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 Zygossaccharomyces rouxii

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ABSTRACT

Three yeasts, Saccharomyces cerevisiae, Zygossaccharomyces rouxii and Candida pelliculosa, were tested for ethanol production on dates' syrup. In batch fermentation, the ethanol concentration depended on the initial sugar concentration and the yeast strain. For an initial sugar concentration of 17.4°Brix, maximum ethanol concentration was 63 g/L during S. cerevisiae growth, higher than the amounts achieved during Z. rouxii and C. pelliculosa growth, 33 g/L and 41 g/L respectively. On 35.8°Brix initial sugar amount, only Z. rouxii was able to grow, resulting in 50 g/L ethanol production, showing an inhibitory effect on S.cerevisiae and C. Pelliculosa due to the osmotic stress resulting from the high sugar concentration.

Keywords: Ethanol, production, Yeasts, Sugar, Dates 'syrup, Osmotic stress

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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 (Abbés 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 on 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 (Rygieliska et al., 2012). Dates are mainly composed of fermentable sugars, like glucose, fructose and sucrose (73-83 %) (Rygieliska 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 vitality 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 osmosylates 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 extracellular medium (Sasano et al., 2012; Thorne et al., 2011). Candida pelliculosa (Xu et al., 2014) and Zygossaccharomyces rouxii (Chniti et al., 2014) can grow under extreme environmental stress conditions, such as low and high pH, low water activity and anaerobic conditions. In this study, a date by-product (of the Deglet-Nour variety) was therefore used as an alternative material for the production of ethanol. This bioproduction was conducted by two osmotolerant yeasts (Z. rouxi and C. pelliculosa) and comparative study was performed with S. cerevisiae.

MATERIAL AND METHODS

Microorganisms

The fermentative yeasts Saccharomyces cerevisiae 522D, Zygossaccharomyces 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 gelled 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 KCl 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, resuspended in 25 mL KCl 150 mmol/L and recentrifuged in similar conditions. The suspension obtained after harvesting cells and re-suspending in 10 mL KCl 150 mmol/L was used to inoculate culture media (Djelal et al., 2005).

Raw material

By-products dates “Deget-Nour”, was obtained from a Tunisian conditioning unit of dates “ALKHALILJ”. 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): CaCl₂·2H₂O, 150; FeSO₄·7H₂O, 100; ZnSO₄·7H₂O, 30; CuSO₄·5H₂O, 0.7; H₂BO₃, 15; KI, 2; NaMoO₂·2H₂O; MnSO₄·H₂O, 32; CoCl₂·6H₂O, 5.6; 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 broths 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 (Aₓ₅₀₀) 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) at 65°C and a refractive index detector; 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 2010 A dual wavelength spectrophotometer (Shimadzu, Kyoto, Japan) 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, about 358 g/L of total sugars, about 2 mol/L of monosaccharides like glucose or fructose, was however not negligible since even if maximal cell density was only slightly lower that the value observed at 17.4°Brix, 13 and 14.83 NTU respectively (Chniti et al., 2014) A decline phase was observed after about two days of culture. The inhibitory effect of the high sugar content was more pronounced for the two other fungi, since a weak growth was only observed about 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 effects and to reach the maximum yield; a part of the assimilated sugar is used for cell 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 strain. For example, Saccharomyces cerevisiae consumed 87H (300x 7.8 mm; Bio-Rad) at 65°C and a refractive index detector; 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 2010 A dual wavelength spectrophotometer (Shimadzu, Kyoto, Japan) 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).

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). For instance, the osmotolerant yeast, Z. rouxii, consumed 35.8°Brix (Figure 2b) from the beginning of growth, while glucose (Figure 2a) was only used during stationary growth phase (Chniti et al., 2014) as an energy source for cell maintenance. These results also showed that growth was obviously not limited by carbon substrate availability. Fructose assimilation by Z. rouxii was especially noteworthy since its total depletions at the end of culture was also observed for 35.8°Brix (Figure 3b), accounting for the noticeable growth observed (Chniti et al., 2014). Fructose was not used by the other yeasts, while only a low glucose assimilation was observed (Figure 2a) accounting for the weak growth observed during S. cerevisiae and C. pelliculosa in the presence of 35.8°Brix in the medium. During the production of biomass by Z. rouxii, the switch from fermentation to fermentation is depressed by glucose or sucrose causes a drop in biomass yield (Leandro et al., 2011). These results indicate that at high concentrations of reducing sugars, 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 Zygascalcaromyces 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 the total sugar content, both ethanol and glycerol 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 (Figure 1a) and 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.

REFERENCES


Figure 3 Concentration of products (Ethanol (a), and Glycerol (b) during the fermentation from concentrated date syrup 17.4°Brix.

Figure 4 Concentration of products (Ethanol (a), and Glycerol (b) during the fermentation from concentrated date syrup 35.8°Brix.


