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1    Residue of dates from the food industry as a new cheap feedstock for bioethanol production

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## ABSTRACT

Syrup resulting from date by-products constitutes a favorable medium for yeast development, owing to its sugar composition; it was hence tested for ethanol production. Three yeasts, *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii* and *Candida pelliculosa*, were selected 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  $174.0 \pm 0.2 \text{ kg m}^{-3}$ , maximum ethanol concentration was  $63.0 \pm 0.1 \text{ kg m}^{-3}$  during *S. cerevisiae* growth, namely higher than the amounts achieved during *Z. rouxii* and *C. pelliculosa* growth,  $33.0 \pm 2.0 \text{ kg m}^{-3}$  and  $41.0 \pm 0.3 \text{ kg m}^{-3}$  respectively. Contrarily, only *Z. rouxii* was able to grow on  $358.0 \pm 1.0 \text{ kg m}^{-3}$  initial sugar amount, resulting in  $55.0 \pm 1.0 \text{ kg m}^{-3}$  ethanol produced.

Keywords: date by-products; *Phoenix dactylifera* L.; batch fermentation; Ethanol production; Yeast strain.

## 1. Introduction

The date palm tree (*Phoenix dactylifera* L.) is a perennial monocotyledonous species adapted to the local conditions of arid and semi-arid areas [1]. Dates, the fruits of the date palm tree, are the major staple food in arid areas of North and Middle East Africa and the date crop plays a central role in the economy and the social life in these regions [2,3].

The date palm tree constitutes the principal source of remuneration and the basis of economy for people living in the Tunisian Sahara [2]. Today, worldwide production, utilization and industrialization of dates are continuously increasing in some countries like Egypt, Saudi Arabia, Iran and Algeria [4]. In Tunisia the number of cultivars is evaluated for over than 250 [5] and is currently the 10<sup>th</sup> world producer and the first exporter of dates in value. During the last five years, Tunisian production has reached an average of 120.000 tonnes per year with the dominance of the “Deglet-Nour” variety constituting about 60 % of the total production [2] and 50 000 farmers are employed in this sectors. In 2011, Excess dates were 50. 000 tonnes, 32 % of which were from low quality dates [6].

This production progress is unfortunately accompanied by a substantial increase of loss during picking, storage, commercialization and conditioning process [7,8]. These lost dates could amount to more than 30. 000 tonnes per year in Tunisia [9]. The lost date commonly named “date by-products”, are not consumed by humans due to fungus and/or infestation by insects or simply due to their low quality.

Presently, by-products of dates are discarded or used in limited cases for animal feed [7,9]. Fermentation technology is one of the technologies employed for deriving value added products from by-products of dates. The various products derived from date fruit by-products are biopolymers [10,11], organic acids [12,13], amino acid [14], baker’s yeast [15], probiotics [16], antibiotics [17] enzymes [18] and biofuels such as hydrogen [19] and butanol [20].

Using date by-products as a feedstock should considerably reduce the cost of production. Dates are rich in sugar ranging from 73% to 83 % on dry weight basis and consisted mostly of the two inverted form, glucose and fructose [20-23]. Fresh varieties have a higher content of inverted sugars, the semi dried varieties contain equal amount of inverted sugars and sucrose, while dried varieties contain more sucrose [11].

Kasavi et al [24] 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 tolerance to ethanol but also on its ability to utilize carbon sources available in agri-food residues.

The aim of this study was to evaluate the feasibility of producing bioethanol from substrate with a high level of sugars like date by-products. For this purpose, bioproduction was conducted by two osmotolerant yeasts (*Z. rouxii* and *C. pelliculosa*) and a comparative study was performed with *S. cerevisiae*.

## **2. Material and methods**

### **2.1. Microorganism**

3 yeast strains were tested, the first *S. cerevisiae* well-known for its ability to produce ethanol, but this yeast is sensitive to osmotic stress; *C. Pelliculosa* has the ability to grow in media of high osmotic pressure induced by sugars or salts; and *Z. rouxii* is well-known for its capacity to grow in rich sugar environments.

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 ( $\text{kg m}^{-3}$ ): 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.

## 2.2. Inoculum preparation

A given number of drops (10) of a yeast suspension in KCl 150  $\text{mol m}^{-3}$  was grown in 25  $\text{cm}^3$  of synthetic medium ( $\text{kg m}^{-3}$ ): glucose, 20; peptone, 10; and yeast extract, 10; in a 250  $\text{cm}^3$  bottle on a rotating shaker (New brunswick, INNOVA 40, NJ, USA) at 20  $\text{rad s}^{-1}$ , 28°C for 18 h. After centrifugation (6000 g, 4°C and 5 min), cells were harvested, resuspended in 25  $\text{cm}^3$  KCl 150  $\text{mol m}^{-3}$  and recentrifuged in similar conditions. The suspension obtained after harvesting cells and re-suspending in 10  $\text{cm}^3$  KCl 150  $\text{mol m}^{-3}$  was used to inoculate culture media [25].

## 2.3. 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 and 20 g date pulp were added to 50 g of hot de-ionised water. The extraction was carried out on hot-plate at 85°C for 45 min. [26]. The juice was filtered and centrifuged at 6000 g for 30 min and then the supernatant was immediately concentrated to achieve a total sugar concentration of  $720.0 \pm 1.0$   $\text{kg m}^{-3}$ . The concentrated date juice was then stored at 4°C until use.

The high sugar content allows storage without significant risk of contamination, which can be advantageous for an industrial application. However, the osmotic pressure induced by high sugar concentrations can inhibit the growth of yeasts used for ethanol production. The concentration of substrate was therefore varied from  $100.0 \pm 1.0$   $\text{kg m}^{-3}$  to  $720.0 \pm 1.0$   $\text{kg m}^{-3}$  (data not shown) and two sugar amounts were considered for this work, 17% and 36% to assess the effect of an osmotic stress.

## **2.4. Ethanol production medium**

Dates Syrup containing  $174.0 \pm 0.2 \text{ kg m}^{-3}$  and  $358.0 \pm 1.0 \text{ kg m}^{-3}$  was supplemented with mineral culture medium as described previously by Djelal et al [24]. The pH was adjusted to 6.0 using KOH  $1000 \text{ mol m}^{-3}$ . The medium was transferred into  $500 \text{ cm}^3$  bottles with a final working volume of  $300 \text{ cm}^3$ , which were autoclaved at  $120^\circ\text{C}$  for 20 min before adding the  $\text{NH}_4\text{Cl}$  sterilized by filtration on a  $0.2 \mu\text{m}$  membrane (Sartorius, Goettingen, Germany).

## **2.5. Fermentation processes**

$300 \text{ cm}^3$  of medium containing sugar concentration of  $174.0 \pm 0.2$  or  $358.0 \pm 1.0 \text{ kg m}^{-3}$  were inoculated with  $100 \mu\text{L}$  of yeast suspension. Batch fermentation was carried out in  $500 \text{ cm}^3$  bottles on an incubator shaker (New Brunswick, INNOVA 40, NJ, USA) at  $20 \text{ rad s}^{-1}$ ,  $28^\circ\text{C}$  for 72 h. All experiments were performed in duplicates and samples ( $5 \text{ cm}^3$ ) were taken from the culture at regular time intervals.

## **2.6. Analytical methods**

The cell density of the culture medium was measured at  $600 \text{ nm}$  ( $A_{600}$ ) using a spectrophotometer (SECOMAM, Alès, France). The culture medium was then centrifuged at  $6000 \text{ g}$ , at  $4^\circ\text{C}$  for 5 min and the supernatant was used for the determination of the various metabolites produced by yeasts including ethanol and glycerol, as well as the residual sugar concentrations by HPLC involving an ion exclusion column HPX-87H ( $300 \times 7.8 \text{ mm}$ ; Bio-Rad, Hercules, CA, USA), maintained at  $45^\circ\text{C}$  (Oven CrocoCil™; Cluzeau-Info-labo, Ste Foy La Grande, France). The elution was performed at a flow rate of  $0.7 \text{ cm}^3 \text{ min}^{-1}$  (waters pump, Milford, MA, USA) using sulfuric acid  $0.5 \text{ mol L}^{-1}$ . A Shimadzu RIO-6A Refractive Index Detector (Japan) was used for the detection of the various compounds (glucose, fructose, sucrose, ethanol and glycerol) [27]. In addition,  $\text{NH}_4\text{Cl}$  concentration was analyzed by the

Mann Method [28]. The total sugar content was expressed in equivalents of glucose (glucose + fructose + 1.05 x sucrose) [29]. The values are the average of two determinations.

### 3. Results and Discussion

The three yeasts *S. cerevisiae*, *C. pelliculosa* and *Z. rouxii* could growth on date syrup containing  $174.0 \pm 0.2 \text{ kg m}^{-3}$  sugar (Fig.1a). But at higher sugar content (  $358.0 \pm 1.0 \text{ kg m}^{-3}$ ), only osmotolerant yeasts can grow; it was the case for *Z. rouxii*, while the other osmotolerant yeast, *C. pelliculosa*, showed only a weak growth after more than two days of culture (Fig.1b).

Examination of Fig.2 clearly shows that nitrogen was the limiting substrate, since it was completely exhausted at the end of growth, namely after about 42 h of culture. Indeed, it was the case for the three yeasts for  $174.0 \pm 0.2 \text{ kg m}^{-3}$  sugar content in the medium (Fig.2a) and only for *Z. rouxii* in the case of  $358.0 \pm 1.0 \text{ kg m}^{-3}$  sugar content in the medium (Fig.2b).

As expected, there was a clear link between sugars consumption and growth since both parameters followed similar trends, namely a higher consumption was recorded for the lowest amount of sugars ( $174.0 \pm 0.2 \text{ kg m}^{-3}$ ) if compared to  $358.0 \pm 1.0 \text{ kg m}^{-3}$  (Table 1). For the non-inhibitory sugar amount ( $174.0 \pm 0.2 \text{ kg m}^{-3}$ ), a high yield of sugars consumption was observed for the three yeasts after three days culture, namely 94, 71 and 67 % for *S. cerevisiae*, *C. pelliculosa* and *Z. rouxii* respectively (Table 1).

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  $174.0 \pm 0.2 \text{ kg m}^{-3}$  in the culture medium (Table 1); while in the presence of  $358.0 \pm 1.0 \text{ kg m}^{-3}$  sugar content in the medium, metabolites production was only observed for *Z. rouxii*. It should be observed that the highest ethanol production was observed for *S. cerevisiae* (Tab.1), in agreement with its well-known use for such production [30], while *C. pelliculosa* and *Z. rouxii* showed roughly similar amounts of ethanol produced. Regarding the osmoprotective



167 metabolite, glycerol, rather similar amounts were produced by the three yeasts in the presence  
 168 of  $174.0 \pm 0.2 \text{ kg m}^{-3}$  sugars (Table 1); while the production was almost twice ( $10 \text{ kg m}^{-3}$ ) for *Z.*  
 169 *rouxii* for a high sugar content ( $358.0 \pm 1.0 \text{ kg m}^{-3}$ ) and hence a high osmotic stress (Table 1).  
 170 These species produce high concentrations of intracellular polyols, such as glycerol, that  
 171 balance the external osmotic pressure. The mechanisms by which some yeast species tolerate  
 172 high salt and high sugar (low activity) environments have been the subject of considerable  
 173 studies [25].

174 Table 1 showed that *S. cerevisiae* has consumed more than 90 % sugars after 72 hours of  
 175 fermentation for  $174.0 \pm 0.2 \text{ kg m}^{-3}$  sugar content. With *Z. rouxii*, and *C. pelliculosa*, the sugar  
 176 consumption yield reached 67 and 71 % respectively (Table 1); in close relation with sugar  
 177 consumption, the highest ethanol yield was obtained for *S. cerevisiae* (38 % – Table 1), as well  
 178 as maximum ethanol productivity ( $0.9 \pm 0.1 \text{ kg m}^{-3} \text{ h}^{-1}$  – Table 1). Contrarily, at high initial  
 179 substrate concentration ( $358.0 \pm 1.0 \text{ kg m}^{-3}$ ), in close link with the inhibitory effect on *C.*  
 180 *pelliculosa* and *S. cerevisiae* growth, there was an almost total absence of substrate utilization,  
 181 and hence no metabolites released (Table 1); while the productivity of the last strain, the  
 182 osmotolerant *Z. rouxii*, increased significantly from  $0.5 \pm 0.1$  to  $0.8 \pm 0.1 \text{ kg m}^{-3} \text{ h}^{-1}$  for an  
 183 increase of the initial sugar content from  $174.0 \pm 0.2$  to  $358.0 \pm 1.0 \text{ kg m}^{-3}$  (Table 1).

184 The comparison of the ethanol production obtained in this study, in the best conditions, to  
 185 those of the literature with other biomasses, such as soybean molasses [31], sugar beet pulp,  
 186 sugar beet molasses, carrot peel waste [24], shows similar production, namely  $63.0 \pm 0.1 \text{ kg m}^{-3}$   
 187 (this study),  $37 \text{ kg m}^{-3}$ ,  $34 \text{ kg m}^{-3}$ ,  $32 \text{ kg m}^{-3}$ ,  $33 \text{ kg m}^{-3}$ , respectively. Date Syrup from  
 188 industrial by-product appears therefore to be an interesting feedstock for ethanol  
 189 bioproduction. It should also be noted that the date-producing countries are conducting  
 190 studies in order to enhance the conservation and the improvement of the local date-palm  
 191 germplasm [1,6]. It would be also interesting to make a study dealing with technical,

economic and social feasibility at the date producers countries, such as the study of Stewart and Lambert [32] on spatial heterogeneity of factors determining ethanol production site selection in the U.S.

### 3. Conclusions

This study established that the three studied yeasts were able to grow on date by-products (an agri-food residues) leading to ethanol production in batch fermentation. However, the choice of the strain affected the bioproduction of ethanol. Production of high levels of ethanol could be achieved by using osmotolerant yeasts, such as *Z. rouxii* from concentrated date syrup. However, it was preferable to use *S. cerevisiae* if the culture medium is less concentrated in sugar.

Results of alcohol fermentation showed that date juice can be a good feedstock for bioethanol production, and it did not negatively affect human food. However, some questions remain to confirm the relevance of the proposed valorization and before any transposition on an industrial scale. Indeed, 30,000 tons per year of “low quality” dates is it sufficient for an industrial production of biofuel? Is it more interesting to produce high added value products like glycerol? An economic study is therefore needed before any industrial scale-up.

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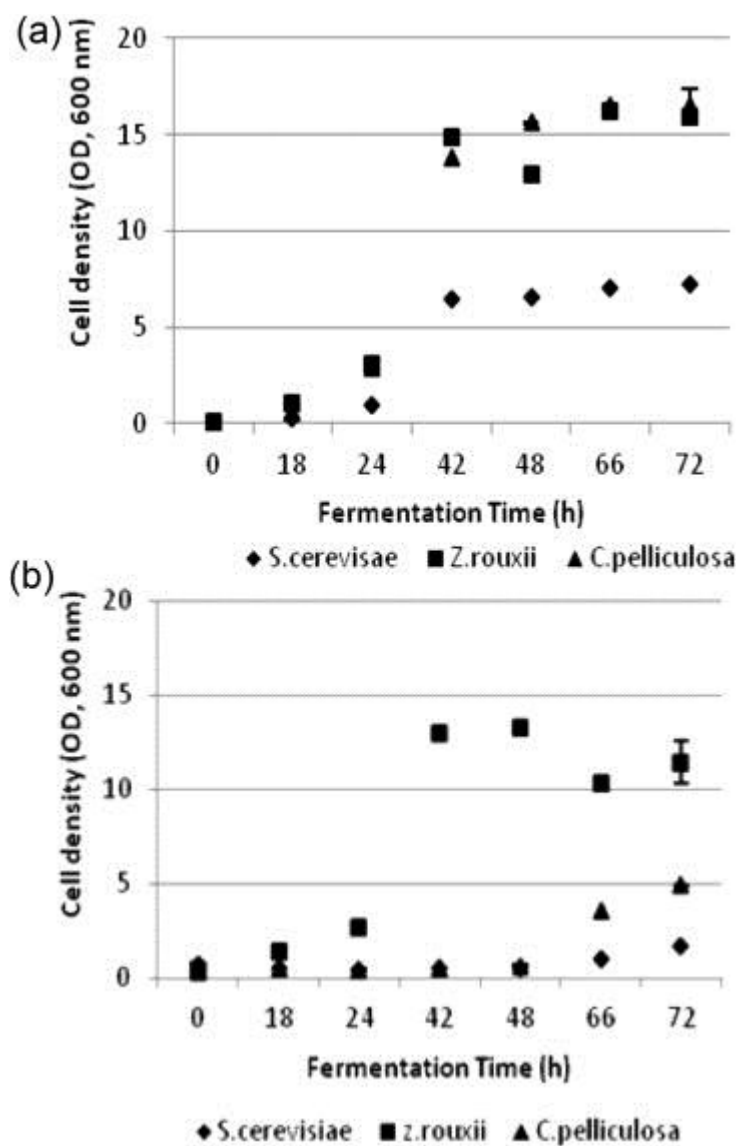
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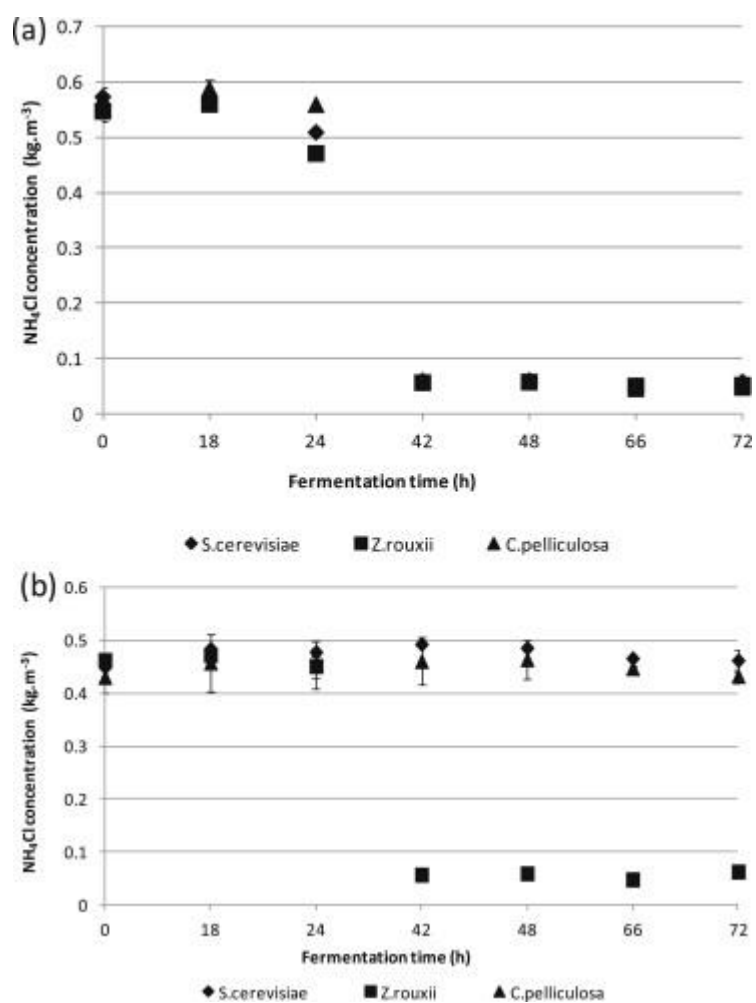
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325 Fig. 1 - Cell density (OD. 600 nm) time-courses during growth of the three considered yeast  
 326 strains in medium containing  $174.0 \pm 0.2 \text{ kg m}^{-3}$  sugar (a) and  $358.0 \pm 1.0 \text{ kg m}^{-3}$  sugar (b).





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328 Fig. 2 -  $\text{NH}_4^+$  concentration time-courses during growth of the three considered yeast strains

329 in medium containing  $174.0 \pm 0.2 \text{ kg m}^{-3}$  sugar (a) and  $358.0 \pm 1.0 \text{ kg m}^{-3}$  sugar (b).

Table 1.

The performance of ethanol production from dates syrup by *S. cerevisiae*, *C. pelliculosa*, *Z. rouxii* after 72 h culture on media containing a sugar amount of 174 kg m<sup>-3</sup> (C<sub>1</sub>) and 358 kg m<sup>-3</sup> (C<sub>2</sub>) expressed as glucose from dates syrup.

| Parameters  | C <sub>1</sub>       |                  |                       | C <sub>2</sub>       |                  |                       |
|---|----------------------|------------------|-----------------------|----------------------|------------------|-----------------------|
|   | <i>S. cerevisiae</i> | <i>Z. rouxii</i> | <i>C. pelliculosa</i> | <i>S. cerevisiae</i> | <i>Z. rouxii</i> | <i>C. pelliculosa</i> |
| Eq Glu (kg m <sup>-3</sup> ) <sup>a</sup>                   | 174.0 ± 0.2          | 169.0 ± 1.7      | 168.0 ± 0.1           | 349.0 ± 0.8          | 357.0 ± 1.0      | 356.0 ± 0.2           |
| Eq Glu consumed (%)   | 94.0 ± 0.2           | 67.0 ± 2.0       | 71.0 ± 0.1            | 4.0 ± 0.1            | 41.0 ± 0.6       | 3.0 ± 0.4             |
| Ethanol (kg m <sup>-3</sup> )                               | 63.0 ± 0.1           | 33.0 ± 2.0       | 41.0 ± 0.3            | — <sup>**</sup>      | 55.0 ± 1.0       | — <sup>**</sup>       |
| Glycerol (kg m <sup>-3</sup> )                              | 5.0 ± 0.1            | 4.6 ± 0.1        | 4.6 ± 0.1             | — <sup>**</sup>      | 10.0 ± 0.1       | — <sup>**</sup>       |
| Y <sub>EtOH/S</sub> (%)                                     | 38.0 ± 0.5           | 29.0 ± 0.1       | 34.0 ± 0.2            | —                    | 38.0 ± 0.1       | —                     |
| Y <sub>Gly/S</sub> (%)                                      | 3.2 ± 0.1            | 4.0 ± 0.1        | 4.0 ± 0.1             | —                    | 7.0 ± 0.1        | —                     |
| Q <sub>Ethanol</sub> (kg m <sup>-3</sup> h <sup>-1</sup> )  | 0.9 ± 0.1            | 0.5 ± 0.1        | 0.6 ± 0.1             | —                    | 0.8 ± 0.1        | —                     |
| Q <sub>Glycerol</sub> (kg m <sup>-3</sup> h <sup>-1</sup> ) | 0.07 ± 0.1           | 0.06 ± 0.1       | 0.06 ± 0.1            | —                    | 0.14 ± 0.1       | —                     |

—<sup>\*\*</sup> < limit of detection.

a Concentration of total sugars in equivalents glucose (kg m<sup>-3</sup>).