Review

Advances in the Study of *Candida stellata*

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Abstract: *Candida stellata* is an imperfect yeast of the genus *Candida* that belongs to the order *Saccharomycetales*, while phylum *Ascomycota*. *C. stellata* was isolated originally from a must overripe in Germany but is widespread in natural and artificial habitats. *C. stellata* is a yeast with a taxonomic history characterized by numerous changes; it is either a heterogeneous species or easily confused with other yeast species that colonize the same substrates. The strain DBVPG 3827, frequently used to investigate the oenological properties of *C. stellata*, was recently renamed as *Starmerella bombicola*, which can be easily confused with *C. zemplinina* or related species like *C. lactis-condensi*. Strains of *C. stellata* have been used in the processing of foods and feeds for thousands of years. This species, which is commonly isolated from grape must, has been found to be competitive and persistent in fermentation in both white and red wine in various wine regions of the world and tolerates a concentration of at least 9% (*v/v*) ethanol. Although these yeasts can produce spoilage, several studies have been conducted to characterize *C. stellata* for their ability to produce desirable metabolites for wine flavor, such as acetate esters, or for the presence of enzymatic activities that enhance wine aroma, such as β-glucosidase. This microorganism could also possess many interesting technological properties that could be applied in food processing. Exo and endoglucosidases and polygalactosidase of *C. stellata* are important in the degradation of β-glucans produced by *Botrytis cinerea*. In traditional balsamic vinegar production, *C. stellata* shapes the aromatic profile of traditional vinegar, producing ethanol from fructose and high concentrations of glycerol, succinic acid, ethyl acetate, and acetoin. Chemical characterization of exocellular polysaccharides produced by non-*Saccharomyces* yeasts revealed them to essentially be mannoproteins with high mannose contents, ranging from 73–74% for *Starmerella bombicola*. Numerous studies have clearly proven that these macromolecules make multiple positive contributions to wine quality. Recent studies on *C. stellata* strains in wines made by co-fermentation with *Saccharomyces cerevisiae* have found that the aroma attributes of the individual strains were apparent when the inoculation protocol permitted the growth and activity of both yeasts. The exploitation of the diversity of biochemical and sensory properties of non-*Saccharomyces* yeast could be of interest for obtaining new products.

Keywords: *Candida stellata*; ecology; taxonomy; metabolism; processing foods; co-fermentation

1. Characteristics of the Genus *Candida*

The genus *Candida* belongs to the order *Saccharomycetales* of the phylum *Ascomycota* and is defined as incerta sedis (of uncertain placement). *Candida* is phylogenetically heterogeneous and included 314 species and the type species *C. vulgaris* (syn. *C. tropicalis*) [1].

Candida are widespread distributed in natural and artificial habitats, being damp and wet with a high content of organic material, including organic acids and ethanol, a broad range of temperatures,

and high salt and sugar osmolarity. Some species have been implicated in the conversion of foods and feeds for thousands of years. Their high biochemical potency makes *Candida* useful for commercial and biotechnological processes.

The diversity of the genus is reflected by an amplitude of unique species with respect to colony texture, microscopic morphology, and fermentation and assimilation profiles. The members of this genus may ferment a lot of sugars, assimilate the nitrate, and form pellicles and films on the surface of liquid media. Extracellular starch-like compounds are not produced. Some species assimilate the inositol and normally the urease is not produced, and gelatin may be liquefied. The reaction with blue of diazonium blue B is negative. The sugars (xylose, rhamnose, and fucose) are not found in cell hydrolysates. The dominant ubiquinones are Q9, Q7, Q8, and Q6. Additionally, the inositol assimilation might be positive or negative; in the case of the inositol-positive response, most strains develop pseudomycelia [2].

2. Ecological and Physiological Properties of Genus *Candida*

Candida covers numerous habitats that determine a wide range of physiological properties. Most of *Candida* is mesophilic, growing well at temperatures of 25–30 °C, with extremes of below 0 °C and up to 50 °C. The genus *Candida* does not have photosynthetic capacity or fix nitrogen and normally cannot grow anaerobically. *Candida* yeasts are employed to obtain a wide variety of biotechnologically interesting compounds like higher alcohols, organic acids, esters, diacetyl, aldehydes, ketones, acids, long chain dicarboxylic acids, xylitol, and glycerol. Other products are nicotinic acid, biotin, and D-β-hydroxyisobutyric acid. Another property exhibited by some strains of *Candida* is the ability to synthesize sophorosides [3] when they are growing on substrates like n-alkanes, alkenes, fatty acids, esters, or triglycerides. Also, the genus *Candida* is able to liberate extracellular enzymes, such as pectinases, β-glucosidases, proteases, invertases, amylases, and lipases, that are of high commercial interest [4]. *Candida* dominates in a vast variety of nutrient-rich habitats. These habitats are associated with plants, rotting vegetation, and insects that feed on plants. Insects (*Drosophila*, bees and bumblebees, etc.) act as vectors, and yeasts are an important food source for both the larval and adult stages of numerous insects [5]. Some species of *Candida* such as *C. famata*, *C. guilliermondii*, *C. tropicalis*, *C. parapsilosis*, and others may be isolated from natural and polluted water or sediments. Other species like *C. glabrata* and *C. parapsilosis* are often isolated from seafood; *Candida inconspicua* and *C. parapsilosis* from fish; and *C. stellata*, *C. sake*, and *C. parapsilosis* from oysters. *C. krusei* and *C. valida* grow better on polluted sediments. The presence of the *C. krusei* complex may be an index of sewage pollution. *C. boidinii* is associated with tanning solutions containing sugars, nitrogenous compounds, and mineral salts (pH 4.0–5.9) [2].

The presence of non-*Saccharomyces* yeasts in wine fermentation process has been widely documented [6,7]. During the early stages of fermentation, a lot of species can grow simultaneously in the grape must; the species *C. stellata* has been described in this stage and can survive even with a high level of ethanol in the medium [8,9], and it is supposed to play an important role in the contribution of aroma properties of certain wines [10]. In recent years, recent taxonomic studies revealed that *C. stellata* can be mistaken for the closely related species *C. zemplinina* [11,12]. This confusion around the taxonomic position of the strains may explain some of the controversial descriptions of the oenological properties of *C. stellata* [13].

3. Methods of Isolation and Identification of Genus *Candida*

In general, the identification and enumeration of microorganisms present in wine involve enrichment techniques [14,15]. These methods are considered indirect, because they do not reflect the number of original cells in the sample, but they are also considered to be progeny, because they are enriched in a selective growth media for cultivating yeast and bacteria from wine. The characterization of *Candida* at the species level is laborious, since they are widely disseminated, highly variable, change their physiology with varying conditions, and normally are associated with other yeasts,

bacteria, and molds. Nonselective media most commonly used for yeast separation, cultivation, and enumeration are composed of glucose such as carbon source, and these media may be employed at the beginning. Examples include dextrose agar (pH 6.9), dextrose broth (pH 7.2), Sabouraud medium, dextrose tryptone agar, rice agar, malt extract medium, or plate count agar. The use of lactic, tartaric, or citric acid (10%, final pH 3.5) for acidification of the media, as well as the incorporation of antibiotics (up to 100 mg/L), such as cycloheximide, streptomycin, chloramphenicol, and gentamycin, enhances their selectivity in order to inhibit the development of acid lactic bacteria and other yeasts. Biphenyl, propionic acid, and dichloran control overgrowth of filamentous fungi. The culture temperatures are also an important factor; those between 25 °C and 30–32 °C should be chosen. Incubation times are fixed in the range of 3–5 days and must be increased for osmotolerant and osmophilic yeasts to 5–10 days and 14–28 days, respectively. Many specific commercial media are available for isolating and enumerating the genus *Candida* in different food products, including the brewing industry and wine industry [12].

Although selecting wine yeast strains have been addressed for decades, the unequivocal characterization has been possible with the knowledge of molecular techniques. Pramateftaki et al. [16] applied the PCR amplification and restriction pattern analysis of the ITS1-5.8S-ITS2 regions of the nuclear ribosomal gene complex for species characterization of isolated yeasts based in the techniques developed by different authors [17–19].

Most PCR-DGGE studies have been employed to discriminate both yeasts and bacteria in wine. Cocolin et al. [20] were the first to apply PCR-DGGE method in wine fermentation, developing primers for the D1/D2 domain of the large-subunit rDNA amplification of the yeast species. That work demonstrated that the population shifts of different wine-related yeasts could be easily followed using PCR-DGGE [21]. This study also confirmed the persistence of *Candida* sp. throughout wine fermentation, detecting populations until 104 days later. Supplementary studies on commercial sweet wine fermentation showed that non-*Saccharomyces* yeasts could be found in late stages of the fermentation process by PCR-DGGE and even a long time after could be cultured on specific media [7]. This fact was particularly evident for the *Candida* sp. population, *C. zemplinina* [11]. DGGE signatures from both RNA and DNA templates directly extracted from wine revealed *C. zemplinina* signatures remained throughout the fermentation, even when direct plating manifested clearly a relative low number of cells. Applying RNA dot blot analysis with *C. zemplinina*-specific probes shows that the size of that population could be relatively high (>10^6 cells per mL) at the end of the fermentation, while only 100–1000 CFU per mL could be detected by plating. These results provided some of the first evidence of the presence of metabolically active but nonculturable yeasts in wine fermentation.

Endpoint PCR assays have been developed and applied for several wine yeast and bacteria. López et al. [22] used a multiplex PCR approach amplifying different segments of the yeast *S. cerevisiae* COX1 gene to enumerate different starter strains. Cocolin et al. [23] also developed 26S rRNA gene PCR primers for specific amplification of *Hanseniaspora uvarum* and *C. zemplinina*. In that experiment, the authors founded a persistence of both RNA and DNA signatures for *H. uvarum* and *C. zemplinina* in sulfited wine, even though no growth of either strain was witnessed on plating media. After 20 days of SO$_2$ addition and without grow on plates, the detection of *H. uvarum* and *C. zemplinina* RNA signatures in wine provides a useful example of how PCR results must be considered with caution, since both live and dead cells may be detected.

The more recent QPCR system is being widely applied in wine fermentation. This technique is used to the exponential amplification of target DNA sequences together with a fluorescent molecule (SYBR Green dye is commonly used by wine-related species) [24]. The application of QPCR to specific bacteria or non-*Saccharomyces* yeasts in wine fermentation allows for their enumeration in combination with high populations of *Saccharomyces*. Organisms such as *Candida* sp. can be detected and quantified in as little as one to two hours, which is a considerable improvement on the five to 10 days necessary to develop the conventional analysis by plates [25,26].

4. Characteristics of *Candida stellata*

The non-*Saccharomyces C. stellata* is an *Ascomycete*, anamorph yeast belonging to the genus *Candida*, with a taxonomic history subject to numerous changes; it is either a heterogeneous species or is easily confused with other yeast species present in the same substrates.

C. stellata, a habitual member of the early yeast strains in both white and red wines in certain wine regions of the world [7,9,27–38], is able to remain active throughout most of the alcoholic fermentation and much longer than most other non-*Saccharomyces* yeasts [29,32,35,39–41]. The habitual presence of *C. stellata* in the samples confirmed that this yeast is frequently associated with overripe and botrytized grape berries and musts proceeding from botrytized grapes [42].

Among the genus/species linked to *Candida stellata*, the most notable are *Saccharomyces stellatus*, *Torulopsis stellata*, *Cryptococcus stellatus*, *Cryptococcus bacillaris*, *Saccharomyces bacillaris*, *Torulopsis bacillaris*, and *Brettanomyces italicus*. The cells are spherical to ovoid; they are usually found as single cells but may be arranged in a star-like configuration of cells; no hyphae or pseudohyphae are formed. Growing in YPD, colonies are grayish-white to brownish, glossy soft, and smooth. In malt agar, there are large, round cream, or white colonies. *C. stellata* does not form spores. A whitish cheese-like film can appear in liquid medium. This non-*Saccharomyces* yeast ferments glucose, sucrose, and raffinose (sometimes it does this slowly). On the other hand, it can assimilate sucrose and raffinose but not nitrate. *C. stellata* uses lysine as sole N source. Its growth requires vitamins such as biotin, pantothenate, inositol, and thiamin. With regard to medium conditions, its growth is variable at 37 °C but is sensitive to heat, while it is able to grow at lower temperatures and higher pH values. Moreover, it is not sensitive to ethanol and under aerobic conditions; by contrast, it is sensitive to cycloheximide, sorbate, DMDC, low pH, and acids.

5. Taxonomic Reclassification of *Candida stellata*

Initially, two types of *Candida* were isolated from a must elaborated in Germany from overripe grape berries and raisins with a high sugar concentration (ca. 60%). One type had elongated cells and was denominated *Saccharomyces bacillaris*. The other type was nominated *Saccharomyces stellatus*, because in liquid media the star-like chains presented cells with spherical shape. Both species were later included in genus *Torulopsis* due to the lack of spores' generation. In another study on taxonomic research carried out in Italy, a third type of species was isolated from grapes and named as *Brettanomyces italicus*. Lastly, these taxa were unified in a single species named *C. stellata*, and the strain originally described as *S. stellatus* was considered as type strain (CBS 157) (Figure 1).

Figure 1. Taxonomic reclassification of *Candida stellata* [13,43,44].

Traditionally, *C. stellata* is associated with overripe and botrytized grape berries [27,29,34,38]. *Candida* is present almost until the final stages of the alcoholic fermentation [29,32,39,41], suggesting that *C. stellata* might significantly take part in the ecology of fermentation and the wine quality. Nevertheless, the role of *C. stellata* in wine attributes seems to be controversial owing to the contradictory enological features attributed to this yeast by several research groups. Some authors report on the high production of acetic acid [45], glycerol [10,46], and succinic acid [47]; conversely, other works found low acetic acid levels and low glycerol production [35]. These controversial results of *Candida* show that *C. stellata* is either a heterogeneous species or is easily confused with other yeast species of *Candida*, which are present in the same substrates. Sipiczki [11] found a new osmotolerant and psychrotolerant species studying four yeast strains isolated from fermenting botrytized grape musts in the Tokaj wine region of Hungary, corresponding to *C. zemplinina*. Traditional taxonomic test shows small differences between these isolates and *C. stellata* strains CBS 157^T and DBVPG 3827 (Dipartimento di Biologia Vegetale, Perugia, Italia) (CBS 843).

The species *C. zemplinina* was discovered among wine yeasts that showed a taxonomic profile characteristic of *C. stellata* [11]. Both species grow in similar environments (must with high sugar concentration) but may form mixed populations in the colonized substrates. Lastly, the strain DBVPG 3827, frequently used to investigate the oenological properties of *C. stellata*, has been reclassified as *Starmerella bombicola* [43] (Figure 1). Considering the recent identification of these new species *C. zemplinina* and *S. bombicola*, they may be confused with *C. stellata* when conventional taxonomic tests and routine PCR-restriction fragment length polymorphism (RFLP) analysis are used for identification [11,43]. In view of these results, Csoma and Sipiczki [13] report that the name of the species *C. stellata* has been used for group yeasts not conspecific with the type of strain of *C. stellata*. Most strains originally identified as *C. stellata* and examined by the authors turned out to belong to species that were not known yet at the time of their isolation, such as *C. zemplinina*, *C. lactis-condensi*, *C. davenportii*, or *S. bombicola*. Csoma and Spiczki [13] studied 41 strains deposited in six culture collections originally identified as *C. stellata* (Figure 1). The ITS1-5.8S rRNA-ITS2 sequence region was studied in all strains by PCR-RFLP. The enzymes MBoI, DraI, and HaeIII were used separately during the digestion of amplified fragments. Digestion with MboI is known to generate specific patterns for each of *C. stellata*, *C. zemplinina*, and *S. bombicola* [12]. As result of the digestion of the fragments of amplification, all strains gave three or four patterns. Thirty-nine out of the 41 strains examined showed combinations of patterns different from those of the type strain of *C. stellata*; this result highlights the fact that only two of investigated strains might belong to *C. stellata*. The digestion of the amplified region with MboI and DraI distinguished *C. stellata* from *C. zemplinina* and the CfoI, HaeIII, and HinfI restriction patterns separated *C. stellata* from *S. bombicola* [43]. Later, the D1/D2 domains of the LSU rRNA gene of all strains studied were amplified and sequenced. The Blast search with the sequences identified high degrees of similarity (98–100%) with the sequences of the type strains of 11 species. Based on these sequences, most strains originally isolated from grapes or wine fermentation belonged to *C. zemplinina* or *S. bombicola* (DBVPG strains). At the same time, the results of the taxonomic physiological test were contrasted with the molecular results and all *C. zemplinina* strains growing in the presence of 1% acetic acid, which inhibited the growth of *C. stellata*. The wine yeasts deposited in DBVPG as *C. stellata* strains turned out to be strains of *S. bombicola*, which were identified species [48], unknown at the time of their deposition.

As a result, it can be concluded that most wine strains preserved in CBS or described in recent publications as *C. stellata* proved to belong to *C. zemplinina* [13]. *C. stellata* was not found among yeasts newly isolated from noble rotted grapes and botrytized wines either, although overripe grapes and fermenting grape musts with high sugar concentrations are environmental conditions in which strains identified as *C. stellata* were frequently detected [27,28,37,38]. *C. stellata* is far less present in grapes and natural wine fermentation than hitherto thought. Regarding botrytized wines, the higher appearance of *C. zemplinina* is ligated to the capacity to resist higher acetic acid concentrations. It is known that *C. zemplinina* can grow in presence of 1% of acetic acid, which is inhibitory to *C. stellata*. The grapes

infected by *Botrytis cinerea* present a high number of acetic acid bacteria, with can grow in grape must with production of gluconic and acetic acids [49].

To probe the hypothesis about the broad presence of *C. zemplinina*, the *C. stellata* LSU rRNA gene sequences published by others authors were reviewed [13]. The results showed that D1/D2 domain sequences of the *C. stellata* strains isolated from French cider by Coton et al. [50] and from Spanish wine by Hierro et al. [38] and the corresponding sequence of *Candida* sp. isolated from Californian sweet botrytized wine by Mills et al. [7] are coincident with those of the type strain of *C. zemplinina*. Moreover, *C. zemplinina*, but not *C. stellata*, was found in fermented red wine from Portugal grape variety Castelao. Besides, *C. zemplinina* was also identified in other Portuguese wine (accession number AY394855) and in Greek botrytized wines (accession number DQ872872).

The results obtained from electrophoretic karyotypes suppose another means for the differentiation of these species. Although both species had three chromosomes and showed length polymorphism, their chromosomes differed in size. *C. stellata* had a somewhat larger genome, and each chromosome differed in size from the comparative used strains, CBS 157^T and CBS 843. *C. stellata* appears to be prone to undergo chromosomal rearrangements. In contrast, the *C. zemplinina* strains did not show chromosomal polymorphism [13].

C. zemplinina also proved to be much more acidogenic; this aspect may significantly affect the quality of the wine. *C. stellata* grew much more slowly at all conditions tested. This observation is in accordance with earlier reports that described *C. stellata* as a slow-growing yeast [29].

On the other hand, different studies about the original type *Saccharomyces bacillaris* described together with *Saccharomyces stellata* from overripe grapes and concentrated musts concluded that this species is not synonymous with *C. stellata*. Different profiles were observed for the type strain of *C. stellata* (CBS 157) for both the isoenzyme and rDNA restriction analysis, and only 91% similarity was found between the D1/D2 sequence of this strain and *S. bacillaris*. In view of the results, *S. bacillaris* has been recently reinstated as *Starmerella bacillaris* comb. nov., with *C. zemplinina* as an obligate synonym [44] (Figure 1). This reorganization is in line with the latest edition of the "International Code of Nomenclature for Algae, Fungi and Plants" [51], which eliminated the rule that was in force for a long time that anamorphic yeasts with ascomycetous affiliation had to be classified to *Candida*; this species was recently moved to the genus *Starmerella*, leaving the name *C. zemplinina* as obligate synonym [52]. Following this reclassification of most of the yeasts, *S. bacillaris*, previously identified as *C. stellata*, became *Starmerella bacillaris* [44].

6. Characteristics of *Candida zemplinina* sp. nov. Sipiczki

C. zemplinina was discovered studying wine yeasts with a taxonomic profile characteristic of *C. stellata* [11]. Both species grown in similar environments (overripe grapes and grape must with high sugar concentration) presumably may form mixed populations in the colonized substrates [13].

C. zemplinina owes its name to the Zemplin mountain range, whose south and south-east facing slopes form the Tokaj wine region. The type strain is 10-372^T (=CBS 9494^T = NCAIM Y016667^T), which was isolated from white wine in Zemplin, Hungary [11]. Growing on morphologic agar, the cells are ellipsoid to elongated (2.2–3.0 × 3.0–5.2 μm) alone and in pairs after 3 days incubation at 25 °C. Their budding is multilateral. In contrast, after 7 days incubation at 25 °C on the same culture media, colonies are low convex with smooth to finely lobed margins, and their texture is butyrose. Neither hyphae nor pseudohyphae are generated. Ascospores formation is not seen after 25 days incubation at 25 °C on the agar culture media for corn-meal, potato dextrose, or Gorodkowa. *C. zemplinina* ferments sugars, glucose, sucrose, and raffinose but does not ferment galactose, maltose, and lactose. On the other hand, it can assimilate glucose, sucrose, L-sorbose (slowly), raffinose, and lysine but does not assimilate the following compounds: galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, maltose, trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, lactose, melezitose, inulin, starch, glycerol, erythritol, ribitol, D-glucitol, D-manitol, galactitol, inositol, D-glucono-1,5-lactone, succinate, citrate, methanol, ethanol, potassium nitrate,

cadaverine, *N*-acetyl-D-glucosamine, and lysine. Vitamins are essential to its growth. Finally, this yeast species is able to grow in the presence of 60% (*w/v*) glucose. Additionally, no growth is observed in the presence of 10 µg/mL cycloheximide or at 37 °C.

C. zemplinina cannot be considered a wine-specific yeast. *C. zemplinina* has been detected in yeast populations associated with Ghanaian cocoa fermentation and two CBS strains identified as *C. zemplinina* originate from soil (CBS 2799) and *Drosophila* sp. (CBS 4729) [53,54]. The association of *C. zemplinina* with *Drosophila* confirms that fruit flies can be important vectors of yeasts from winery to ripening grapes in the vineyard. The related species as *C. bombi*, *C. lactis-condensis*, and *S. bombicola* are also associated with insects [55,56].

The genome of *C. zemplinina* is similar in size to the genome of *C. stellata* and the genomes of the other related species, *C. bombi*, *C. lactis-condensi*, and *Starmerella bombicola*, but appears to differ from it in stability. The *C. zemplinina* chromosomes show less variability than those of *C. stellata*. This stability indicates that chromosome rearrangements may not be as important in this species as in *S. cerevisiae* [57] for adaptation conditions during wine fermentation.

Kurtzman and Robnett [58] observed that strains showing greater than 1% difference in the D1/D2 domain of the 26S rRNA are usually different species. The D1/D2 domains of the 26S rDNA of four isolates of *C. zemplinina* and the control strain *C. stellata* CBS 157^T were amplified and sequenced to confirm the taxonomic separation of *C. zemplinina* from *C. stellata*. The amplified fragments of the *C. zemplinina* strains had identical nucleotide sequences, which differed from the homologous sequence of *C. stellata* 157^T at 39 positions (8.1% sequence difference).

C. zemplinina stand out against *C. stellata* for being osmotolerant and psychrotolerant and thus could be better adapted to grow under high sugar concentrations and at low temperatures. These physiological attributes can be especially favorable for propagating botrytized grape musts, which normally contain high sugar content and are fermented at low temperatures, as in the case of Tokaj wines generally below 15 °C.

7. Characteristics of *Starmerella bombicola*

Starmerella bombicola is the type species of genus *Starmerella* (Rosa and Lachance, 1998) [48]. The strain studied, CBS 6009 (type strain), was isolated from honey of bumble bee (*Bombus* sp.).

S. bombicola is the anamorph of *C. bombicola* and the synonym of *Torulaspora bombicola* and *C. bombicola* [59]. On YM agar after 3 days at 25 °C, the cells are ovoidal to elongated, 1–2 × 2–4 µm, and occur singly and in pairs. The colonies are small, convex, and white and have an entire margin. In glucose-yeast extract broth, a ring forms after 1 month. In Dalmau plate culture on corn meal agar, pseudohyphae and true hyphae are not formed. Positive formation of ascospores after 1 day on YCBAS (yeast carbon base, Difco, with. 0.01 % ammonium sulphate) agar mixed compatible mating types fuse in pairs. After 3 days, the conjugated asci contained a single spherical ascospore with a convoluted wall and a membranous basal ledge. The ascospores are released terminally and tend to agglutinate. This species presents positive fermentation of glucose and sucrose, and variable to raffinose. The fermentation of galactose, maltose, lactose, and trehalose is negative. It can grow on agar media of glucose, ethanol, glycerol, and mannitol, and provides a positive answer to the additional growth test of glucono-δ-lactone, cadaverine, 50% glucose, amino acid-free, and 30 °C CoQ 9.

This yeast had been previously assigned to other species now known to be members of the *Starmerella* clade. This species shows the ability to excrete extracellular hydroxyl fatty acid sophorosides. *S. bombicola* is associated with bees and flowers, with the bees as the principal vector. Sophorolipid biosynthesis by *S. bombicola* may be industrially useful for the production of biodegradation detergents [60].

To confirm the taxonomic affiliation of species of *Candida* deposited in DBVPG, their growth under various conditions was studied. *C. zemplinina* and *C. stellata* differed from *Candida* strains deposited in DBVPG with regard to temperature profile, osmotolerance, and greater sensitivity to ethanol compared with these two *Candida* species. Comparing its electrophoretic karyotype, *Candida*-type strain differed

in the banding pattern; although both had three chromosomal bands, their chromosomes differed in size, and the genome of *C. zemplinina* was smaller than the genome of *C. stellata*. The karyotype of DBVPG 3827 was indistinguishable from that the karyotype of *S. bombicola* CBS 6009[T], which only had two bands, one of which corresponded in size to one of the *Candida* chromosomal bands.

Table 1 shows the principal aspects that can help to distinguish between *Candida stellata*, *Candida zemplinina*, and *Starmerella bombicola* to achieve a correct identification of these species.

Table 1. Main differential characteristics of species *Candida stellata* (CBS 157), *Candida zemplinina*, and *Starmerella bombicola*.

	C. stellata (CBS 157)	*C. zemplinina*	*S. bombicola*
Growth in high sugar concentration	+	+	+
Growth in botrytized grape berries	−	++	+
Growth in presence of 1% of acetic acid	−	+	v [1]
Formation of ascospores	−	−	+
Banding pattern (electrophoretic karyotype)	3	3	2
Chromosomal polymorphism	yes	no	no
% D1/D2 sequence in difference with *C. stellata* (CBS 157)		8.1	nd [2]
MboI and DraI digestion distingue between the species	yes	yes	
CfoI, HaeIII, and HinfI digestion distingue between the species	yes		yes

[1] v, variable; [2] nd, not determined.

8. Characteristics of *Starmerella bacillaris* (synonym *C. zemplinina*)

Starmerella bacillaris (synonym *Candida zemplinina*) [52] is a non-*Saccharomyces* yeast, isolated for the first time in Napa Valley (Napa, CA, USA) in 2002, under the name EJ1 [7]. This yeast is characterized by ellipsoid to elongate cells upon growth in yeast malt agar. It ferments glucose, sucrose, and raffinose, but not galactose, maltose, or lactose. It assimilates very few carbon and nitrogen sources, namely, glucose and L-lysine, and it experiences no growth in the presence of high glucose concentration. Additionally, it presents high fructophily, average volatile acidity and alcoholic degree production, and high glycerol production [52].

Starm. bacillaris can be distinguished from the closely related species *C. stellata* by EST, G6PD, ACP, LDH, and ADH isoenzymes profiles; restriction profiles of a region of 26S rDNA digested with endonucleases HinfI, MseI, CfoI, and HaeIII clearly distinguish both species. The nucleotides sequence of D1/D2 region of 26S rDNA of *Starm. bacillaris* shows an 8% difference at 39 positions [52] thereby justifying the separation of the two species.

From the point of view of its enological application, this strain was able to ferment exclusively the fructose from Chardonnay must without affecting the concentration of glucose. *Starm. bacillaris* has since been reported to have a potentially important role in the winemaking industry, due to its extremely fructophilic character and the poor ethanol yield from sugar consumed [61,62].

Starm. bacillaris presents other interesting characteristics, such as growth at high concentrations of sugars and low temperatures [11,63] and production of low levels of acetic acid and acetaldehyde and significant amounts of glycerol from consumed sugars [64].

9. Metabolic Features and By-Products from *Candida stellata* Activity

Candida spp. are found as food-associated and beverage-associated yeasts. In particular, *C. stellata* has been typically isolated during must fermentation process in different wine regions worldwide, where this yeast species is normally associated with the fermentation of botrytized wines and other wines produced from overripe grapes in cooked musts and in traditional balsamic vinegars [2,3,28,38,65]. Thus, there are several studies that allow one to better understand the metabolic

characteristics of *Candida* spp. with interesting applications in food and fermented beverages industries, with special focus on wine elaboration.

9.1. Fructophilic Character

The strong fructophilic character of *Candida* is one of distinctive features of this yeast genera. Several studies have described sugar depletion (glucose and fructose) during grape juice fermentation [10,64,66–68]. All *C. stellata* strains studied in these works showed a significant lower fermentation rate for glucose than the rate measured for the fructose. In the work with *C. stellata* CBS 2649 strain [10], the extreme fructophilic nature of this strain has been reported, since glucose was not consumed until the fructose was completely depleted. Similar behavior was observed by Mills et al. [7] when studying a *Candida* sp. isolate (EJ1) in Chardonnay wine elaboration. However, it is still unknown how preferential consumption of fructose can be beneficial, since vigorous growth on glucose has been observed when this sugar is the only energy and carbon source available in fructophilic yeasts [63,69]. As previously observed in *Zygosaccharomyces bailii* [70] and *Z. rouxii* [71], Gonçalves et al. [72] have noted the presence of the transporter Ffz1 as a prerequisite for fructophily in *S. bombicola*. This Ffz1 is a specific fructose transporter codified by FFZ1 gene [73]. The reason for the preferential use of fructose by *C. stellata* may be result of a wider remodeling of central carbon metabolism, together with an adaptation to high-sugar environment [72,74].

9.2. Alternative Carbon Metabolism: Glycerol Production

Glycerol can be used as food additive produced from fats and oils, from chemical synthesis, or by microbial fermentation [75]. Glycerol biosynthesis is an important side-reaction of glycolysis pathway produced by reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) and by dephosphorylation of G3P to glycerol (Figure 2). The first step of this conversion is catalyzed by the enzyme NAD-dependent glycerol-3-phosphate dehydrogenase (Gpd) and, subsequently, the glycerol is formed by glycerol-3-phosphatase (Gpp). The enzyme Gpd is encoded as two isoforms by the GPD1 and GPD2 genes [76]. Yeast growth under hyperosmotic stress situation leads to the expression of GPD1 through the so-called HOG (High Osmolarity Glycerol) signaling pathway [77–79]. On the other hand, GPD2 is believed to help maintain the cell's intracellular redox balance.

As mentioned previously, *C. stellata* species exhibits unusual metabolism of sugar; it is usually considered a facultatively fermentative yeast characterized by a very low fermentation rate and high production of secondary metabolites as glycerol, acetaldehyde, acetoin, and succinic acid [67,80]. In regard to glycerol formation, this behavior of *C. stellata* is probably owing to low alcohol dehydrogenase activity (4-fold lesser than *S. cerevisiae*) and high glycerol-3-phosphate dehydrogenase activity (40-fold higher than *S. cerevisiae*); thus, this higher Gpd activity causes a strong deviation towards glycerol production [67] (Figure 2, in red).
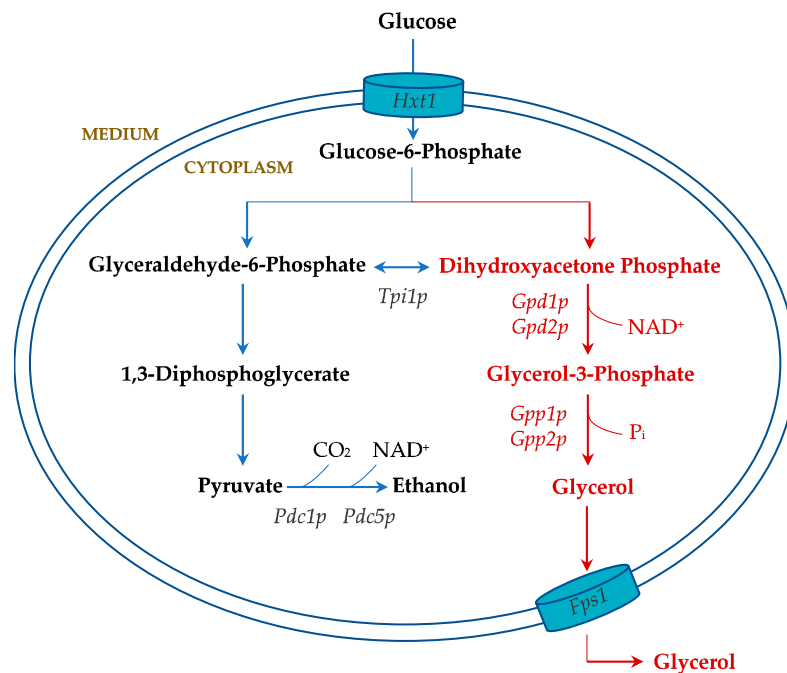
Figure 2. Glycerol biosynthesis in yeasts. Glycolysis and the reduction of intermediate DHAP to G3P, followed by oxidation of NADH to NAD$^+$ leads to glycerol formation (adapted from Scanes et al. [81]).

In oenology, the glycerol content is appreciated, because it imparts some sensory attributes to the wine. It is an important alcohol with a slightly sweet taste and viscous nature that contributes to the smoothness, consistency, and overall body in wine [82,83]. Typically, glycerol concentration is higher in red than in white wines ranging from 1 to 15 g/L. The threshold taste level of glycerol is observed to 5.2 g/L in wine, whereas a change of viscosity is only perceived at 25 g/L of glycerol [84]. Also, it is known that its production is raised by the presence of sulfur dioxide, higher incubation temperature, and high-sugar concentration, but it is significantly influenced by yeast strain and species [85]. In particular, *C. stellata* has typically been described as glycerol producer in wine elaboration [46,68,86,87]. Glycerol concentrations between 9 and 14 g/L have been reported in wines elaborated with *C. stellata*, in contrast with lower amounts produced by *S. cerevisiae* monoculture [46,64,87]. However, glycerol and ethanol content are inversely related; as consequence, the tendency of *C. stellata* to form glycerol seems to be the reason for its low growth and fermentation rate [67,81]. Other authors found an ethanol yield produced by *C. stellata* comparable with that of *S. uvarum/bayanus* strains, although both produced significantly lower ethanol than *S. cerevisiae* [64]. By contrast, Gobbi et al. [88] reported one *C. stellata* strain with an ethanol yield (9.09 g/100 mL) and fermentative power (19 g CO_2 evolved) without significant differences from *S. cerevisiae* (9.05 g/100 mL and 19.2 g CO_2 evolved, respectively).

9.3. Biotechnological Application of Extracellular Enzymes Secreted by Candida stellata

Enzymes are the bio-catalysts that play an important role in metabolism and biochemical reactions [89]. Microorganisms are the primary source of enzymes that have a more active and stable nature than those of plants and animals [90]. Specifically, yeast strains with enzymatic activity could be a potential source of commercial enzymes and an important factor with which to improve the food and beverages processing. The *Saccharomyces* genus is not considered as a good producer of exogenous enzymes. Instead, several non-*Saccharomyces* yeast species exhibit natural enzymatic activities [91]. The enzymes of interest produced by these yeasts include esterases, lipases, glycosidases, proteases, and cellulases usually related to hydrolysis of structural components [4,92].

Specifically, *Candida* spp. have been described as extracellular enzymes producer. The enzymatic capacities of this non-*Saccharomyces* genus have been widely researched in oenology, as they can improve the process of winemaking and enhance wine quality [93,94]. However, it is well known that the secretion of enzymes with technological interest is not characteristic of a particular genus or species but depends specifically on yeast strain analyzed [33,92]. In the following paragraphs, a brief overview will be given of enzymes used in oenology with a special focus on those produced by *C. stellata*.

9.3.1. Pectinases

Pectic substances are the major component of the plant cell wall and comprise a network in which cellulose microfibrils are linked [95]. The high viscosity of pectin prevents juice extraction, clarification, and filtration when it is dissolved after berry crushing. Furthermore, pectin impedes the phenolic and aroma compounds' diffusion into the must during wine fermentation [94]. Thus, pectinases such as polygalacturonase, pectin lyase, pectin methyl esterase, and polygalactosidase have the capacity to reduce the molecular size of pectin polymers by cleaving neutral side chain residues, facilitating the pressing and filtration processes of wines and ciders [96,97]. In addition to their use in winemaking, these enzymes are also utilized in oil extraction [98], coffee and cocoa curing [99], the extraction and clarification of fruit juices, and the retting of textile fibers [100].

Several authors have reported the production of polygalacturonase and pectin methyl esterase by *Candida* in wine [4,101,102]. In a study realized by Cordero-Bueso et al. [103], *C. stellata* CLI 920 strain, which was isolated during spontaneous fermentation in Malvar (*Vitis vinifera* cv. L.) must, produced the highest quantity of pectinases (polygalacturonases) in comparison with other non-*Saccharomyces*. This pectinase activity of *C. stellata* CLI 920 could be correlated with the higher galacturonic acid content observed into the oligosaccharides fraction of the wine produced with this strain alone [104]. Also, polygalactosidases enzymes produced by *C. stellata* together with exo and endoglucosidases are important in the degradation of the β-glucans by *Botrytis cinerea* [2].

9.3.2. Proteases

Protein haze supposes the most common physical instability in white wine and fruit juices. Proteases activity hydrolyzes the proteins into smaller stable molecules promoting clarification and stabilization of beverages and helping to prevent stuck and sluggish fermentations due to low level of assimilable nitrogen in the must [101,105]. Yeast producers of proteases can be a good substitute with which to bentonite for removal undesirable wine proteins [106]. In the study of Strauss et al. [4], 38% of *C. stellata* yeast strains presented protease activity. Also, other works have recorded protease activity in several strains of *Candida* species [101,107].

9.3.3. Cellulases and Hemicellulases

Hemicelluloses are a group of polysaccharides strongly bound to cellulose in plant cell walls. In winemaking, cellulases (glucanases) and hemicellulases (xylanases) enzymes have an impact on organoleptic properties of wine by promoting extraction of pigments and volatile compounds from grape skins, thus improving the filtration and clarification processes and reducing the time of maceration [4,108]. Only a few yeast strains have been known as major producers of these enzymes, but *Candida* species have been reported as able to produce cellulases and hemicellulases [4,102,109,110].

9.3.4. Glycosidases

The organoleptic characteristics of beverages (taste and aroma) can be enhanced by glycosidases that hydrolyse odourless and non-volatile glycosidic precursors of the fruits [111]. Glycosidase activities comprise β-D-glucosidase, β-D-xylosidase, β-D-apiosidase, α-L-rhamnosidase, and α-L-arabinofuranosidase. The bound aroma complex includes glucosides and diglycosides, and compounds such as terpenols, terpene diols, benzene derivatives, aliphatic alcohols, phenols, and C-13 norisoprenoids; additionally, the enzymatic hydrolysis of these sugar-conjugated precursors released

very aromatic volatile monoterpenes (aglycons) through two-step reaction [112]. Numerous works have been based on glycosidase activities in yeasts of an oenological origin; in particular, some of them have observed β-glucosidase activity in *C. stellata* strains possibly related to the fruity and floral aroma found in the wines elaborated with these strains [4,87,103,113,114]. Hock et al. [115] had already documented the terpenes production (β-myrcene, limonene, linalool, α-terpineol, and farnesol) of *C. stellata*. Another study using one *C. stellata* strain isolated from Denomination of Origin (D.O.) "Vinos de Madrid" showed the highest concentration of β-phenylethyl alcohol (roses) in wine compared to other *Saccharomyces* and non-*Saccharomyces* strains analyzed [87]; the flowery and fruity aroma of pure culture with this *C. stellata* strain could be related to β-glucosidase activity previously documented by Cordero-Bueso et al. [103]. Similar results were obtained by other authors [105,116]; they concluded that the use of *C. stellata*, alone or combined with *S. cerevisiae*, enhanced the final quality and complexity of wines.

9.3.5. Invertases

Invertase enzyme, also known as β-D-fructofuranosidase, is commonly used in industries with numerous applications as production of lactic acid [117], fermentation of sugarcane to ethanol [118], and production of fructose syrup. Furthermore, it is employed in pharmaceutical industry, child nutrition, and fortified wines [119]. These enzymes hydrolyse the glycosidic linkage from sucrose in its respective monomers, glucose and fructose, to form "inverted sugar syrup" with special characteristics: 40% sweeter than sucrose, stable at high temperatures, more soluble than sucrose and higher point of boiling and lower of freezing [119]. Yeast production of these enzymes is typically studied in *S. cerevisiae* [120]. Recently, Gargel et al. [121] have observed that one *C. stellata* strain (N5) isolated from Brazilian grapes is a potential invertase producer. They propose this new invertase as a promising catalytic agent for use in biotechnological processes in the food industry and alcoholic fermentations.

9.4. Production of Sophorolipids Biosurfactants by Candida

The worldwide production of surfactants is about 10 million tons per year, divided between domestic and laundry detergents and different industrial applications. Currently, the surfactants are usually petroleum-derived, although the aim is to produce these compounds from renewable substances. Sophorolipids (SLs), which are composed of sophorose (a dimeric sugar) linked to a long-chain hydroxy fatty acid, are good candidates as surfactant product from renewable sources. These molecules are produced in high concentrations by phylogenetically diverse group of yeasts [122], and their biosynthesis is clearly influenced by aeration, initial glucose concentration, and pH values [123,124]. SLs present two different forms: a closed lactone and an open acidic form. Each form has different properties: Lactonic SLs have antimicrobial activity and are better in surface tension reduction, while acidic SLs have better foaming attributes [122].

The yeast *S. bombicola* has been widely studied as a major producer of SLs together with *Candida apicola* within *Starmerella* clade [122]. The highest *C. bombicola* (ATCC 22214) SL yield of 400 g/L was obtained when corn oil and honey served as the carbon sources [125]; also, Cavalero and Cooper [126] showed that the same strain synthetized SLs with antibacterial activity mainly against Gram-positive bacteria. In a study with 19 species of *Starmerella* yeast clade [123], *C. stellata* NRRL Y-1446 strain from Rovello bianco grape variety was one of 19 species with a significant production of SLs with 11.9 g/L predominantly as di-*O*-acetyl free-acid form, plus lesser amounts of mono-*O*-acetyl and non-acetyl SLs. Parekh et al. [124] obtained similar SLs concentration (18.2 g/L) using *S. bombicola* NRRL Y-17069 and determining the optimal fermentation method to generate these surfactant compounds. Recently, a novel lactone esterase enzyme from *S. bombicola*, which catalyzes the intramolecular lactonization of acidic SLs in an aqueous environment, is being investigated to become an ecological tool in industry applications [127].

10. Co-Fermentations between *Candida stellata* and *Saccharomyces cerevisiae*: A Way against Standardized Wines

The use of co-fermentation strategies between non-*Saccharomyces* and *S. cerevisiae* yeast species in a controlled manner can be a useful tool for wine production. Several aspects support this consideration, such as

1. Effect on some analytical compounds as increased glycerol concentration, enhanced total acidity, and reduced acetic acid concentration of wine.
2. Enhancement of desirable aromatic compounds (esters, volatile thiols).
3. Reduction of final ethanol content of the wine.
4. Improvement of complexity and overall quality of wine.
5. Larger release of polysaccharides (mannoproteins).

In the last few years, the use of *C. stellata* yeast in multi-starter fermentations with *S. cerevisiae* has been widely investigated for its ability to increase the glycerol content in wines and their special fructophilic character [66], its capacity to contribute to greater aroma complexity of the wine [128], and its capacity to minimize the risk of fermentation problems [68]. Ciani and Ferraro [66] carried out mixed and sequential fermentations with *C. stellata* and *S. cerevisiae*; the final wines were rich in glycerol and succinic acid, and with less alcohol and acetic acid in comparison with the mono-inoculated *S. cerevisiae* control. Milanovic et al. [68] concluded that *S. bombicola* influenced the alcohol production ability of *S. cerevisiae* under mixed inoculation, since pyruvate decarboxylase (Pdc1) activity in mixed fermentation was lower than pure culture of *S. cerevisiae*, while alcohol dehydrogenase (Adh1) activity showed opposite behavior.

The wines made through *C. stellata*/*S. cerevisiae* co-fermentations usually present higher aroma complexity and overall quality. In a study using Malvar white grape [87], an autochthonous grape variety from Madrid (Spain), different inoculation strategies were applied with *C. stellata* CLI 920 (Cs) and *S. cerevisiae* CLI 889 (Sc). Mixed and sequential were significantly different with regard to their volatile composition and the control of *S. cerevisiae*. These wines were characterized by increased esters concentration and β-phenylethyl alcohol (Figure 3). After sensory analysis, the sequential inoculation was well appreciated by tasters for its pleasant fruity (green apple, grapefruit) and floral aroma and its freshness and full-bodied on the palate. These results were corroborated by pilot scale fermentations [26].
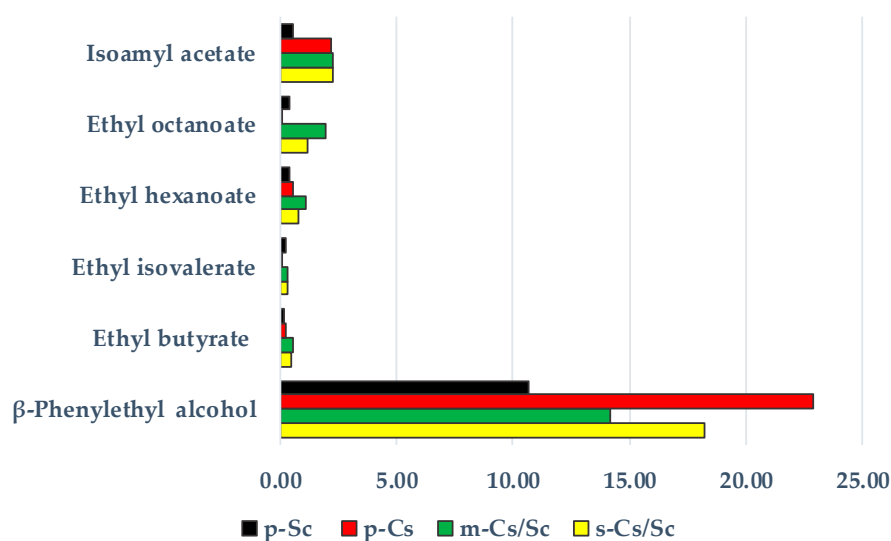

Figure 3. Relevant volatile compounds (mg/L) of pure (p), mixed (m), and sequential (s) fermentations made with *C. stellata* CLI 920 (Cs) and *S. cerevisiae* CLI 889 (Sc) native strains (adapted from García et al. [87]).

In agreement with above, Soden et al. [10] described the aroma of banana, flowers, and lime in wines conducted by sequential inoculation in comparison with the control of *S. cerevisiae*. Other works have also shown the fruity and flowery aroma in cocultures between *C. stellata* and *S. cerevisiae*, which is the result of greater concentration of desirable aromatic compounds including some higher alcohols; β-phenylethyl alcohol and ethyl esters correlated well with its medium-chain fatty acids [26,64,105,116,129].

In recent years, multiples studies have focused on polysaccharides content in wines, giving special attention to the mannoproteins. These molecules are one of the major polysaccharide groups in wines from yeast cell walls [130], and they are secreted into wine during alcoholic fermentation and yeast autolysis during ageing on lees [131]. Mannoproteins composition consists mainly of mannose (80 to 90%) and small amounts of glucose, associated with 10–20% of protein. Numerous investigations have clearly confirmed that these macromolecules are related to technological and sensorial properties in wines, such as prevention of protein haze in white wines [132], protection against crystallization of tartrate salts [133], interaction with aroma compounds [134], improvement of foam stability and flocculation in sparkling wines [135], reduction of astringency and increased body and mouthfeel [136], and increase of the growth of malolactic bacteria [137]. Moreover, it has been noted that the utilization of *Saccharomyces*/non-*Saccharomyces* co-fermentations results in increased release of polysaccharides into the wine, since the high capacity of non-*Saccharomyces* wine yeasts to release polysaccharides (including mannoproteins) has been verified [104,138–140]. Giovani et al. [139] characterized the monosaccharide composition of mannoproteins produced by *S. bombicola* 3827; they noted that the polysaccharides produced by *S. bombicola* were essentially mannoproteins with 73–74% of mannose residues.

In the previously mentioned study [87] (Figure 3), the polysaccharides' content and structure were studied in Malvar wines elaborated with *C. stellata* CLI 920 and *S. cerevisiae* CLI 889. The greater content of arabinose, galactose, and mannose in the total colloids means that mannoproteins from yeast cell walls and Polysaccharides Rich in Arabinose and Galactose (PRAGs) were the main macromolecules in Malvar wines regardless of the inoculation strategy used (Figure 4a). The high content of galactose observed, especially in *C. stellata* pure culture (p-Cs), could also be explained by the presence of this monosaccharide-like galactomannan in yeast cell walls, as in *Schizosaccharomyces pombe*. However, a phylogenetic study with 33 species of *Candida* carried out by Suzuki et al. [141] determined that the cell wall of *C. stellata* lacked galactose.
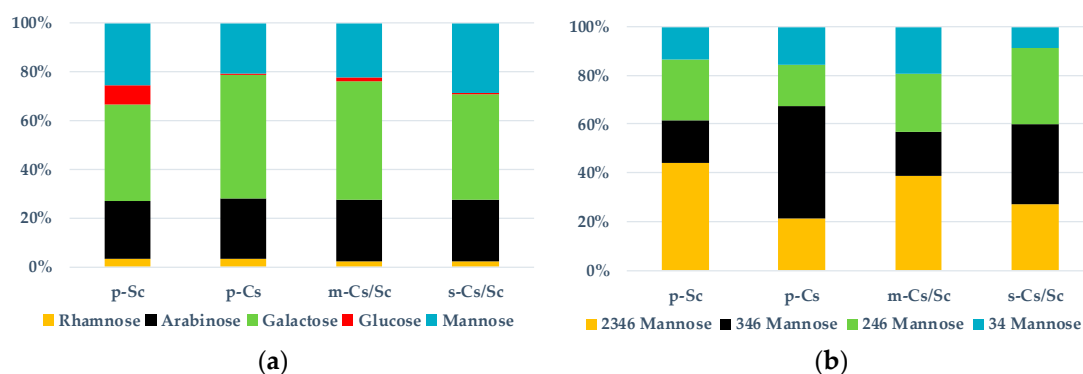

Figure 4. Study of polysaccharides content and structure in Malvar wines elaborated under different inoculation strategies with *C. stellata* and *S. cerevisiae* native strains *: (**a**) Glycosyl residue composition of polysaccharides from Malvar white wines and (**b**) Glycosil-linkage composition of mannose residue isolated from Malvar white wines. * Abbreviations associated with type of fermentation and yeast strains are explained in Figure 3.

Mannose residues are larger in *C. stellata*/*S. cerevisiae* sequential fermentation (s-Cs/Sc) than control (Figure 4a); s-Cs/Sc could be the best combination for mannoproteins release into the wine using

these yeast strains. Other studies also showed that mixed inoculations with *C. zemplinina*/*S. cerevisiae* supposed an increase of polysaccharides mainly mannoproteins in the final wines [142,143]. Regarding the structure of mannose residues from mannoproteins (Figure 4b), these results are consistent with the *Candida* mannoproteins structure described by Ballou [144]. The structure of mannoproteins consists of a 6-linked backbone, substituted on the 2-position with 2- and 3- linked mannose. This 3-linked mannose (2,4,6-tri-*O*-mannose) proportion is substantially lower in p-Cs than in the control, which agrees with the results previously reported [144]. The high proportion of 3,4,6-tri-*O*-methyl mannose (2-linked mannose) in p-Cs can be observed in comparison with the control (p-Sc); therefore, the *C. stellata* mannoproteins released into the wine present a greater branched structure than those released by the control (Figure 4b). Also, sequential fermentation contained mannoproteins structurally similar to those in the monoculture with *C. stellata*. This could be explained by the important contribution of *C. stellata* strain to wine composition before the inoculation of *S. cerevisiae* strain.

11. Conclusions

At present, a preliminary genetic study needs to be used before the application of *Candida stellata* in food and beverage processing. This research should help to distinguish it from other closely related species within *Starmerella* clade.

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