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Research Article

Impact of Yeast Flocculation and Biofilm Formation on Yeast-Fungus Co-Adhesion in a Novel Immobilization System

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Abstract: A novel method of yeast immobilization, called biocapsules, has been developed in which cells of the yeast *Saccharomyces cerevisiae* become attached to the hyphae of the fungus, *Penicillium chrysogenum,* remaining adhered following loss of viability of the fungus. Yeast immobilization facilitates higher cell densities than traditional fermentation methods, improves yield and allows the reutilization of the biocatalyst. Yeast cells may be adherent to each other via specific cell surface molecular interactions (flocculation) or attach to surfaces (biofilm formation), and the role of these two distinct mechanisms of attachment in biocapsule formation is unknown. To elucidate the influence of biofilm formation versus flocculation on the yeast-fungus co-immobilization, a screening of selected strains from the Viticulture and Enology Department collection at University of California, Davis was carried out and their ability to flocculate and form biofilm was quantified. Eighteen yeast strains capable

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of flocculation and biofilm formation were concluded from this screening. Strains displaying differential capabilities in flocculation or biofilm formation plus two control strains were further evaluated for their ability to specifically immobilize with *P. chrysogenum*. Seven strains were found to show different patterns of flocculation and biofilm formation. Yeast strains able to form biofilm displayed higher rates of immobilization with *P. chrysogenum* and formed more consistent biocapsules. In contrast, strains able to flocculate developed smaller, inconsistent biocapsules. Although the size and number of biocapsules formed varied by yeast strain, the total mass of biocapsules generated was similar for all strains. These results shed light on parameters that influence yeast-fungus co-immobilization, which may lead to an improvement of biocapsule consistency and further the field of application for this new immobilization system.

Key words: biocapsule, fermentation, *P. chrysogenum*, *S. cerevisiae,* yeast immobilization

Introduction

The use of immobilized microbial systems in the production of fermented beverages offers many advantages over conventional free cell fermentations. These advantages include: high yeast cell densities, product yield improvement, lowered risk of microbial contamination and reuse of the biocatalyst (Kourkoutas et al. 2004). Various supports have been used for cell immobilization including inorganic, organic polymers, natural matrices as well as membrane systems (Kourkoutas et al. 2010, Nedović et al. 2010). Natural supports derive from materials that are generally food grade with minimal or no pre-treatment intervention such as components of fruit, wood, or sawdust (Kourkoutas et al. 2004). Immobilization supports and techniques have been applied to alcoholic beverage as well as fuel ethanol production.

A novel yeast immobilization system, termed “biocapsules”, is based on a natural support consisting of the hyphae of a filamentous fungus *Penicillium chrysogenum*. The hyphae of this fungus

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serve as a platform for yeast cell attachment and adherence (Peinado et al. 2004). Biocapsules are hollow, spherical bodies that constitute a natural matrix system which eliminates the cost of inert supports, since it takes advantage of natural adhesion properties of yeast and filamentous fungus cell walls, minimizes changes to the yeast metabolism and/or yeast viability. Additionally, biocapsules enable diffusion of nutrients and end products to and from the biocapsules due to the porous structure of the filamentous fungus (Peinado et al. 2004, García-Martínez et al. 2011). The rapid diffusion of carbon dioxide is an important feature as it prevents bubbles from building up within the matrix and breaking the matrix, which has been an issue with other types of support systems. When the yeast and fungus are co-cultivated in a medium supporting hyphal growth under agitation, visible ball structures form. The yeasts are attached to the hyphae within the ball structures in a stable manner. Subsequent incubation of the biocapsules in media supporting yeast fermentation enables yeast growth and metabolism, thereby causing the fungus to die most likely from the combination of ethanol and lack of oxygen, and the hyphal structure remain as a mere support for the attached yeast cells (Peinado et al. 2006). Given their size, biocapsules can be easily recovered from fermentation and the yeast retain viability and fermentative capacity over multiple rounds of reuse (Peinado et al. 2004). Because of these features, biocapsules have been considered a promising technique for industrial-scale fermentation purposes and have already been utilized in production of white wine, sparkling wine and natural sweet wine as well as for bioethanol from starch and molasses (Peinado et al. 2005, 2006, García-Martínez et al. 2012, Puig-Pujol et al. 2013, García-Martínez et al. 2015).

Peinado et al. (2004) observed that when co-inoculated with the fungus, flor yeast strains form biocapsules with relatively high consistency and mechanical resistance; defined by the biocapsule's ability to withstand compression force. Flor yeasts differ from other yeast in their capacity to auto-immobilize forming biofilm aggregates at liquid-air interfaces under certain conditions (Esteve-Zarzoso et al. 2001, Aranda et al. 2002, Alexandre, 2013). Flor yeasts are used for the elaboration of Sherry wines

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due to their ability to survive in a post- fermentation environment where fermentable carbon sources are nearly exhausted and only ethanol and glycerol remain (Esteve-Zarzoso et al. 2001). Oxygen is required to metabolize these carbon resources. The biofilm formation process allows the flor yeasts to access regions where oxygen is rich, the wine-air interface. Zara el al. (2009) demonstrated the existence of an extracellular matrix among flor yeast forming the velum. The composition of the velum is unknown.

The interactions of flor yeast with the fungal hyphal matrix within a biocapsule has been investigated using transmission electron microscopy (García-Martínez et al. 2011). Yeasts were observed to be directly attached to the cell surface of the fungus and the integrity of the biocapsules was retained following fermentations (Peinado et al., 2006) enabling reuse of the capsules. García-Martínez et al. (2011) established that biocapsules formed naturally, stabilized yeast fermentative activity and viability, and retained integrity even after loss of viability of the fungus due to the nature of the cell-hypha contact. The yeasts were able to adapt to high ethanol conditions and complete fermentations. However, previous works were largely conducted with only one strain of flor yeast which may produce a spectrum of aroma compounds not normally found in table wine yeast (Peinado et al., 2005, 2006, García-Martínez, 2015).

The main focus of this work was to study the properties of yeast strains that affect co-adhesion with *P. chrysogenum* when forming biocapsules. Although effectiveness of biocapsule immobilization made with non-flor yeast versus flor yeast has been compared (García-Martínez et al. 2012), specific yeast properties such as flocculation and biofilm forming abilities and their effects on the formation of co-adhesion has not been systematically investigated. In this work, we defined methodologies for assessment of flocculation and biofilm forming ability and assessed the impact of these properties on biocapsule formation. Known flocculent and biofilm forming yeast strains were initially screened to observe and quantify flocculation and biofilm formation phenotypes and strains displaying differences in these phenotypes were selected for subsequent analysis. The ability of these yeasts to form biocapsules was assessed and biocapsule parameters like percentage of immobilized yeasts, number of biocapsules, total

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volume, diameter, consistency and dry weight, were evaluated.

Materials and Methods

Microorganisms and growth media. *Saccharomyces cerevisiae* yeast strains from the Department of Microbiology (University of Cordoba, Spain (UCO)) and the Department of Viticulture and Enology (University of California, Davis (UCD)) collection were used in this work (Table 1). *S. cerevisiae* G1 and UCD932 were utilized as positive and negative controls for flocculation/biofilm formation, respectively. Eighteen strains were chosen from the UCD collection due to their reported ability to form flocs and/or biofilms. This strain set included *S. cerevisiae* yeasts isolated from sherry, sparkling, dry and standard wine, beer and must, as well as commercial strains (Table 1). After analyzing and quantifying strain flocculation and biofilm formation phenotypes, seven strains showing different flocculation/biofilm formation patterns were selected together with G1 and UCD932 control strains, for co-immobilization experiments with the filamentous fungus and biocapsule formation. Cell population size for strains forming flocs in liquid media are difficult to quantify under the microscope or spectrophotometrically, producing false values. Therefore yeasts were pre-grown on solid media, YPD-agar (1% yeast extract: 2% peptone, 2% glucose and 2% agar), and the colonies transferred to an Eppendorf tube containing the liquid growth medium and agitated for 5 minutes to avoid flocs and generate the inocula.

Yeasts were co-immobilized with the filamentous fungus strain *P. chrysogenum* H3 from UCO collection. The fungus was pre-grown in a sporulation medium (SM) containing 1.7% corn meal agar, 0.1% yeast extract, 0.2% glucose and 2% agar for seven days at 28 ºC.

Flocculation assessment. To assess flocculation ability, 4 x 10^6 yeasts cell/mL were inoculated into 5 mL of synthetic grape juice medium "minimal must medium" (MMM) (Giudici and Kunkee 1994, Spiropoulos et al. 2000). The synthetic grape juice medium has the following composition: 11% (w/v) D-

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fructose, 10% (w/v) D-glucose, 0.3% (w/v) L(-)malic acid, 0.3% (w/v) citric acid, 0.17% (w/v) YNB (Difco Yeast Nitrogen Base) without amino acids and ammonium sulfate, 10 mg/L ergosterol and 1 mg/L Tween 80. The nitrogen equivalents were 123 mg/L. The media pH were adjusted to 3.5 with KOH or NaOH. The cells were then incubated at 25ºC under agitation conditions, on a rotary drum for 5 days. For the semi-quantitative analysis, pictures were taken at day five after the inoculum both macroscopically and microscopically, the later by using the Celestron microscope. To quantify non-flocculated versus flocculated yeast population sizes, cultures were filtered through a Hydrophilic Nylon membrane filter 30 µm ⌀ pore size (NY3004700 | Nylon mesh filter, hydrophilic, 30 µm, 47 mm) that allowed passage of free or suspended cells but retained flocs of 6 or more cells on the filter matrix. The strategy was verified by observing samples under the microscope before and after the filtration (no flocs were observed after the filtration). The dry weight of the suspended yeasts and the flocculent yeasts were then measured following drying to a constant weight. The filters were dried in an oven at 80 ºC constant temperature overnight. Non-flocculant control strains showed some adherence to the membrane and this value was considered background binding of single cells or cells in aggregates of less than 6.

Biofilm formation and assessment. Approximately 6 x 10^7 yeasts cells/mL were inoculated into 10 mL of flor medium in a glass test tube containing 0.67% YNB without amino acids and 3% ethanol adjusted to pH 3.5; and incubated at 21 ºC for 5 days (Ishigami et al. 2004, Govender et al. 2010). Biofilms were defined as layers on solid surfaces or across the air/liquid interface. Pictures were taken macroscopically at day 5 from inoculation. For quantification, biofilm forming yeast and non-biofilm forming yeasts (precipitated and suspended) dry weight were measured. Biofilms were carefully extracted with a 5 mL pipette and a spatula when they were completely covering the medium surface at day 5 (Govender et al. 2010) while non-biofilm forming yeasts were collected by centrifugation (4500 rpm for 15 minutes).

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Biocapsule formation and assessment of biocapsule properties. *S. cerevisiae* strains were pre-grown for 3 days in YP + 3% glycerol medium (175 rpm, 28 ºC). Yeast nitrogen base medium without amino acids (Difco); containing 5 g/L gluconic acid as a carbon source and buffered to pH 7 with sodium and KH_2PO_4, was used as a biocapsule formation medium (BFM). The medium used for the co-immobilization is suitable for yeast to express its flocculation (Miki et al. 1982a, 1982b, Stratford 1992, 1996, Dengis et al. 1995, Kida et al. 1989, Soares et al. 1991, Straver et al. 1993, Soares and Seynaeve 2000, Verstrepen and Klis 2006, Soares, 2011) and biofilm formation phenotypes (Esteve-Zarzoso et al. 2001, Aranda et al. 2002, Alexandre, 2013; Zara et al. 2010).

Three flasks per yeast strain, each containing sterile, autoclaved 150 mL BFM, were inoculated with 4 x 10^6 yeast cells/mL and 4 x 10^6 *P. chrysogenum* spores. The flasks were then shaken at 175 rpm and at 28 ºC for 6 days. Under these conditions, spontaneous immobilization occurred and yeast biocapsules were produced. The immobilization procedure used is the same as that patented by Peinado et al. (2004). The capacity of cells to be immobilized in the co-adhesion assay was determined by quantifying the following parameters: number of non-immobilized yeasts, immobilized yeasts, % yeast immobilized, number of biocapsules, diameter, total volume, consistency and dry weight. Yeast biocapsules were separated from the medium and washed with distilled water prior to their analysis. Biocapsules were counted in each of the flasks, and then the diameter size and the volume occupied by all biocapsules were measured. The total volume of biocapsules formed was calculated by submerging all biocapsules in water from each sample (flask) in a 50 mL graduated cylinder and measuring the difference in volume before and after submersion. Biocapsule consistency was quantified by a TA.XT2 texture analyzer which measures the force required to compress biocapsules to a 2 mm thickness.

For the immobilized yeasts counting, ten biocapsules out of the total from each flask were disrupted with salt to separate yeast cells from the fungal hyphae. Cells were counted and normalized to the total number of biocapsules in each replicate. Biocapsules were broken by placing them into a NaCl

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solution (100 mM), crushing with a pestle and mortar for 2 minutes, then transferred to a test tube and vortexed for 20 seconds. As a result, a mixture of yeast cells and *P. chrysogenum* hypha segments was obtained. Successive differential filtrations were carried out to obtain the released yeast cells for quantitation: i) by using a colander to remove large biocapsule fragments; ii) a 180 µm ⌀ filter (NY8H04700 | Nylon mesh filter, hydrophobic, 180 µm, 47mm) and iii) a 30 µm ⌀ filter (NY3004700 | Nylon mesh filter, hydrophilic, 30 µm, 47 mm). Yeast population sizes (non-immobilized and immobilized yeasts) were determined by direct counting using a Haemocytometer grid under the microscope at 40x objective. The remaining biocapsules were used for the measurement of the dry weight (80 ºC constant temperature overnight) and used to obtain an average dry weight of the biocapsules.

Statistical analyses. Data obtained from the quantified yeast strain and biocapsule parameters were subjected to statistical analyses through the software package Statgraphics Centurion XVI (Manugistics, Inc., Rockville, MD, USA). ANOVA and the Kruskal-Wallis tests were used to detect parameters which quantified values depending on the yeast strain analyzed. Further, to detect correlations among yeast strain and biocapsule parameters, a multivariate analysis was performed and a Pearson coefficient and *p*-value were provided for each couple of variables (Table 2). The range of the Pearson coefficient is from -1 to +1, and it measures the linear relation among variables: 1 is total positive linear correlation, 0 is no linear correlation, and −1 is total negative linear correlation. The *p*-value tests the statistical significance of the estimated correlations.

Results

Screen of flocculation and biofilm formation phenotype. Eighteen yeast strains were selected from the UCD collection to screen for flocculation and/or biofilm formation potential (Table 1). Yeast were categorized visually for the formation of cell aggregates with constant mixing (Figure 1). Biofilm formation capacity was evaluated by assessing the formation of a film on the surface of the tube or

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growing on the side of the tube for non-agitated samples. Five of the 18 selected strains were flocculent to some degree and 12 were able to form a visible biofilm, also to different degrees (Figure 1). Seven out of the 18 yeast strains tested were selected for further analysis in quantification of flocculation/biofilm formation and *P. chrysogenum* co-adhesion assays (UCD77, UCD519, UCD580, UCD804, UCD854, UCD1109 and UCD1162 plus the controls G1 and UCD932) based on different flocculation/biofilm patterns (see phenotypic qualification and physical aspect in Figure 1). G1, the original strain evaluated by Peinado et al. (2004) for biocapsule formation displaying both flocculation and biofilm formation, and UCD932, a non-flocculating strain that was not able to form a biofilm, were also included in subsequent assays as controls.

Quantification of flocculation and biofilm formation. The set of 9 strains were evaluated in quantitative assays for assessment of flocculation ability and biofilm formation capacity (Figure 2). In brief, differential filtration was used to separate free or planktonic cells from adhered cells. The separated populations were then harvested by centrifugation and dry weight of the two populations determined. It should be noted that in the case of non-flocculating strains, a portion of cells were retained on the filter through the filtration process. This weight was considered as background value. Yeast strain UCD854 originally isolated from British Ale beer displayed the highest flocculation capacity (Figure 1 and 2 a), with adhered cells comprising 39.83 out of a total dry weight of free and adhered cells of 40.96 mg, or 97.5%. UCD854 flocs could attain sizes at macroscopical dimensions. UCD580 does not form visible flocs in the qualitative assay, but showed a high biomass that was attached to the filter (Figure 2a). UCD580 is a strong biofilm forming yeast strain. Such strains synthesize proteins essential for the biofilm formation, like Flo11p, which may have caused attachment to cells to the material of the membrane filter used (Fidalgo et al. 2008).

UCD580 displayed the highest biofilm formation capacity among the screened yeast strains (Figure 2b), both in terms of biofilm total dry weight and biofilm dry weight percentage, reaching up to

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7.17 mg of biofilm out of 29.67 mg of total cells or 24.05%. This strain was closely followed by G1 with a 6.5 mg biofilm dry weight out of a total of 31 mg (20.94%) and UCD804 with 5.17 out of 25.33 mg (20.33%). Those strains, UCD580 and G1, are both flor yeasts used for Sherry wine elaboration. Although G1 demonstrated the formation of flocs when grown in MMM under agitation, it shows low retained biomass values. It might be that G1 flocs are less adherent than others and yeast cells were disaggregated during the filtration thus yielding low retained dry weight values. UCD1109 isolated from must and the Sherry wine yeast UCD519 grew better in the flor medium than the rest of the strains reaching total dry weights of 52.33 and 42 mg, respectively. UCD1162 and UCD77 displayed the lowest biofilm percentage values: 0.5 out of 10.33 mg (4.85%) and 1 out of 18.17 mg (5.41%), respectively.

Biocapsule formation. The selected yeast strains were then evaluated for biocapsule formation and biocapsule properties (Figure 3). The percent of immobilized cells of the total population present during biocapsule formation was measured in addition to number, diameter, and dry weight of the biocapsules formed. Biocapsule consistency was defined as resistance to compression using a texture analyzer. Using the Kruskal-Wallis test all parameters excluding "biocapsule total volume", were found to be dependent on the strain of yeast with a p-value < 0.01 (biofilm formation, yeast immobilization, biocapsule number and consistency and dry weight) and < 0.05 (flocculation and biocapsule diameter). Biocapsule total volume (Figure 3d) seems to be dictated by the amount of growth of the fungus which was uniform for all samples and replicates while other biocapsule parameters are more yeast strain dependent.

The highest immobilization (Figure 3a) capacity was observed for the positive control G1 flor yeast with 93.2% of the yeasts cells adhering to the filamentous fungus hyphae. Another strain of flor yeast, UCD580, showed the second highest adherence percentage with 85.0% of the cells attached to the *Penicillium* hyphae. Both strains showed high biofilm forming capacity. G1 total yeast cells in the BFM were more abundant than UCD580, 4.0 vs. $1.7 \bullet 10^7$ total cells in 150 mL, respectively. The highest

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number of immobilized yeast cells were found with strain UCD1109 reaching 1.1 x 10^8 cells, however, the percentage of immobilization was lower, 83.7% (Figure 3a). It should be noted that this strain reached the highest cell population under biofilm formation, though possessed relatively low biofilm formation percentages and low total cell mass under the flocculation condition. UCD77, a yeast strain isolated from sparkling wine production, showed the lowest immobilization efficiency (12.08%) but the highest total population (2.2 x 10^8 cells) in BFM (Figure 3a). Abundant yeasts found dispersed in the medium and with sparse cells attached to the fungus may indicate that the strain does not need the attachment to the fungus to grow in a medium like BFM. The major carbon source in BFM is gluconic acid, which, although can be utilized by *S. cerevisiae* as the only carbon and energy source -which seems to be the case of UCD77-, many other yeast strains do not grow well on this substrate. It should be noted that UCD77 is a strain with a very low ability to form biofilm, only 5.41% of the total biomass and flocculation close to the strain average value (79.28 ± 4.53%).

The number of biocapsules formed varied ten-fold from a low of 100 to over 1000 with the average number formed being around 360 (Figure 3b). The highest values were obtained with UCD1162 (1198 biocapsules). This value was significantly above those of all other strains. UCD1162 is characterized mainly by its scarce ability to form biofilm (4.85%) and values of flocculation below average. UCD1109 also formed nearly twice the average number of biocapsules (632). UCD1162, UCD1109, UCD854 and UCD77 formed more biocapsules and exhibited less ability to form biofilm than the biofilm-forming yeast strains G1, UCD519, UCD580 and UCD804. This observation suggests that yeast biofilm formation is inversely related with the number of biocapsules formed. Furthermore, yeast strains with lowest biocapsule diameters (Figure 3c) coincide with those with highest number of biocapsules, thus indicating that there may be a correlation between “biocapsule diameter” and “biocapsule number” (Figure 4) and both may be functions of the biofilm forming capacity of the cells.

Biocapsule total volume (Figure 3d) was not significantly different among the yeast strains. This

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study showed that those strains with higher capacity to form biofilm produce larger but fewer biocapsules than those made with yeast strains with lower capacity to form a biofilm. The yeast impacts the dimensions of the biocapsules in a way that is correlated with ability to form a biofilm.

In addition, strains with the highest biofilm forming ability G1, UCD580 and UCD804 formed biocapsules that resisted deformation in the texture assay and less resistant biocapsules were obtained by immobilizing the strains with lower biofilm forming capacity UCD77, UCD854, UCD1109 and UCD1162. UCD519 which shows a high ability to form a heavy but fragile biofilm (fragments detached from the biofilm were observed), formed biocapsules with low consistency (similar to UCD932) (Figure 3e). This may indicate that in addition to the biofilm weight, other factors (i.e. fragility) also affect the consistency of biocapsules formed. Similarly, heaviest biocapsules were obtained with the flor yeast G1 yeast strain while lightest with the non-biofilm forming UCD77 and UCD1162 (Figure 3f). The rest did not differ significantly in dry weight with an average of 0.07 g.

After applying the Pearson test to detect correlations among yeast strain and biocapsule parameters, lowest *p*-values were obtained among the following parameter couples: “biocapsule diameter” - “yeast forming biofilm (%)”, “biocapsule consistency” - “biofilm yeast weight” and “biocapsule consistency” - “yeasts forming biofilm (%)” (Table 2). For all these couples of parameters, the Pearson coefficients were over 0.7, indicating that a positive correlation exists for each, reaching 0.93 in the case of “biocapsule consistency” and “yeasts forming biofilm (%)”. Bigger and more consistent biocapsules are obtained by immobilizing biofilm forming yeast strains. This result is consistent with the observation made by Peinado et al. (2004) who proposed flor yeasts as better candidates to make biocapsules (in terms of consistency) than those without the ability to form biofilm. On the other hand, flocculation appears to be inversely correlated with the biocapsule consistency: “non-floc yeast weight”- “biocapsule consistency” showed a Pearson value of 0.4274 and a *p*-value of 0.0262. However, a positive correlation (Pearson coefficient = 0.6849; *p*-value = 0.0001) among the number of yeasts immobilized

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and the amount of yeasts that did not make biofilm in the flor medium (Table 2) may indicate that the yeast's ability to form biofilm and the number of yeast immobilized in biocapsules are negatively correlated.

Biocapsule dry weight (Figure 3f) is both positively correlated with the biofilm yeast weight plus the weight of non-biofilm forming yeasts. Not surprisingly, this indicates that biocapsule weight is positively correlated with the total amount of yeast growing under the biofilm forming conditions. However, it should be considered that: i) the *p*-value for "biofilm yeast weight" was lower than that for "non-biofilm yeast weight", and ii) the "biocapsule dry weight" is also positively correlated with a *p*-value < 0.005 to the percentage of yeast forming biofilm, meaning that biofilm formation positively affects the weight of the biocapsules. The same was true for the percentage of yeasts immobilized within biocapsules.

Lastly, the Pearson test also showed that the number of biocapsules obtained at day 6 of the fungus-yeast co-inoculation is negatively correlated to biofilm formation (as previously mentioned) but positively correlated in a significant level to the yeast capacity to flocculate under agitation.

Discussion

Flor yeast strain G1 was shown to form biocapsules that display fermentative capacity and retain yeast cell viability (García-Martínez et al. 2012). This strain both flocculates, forms a velum biofilm during sherry wine production. It is unclear if any or all of these properties impact the co-adhesion to fungal hyphae. To determine what cellular properties were important in biocapsule formation and functionality, we screened a set of wine/beer yeast strains for flocculation and biofilm phenotypes and then evaluated a subset for biocapsule formation. It was shown that these yeasts immobilize differently with the filamentous fungus *P. chrysogenum*. Values from all parameters evaluated differed between strains with a *p*-value < 0.05, with the exception of "total volume of biocapsules".

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316 Biofilm formation is defined as a biological process where planktonically growing
317 microorganisms grow at a liquid-air interface, which is the case for flor or velum; or on a solid substrate
318 under the flow of a liquid, like the case of filamentous fungus hyphae surface (Kuchin et al. 2002,
319 Ishigami et al. 2006, Fidalgo et al. 2008). This means that flor yeast strains capable of biofilm formation,
320 can further extend a film on a solid substrate which can be the hyphae surface of filamentous fungus. It
321 must also be considered that the matrix formed by the filamentous fungus can trap air during agitation.
322 Accordingly, flor yeast may encounter an environment in the hyphae matrix similar to that during
323 biological aging (i.e. Sherry wine elaboration) where oxygen is necessary to metabolize the carbon source
324 present in the medium. Both conditions promote the onset of biofilm formation in these yeast. Once the
325 biofilm forming yeasts are attached to the hyphae, they can form an extracellular matrix as previously
326 revealed in the velum formed by some strains (Zara et al. 2009). This extracellular matrix was later
327 detected in the biocapsule walls by Peinado et al. (2006) who observed this connection of yeasts to
328 hyphae. The ability of flor yeasts to form this fibrillary material, which until now is of unknown
329 composition, may be encompassing the biocapsules and therefore explain the larger size of the
330 biocapsules.

331 Biocapsule dry weight and percentage of yeasts immobilized were both directly correlated with
332 the biofilm yeast weight and the weight of the non-biofilm forming yeasts. Nonetheless, they were also
333 positively correlated with a p-value < 0.005 to the percentage of yeast forming biofilm, thus the formation
334 of the biofilm positively affected the weight of the biocapsules. The number and diameter of the
335 biocapsules appear inversely correlated: the more abundant the number of biocapsules, the smaller the
336 biocapsule diameter. Consequently, the total volume remains consistent (Figure 3d). It was also observed
337 that the smaller biocapsules in larger quantities were formed with the non-biofilm forming strains while
338 biofilm forming yeasts developed biocapsules that are larger in diameter but smaller in quantity (Figure
339 4). This fact is further confirmed by a negative significant Pearson coefficient.

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Unlike biofilm formation, the yeast's ability to flocculate allows for a greater number of biocapsules formed but smaller average diameter of each biocapsule. Flocculation and biofilm are two different types of cell aggregation. Flocculation is a homotypic process, involving only one type of cell in the interaction (Stratford 1992, Stewart 2009) and all cells have components on their cell surfaces (lectins and α-mannans) that participate in the adhesion reaction (Miki et al. 1982a). Our data suggest that yeast cell self-adhesion may compete with adhesion to the hyphae of the fungus (non-self adhesion) -more yeast cell surface used for adhesion to other yeast cells rather than to the fungus and consequently not letting the hyphae grow in the same biocapsule. Thus, small biocapsules resulting from use of flocculant yeasts should proportionally contain less *P. chrysogenum* hyphae. In contrast, cell surface changes associated with biofilm formation are to enable attachment to a wide array of surfaces and confining the population to the surface-air interface. The stronger correlation of biofilm forming capacity to adhesion to the fungus suggests that factors driving cell-cell self-interactions may block interaction with the fungal hyphae.

Biocapsule consistency was shown to be yeast strain dependent (Figure 3e). This parameter also strongly relates with the yeasts' ability to form biofilm, with Pearson coefficients of over 0.7 for the correlations "Biocapsule consistency"-"Biofilm yeast weight" and "Biocapsule consistency"-"Yeast forming biofilm (%)". High biofilm weight yeast showing a low biocapsule consistency (UCD519) indicates that there may be other factors in addition to biofilm weight that influence the consistency of the biocapsules, such as biofilm fragility. Biocapsule consistency is also inversely correlated by the capacity of flocculation. The yeast's competence to form biofilm, allowing it to strongly attach to the fungus hyphae and produce extracellular polymers that facilitate matrix formation, may explain the strong resistance conferred by the biocapsules to withstand the forces applied without breaking. Contrary, yeasts that flocculate congregate with each other and form flocs that can potentially interfere with attachment to the fungus and make less deformable biocapsules.

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Yeast ability to co-adhere with the filamentous fungus *P. chrysogenum* may be due to multiple factors. The biocapsule matrix can be considered a place that entraps yeast cells in co-culture and allows air to be stored in their matrix gaps. This matrix is beneficial for yeast and makes it a suitable environment to oxidatively metabolize the non-fermentable carbon source and gluconic acid when attached to the hyphae. Further, similar to flocculation, the main purpose of yeast-hyphae association could be a community mechanism for survival: the external cells from the floc structure can directly protect the internal cells against harmful environments by physically shielding them. Flocculent yeasts when exposed to several negative conditions such as nutrients starvation, ethanol toxicity, cold-shock, and osmotic stress induce the onset of flocculation (Gibson et al. 2007). Thus, biocapsule formation provide similar protections to floc formation and make it possible for long-term survival of a cellular community of yeast cells in an unfavorable environment. Another hypothetical reason is that yeast and fungus establish a symbiotic relationship because the yeast (which catabolize gluconic acid less easily than the fungus) may obtain gluconic acid subproducts from the fungus that are easier to utilize as a carbon and energy source. Peinado et al. (2006) observed through transmission electron microscopy, within zones of contact with hyphae, vesicular structures are located near the contact zone in the cytoplasm of yeast cells, thus providing a polarized aspect to these cells. Cross-feeding may be a strong selective pressure for the communal association of the cells.

Even both, *S. cerevisiae* and *P. chrysogenum* can utilize gluconic acid as a carbon source and incorporate it to the carbohydrate acid metabolism, *S. cerevisiae* catabolizes it in a later stage. In the case of flor yeasts, it has been observed that gluconic acid in contents lower than 5 g/L is assimilated during the aerobic biological aging process of sherry wines (Cortés et al. 1999) where consumption is initiated after 18 days (Peinado et al. 2003). This delayed time may occur because the protein that catalyzes the phosphorylation, the cytoplasmic putative gluconokinase *YDR248C*, is up-regulated in amino acid starvation (Gasch et al. 2000). Aversely, Schmitz et al. (2013) reported that *P. chrysogenum* has higher

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uptake rates for gluconate than for glucose. Hence, during the biocapsule formation, the first 6 days after the yeast-fungus co-inocculation, *YDR248C* may not yet be present, and yeasts are forced to establish a symbiotic relationship with the fungus to obtain an alternate carbon source from the fungal gluconic acid degradation.

Conclusion

S. cerevisiae immobilization in the filamentous fungus *P. chrysogenum* and the consequent formation of biocapsules was found to depend on the yeast ability to flocculate and aggregate into biofilms. Biofilm forming yeast strains had higher rates of immobilization and formed larger, more consistent biocapsules, while strains able to flocculate formed more abundant biocapsules that were smaller in size and less uniform.

Understanding the relationship of flocculation and biofilm formation with the yeast immobilization in *P. chrysogenum* is not only important in terms of potential industrial application, but also for our understanding of possible evolutionary mechanisms linked to physical interactions between different organisms in shared ecological niches. The way in which mixed species communities control their cell-cell interactions in complex habitats may provide novel insights into ecosystem evolution. Such physical associations can increase the probability of metabolic exchange between cells of different species, and support symbiotic associations as conditioned by the selective pressures of various fermentation environments.

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Table 1 Yeast and fungus strains used in the experiment.

Strain	Strain details	Collection
G1 (positive control)	*Saccharomyces cerevisiae* (Sherry wine (Spain))	UCO
932 (negative control)	*Saccharomyces cerevisiae*	UCD
77	*Saccharomyces cerevisiae* (wine, champagne)	UCD
519	*Saccharomyces cerevisiae* (Sherry wine)	UCD
580	*Saccharomyces cerevisiae* (Sherry wine)	UCD
595	*Saccharomyces cerevisiae* (commercial dry wine yeast)	UCD
661	*Saccharomyces cerevisiae* race *bayanus* (wine, champagne)	UCD
662	*Saccharomyces cerevisiae* race *bayanus* (wine, champagne)	UCD
726	*Saccharomyces cerevisiae* (wine)	UCD
775	*Saccharomyces cerevisiae* (wine, Champagne)	UCD
777	*Saccharomyces cerevisiae* race *bayanus* (commercial wine yeast)	UCD
804	*Saccharomyces cerevisiae* (commercial wine yeast, champagne)	UCD
814	*Saccharomyces cerevisiae* race *bayanus* (commercial wine yeast)	UCD
854	*Saccharomyces cerevisiae* (Ale, England)	UCD
1109	*Saccharomyces cerevisiae* (must)	UCD
1162	*Saccharomyces cerevisiae* (unknown)	UCD
2034	*Saccharomyces cerevisiae* (Commercial yeast)	UCD
2547	*Saccharomyces cerevisiae* (Wine Spain)	UCD
2865 B11 or BA11	*Saccharomyces cerevisiae*	UCD
Original 594 (prise di mousse)	*Saccharomyces cerevisiae*	UCD
H3	*Penicilliun chrysogenum*	UCO

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Table 2 Yeast and biocapsule parameters correlation.

		Floc weight	Non floc yeast weight	Yeasts floculated (%)	Biofilm yeast weight	Non biofilm yeast weight (mg)	Yeasts forming biofilm (%)
Free yeasts (10^6 cells in 150 mL)	Pearson coefficient	-0.1397	0.2148	-0.1749	-0.3451	-0.2548	-0.3298
	p-value	0.4871	0.2820	0.3829	0.0779	0.1996	0.0930
Immobilized yeasts (10^6 cells/mL)	Pearson coefficient	-0.0905	-0.2767	0.2744	0.1185	0.6849	-0.1088
	p-value	0.6534	0.1623	0.1660	0.5561	**0.0001**	0.5890
Yeasts immobilized (%)	Pearson coefficient	-0.0222	-0.0570	0.0717	0.5222	0.4626	0.4387
	p-value	0.9123	0.7775	0.7222	**0.0052**	**0.0151**	**0.0221**
Biocapsule number	Pearson coefficient	-0.2788	-0.4024	0.0601	-0.6190	-0.1988	-0.6501
	p-value	0.1591	**0.0375**	0.7658	**0.0006**	0.3202	**0.0002**
Biocapsule total volume (mL)	Pearson coefficient	0.1646	-0.1870	0.2973	-0.2006	-0.2406	-0.1532
	p-value	0.4119	0.3503	0.1320	0.3157	0.2267	0.4456
Biocapsule diameter (mm)	Pearson coefficient	0.1991	0.4021	-0.0859	0.5613	-0.0764	0.7197
	p-value	0.3193	**0.0376**	0.6701	**0.0023**	0.7048	**0.0000**
Biocapsule consistency	Pearson coefficient	0.0178	0.4274	-0.2290	0.7581	-0.0687	0.9288
	p-value	0.9297	**0.0262**	0.2506	**0.0000**	0.7335	**0.0000**
Biocapsule dry weight	Pearson coefficient	0.3166	0.0412	0.1399	0.6095	0.5249	0.5285
	p-value	0.1076	0.8383	0.4865	**0.0007**	**0.0049**	**0.0046**

*Pearson coefficient and *p*-value were provided for each couple of parameters. Correlations with a *p*-value lower than 0.05 are shown in bold.

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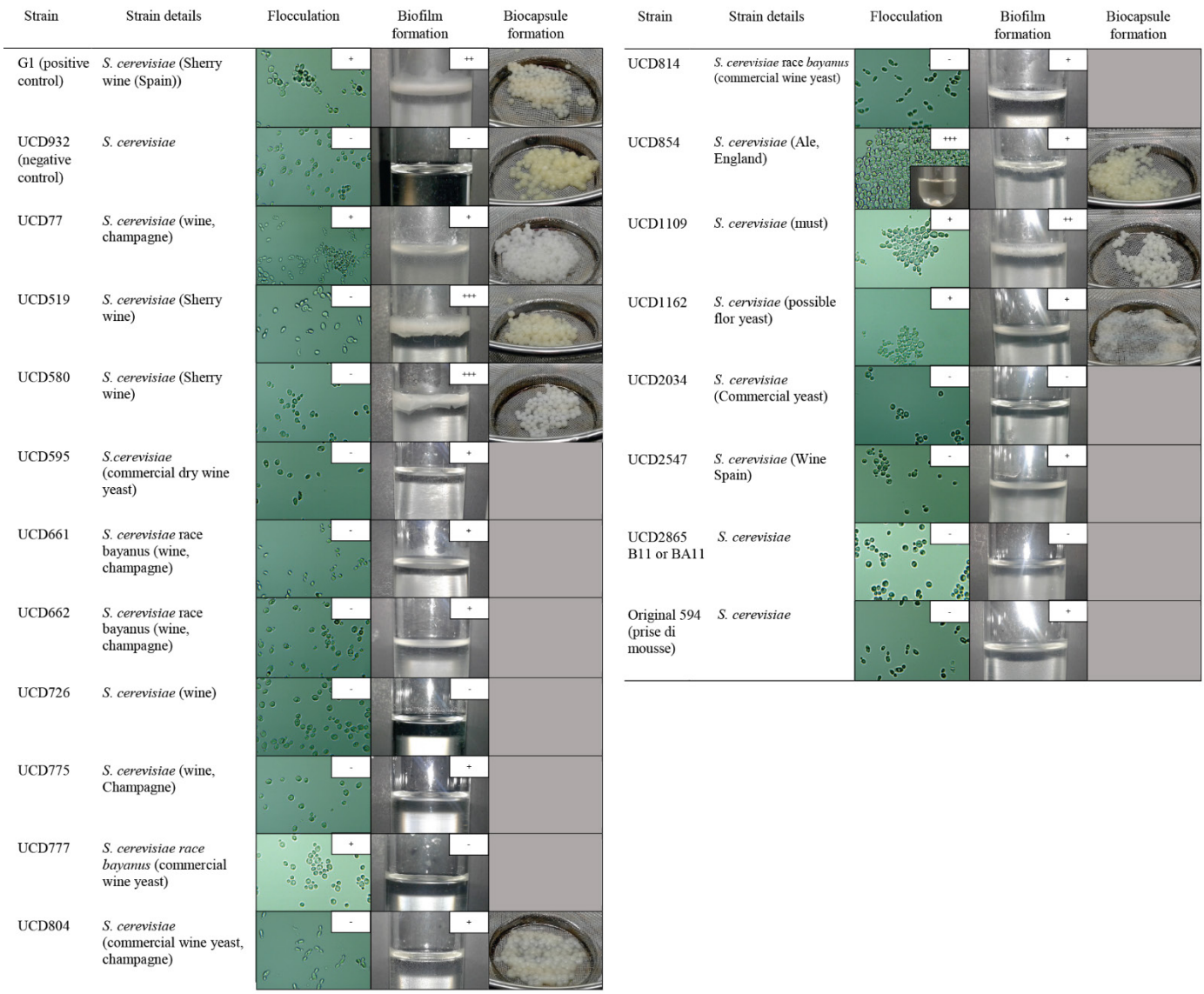

Figure 1 Results from the flocculation, biofilm and biocapsule formation semi-quantitative assays of the yeast strains. Symbols -, +, ++ and +++; indicate the phenotype qualification. Biocapsules were only made with those yeast strains showing different flocculation/biofilm formation patterns.

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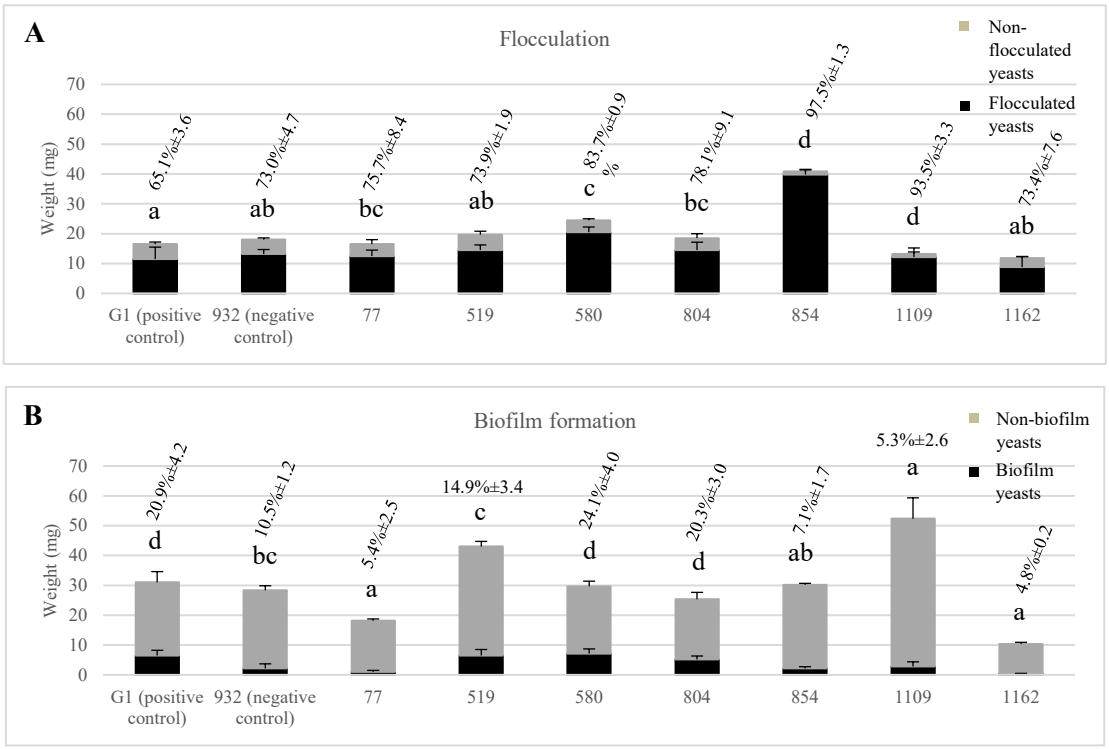

Figure 2 Yeast strains flocculation (**A**) and biofilm formation (**B**). In **A**, black bars represent yeasts flocculated, in **B**, biofilm yeasts. Grey bars represent yeasts non-flocculated and non-biofilm yeasts in **A** and **B**, respectively. ± indicates standard deviations. Different letters indicate different homogeneous groups considering percentages of flocculation and biofilm formation among the strains with significant differences at 0.05 level according to the F-test. The alphabetical order indicates an increasing value.

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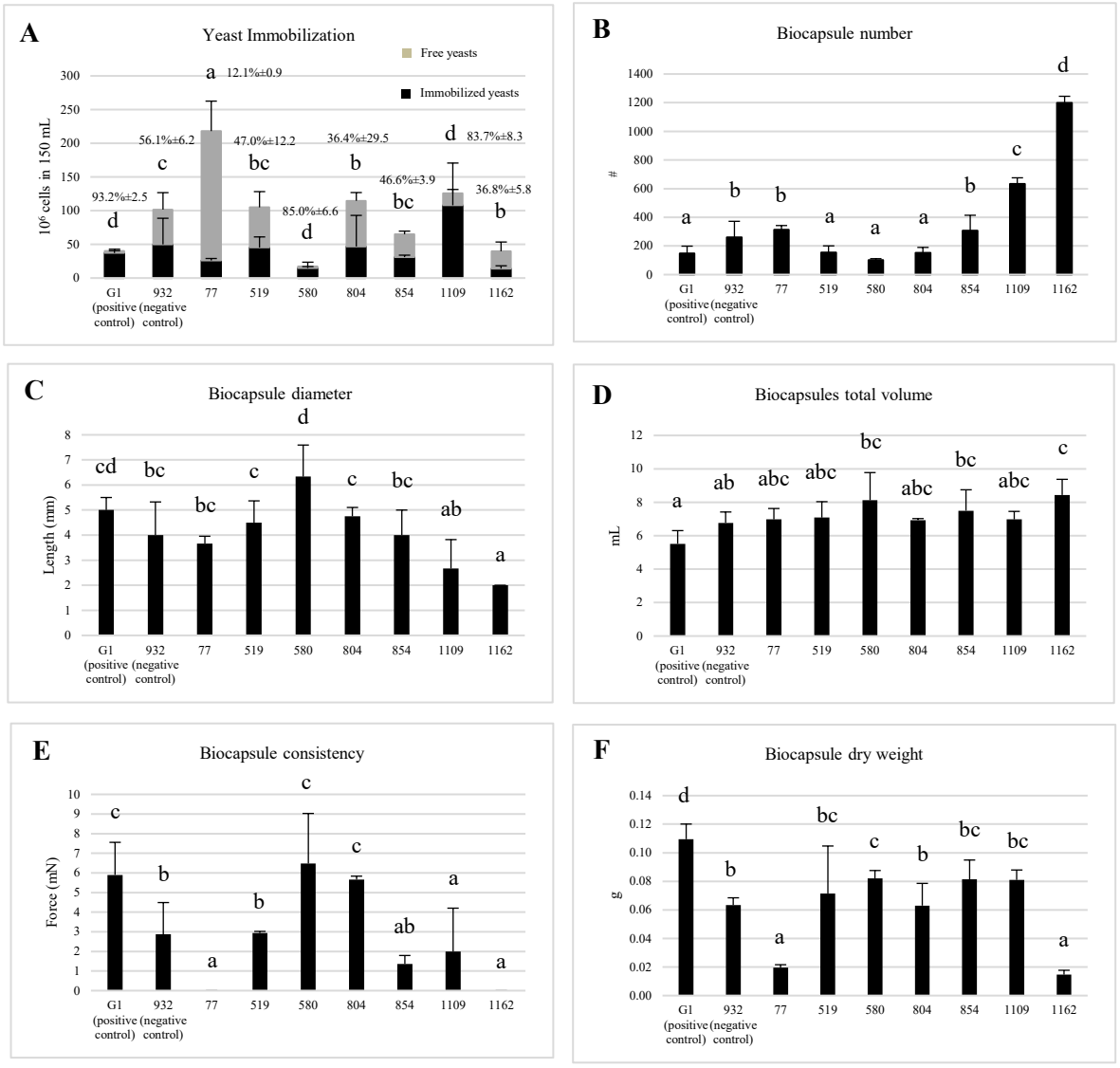

Figure 3 Yeast strains biocapsule related parameters. In **A**, black bars represent yeasts immobilized in biocapsules. Grey bars represent yeasts non-immobilized or free yeasts. In **E**, strains with no values, UCD77 and UCD1162, showed a consistency below the measurable range. ± indicates standard deviations. Different letters indicate different homogeneous groups considering different parameters among the strains with significant differences at 0.05 level according to the F-test. The alphabetical order indicates an increasing value.

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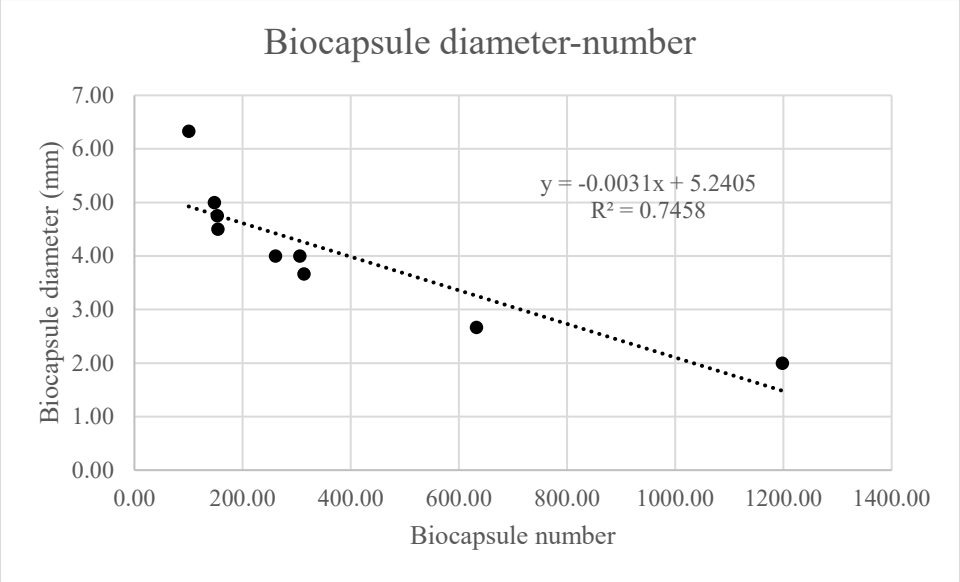

Figure 4 Linear regression among the "biocapsule diameter" and "biocapsule number" parameters.