Chapter

# Yeast from Distillery Plants: A New Approach

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## Abstract

Nowadays, there is more and more interest in the microbiological resources from different ecosystems, not only because this would allow knowing more about the microbial biodiversity related with these substrata but also because it provides an opportunity to study their characteristics and technological properties which may be of potential interest. This knowledge may allow finding future biotechnological applications for these microorganisms on bio-conservation and reuse of agricultural by-products and may also lead to studies on the improvement of raw material processing. Some raw materials and processing plants in wine and related industries constitute a suitable place for yeast growth; for example, musts, wines in cellars, piquettes, bagasse, pomace, grape skins and yeast lees in the ethanol industry all provide an inexhaustible supply of yeasts. Few microbiological studies have been published so far about the biodiversity of the yeast population in distillery plants. For that reason, the aim of this research was to determine yeast biodiversity and their distribution in different distillery plants in the La Mancha region which are at least 100 years old.

**Keywords:** distilleries, non-*Saccharomyces*, *Saccharomyces* spp., wine by-products, cell vitality, biocontrol

## 1. Introduction

Agricultural residues from food industries are an important raw material involved in bioethanol production. Traditionally, residual juice, molasses and pomace from sugarcane, agave and sugar beet have been widely used in South America for obtaining distilled beverages such us cachaça, tequila and rum. The distillation process is used to isolate, select and concentrate pleasant volatile compounds from the previously fermented liquids and concentrate the alcohol content. Additionally, certain long esters from yeast cells are extracted by distillation and transferred to the final product [1].

Microbial communities from these raw materials and their fermented and distillate beverages not only are interesting due to their role in the aroma production, but their biodiversity and other biotechnological properties are also important. Yeast populations from these ecosystems have not been studied very much, and any studies on them have normally been focused on tequila [2–5], rum [6] and cachaça beverages [7–10].

Yeasts are able to spread from diverse niches to many environments, especially in the vegetable world [11]. Crops and processing plants provide a good niche for yeast growth. In fact, grape crops, musts and wines have been thoroughly studied [12–14], although distillate products and their industry have not been analyzed in Spain in spite of the fact that it is believed to be a new environment for yeast biodiversity study and its biotechnological applications.

In recent years, Spain has been established as the vineyard of the world, presenting the largest surface area (13%) dedicated to this crop [15]. The wine industry is an important sector in Spain which grew considerably throughout 2018. The number of cellars has increased by 6.8% with wine production also increasing (26%) and current production being 40.9 million hectolitres.

Castilla-La Mancha is the world's largest vine-growing region with an annual wine production of around 17 million hectolitres in the 2017–2018 vintage, which accounted for nearly 50% of the total Spanish production. Part of this large production is derived from the distillery industry; in the last year, nearly 250,000 hectolitres were transformed into alcoholic derivatives (16). There is a total of 33 authorized distilleries for wine by-product distillation, 13 of which are located in the La Mancha region. These industries process not only wine but also sweet grape pomaces and its fermented products, obtaining around 4–4.5 million hectolitres [16].

Wine production generates around 600,000 tons of grape derivatives annually such as fermented red skins, which still contain reducing sugars and ethanol, and sweet pomace (from white wine vinification). These by-products, as well as yeast lees and flocculated yeasts, are transported to distilleries where the ethanol is extracted. As **Figure 1** shows, sweet pomaces are mixed and stored for 10–15 days, starting a spontaneous fermentation process. Then, pomace and grape skins are washed with water at 50°C in a heat diffusion system in order to extract the residual sugars and ethanol. After that, a liquid is obtained which is a mixture of alcohol (3–4%) (V/V), water and sugar and is called fermented or sweet piquette. On the other hand, a liquid is drained during the storage of solid organic waste which is mixed with the piquette and fermented for 2–3 days in a stainless-steel container, obtaining a higher alcohol

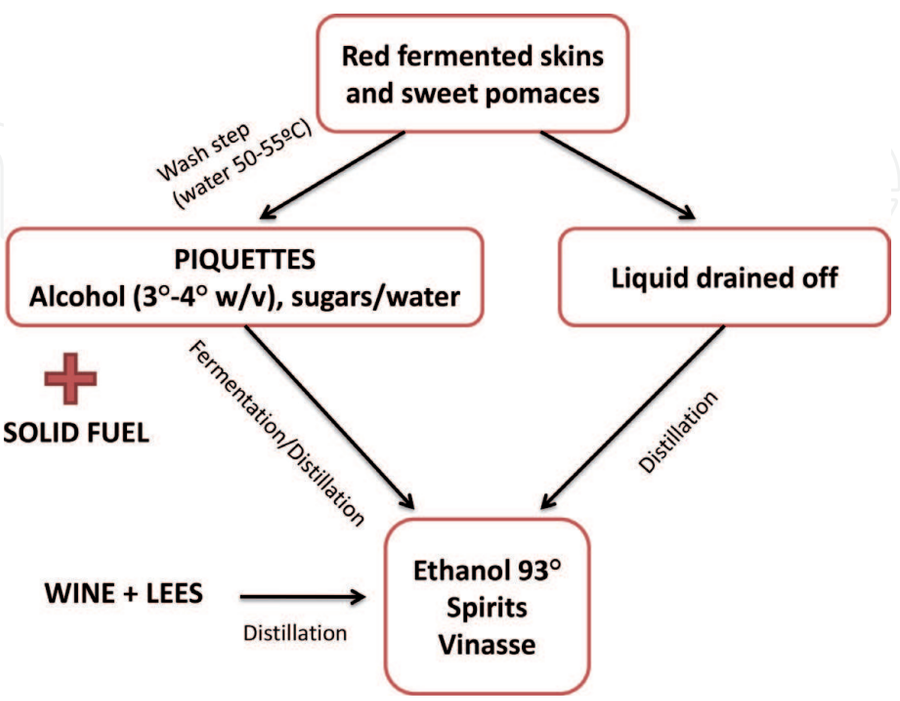


**Figure 1.**
*Flowchart that shows all the steps involved in alcohol production from skin, lees and pomace.*

content (4–5% V/V). Finally, red fermented skins are washed at a lower temperature with the aim of extracting the residual ethanol [17, 18].

The fermented piquettes and the drained liquid are distilled, producing a 93% (V/V) alcohol content product. Then, a dehydration process is carried out until the ethanol concentration of 99.9% is reached. This is mainly used in gasoline as an anti-detonating additive. Residues from distillation can be used as solid fuel (solid residue or "bagasse") or as fertilizer (liquid residue or "vinasse") [17].

Spontaneous fermentations during this last process are produced by non-*Saccharomyces* and *Saccharomyces* biota present in the environment whose biodiversity has not been widely studied [19].

The lack of information about yeast ecology in this habitat and, more specifically, in this territory has prompted the aim of this research.

## 2. Sample collection

Six of the largest distilleries in Europe, which are at least 100 years old and are found in the towns of Argamasilla de Alba (A), Campo de Criptana (B), Madridejos (C), Villarrobledo (D), Daimiel (E) and Tomelloso (F) in the La Mancha region (**Figure 2**), were selected to carry out the study. La Mancha is the principal area for the production of bioethanol and distillates in Spain.

A total of 47 samples were randomly collected from sweet piquettes [20], fermented piquettes [19], flocculated lees [7] and plant oil [1] throughout the pomace-based ethanol production process, and they were transported to the laboratory under aseptic and refrigerated conditions.

Samples and/or their dilutions were spread on YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar); chloramphenicol and sodium propionate were added to inhibit bacteria and mold growth, respectively. Plates were incubated at 28°C/72 hours. Then, samples displaying fewer than 30 colonies were centrifuged to concentrate the cells, and the pellet was directly spread

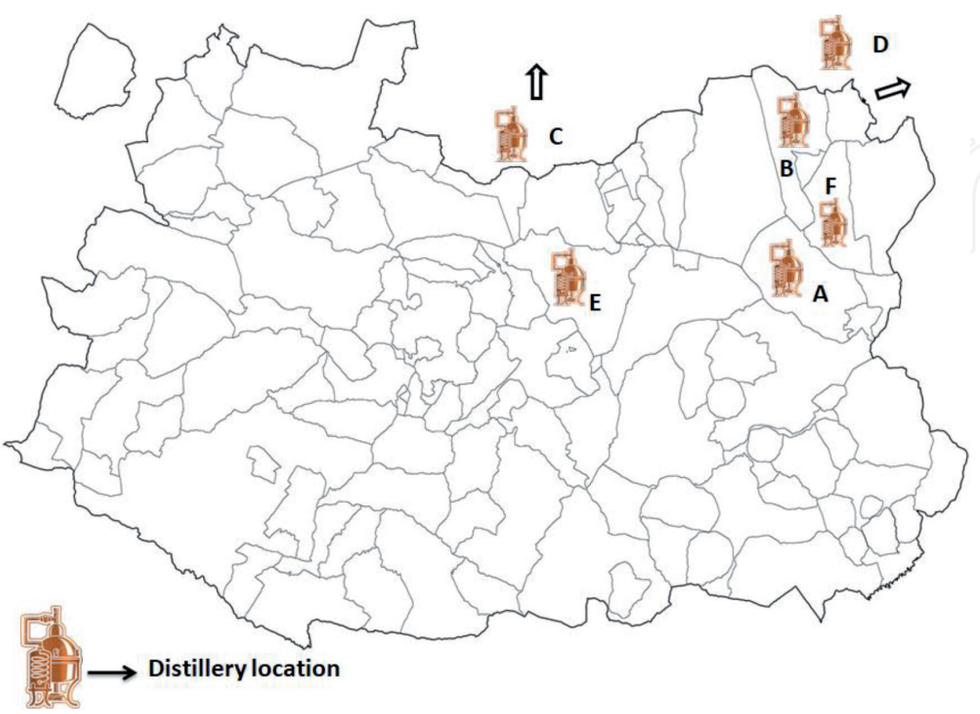


**Figure 2.**
*Location of the distilleries included in this research in the La Mancha region (Spain).*

on YPD agar. Plates with sufficiently separated colonies were replicated onto lysine agar medium (Oxoid, Basingstoke, UK) to distinguish between *Saccharomyces* sp. and non-*Saccharomyces* sp.

The isolates were obtained from 19 samples. A sample was not taken from distillery F, which is possibly due to the hot washing of the skins which would drastically decrease the number of cells.

A total of 210 purified isolates were obtained, 144 *Saccharomyces* and 66 non-*Saccharomyces*, and were stored in 15% glycerol at −80°C until they were studied.

## 3. Yeast classification by genetic identification

*Saccharomyces* spp. yeasts were the predominant profile in all distillery plants. However, the number of non-*Saccharomyces* species varied between distilleries.

### 3.1 Non-*Saccharomyces* yeasts

Genetic species identification was done using the polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) technique, by amplifying the 5.8S rRNA gene using ITS1 and ITS4 [20]. Amplified products were digested (37°C for 7 h) with the three restriction endonucleases *Hinf* I, *Hae* III and *Cfo* I.

Both PCR products and their restriction fragments were separated on agarose gel with GelGreen™ (Biotium), and the results were visualized using a GeneFlash documentation system. For those isolates that could not be identified by PCR-RFLP analysis, the region D1/D2 from the domain 26D rRNA gene was sequenced using NL1 and NL4 primers. If any variation existed due to the action of the NL4 primer, LR6, NL3A and NL2A primers were used as alternatives. Finally, for those samples in which the percentage of identity at species level was less than 99%, the ITS region was sequenced using ITS1 and ITS4 primers [17]. In **Table 1** all isolates are shown, classified at the species level with 99% similarity and the NCBI accession number obtained. A percentage of similarity lower than 99% was obtained with isolates 23, 33, 48 and 62 using the primers NL1/NL4. Sequencing of the 5.8S rRNA + ITS region confirmed this with a similarity of 99%.

Non-*Saccharomyces* yeasts were mainly distributed in sweet piquettes (45.5%) without ethanol, 43.3% were found in fermented piquettes, where the ethanol concentration varied between 4% and 5% (v/v). Finally, 18.2% and 3% were isolated from plant soil and sedimented yeast lees, respectively (**Figure 3**).

As can be observed in **Figure 4**, non-*Saccharomyces* yeasts were more present in plant C (14%) and in plant D (47%) due to the difference in the age of the distilleries and the specific elaboration process followed.

The 66 isolates were cataloged as 8 genera and 20 species, which belonged mainly to the genera *Pichia* (38.0%), *Candida* (22.7%), *Hanseniaspora* (18.2%) and *Torulaspora* (10.6%). The remaining 10% belonged to *Zygosaccharomyces*, *Lachancea*, *Ogataea* and *Saccharomycodes*.

There were four predominant species that were identified as *Pichia galeiformis*, *Torulaspora delbrueckii*, *Hanseniaspora osmophila* and *Candida lactis-condensi*. All these results showed that a considerable diversity exists in this environment, unlike in grape must fermentations [21].

With regard to the substrata of isolation (**Figure 3**), *T. delbrueckii*, *H. osmophila*, *P. kudriavzevii*, *C. lactis-condensi* and *P. anomala* were isolated from sweet piquettes, while *P. galeiformis* and *C. ethanolica* were found in fermented piquettes, from which other species, such us *S'codes ludwigii*, *P. bimundalis*, *Zygosaccharomyces bailii* and *C. sake*, were also isolated but at a very low percentage. Only two species, *L. thermotolerans* and

| Species | Isolates Nº | Accession number (NCBI) |
|---|---|---|
| *Candida ethanolica* | 35a, 36c, 40b 41a, 48a, | 35/JX880409 |
| | | 40/JQ073769 |
| | | 41/JX880400 |
| | | 48/JQ410478 |
| *Candida lactis-condensi* | 50c, 51c, 52c 53c, 54c, 55a 56a, 57a | 55/JN248610 |
| | | 56/JN248614 |
| | | 57/JN248611 |
| *Candida sake* | 44a | JX880410 |
| *Candida viswanathii* | 39a | JQ512833 |
| *Hanseniaspora meyeri* | 7a | JN248602 |
| *Hanseniaspora osmophila* | 4b, 26a, 58a 59a, 62 a, d, 65a, 66a | 4/JQ073772 |
| | | 26/JQ512831 |
| | | 59/JQ512840 |
| | | 58/JQ512840 |
| | | 62a/JQ410479 |
| | | 62d/JQ410479 |
| | | 65/JQ512841 |
| | | 66/JQ780464 |
| *Hanseniaspora uvarum* | 11a, 28 a, d | 11/JN248600 |
| | | 28/JN512834-9 |
| *Hanseniaspora valbyensis* | 5a | JN248613 |
| *Hanseniaspora vineae* | 2a | JN248606 |
| *Lachancea thermotolerans* | 25 a, b, 46a | 25/JQ073770 |
| | | 46/JN248601 |
| *Ogataea polymorpha* | 19a | JN248599 |
| *Pichia anomala* | 10c, 20a, 21a, 22a, 27a, 32a | 20/JX880399 |
| | | 21/JX880404 |
| | | 22/JN248608 |
| | | 27/JX880405 |
| | | 32/JX880406 |
| *Pichia bimundalis* | 43b | JQ073768 |
| *Pichia galeiformis* | 9c, 37a, 38a, 45b, 47c, 49c, 68c, 69c, 70c, 71c, 74b, 76c | 37/JX880397 |
| | | 38/JX880398 |
| | | 45/JQ073767 |
| | | 74/JQ073765 |
| *Pichia kudriavzevii* | 3 a, b, 8a, 13a, 14b, 24b | 3/JN248607 |
| | | 8/JN248609 |
| | | 13/JX880402 |
| | | 14/JQ073771 |
| | | 24/JQ073766 |
| *Pichia membranaefaciens* | 23 a, d | 23a/JQ410476 |
| | | 23d/JQ410476 |

| Species | Isolates Nº | Accession number (NCBI) |
|---|---|---|
| *S'codes ludwigii* | 72a, 77a | 72/JX880401 |
| | | 77/JQ512842 |
| *Torulaspora delbrueckii* | 1a, 6a, 60a, 61a, 64a, 67a, 75a | 1/JN248605 |
| | | 6/JQ780463 |
| | | 60/JX880407 |
| | | 61/JQ512830 |
| | | 64/JX880408 |
| | | 67/JQ512843 |
| | | 75/JQ780465 |
| *Zygosaccharomyces bailii* | 34a | JN248597 |
| *Zygosaccharomyces fermentati* | 15a | JX880403 |

*Technique that allowed identification: (a) NL1/NL4 primers; (b) NL2A/LR6 and NL2A/NL3A primers; (c) PCR-RFLP; (d) 1.8S–5.8S rRNA region sequence (ITS1/ITS4 primers).*

**Table 1.**
*Yeast isolates identified in the different distilleries studied and accession number (NCBI).*

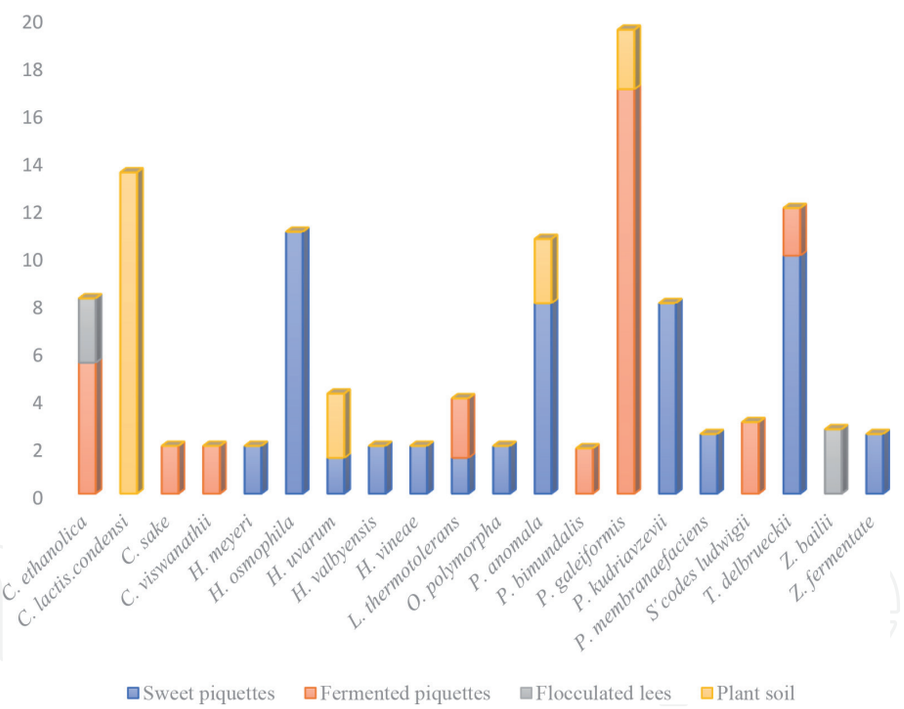


**Figure 3.**
*Percentage of yeast species isolated in sweet and fermented piquettes, lees and plant soil.*

*T. delbrueckii*, were found equally frequently in both sweet and fermented piquettes. Having analysed all these results, a large biodiversity of yeasts was found in the studied substrata, as was documented for grape marc by Bovo et al. [22, 23].

On the other hand, the distribution of genera (**Figure 4a**) and species (**Figure 4b**) in the studied distilleries was also analysed. *Candida* and *Pichia* genera were found in almost all of them, and *Torulaspora* and *Hanseniaspora* were found in three of the five plants in which yeasts were isolated. *P. galeiformis*, *P. kudriavzevii*, *T. delbrueckii* and *H. osmophila* were the species identified in most of the ethanol plants, with plant A being the only one where no major species were found, which contrasts with the results for the other plants (**Figure 4b**).

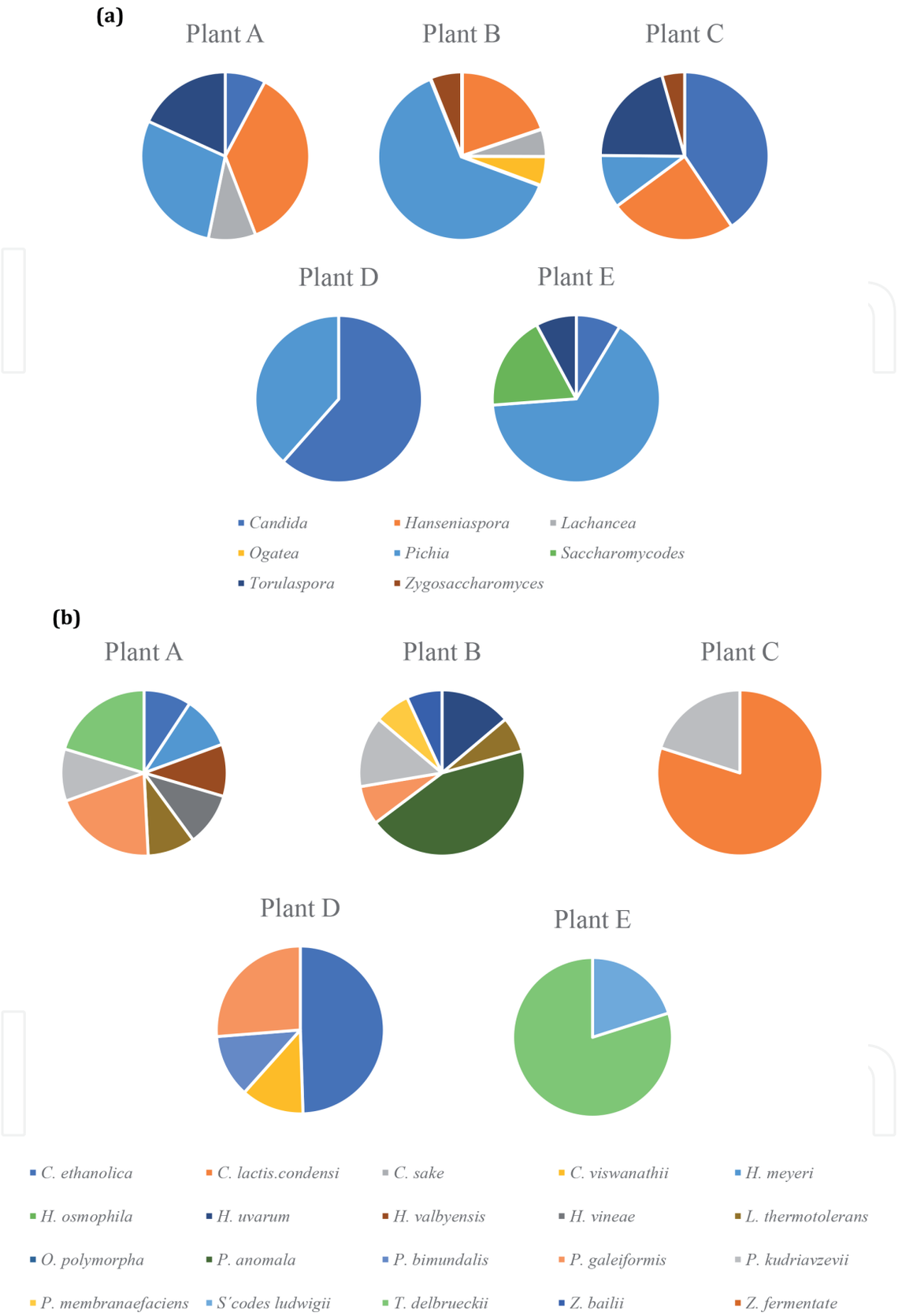


**Figure 4.**
*Distribution of genus (4a) and species (4b) in distilleries studied.*

The presence of *Candida* species (*C. sake*, *C. sorbosa*, *C. stellata*, *C. guilliermondii*, *C. karawaiewii* and *C. citrea*), *P. membranaefaciens*, *P. guilliermondii*, *K. marxianus* and large *Saccharomyces* spp. populations has been previously documented in Brazilian distilleries [7, 8]. These results confirmed that the yeast profiles in the distilleries of the two regions are very different and it is evident that the

Spanish industry is an interesting yeast niche. Additionally, some of these genera and species were also found by Amaya-Delgado et al. [5] and Lappe-Oliveras et al. [4] in tequila and agave beverages.

### 3.2 *Saccharomyces* yeasts

For *Saccharomyces* isolate characterization, a PCR-RFLP analysis was done, and the results showed that 95% of the isolates belonged to *Saccharomyces cerevisiae*, while only 3% and 2%, respectively, were identified as *S. paradoxus* and S. *bayanus*.

However, to discriminate isolate samples within the *Saccharomyces* sensu stricto group, a mitochondrial DNA restriction analysis [13] was carried out by digestion with the restriction endonuclease enzyme *Hinf* I. Restriction fragments were separated by electrophoresis on agarose gel with GelGreen™ (Biotium), and the results were visualized using a GeneFlash documentation system.

The *Saccharomyces* isolates were clustered in 105 different mtDNA patterns (**Table 2**), reflecting a variability of nearly 73% which is very high if it is compared to their variety in cellars [24–26].

Genetic patterns which involved at least 20% of the isolates were named as the "majority profile". At plants A and C, two majority profiles were characterized; at B and D, there was only one; and none was found at plant E. In addition, sweet and fermented piquettes were the substrata from which the most profiles were identified. Although patterns tended to be typical of each plant, the majority profiles accounted for 57% of the isolates at plant B and 33% and 30% at plants C and A, respectively.

Fermented piquettes presented the greatest degree of *Saccharomyces* variability, although several strains coexisted in both lees and sweet piquettes.

| Plants | Sample | Isolates | Strains | Variability | Majority profile |
|---|---|---|---|---|---|
| | Fresh piquette | 28 | 16 | 57 | — |
| A | Fermented piquette | 11 | 5 | 45 | 27% |
| | Lees | 10 | 5 | 50 | 30% |
| | Fresh piquette | 9 | 8 | 89 | — |
| B | Fermented piquette | 13 | 12 | 92 | — |
| | Lees | 7 | 4 | 57 | 57% |
| | Fresh piquette | 27 | 22 | 81 | 22% |
| C | Fermented piquette | — | — | — | — |
| | Lees | 9 | 7 | 78 | 33% |
| | Fresh piquette | — | — | — | — |
| D | Fermented piquette | 8 | 7 | 88 | 27% |
| | Lees | — | — | — | — |
| | Fresh piquette | — | — | — | — |
| E | Fermented piquette | 22 | 19 | 86 | — |
| | Lees | — | — | — | — |

**Table 2.**
*Distribution of Saccharomyces isolates and strains in sweet and fermented piquettes and lees at the ethanol plants studied.*

## 4. Biotechnological properties of non-*Saccharomyces*: fermentation and assimilation of carbon compounds

Fermentation of carbon compounds is particularly useful for identifying isolates with new fermentation profiles for potential applications in various fields. The carbon compounds assayed were D-glucose, D-galactose, L-arabinose, L-rhamnose, melibiose, lactose, raffinose, xylose, maltose, mannose, saccharose and cellobiose. The tests were carried out on a 96-well microtiter plate. Each well was filled with sugar solution, bromocresol green and cell suspensions (exhausting the endogenous carbon compound reserves). Finally, the wells were sealed with sterile vaseline, and the plates were incubated at 28°C/5 days. Depending on the time of the change and the intensity of colouration (from blue to yellow or yellow green), a classification system was established [27, 28].

The majority of the isolates (*Torulaspora*, *Lachancea* and *Saccharomycodes* species and *C. lactis-condensi*) fermented D-glucose either in the first 12 h or on the 5th day. D-mannose and saccharose were fermented to a lesser extent.

None of the isolates fermented xylose, lactose, arabinose, melibiose and rhamnose, and some only weakly fermented galactose, maltose and raffinose.

*C. lactis-condensi* fermented the majority of the sugars at a major or minor intensity. On the other hand, for galactose, raffinose and saccharose fermentation, variability was observed in species such as T. *delbrueckii*, *C. lactis-condensi*, *P. galeiformis* and *C. ethanolica*.

Only one *H. uvarum* isolate and one *H. vinae* isolate weakly fermented cellobiose, which is a sugar of great biotechnological interest in the production of bioethanol from agricultural and forest by-products.

The compounds used for the assimilation assay were mono- and disaccharides (D-glucose, maltose, lactose, L-rhamnose, xylose and cellobiose), polysaccharides (starch, carboxymethylcellulose and lignin) and alcohols (ethanol and methanol).

The tests were carried out in agar plates containing the carbon source and YNB without amino acids (Difco™). The assimilation profile was noticed as (++) abundant growth, (+) normal growth and (−) absence of growth.

Assimilation of carbon compounds, glucose and maltose were the most commonly used and, to a lesser extent, xylose and methanol. Three species of *Candida*, *C. viswanathii, C. ethanolica* and *C. sake*, and one *P. galeiformis* isolate assimilated carboxymethyl cellulose, while three *Pichia* isolates used starch. The majority of *Torulaspora* isolates and a few isolates of *P. kudriavzevii*, *P. galeiformis* and *H. osmophila* assimilated xylose. All of the *H. osmophila, H. uvarum* and *S'codes ludwigii* isolates effectively assimilated cellobiose. Ethanol was assimilated by a few *P. galeiformis* and *P. anomala* isolates. Finally, only some *L. thermotolerans, P. kudriavzevii*, *C. sake* and *C. viswanathii* isolates assimilated methanol. Thus, differences between isolates of the same species were observed, as can be seen in the fermentation tests.

## 5. Biotechnological properties of *Saccharomyces*: cell vitality and growth rate at different temperatures

Cell vitality and growth rates at different temperatures were carried out with the 105 strains. These properties were selected because they are considered a relevant characteristic in a fermentation process.

Cell vitality was evaluated as a measure of fermentative activity by an indirect electrical method [29].

Detection time (DT), expressed in hours, was obtained by impedance measured. It was considered that strains with lower DT presented high vitality.

DT results were clustered in five groups, as can be observed in **Figure 5**. In the interval 0.61–0.95 h, 10% of the strains studied were included, suggesting the highest vitality. Other yeasts (27%) were involved in the range between 0.96 and 1.29 h, indicating a fast cell vitality. Nevertheless, most stains (40%) were comprised between 1.30 and 1.64, and only 3% showed a low cell vitality (1.93–2.33 hours). These results indicate that yeasts from distillery plants have adequate vitality and probably they can displace the slower strains. In studies carried out by Ortíz et al. [13] and Barrajón et al. [29], it was noticed that DT of *Saccharomyces* wine strains oscillated from 0.67 to 1.80 h, although most of the strains showed a DT higher than 1.5 h.

The kinetic parameters (the maximum growth rate, generation time and maximum optical density) were studied at different temperatures (18, 24, 28, 38, 40 and 42°C) using a hurdle selection criteria. All strains were evaluated at 28°C and, depending on their specific growth rate ($h^{-1}$), were distributed into three groups: higher rate values correspond to the first group and the lowest to the third group.

Strains in the top range were assayed at 38 and 24°C. Likewise, strains with the best rate at 38°C were then tested at 40°C, and those which showed the best rate were again tested at 42°C. Similarly, the best strains at 24°C were also tested at 18°C.

At 28°C, 41 of the 105 evaluated strains were in the first group with the best-performing growth rates (0.25–0.32 $h^{-1}$), and 46 and 21 strains, respectively, were categorized in the second and third groups.

At 38°C, the groups presented 14, 13 and 14 strains, respectively, with homogenous results. Afterwards, assays were performed at 40 and 42°C, based on the same criteria. It is remarkable that at 42°C the duration of lag phase was higher than 45.5 hours. Nevertheless, at 40°C, 13 strains from the 25 studied gave the worst growth rate, which constitutes an expected result since this temperature is suboptimal.

In **Figure 6** the percentage of strains in every group was showed. It can be observed that at 38°C, strains were dispersed among three groups. Nevertheless at 18 and 40°C, most strains were included in the worst group.

In the majority of the cases, growth rates at low temperatures (≤0.2 $h^{-1}$) were worse than those gotten at 40°C (≥0.25 $h^{-1}$); this fact confirms that the microbial growth (outside the optimal temperature interval) is better at higher temperatures (**Table 3**). This is a logical outcome, because in distillery plants the substrate is

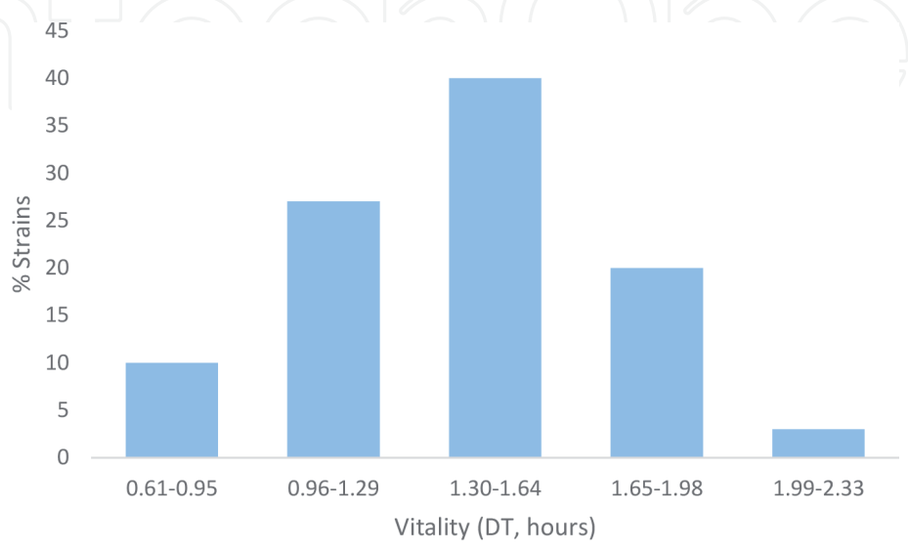


**Figure 5.**
*Saccharomyces spp. strains grouped by their vitality according to the measurement of impedance expressed as detection time (DT, hours).*

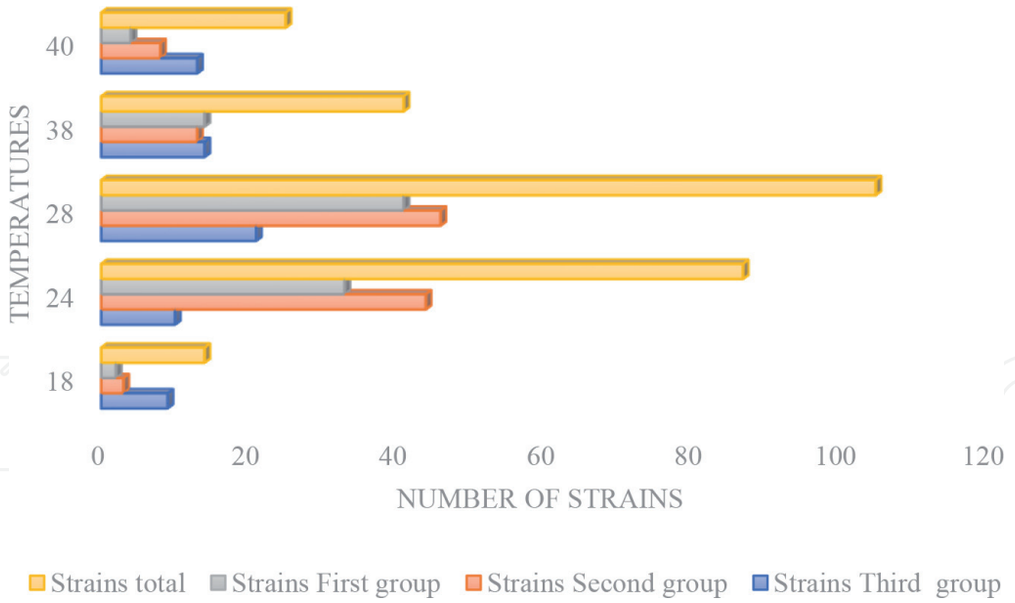


**Figure 6.**
*Number of strains presented in each temperature range (18–40°C) based on their growth rate value. Values are means of n = 3.*

| | Temperatures (°C) | | | | |
|---|---|---|---|---|---|
| **Groups** | **18** | **24** | **28** | **38** | **40** |
| First | 0.17 ± 0.14 | 0.10 ± 0.02 | 0.32 ± 0.06 | 0.26 ± 0.06 | 0.32 ± 0.07 |
| Second | 0.13 ± 0.03 | 0.064 ± 0.01 | 0.21 ± 0.05 | 0.20 ± 0.04 | 0.23 ± 0.07 |
| Third | 0.09 ± 0.03 | 0.06 ± 0.01 | 0.14 ± 0.04 | 0.13 ± 0.07 | 0.13 ± 0.09 |

**Table 3.**
*Distribution of Saccharomyces spp. strains based on their maximum growth rate ($h^{-1}$) at each temperature.*

washed with warm water, and the yeasts isolated from there will grow better at higher temperatures.

The thermal washing process for the extraction of alcohol contributes to the presence of *Saccharomyces* strains with technologically interesting properties, especially in terms of vitality and resistance to high temperatures.

## 6. Biocontrol activity of yeast against epiphytic molds

The molds were provided from the culture collection of the University of Castilla-La Mancha (UCLM) and IVICAM (Grapevine and Wine Institute of Castilla-La Mancha). They were *Phaeomoniella* (*Pa.*) *chlamydospora*, *Neofusicoccum parvum*, *Diplodia seriata*, *Phaeoacremonium* (*Pm.*) *aleophilum* and *Aspergillus niger*.

Fungi were grown in YM agar, and pieces of agar with fungal mycelium were inserted in wells excavated in the YM agar which had been previously inoculated with yeast strains.

The results showed that there were both inter- and intraspecific variabilities.

*H. meyeri*, *H. uvarum*, *H. vineae* and *H. valbyensis* scarcely controlled fungal growth, and mycelium grew as in the control except for six *H. osmophila* which showed a good action against them.

However, *P. anomalous*, *P. galeiformis* and *P. kudriavzevii* effectively controlled all fungal strains including *A. niger*. Also, all *S. cerevisiae* strains except one presented good

fungal growth control behaviour towards all the molds, and *A. niger* was inhibited effectively by only one of these strains. Additionally, the different *C. ethanolica* and *C. sake* have an effective action on the fungal growth, except in the case of *C. lactis-condensi*.

Finally, *T. delbrueckii* and *S'codes ludwigii* strains proved to have a large biocontrol effect not only because of their action against the growth but also because they affected every mold.

Most of the yeasts grew rapidly, forming a very dense lawn after 2 days of growth, suggesting that the mechanism of control might be based on a competition for space and nutrients. To qualitatively analyse the degree of competition between yeast and mold, the 0-day test was carried out afterwards. The assay was carried out with the yeast species which presented the best result in the previous experiment (**Figure 7**), allowing the detection of a high degree of competition between the two microorganisms.

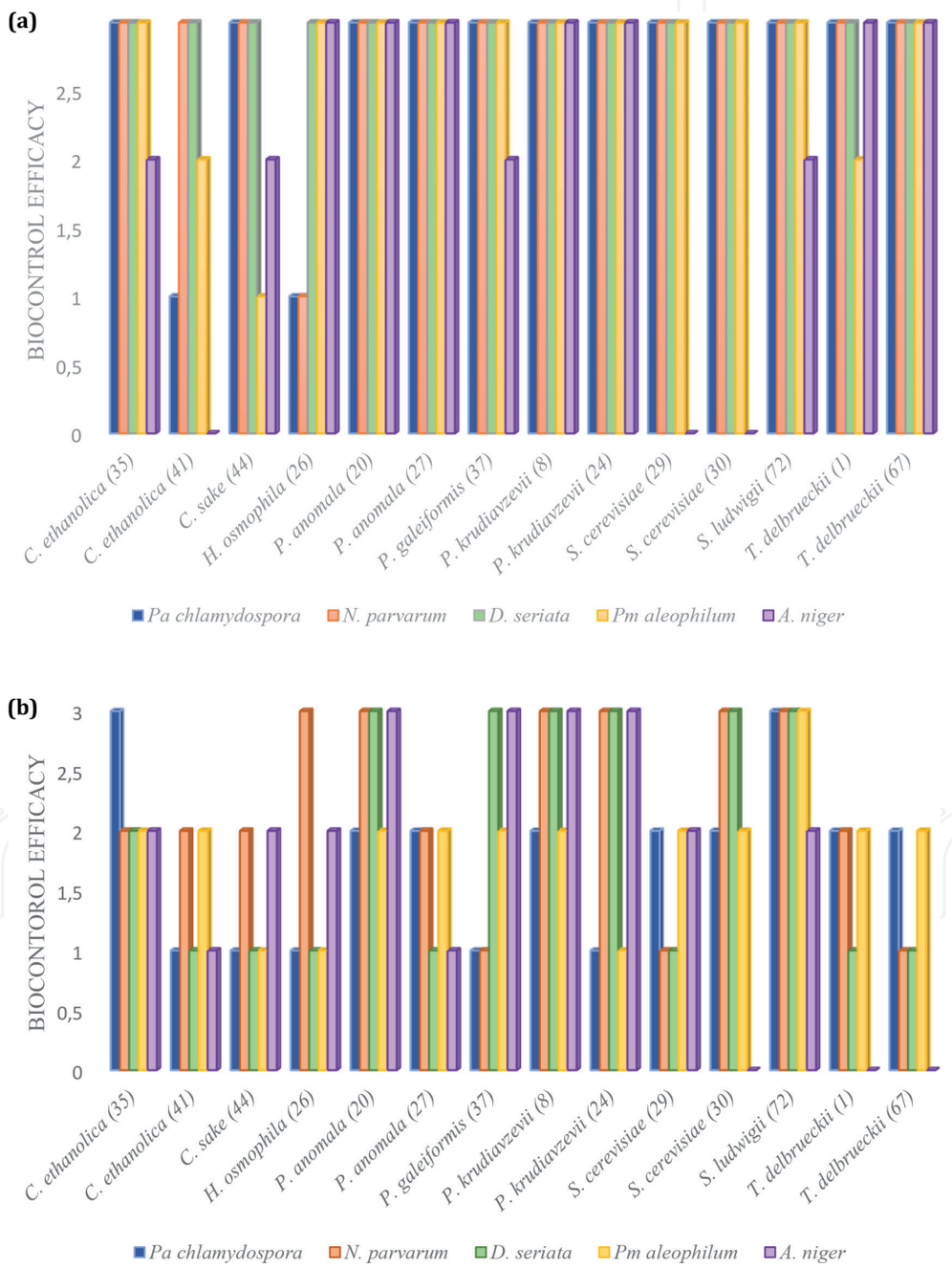


**Figure 7.**
*Biocontrol efficacy of yeast species with 2 days (a) and 0 days (b) of preincubation time. 3: Very effective control, 2: Effective control (fungal mycelium growing slightly beyond the plug), 1: Slight control (fungal mycelium spreading in an evident form), 0: With fungal mycelium spreading similarly to the control.*

*The Pichia* species and the only *S'codes ludwigii* assayed offered a high degree of control. One of the conclusions given by these trials is that the competition between yeast and mold for nutrients and space appeared from the first moment of contact, probably due to the very different growth rates, i.e. the yeasts have a high rate and rapidly colonize the medium preventing the development of molds. However, the inhibition mechanism may be associated with other antagonistic or enzymatic activities occurring via the production of some active compounds.

With the aim of verifying if the inhibition mechanism was produced by cell metabolites or cell wall components, the biocontrol assays were carried out with viable yeast cells, cell extract and filtered supernatant. To carry out the experiment, four wells were excavated at different points on growth fungal plates and were filled with each faction and a negative control (lysis buffer). All of them were incubated at 30°C for a maximum of 5 days in a wet chamber [30].

In most of the tests, an inhibition halo was observed with cell extracts, but when compared to the control (lysis buffer), it was difficult to identify a clear discrimination. Nevertheless, with some cell extracts, an inhibition halo slightly larger than that of the control was observed but only related to *Pm. aleophilum*. No supernatant showed antifungal activity except *H. uvarum* against *A. niger* (**Figure 8**). Finally, whole cells inhibited the molds in most cases, which is consistent with previous results except for *A. niger* which was tested with *H. osmophila*.

On the other hand, enzymatic activity such as in pectinolytic enzymes and chitinase was studied. The tests were carried out to know if the yeasts were able to degrade polygalacturonic acid and chitin. For both activities, the presence of a hydrolysis halo around the colony was considered a positive result; nevertheless, chitinolytic and pectinolytic activities were not observed in the yeasts assayed in the conditions tested.

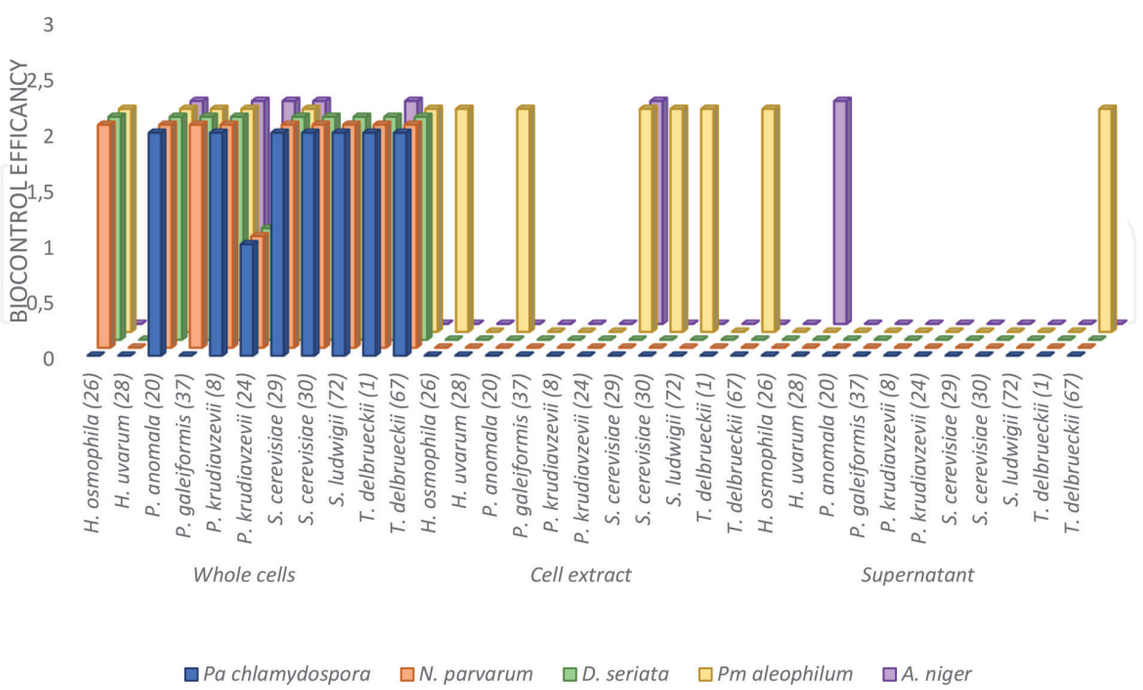


**Figure 8.**
*Biocontrol efficacy of whole cells, cell extracts and supernatants from yeast species. 3: Very effective control, 2: Effective control (fungal mycelium growing slightly beyond the plug), 1: Slight control (fungal mycelium spreading in an evident form), 0: With fungal mycelium spreading similarly to the control.*

## 7. Bioaccumulation of heavy metals

For bioremediation proposals, a selective elimination of metals using yeasts combined with other processes could be a feasible strategy.

Different metallic ions were tested [Cr (VI), Pb (II), Cd (II)]. Metal solutions added to inactivate biomass (obtained by thermal treatment, 5 min/121°C) were incubated at 20°C with horizontal shaking (150 rpm). Aliquots before inoculation and at time 0, 0.2, 3, 6, 24 and 48 hours were taken.

Metallic ion determination was performed by means of an inductively coupled plasma optical emission spectrometer (ICP-OES: Varian Vista-Pro, Mulgrave, VIC, Australia). Tests were semiquantitative.

Very different results were obtained depending on the yeast species as well as the metal tested for the bioaccumulation experiment (**Table 4**). The greatest metal elimination took place for Pb (II) with *H. meyeri, Z. bailii*, *P. membranaefaciens, P. kudriavzevii* and *S'codes ludwigii*, which presented an elimination range of around 20%, reaching 30% in some cases.

This percentage diminished by nearly half for Cd (II), with *P. kudriavzevii* having produced the highest elimination, followed by *Z. fermentati*.

Cr (VI) was eliminated in a much lower proportion, highlighting only *P. membranaefaciens* with 10% elimination, followed by the majority of the yeasts in which adsorption was not detected or was very low.

In general, the metal removal was instantaneous, and during the first 10 min of contact, no additional adsorption was observed. However, in some cases, *S'codes ludwigii* for Pb (II) and *H. uvarum* for Cd (II), the adsorption was progressive, possibly due to the different compositions of polysaccharides and proteins in the cell wall [31]. Unfortunately, *S. cerevisiae*, a by-product of the wine industry and suitable for this type of process, offered a low percentage of elimination for Pb (II) and a medium percentage for the other two metals compared with the rest of the yeasts of the same group. Appreciable desorption processes were not observed, although *P. kudriavzevii* released Cr (VI) into the media after 6 h of contact.

## 8. Conclusions

This initial study of yeast populations isolated from very old distilleries reflects the great existing biodiversity of this valuable yeast niche. This contrasts with what occurs in wine cellars, where the intra and interspecific variability of yeasts have been reduced drastically due to the starter use. *Saccharomyces*, *Pichia* and *Candida* are the genera found in large proportions. Some species were only isolated for certain substrates, like *T. delbrueckii* in sweet piquettes and *P. galeiformis* in fermented piquettes.

The yeast biota of these environments is varied, so these ecological niches are microbial reserves of undoubted biotechnological interest.

In fact, a great number of thermophilic *Saccharomyces* strains with a great cell vitality were found to have potential use as starters in distillery plants.

On the other hand, yeasts coming from very old distilleries might be used as biocontrol and bioremediation agents. *Pichia* sp. inhibited all molds effectively and might be produced in an aerated fermentation process and used as an antifungal postharvest treatment of fruits. In the case of *S'codes ludwigii*, *P. membranaefaciens* and *P. kudriavzevii*, the elimination of Pb (II) was achieved, with the adsorption being almost instantaneous.

*P. kudriavzevii* is a good candidate for both biocontrol and bioremediation because it efficiently inhibited molds and had the highest accumulation average of the tested metals.

| Yeast species | Pb (II) | | | | | Cd (II) | | | | | Cr (VI) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time (h) | | | | | | | | | | | | | | |
| | 0.2 | 3 | 6 | 24 | 48 | 0.2 | 3 | 6 | 24 | 48 | 0.2 | 3 | 6 | 24 | 48 |
| *C. ethanolica* | 2.0 | 1.2 | 2.2 | 2.9 | 4.0 | 2.6 | 0.9 | 2.7 | 1.8 | 2.9 | - | - | - | 0.6 | 0.2 |
| *C. lactis-condensi* | 10.9 | 10.6 | 10.6 | 9.6 | 9.8 | 0.7 | 0.4 | 1.5 | -0.2 | 5.0 | - | - | - | - | - |
| *C. sake* | 4.7 | 10.1 | 8.0 | 10.5 | 10.2 | 2.4 | 2.4 | 0.9 | 2.3 | -0.1 | - | - | - | - | - |
| *H. meyeri* | 16.8 | 20.7 | 20.5 | 21.4 | 14.2 | - | - | - | - | - | - | 0.6 | 1.2 | 0.5 | 0.9 |
| *H. osmophila* | 5.6 | 6.6 | 7.4 | 5.0 | 5.8 | 6.4 | 4.9 | 5.4 | 6.1 | 5.3 | - | - | - | 0.9 | - |
| *H. uvarum* | 5.3 | 5.8 | 9.4 | 9.6 | 10.4 | 3.9 | 6.8 | 6.4 | 8.2 | 8.7 | 4.6 | 4.8 | 6.3 | 4.9 | 1.4 |
| *H. valbyensis* | 3.8 | 9.2 | 5.7 | 6.4 | 5.0 | 0.3 | 0.6 | 0.9 | - | - | 3.3 | 3.4 | 2.5 | 3.8 | 1.3 |
| *H. vineae* | 9.9 | 9.8 | 9.5 | 8.6 | 9.4 | 5.2 | 5.6 | 7.4 | 5.8 | 4.5 | 1.9 | 2.6 | 2.4 | 3.0 | 3.3 |
| *L. thermotolerans* | 1.3 | 1.3 | 2.3 | 0.4 | 18.2 | 2.7 | 4.0 | 3.1 | 1.6 | 1.9 | - | 2.3 | 1.4 | 2.2 | 3.7 |
| *O. polymorpha* | 10.8 | 10.0 | 9.9 | 11.1 | 10.5 | 4.5 | 4.3 | 5.0 | 4.4 | 4.2 | - | - | - | - | - |
| *P. anomala* | 5.1 | 5.7 | 9.6 | 10.3 | 10.7 | 5.1 | 4.9 | 5.1 | 6.0 | 5.8 | - | 2.2 | 2.7 | 3.4 | 3.4 |
| *P. galeiformis* | 0.9 | 3.0 | 2.3 | 2.0 | 1.7 | 3.3 | 6.8 | 6.7 | 7.1 | 7.0 | - | - | - | - | 1.8 |
| *P. kudriavzevii* | 18.5 | 19.6 | 21.5 | 19.2 | 19.3 | 10.5 | 11.3 | 11.2 | 12.3 | 12.8 | 7.1 | 7.4 | 2.1 | 0.8 | 0.2 |
| *P. membranaefaciens* | 20.7 | 20.4 | 20.9 | 20.1 | 20.2 | 2.8 | 3.2 | 3.3 | 2.6 | 2.3 | 9.5 | 9.7 | 11. | 8.9 | 8.2 |
| *S. cerevisiae* | 6.0 | 6.6 | 9.0 | 8.1 | 10.7 | 5.2 | 6.1 | 6.1 | 6.1 | 7.6 | 2.0 | 3.8 | 5.7 | 4.4 | 4.8 |
| *S. ludwigii* | 19.7 | 22.7 | 28.1 | 27.8 | 30.1 | 1.0 | 2.6 | 3.3 | 2.3 | 0.2 | 4.1 | 5.6 | 4.5 | 7.5 | 5.7 |
| *T. delbrueckii* | 3.2 | 4.2 | 5.0 | 7.7 | 8.6 | 2.2 | 2.2 | 3.3 | 3.1 | 3.4 | 0.9 | 3.5 | 3.8 | 5.3 | 4.2 |
| *Z. bailii* | 19.4 | 19.1 | 19.5 | 13.5 | 17.0 | 0.4 | 0.8 | 0.5 | 2.0 | 2.8 | - | 0.2 | - | 1.1 | 1.2 |
| *Z. fermentati* | 6.9 | 7.8 | 9.9 | 7.9 | 7.6 | 7.6 | 8.2 | 10.4 | 11.4 | 13.7 | 3.5 | 3.2 | 3.1 | 2.4 | 3.3 |

**Table 4.**
*Percentage elimination of Pb (II), Cd (II) and Cr (VI) by different yeast species compared to the control*

## Conflict of interest

None of the authors have any conflict of interest with respect to the material contained in this manuscript.





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