L]
Caproic acid	1.3	Butyric acid ethyl ester	0.07	Isoamyl acetate	1.1
Caprylic acid	2	Decanoic acid ethyl ester	0.1	Ethyl acetate	14.1
Decanoic acid	0.69	Acetic acid 2-phenylethyl ester	0.26		
Isovaleric acid	1.2	Acetic acid isobutyl ester	0.03		
		Caproic acid ethyl ester	0.1		
		Caprylic acid ethyl ester	0.26		

Table 6 **Higher alcohols and fermentation by-products detected in the beer sample fermented with TUM 35**

Higher alcohols	Content [mg/L]	Fermentation by-products	Content [mg/L]
n-propanol	15.0	Diacetyl	0.05
i-butanol	18.1	2,3-pentanedione	0.01
Amyl alcohol (2,3-methylbutanol)	82.3	Acetaldehyde	5.3
Phenyl ethanol	27.9	Acetoin	1.2

Table 7 **Mean results of the sensory evaluation by DLG-scheme for the beer produced with TUM 35 and a mean of 480 lager beers**

Attributes	Mean TUM 35	Mean of 480 lager beers
Smell	4.4	4.2
Taste	4.4	4.2
Quality of bitterness	4.4	4.2
Mouthfeel	4.5	4.5
Carbonization	4.5	4.5

12 °P lager beer [39, 40]. As lager beer is known for its clear taste and flavor, these analyses show exactly what is expected from a lager beer. Diacetyl, an indicator of maturation, is well below the threshold of 0.1 mg/L [41].

In his study in 2002, Wagner determined the following values for the final beer fermented with strain TUM 35: pH value 4.4, diacetyl 0.06 mg/L, sum of higher alcohols 72 mg/L, sum of acetate esters 13 mg/L, acetaldehyde 13 mg/L [29]. In our study, the pH value, sum of higher alcohols, and sum of acetate esters are slightly higher than in Wagner's study. Diacetyl and acetaldehyde were slightly lower in this study. The fermentation degree in Wagner's study is not comparable due to a different time of sampling. All values in both studies are within the range of values for lager beers. The slight differences can be explained by different substrates, fermentation parameters and pitching rates.

3.6 Sensorial evaluation

The beer was judged by ten trained panelists and a mean of the ten results was calculated. The results were compared with a mean value of 480 lager beers, which were also tasted by the tasting panel over one year (2018). The results shown in table 7 indicate that the beer fermented with TUM 35 was above average in flavor, taste and quality of the bitterness in comparison with the mean of 480 judged lager beers. The DLG evaluation of the beer made with TUM 35 in the study of Wagner was 4.1 for smell and taste in 2002 [29]. This is a difference of 0.3 DLG points. In Wagner's study, only 5 out of 41 beers made with different lager strains had evaluations above 4.2. One possible reason for the shift could be the differing wort substrate and the differing testing panel.

3.7 Sensory description

The beer was described in smell as pure and delightful, fresh and yeast-typical. The taste was described as being pleasantly fresh-yeast-typical, full-bodied with a slight bitterness, well balanced and soft. No sulfuric aromas could be detected in the smell or taste. Fresh sulfuric aroma profiles are often typical of beers produced with other lager strains.

4 Conclusion/Summary

The strain history of *Saccharomyces pastorianus* TUM 35 could be traced back via Nuremberg to its origin in the city of Coburg in the Upper Franconia district. It was a dominant and widespread strain after World War II in Franconia (northern Bavaria) and lost its impact as its fermentation performance was very susceptible to the influence of bad malt quality. In most cases it was replaced by other more robust lager strains and completely disappeared as an industrial lager strain. In 2018, a freeze-dried stock could be reactivated applying a tailored cultivation/incubation procedure in liquid wort. Using specific qPCR systems the reactivated lager strain TUM 35 was confirmed to belong to the species *S. pastorianus*. A fingerprint analysis confirmed its close relation to the well-characterized strain *S. pastorianus* TUM 34/70 with subtle differences observed in the IGS2-314 rDNA fingerprint pattern. Phenotypic trials revealed that TUM 35 is POF (phenolic-off-flavor) negative and in 30-L pilot fermentations, the strain performs comparably to a typical flocculant Frohberg-type lager strain. The analysis of the fermentation by-products and main beer parameters were comparable to standard lager beers. The sensory evaluation of the beer produced with TUM 35 achieved higher scores than the mean of the sensory evaluation points of 480 lager beers in the database. The descriptive sensory evaluation confirmed the high point ranking as the beer was described as an exceptionally neutral, soft and harmonic beer comprising a typical lager beer aroma profile. This study is a first blueprint for the reactivation and characterization of 'old, historic' lager strains. Due to its origin, the strain TUM 35 is now deposited under the brand name 'Franconia – TUM 35' at the Research Center Weihenstephan for Brewing and Food quality and can be requested for further scientific studies or for industrial applications.

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