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An effective acid pretreatment of agricultural biomass residues for the production of second-generation bioethanol

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2 An effective acid pretreatment of agricultural biomass residues 3 for the production of second-generation bioethanol

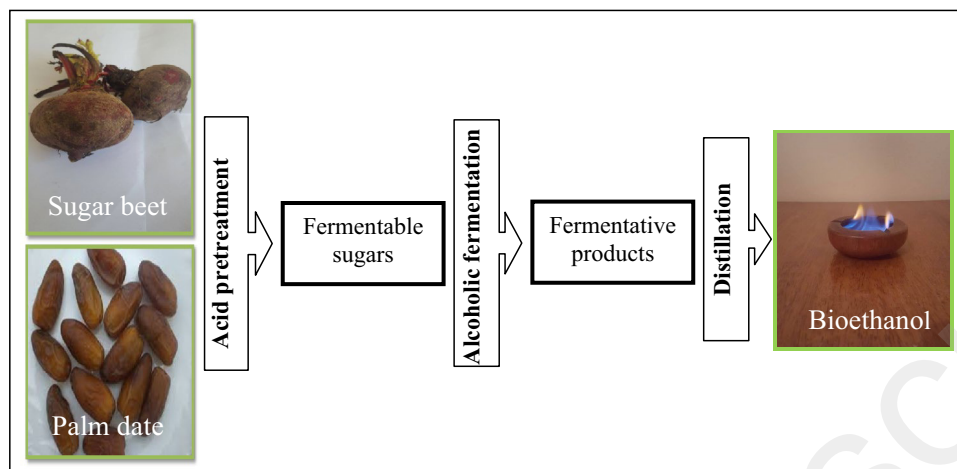
4 Ibtissem Fennouche¹ · Nabila Khellaf¹  · Hayet Djelal² · Abdeltif Amrane³

6 Abstract

7 Currently, the potential for energy recovery of plant biomass by biotechnological processes is a preferred solution for the
8 use of agricultural products of low commercial value in order to produce bioenergy that is alternative to fossil fuels. The
9 objective of this study was to obtain second-generation bioethanol by valorization of sugar beet and common dates of
10 low quality. This involved separate hydrolysis and anaerobic fermentation process using the yeast strain *Saccharomyces*
11 *cerevisiae*. Physicochemical and biochemical analyzes were carried out on beet and date substrates before and after alco-
12 holic fermentation to determine their effect on yeast activity. The results showed that palm date was a good substrate
13 for the microorganisms in contrast to sugar beet which required a high pretreatment in order to hydrolyze sucrose into
14 fermentable sugars. After distillation, it was possible to recover bioethanol with a quality and a concentration depend-
15 ing on the substrate nature. For an initial sugar concentration of 12.0 °Brix, 74.7 g/kg DM of bioethanol (92.4 g/L) was
16 produced by *Saccharomyces* from date syrup. In the presence of concentrated date syrup (22.2 °Brix), a lower efficiency
17 of ethanol production was observed (78.2 g/L). It can be conclude that the diluted date substrate led to a good quality
bioethanol with a high production yield.

A1 *Statement of novelty* In this project, we tried to valorize agricultural residues (Algerian sugar beet and palm date of low quality) for the
A2 production of bioethanol which can substitute fossil energies. Algeria is considered as one of the major producers and exporters of palm
A3 dates in the world, and thousands of tons of dates remain unused. Thus, these agricultural by-products could be recovered instead of
A4 being released into the environment by polluting it. In order to produce bioethanol, an effective pretreatment and optimal fermentation
A5 conditions were used. This project is part of a strategy to increase the feedstock exploitation and decrease the residues while producing
A6 clean and renewable energy.

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20

21 **Keywords** Alcoholic fermentation · Beet juice · Bioethanol · Date syrup · Energy recovery · *Saccharomyces cerevisiae*

1 Introduction

Fossil fuels (oil and natural gas) provide fast and efficient tools of transport as well as a good source of several industrial activities. However, their reserves are limited and security of supply is problematic for many countries importing this kind of fuels. On the other hand, their contribution to environmental pollution is a major problem [1]. Contemporary societies are always interested in finding substitutes for these fuels which must be renewable and environmentally sustainable. Thus, the waste-based energy rich in renewable organic matter (biomass) constitutes good raw materials for many industries and the recovery of biomass by biotechnological processes would be an economical and sustainable solution of energy supply. Special attention should be paid to agricultural by-products (sugar beet, palm date, sugar cane, wheat, fruits, vegetables, etc.) since some fruits and vegetables of low quality cannot be integrated into human nutrition as they may have colors, tastes and aromas that are not appreciated [2–4]. Thus, a considerable part of this biomass is recognized as waste and not conflicting with food availability.

Agricultural residues are rich in organic matter but are unfortunately underutilized and often end up being pollutants of the environment. These by-products can be valorized in order to produce bioethanol which can be used as alone or can be blended with gasoline as transportation fuels [5–7]. Bioethanol can be produced through fermentation of any raw materials as long as it contains sugar [8, 9]. Vegetables and fruits are very rich in carbohydrates. A successful conversion of these carbohydrates is considered

as the most crucial step for bioethanol production. Thus, an efficient pretreatment should increase the content of fermentable sugars (glucose, fructose, galactose, etc.) and will result in a good yield of fermentative bioethanol [6, 10, 11]. Palm date is very rich in sucrose, fructose and glucose. Other simple sugars (oses) were identified in some date varieties; it is arabinose, xylose, galactose and mannose present at very low concentrations [12]. Palm date can therefore be good candidates for the production of bioenergy. In Algeria, one of the leading countries in the production and export of dates in the world, palm date cultivars are numerous and poorly exploited, with the exception of Deglat-Nour, Ghars, Deglat- Beida and Mech-Deglat, which are of major economic importance [12]. Thousands of tons of dates remain unused and can exceed 50% of year-round production, which could be valorized [13, 14]. Sugar beet is also a typical feedstock for bioethanol production which is rich in hemicelluloses (24–32%), and cellulose (22–30%) (It has very low lignin content) [15]. The juice is fermented by yeast or bacteria. The chemical composition of sugar beet reveals a high content of fermentable sugars (arabinose, mannose and xylose) [16]. The pulp, once drained, is used as animal feed or sold to the chemical, pharmaceutical or food industry.

The yeast *Saccharomyces cerevisiae* has long been an efficient agent for ethanol production at laboratory and industrial level with high efficiency [17–19]. It is considered as the world's premier industrial microorganisms being the best exploited microorganism in terms of both old and new biotechnologies [20]. The yeast tolerates a wide range of pH with an optimum under acidic

83 conditions, which makes its fermentation less susceptible
84 to infection than bacteria [21]. It also tolerates ethanol and
85 inhibitory compounds better than other ethanol produc-
86 ing microorganisms. *S. cerevisiae* is considered as of major
87 economic and social significance in human culture since it
88 has long been used to produce alcoholic beverages (beer
89 and wine) and ferment bread.

90 The objective of the present project was to propose a
91 method of valorization of agricultural products in order
92 to produce second-generation bioethanol. We opted for
93 Algerian palm dates and sugar beets of low quality. *Sac-*
94 *charomyces cerevisiae* was the microorganism used to
95 perform the alcoholic fermentation. The main goal was
96 to develop an effective pretreatment of the selected raw
97 material and alternative and efficient process for bioetha-
98 nol production from a large variety of agro-industrial
99 wastes.

100 2 Materials and methods

101 2.1 Reagents

102 All the chemicals used in the present work (acids, nutrient
103 salts, etc.) were purchased from Merck.

104 2.2 Selection and pretreatment of raw material 105 (palm date and sugar beet)

106 The sugar beet, *Beta vulgaris* from Algeria was used in the
107 present work. It is a vegetable with a tuberous root, in
108 which high amounts of sugar accumulate. The palm date
109 used was Deglet-Nour which is the edible fruit of the date
110 palm *Phoenix dactylifera*. It is highly coveted inside and
111 outside the country because of its flavor and nutritional
112 quality; it is a fleshy fruit containing an elongated core
113 marketed mostly in the form of dry dates. The two prod-
114 ucts used in this work are of lower quality to be taken as
115 residues of agriculture.

116 For the preparation of date syrup and beet juice, some
117 pretreatments were carried out. They differ according to
118 the nature of the raw material; the protocol was described
119 in Fig. 1. In the case of sugar beet, two kinds of juice were
120 prepared: beet juice with liming (BJL) and beet juice with-
121 out liming. The operation of liming consists of a clarifica-
122 tion of beet juice by calco-carbonic purification (addition
123 of CaCO_3) in order to precipitate impurities. To obtain BJL,
124 40 ml of CaCO_3 (10 g/L) was added to the filtrate. Calcium
125 carbonate fixes and precipitates impurities. The precipitate
126 was then removed by a second filtration which led to a
127 clear beet juice. Hydrolysis and neutralization were then
128 carried out in the same way as for pretreatment without

liming. The extracted date syrups and beet juices were
stored in hermetic flasks at 4 °C until use.

The extraction was operated in a 500-ml flask equipped
with a reflux condenser at a temperature of 65 °C during
30 min. This allowed a better extraction of sugars and a
good consumption of the vegetable pulp.

2.3 Characteristics of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae selected in the present work
was that marketed in Algeria. It was inoculated in nutri-
ent broth (supplemented with glucose and salts) at 30 °C
under shaking conditions to ensure the growth and activ-
ity conditions of the yeast. Cell density referred to the num-
ber of living cells per unit volume was measured at 600 nm
(see Analytical techniques).

2.4 Alcoholic fermentation experiments

The fermentation assays were carried out in a 1 L-batch
bioreactor under magnetic stirring. The temperature was
maintained at 30 ± 2 °C. A volume of 500 ml of slightly
heated juice or syrup (pH=4.5) was placed in the fer-
menter supplemented with nutrients necessary to pro-
mote rapid cell growth. Two grammes of yeast were then
added to the reactor which must be quickly closed with a
hermetic plug to ensure anaerobic conditions. Fermenta-
tion began once the release of CO_2 was observed. The fer-
mentation time was fixed at 72 h after which the bioetha-
nol was recovered by distillation of the fermented mixture.
The distillation temperature was of 78 °C. It was carried out
in conventional equipment containing a heating bottle,
refrigeration columns and a distillate recovery bulb.

Liquid samples for bioethanol determination were col-
lected at the end of fermentation and filtered by a 0.45 μm
Millipore hydrophilic filter. The amount of CO_2 released
was measured by the liquid displacement method [13]
(Fig. 2).

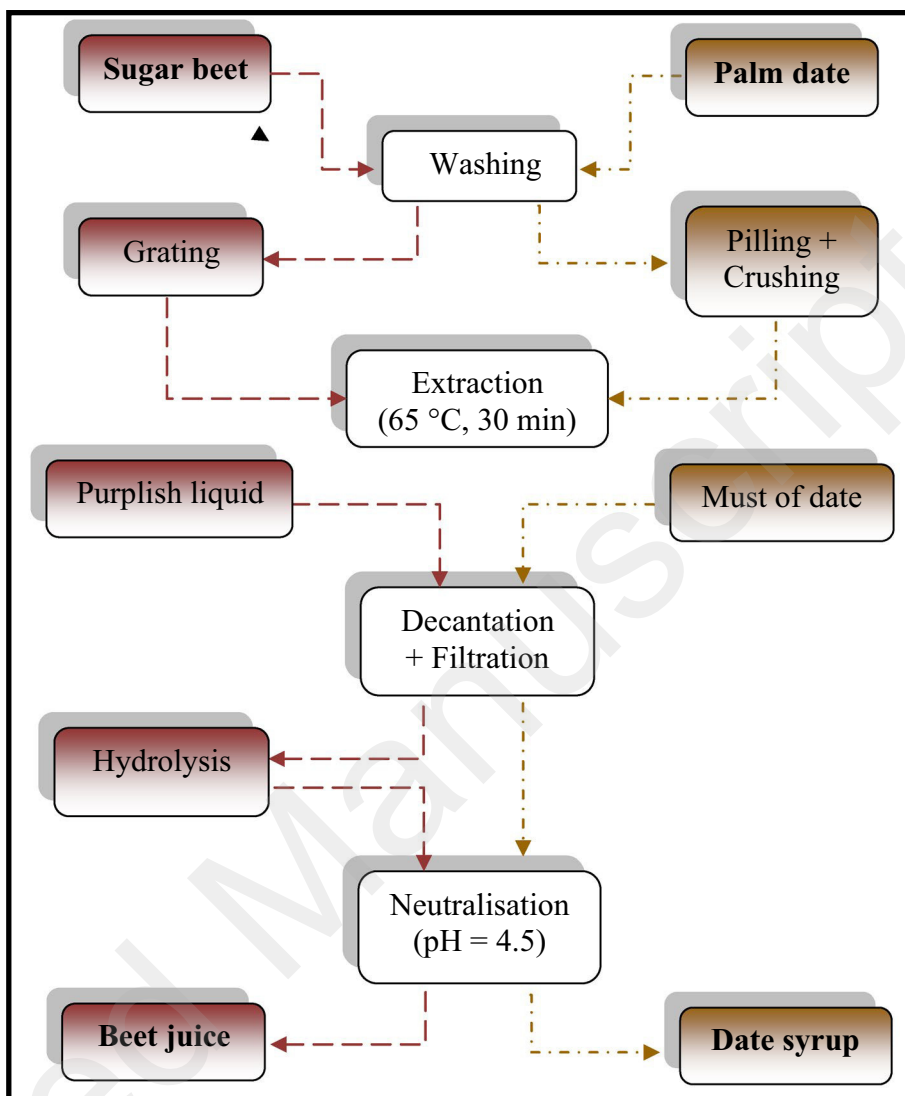
2.5 Analytical techniques

2.5.1 Characterization of beet juice and date syrup

Juice and syrup produced from raw materials were charac-
terized by some physicochemical and biochemical param-
eters such as pH, mass soluble solids (Brix), dry matter,
total sugars and cell density.

- The Brix, measured by a refractometer, is a measure
of total soluble solids (including sugars). It is based
on the ability of a juice's sugar to deflect light. In the
present work, the Brix of beet juice and date syrup

Fig. 1 Pretreatments of sugar beet and palm date for the extraction of beet juice and date syrup



173 was made by measuring the refractive index at 20 °C
174 using an ATAGO RX-5000 type refractometer.

- 175 • The pH which is essential for the control of the sub-
176 strate before and during fermentation is an indica-
177 tor of the metabolic activity of the yeast during the
178 transformation of sugars. Its measure was carried out
179 by a pH-meter Inolab pH 7110.
- 180 • The dry matter (DM) was determined by drying in an
181 oven at a temperature of 105 °C until a constant weight
182 a sample of 10 ml of juice or syrup. It is expressed as:

$$183 \quad DM(\%) = \frac{M_1 - M_0}{M_2 - M_0} \times 100$$

184 M_0 is the mass of the empty capsule (g), M_1 is the mass
185 of the capsule and the residue after desiccation (g), M_2 is
186 the mass of the capsule and the sample (g).

- Total sugars were determined by the phenol–sulfuric acid
method based on the absorption of light at 448 nm [22].
A Secomam Prime-type spectrophotometer was used for
this purpose.

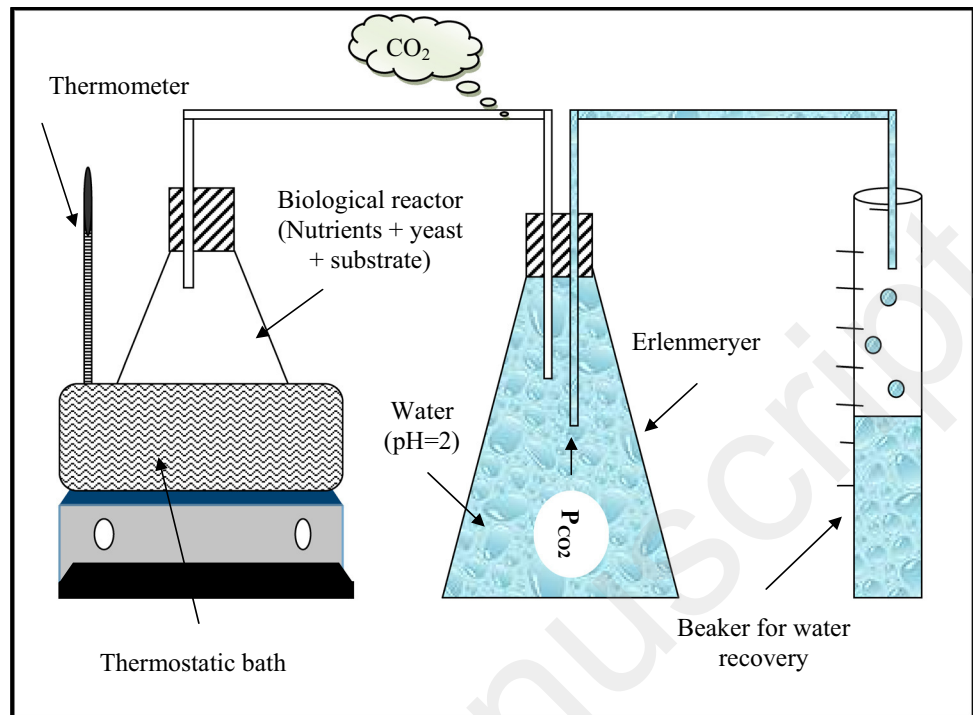
2.5.2 Cell density

Cell density was measured at 600 nm (A600) using a Seco-
mam Prime-type spectrophotometer. A600 values were
converted into cell density (10³ kcell/mL) by using a stand-
ard curve [23].

2.5.3 Characterization of bioethanol

The fermentative bioethanol was characterized by its
physical appearance, smell and flammability. Its density,
weight and concentration were also measured. Common
techniques were used for this purpose.

Fig. 2 Experimental setup of alcoholic fermentation



201 **2.5.4 Pressure of CO₂**

202 The biological conversion was followed by measuring the
 203 CO₂ release resulting from the fermentation following the
 204 degradation of sugars; CO₂ production was measured by
 205 displacement of water (Fig. 2). The reactor was connected
 206 via a tube to an Erlenmeyer flask filled with water at pH 2.
 207 The latter was connected by a second tube to an empty
 208 container. The CO₂ produced in the reactor enters the flask
 209 exerting a pressure that will expel a quantity of water pro-
 210 portional to this pressure. This quantity of water recovered
 211 in the second container has a volume equivalent to the
 212 CO₂ pressure.

213 The results were analyzed using a one-way analysis
 214 of variance (ANOVA). Comparison between the differ-
 215 ent treatments was statistically analyzed and the valid-
 216 ity of investigation was expressed as probability value of
 217 $p < 0.05$.

3 Results and discussion

3.1 Physicochemical proprieties of beet juice and date syrup

221 Pretreatment is an important step in the conversion of
 222 biomass to bioethanol. In this study, an effective acid
 223 pretreatment was chosen to treat date and beet in order
 224 to convert the biomass into fermentable products. The
 225 different proprieties of beet juice and date syrup before
 226 conducting fermentation are given in Table 1. The five
 227 substrates appeared syrupy and viscous with a density
 228 varying between 0.99 and 1.05. Their color differs accord-
 229 ing to the nature of the raw material. Liming operation
 230 led to a beet juice with a lighter red color probably due
 231 to the elimination of impurities and some pigments. The
 232 pH of concentrated and diluted beet juice was around

Table 1 Physicochemical proprieties of beet juice and date syrup before fermentation

Parameter	Beet juice			Date syrup	
	4.8 °Brix	3.1 °Brix ^a	2.2 °Brix	22.1 °Brix	12 °Brix
pH (20 °C)	4.18	6.66	4.29	6.30	4.10
Density (20 °C)	1.01	1.05	0.99	0.99	0.99
Total sugars (mg/L)	47.14	30.07	17.21	220.1	110.8
Color	Dark red	Very light red	Light red	Dark brown	Light brown

Three independent experiments were performed for each assay. The values are expressed as mean ± SD where $0.01 \leq SD \leq 0.5$

^aBeet juice with liming

233 4.2 which is an optimal value for the development of
 234 *S. cerevisiae*. The beet juice with liming had a pH value
 235 close to neutrality due to CaCO_3 supplied during the liming
 236 operation. Concentrated beet juice was richer in total
 237 sugars than the two others with a value of 47.14 mg/L.
 238 The liming operation was probably responsible for the
 239 lost of sugar amount from the juice (30.07 mg/L). Con-
 240 centrated date syrup containing 220.1 mg/L of total
 241 sugar is considered as the most sugar-rich raw mate-
 242 rial used in the present study with a concentration of
 243 220.1 mg/L.

244 3.2 Fermentation monitoring

245 *Saccharomyces cerevisiae* represents the organism of
 246 choice for the production of ethanol at laboratory and
 247 industrial scale [23]. In this work, the time course of
 248 fermentation process was studied using the five sub-
 249 strates as a carbon source during 72 h. The medium
 250 was, as described above, adjusted to initial pH of 4.5
 251 and fermented at temperature of 30 ± 2 °C. The Yeasts
 252 also require appropriate supplies of nutrients, in order
 253 to efficiently carry out fermentation. Typical profiles of
 254 CO_2 production were obtained for all the five substrates
 255 (Fig. 3). It was possible to define three standardized
 256 phases in the curves: (1) a lag phase (of about a few tens

of minutes) during which the yeasts adapt to the process
 conditions, (2) an exponential phase leading to assess
 the maximal biomass, and (3) a stationary phase. The
 highest biomass was reached at 3.0, 3.5 and 3.6 h in the
 presence of beet juice with 2.2, 4.8 and 3.2 °Brix, respec-
 tively (Fig. 3a). The yeast reached its highest biomass in
 the presence of date syrup (12 °Brix) after 10 h of fermenta-
 tion (Fig. 3b). The concentrated syrup appeared to be
 less favorable than the diluted one for the development
 of yeast; this effect is probably attributed to the osmotic
 stress resulting from high sugar concentration of con-
 centrated date syrup [23] or caused by some by-products
 (acids, aldehydes, esters, etc.) and/or non-metabolized
 components which can inhibit the yeast growth [24].
 From these results, it is expected that the diluted date
 syrup would give a good quality bioethanol since alco-
 hol production and yeast growth are inextricably linked
 [25].

275 3.3 Physicochemical and biochemical 276 characterization of fermentative products

277 The fermentable sugars are metabolized by the yeasts
 278 that carry out bioconversion into bioethanol via fermenta-
 279 tion. As well known, *S. cerevisiae* conduct fermentative
 280 metabolism to ethanol and carbon dioxide (as the primary
 281 fermentation metabolites) and also produce numerous
 282 secondary metabolites [25]. After alcohol fermentation, it
 283 was observed a decrease in the pH for all the substrates
 284 used in the resent work. This decrease was due to the for-
 285 mation of the carbon dioxide following the conversion of
 286 sugars into ethanol and making the medium more acid.
 287 This result was reported in several studies related to fer-
 288 mentation process with yeasts [3, 26]. In a similar trend,
 289 total soluble solids (Brix) and total sugars decreased for
 290 the five substrates. Total soluble solids decreased by more
 291 than 60% in the case of beet juice (4.8 °Brix) and date syrup
 292 (12.0 °Brix). The yeast consumed more than 50% sugars of
 293 beet after 72 h of fermentation. Total sugars contained in
 294 concentrated syrup date were not consumed more than
 295 18% while those contained in the diluted syrup date were
 296 converted to more than 63%. The cell density of yeast led
 297 to estimate the quality of the fermentation process. The
 298 maximum cell density of *S. cerevisiae* in the present fer-
 299 mentation conditions was $8.4 \cdot 10^3$ kcell/mL with 12 °Brix of
 300 date syrup while the minimum cell density ($3.6 \cdot 10^3$ kcell/
 301 mL) was obtained for concentrated date syrup. This result
 302 was confirmed by the formation of dense foam during the
 303 fermentation process. Finally, the results summarized in
 304 Table 2 show that the diluted syrup date represents the
 305 best substrate for the development of *S. cerevisiae* and the
 conversion of sugars.

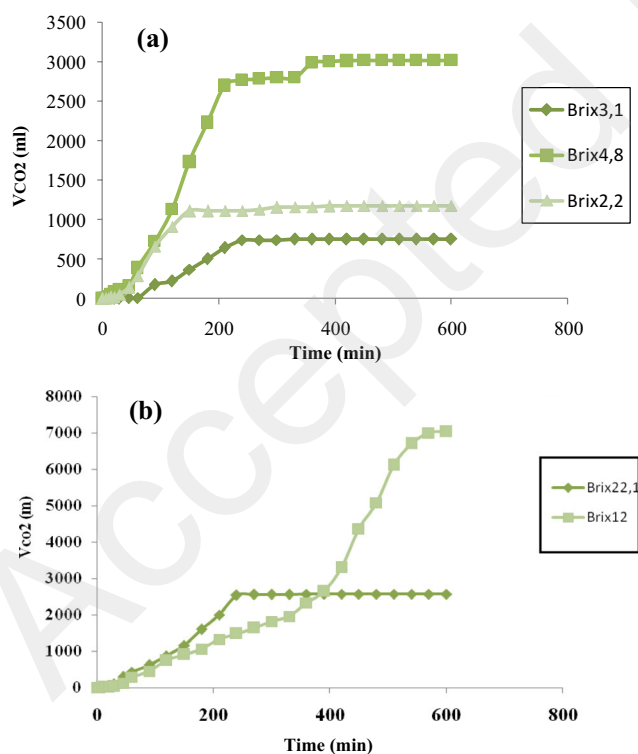


Fig. 3 Time course of fermentation for a beet juice and b date syrup for different Brix values

Table 2 Physicochemical and biochemical characteristics of fermentative products

Parameter	Beet juice			Date syrup	
	4.8 °Brix	3.1 °Brix ^a	2.2 °Brix	22.1 °Brix	12 °Brix
pH (20 °C)	3.54	4.06	3.70	4.70	3.90
Density (20 °C)	0.99	1.04	0.97	0.99	0.96
Total soluble solids (°Brix)	1.8	2.4	1.5	19.5	4.5
Total sugars (mg/L)	21.93	19.57	5.14	180.5	40.5
Cell density (10 ³ kcell/mL)	6.7	5.6	6.0	3.6	8.4
Dry matter (g/L)	6.7	2.5	4.9	21.3	43.5

Three independent experiments were performed for each assay. The values are expressed as mean ± SD where 0.01 ≤ SD ≤ 0.5

^aBeet juice with liming

Table 3 Characterization of bioethanol produced by distillation

Substrate	d (/)	Volume (ml)	Yield ^b (g/kg DM)	Concentration (g/L)
Beet juice (4.8 °Brix)	0.86	17	28.95	36.5
Beet juice ^a (3.1 °Brix)	0.85	14.5	24.40	30.8
Beet juice (2.2 °Brix)	0.88	8.0	14.22	17.6
Date syrup (22.1 °Brix)	0.86	36.4	63.24	78.2
Date syrup (12 °Brix)	0.88	42.0	74.67	92.4

^aBeet juice: Beet juice with liming

^bThe yield is defined as ratio of bioethanol to the mass of substrate

3.4 Bioethanol yield and concentration

The physical and chemical pretreatments performed on beet and date materials were required to reduce recalcitrance biomass for hydrolyzing complex sugars (mainly sucrose) into simple intermediate products and increase the reactive surface area [2–21]. A comparison of the yield and concentration of ethanol of all fermentation processes is presented in Table 3. The values showed that alcohol obtained from the three substrates extracted from sugar beet had low concentration and yield (< 29 g/kg DM) suggesting that this raw material requires high pretreatments to hydrolyze sucrose into fermentable sugars. In parallel, for an initial soluble solids concentration of 12.0 °Brix, 92.4 g/L of bioethanol was produced by *S. cerevisiae* from date syrup. This concentration corresponded to an ethanol yield of 74.67 g/kg DM. In the presence of concentrated date syrup (22.2 °Brix), a lower efficiency of ethanol production was observed (78.2 g/L). These results corroborate with those of Chniti et al. [23] which attributed this effect to the osmotic stress resulting from high sugar

concentration. Joshi et al. [24] on the other hand, assessed that the low efficiency of ethanol production may be due to an inhibition of the yeast growth, and consequently alcohol fermentation caused by some by-products or non-metabolized components. A flammability test was conducted with this bioethanol; a very intense and sustainable flame was obtained proving its good quality as a fuel. Although alcoholic fermentation with *S. cerevisiae* has long been improved, the present results showed that lower quality of Algerian dates, which cannot be integrated into human nutrition and are useless as animal feed due to low protein content [27] can be valorized for the production of ethanol with good quality that could be used as fuel. On the other hand, sugar beet requires high pretreatments to hydrolyze some complex molecules (starch, cellulose, etc.) into fermentable sugars.

4 Conclusion

Regarding the efficiency of the fermentation, in respect to *S. cerevisiae* growth and ethanol yield, the results seemed to be promising. The ethanol yield was 74.67 g/kg dry matter (DM) of original date material with a concentration of 92.4 g/L. Syrup extracted from Algerian dates of lower quality has the potential to be an industrially useful substrate for producing second-generation bioethanol. However, sugar beet requires high pretreatments to improve biomass digestibility by hydrolyzing complex sugars into fermentable sugars. Finally, to increase even more the productivity of this alcoholic fermentation process, factors inhibiting the bioethanol production should be identified and overcome. Thus, the extrapolation of the present process to the semi-pilot and pilot scale will be possible. Also, the search for other microorganisms capable of accelerating the process of alcoholic fermentation, increasing the conversion rate and producing a good ethanol is strongly encouraged.

363 **Author contributions** All authors contributed to the study concep-
364 tion and design. Material preparation, data collection and analysis
365 were performed by Ibtissem FENNOUCHE and Nabila KHELLAF. The
366 first draft of the manuscript was written by Nabila KHELLAF and all
367 authors commented on previous versions of the manuscript. All
368 authors read and approved the final manuscript.

369 Compliance with ethical standards

370 **Conflict of interest** The authors declare that there is no conflict of
371 interest.

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