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

2

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26

27 **ABSTRACT**

28 Syrup resulting from date by-products constitutes a favorable medium for yeast development,
29 owing to its sugar composition; it was hence tested for ethanol production. Three yeasts,
30 *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii* and *Candida pelliculosa*, were selected
31 for ethanol production on dates syrup. In batch fermentation, the ethanol concentration
32 depended on the initial sugar concentration and the yeast strain. For an initial sugar
33 concentration of $174.0 \pm 0.2 \text{ kg m}^{-3}$, maximum ethanol concentration was $63.0 \pm 0.1 \text{ kg m}^{-3}$
34 during *S. cerevisiae* growth, namely higher than the amounts achieved during *Z. rouxii* and *C.*
35 *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.*
36 *rouxii* was able to grow on $358.0 \pm 1.0 \text{ kg m}^{-3}$ initial sugar amount, resulting in $55.0 \pm 1.0 \text{ kg m}^{-3}$
37 ethanol produced.

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

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49 **1. Introduction**

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

54 The date palm tree constitutes the principal source of remuneration and the basis of economy
55 for people living in the Tunisian Sahara [2]. Today, worldwide production, utilization and
56 industrialization of dates are continuously increasing in some countries like Egypt, Saudi
57 Arabia, Iran and Algeria [4]. In Tunisia the number of cultivars is evaluated for over than 250
58 [5] and is currently the 10th world producer and the first exporter of dates in value. During the
59 last five years, Tunisian production has reached an average of 120.000 tonnes per year with
60 the dominance of the “Deglet-Nour” variety constituting about 60 % of the total production
61 [2] and 50 000 farmers are employed in this sectors. In 2011, Excess dates were 50. 000
62 tonnes, 32 % of which were from low quality dates [6].

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

68 Presently, by-products of dates are discarded or used in limited cases for animal feed [7,9].
69 Fermentation technology is one of the technologies employed for deriving value added
70 products from by-products of dates. The various products derived from date fruit by-products
71 are biopolymers [10,11], organic acids [12,13], amino acid [14], baker’s yeast [15], probiotics
72 [16], antibiotics [17] enzymes [18] and biofuels such as hydrogen [19] and butanol [20].

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

78 Kasavi et al [24] clearly established the importance of choosing the appropriate yeast strain to
79 be used in ethanol production from biological residues; the choice will not only depend on a
80 strain's tolerance to ethanol but also on its ability to utilize carbon sources available in agri-
81 food residues.

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

86

87 **2. Material and methods**

88 **2.1. Microorganism**

89 3 yeast strains were tested, the first *S. cerevisiae* well-known for its ability to produce ethanol,
90 but this yeast is sensitive to osmotic stress; *C. Pelliculosa* has the ability to grow in media of
91 high osmotic pressure induced by sugars or salts; and *Z. rouxii* is well-known for its capacity
92 to grow in rich sugar environments.

93 The fermentative yeasts *Saccharomyces cerevisiae* 522D, *Zygosaccharomyces rouxii* (IP
94 2021.92) and *Candida Pelliculosa* (IP 820.63) were obtained from the culture collection of
95 the Pasteur Institute (Paris, France). Stock cultures were maintained on a gelified medium

96 whose composition was (kg m^{-3}): glucose, 20; peptone, 10; yeast extract, 10; and agar, 10. In
97 all cases, cultures were maintained at 28°C for 24 h and then stored at 4°C .

98 **2.2. Inoculum preparation**

99 A given number of drops (10) of a yeast suspension in $\text{KCl } 150 \text{ mol m}^{-3}$ was grown in 25 cm^3
100 of synthetic medium (kg m^{-3}): glucose, 20; peptone, 10; and yeast extract, 10; in a 250 cm^3
101 bottle on a rotating shaker (New brunswick, INNOVA 40, NJ, USA) at 20 rad s^{-1} , 28°C for 18
102 h. After centrifugation (6000 g , 4°C and 5 min), cells were harvested, resuspended in 25 cm^3
103 $\text{KCl } 150 \text{ mol m}^{-3}$ and recentrifuged in similar conditions. The suspension obtained after
104 harvesting cells and re-suspending in $10 \text{ cm}^3 \text{ KCl } 150 \text{ mol m}^{-3}$ was used to inoculate culture
105 media [25].

106 **2.3. Raw material**

107 By-products dates “Deglet-Nour”, was obtained from a Tunisian conditioning unit of dates
108 “ALKHALIJ”. The fruits were pilled, crushed with a sharp knife and 20 g date pulp were
109 added to 50 g of hot de-ionised water. The extraction was carried out on hot-plate at 85°C for
110 45 min. [26]. The juice was filtered and centrifuged at 6000 g for 30 min and then the
111 supernatant was immediately concentrated to achieve a total sugar concentration of 720.0 ± 1.0
112 kg m^{-3} . The concentrated date juice was then stored at 4°C until use.

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

119 **2.4. Ethanol production medium**

120 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
121 mineral culture medium as described previously by Djelal et al [24]. The pH was adjusted to
122 6.0 using KOH 1000 mol m^{-3} . The medium was transferred into 500 cm^3 bottles with a final
123 working volume of 300 cm^3 , which were autoclaved at 120°C for 20 min before adding the
124 NH_4Cl sterilized by filtration on a $0.2 \text{ }\mu\text{m}$ membrane (Sartorius, Goettingen, Germany).

125 **2.5. Fermentation processes**

126 300 cm^3 of medium containing sugar concentration of 174.0 ± 0.2 or $358.0 \pm 1.0 \text{ kg m}^{-3}$ were
127 inoculated with $100 \text{ }\mu\text{L}$ of yeast suspension. Batch fermentation was carried out in 500 cm^3
128 bottles on an incubator shaker (New Brunswick, INNOVA 40, NJ, USA) at 20 rad s^{-1} , 28°C
129 for 72 h. All experiments were performed in duplicates and samples (5 cm^3) were taken from
130 the culture at regular time intervals.

131 **2.6. Analytical methods**

132 The cell density of the culture medium was measured at 600 nm (A_{600}) using a
133 spectrophotometer (SECOMAM, Alès, France). The culture medium was then centrifuged at
134 6000 g , at 4°C for 5 min and the supernatant was used for the determination of the various
135 metabolites produced by yeasts including ethanol and glycerol, as well as the residual sugar
136 concentrations by HPLC involving an ion exclusion column HPX-87H ($300 \times 7.8 \text{ mm}$; Bio-
137 Rad, Hercules, CA, USA), maintained at 45°C (Oven CrocoCil™; Cluzeau-Info-labo, Ste Foy
138 La Grande, France). The elution was performed at a flow rate of $0.7 \text{ cm}^3 \text{ min}^{-1}$ (waters pump,
139 Milford, MA, USA) using sulfuric acid 0.5 mol L^{-1} . A Shimadzu RIO-6A Refractive Index
140 Detector (Japan) was used for the detection of the various compounds (glucose, fructose,
141 sucrose, ethanol and glycerol) [27]. In addition, NH_4Cl concentration was analyzed by the

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

144 3. Results and Discussion

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

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

154 As expected, there was a clear link between sugars consumption and growth since both
155 parameters followed similar trends, namely a higher consumption was recorded for the lowest
156 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-
157 inhibitory sugar amount ($174.0 \pm 0.2 \text{ kg m}^{-3}$), a high yield of sugars consumption was observed
158 for the three yeasts after three days culture, namely 94, 71 and 67 % for *S. cerevisiae*, *C.*
159 *pelliculosa* and *Z. rouxii* respectively (Table 1).

160 The production of the main metabolites was also and as expected linked to growth, since both
161 ethanol and glycerol productions were observed for the three yeasts for a sugar content of
162 $174.0 \pm 0.2 \text{ kg m}^{-3}$ in the culture medium (Table 1); while in the presence of $358.0 \pm 1.0 \text{ kg m}^{-3}$
163 sugar content in the medium, metabolites production was only observed for *Z. rouxii*. It
164 should be observed that the highest ethanol production was observed for *S. cerevisiae* (Tab.1),
165 in agreement with its well-known use for such production [30], while *C. pelliculosa* and *Z.*
166 *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 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 ± 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 ± 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 kg m^{-3} , 34 kg m^{-3} , 32 kg m^{-3} , 33 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,

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

195

196 **3. Conclusions**

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

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

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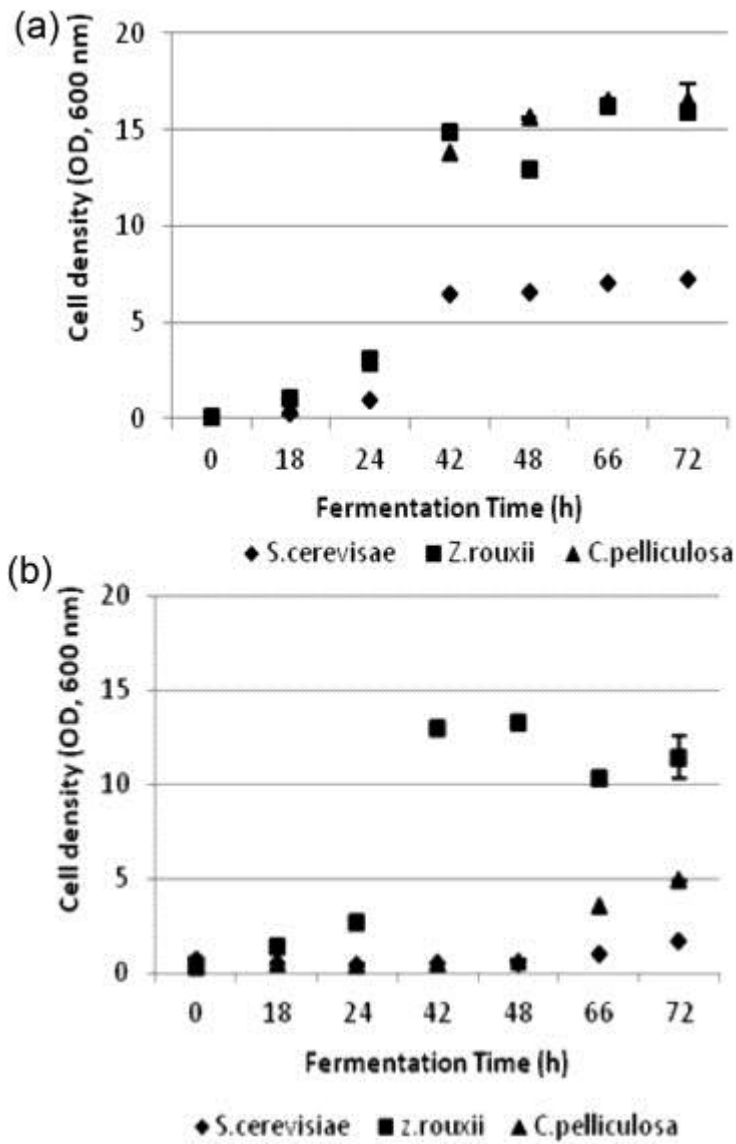
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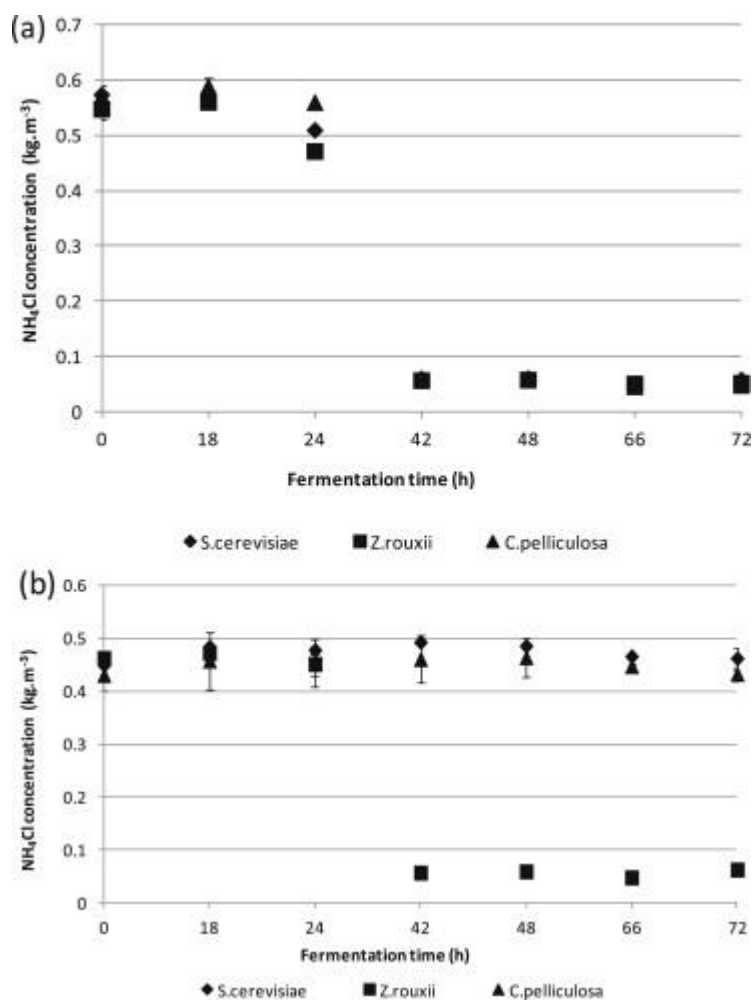
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323 **Figure captions**



324

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).



327

328 Fig. 2 - 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⁻³ (C₁) and 358 kg m⁻³ (C₂) expressed as glucose from dates syrup.

Parameters	C ₁			C ₂		
	<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 ⁻³) ^a	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 ⁻³)	63.0 ± 0.1	33.0 ± 2.0	41.0 ± 0.3	–**	55.0 ± 1.0	–**
Glycerol (kg m ⁻³)	5.0 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	–**	10.0 ± 0.1	–**
Y _{EtOH/S} (%)	38.0 ± 0.5	29.0 ± 0.1	34.0 ± 0.2	–	38.0 ± 0.1	–
Y _{Gly/S} (%)	3.2 ± 0.1	4.0 ± 0.1	4.0 ± 0.1	–	7.0 ± 0.1	–
Q _{Ethanol} (kg m ⁻³ h ⁻¹)	0.9 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	–	0.8 ± 0.1	–
Q _{Glycerol} (kg m ⁻³ h ⁻¹)	0.07 ± 0.1	0.06 ± 0.1	0.06 ± 0.1	–	0.14 ± 0.1	–

–** < limit of detection.

a Concentration of total sugars in equivalents glucose (kg m⁻³).

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