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Effect of the Nitrogen Source on Bioethanol Production from Syrup Dates by *Saccharomyces cerevisiae*

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***Abstract* – Syrup resulting from byproducts of dates' fruit (*Phœnix dactylifera, L.*) constitutes a favorable medium for ethanol production by *Saccharomyces cerevisiae*, according to its content in simple sugars. However, it is limited by its content in nitrogen and nutrients, and hence must be accordingly supplemented. Yeast extract and ammonium were therefore tested as nitrogen sources for ethanol fermentation from date syrup by S. cerevisiae. The results showed that both nitrogen sources promoted ethanol production efficiencies. Even if yeast extract appeared the most efficient nitrogen source if compared to the same amount of ammonium, the Duncan's test showed no significant difference of the ethanol on sugar yields for broths enriched with NH_4Cl (0.45 g g^{-1}) and Yeast extract (0.51 g g^{-1}), while the difference was significant in the case of an absence of enrichment (0.30 g g^{-1}). Therefore and from an economical point of view, ammonium remains an interesting alternative. These results clearly indicate the high potential of date syrup supplemented with nitrogen for ethanol production by *S. cerevisiae* for subsequent industrial applications.**

***Keywords* – Ethanol Fermentation, *Saccharomyces cerevisiae*, Nitrogen Sources, Date Syrup.**

I. INTRODUCTION

The increasing demand for ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleaning agents and preservatives, has induced increased production of this alcohol. Owing to the depleting reserves and competing industrial needs of petrochemical feed stocks, there is global emphasis in ethanol production by fermentation process.

The use of energy derived from biological reactions (bio-energy) provides many advantages; perhaps the most important being the reduced dependence on a non-renewable fossil fuel sources. It can also provide good opportunities to convert renewable organic waste materials into energy [1].

The world production of dates has increased from 6,723,118 tons in 2002 to 7,548918 tons in 2010 (FAO 2013). Tunisia is the 9th world producer [2], and the first exporter of dates in value [3]. The date palm tree (*Phonix dactylifera L.*) constitutes the basis of economy for people living in Tunisian Sahara [3]. Tunisian production has reached 190 000 tons per year [2] with a dominance of the "*Deglet-Nour*" variety constituting about 60% of the total production [3]. This production progress is unfortunately accompanied by a substantial increase of loss during harvest, storage, commercialization and conditioning process [4]. These lost dates could amount to more than 30 000 tons per year in Tunisia [3]. Date byproducts, are not consumed because of their low quality (inadequate texture, contamination with microbes and / or infestation by insects).

Presently, very little use of these byproducts is made; they are discarded or used in limited cases for animal feed [5]. Research into date byproducts has not been a true reflection of the importance and potential of this crop, since dates are a rich source of certain nutrients and sugars (70-80%) [6] [5], in the form of glucose, fructose and sucrose. It is therefore of a particular interest as a substrate for ethanol production.

Production of ethanol from "dates' byproducts" by *Saccharomyces cerevisiae* fermentation has been therefore examined in this study. Syrup resulting from dates, according to its content in simple sugar, constitutes a favorable medium for yeast development. But it is limited by the low content of nitrogen and nutrients. It must be therefore supplemented by mineral salts and nitrogen sources.

Nitrogen sources that are widely used to stimulate fuel alcohol, brewing, or winery fermentations are yeast extract and peptones [7-12], ammonium [12, 13], and urea [14]. They are employed to increase yeast growth or viability and the rate of sugar utilization, and to reduce fermentation time. However, several investigators reported negative effects of using ammonium and urea as nitrogen supplements in ethanol fermentation [11, 16-18]. Contrarily, yeast extract and peptones are nitrogen supplements, which are the most widely used at laboratory scale.

This investigation was therefore designed to evaluate the effect of different nitrogen sources in ethanol production from syrup of byproduct of date's fruit, by S. cerevisiae. NH_4Cl a mineral nitrogen source was compared with Yeast extract as an organic nitrogen source.

II. Materials and Methods

A. Raw material treatment

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 pulpe 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 [19]. 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.

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B. Medium preparation

Dates syrup containing 10°Brix was supplemented with (g L^{-1}): KH_2PO_3, 3; $(NH_4)_2SO_4$,3; $MgSO_4$, $7H_2O$, 0.4; Na_2HPO_4,$12H_2O$, 3 g L^{-1}; $CaCl_2$, $2H_2O$, 6.9; $ZnSO_4$,$7H_2O$, 12; $CaCl_2$, $6H_2O$, 0.15; H_3BO_3, 0.9; $CuSO_4$,$5H_2O$, 0.3; $MnSO_4$,H_2O, 11.4, the pH was adjusted to 4.5 using HCl 1 N [20]. The medium was transferred into a 500 mL bottle to achieve a final working volume of 300 mL and was then autoclaved at 120°C for 20 min after adding 16 g L^{-1} of nitrogen source.

C. Yeast strain and inoculation

The fermentative yeast *S. cerevisiae* was obtained from the STL (yeast Tunisian Society, Beja, Tunisia). The yeast strains were maintained in a synthetic medium whose composition (in g L^{-1}) was glucose, 20; yeast extract, 10; peptone, 10 and agar, 10. In all cases, cultures were maintained at 28 °C for 24 h and then stored at 4°C. Subculture was done every two months. The composition of the pre-culture medium for yeast was (in g L^{-1}): glucose, 20; yeast extract, 1 and peptone, 10. The cells were cultivated in the pre-culture medium at 28°C, on a rotating shaker (INNOVA 40) at 180 rpm for 18 h in order to obtain high cell density. At the end of the incubation period, cells were centrifuged aseptically (3000 rpm, 4°C and 5 min), and resuspended in 25 mL KCl (150 mM) and then centrifuged again in similar conditions. The suspension obtained after harvesting cells and re-suspending in 10 mL of KCl 150 mM was used for inoculation [20, 21].

D. Ethanol fermentation

To evaluate the effects of the nitrogen source, organic, Yeast extract (BIO BASIC INC) (M_YE), or mineral, NH_4Cl (Carlo ERBA) (M_NH_4Cl), on ethanol production, a batch fermentation was inoculated with 200 µL of yeast suspension and was carried out in an incubator Shaker (New Brunswick, INNOVA40, NJ, USA) at 28° C. In addition, a control (M_C) without any feeding source was also carried out under the same conditions. All fermentations were performed in duplicate. After inoculation, 5 mL samples were withdrawn aseptically from the fermentation broths after yeast addition, and after 18, 24, 42 and 55 h for analysis.

E. Analytical methods

Dry matter was determined according to the AOAC method [22]; data were expressed as percentage of dry weight. Proteins were determined by the method of [23] and ashes by combustion of the sample in a muffle furnace at 550°C for 8 h [22]. The residue was dissolved in HNO_3 (14.4 mol L^{-1}) and the mineral constituents (Ca, Mg, Fe and Zn) were analyzed separately, using an atomic absorption spectrophotometer (Nova 400, Analytic Jena, Germany). Sodium (Na) and Potassium (P) contents were determined by flame photometer (Scherwood 410, Cambridge, UK).

The concentration of yeasts in the samples was measured as OD600 nm using a Jasco-V-530 UV/VIS spectrophotometer (Jasco, Japan) after vigorous shaking for 3-5 seconds. Serial dilutions of inoculums were made; then 100 µL of each dilution was plated onto Sabouraud Agar. Plates were incubated at 30°C for 72 h; two plates were used for each dilution to determine the UFC mL^{-1}. The measured OD600 was then correlated to UFC mL^{-1}.

During the fermentation period, a 5.0 mL sample was taken on 0, 18, 24, 42 and 55 h and centrifuged at 3000 rpm, 4°C for 5 min after pH and OD600 nm measurements. The supernatant was used to analyze ethanol and residual sugar concentrations. Ethanol concentrations were determined by gas chromatography (Agilent 6890 N) equipped with flame ionization detector (FID) and HP-INNOWAX column (30 m × 0.25 mm × 0.25 µm). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. The operating temperature at the injection port and detector were 250°C and 280°C. The initial temperature of the oven was 120°C for 1 min; it was then increased to 250°C at a rate of 25°C/min and maintained for 2 min.

Sugars were determined using high performance liquid chromatography Agilent 1200 (HPLC), with RID (Refractor Index Detector). A solution of acetonitrile and water in a ratio 82/18 was used as a mobile phase. An amino kind column was used (Teknokroma, Kromasil 100 NH_2, 5 µm 25×0.46 cm^2), thermostated at 30°C.

The total sugar content was expressed in equivalents of glucose (glucose +fructose + 1.05 × sucrose) [24] 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% Brix).

The ethanol yield ($Y_{P/S}$) was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized (g g^{-1}). The volumetric ethanol productivity (Q, g L^{-1} h^{-1}) was determined from the concentration produced (P, g L^{-1}) divided by the fermentation time (t, h) giving the highest ethanol concentration.

F. Statistical Analysis

Each analysis was done in duplicate and the results were expressed as mean and standard deviation (SD). The Duncan's test was used to compare all mean pairs in conjunction with the analysis of variance (ANOVA) using XLSTAT software, version 2013.3.01 (Addinsoft). Differences between means were considered as significant when $p < 0.05$.

III. Results and Discussion

A. Physico-chemical analysis of date syrup

The composition of the syrup was summarized in Table

1. Carbohydrate was the predominant component (70% in dry weight), followed by moisture, along with small amounts of protein (1%) and ash (1.5%).

Table 1: Composition of the date syrup (72°Brix) used for experiments

Moisture [2]	26.3	**Na**[2]	58.51 ± 7.60
Glucose (g L^{-1})	226	**K**[2]	494.05 ± 18.70
Fructose (g L^{-1})	220	**Mg**[2]	91.27 ± 7.06
Sucrose (g L^{-1})	170	**Cu**[2]	<0.1
Protein content [1]	1	**Fe**[2]	0.55 ± 0.04
Ash[1]	1.5	**Ca**[2]	34.51 ± 1.08
pH	4.7	**Zn**[2]	0.5 ± 0.074

[1]Data are expressed as g/100 g ± SD (n=3) on a dry weight basis

[2]Data are expressed as mg/100 g ±SD (n=3) on a fresh weight basis

In fermentation industry, the reducing sugar content of raw material is an important parameter which reveals the fermentative sugars contained in the feedstock. Glucose and fructose contents in date syrup were around 226 g L^{-1} and 220 g L^{-1}, respectively.

Date syrup (72°Brix) contained significant amount of minerals (Table 1). Potassium concentration was the highest (494 mg/100 g fresh weight basis), followed in decreasing order by Magnesium (91.3 mg/100 g fresh weight basis). These results were in close agreement with the findings of [5] for *Deglet-Nour* date palm fruit from Tunisia.

B. Effecy of nitrogen source supplementation

1. Biomass

In order to study the effect of nitrogen sources NH_4Cl or Yeast extract on ethanol production, fermentation progress was recorded routinely by measuring the viable cell numbers and ethanol concentrations. The cell number corresponding to the OD 600 reading was calculated from a calibration curve obtained for *S. cerevisiae* (Fig 1).

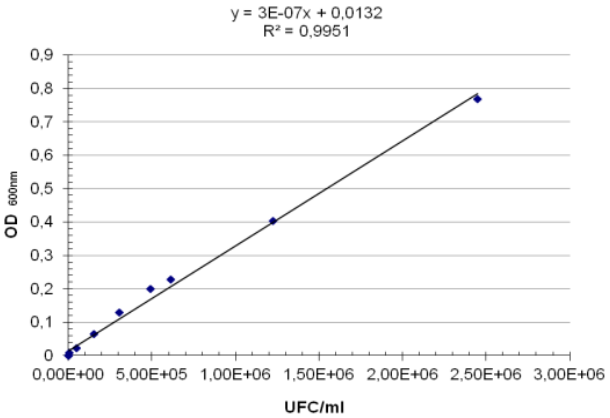

Fig.1. Calibration curve used for the determination of the cell number corrsponding to the OD 600 reading.

From the first sampling, after 18h, viable cell number was higher in the presence of a nitrogen supplementation if compared to the control and the trend was especially pronounced in the presence of yeast extract if compared to ammonium addition (Fig. 2). Viable cell number reached a maximum of 5.7×10^8 UFC mL^{-1} in the fermentation broth supplemented with yeast extract (M_YE) as nitrogen source, which can be compared to the medium supplemented with NH_4Cl as nitrogen source showing 4.5×10^8 mL^{-1} of viable cells and the control giving 2.1×10^8 UFC mL^{-1} at the end of culture, namely after 55 h (Fig. 2).

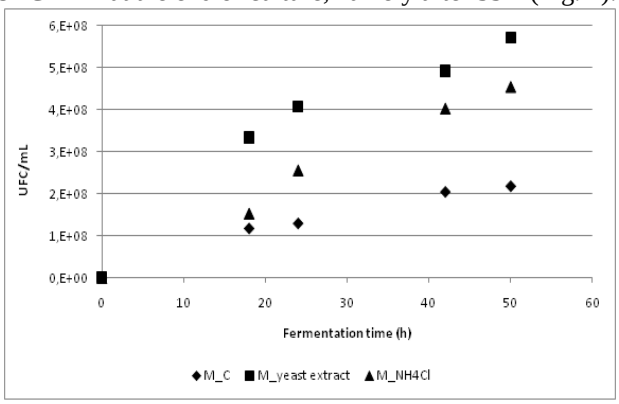

Fig.2. Yeast viability during batch ethanol fermentation by S. cerevisiae.

Fig 2 indicated that Nitrogen source promote growth of *S. cerevisiae*. At the end of the fermentation (55 hours), cell concentration in the broth containing yeast extract was approximately 3 times higher than that in the broth without nitrogen source.

2. Sugars Consumption

The uptake of sugars differed according to the considered nitrogen source (Fig. 3); for instance when yeast extract was used as the nitrogen source, carbon metabolism flux was different from that observed for ammonium as a nitrogen source.

In the presence of yeast extract (Fig. 3b), sucrose is completely consumed by *S. cerevisiae*, contrarily to NH_4Cl supplementation (Fig. 3c) or an absence of supplementation (Fig. 3a). This can be most likely attributed to the nutrients content of yeast extract, which allows a rapid adaptation of *S. cerevisae* to the availability of sucrose in the medium; indeed, *S.cerevisiae* is able to produce invertase to hydrolyse sucrose to glucose and fructose.

S. cerevisiae showed a preference for glucose over fructose. Although fructose was used concomitantly with glucose, this latter was depleted first from the medium. Glucose and fructose are transported by *Hxt* carriers in *S. cerevisiae* [25].

The highest sugar consumption was found in the medium supplemented with yeast extract (Fig. 3b). This finding was supported by [26] who reported that when the concentrations of yeast extract in the medium were increased, yeast could better tolerate the osmotic pressure. Our studies indicated that yeast extract stimulated the rates of sugar utilization (especially sucrose); the sugars were totally consumed before 42 hours of the fermentation (Fig 3b). The results were in agreement with [27] who reported that the fermentation time of wheat mash containing 350 g L^{-1} dissolved solids was reduced from 8 days at 3 days when the medium was supplemented with 0.9 % yeast

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extract.

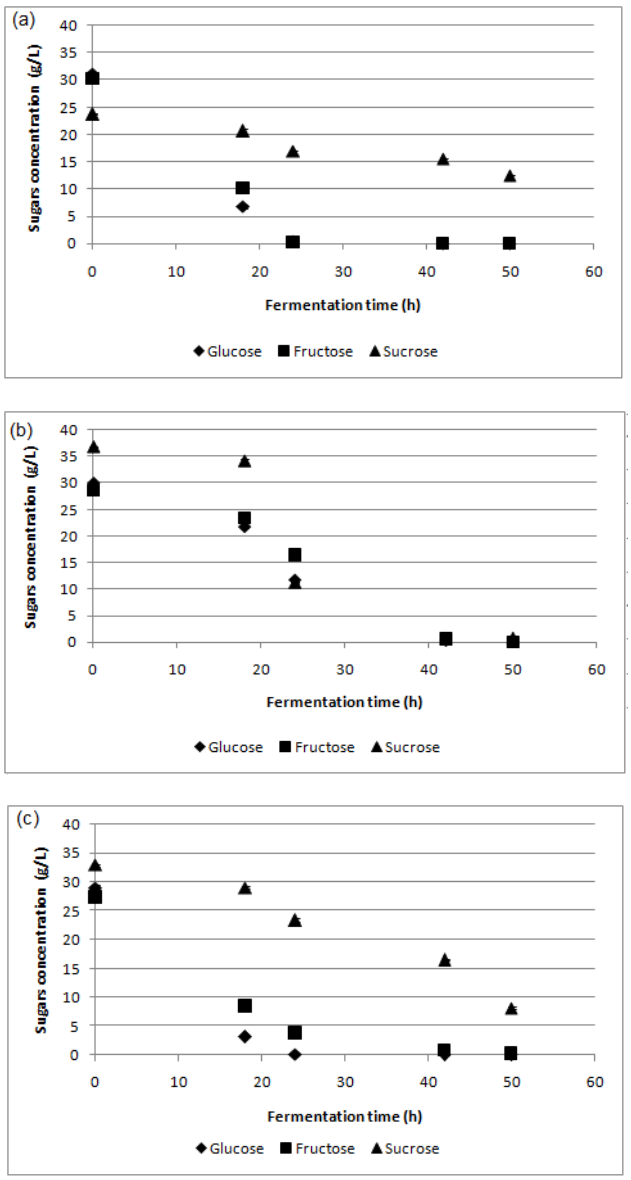

Fig. 3. Sugar consumption during batch ethanol fermentation by *S. cerevisiae* (a) M-C, (b) M_YE, (c) M_NH_4Cl.

Sucrose has been widely used in the broth supplemented with yeast extract; while its consumption was 2.5 and 1.5 times lower in the case of NH_4Cl supplementation and in the absence of additional nitrogen source, respectively.

3. Ethanol Production

With yeast extract as nitrogen source, maximum ethanol concentration reached 41.4 g L^{-1} after 55 h (Fig. 4); while final ethanol concentrations of the control and in the case of NH_4Cl as a nitrogen source were 24.8 g L^{-1} and 37.2 g L^{-1} respectively. Organic nitrogen supplementation improved therefore ethanol production.

Yeast extract efficiency for ethanol production was in agreement with the finding of Turhan et al. [28], who found that cultures of carob extract supplemented with yeast extract led to the highest ethanol yield with almost total sugar consumption. Indeed, yeast extract comprises the water soluble components of the yeast cell, including primarily amino acids, peptides, carbohydrates and salts. Yeast extracts are therefore rich in nitrogen, vitamins and other growth stimulating compounds [29].

Metabolizable nitrogen is needed for growth and hence has an impact on ethanol production. However, date syrup can be fermented without any nitrogen source addition. [30] have also used NH_4Cl as nitrogen source for ethanol production from molasses (35 g L^{-1}), showing 14.1 g L^{-1} of ethanol produced after 28 hours of fermentation for an initial NH_4Cl concentration of 1.5 g L^{-1}.

The ethanol concentration obtained after 55 h of fermentation was in the range 24.8 – 41.4 g L^{-1} (Table 2), with a significant difference related to the nitrogen source ($p < 0.05$). However, Duncan's test showed that there was no significant difference among the ethanol yields obtained for broths enriched with NH_4Cl (0.45 g g^{-1}) and Yeast extract (0.51 g g^{-1}), while the difference was significant in the case of an absence of enrichment (0.30 g g^{-1}) (Table 2).

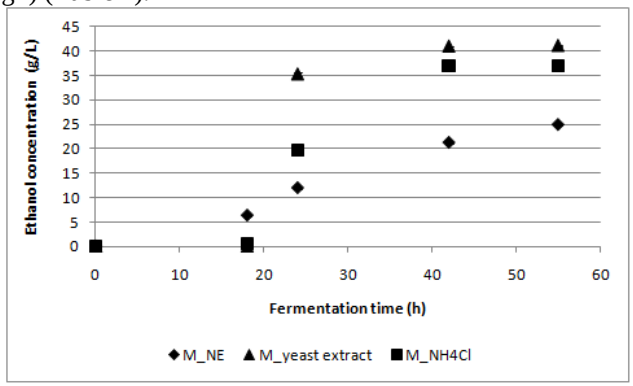

Fig.4. Ethanol production during batch ethanol fermentation by *S. cerevisiae*.

The results obtained from this study indicated that the byproduct of date fruit was one of the most promising raw materials for ethanol production by *Saccharomyces cerevisiae*, in terms of ethanol yield when compared with other potential raw materials. Indeed, if compared to other findings obtained with raw materials containing sugars, ethanol yields obtained in the present work in the case of M_NH_4Cl (0.45) and M_yeast extract (0.44) supplementations were similar to the use of sweet sorghum juice (ranging from 0.44 to 0.51) [31] and higher than the yields obtained using sugar beet juice ($Y_{P/S}$ = 0.42), [32] and soybean molasses ($Y_{P/S}$ = 0.25) [33].

Table 2: Kinetic parameters of ethanol production from date syrup supplemented with different nitrogen source by *S. cerevisiae*

	M_C	M_NH_4Cl	M_ yeast extract
Consummed sugars (Equi Glu)	82.6 [a]	82.09 [b]	96.1 [b]
EtOH (g L^{-1})	24.8 [a]	36.8 [b]	41.9 [c]
Y $_{(P/S)}$ g g^{-1}	0.30 [b]	0.45 [a]	0.44 [a]
Q (g L^{-1} h^{-1})	0.45 [a]	0.67 [b]	0.76 [c]
UFC mL^{-1}	2.2 10^{8} [a]	4.5 10^{8} [b]	5.7 10^{8} [c]

Means followed by the same letter within the same line are not significantly different using the Duncan's multiple range test at the level of 0.05; Productivities were calculated according to the final fermentation times (55 h)

IV. Conclusion

Results showed that date syrup can be a good feedstock for ethanol production by *Saccharomyces cerevisiae* using batch fermentation. The nitrogen source has a significant impact on ethanol production by *S. cerevisiae* and allows to improve the profitability of fuel alcohol production from date syrup. Yeast extract was shown to be preferred over ammonium as a nitrogen source during ethanol fermentation from date syrup. However, from an economical point of view, this latter remains an interesting alternative.

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