Kinetic of sugar consumption and ethanol production on very high gravity fermentation from syrup of dates by-products (Phoenix dactylifera L.) by using Saccharomyces cerevisiae, Candida pelliculosa and Zygosaccharomyces rouxii

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INTRODUCTION

Biomass, or biomass-derived products, is considered to be one of the most promising alternatives to the use of conventional fossil fuels, due to the foreseeable low cost and abundant resource (Adaganti et al., 2014; Lynd et al., 1999). Moreover, production of renewable fuels from biomass offers benefits in terms of sustainable resource supply, energy security and rural economic development.

Tunisia is currently the 10th world producer and the first exporter of dates (Phoenix dactylifera L.) in value (Besbes et al., 2008). Tunisian production has reached an average of 190 000 tons per year (FAO, 2014) with dominance of the “Deglet-Nour” variety constituting about 60 % of the total production (Besbes et al., 2008). This production is unfortunately accompanied by a substantial increase of loss during picking, storage, commercialization and conditioning processes (Abbes et al., 2011; Masmoudi et al., 2008). The lost dates commonly named “date by-products” are not consumed by humans due to microbes and/or infestation by insects simply due to their low quality. Ethanol production from date by-products is an attractive option for the sustainable production of fuels. In many developed countries like Brazil and USA, the commercial ethanol is produced mainly by the fermentation of sucrose from sugarcane, or from glucose derived from starch-based biomass such as corns (Bhatia et al., 2015), potato Ben (Tahar et al., 2016) and cereals (Rygielska et al., 2012). Dates are mainly composed of fermentable sugars, like glucose, fructose and sucrose (73-83 %) (Rygielska et al., 2012) and it can be a good feedstock for ethanol production (Chniti et al., 2014). Kasavi et al. (2012) clearly established the importance of choosing the appropriate yeast strain to be used in ethanol production from biological residues; the choice will not only depend on a strain’s ethanol tolerance but also its ability to utilize carbon sources available in agri-food residues. Saccharomyces cerevisiae, is traditionally used for alcoholic beverage and bioethanol production; however, its performance during fermentation is compromised by the impact of variable environmental factors (Li et al., 2011) such as high temperature (Kim et al., 2006), aeration (Djelal et al., 2006), the increasing ethanol concentration medium (Aguilera et al., 2006), hyperosmolarity due to high product concentrations (Hohmann et al., 2002) and the large amount of sugar (Carrasco et al., 2001). A high sugar concentration in the culture broth is a significant stress factor during fermentation. It is an inhibitor of yeast growth at relatively high concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentration reduces cell viability and increase cell death (Djelal et al., 2005; Djelal et al., 2006). The osmotic stress response is a crucial mechanism in the survival of yeasts to variations of their external environment. In the case of hyper-osmotic stress, fungal cells must react to the presence of external osmolites that alter the osmotic pressure acting on the cell. Part of the response consists of the production of intracellular osmolyte glycerol to increase the internal osmolarity of the cell; a fraction of glycerol is excreted into the environment by the yeast (Climent et al., 1998; Lartigue and Leclercq, 1999).

MATERIAL AND METHODS

Microorganisms

The fermentative yeasts Saccharomyces cerevisiae 522D, Zygosaccharomyces rouxii (IP 2021 92) and Candida Pelliculosa (IP 820.63) were obtained from the culture collection of the Pasteur Institute (Paris, France). Stock cultures were maintained on a gelified medium whose composition was (in g/L): glucose, 20; peptone, 10; yeast extract, 10; and agar, 10. In all cases, cultures were maintained
at 28°C for 24 h and then stored at 4°C (Chniti et al., 2014). Subculture was done every two months.

**Inoculum preparation**

A 1 mL of a yeast suspension in KCI 150 mmol/L was grown in 25 mL of synthetic medium (g/L): glucose, 20; peptone, 10; and yeast extract, 10; in a 0.25 L bottle on a rotating shaker (New brunswick, INNOVA 40, NJ, USA) at 180 rpm, 28°C for 18 h. After centrifugation (3000 rpm, 4°C and 5 min), cells were harvested, re-suspended in 25 mL KCI 150 mmol/L and re-centrifuged in similar conditions. The suspension obtained after harvesting cells and re-suspending in 10 mL KCI 150 mmol/L was used to inoculate culture media (Djelal et al., 2005).

**Raw material**

By-products dates “Deglet-Nour”, was obtained from a Tunisian conditioning unit of dates “ALKHALIJ”. The fruits were pilled, crushed with a sharp knife. The juice was then extracted with distilled water (1:2.5 w/v), at 85°C for 45 min (Acourene et al., 2011). The juice was filtered and centrifuged at 5000 rpm for 30 min and then the supernatant was immediately concentrated to achieve a total sugar concentration of 72°Brix. The concentrated date juice was stored at 4°C until use.

**Ethanol production medium**

Dates Syrup containing 17.5 and 35.8°Brix was supplemented with (mmol/L): NH₄Cl, 10; KH₂PO₄, 3.7; MgSO₄.7H₂O, 4; as well as an EDTA mineral solution, derived from the Wikerham medium (mg/L): CaCl₂.6H₂O, 150; Fe₂(SO₄)₃.7H₂O, 100; ZnSO₄.7H₂O, 30; CuCl₂.2H₂O, 0.7; HBO₂, 15; KI, 2; Na₂MoO₄.2H₂O; MnSO₄.7H₂O; 32; CoCl₂.6H₂O; EDTA, 100. The pH was adjusted to 6.0 using KOH 1 mol/L. The medium was transferred into a 500 mL bottle with a final working volume of 300 mL and was autoclaved at 120°C for 20 min before adding the NH₄Cl sterilized by filtration on a 0.2 μm membrane (Sartorius, Goettingen, Germany) (Djelal et al., 2012).

**Fermentation processes**

A 300 mL of medium containing sugar concentration of 17.4 or 35.8°Brix were inoculated with 200 μL of yeast suspension. Batch fermentation was carried out in 500 mL bottle on an incubator shaker (New brunswick, INNOVA 40, NJ, USA) at 28°C for 72 h. All fermentations were performed in duplicate. After inoculation, samples of 5 mL were withdrawn aseptically from the fermentation broth after yeast addition, and after 18, 24, 42, 48, 66 and 72h, for analysis.

**Analytical methods**

The cell density of the fermentation broth was measured at 600 nm (A₆₀₀) using a spectrophotometer (SECOMAM, Alès, France). The fermentation broth was centrifuged at 3000 rpm, at 4°C for 5 min. The supernatant was used for the determination of the various metabolites produced by yeasts including ethanol and residual sugar concentrations by HPLC involving an ion exclusion column HPX-87H (300x 7.8 mm; Bio-Rad, Hercules, CA, USA), maintained at 45°C (Oven CrocorCol 181e, Chueze-Info-labo, Ste Foy La Grande, France). The elution was performed at a flow rate of 0.7 mL/min (waters pump, Milford, MA, USA) using sulfuric acid 1 N. A Shimadzu RIO-2000 HPLC (Sousa et al., 2011) was used for the detection of the various compounds (glucose, fructose, sucrose, ethanol, glycerol) (Djelal et al., 2006). In addition, the total sugar content was expressed in equivalents of glucose (glucose + fructose + 1.05 × sucrose) (Guigou et al., 2011) and one-degree Brix is 1 gram of sugar in 100 grams of solution. The °Brix of the extracted juice was determined by refractometry (AUXILAB S.L. 0-90 % ± 0.2.

**RESULTS AND DISCUSSION**

**Yeast growth**

Saccharomyces cerevisiae, Candida pelliculosa and Zygosaccharomyces rouxii could tolerate sugar concentrations of 17.4°Brix (Chniti et al., 2014) at higher initial sugar content (35.8°Brix), Zygosaccharomyces rouxii showed nearly similar trend, since after less than one-day lag time significant growth was observed, which reached stationary growth phase after about 40 h of culture (Chniti et al. 2014). The inhibitory effect of the high sugar content was more pronounced for the two other fungi, since a weak growth was only observed after 60 h of culture, which was however slightly higher for the osmotolerant yeast, Candida pelliculosa, if compared to Saccharomyces cerevisiae, 4.89 and 1.72 NTU respectively (Chniti et al., 2014).

**Sugars consumption by yeasts.**

As expected, there was a clear link between sugar consumption and growth since a higher consumption was recorded for the lowest amount of sugars (17.4°Brix) if compared to 35.8°Brix (Chniti et al., 2014). Jiménez-Martí et al. (2011), indicated that, under particular environment yeasts have to cope with osmotic stress, which can cause a high symptoms of toxic stress and can reduce the ability of the yeast cells for maintenance (Djelal et al., 2005), and the production of osmoprotective metabolites increases, as shown in this work for glycerol and discussed below. Examination of sugar consumption during cultures also showed different trends regarding on the one hand the considered sugar and on the other hand the yeast species (Figure 1a-c). Contrarily, yields of fructose consumption were high for S. cerevisiae and Z. rouxii (Figures 1a and 2a) but decreased significantly during C. pelliculosa culture, 39.53% (Figure 1b); while for sucrose, high yields of consumption were observed for S. cerevisiae, C. pelliculosa, 91.53 and 93.30% (Figure 1c) and was only 11% for Z. rouxii (Figure 1c).

If time-courses of sugars consumption are considered for each yeast individually, it can be seen that monosaccharides, glucose and fructose, were assimilated since the beginning of growth by S. cerevisiae (Figure 1), while the consumption of the disaccharide, sucrose (Figure 1c), appeared significant only during stationary growth phase (Chniti et al., 2014) showing its use mainly as an energy source for cell maintenance. Regarding C. pelliculosa, it is noteworthy that high yields of glucose and sucrose consumption were observed, while significant fructose assimilation (Figure 1b) was only observed during stationary growth phase (Chniti et al., 2014). A continuous sucrose hydrolysis and assimilation of the resulting glucose accounted mostly likely for this behavior, in agreement with the available literature (Jiménez-Martí et al., 2011; Stambuk et al., 2010). S. cerevisiae for example, can metabolize sucrose, in two ways. In the first and predominant mechanism, sucrose is hydrolyzed by an extracellular invertase. Hydrolysis yields glucose and fructose, which enter into the cell by facilitated diffusion via hexose transporters. In the second mechanism sucrose can be actively transported in the cells by a proton-symport mechanism and hydrolyzed intracellularly (Jiménez-Martí et al., 2011; Stambuk et al., 2010).

Z. rouxii consumed fructose faster than glucose and sucrose, in agreement with its fructophilic character (Sousa-Dias et al., 1996). At high concentrations (35.8°Brix), fructose significantly inactivated the glucose transporter, preventing the uptake of this sugar. Fructose was able to utilize the glucose transporter, by competing with glucose. The pattern of glucose inhibition by fructose is similar to that described by Sousa-Dias et al. (1996), for Zygosaccharomyces bailii. Transport systems for a given sugar depend on the yeast strain, growth conditions, experimental conditions and the nature of the carbohydrate.

**Comparison of products formation**

The production of the main metabolites was also and as expected linked to both ethanol and glucose productions, were observed for the three yeasts for a sugar content of 17.4°Brix in the culture medium (Figure 3); while in the presence of 35.8°Brix sugar content in the medium, metabolites production was only observed for Z. rouxii and no noticeable amount of ethanol and glycerol were produced by S. cerevisiae and C. pelliculosa (Figure 4). It should be observed that the highest ethanol production was observed for S. cerevisiae (2011) only when the ethanol production was measured on a w/v basis with a high sugar concentration (Stambuk et al., 2010), while the osmotolerant yeasts C. pelliculosa and Z. rouxii showed nearly similar amounts of ethanol produced (Figure 3).

Regarding the osmoprotective metabolite, glycerol, rather similar amounts were produced by the three yeasts in the presence of sugars (17.4°Brix) (Figure 3); while the production was almost twice (10 g/L) for Z. rouxii for a high sugar content (35.8°Brix) and hence a high osmotic stress and it was observed until the end of culture (Figure 4), while it ceased at the end of growth for a lower sugar
content (17.4°Brix) (Figure 3) (Sasano et al., 2012; Thorne et al., 2011). These species produce high concentrations of intracellular polyols such as glycerol that balance the external osmotic pressure.

Figure 1 Sugars consumption (Glucose (a), Fructose (b) and Sucrose (c) by yeasts in batch fermentation of date syrup at initial sugar concentration of 17.4°Brix.

Figure 2 Sugars consumption (Glucose (a), Fructose (b) and Sucrose (c) by yeasts in batch fermentation of date syrup at initial sugar concentration of 35.8°Brix.
CONCLUSION

This study established that the three yeasts studied were able to grow on date by-products (an agri-food residue) leading to ethanol production. However, the choice of the strain affected the bio-production of ethanol. Production of high levels of ethanol could be achieved by using osmotolerant yeasts, such as Z. rouxii, during batch ethanol fermentation from concentrated date syrup, and the effect of osmotic stress, resulting from high sugar concentrations, decreased the efficiency of ethanol production by both S. cerevisiae and C. pelliculosa. Other fermentation systems such as continuous systems (3 L) should be investigated, to improve ethanol fermentation with osmotolerant yeasts, like Z. rouxii.

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