

A Case-study on the Selection of Promising Functional Starter Strains from Grape Yeasts: A Report by Student of Food Science and Technology Degree, University of Foggia (Southern Italy)

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Abstract

The main aim of this research, performed by some students in Food Science and Technology of Foggia University, is to show how perform the selection of a functional starter through a step-by-step procedure. Fifteen yeast strains were studied in order to assess their biotechnological traits, e.g. catalase, urease, β -glucosidase, pectolytic and xylanolytic activities, production of H₂S, resistance to copper, SO₂ and acetic acid, growth at different temperatures, alkaline pH, in presence of different amounts of ethanol and glucose, and some probiotic properties. After studying these abilities, yeasts were identified through the miniaturized system API 20 C AUX and two kinds of multivariate analyses (Cluster Analysis and Principal Component Analysis) were performed to highlight the best strains.

Keywords: grape, functional starter, yeast, selection

1. Introduction

Yeasts are eukaryotic microorganisms classified in the kingdom of Fungi, with 1,500 species currently described (estimated to be only 1% of all fungal species). Yeasts are unicellular, although some species with yeast forms may become multicellular through the formation of a string of connected budding cells known as *pseudohyphae*, or *falsae hyphae*. Yeast size can vary greatly depending on the species, typically measuring 3-4 μ m in diameter, although some yeasts can reach over 40 μ m. Most yeasts reproduce asexually by mitosis, and many do so by an asymmetric division process called budding (Kurtman & Fell, 2006). Yeasts do not form a single taxonomic or phylogenetic grouping; although the term yeast is often taken as a synonym for *Saccharomyces cerevisiae*, the phylogenetic diversity of yeasts is shown by their placement in two separate phyla: *Ascomycota* and *Basidiomycota*. The budding yeasts ("true yeasts") are classified in the order of *Saccharomycetales* (Kurtman & Fell, 2006).

The most common yeast associated with winemaking is *Saccharomyces cerevisiae* which has been favored due to its predictable and vigorous fermentation capabilities, tolerance of relatively high levels of alcohol and sulfur dioxide as well as its ability to thrive in normal wine pH between 2.8 and 4. Despite its widespread use which often includes deliberate inoculation from cultured stock, *Sacch. cerevisiae* is rarely the only yeast species involved in a fermentation. Grapes brought in from harvest are usually teeming with a variety of "wild yeast" from *Kloeckera* and *Candida* genera (Vincenzini et al., 2006). In addition to *Sacch. cerevisiae*, other species within the *Saccharomyces* genus that may be involved with winemaking include *Sacch. bayanus*, *Sacch. paradoxus*, and *Sacch. pastorianus* (Vincenzini, Romano, & Farris, 2006).

The role of the yeast population on the fermentation process, final chemical composition and sensorial properties of wine has been widely reported (Capece, Pietrafesa, & Romano, 2011). Louis Pasteur had imagined that the taste and properties of the wine could depend on the special nature of yeasts which develop during the fermentation of the grapes (Dubourdiou et al., 2006), thus he highlighted the role of yeasts in alcoholic fermentation. The basic principles involved in alcoholic fermentation are relatively simple: yeasts convert grape sugar into alcohol; however, many other processes can take place as yeast population is complex and variable

(Howell, Cozzolino, Bartowsky, Fleet, & Henschke, 2006).

Recently, the use of functional starter cultures with an industrially or nutritionally important functionality is being explored. Functional starter cultures represent a new frontier for the development of food products, as they show the benefits of traditional starters and have an additional function health- or product-focused. They are preparations that contain live and viable microorganisms and are employed with the aim of using microbial metabolism to achieve specific technical objectives. A particular kind of functional starter cultures are those with probiotic properties (Bevilacqua et al., 2010 & 2012).

The functional traits of a starter could be different, e.g. improvement of aroma, production of bacteriocins, enrichment of food with micronutrients, ability to control the growth of undesirable microorganisms, both spoilage and pathogens, to eliminate ochratoxin from wine, polyphenols from waste, oil from water, oleuropein from olives and purify olive mill waste water.

In this paper 15 oenological yeasts were studied in relation to their technological performances and functional traits, in order to select some promising functional strains for wine-making; a step-by step protocol was used assessing some technological performances e.g. catalase, urease, β -glucosidase, pectolytic and xylanolytic activities, production of H_2S , resistance to copper, SO_2 and acetic acid, growth at different temperatures, alkaline pH, in presence of different amounts of ethanol and glucose, and some probiotic properties.

Table 1. Criteria for yeast taxonomy

Morphological traits	Physiological characteristics
Sexual reproduction	Fermentation of sugar
Mode of conjugation	Assimilation of nitrogen
Vegetative reproduction	Vitamin requirements
Formation of basidiospores	Temperature of growth
Budding	Growth at low water activity
Fission	Resistance to cycloheximide
Microscopic growth	Biochemical traits
Colonies on solid media	Production of acetic acid
Liquid culture	Urease reaction
Size and shape of cells	

2. Method

2.1 Strains

15 yeast strain, isolated from Bombino Bianco and Nero di Troia, two grape varieties of Apulian region (Southern Italy), were studied throughout this research. They were identified through the miniaturized system API 20 C AUX (Biomerieux, Marcy L'Etoile, France) and then maintained on YPD agar (bacteriological peptone, 20 g/l; yeast extract, 10 g/l; glucose, 20 g/l; agar, 15 g/l; the ingredients were purchased from Oxoid, Milan, Italy) at 4°C. Before each assay, yeast were grown in YPD broth, incubated at 30°C for 24 h.

2.2 Catalase Activity

Catalase activity of yeasts was evaluated by adding 3% (v/v) of hydrogen peroxide (Sigma-Aldrich, Milan) as reported by Whittenbury (1964).

2.3 Pectolytic and Xylanolytic Activity

The extracellular pectolytic activity was assessed as reported by Hernandez, Martin, Aranda, Pérez-Nevado and Cordoba (2007); yeasts were streaked onto the surface of YM agar (universal medium for yeasts, medium n. 186: yeast extract, 3.0 g/l; malt extract, 3.0 g/l; peptone from soybeans, 5.0 g/l) (DSMZ, www.dsmz.de) without glucose and supplemented with 12.5 g/l of apple pectins (Sigma-Aldrich) and adjusted to pH 4.0 with HCl 1.0 N. The plates were incubated at 25°C for 7 days.

The ability of yeasts to produce an extracellular xylanase was measured on yeast extract agar (YEA) (Sigma-Aldrich), added with 5 g/l of xylan (Sigma-Aldrich), 5 g/l of bacteriological peptone and 5 g/l of NaCl. After streaking the strains onto the surface of the medium, the plates were incubated at 25°C for 7 days; the

xylanolytic activity was evidenced by a clear halo around the colonies (Hernandez et al., 2007).

2.4 β -glucosidase Activity

This trait was assessed using the method by Caridi, Pulvirenti, Restuccia and Sidari (2005) on a medium containing arbutin (0.5%) (Sigma-Aldrich), yeast extract (1%) and agar (2%). After sterilization, the medium was added with 10 ml/l of a 1% solution of ferric ammonium citrate (C. Erba, Milan); after inoculation of microorganisms, the plates were incubated at 25°C for 7 days. Positive colonies assume a brown colour, more or less pronounced depending on the intensity of activity.

2.5 Urease

This test was performed in Christensen's urea agar (Oxoid), containing phenol red as pH indicator. After yeast inoculum, plates were incubated at 25°C for 2-7 days; colour turning to purple highlighted urea hydrolysis and pH increase (McTaggart et al., 2011).

2.6 Production of H₂S

Biggy Agar (Bismuth glucose glycine yeast agar, Oxoid) was used for the evaluation of yeast ability to produce H₂S. Yeasts were inoculated onto the surface of Biggy agar, then incubated at 25°C for 2-4 days: colour turning to black revealed the production of H₂S (Vincenzini, Romano, & Farris, 2006).

2.7 Resistance to Acetic Acid, Cu and SO₂

Yeasts were inoculated onto the surface of YPD agar, containing acetic acid (0.3%) (J. T. Baker), CuSO₄ (50, 100, 150 ppm) (J. T. Baker) or SO₂ (50, 100 and 150 ppm) (J. T. Baker). Plates were incubated at 25°C for 2-4 days; yeast growth revealed resistance.

2.8 Growth Assays

YPD broth, adjusted to pH 8 and 9, containing glucose (10, 20 or 30% w/v) or ethanol (3, 9 and 15%, v/v), was inoculated with yeasts at 5 log cfu/ml; then, samples were incubated at 15, 25 and 37°C. Aliquots of not-modified YPD broth, inoculated with yeasts and incubated at 25°C were used as controls. Yeast growth was evaluated after 24, 48, and 96 h through absorbance measurement at 600 nm; data were modeled as Growth Index, as reported by Blaszyk and Holley (1998) and Bevilacqua, Perricone, Cannarsi, Corbo and Sinigaglia (2009):

$$GI = (Abs_s / Abs_c) * 100\% \quad (1)$$

where, for each time of analysis Abs_s is the absorbance of the samples and Abs_c the absorbance of positive controls.

2.9 Strain Selection

Strain selection was performed through two different kinds of multivariate approaches: Cluster Analyses and Principal Component Analysis through the software XLSTAT (Addinsoft, Paris, France).

For the statistical analyses, both the results of enzymatic traits and growth assays were converted into qualitative codes (0, 1, 2, and 3). For the enzymatic traits (catalase, pectolytic and xlanolytic activities, β -glucosidase, urease, production of H₂S, resistance to Cu, acetic acid and SO₂), code setting was performed as follows:

0, “-” (negative assay, i.e. absence of the enzymatic trait);

1) “-/+” (variable results, i.e. the same strain in a sample did not possess the trait, while the activity was recovered in another sample containing the same microorganism);

2) “+” (the microorganism possessed the trait but at moderate levels);

3) “++” (strong enzymatic activity).

In addition, the results of growth assays were converted as follows (Bevilacqua et al., 2009):

0, GI < 25% (inhibition);

1) 25% < GI < 50% (growth at moderate-to-low levels)

2) 50% < GI < 75% (growth at moderate-to-high levels)

3) GI > 75% (growth similar to that recovered in the control).

2.10 Resistance to Bile Salts

Bile tolerance was examined in sterile distilled water, containing 0.3% of bile salts (Oxoid) and inoculated at 5 log cfu/ml, then incubated at 25°C for 3 h. The viability of yeast strains was evaluated through spread plate

method on YPD agar, incubated at 25°C for 2-5 days. Aliquots of distilled water inoculated with yeasts but not containing bile salts were used as controls. Data were modelled as follows:

$$V = N_{t_0} - N_{t_3} \quad (2)$$

where N_{t_0} and N_{t_3} were respectively cell counts (log cfu/g) at the beginning and after 3 h in the sample containing bile salts.

2.11 Resistance to Cycloheximide

YPD broth containing 50, 100, 200 and 300 ppm of cycloheximide (Sigma-Aldrich) was inoculated at 5 log cfu/ml of each strain (Bolcato, Spettoli, & Dal Belin Peruffo, 1974). Then, samples were incubated at 25°C and yeast growth was evaluated after 24, 48 and 72 h through absorbance measurement at 600 nm.

Aliquots of YPD broth, inoculated with yeasts but not containing the antibiotic, were used as controls. Data were modeled as Growth Index.

2.12 Statistical Analysis

The experiments were performed in duplicate over two different batches; data were analyzed through One-Way Analysis of Variance and Tukey's test ($P < 0.05$).

3. Results and Discussion

3.1 Identification and Strain Selection

As the first result of this research, Table 2 shows strain identification through phenotyping; yeasts were identified as *Kloeckera* spp., *Candida* spp. and *Hansenula polymorpha*. Then, data of technological characterization, were used to perform a multivariate analysis and group yeast, in order to point out similarities/dissimilarities.

Cluster analysis divided yeasts in 4 different groups and this kind of division confirmed phenotypical identification; namely the group I includes the two strains identified as *H. polymorpha*, while the group IV, characterized by a kind of heterogeneity inside, includes 4 strains identified as *Kloeckera* spp. The group II and III were quite heterogeneous as they included *Candida* spp. and *Kloeckera* spp (Figure 1A).

Concerning PCA, the percentage of variability is quite low (ca. 54%), thus suggesting a kind of homogeneity inside data; however, it pointed out that none of the studied strains showed all the traits at the optimal level, thus the selection of a suitable starter is a kind of risk/benefit analysis. Statistic pointed out some promising and interesting strains: the strain 13, which showed all classical oenological traits at good levels (growth in presence of EtOH; production of SO₂), but unfortunately it produced high amounts of H₂S and acetic acid; a second interesting group includes some osmo-tolerant strains, able to grow with 20% of glucose added (strains 1, 2 and 18). Finally in the left side of the Figure there are some strains resistant to 150 ppm of Cu and able to grow at 37°C (for example the strain 11) (Figure 1B).

Table 2. Phenotypic identification

Strain	Identification
1	<i>Candida sphaerica</i>
2	<i>C. sphaerica</i>
3	<i>C. famata</i>
5	<i>Candida</i> spp.
9	<i>Kloeckera</i> spp.
10	<i>Kloeckera</i> spp.
11	<i>Kloeckera</i> spp.
12	<i>Kloeckera</i> spp.
13	<i>Hansenula polymorpha</i>
14	<i>Kloeckera</i> spp.
15	<i>Kloeckera</i> spp.
16	<i>Kloeckera</i> spp.
17	<i>Kloeckera</i> spp.
18	<i>Candida</i> spp.
19	<i>Hansenula polymorpha</i>

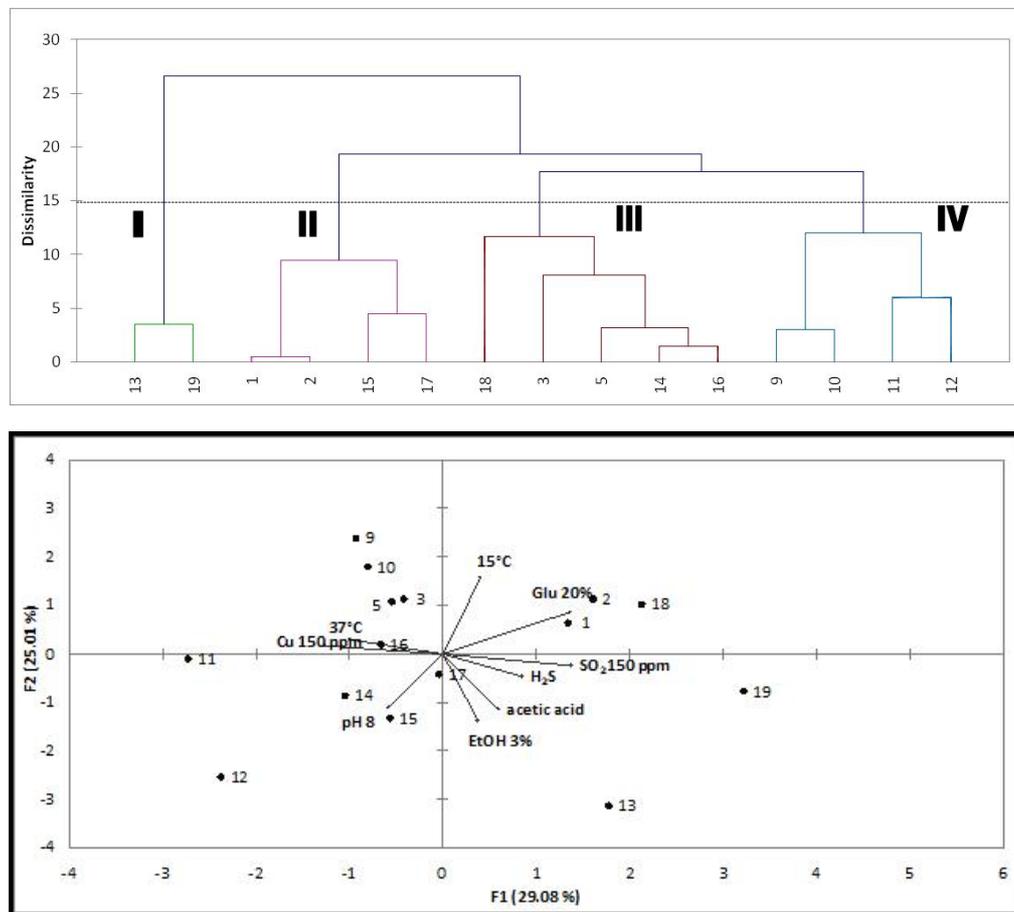


Figure 1. Cluster analysis and principal component analysis for yeast strains

3.2 Enzymatic Traits

Yeasts showed significant pectolytic and catalase activity as typical of wine starters (Figure 2). All the strains except for one isolate (strain 14) had catalase activity (moderate-code 2- or high- code 3). High levels of pectolytic activity were detected and only 4 strains showed a moderate activity; on the other hand, xylanolytic activity was negative for all the strains.

Pectolytic and xylanolytic activity could be related to the possibility of degrading grape skin and promote the extraction of pigments (Vincenzini et al., 2006); if this trait is beneficial or detrimental relies upon the kind of wine to be produced (white or red). Generally, it has been referred as good for the production of red wines, where producers and consumers required a strong colour.

Another topic of great importance for yeasts intended as starters is the β -glucosidase activity, here assessed through the ability to degrade arbutin; Figure 2 shows that the most of the strains did not possess an active metabolism of this compound, except for a single isolate (the strain 3). Terpenic compounds are responsible for the varietal aroma of many grape varieties. Normally these compounds are synthesized as non volatile-glycosides since the better solubility of these derivatives in aqueous solution facilitates their translocation in plant tissues. Glycosides of terpenes are less volatiles than aglycones; thus the hydrolysis of the sugar group to release the volatile aglycones increase the aromatic nature of wine (Suárez-Lepe & Morata, 2012). β -glucosidase is an activity of some strains of *Saccharomyces* (Hernández, Espinosa, Fernandez-Gonzalez, & Briones, 2003), as well as recovered in some non-*Saccharomyces* strains (Mendes-Ferreira, Climaco, & Mendes Faia, 2001). Yeasts from grape skin with this activity are of interest, as they act as a powerful mean to increase varietal aroma, thus the strain 3 could be a promising isolate for this trait.

Concerning the production of H_2S , the qualitative assay (Figure 2) highlighted 4 different situations: 1) yeasts did not grow or show white colonies (no production of H_2S); 2) colonies from yellow to black (low production of H_2S); 3) brown colonies (moderate production of H_2S); 4) dark or black colonies (strong production of H_2S).

Thus this qualitative responses were converted into codes (from 0 to 3), as reported in Figure 1: 7 strains did not produce H₂S, while the remaining 8 isolates did it; in particular 5 strains produced moderate amount of this compound and 3 showed a strong production.

Sulphur metabolism leads to the production of many volatile compounds with negative organoleptic impacts; these compounds, namely hydrogen sulphide, have descriptors of cabbage, garlic, onion, rotten eggs (Suárez-Lepe & Morata, 2012). Thus, the selection of yeasts with a low propensity to produce H₂S can minimize the production of off-flavours and preserve wine quality.

The last enzymatic trait assessed in this research was the urease activity; only 4 strains showed a significant level for this ability (the strains 13 and 19-code 2- and 3 and 18-code 3) (Figure 2).

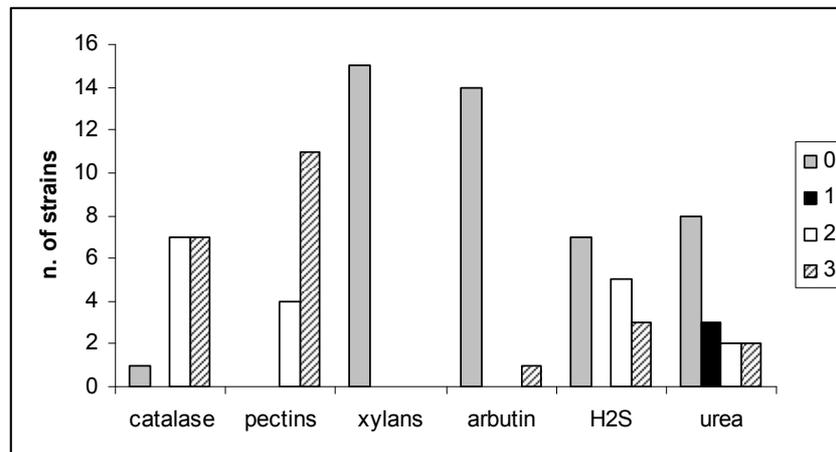


Figure 2. Enzymatic traits. 0, negative assay (“-”); 1, variable results (“-/+”); 2, moderate level of enzymatic trait (“+”); 3, strong enzymatic activity (“++”)

3.2 Resistance to SO₂, Cu, and Acetic Acid

Figure 3A shows the results of strain resistance towards SO₂. Yeasts were resistant to 50 ppm of SO₂ and the number of strains able to grow decreased with increasing the concentration of this antimicrobial, thus at the highest concentration only 4 strains were positive to this assay.

As expected, Cu exerted an antimicrobial effect and its bioactivity relied upon its concentration; however, some strains were resistant also at the highest concentration used in the lab medium (150 ppm), namely 7 strains (codes 2 and 3) (Figure 3B).

Concerning resistance to acetic acid, the most of yeast population was able to grow (11 strains, codes 2 and 3) and only 4 strains did not grow (code 0) or showed a stunted growth (code 1) (Figure 4).

Generally *Saccharomyces cerevisiae* is more resistant to SO₂ than yeasts present in the first stages of fermentation (*Pichia*, *Candida*, *Hansenula*); however, a wide range of variability exists amongst wine yeasts, being some strains inhibited at 50 ppm and other more resistant (Suárez-Lepe and Morata, 2012; Ubeda, Briones, Izquierdo, & Palop, 1995). Our data confirmed literature reports and highlighted that some non-*Saccharomyces* strains are very resistant to this preservatives.

Copper resistance is another trait for yeast selection, as a high level of Cu in must could be the result of agronomic practices (Vincenzini et al., 2006); thus, a starter should be resistant to this compound. The strains under investigation could be easily divided into two different groups, i.e. the sensitive and resistant strains, thus suggesting that a starter could be selected within the second group.

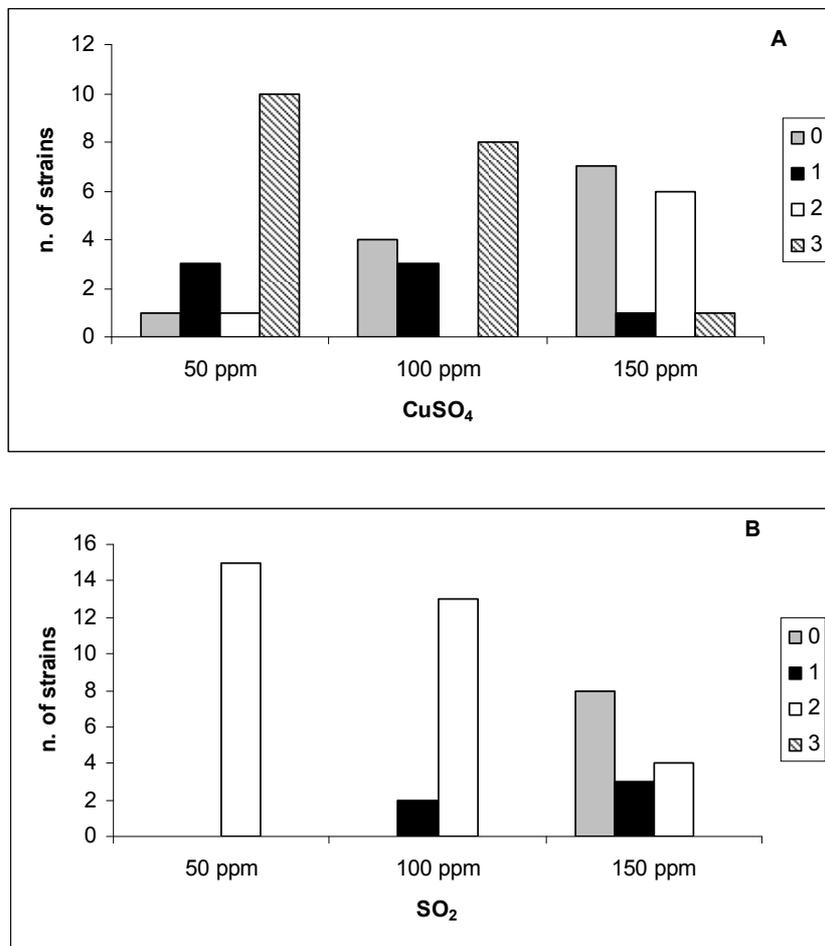


Figure 3. Resistance to SO₂ (A) and Cu (B): frequency histograms. 0, no growth (“-“); 1, variable growth (“-/+”); 2, moderate growth (“+”); 3, enhanced growth (“++”)

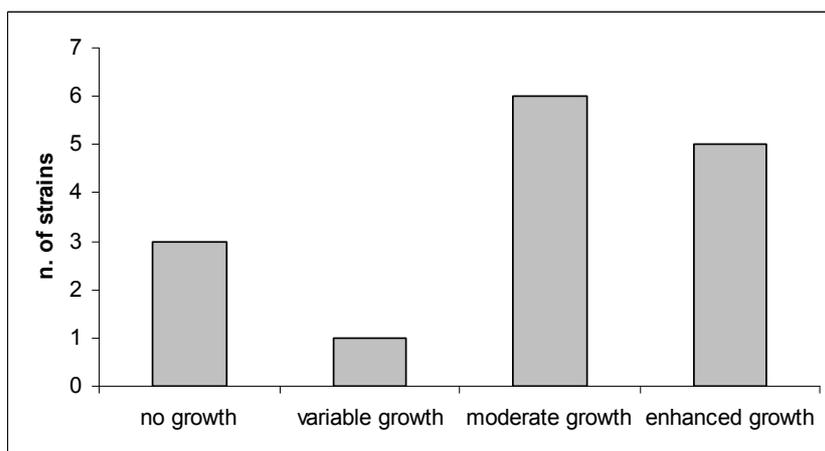


Figure 4. Growth on YPD agar containing acetic acid (0.3%): frequency histogram. no growth (“-“, code 0); variable growth (“-/+”, code 1); moderate growth (“+”, code 2); 3, enhanced growth (“++”, code 3)

3.3 Growth Assays

Other traits for technological characterization are the ability of growing at low temperatures (15°C) for a controlled fermentation and osmotolerance (Suárez-Lepe & Morata, 2012), whereas the growth at 37°C shows a taxonomic value (Bevilacqua et al., 2009).

Yeasts were able to grow at 15°C, although some strains were inhibited in strong or a moderate way (codes 1 and 2); otherwise some strains did not grow as well at 37°C (8 strains) (Figure 5A). Concerning pH, the most of yeast population did not grow at pH 9.0, whereas we recovered growth at pH 8, although some strains were significantly inhibited (codes 1 and 2) (Figure 5A).

Glucose exerted a kind of effect both at 10% and 20%; at the lowest concentration a strain was inhibited in a significant way ($25\% < GI < 50\%$) and 7 moderately ($50\% < GI < 75\%$). Increasing the concentration of glucose, yeast delay was more pronounced, as only 1 strain after 24 h showed a growth similar to the control.

The lowest amount of ethanol exerted a partial (GI of 50-70%) or a strong effect (GI of 25-50%) on yeasts after 24 h, as only 4 strains showed a GI similar to the control ($GI > 75\%$); in addition, higher amounts of ethanol inhibited all the strains (Figure 5B).

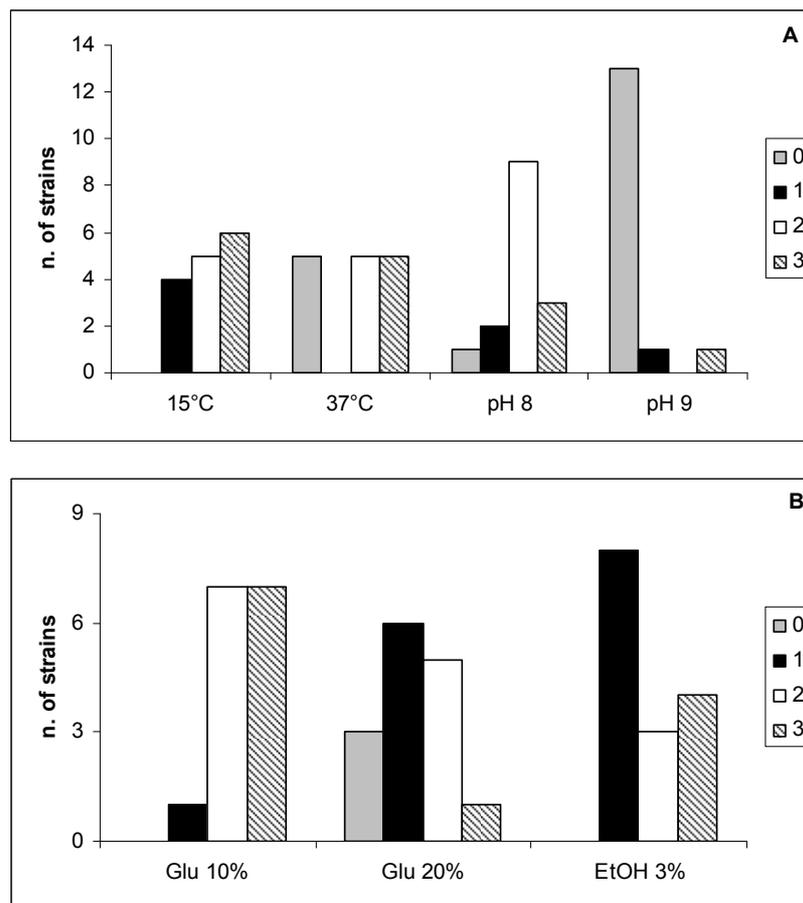


Figure 5. Growth after 24 h of yeasts 37°C and at pH 8/9, growth at 15°C after 96 h (A) and growth after 24 h with glucose and ethanol added (B). Frequency histogram. 0, Growth Index (GI), $< 25\%$; 1, $25\% < GI < 50\%$; 2, $50\% < GI < 75\%$; 3, $GI > 75\%$

Oenological yeasts are microorganisms adapted to very demanding nutritional conditions (Suárez-Lepe & Morata, 2012); they are normally able to use sugars up to critical level of 22-24%. Generally, data for growth with glucose partially confirmed literature reports as the concentration of 20% is a critical break-point, as some strains appeared inhibited (strongly or in a moderate way).

3.4 Some Probiotic Traits

Different probiotic traits should be assessed; in this paper, as an example, we focused only on the survival at 0.3% of bile salts and on the resistance to cycloheximide, as a representative compound for antibiotics.

Cycloheximide inhibits protein biosynthesis in yeasts and its toxicity relies upon its concentration and yeast species; its effect was strongly strain-dependent, as the strain 1, 2, 3, 5 and 19 appeared completely inhibited also at the lowest amount (50 ppm), as well as a strong effect was recovered for the strains 9 and 15, that experienced a GI of ca. 20%. The other strains showed a variable sensitivity; generally they exhibited a higher GI value at the lowest concentration of the antibiotic, then this parameter decreased at 300 ppm (Figure 6).

Concerning bile salts, only three strains (10, 11 and 18) were highly sensitive, as they were reduced below the detection limit; the other isolates appeared to be resistant (Figure 7). Other probiotic traits should be assessed, like survival in simulated gastro-intestinal conditions, adhesion to intestinal mucosa, functional effects, antimicrobial activity, antibiotic resistance (van der Aa Kühle, Skovgaard, & Jespersen, 2005).

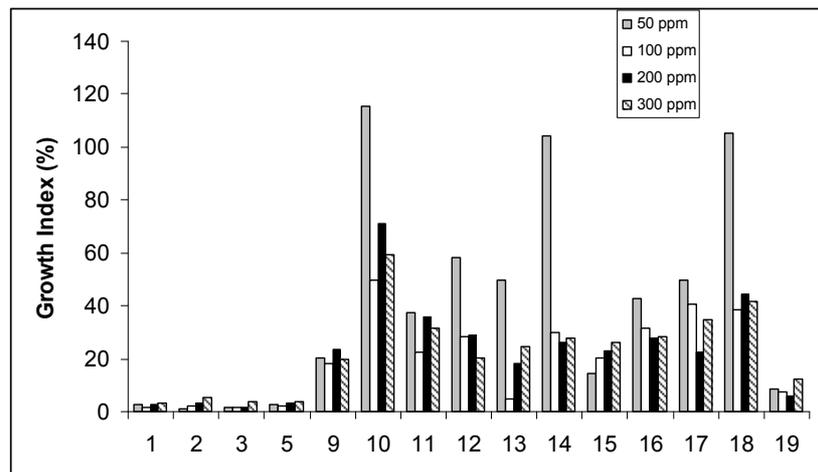


Figure 6. Growth Index at 24 h of yeasts in YPD broth containing different amounts of cycloheximide (from 50 to 300 ppm). Data are the mean of two replicates

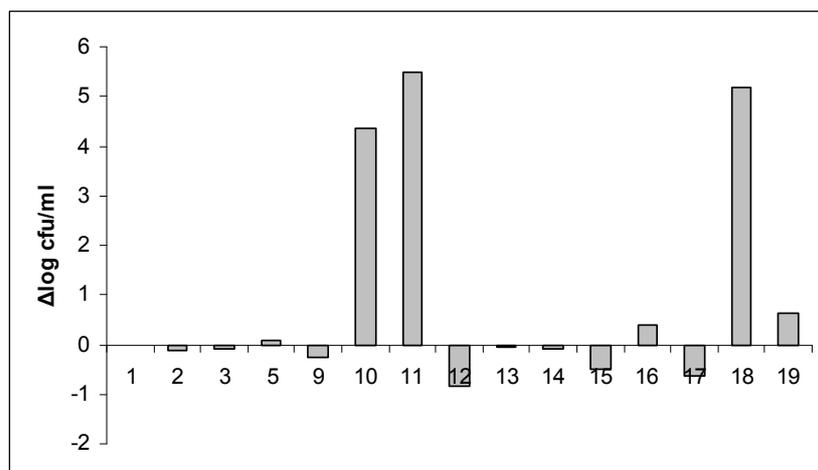


Figure 7. Decrease of cell counts of yeast after 3 h in distilled water containing 0.3% of bile salts. Data are the mean of two replicates

4. Conclusions

Starter selection is a complex process, as it involves many steps; many times this is a never-ending process. It's a

common and well-accepted idea that starter selection should begin with a wide bouquet of strains (200-300) and then continue with some intermediate selection steps and obtain at the end of the process 2-5 promising strains for the validation in food. This research is only a case study, showing how to select a starter with functional trait.

Our starting question was the following: how to select a functional starter? An easy way could be the overlapping of multivariate analysis and the results of the probiotic traits; as an example, we can report the strain 13, with some interesting technological traits, partially resistant to cycloheximide and able to survive in presence of bile salts.

5. Note by Supervisor

Biotechnology of Functional Starter is a class for MSc and Bachelor students of Food Technology degree (University of Foggia). In the past students attended some other classes on Food Microbiology and Microbiology of Food Fermentation, as well as many lessons on lactic acid bacteria. Therefore, I decided to focus on yeasts and on their impact as functional starter cultures and organize this class without lessons but in lab, taking into account that "learning by doing" is a basic rule for future food technologists.

Generally, each meeting was organized as follows:

- (1). a brief introduction on the impact and importance of the particular technique or protocol under investigation;
- (2). the experiment (students were responsible for media preparation, result interpretation and data modeling)
- (3). a final brain-storming on the protocol, as well as a discussion on how to model and write in a clear way the results of the experiments.

The last meeting of the class was used to plan the present research paper; then, students were divided into some groups, each of them involved in writing a part (Introduction, Materials and Methods, Results and Discussion). The present paper is the result of their "learning by doing", after a critical review and correction.

Antonio Bevilacqua: student supervisor-researcher;

Francesco Pio Casanova: PhD student;

Ersilia Arace, Salvatore Augello, Rosangela Carfagna, Annamaria Cedola, Susanna Delli Carri, Fabio De Stefano, Grazia Di Maggio, Valeria Marinelli, Arcangela Mazzeo, Angela Racioppo: students;

Maria Rosaria Corbo: Associate Professor of Food Microbiology;

Milena Sinigaglia: Full Professor of Microbiology.

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