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BY-PRODUCTS OF DATES: OPTIMIZATION OF THE EXTRACTION OF JUICE USING RESPONSE SURFACE METHODOLOGY AND ETHANOL PRODUCTION

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ABSTRACT

The optimal extraction conditions were determined for by-product of date fruit by using the response surface design method. The obtained juice was used for the production of ethanol by fermentation of free cells of *Saccharomyces cerevisiae*. Optimal conditions for date juice extraction were found to be 80°C, 60 min, 1:2 dilution (fruit on water ratio) according to the result of response surface analysis (Equivalents glucose: 219 $g.L^{-1}$). *Saccharomyces cerevisiae* showed a preference for glucose over fructose and among the tested total sugar concentrations, namely 50, 100, 174 and 358 $g.L^{-1}$, 174 $g.L^{-1}$ appeared to be the optimal amount, leading to 70 $g.L^{-1}$ ethanol concentration after 66 h of fermentation,; while an inhibitory effect of a high sugar content, 358 $g.L^{-1}$ of total sugars, namely about 2 mol/L of monosaccharide like glucose or fructose was also shown. Overall, this study suggested that date juice can be utilized for ethanol production.

Keywords: by-product, optimal extraction, response surface analysis, fermentation, *Saccharomyces cerevisiae*, ethanol

INTRODUCTION

Date, the fruit of the date palm tree (*Phoenix dactylifera* L.), is one of the oldest fruit crops grown in arid areas of North Africa and Middle East. **(Chandrasekaran and Bahkali, 2013)**.
Tunisia is the 9th word producer **(FAOSTAT, 2015)** and the first exporter of dates in value **(Besbes *et al.*, 2009)**. The date palm tree *Phoenix dactylifera* L. constitutes the basis of economy for people living in Tunisian Sahara **(Besbes *et al.*, 2009)**. Tunisian production has reached 120 000 tons per year with the dominance of the "*Deglet-Nour*" variety constituting about 60% of the total production **(Besbes *et al.*, 2009)** and **(Kchaou *et al.*, 2013)**. This production progress is unfortunately accompanied by a substantial increase of loss during storage, commercialization and conditioning process **(Abbès *et al.*, 2011)**. Date by-products, are not consumed because of their low quality (inadequate texture, microbes contamination and/or insect infestation).
Presently, very little use is made of these by-products and they are discarded or used in limited cases for animal feed **(Abbès *et al.*, 2011)**. Research into date by-products has not been a true reflection of the importance and the potential of this crop, while dates are a rich source of some nutrients and sugars (70-80%) such as glucose, fructose and sucrose **(Al-Farsi *et al.*, 2011; Elarem *et al.*, 2008; Elleuch *et al.*, 2008**. Due to the high mineral and carbohydrate content of dates, juice has been utilized for the production of value added products, especially by fermentation.
Recently, there has been an increased attention in the field of bioenergy as world energy consumption has increased. Ethanol is a renewable energy with a high efficiency and a low environmental impact. Bioethanol can be obtained from a variety of feedstocks using cellulosic, starchy and sugar sources. These feedstocks include corn, sugar cane, bagasse, sugar beet, sorghum, barley, potatoes, wheat, wood and other biomass materials **(Ibeto *et al.*, 2011)**. Date's fruit can also be used for bioethanol production after sugar and nutriments extraction. One of the most expensive steps of bioethanol production from biomass is the pretreatment; so it is necessary to propose an easy process in accordance with economic and environmental aspects. In general, pretreatment technologies are divided into four major groups i.e. physical, chemical, physico-chemical and biological. Although each extraction method has some advantages, one method cannot be the most relevant for all types of biomasses and products. For Date juice different methods were tested like soxhlet and solvent extraction **(Louhichi *et al.*, 2013)**. The energy consumed by the soxhlet technique was too important for an eco-friendly process; while the use of solvent extraction can present some risks. Otherwise enzymatic extraction gave good results but this process appeared too expensive for an industrial application **(Chandrasekaran and Bahkali, 2013)**, therefore, it's necessary to optimize this step.
Response surface methodology (RSM) is a collection of statistical and mathematical techniques that has been successfully used for developing, improving and optimizing biochemical process **(Chandrika and Freidoon, 2005; Lee *et al.*, 2011; Luo, 2012)**. When many factors and interactions affect the desired response, RSM is an effective tool for optimizing the process **(Cai *et al.*, 2007)**. Response surface methodology is often considered, since it allows determining the effect of factors on characteristic properties, the best optimal conditions of process and parameter interactions **(Cai *et al.*, 2007)**. Therefore, it is less laborious and more informational than other approaches **(Wang *et al.*, 2007)**. Box-Behnken (BBD) is a type of response surface design. It is an independent quadratic design, since it does not contain an embedded factorial or fractional factorial design. In this design the treatment combinations are at the midpoints of edges of the process space and at the center. This design is rotatable (or near rotatable) and require 3 levels of each factor. It is more efficient and easier to arrange and interpret experiment in comparison with others. It is widely used in many researches **(Zhu and Liu, 2013; Khajet, 2011; Sun *et al.*, 2010; Zhao *et al.*, 2009)**.
In this paper, RSM was employed for the extraction of sugars from by-products of dates "*Deglet-Nour*". The aim of this research was to develop an approach that would bring a better understanding of the combined effects of the key processing variables (extraction time, extraction temperature and ratio of raw material to water) on the desired response (Equivalents glucose on date juice), as well as to look for optimal conditions of sugars extraction from by-products of dates. Secondly, the obtained juice was used to produce ethanol by fermentation of free

cells of *Saccharomyces cerevisiae*, since it is traditionally used for alcohol beverage and bioethanol production; however, its performance during fermentation is compromised by the impact of sugar concentration.

MATERIAL AND METHODS

Extraction and response surface analysis

Byproduct dates of "*Deglet-Nour*" were supplied from Tunisian conditional unit of dates "ALKHALIJ". The fruits were pitted and cut in small pieces with a knife. Date pulp was added to hot distilled water at a weight to volume ratio of 1:2.5. The extraction was carried out on hot-plate at 85°C for 45 min **(Acourene *et al.*, 2011)**. The juice was filtered and centrifuged at 5000 rpm, at 4°C for 30 min and then the obtained supernatant was immediately concentrated to a total sugar concentration of 720 $g.L^{-1}$ (72 °Brix) on a hot plate at 80°C and then stored at 4°C.

In order to extract sugar from dates, extraction conditions were determined by Box-Behnken response surface method (Box and Behnken, 1960) to examine the relationship between one or more response variables. The advantages of this method are a reduced number of samples and replicates (12 edges for three factors with three levels, for a total of 12 data points) and the center of the factor space (center point is replicated three times; totally 12+3 = 15 data points), whereas the full factorial design has 27 data points (3 factors x 3 levels x 3 replicates). JMP 10, statistical software (Sas Institute, Cary, NC, USA) was used to design the best extraction conditions for date by giving the minimum and maximum values of determined parameters (Table 1).

Table 1 Box-Behnken response surface method design extraction parameters

Variable	Minimum	Maximum
Temperature (°C)	70	90
Time (min)	30	90
Dilution*	1:2	1:4

* dilution 1:2 means a mixture of 20 g of pulp dates and 40 g of water

Microorganism and inoculum preparation

The fermentative yeast, *Saccharomyces cerevisiae* 522D, was obtained from the culture collection of Pasteur Institute, Paris, France. Stock cultures were maintained on a gelified medium ($g.L^{-1}$): glucose, 20; peptone, 10; yeast extract, 10; and agar, 10; and were stored at 4°C. The inoculum preparation was previously described by **Chniti *et al.* 2014**).

Ethanol production medium

Date Syrup containing 50, 100, 174 and 358 $g.L^{-1}$ was supplemented with mineral culture medium as described previously by **Chniti *et al.* 2014**. The ethanol production medium (EPM) was transferred into a 500 mL bottle with a final working volume of 300 mL and was autoclaved at 120°C for 20 min. KOH 1 mol/L was used to adjust the pH to 6. Medium was sterilized by filtration on a 0.2 µm membrane (Sartorius, Goettingen, Germany).

Fermentation processes

The sterile EPM medium containing sugar concentration in the range of 50 to 358 $g.L^{-1}$ was inoculated with *Saccharomyces cerevisiae* (200 µL of inoculum). Batch fermentation was carried out in 500 mL bottle containing 300 mL of medium in an incubator shaker (New Brunswick, INNOVA 40, NJ, USA) at 28°C for 72 h. All experiments were performed in duplicate and samples (5 mL) were taken from the culture at regular time intervals.

Analytical methods

The cell density of the fermentation broth was measured at 600 nm (A600) using a spectrophotometer (SECOMAM, Alès, France). The fermentation broth was centrifuged at 350 rad/s for 5 min. The supernatant was used to analyse ethanol and residual sugars concentration by HPLC **(Chniti *et al.*, 2014)**. In addition, NH_4Cl concentration was analysed by the Method of Mann, 1963. The total sugar content was expressed in equivalents of glucose (glucose + fructose + 1.05 x sucrose) **(Guigou *et al.*, 2011)**.

Statistical analysis

All fermentation and date extractions were duplicated. In order to evaluate the significant differences between results, the Generalized Linear Model (GLM; with $p < 0.05$) and Tukey's honestly significant differences (HSD) multiple comparison module within JMP statistical software package were used.

RESULTS AND DISCUSSION

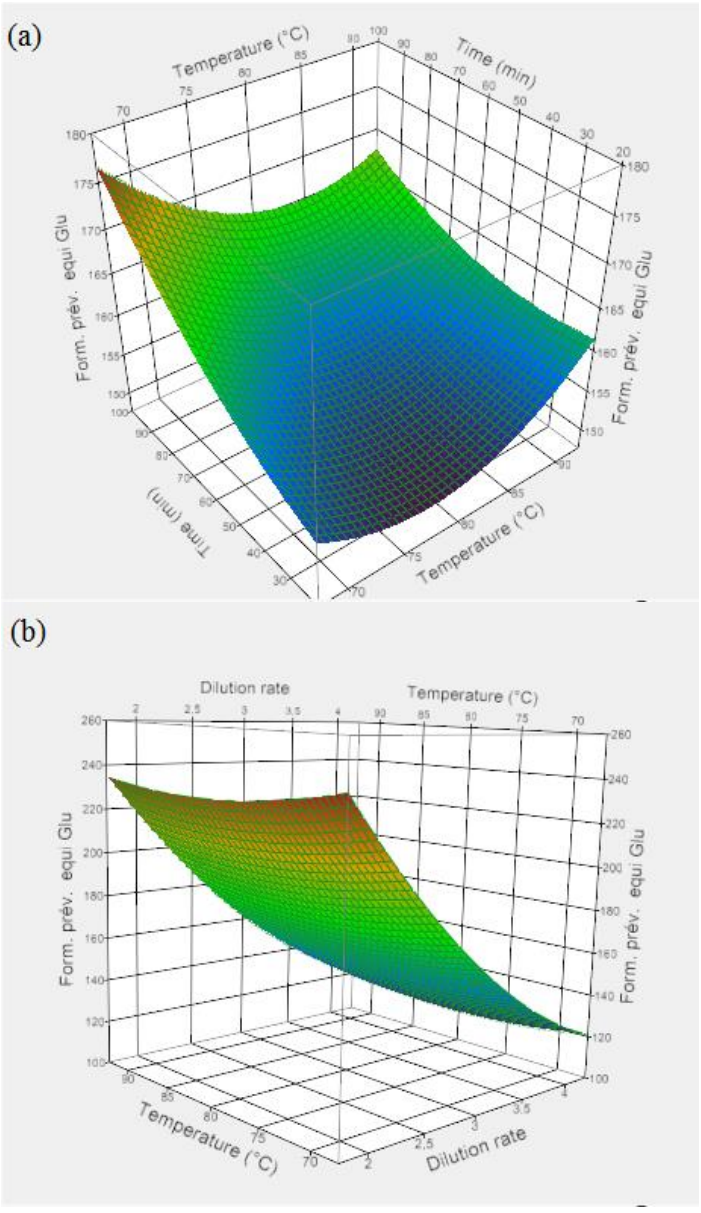
Date extract optimization

Optimization was performed by using Box-Behnken response surface method in terms of temperature, extraction time and dilution (ratio of date to water).

Effect of temperature on the extraction

When the temperature of the extraction increased, the sugar concentration in the juice increased slightly during the 3-extraction times (Figure 1). For instance, when compared with 70 and 90°C extraction temperature at 1:4 dilution and for the same extraction time (60 min), the sugar concentration in the juice obtained at 90°C (126 $g.L^{-1}$) was almost the same to that obtained at 70°C (122 $g.L^{-1}$). However the temperature effect depends significantly upon the dilution, for instance, juice obtained at 70°C and at 60 min extraction were 18.8% and 33.6% for 1:4 and 1:2 dilution (Table 2). Therefore, the extraction temperature * the dilution interaction were found to be statistically significant ($p<0.05$).

As can be seen from Figure 1, the residual sugars increased only slightly with the temperature. Based on these results, it can be deduced that the temperature did not play a significant part in the extraction of residual sugars from dates' fruit.



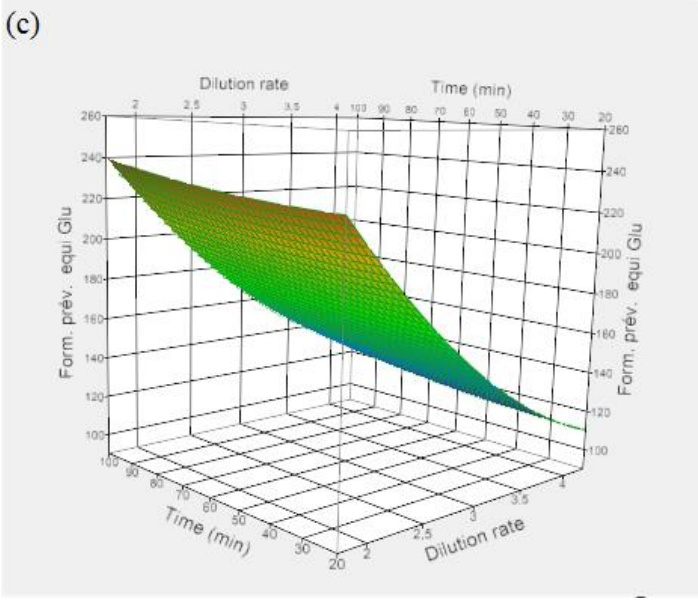


Figure 1 Surface plots of total sugar ($g.L^{-1}$) for dates' extraction.

Effect of the extraction time

Extraction is a contact-equilibrium process. When the soluble solids in the juice reach equilibrium, there is no diffusion of soluble solids from the raw material into the juice. Fig 1 showed that extraction time was not effective by itself, but also directly depends upon dilution and temperature. While the extraction time was at a low level, the effect of temperature on the response was insignificant (Figure 1a). When the dilution was kept low, the amount of soluble solids in the juice was highly independent of time (Figure 1c). According to the results, the extracted amount of sugars was significant ($p < 0.05$), and was affected by the extraction time and the dilution (Table 2).

Table 2 Results of dates' extraction obtained by the response surface method

Sample N°	Temperature (°C)	Time (min)	Dilution	Sugar Amount of the juice ($g.L^{-1}$)
1	80	60	1:3	156
2	90	30	1:3	154
3	70	90	1:3	174
4	80	90	1:2	216
5	90	60	1:2	218
6	90	60	1:4	126
7	80	60	1:3	156
8	70	60	1:4	122
9	80	30	1:2	212
10	80	60	1:3	156
11	80	30	1:4	120
12	90	90	1:3	156
13	70	60	1:2	218
14	70	30	1:3	154
15	80	90	1:4	126

Effect of the dilution on the extraction

Sugar concentration in the juice was the lowest when the largest dilution was considered (Table 2). It was also observed that the sugar concentration reached equilibrium level depending on the temperature and the time. It could be seen from Fig. 1 that the residual sugar concentration decreased gradually with increasing dilution. In other words, a low dilution decreased the time needed to reach equilibrium (Table 2). The lowest sugar concentration (120 $g.L^{-1}$) was obtained from the 1:4 dilution at 80°C and 30 min. In contrast, the highest sugar concentration was obtained from the 1:2 dilution at 90°C and 60 min. These data showed therefore that the dilution was among the major extraction parameters. As shown by the model (Figure 1), the concentration of residual sugars in the juice showed a maximum at 1:2 dilution (217 g/L).

Response surface modeling

A full quadratic response surface model was developed. Variables used in the model were temperature (°C), time (min) and dilution (w/v). The response surface model was generated using JMP with uncoded units and the following regression equation was obtained.

Y : Residual sugar concentration in dates' juice

$$Y = \beta0 + \beta1T + \beta2\ t + \beta3\ D + \beta22\ t2 + \beta12\ Tt + \beta13\ TD + \beta23\ tD + \varepsilon. \quad (1)$$

Where, ε = Error and $\beta0$, $\beta1$, $\beta2$, $\beta3$, $\beta22$, $\beta12$, $\beta13$, and $\beta23$ are coefficients (Table 3).

T2, t2 and D2 terms were removed from the model because they were not significant ($p < 0.05$). An R2 value (coefficient of determination) of 0.98 was obtained, which shows that 95% of the samples variation was taken into account by the model. It showed that extraction time (t) and dilution rate were significant ($p < 0.05$) (Table 3).

The model predicted that the best extraction conditions were 80°C, 60 min and 1:2 dilution, leading to 219 $g.L^{-1}$ sugar concentration. For given conditions, when the dilution increased, the equilibrium point of the juice also increased. It was also previously reported that the final sugar concentration of dates' extract produced in hatch system was directly linked to temperature and time **(Turhan *et al*., 2006)**. Application of high temperatures showed higher rates of soluble solids in the juice which contained more sugar; temperatures such as 80-90°C may be applied to obtain high sugar concentrations **(Berthels *et al*., 2004)**. Based on the optimal conditions obtained, it can be concluded that there was no need for the highest temperature, the longest time and the largest dilution which means less waste of energy, time and materials.

Verification of the models

The suitability of the model equation for predicting the optimum response values was tested by using the selected optimal conditions. The maximum predicted concentration and experimental concentration of Equi Glucose were given in Table 4. Additional experimental by using the predicted optimum condition for syrup extraction were carried out: extraction temperature 80°C, extraction time of 60 min, ratio of raw material to water 1:2, and the model predicted a maximum response of 219 $g.L^{-1}$. To ensure the predicted result was not biased toward the practical value, experiment rechecking was performed by using these modified optimal conditions: extraction time of 60 min, extraction temperature 80°C, ratio of material to water 1:2. A mean value of 218.4 ± 1.2 $g.L^{-1}$ (n=3) was gamed, obtained from real experiments, demonstrated the validation of RSM model. The result of analysis confirmed that the response model was adequate for reflecting the expected optimization, and the model was satisfactory and accurate.

Fermentation Kinetics

To investigate the influence of initial sugar concentration on end-product formation, *S. cerevisiae* was cultivated with Date juice at various substrate concentrations.

Yeast growth

The profile of cell growth is presented in Figure 2. Until 174 $g.L^{-1}$ cell growth ceased after 48 h culture. The viable cell numbers reached a maximum OD value at 600 nm of 14 for 100 and 174 $g.L^{-1}$, and around 10 OD value for 50 $g.L^{-1}$; while an almost total absence of growth was observed for 358 $g.L^{-1}$, showing the inhibitory effect of a high sugar content, 358 $g.L^{-1}$ of total sugars, namely about 2 $mol.L^{-1}$ of monosaccharide like glucose or fructose; at such high sugar content, only osmotolerant yeasts can grow **(Passoth *et al*., 2006; Leandro *et al*., 2011)**.

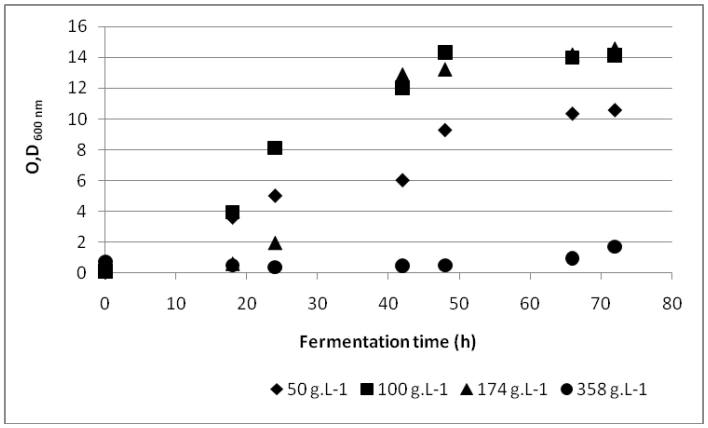


Figure 2 Cell density (OD 600 nm) in media containing 50, 100, 174 and 358 $g.L^{-1}$ sugars.

Sugar consumption

Dates juice contains almost equal amounts of glucose and fructose. The data clearly show that, for 50, 100 and 174 $g.L^{-1}$, sugars were almost completely fermented by *S. cerevisiae* (Figures 3a-c), which showed a preference for glucose over fructose; although it was shown that fructose can be used concomitantly with glucose **(Berthels *et al*., 2008)**. Glucose and fructose are transported by the same carriers in *S. cerevisiae* **(Berthels et al., 2008; Rogers et al.,1979)**.

Structural analysis of sucrose shows a β-1,2 bond, which is cleaved by the enzyme invertase (β-D-fructofuramoside fructohydrolase, EC.3.2.1.26). It is known that the yeast *Saccharomyces cerevisiae* produces intra and extracellular invertase.

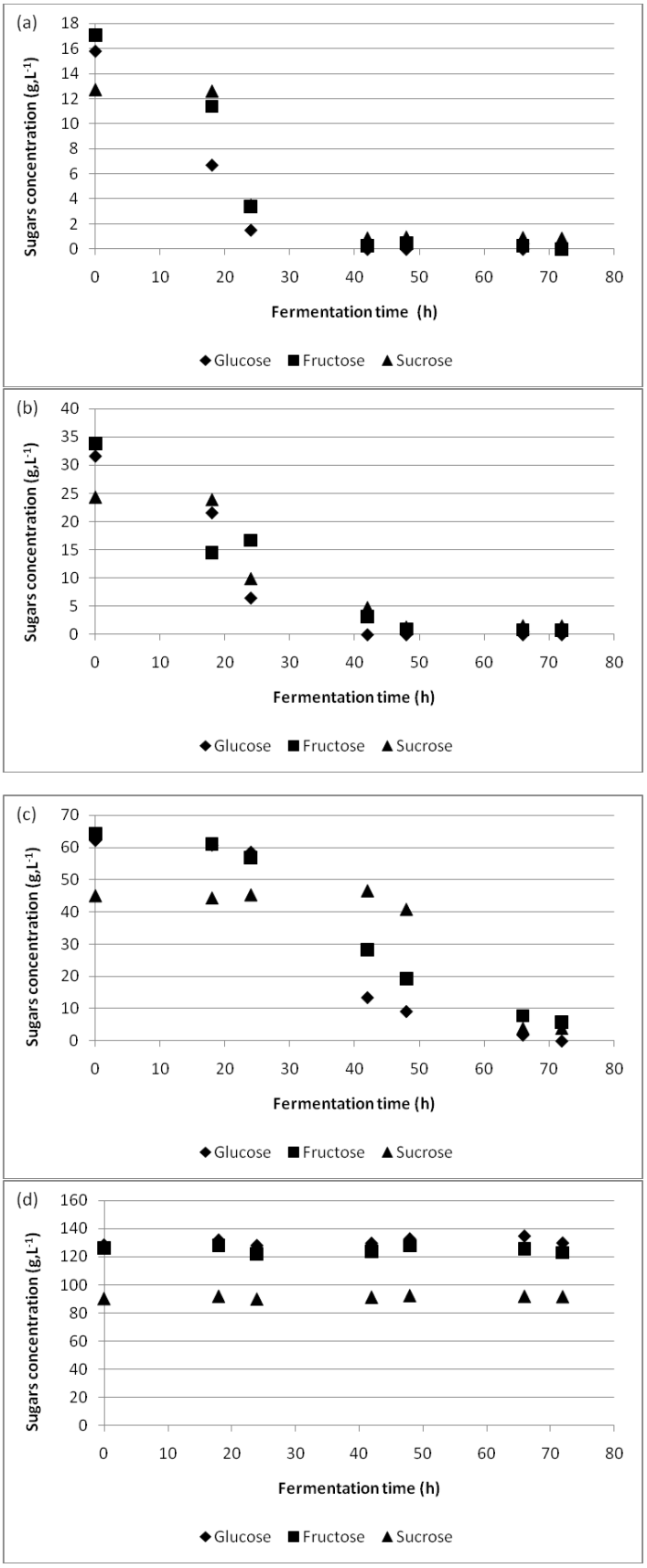


Figure 3 Sugar consumption during batch fermentation at initial sugar concentrations of 50 g.L^{-1} (a), 100 g.L^{-1} (b), 174 g.L^{-1} (c), 358 g.L^{-1} (d)

In close linking with growth (Figure 2), sugars consumption appeared almost negligible for 358 g.L^{-1} (Figure 3d), confirming that *S. cerevisiae* could not tolerate such high sugar content. This result appears in agreement with **Rogers et al., 1979 and Chniti et al., 2014**, which showed that at high glucose concentration yield of ethanol production by *S. cerevisiae* remained almost unaffected by initial glucose concentration up to approximately 20% and declined beyond that.

Ethanol production

Ethanol (Figure 4) production increased with the increase of sugar concentration up to 174 g.L^{-1}, while no production was observed for 358 g.L^{-1} (Figure 5), in agreement with the absence of growth (Figure 2). Ethanol production increased with the sugar content until 62.9 g.L^{-1} (Figure 4) produced for 174 g.L^{-1}, after 66 h of fermentation. Similar results were found by **Laluce *et al*., 2009**.

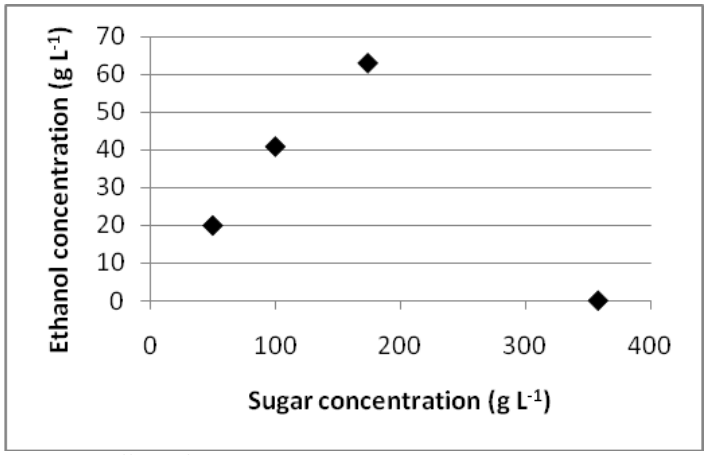


Figure 4 Effect of initial sugar concentration on ethanol production by *Saccharomyces cerevisiae*.

Up to 174 g.L^{-1}, Table 4 shows that *S. cerevisiae* has consumed more than 90% sugars after 72 h of fermentation. For 50 and 100 g.L^{-1} initial sugar amount, the ethanol yield ($Y_{Ethanol/Glu}$) reached 43% and 45% respectively, after 72 h, while 38% yield was observed for 174 g.L^{-1}. High initial substrate concentration may inhibit substrate utilization and decrease product yields.

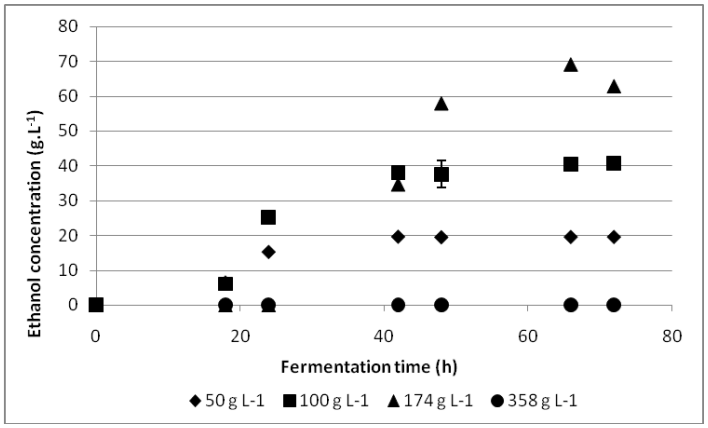


Figure 5 Ethanol production during batch fermentation by *Saccharomyces cerevisiae* on dates' juice.

A maximum ethanol productivity of 1.1 g. L^{-1}.h^{-1} ($Q_{Ethanol}$) was obtained for 174 g.L^{-1}, at the end of fermentation (72 h). For the highest sugar content (358 g.L^{-1}), yield as well as productivity appeared negligible, several studies have reported that high substrate concentration inhibit growth and fermentation of yeasts in industrial ethanol production as the result of high osmotic pressure **(Guigou *et al*., 2011)**. Ethanol productions increased from 20 to 63 g.L^{-1} for an increase in the sugar amount from 50 to 174 g.L^{-1}. A low dilution represents an economy of equipment and process costs (e.g distillation costs), which appears appropriate for an industrial purpose.

CONCLUSION

The various examined parameters (temperature, extraction time and dilution) involved in hot water extraction of dates' juice showed that all these variables markedly affect the total sugars concentration; it was related to the extraction conditions by using second order polynomials. The optimal conditions for sugar extraction were found to be a solid:liquid ratio 1:2, 60 min time extraction and 80°C, leading to a sugar concentration of 219 g.L^{-1}. Optimal ethanol concentration and yield were obtained for 174 g.L^{-1}. Results of alcohol

fermentation showed that date juice can be a good feedstock for ethanol production by *S. cerevisiae* in batch fermentation after pretreatment without utilization of chemicals materials. However, an economic study is necessary to evaluate the energy input for pretreatment step and the total yield of bioethanol production.

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