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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JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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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doi: 10.15414/jmbfs.2017.7.2.199-203

ARTICLE INFO

Received 24. 5. 2017
Revised 12. 7. 2017
Accepted 24. 8. 2017
Published 1. 10. 2017

Regular article

OPEN ACCESS

ABSTRACT

Three yeasts, *Saccharomyces cerevisiae, Zygosaccharomyces 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.cerevisae* and *C. Pelliculosa* due to the osmotic stress resulting from the high sugar concentration.

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

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 (**Abbès *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 us 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 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 osmolytes 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 *Zygosaccharomyces 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. rouxii* and *C. pelliculosa*) and comparative study was performed with *S. cerevisiae*.

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 KCl 150 mmol/L was grown in 25 mL of synthtetic 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 "*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): NH_4Cl, 10; KH_2PO_4, 3.7; $MgSO_4$. $7H_2O$, 4; as well as an EDTA mineral solution, derived from the Wikerham medium (mg/L):[23] $CaCl_2.6H_2O$, 150; $FeSO_4.7H_2O$, 100; $ZnSO_4.7H_2O$, 30; $CuSO_4.5H_2O$, 0.79; H_3BO_3, 15; KI, 2; $NaMoO_4.2H_2O$; $MnSO_4.H_2O$, 32; $CoCl_2.6H_2O$, 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 NH_4Cl 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 (A_{600}) 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 CrocoCil $_{TM}$; Cluzeau-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-6A Refractive Index Detector (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 cerevisae, *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-Marti et al. (2011)**. indicated that, under particular environment yeasts have to cope with osmotic stress caused by high sugar concentration; 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 considered yeast (Figures 1 and 2). For the non-inhibitory sugar amount (17.4°Brix), a high yield of consumption was observed for the three yeasts after three days culture only for glucose, namely 100, 86.7 and 78.4 % for *Saccharomyces cerevisiae*, *Candida pelliculosa* and *Zygosaccharomyces rouxii* respectively (Figures 1a-c). Contrarily, yields of fructose consumption were high for *S. cerevisiae* and *Z. rouxii*, 91 and 100% (Figure 1b) 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 most likely for this behavior, in agreement with the available literature (**Jiménez-Marti *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-Marti *et al*., 2011; Stambuk *et al*., 2010**)

Concerning *Z. rouxii*, it should be observed the assimilation of fructose (Figure 1b) 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 depletion 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, the switch from respiration to fermentation induced 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 *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 growth, since 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 3), in agreement with its well-known use for such production (**Stanley *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.

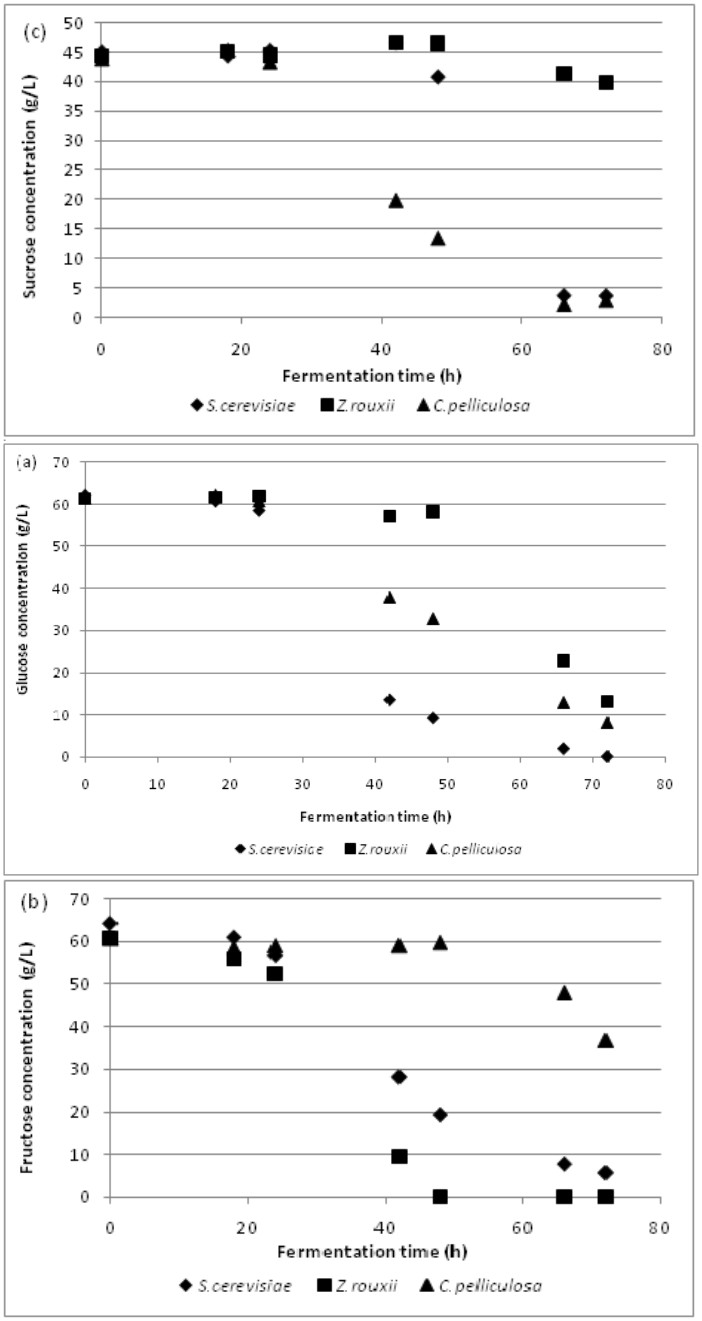

Figure 1 Sugars consumption (Glucose (a), Fructose (b) and Sucrose (c) by yeasts in batch fermentation of date syrup at initial sugar concentration of 17.4°Brix.

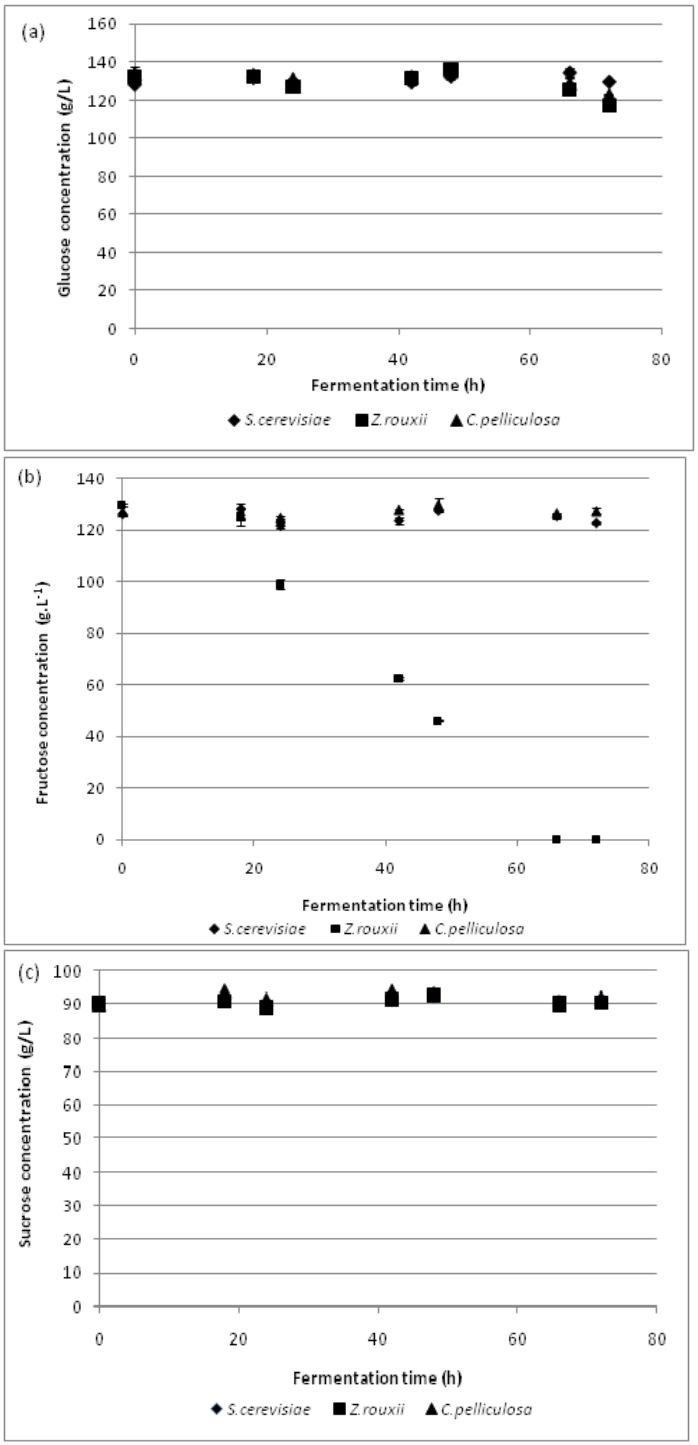

Figure 2 Sugars consumption (Glucose (a), Fructose (b) and Sucrose (c) by yeasts in batch fermentation of date syrup at initial sugar concentration of 35.8 °Brix.

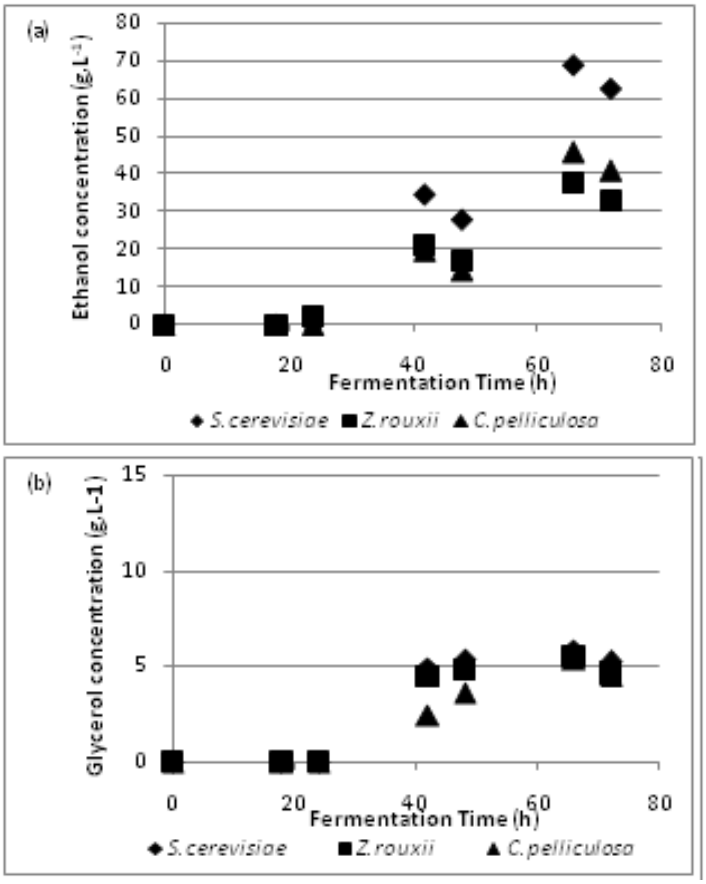

Figure 3 Concentration of products (Ethanol (a), and Glycerol (b) during the fermentation from concentrated date syrup 17.4°Brix.

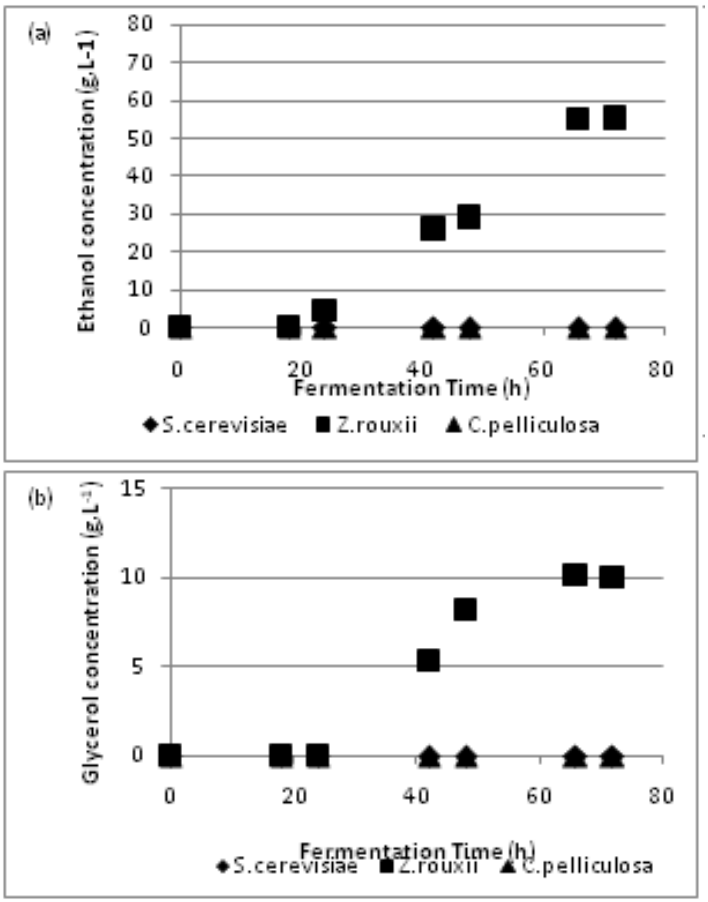

Figure 4 Concentration of products (Ethanol (a), and Glycerol (b) during the fermentation from concentrated date syrup 35.8°Brix.

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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