**Application of non-conventional yeasts to improve the quality of innovative tropical fruit beverages**

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The present PhD thesis dealt with innovations in technology of production of tropical fruit beverages. In the first part of the PhD programme, ecological niches associated with sugar-rich sources such as manna and fermented honey by-products were investigated. The high sugar content (about 80% w/w) makes these matrices extremely selective for microorganisms with potential food applications. Yeast strains have been isolated, characterized and applied as starter and co-starter cultures in fruit beer production. To this purpose, *Eriobotrya japonica* fruits were used. The improvement of physicochemical and sensory quality of the final product have been evaluated.

**Applicazione di lieviti non convenzionali per migliorare la qualità di bevande innovative a base di frutta tropicale**

La presente tesi di dottorato si è occupata dello sviluppo tecnologico di bevande innovative a base di frutta tropicale. Nella prima parte del dottorato sono state studiate le nicchie ecologiche associate alle fonti altamente zuccherine rappresentate dalla manna e dai sottoprodotti del miele fermentato. L'elevato contenuto di zuccheri (circa l'80% in peso) rende queste matrici estremamente selettive per i microrganismi con potenziali applicazioni alimentari. I ceppi di lievito sono stati isolati, caratterizzati e applicati come colture starter e co-starter nella produzione di birra alla frutta. A questo scopo sono stati utilizzati i frutti di *Eriobotrya japonica*. È stato valutato il miglioramento della qualità fisico-chimica e sensoriale del prodotto finale.

**Key words**: Alcoholic fermentation; *Eriobotrya japonica*; non-*Saccharomyces* yeast; *Saccharomyces cerevisiae*; sugar-rich matrix.

**1. Introduction**

In accordance with the PhD thesis project summarized above, the present oral communication reports the main results of the following five activities:

(A1) *in vitro* evaluation of *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* strains,isolated from sugar-rich matrices, such as honey and manna ash, for producing experimental fruit beers. The strains were selected as a result of a preliminary investigation (e.g. ethanol resistance, H2S production, hop resistance or consumption of sugars);

(A2) technological screening to evaluate the beer wort fermentation capacity of *Lachancea thermotolerans* strains isolated from manna ash;

(A3) application of *S. cerevisiae* and *L. thermotolerans* strains under medium scale level conditions to several experimental beer productions including addition of loquat, mango and blackberry fruits;

(A4) microbiological analysis and determination of the main physicochemical parameters of samples collected during the different stages of beer production. Finally, volatile organic compounds (VOCs) and sensory analyses of the fruit beers produced were carried out to evaluate the effect of yeast strains applied.

**2. Microbiological study on sugar-rich matrices**

The increasing interest in novel beer productions focused on non-*Saccharomyces* yeasts in order to pursue their potential in generating groundbreaking sensory profiles. In fact, fermenting yeasts mostly influence beer production. These agents have a direct effect on flavour and quality of the final beers (Larroque et al., 2021). This research focused on the selection of yeasts from sugar-rich matrices with the aim to select new yeast strains capable of producing innovative fermented alcoholic beverages. In particular, the ecological niches associated with highly sugary sources were investigated. In particular, manna, the sugar product obtained from the solidification of processed sap from different *Fraxinus* sp. (Schicchi et al., 2007), and honey by-products used to process a highly alcoholic beverage (Guarcello et al., 2019; Matraxia et al., 2021) were the two sources object of investigation. Both sources, due to the high sugar content, host osmophilic microorganisms. The study resulted in the isolation of several yeast species showing useful characteristics to act as starters or co-starters in food applications such as fruit beer production.

**3. Preliminary study and technological screening of selected yeast strains**

The yeasts present in fermented honey by-products were identified as *S. cerevisiae*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *H. uvarum* (Matraxia et al., 2021). Manna ash hosted the following species: *Candida aaseri*, *Candida lactis-condensi*, *Citeromyces matritensis*, *L. thermotolerans*, *S. cerevisiae* and *Zygosaccharomyces bailii* (Guarcello et al., 2019) with *L. thermotolerans* being the most represented species. The study was firstly focused on *H. uvarum* YGA 34 and *S. cerevisiae* MN113, isolated from honey by-products and manna ash, respectively. They were applied for preliminary experimental trials after being evaluated and selected for ethanol resistance, H2S production, hop resistance or consumption of major sugars. Subsequently, fifteen strains of *L. thermotolerans*, all isolated from manna ash, were examined for their ability to produce light sour beer. Interestingly, all *L. thermotolerans* strains showed growth in presence of ethanol and hops and evidenced excellent beer wort fermentation performances. The strain showing the most significant brewing aptitude was selected for further experimental applications.

**4. Materials and Methods**

**4.1 Assays in synthetic beer wort**

A craft beer was produced with the addition of loquat (*Eriobotrya japonica* Lindl) juice extracted from local cultivars. The strains *S. cerevisiae* MN113 and *H. uvarum* YGA34 in combination with *S. cerevisiae* MN113 and US-05 strains, were investigated in this study. All trials were fermented in synthetic beer wort. At the end of alcoholic fermentation (AF), an amount of loquat juice was added to the must up to 20 % (v/v) of total volume. A wort medium designed for fermentations was used and prepared according to the wort composition reported by Larroque et al. (2021). Experimental fermented beers were produced at laboratory-scale level (0.75 L sterilised batch). Loquat fruits were harvested at mature stage from a commercial orchard in Sicily. Fruit juice was extracted, filtered and immediately frozen at -20 °C (Tarantino et al., 2021). Loquat juice was added to all trials at the end of AF (day 10) as reported by Gasiński et al. (2020). All trials were inoculated with approximately 2.0 × 106 cells/mL of each yeast strain. The fermentation was carried out at 18 °C under static condition. Planned analysis: microbiological counts; determination of physicochemical parameters (as sugars, main acids and glycerol); determination of VOCs and sensory analysis. The experimental plan included four trials: T1, inoculated with *S. cerevisiae* US-05, used as control; T2, inoculated with *S. cerevisiae* MN113; T3, inoculated sequentially with strain YGA34 and, after 48 h, with strain MN113; T4, inoculated sequentially with strain YGA34 and after 48 h with strain US-05.

**4.2 Application of yeast strains in craft beer productions**

Unconventional yeasts and loquat juice were added to a wort produced under medium scale level conditions in order to assess the industrial scale-up of the process. Yeast strains used in this study were *S. cerevisiae* MN113 and *L. thermotolerans* MNF105, both isolated from manna ash and selected for their optimal characteristics after preliminary studies. The wort was made using only Pilsen malt (BestMalz, Heidelberg, Germany) to better understand the effect of yeast inoculum. Experimental top-fermented beers were produced at a medium-scale level (5 L batch) using four different inocula. Loquat juice was squeezed from fruits of the white-fleshed local cultivar “Claudia” (*Eriobotrya japonica* Lindl) following the procedure reported at the previous paragraph. As reported by Gasiński et al. (2020), 20 % (v/v) loquat juice was added to all trials at the end of AF (day 10). Samples were collected at different stages of beer production: 0, 3, 6, 10, 11 and 16 days. The fermentation was carried out at 18 °C under static condition. Planned analysis: microbiological counts; determination of physicochemical parameters (as sugars, main acids, glycerol and alcohol); determination of volatile organic compounds and sensory analysis (aroma and taste). Experimental design: TF1, inoculated with *S. cerevisiae* MN113; TF2, inoculated with commercial *S. cerevisiae* US-05, used as a control; TF3, inoculated with *L. thermotolerans* MNF105; TF4, inoculated with commercial strain of *L. thermotolerans* Philly sour (Lallemand brewing), used as a control.

**5.** **Results**

**5.1 Experimental study with synthetic beer wort**

The initial wort had a pH value of 3.00 and 9.70 °Bx, whereas loquat juice was characterized by pH 3.75 and 11 °Bx. The values of pH registered at the end of AF ranged between 3.28 and 3.55. Interestingly, *S. cerevisiae* MN113 in T2 trial showed a more rapid sugar consumption kinetics than control strain *S. cerevisiae* US-05 in T1, including maltose, after 2 days of fermentation. This strain showed an excellent ability to consume sugars in short time. After the sequential inoculum, the concentration of maltose decreased. In fact, at the consecutive sampling day, MN113 together with YGA34 (T3) consumed sugars faster than YGA34 with US-05 (T4). All strains showed their levels in the range 5.0 – 8.0 Log cycles during fermentation. The absence of off-odours and the improvement of aromatic perception were observed in experimental trials involving the use of *S. cerevisiae* MN113 as a monoculture and in sequential inoculum with *H. uvarum* YGA34. Esters and alcohols were the most abundant compounds emitted from the beers. The beers produced with sequential inoculation of *H. uvarum* YGA34 and *S. cerevisiae* MN113 or US-05 were characterised by higher ester and lower alcohol concentrations. These two unconventional yeast strains, isolated from sugar-rich matrices, showed great technological properties, representing promising co-starters and starter during craft fruit beer production.

**Figure 1** *Monitoring of yeast concentrations during AF. Beer fermented by strains: US-05 [T1]; MN113 [T2]; sequential inoculum with YGA34 and MN113 [T3]; sequential inoculum with YGA34 and US-05 [T4]. Different superscript letters indicate significant differences on microbial concentrations were performed at each sampling time according to Tukey's test for P < 0.05. Abbreviations: WL, Wallerstein nutrient agar for yeasts; LA, Lysine Agar for non-Saccharomyces group.*



**5.2 Technology screening of *Lachancea thermotolerans* strains from manna ash**

The results obtained showed that all strains were able to consume the main sugars present in the wort, low production of hydrogen sulphide. However, but not strains were able to resist to the different levels of ethanol.

**Table 1** *Results of fermentation capacity of selected yeast strains.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|   | **Strain code** | **H2S Productiona** | **Growth on LA** | **Growth on WLD** | **Ethanol tolerance 5/10 % (v/v)** | **Sugar fermentation** |
| **Maltose**  | **Glucose**  | **Fructose** |
| ***Lachancea thermotolerans*** | **MN28** | 0 | + | - | 10 | + | + | + |
| **MN136** | 0 | + | - | 5 | + | + | + |
| **MN93** | 0 | + | - | 10 | + | + | + |
| **MN400** | 0 | + | - | 10 | + | + | + |
| **MNF104** | 0 | + | - | 5 | + | + | + |
| **MNF105** | 0 | + | - | 10 | + | + | + |
| **YS186** | 0 | + | - | 5 | + | + | + |
| **YS1** | 0 | + | - | 5 | + | + | + |
| **YS42** | 0 | + | - | 5 | + | + | + |
| **YS45** | 0 | + | - | 5 | + | + | + |
| **YS55** | 0 | + | - | 5 | + | + | + |
| **XV11** | 0 | + | - | nd | + | + | + |
| **XV22** | 0 | + | - | 5 | + | + | + |
| **XV34** | 0 | + | - | 5 | + | + | + |
| **XV47** | 0 | + | - | 10 | + | + | + |

Symbols: +, positive growth; -, no growth; +/-, weak growth. Abbreviation: H2S, hydrogen sulfide.

aColor of colony on Biggy agar plates: 0 = white; 1 = beige; 2 = light brown; 3 = brown; 4 = dark brown; 5 = black.

Subsequently, yeast strains resistant to 10 % (v/v) ethanol were examined and subjected to further analyses including flocculation, hop and ethanol resistances, fermentation activity (fermentation rate and power, lactic and acetic acid production) during micro-fermentation. The strain MNF105 showed the best technological performances for brewing application.

**Table 2** R*esults of hop resistance and cross resistance (hop and ethanol) of the yeast strain studied.*

|  |  |  |
| --- | --- | --- |
|  | **Hop resistance** | **Cross resistance (hop and ethanol)** |
| **Strain code** | 0 IBU | 25 IBU | 50 IBU | 90 IBU | 0 IBU/5% | 25 IBU/5% | 50 IBU/5% | 90 IBU/5% |
| **MN28** | + | + | + | - | + | + | + | - |
| **MN93** | + | + | + | + | + | + | + | + |
| **MN400** | + | + | + | - | + | + | + | - |
| **MNF105** | + | + | + | + | + | + | + | + |
| **XV47** | + | + | + | + | + | + | + | + |

Symbols: +, positive growth; -, no growth; +/-, weak growth. Abbreviations: IBU, International Bitterness Unit.

**5.3 Application of yeast strains to experimental real beer productions**

The initial must showed a pH value of 5.25 and 12 °Bx, whereas the loquat juice was characterized by pH 3.65 and 10.9 °Bx. The pH values recorded at the end of AF were between 3.78 and 3.44 for TF3 and TF4 trials, highlighting the ability of *Lachancea* to acidify the must. As a result, *L. thermotolerans* MNF105 yeast strain showed a low lactic acid production and a marginal influence on the decrease of pH compared to the commercial strain (0.52 g/L and 2.25 g/L respectively). The evolution of yeast populations during the AF is reported in Fig. 2. After inoculation, yeast cell densities ranged between 6.20 and 6.95 Log CFU/mL. The persistence of the strains inoculated was phenotypically investigated by means of colony shape and cellular morphology to recognize typical members of *Lachancea* and *Saccharomyces* genera (Iris et al., 2020). Starter yeast levels increased about 0.5 Log cycles after 3 d for all trials and these results follow the general dynamics of yeast growth in fermenting must-beer. At day 3, trials TF3 and TF4 showed a decrease of presumptive *Lachancea* spp. populations. After loquat juice addition (day 11 of AF), yeast populations levels increased for all trials. At the end of the AF, the highest cell counts were registered for *S. cerevisiae* MN113 in trial TF1 (6.80 Log CFU/mL). Instead, *L. thermotolerans* MNF105 in trial TF3 (6.05 Log CFU/mL) showed values higher than those observed for control trial (5.80 Log CFU/mL).

**Figure 2** *Monitoring of yeast concentrations during AF. Beer fermented by: S. cerevisiae MN113 (TF1); S. cerevisiae US05 (TF2); L. thermotolerans MNF105 (TF3); L. thermotolerans Philly sour (TF4).*



The overall organoleptic investigation showed a preference for *S. cerevisiae* MN113 (TF1) (values of 5.65 for aroma and 5.56 for taste). Experimental trials conducted with the selected strains demonstrated the absence of off-odour and off-flavour and an improved aroma perception. In addition, beers produced with *L. thermotolerans* MNF105 were more balanced than controls, especially in terms of perceived acidity during sensory analysis (values of 5.60 and 6.38, respectively). This could be due to the lower lactic acid production (0.49 g/L) compared to the control trials (1.74 g/L). Beers produced with *S. cerevisiae* MN113 were characterized by the highest concentrations of alcohols, ketones and carboxylic acids (100.63 ppm, 0.78 ppm and 1.91 ppm). In particular, ethyl acetate, a secondary metabolite of AF responsible for the fruity aroma, was also at high levels (1.27 ppm). Interestingly, the trials inoculated with *Lachancea* strains (TF3 and TF4) showed the highest ethyl lactate content (0.69 ppm and 2.06 ppm, respectively), a compound produced by this species. These results show that novel yeast strains from unconventional matrices, both *Saccharomyces* and non-*Saccharomyces*, could increase flavour complexity, in agreement with some studies.

**Figure 3-4** *Sensory analysis performed on experimental beers: spider plots of average scores for aroma, taste attributes and overall quality of bottled craft fruit beers, determined by judges during tasting sessions.* *Symbols: \*\*\*, P < 0.001; \*\*, P < 0.01; \* P <0.05. Abbreviations: DMS, dimethyl sulphide.*



In conclusion, sugar-rich matrices for the selection of yeast starters were explored for the first time and scientific data were provided on their technological feature useful for brewing applications. This work enriches the very limited scientific knowledge on the role of the yeasts *H. uvarum* and *L. thermotolerans* as potential co-starters and starters and also on the effect of loquat fruit as ingredient for brewing. However, further investigations are underway to assess the role of these strains at industrial scale level. Technology transfer trials are in progress at the commercial brewery Epica srl. This research was partially financed by the research project of the Region of Sicily for the support of inland areas.

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