Mead production: Selection and characterization assays of *Saccharomyces cerevisiae* strains

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A B S T R A C T

Mead is a traditional drink, which results from the alcoholic fermentation of diluted honey carried out by yeasts. However, when it is produced in a homemade way, mead producers find several problems, namely, the lack of uniformity in the final product, delayed and arrested fermentations, and the production of "off-flavours" by the yeasts. These problems are usually associated with the inability of yeast strains to respond and adapt to unfavourable and stressful growth conditions. The main objectives of this work were to evaluate the capacity of *Saccharomyces cerevisiae* strains, isolated from honey of the Trás-os-Montes (Northeast Portugal), to produce mead. Five strains from honey, as well as one laboratory strain and one commercial wine strain, were evaluated in terms of their fermentation performance under ethanol, sulphur dioxide and osmotic stress. All the strains showed similar behaviour in these conditions. Two yeasts strains isolated from honey and the commercial wine strain were further tested for mead production, using two different honeys (a dark and a light honey), enriched with two supplements (one commercial and one developed by the research team), as fermentation media. The results obtained in this work show that *S. cerevisiae* strains isolated from honey, are appropriate for mead production. However it is of extreme importance to take into account the characteristics of the honey, and supplements used in the fermentation medium formulation, in order to achieve the best results in mead production.

1. Introduction

Mead is a traditional drink, containing 8–18% (v/v) of ethanol, which results from the alcoholic fermentation of diluted honey carried out by yeasts. Mead fermentation is a time-consuming process, often taking several months, and the fermentation rate depends on several factors, especially on honey variety, yeast strain, yeast nutrition, control of pH (Navrátil et al., 2001).

Honey is a natural product, mainly composed of a complex mixture of carbohydrates and other minor substances, such as organic acids, amino acids, proteins, minerals, vitamins, and lipids (Finola et al., 2007). In almost all honey types, fructose and glucose predominate. These two sugars account for nearly 85–95% of the honey carbohydrates (Finola et al., 2007). However, the composition of honey is rather variable and primarily depends on the floral source; in addition, certain external factors also play an important role, such as seasonal and environmental factors, as well as the processing method (Arráez-Román et al., 2006). Some reports show possible correlations between floral origin and flavonoid profiles (Anklam, 1998; Yao et al., 2004). In relation to honey colour, it depends on the potential alkalinity and ash content, as well as on the antioxidatively active pigments, such as carotenoids and flavonoids (Baltrušaitytė et al., 2007).

Honey production is an activity with significant economic importance in several regions of Portugal. However, nowadays in the northeast of Portugal, there is an excess of honey that is being sold below production prices, making it imperative to found new ways to make apiculture a viable enterprise. One possible solution for this problem could be mead production. However, when it is produced in a homemade way, the beekeepers and mead producers find several problems, namely, lack of uniformity in the final product, since the water content of honey changes every year, (20% maximum, except for *Calluna* honey which is 23%) (Decreto-Lei n° 214/2003 de 18 de Setembro), that can induce not only refermentations by yeasts, but also metabolisation of residual sugar by acetic acid bacteria and lactic acid bacteria. This increases volatile acidity and produces abnormal esters, changing the organoleptic quality of the final product (O'Connor-Cox and Ingledew, 1991). Delayed and arrested fermentations are other problems found in mead production, causing significant delays in the marketing of mead, being sold one year after its production. Finally, the stages of clarification and filtration, that are desirable, make the production process extremely expensive.

In wine production, some similar problems are also encountered. Delayed and arrested fermentations, as well as the

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production of off-flavours by the yeasts, are usually associated with the inability of yeast strains to respond and adapt to unfavourable stressfull growth conditions (Attfield, 1997; Bisson, 1999). Some possible stress factors are temperature (heat or cold) shock stresses, limitations in essential nutrients, osmotic stress, ethanol toxicity (Bauer and Pretorius, 2000; Hohmann and Mager, 2003). Analysis of stress resistance has been proposed as a suitable criterion for wine yeast selection (Zuzuarregui and del Olmo, 2004a,b).

Yeasts used in mead production are starter yeasts, such as strains of *Saccharomyces cerevisiae* used in wine, beer, and champagne production. However, regarding the composition of honey and wine must, namely the higher sugar levels (>60% *versus* 20–25%) (Decreto-Lei n°214/2003 de 18 de Setembro) and lower nitrogen concentrations (0.04% average *versus* 4–5% optimum) (Anklam, 1998) present in honey, it was thought that these strains might not be the most suitable for mead production. In previous studies performed by our team, some strains of *S. cerevisiae* have been isolated from honey, showing high fermentative capacity in wine making and revealing high potential for mead production.

Owing to this, the aim of this work was to select the most appropriate yeasts isolated from honey for mead production and to optimize the conditions for its production. The *S. cerevisiae* strains isolated were evaluated in terms of their fermentation performance under ethanol, sulphur dioxide, osmotic stress. In order to characterize the yeast strains in terms of their suitability for mead production and fermentation performance, several batch fermentations were carried out. Each strain was subjected to two different honeys enriched with two supplements, in order to evaluate the effect of the honey source and the quantity and type of nutrients. Then the fermentation kinetics, the yeast growth and the production of ethanol, glycerol, and acetic acid were determined.

2. Materials and methods

2.1. Yeast strains, media, and growth conditions

Seven strains of *S. cerevisiae* were selected, namely: Five strains isolated from Portuguese honeys and identified in a previous work (Carvalho et al., 2005), one laboratory strain (W303-1A) and one commercial wine yeast strain (Active Dry Wine Yeast (Premier cru)).

Yeast cells were grown in YPD liquid medium (2% (w/v) glucose, 1% (w/v) peptone, and 0.5% (w/v) yeast extract). Incubation overnight was carried out at 25 °C prior to the application of stressful conditions.

2.2. Stress treatments

For the analysis of ethanol, sulphur dioxide, and osmotic stresses, an initial concentration of yeasts of 1×10^5 cells/mL was used. In all cases, the fermentations were carried out with orbital agitation (Stuart Scientific SI50 model, 2001) at 25 °C for 168 h. Yeast cell growth was followed through by measuring the optical density at 640 nm in a UV–visible spectrometer (Unicam Heλios, 1997) and by counting the colony-forming units (CFU) in solid YPD medium.

For ethanol stress analysis, the seven yeast strains were pre-cultured in YPD media containing 5% (v/v) ethanol and afterwards inoculated in YPD media containing different ethanol (Sigma–Aldrich) concentrations, namely 10%, 15%, and 20% (v/v).

For the sulphur dioxide resistance, fermentations were carried out in YPD media supplemented with sulphur dioxide to concentrations of 100, 250, and 500 mg/L.

To induce osmotic shock, cells were transferred to YPD liquid medium containing 40% of sugars (20% (w/v) glucose + 20% (w/v) fructose).

Periodically, samples were taken in order to quantify glucose, fructose, ethanol, glycerol, and acetic acid.

In all cases, a control was carried out with yeast cells grown in YPD media at 25 °C, not exposed to any stress condition.

2.3. Physico-chemical characterization of honey samples

In the experiments involving mead production, two honeys were used. Both were obtained directly from beekeepers from Northeast Portugal (Trás-os-Montes region) but they differ in colour, one being dark and the other clear.

In order to characterize these honeys, the water content, diastase index, and hydroxymethylfurfural (HMF) content were determined according to Anonymous (1986); pH, acidity and reducing sugars (fructose and glucose) as described by Bogdanov et al. (1997); and electric conductivity and ashes content according to the procedure of Sancho et al. (1991). The polinic analysis of both honeys was also performed, according to the acetolitic method (Anonymous, 1986).

2.4. Mead fermentations with selected yeast strains

Three strains of *S. cerevisiae*, two isolated from honey and one commercial wine yeast strain (Active Dry Wine Yeast (Premier cru)), selected from a previous study relative to the evaluation of stress resistance, were inoculated in media prepared by mixing of honey together with a nutrient supplement.

Both honeys described in the previous section were used, as well as two nutritive supplements, namely:

- Supplement 1: 0.4 g/L commercial nutrients (Enovit®); 1 mL/L of 6% (v/v) SO_2 and 2.5 g/L tartaric acid (Sigma–Aldrich).
- Supplement 2: developed by the team, taking into account data described in the literature about alcoholic fermentations performed by *S. cerevisiae*. It consisted of: 0.4 g/L ammonium dihydrogen phosphate (Merck, Darmstadt); 3.8 g/L potassium sodium tartrate 4-hydrate (Panreac); 0.08 g/L magnesium sulphate heptahydrate (Merck, Darmstadt); 0.2 g/L calcium sulphate (Merck, Darmstadt); 67 μL/L of 6% (v/v) SO_2; 1 g/L tartaric acid (Sigma–Aldrich) and 0.3 g/L bentonite sodium form (Alfa Aesar GmbH & Co).

In all cases, growth media was inoculated in order to obtain an initial population of 10^5 cells/mL and incubated at 27 °C with gentle orbital agitation (120 rpm) (Stuart Scientific SI50 model, 2001) for 8 or 13 days. Along the fermentations, yeast cell biomass was determined by measuring the optical density at 640 nm in a UV–visible spectrometer (Unicam Heλios, 1997). Glucose, fructose, ethanol, glycerol, and acetic acid were also quantified by HPLC.

2.5. Glucose, fructose, ethanol, glycerol, and acetic acid quantification

Glucose, fructose, ethanol, glycerol, and acetic acid were analysed using a Varian HPLC system, equipped with a 20 μL Rheodyne injector, a Supelco Gel C-610H column (300 × 17.8 mm) at 35 °C and a refractive index detector RI-4 (Varian). Isocratic elution was employed with a mobile phase consisting of HPLC grade 0.1% (v/v) phosphoric acid at a flow rate of 0.5 mL/min. Data was recorded and integrated using the Star Chromatography Workstation software (Varian). Glucose, fructose, ethanol, glycerol, and acetic acid were quantified on the basis of their peak areas and comparison with the calibration curves obtained with the corresponding standards.

All values in own work, are averages of the results obtained from triplicate assays and data variation was less than 5% (percentage relative standard deviation).

3. Results and discussion

3.1. Evaluation of the stress resistance of the selected yeasts strains

Osmotic stress is an adverse condition for yeast cells that occurs at the beginning of the fermentation and more precisely in mead production, as honey has a high content on sugars (>60%) (Decreto-Lei n° 214/2003 de 18 de Setembro). Analysis of yeast strain survival under this stress condition could provide useful information about the ability of the yeast to start growth and carry out fermentation. In order to evaluate the behaviour of the seven *S. cerevisiae* strains to osmotic stress, 20% (w/v) glucose plus 20% (w/v) fructose were added to YPD liquid medium in order to simulate as closely as possible the concentration of the sugars present in must honey. The results obtained and described in Fig. 1 show that all the strains had a similar behaviour when sugars were added. The specific growth rates varied between 0.18 (strain 1) and 0.20 (strains 2 and 4) h^{-1}. When compared with the control (Fig. 2), neither was a decrease in growth rates observed.

Ethanol stress is probably one of the most interesting conditions to analyze, since one of the traditional criteria used to select yeast strains for production of alcoholic drinks is their tolerance to ethanol, owing to the high concentrations of this alcohol reached during fermentation. To better observe the effect of this kind of stress on cell viability, ethanol was exogenously added to the cells in a single pulse. All yeast strains showed the same behaviour at 10%

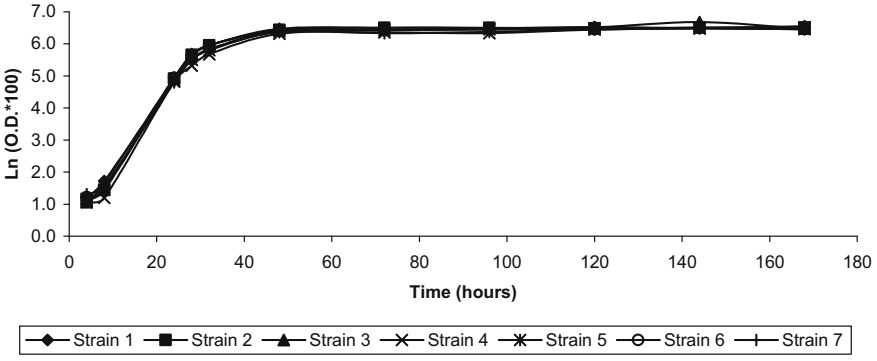

Fig. 1. Cell growth ($O.D._{640\ nm}$) of the seven strains submitted to osmotic stress (20% (w/v) glucose + 20% (w/v) fructose): strains 1 ($\mu = 0.18\ h^{-1}$), 2 ($\mu = 0.20\ h^{-1}$), 4 ($\mu = 0.19\ h^{-1}$), 5 ($\mu = 0.18\ h^{-1}$), and 6 ($\mu = 0.18\ h^{-1}$) – strains isolated from honey; strain 3 ($\mu = 0.18\ h^{-1}$) – reference strain; strain 7 ($\mu = 0.19\ h^{-1}$) – commercial strain.

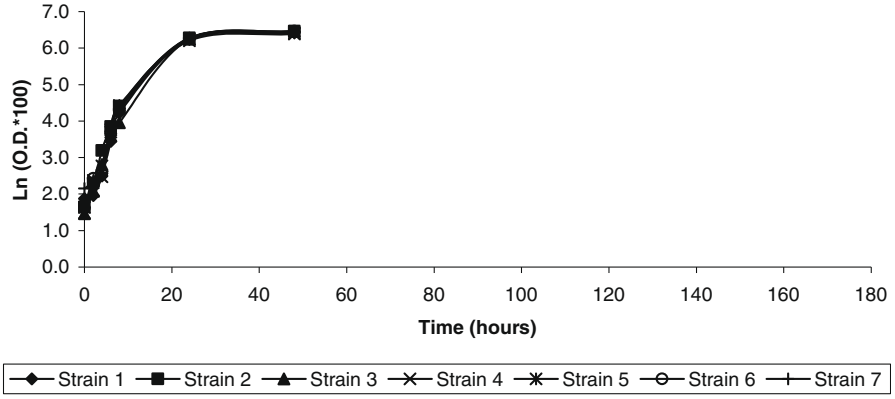

Fig. 2. Cell growth ($O.D._{640\ nm}$) of the seven strains not submitted to stress conditions: strains 1 ($\mu = 0.18\ h^{-1}$), 2 ($\mu = 0.19\ h^{-1}$), 4 ($\mu = 0.19\ h^{-1}$), 5 ($\mu = 0.18\ h^{-1}$), and 6 ($\mu = 0.18\ h^{-1}$) – strains isolated from honey; strain 3 ($\mu = 0.18\ h^{-1}$) – reference strain; strain 7 ($\mu = 0.18\ h^{-1}$) – commercial strain.

(v/v) ethanol (Fig. 3), but there was a decrease on cell viability. Another interesting fact observed was that the specific growth rates decreased by half. It was also observed that none of the stains was able to grow at concentrations of 15% (v/v) and 20% (v/v) ethanol (data not shown). Similar results were obtained by Carrasco et al. (2001), who observed that all commercial wine yeast strains studied were tolerant to 10% (v/v) ethanol, but most of them were significantly affected by concentrations of 12% (v/v).

Another desirable trait for fermentation yeast strains is a high tolerance to SO_2. In respect to SO_2 tolerance the results described in Table 1 show that the specific growth rate of all the studied strains was not affected by concentrations until 250 mg/L. In fact, the μ values of each studied strain were identical in the media supplemented with 100 and 250 mg/L of SO_2. The presence in culture medium of SO_2 concentrations of 500 mg/L inhibited the growth of all the strains. Although the specific growth rate has not been affected by SO_2 concentrations of 250 mg/L, there was an increase on the lag phase duration of about 8 h (data not shown). These results are in accordance with the ones reported by Nikolaou et al. (2006), who tested the resistance of six *S. cerevisiae* strains, isolated from wine must, and subjected to various concentrations of sulphur dioxide (50–300 mg/L), observing that only the growth of one strain was affected by SO_2 concentrations of 300 mg/L. In all studies of our work the population growth was also confirmed by counting the unit forming colonies (UFC) (data not shown).

Since all studied strains exhibited the same behaviour to the stress conditions, two strains were randomly selected from the five

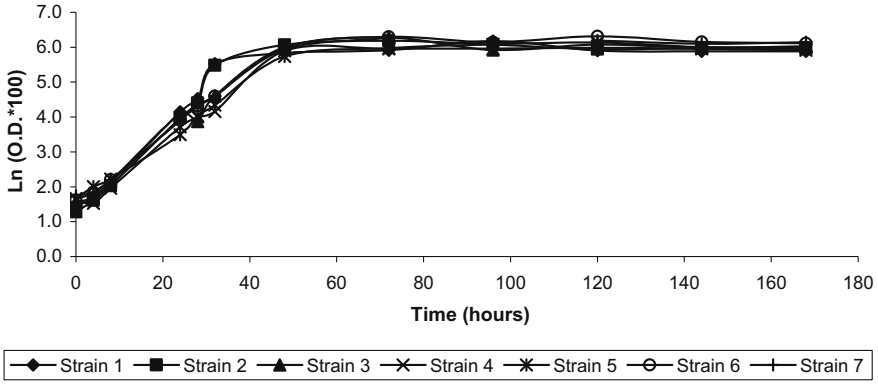

Fig. 3. Cell growth ($O.D._{640\ nm}$) of the seven strains submitted to ethanol stress (10% (v/v)): strains 1, 2, 4, 5, and 6 – strains isolated from honey; strain 3 – reference strain; strain 7 – commercial strain.

Table 1
Specific growth rates of *S. cerevisiae* strains in the presence of SO_2 concentrations of 100 and 250 mg/L.

Strain	μ_c (h^{-1})		
	0 mg/L	100 mg/L	250 mg/L
1 (Honey)	0.18	0.18	0.18
2 (Honey)	0.16	0.17	0.17
3 (Laboratory strain)	0.18	0.17	0.17
4 (Honey)	0.18	0.18	0.18
5 (Honey)	0.17	0.18	0.18
6 (Honey)	0.17	0.17	0.17
7 (Commercial wine strain)	0.17	0.18	0.17

isolated from honey, namely, the last two strains (5 and 6) for a first stage of mead production. The commercial wine strain (7) was also selected in order to perform posterior comparisons between the strains isolated from honey with the one used in oenology in terms of their suitability for mead production.

3.2. Fermentation behaviour of the selected yeast strains

In order to detect more readily the differences between the strains selected during the fermentation process, an analysis was first carried out on the fermentation performance of the three selected strains in synthetic medium containing 20% (w/v) of glucose plus 20% (w/v) of fructose (Table 2). For these strains the fermentation yield and the production of glycerol and acetic acid were determined. The results show that the fermentation yield was similar for all the strains and the strain 5 had the highest glycerol production (10.54 g/L). There was also simultaneous consumption of glucose and fructose during the exponential and stationary phases (Fig. 4). However, in the exponential phase there was a preferential consumption of glucose over fructose. The acetic acid production was similar for all the strains. These results are in accordance with the studies performed by Bely et al. (2008), who verified that the growth of *S. cerevisiae* was not affected by glucose concentrations of 360 g/L. In these conditions the fermentation was completed after 11 days after inoculation, being the ethanol production of 14% (w/v).

Our results also suggest a similar behaviour of all the strains studied, so all of them were used in the further studies related to mead production.

3.3. Physico-chemical characterization of honey samples

Since mead is an alcoholic beverage obtained from honey, the composition of honey will influence the quality of the final product. Hence, before starting the tests, the honeys used on mead production were physico-chemical and polinic characterized and the results are shown in Table 3. In relation to the polinic characterization, both honeys differ in the quantity and type of pollens present. When a sample of honey contains at least 45% of pollen grains of a certain species, as for example of *Erica* sp., it can be considered a monofloral honey of that species. However, with other species, such as *Lavandula* sp., for the honey to be considered monofloral, it only needs 15% of pollen grains of this species (Russo-Almeida and Paiva, 1996; Maia et al., 2003). Owing to this, both honeys analyzed are monofloral, being the light honey of pollen of *Lavandula* sp. and the dark honey of pollen of *Erica* sp.. In relation to the composition criteria of honeys, both light and dark honeys from the Trás-os-Montes region are in accordance with the values established in Portuguese legislation (Decreto-Lei n° 214/2003 de 18 de Setembro). In relation to the physicochemical differences between the two honeys it was observed that the light honey had lower pH (3.84 *versus* 4.90), as well as lower acidity (23.00 *versus* 30.00 meq.Ac/kg), lower diastase index (8.65 *versus* 14.60) and higher HMF content (16.02 *versus* 3.59 mg/kg) than the dark honey. The electrical conductivity and the ashes content (0.32 *versus* 0.77 mS cm^{-1} and 0.17% *versus* 0.55%, respectively) were also lower in the light honey than the dark one. In contrast, the reducing sugars content was similar in both honeys, meaning that there were not significant differences in the concentrations of glucose and fructose in the light and dark honeys.

3.4. Mead production

The previous selected strains (strains 5 and 6) and the commercial wine strain were used to optimize the conditions for mead production. In order to characterize the yeast strains and to study how they behave in mead production, several fermentations were carried out. Each strain was subjected to two different honeys enriched with two supplements. In this way it was possible to evaluate the role of the type of honey used, as well as the added nutrients, on the fermentation kinetics and yeast growth, and on production of ethanol, glycerol, and acetic acid. The first supplement added to the honeys contained commercial nutrients and the second was developed by the research team.

Concerning the mead fermentation with the dark honey enriched with supplement 1, Fig. 5A shows the fermentation behaviour of strain 6. Similar behaviours were observed for the other two tested strains. The fermentations ended at about 200 h for all the strains and there was a progressive increase in the consumption of glucose and fructose with ethanol production (maximum obtained at 150 h) and glycerol (around 5 g/L). Volatile acidity increased during fermentations, mainly as a result of acetic acid synthesis, reaching a maximum of 0.3 g/l at the end of the fermentation. As high concentrations of sugars were used, the carbon source was not the limiting substrate for growth, and fermentations continued even after growth arrest.

Table 2
Quantification of the fermentation products (ethanol, glycerol, acetic acid (g/L)) and sugars (glucose and fructose (g/L)), obtained during the fermentations performed by the three selected yeast strains, grown in YPD culture media, containing 20% (w/v) of glucose, and 20% (w/v) of fructose.

Time (hours)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)	$Y_{Ethanol/Sugars\ consumed}$ (%)	Glycerol (g/L)	$Y_{Glycerol/Sugars\ consumed}$ (%)	Acetic acid (g/L)
Strain 5							
0	197.77	199.58	n.d.	42.38	n.d.	3.38	n.d.
168	17.45	67.72	132.29		10.54		1.64
Strain 6							
0	197.77	199.58	n.d.	41.34	n.d.	3.21	n.d.
168	23.99	82.43	120.26		9.35		1.66
Strain 7							
0	197.77	199.58	n.d.	42.41	n.d.	3.26	n.d.
168	25.32	83.20	122.49		9.41		1.70

n.d. – Not detected.

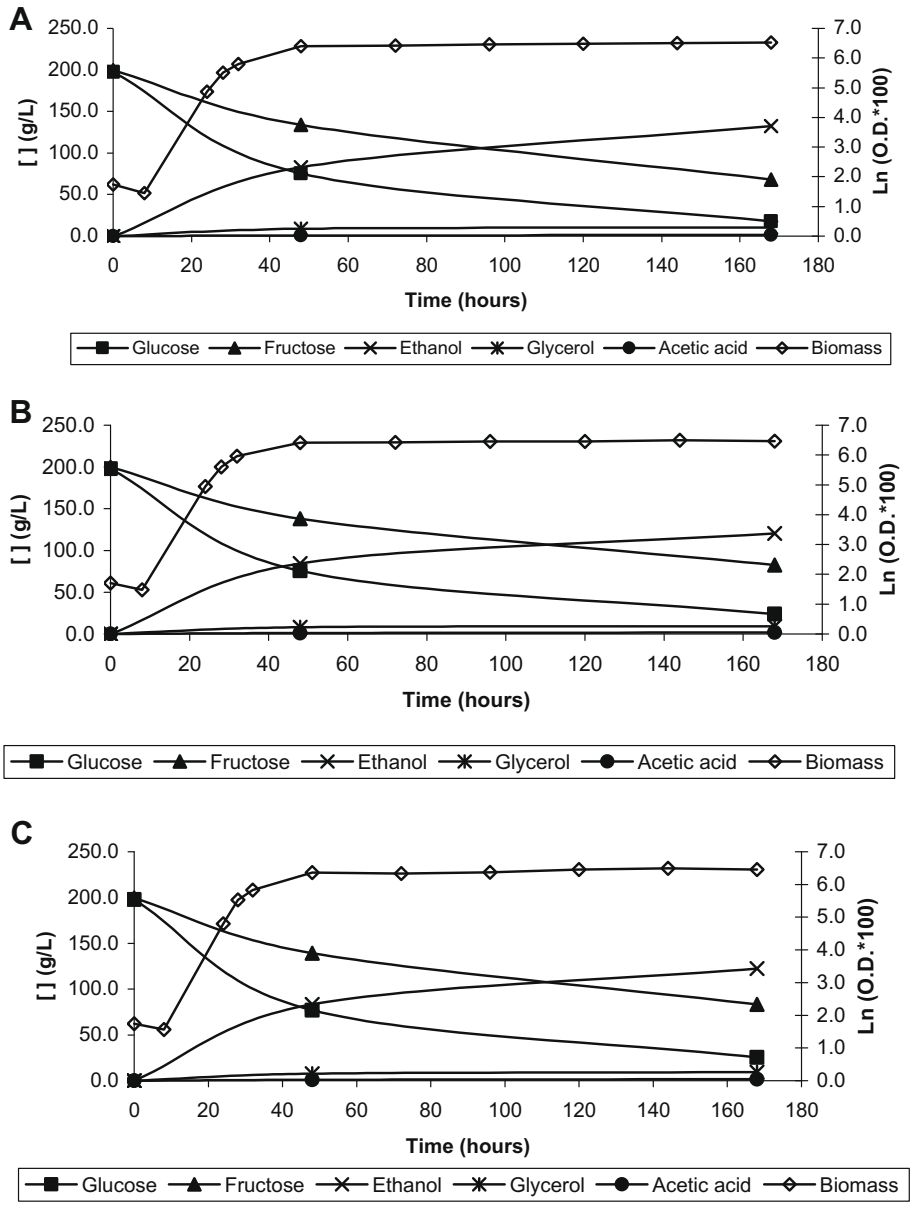

Fig. 4. Fermentation performance of strains 5 (A), 6 (B), and 7 (C) grown in YPD culture media containing 20% (w/v) of glucose and 20% (w/v) of fructose.

Table 3
Physicochemical characterization of the honey samples used in mead production.

	Dark honey	Light honey
Polinic	*Erica* (61.91%) *Castanea* (14.28%) *Lavandula* (14.28%) *Rubus* (9.53%)	*Lavandula* (52.0%) *Trifolium* (24.0%) *Rubus* (16.0%) Others (8.0%)
Physico-chemical		
Moisture (%)	16.80	16.20
pH	4.90	3.84
Acidity (meq.Ac/kg)	30.00	23.00
Diastase index	14.60	8.65
HMF (mg/kg)	3.59	16.02
Electrical conductivity (mS cm^{-1})	0.77	0.32
Total ashes (%)	0.55	0.17
Reducing sugars (%)	71.43	68.03

The fermentation of light honey supplemented with commercial nutrients (supplement 1) stopped approximately after 50 h (Fig. 5B). Sugars were little used by the yeasts and as the glycerol production (measured by the yield of glycerol *versus* sugars) was two times higher than in the fermentation of dark honey (7.60–9.27 g/L *versus* 2.77–3.01 g/L, respectively, these suggest that the strains were under stress conditions. The starvation of nitrogen could be a possible explanation for the stopping of fermentation. In fact, the light honey has a lower proportion of pollen than the dark honey and as the nitrogen compounds are present in pollen, the nitrogen content could be the limiting factor. Moreover, the light honey has a lower pH and a small content of minerals, expressed by the low content of ashes (Table 3), factors that can also decrease the growth of yeast.

With supplement 2, both fermentations performed with dark and light honeys, reached the end at about 200 h (Fig. 5C and D, respectively). Low residual sugar levels and ethanol production indicates complete fermentation. Glycerol production ranged between 4.17 and 5.67 g/L, values that are in agreement with concentrations normally reported for *S. cerevisiae* strains isolated from wine (Nikolaou et al., 2006). This major component of wine improves quality by influencing sweetness, fullness, smoothness. In

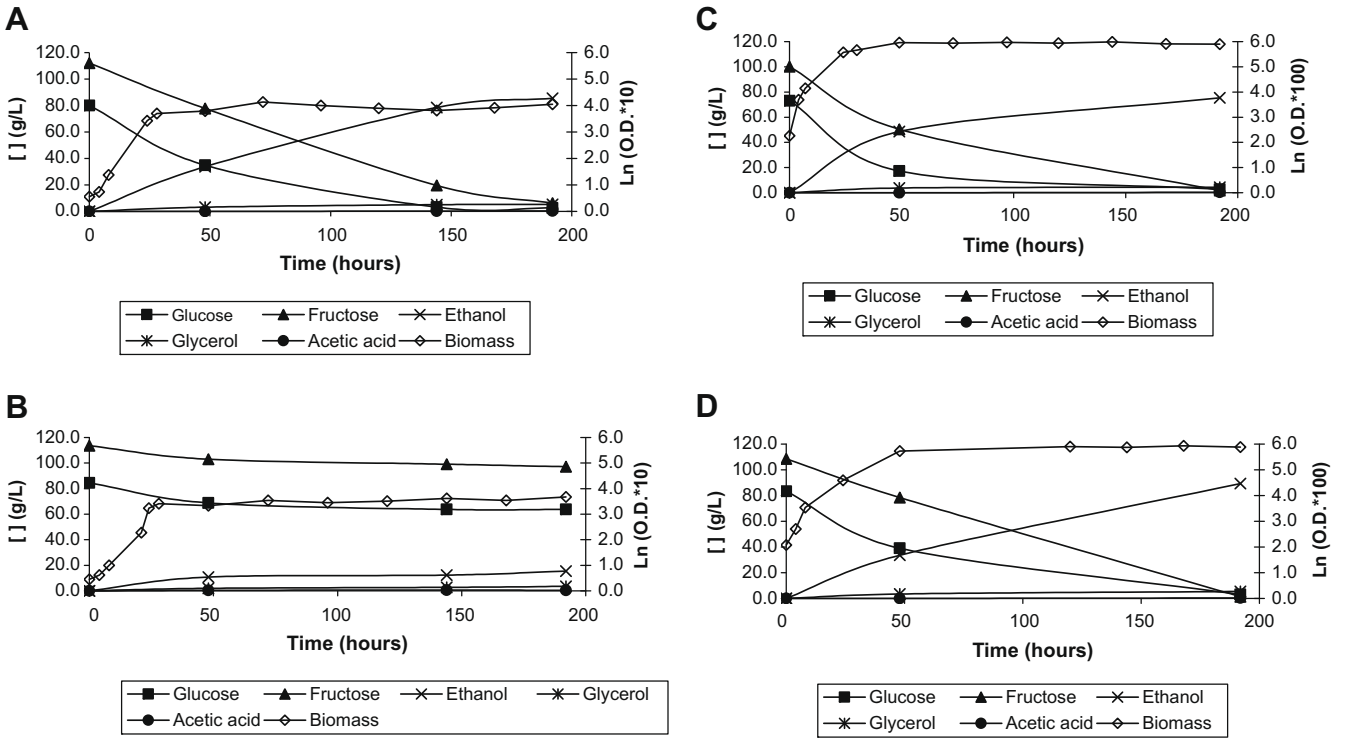

Fig. 5. Fermentation performance of strain 6 during mead production with dark honey enriched with supplement 1 (A), light honey enriched with supplement 1 (B), dark honey enriched with supplement 2 (C), and light honey enriched with supplement 2 (D).

contrast, the formation of acetic acid during fermentation is highly undesirable. Nevertheless, all the strains studied produced low amounts of this volatile acid, less than 0.55 g/L. These values are also in agreement with the ones normally reported for *S. cerevisiae* in wine must fermentations, namely 0.4–0.5 g/L (Nikolaou et al., 2006). However, Sroka and Tuszyński (2007), when studying the influence of organic acids present in honey in mead fermentation, verified that at 7 days of fermentation the concentration of acetic acid was, approximately, 0.75 g/L.

With dark honey enriched with supplement 2, a typical fermentation behaviour was observed. The highest rate of ethanol production occurred in the first 48 h, during which time about 65% of the total ethanol had already been produced. Ilha et al. (2000) obtained similar results, observing higher alcohol production in a period of up to 36 h of alcoholic fermentation, when they used honey to produce vinegar. Concerning light honey, there was a progressive increase in ethanol production up to 192 h, suggesting that the fermentation did not occur in the normal way and so the honey, as well as the supplement used, were not adequate for mead production.

As already mentioned, the fermentative process was similar for the three strains, however the mead produced by strain 5 revealed an aroma and unpleasant flavour. It is suspected that the compounds responsible for these changes are phenolic compounds, such as ethilphenol or ethilguaiacol or hydrogen sulphide, and further studies will be done in order to identify them. Since for light honey none of the supplements was suitable for mead production, probably due to the limitation of nutrients, new medium formulations are being tested.

4. Conclusions

The conditions of stress studied, namely, ethanol, sulphur dioxide, and osmotic stresses, did not permit the selection of any strain from honey that was more favourable to mead production. Thus, the selection must take into account the final organoleptic characteristics of mead, depending on the production of H_2S, aromatic compounds and volatile acidity. In this work it was also shown that mead production depends on the composition of the fermentation medium, namely on the type of honey used, as well as on the supplements added to it. The best results were obtained with the honey that had higher mineral content and pH (dark honey), and with the supplement being prepared by the team, taking into account the yeast's requirements. Furthermore, detailed studies on the mineral composition of honey and its role in fermentation performance should be performed in order to establish a "standard recipe" to be used by mead producers. In comparison to strain 7 (commercial wine strain used in oenology), the strains isolated from honey showed a similar behaviour to the former and so with potential for mead production.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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