Impact of Calcium and Nitrogen Addition on Bioethanol Production by \emph{S. cerevisiae} Fermentation from Date By-Products: Physicochemical Characterization and Technical Design

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Abstract: Given crude oil prices and their environmental impacts, the use of sustainable renewable alternative energies such as biofuels is rapidly progressing in numerous countries. Among biofuels, bioethanol is a renewable and clean fuel that can be obtained from the fermentation of several raw agricultural materials, including date fruit. However, the low product yield, mainly due to the low-grade nutrient content, limits its use as a promising alternative biofuel. This current study investigated bioethanol production from date by-products in Saudi Arabia and examined the impact of calcium and nitrogen sources added at different concentrations (0 to 1 g/L) on the productivity and ethanol concentration using \emph{Saccharomyces cerevisiae}. Yeast extracts and ammonium chloride (NH$_4$Cl) were tested as nitrogen sources for bioethanol fermentation from date juice. Calcium chloride (CaCl$_2$) and calcium carbonate (CaCO$_3$) were evaluated as calcium sources for the same purpose mentioned above. The results showed that both calcium and nitrogen sources improved ethanol production efficiencies. The addition of calcium sources such as CaCl$_2$ at 0.4 g/L resulted in maximum ethanol concentration (41.5 ± 0.85 g/L) and the highest productivity of 0.511 g/L/h. Thus, an increase of 31.3% compared to the control sample was acquired. Ammonium chloride was found to be the best nitrogen supplement among them. Indeed, supplementing the fermentation medium with 1 g/L NH$_4$Cl gave an optimal ethanol concentration and productivity, reaching more than 65 g/L and 0.83 g/L/h, respectively. This is an increase of 106.6%. The functional group of ethanol (C$_2$H$_5$OH) for all the elaborated samples was confirmed by Fourier-transform infrared spectroscopy (FTIR) and NMR analyses. Moreover, the results confirmed the high quality and purity of the bioethanol products. Thus, the “Khodhari” date variety of low market value is a privileged substrate for industrial bioethanol production. For this reason, a proposed flow diagram of a designed plant for bioethanol industrialization is provided and detailed.

Keywords: date fruit; \emph{Saccharomyces cerevisiae} fermentation; bioethanol; calcium source; nitrogen addition; scale production design

1. Introduction

Energy is a foundational stone of the modern industrial economy. Energy provides an essential ingredient for almost all human activities. As a result of industrial growth and population evolution, there has been a massive rise in energy consumption in the world. The worldwide energy consumption has increased 17-fold in the past decade [1]. However, the existing conventional energy resources, such as fossil fuels, are no longer...
able to cope with the evolution and the growing energy needs. Therefore, the uncertainty in its availability has rekindled an interest in alternative renewable and sustainable biofuels. Likewise, compared to raw, non-renewable fossil materials, biofuels are generally considered to offer many benefits, including sustainability, reduction of greenhouse gas emissions, regional development, and social structure. Ethanol, or ethyl alcohol, is one of the most promising biofuels from renewable resources. Ethanol fermentation, also called alcoholic fermentation, produces bioethanol and can use numerous renewable resources, including sugar-rich fruits, corn, molasses, and agricultural residues [2–4].

In Saudi Arabia, one of the major crops produced is the date palm tree, *Phoenix dactylifera* L. [5]. The Saudi production of date fruit has grown since 2014, recording a significant evolution of 18.6% to exceed 1,539,756 metric tons in 2019 [6]. This considerable date palm production allows us to consider dates as an alternative energy feedstock. These fruits can be efficiently converted into fermentable sugars for fuel production.

However, the main hurdle in bioethanol production from agricultural raw materials (plant or fruit) is its high cost of manufacture, which includes a low product yield, low reactor productivity, and, most of the time, the need for high energy for distillation owing to low product concentration in the broth. Thus, several approaches have been used to overcome these obstacles and increase bioethanol production using yeasts. One of the potential methods to increase productivity and consequently reduce production costs is to enrich the medium composition with nutrient components. These nutrients have demonstrated an efficient protective effect on growth, fermentation, and feasibility, which substantially boosts the yield of ethanol production [7]. Many inorganic compounds, such as calcium, sodium, potassium, and magnesium, have been used to increase fermentation productivity and ethanol concentration. Pejin et al. achieved more excellent ethanol production by adding calcium and magnesium ions to triticale mashes. The results revealed that when calcium or magnesium ions were added at a concentration of 160 mg/L, bioethanol production increased by 31.22% or 21.04%, respectively, compared to that of the control extract [8]. In another study, Azam et al. reported that adding CaCO$_3$ or CaCl$_2$ to a fermentation medium of a glucose solution (250–300 g/L) led to a significant augmentation of productivity and ethanol concentrations. Their study showed a 500% increase in productivity when the solution was supplemented with 0.40 g/L of a CaCl$_2$ solution [9]. In addition, Nabis et al. carried out related studies on improving ethanol production by adjusting the type of yeast [10]. In the same context, Sreekumar et al. found that the introduction of calcium carbonate to concentrations of glucose and sucrose improved ethanol production by *Zymomonas mobilis* ZM4 and a mutant (ZMI2) [11].

Similarly, the nitrogen source is considered an essential nutrient for yeast during alcoholic fermentation. This element is a vital nutrient for yeast and plays a key role in protein synthesis, amino acids, nucleotides, and sugar transport [12]. Indeed, several studies have highlighted the crucial role of nitrogen in ethanol productivity using *S. cerevisiae* and its involvement in the molecular mechanism. It has also been shown that several azote compounds, such as ammonium ions [13], urea [14], peptone [15], yeast extract [15], and other nitrogen sources [16], can lead to improvements in yeast cell development and alcohol fermentation productivity. In this context, A. Sheikh et al. [17] investigated the impact of yeast extracts on the production of ethanol from potato peel wastes using commercial and genetically modified *Saccharomyces cerevisiae*. They demonstrated that the highest bioethanol production was observed when a solution of yeast extracts at 2 g/L was added to the fermentation medium. Tareen et al. [18] compared the impact of two nitrogen compounds, urea and YP medium (mixtures of yeast extract and peptone), for ethanol production using the yeast *Saccharomyces cerevisiae* SC90. The results showed significant enhancement of the ethanol concentration and productivity with nitrogen sources; however, with urea, the productivity and ethanol yield were comparatively lower than those obtained when YP was used as a nitrogen source.
The impact of an ammonium source in bioethanol has been carefully tested by Yue et al. [13]. The work of this team highlighted the boon effect of ammonium to improve ethanol production by yeast from concentrated sweet sorghum juice.

As a summary of these reported bibliographic data, we deduced the critical role of adding different minerals as nutrients in enhancing cell growth during the fermentation process, significantly increasing ethanol production yield. Thus, the current study aimed to evaluate the impact of different nitrogen (yeast extract and NH4Cl) and calcium (CaCl2 and CaCO3) sources supplemented during date juice fermentation using S. cerevisiae, thereby quantifying the improvement of bioethanol production.

2. Materials and Methods

2.1. Raw Materials

The raw material was “Khodhari” dates from Saudi Arabia (Qassim region, Buraidah city; 26°20′ N 43°58′ E). Khodhari dates are dark brown and have a dry texture, but are not too wrinkly. Khodhari dates, as with most varieties of dates, are very rich in sugar. However, they are characterized by their low price, and they are abundant in the Qassim region. Therefore, they are good candidates for date juice extraction and the biological production of bioethanol. The fruit samples were purchased from a local supermarket in Saudi Arabia. However, in the future, for the industrial scale, the raw material would be collected directly from the oasis, including defective date scraps and those with low market value, to minimize the manufacturing cost. The selected dates were preserved at 4 °C before subsequent experiments.

2.2. Extraction and Preparation of Date Juice for Fermentation

Date palm fruit was used in the experiment to produce bioethanol. Before the extraction process, the dates were cleaned, pitted, and cut into small pieces with a knife. Then, date pulp was added to hot distilled water at a weight-to-volume ratio of 1:3. Next, the blend was heated on a hot plate and mixed using a hand-held blender (Phillips, Holland). As the extraction temperature increased (from room temperature to 80 °C), the sugar concentration in the date juice increased. After 40 min and at a temperature of around 80 °C, the sugar was entirely extracted. Afterward, the mixture was filtered, centrifuged, and diluted to reach a concentration of 17% TSS, equivalent to approximately 162.2 g/L of total sugar. This last parameter was determined according to the Dubois method [19].

Mineral solutions (nitrogen and calcium sources) were then added, and the pH of the raw juice was adjusted to 5 using 1 M sulfuric acid or sodium hydroxide solution. Finally, the prepared date juice was sterilized at 120 °C for 20 min.

2.3. Microorganisms

Yeast plays a vital role in producing all kinds of alcohol [20]. Therefore, selecting appropriate yeast strains is essential not only to maximize the alcohol yield, but also to generate pure, high-quality alcohol, especially ethanol. Numerous studies have confirmed the effectiveness of S. cerevisiae as the most suitable yeast for alcoholic fermentation [12,21]. For this reason, fresh baker’s yeast, Saccharomyces cerevisiae, was selected and used for bioethanol production. Fresh S. cerevisiae was procured from the “Rayen Food Industries” company (Tunis, Tunisia). At the time of operation, 15 g/L of S. cerevisiae was added to the medium directly as an inoculum without any pretreatment, in reference to our previous study [21]. Each packet of yeast was only used on the day it was opened.

2.4. Fermentation and Fractional Distillation

2.4.1. Fermentation Procedure

Fermentation tests were performed using the selected yeast, Saccharomyces cerevisiae. Alcoholic fermentation experiments were carried out under anaerobic conditions in a round bottom flask (Florence Flask) at a constant temperature of 30 °C and rigorous agitation of
200 rpm for 72 h. Liquid samples (wine) were withdrawn at appropriate time intervals for analysis.

To improve the bioethanol production by *S. cerevisiae*, the date juice was enriched with different concentrations of two kinds of nitrogen sources: yeast extract and ammonium chloride, at different concentrations: 0.2, 0.4, and 1 g/L.

In the same way, the effect of calcium sources was tested by adding various concentrations to the fermentation medium: 0.2, 0.4, and 1 g/L of CaCl$_2$ and CaCO$_3$.

The bioethanol concentration for each sample was determined according to the following equation:

$$\text{Bioethanol concentration (g/L)} = 10 \times \text{alcohol degree (°)} \times \rho$$

(1)

where $\rho = \text{ethanol density} = 0.789 \text{ g/L at 20 °C}$.

The ethanol productivity was calculated according to the following equation:

$$\text{Ethanol productivity } Q (\text{g/L/h}) = \frac{E_f - E_i}{t_f - t_i}$$

(2)

where $E_i$ and $t_i$ are the initial ethanol concentration and time, respectively, and $E_f$ and $t_f$ are the same parameters at the final stage of fermentation.

2.4.2. Fractional Distillation

After completing the alcoholic fermentation, the suspension (wine and yeast biomass) was centrifuged to clarify the wine. The recuperated wine was then poured into different flasks connected to a distillation column enclosed in runoff water. The round-bottomed flask containing the fermented broth was heated to 78 °C using a suitable heating mantle. The distillate was finally collected from the other end of the column in a conical flask. Samples of very pure bioethanol were recovered at the end of the distillation.

2.5. Chemicals

All chemical reagents supplied by Aldrich (St. Louis, MO, USA) were analytical-grade and used without further purification, including sodium hydroxide (NaOH), sulfuric acid (H$_2$SO$_4$), absolute ethanol (C$_2$H$_5$OH), extra pure yeast extract, calcium chloride (CaCl$_2$), calcium carbonate (CaCO$_3$), and ammonium chloride (NH$_4$Cl).

2.6. Analysis

All the experiments were carried out under rigorously identical experimental conditions. The pH, °Brix, and alcohol degree values during fermentation were determined daily (every 24 h).

A digital thermometer from Gerber Instruments AG (Gerber Instruments AG, Effretikon, Switzerland) was used to monitor the temperature during the fermentation process.

The pH values of the date juice and wine were determined at room temperature via an Oakton PH 550 Benchtop pH meter Kit (Parmer, Vernon Hills, IL, USA), while the total soluble solids (TSS) in the date juice were measured using a handheld refractometer from the HHTEC sugar °Brix refractometer model (HHTEC, Heidelberg, Germany).

The degree of alcohol of the elaborated bioethanol samples before and after the fraction distillation was measured using an alcoholmeter from Gerber instruments AG (Gerber Instruments AG, Effretikon, Switzerland).

The infrared (IR) spectra of all bioethanol samples were obtained by a Fourier-transform infrared spectrophotometer BX FTIR system spectrometer (PerkinElmer, Waltham, MA, USA). Liquid samples were analyzed in the transmittance mode, and the spectra were recorded in the range of 4500–400 cm$^{-1}$. The system was equipped with a deuterated triglycine sulfate (DTGS) detector and a diamond (single reflection) UATR. For each spectrum recorded, 64 scans were collected with a resolution of 4 cm$^{-1}$ and 1 cm/s as the scanning speed, referring to previous work [21].
In addition, the bioethanol obtained in each case was analyzed using 1H NMR to identify the ethyl alcohol. An AVANCE-300 NMR spectrometer (Bruker Inc., Rheinstetten, Germany) was used. For the preparation of the NMR tests, each liquid sample was dissolved in a 0.6 mL/mL solution comprising deuterated chloroform (CDCl₃ (99.96%)) [21]. NMR analyses were performed at 40 °C. Each sample analysis was repeated twice.

3. Results
3.1. Effect of CaCO₃ and CaCl₂ on Fermentation

To investigate the influence of calcium source types on the fermentation process and bioethanol production, calcium carbonate (CaCO₃) and calcium chloride (CaCl₂) were used at different concentrations ranging from 0 to 1 g/L. A fermentation medium without added calcium carbonate or chloride was considered as a control. The results recorded in Figure 1 and Table 1 confirmed that adding calcium sources moderately enhanced bioethanol production, and a more significant effect was detected with calcium chloride (Figure 1B and Table 1). Indeed, at a CaCl₂ concentration of 0.4 g/L, the maximum ethanol concentration (41.5 ± 0.85 g/L) and productivity (0.511 g/L/h) was achieved. Thus, an increase of 31.3% compared to the control sample was acquired.

![Figure 1](image)

Figure 1. Bioethanol concentration during alcoholic fermentation: (A) effect of CaCl₂ and (B) effect of CaCO₃.

Then, experiments with calcium carbonate (CaCO₃) supplementation were performed, where different concentrations of CaCO₃ were added to the fermentation medium. The data collected in Figure 1A show that when the concentration of CaCO₃ increased from 0.2 to 1 g/L, the ethanol concentration and productivity also increased slightly from 31.6 to 38 g/L and from 0.4 to 0.48 g/L/h, respectively. In this context, numerous research studies have evaluated the impact of calcium sources on the fermentation medium driven by various feedstock. However, no one has examined this effect in date juice.

For example, Bajpai and Margaritis [22] tested the effect of CaCl₂ on ethanol fermentation using a bacterial strain of Zymomonas mobilis. The results of this study indicated no significant change in the cell mass and ethanol production rates by supplementing the medium with a concentration of 2.0 g/L of CaCl₂. Another study conducted by Azam et al. [9] reinforced the results of these latest research teams. Indeed, the results proved that CaCl₂ and CaCO₃ supplementation of a fermentation medium increased the ethanol...
production. The optimal ethanol concentration was reached when 0.4 g/L of CaCl$_2$ was introduced into the fermentation medium. However, the impact of the CaCO$_3$ addition was not as conspicuous as that of the CaCl$_2$ addition. The concentration of glucose present in the fermentation medium was the main difference between the studies conducted by these two research teams.

Table 1. Effect of calcium and nitrogen sources on bioethanol fermentation of dates jus medium by S. cerevisiae at 30 °C after 72 h.

<table>
<thead>
<tr>
<th>Calcium Source</th>
<th>Amounts (g/L)</th>
<th>pH$_i$</th>
<th>pH$_f$</th>
<th>Bioethanol concentration (g/L)</th>
<th>Ethanol productivity (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCO$_3$</td>
<td>0.0 (Control)</td>
<td>5 ± 0.2</td>
<td>3.91 ± 0.1</td>
<td>31.6 ± 0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>5 ± 0.2</td>
<td>3.91 ± 0.1</td>
<td>32.4 ± 0.8</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>5 ± 0.1</td>
<td>4 ± 0.1</td>
<td>36.5 ± 1</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5 ± 0.15</td>
<td>4.05 ± 0.1</td>
<td>38 ± 0.9</td>
<td>0.48</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.2</td>
<td>5 ± 0.2</td>
<td>3.81 ± 0.11</td>
<td>33.4 ± 0.8</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>5 ± 0.15</td>
<td>3.84 ± 0.15</td>
<td>41.5 ± 0.85</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5 ± 0.2</td>
<td>3.81 ± 0.15</td>
<td>39.9 ± 0.9</td>
<td>0.511</td>
</tr>
<tr>
<td>Nitrogen Source</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.2</td>
<td>5 ± 0.2</td>
<td>4.01 ± 0.1</td>
<td>39.4 ± 0.9</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>5 ± 0.1</td>
<td>4.07 ± 0.1</td>
<td>47.4 ± 1</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5 ± 0.1</td>
<td>4.08 ± 0.2</td>
<td>55.3 ± 1</td>
<td>0.70</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>0.2</td>
<td>5 ± 0.2</td>
<td>3.95 ± 0.1</td>
<td>47.4 ± 0.9</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>5 ± 0.1</td>
<td>3.75 ± 0.1</td>
<td>50.9 ± 0.8</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>65.3 ± 1</td>
<td>0.83</td>
</tr>
</tbody>
</table>

On the other hand, it is essential to draw attention to the evolution of the °Brix (TSS) values, which are equivalent to the sugar concentration, during fermentation. Certainly, as shown in Figure 2A,B, the increase in ethanol concentration was accompanied by a sharp decrease in the TSS during the fermentation process. However, the sugar (°Brix) was not entirely consumed, and it stabilized between 4.5 and 5 °Brix in all the investigated samples. This phenomenon is probably due to two reasons: First, toxic substances in wine, in particular octane and decane, would have aggregated and accumulated in the medium of fermentation [23]. Second, ethanol is known to inhibit the growth of microbes. Indeed, a recent study confirmed the ability of ethanol to destroy the DNA of yeast cells and to inactivate numerous enzymes [24].

Similarly, during the ethanol fermentation process, we observed that the pH of the fermentation medium decreased during the first 24 h (Figure 3). Then, after 48 h of fermentation, the pH gradually increased. This could be due to the presence of carbonic acid (H$_2$CO$_3$) formed in the medium from carbon dioxide (CO$_2$) and water (H$_2$O).
Figure 2. Evolution of total soluble solids (TSS) during anaerobic fermentation: (A) effect of CaCl$_2$ and (B) effect of CaCO$_3$.

Figure 3. pH evolution during anaerobic fermentation: (A) effect of CaCl$_2$ and (B) effect of CaCO$_3$.

3.2. Effect of Yeast Extract and NH$_4$Cl on Fermentation

Yeast extract is a natural ingredient extracted from selected autolyzed yeast cells. This compound is composed of a variety of amino acids, carbohydrates, inorganic salts, and vitamins, and it is rich in high-quality proteins. The effect of yeast extract was studied by varying its concentration from 0 to 1.0 g/L. As shown in Figure 4A and Table 1, yeast extract potentially increased the ethanol production. A higher ethanol concentration was achieved by introducing 1 g/L of yeast extract. In this case, the ethanol concentration and productivity reached approximately 55.3 ± 1 g/L and 0.7 g/L/h, respectively, while the final ethanol concentrations and productivity of the control did not exceed 31.6 ± 0.5 g/L.
and 0.4 g/L/h, respectively. Thus, compared to the control samples, a positive effect was obtained by adding yeast extract as a nitrogen source. This is probably due to the fact that the nutrients in yeast extract include more assimilable nitrogen, vitamins, and other growth factors necessary to improve yeast cell viability, and hence the productivity rate of ethanol was improved [25]. Furthermore, the efficacy of yeast extract for ethanol production in this present work was in good agreement with the finding of Turhan et al. [26]. They demonstrated that supplementing yeast extract to a carob extract culture improved ethanol production. These studies were followed by Chniti et al. [27]. They found that yeast extract had a significant impact on ethanol production by *S. cerevisiae* to improve the profitability of fuel alcohol production from date syrup.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 4.** Bioethanol concentration during alcoholic fermentation: (A) effect of yeast extract and (B) effect of NH₄Cl.

To confirm the excellent effect of nitrogen sources on alcoholic fermentation, another experiment was conducted with ammonium chloride as the second type of nitrogen source. The results reported in Figure 4B and Table 1 reveal that considerable enhancements to ethanol concentration and productivity were noticed upon the addition of a small amount of NH₄Cl (0.2 g/L). An increase in the ammonium level induced a significant improvement in bioethanol production. Indeed, supplementing the fermentation medium with 1 g/L of NH₄Cl gave an optimal ethanol concentration and productivity, reaching more than 65 g/L and 0.83 g/L/h, respectively. This is an increase of 106.6%. Therefore, a synergetic action of the ammonium effect was observed compared to fermentation without an additional source of nitrogen. Numerous studies have proven that in must or synthetic must, ammonium chloride supplementation generally lowers the fermentation’s duration and increases the rate of fermentation [28]. Ter Schure et al. [29] reported that growth in the presence of high nitrogen sources, including ammonia, asparagine, and glutamine, had relatively higher growth rates than on poor sources, such as proline and urea. Chniti et al. [27] obtained a higher ethanol yield of 37.2 g/L by supplementing date syrup with ammonium chloride (1.0 g/L). Shafaghat et al. [30] reported that using NH₄Cl with an initial concentration of 1.5 g/L produced 14.1 g/L of ethanol from molasses (35 g/L). These values of bioethanol concentration are lower than those obtained in the current work, showing the efficiency of the “Khodhari” dates as a promising feedstock for bioethanol production by *S. cerevisiae*, especially in Saudi Arabia. It is now well-admitted
that ammonium sources have a significant effect on alcoholic fermentation, and then bioethanol production. Nevertheless, according to Moreno et al. [31], adding an ammonium source is not always beneficial to fermentation. This contrast in the results may be linked in the first place to the diversity of the matrix, the *S. cerevisiae* strain, and the assimilation of the ammonium source by the microorganism *S. cerevisiae*, which directly influences the biomass and leads to a good increase in the yeast population during the growth phase, therefore resulting in a high production of ethanol [32]. Second, during the fermentation process, nitrogen is extracted from the medium by the cell and directly incorporated into proteins and amino acids, improving both the rate and yield production [33]. Thus, differences in nitrogen sources affect this latter correlation. Consequently, evaluating the impacts of all ammonium salts on the various *S. cerevisiae* strains in a single conventional synthetic medium could be an excellent methodology to test the effect of nitrogen supplementation.

Moreover, by increasing the concentration of ethanol, a remarkable reduction in the level of the TSS (°Brix) was observed (Figure 5). This result is very reasonable and expected, since during the alcoholic fermentation process, the *saccharomyces* yeast consumed sugar (°Brix) to produce bioethanol and CO₂ at the end.

![Figure 5](image)

**Figure 5.** Assessment of total soluble solids (TSS) during anaerobic fermentation: (A) effect of yeast extract and (B) effect of NH₄Cl.

On the other hand, a careful analysis of the pH profile of the date wine extracted during the fermentation process showed that the pH in the media decreased quickly within the first 24 h of fermentation (Figure 6). The cultures containing ammonium chloride exhibited a lower final pH than those revealed in media with only yeast extract (Table 1). This can be explained by the action of the central carbon metabolism, which reduces the pH, but mainly by the excretion of various organic acids generated as by-products of this metabolism. However, for ammonium added as a second nitrogen source, a proton has to be exported to maintain the proton motive force homeostasis [34], which caused a decrease in the pH medium to 2.9 after 24 h (Table 1). Moreover, the addition of ammonium as a nitrogen source caused an increase in the activity of G3PDH, suggesting that the metabolic shift could be induced by pH degradation [35].
Figure 6. pH evolution during anaerobic fermentation: (A) effect of yeast extract and (B) effect of NH$_4$Cl.

In order to further evaluate the bioethanol production and to emphasize the impact of the different added sources of nitrogen and calcium, the following Table 2 is an overview to compare our bioethanol production with other date varieties in the literature.

Table 2. Comparison of production conditions and performance of our produced bioethanol with other varieties in the literature.

<table>
<thead>
<tr>
<th>Date by-products (varieties)</th>
<th>Time (h)</th>
<th>pH</th>
<th>Yeast Without *</th>
<th>Ethanol concentration (g/L)</th>
<th>Increase (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date juice (Kunta, Eguoua, and Bouhatem)</td>
<td>72</td>
<td>6</td>
<td>S. cerevisiae</td>
<td>50</td>
<td>-</td>
<td>[36]</td>
</tr>
<tr>
<td>Date syrup (Deglet-Nour)</td>
<td>72</td>
<td>6</td>
<td>S. cerevisiae</td>
<td>63</td>
<td>-</td>
<td>[37]</td>
</tr>
<tr>
<td>Date molasses (commercial molasses from Saudi)</td>
<td>96</td>
<td>4</td>
<td>H. guilliermondii</td>
<td>11</td>
<td>-</td>
<td>[38]</td>
</tr>
<tr>
<td>Syrup dates (Deglet-Nour)</td>
<td>72</td>
<td>-</td>
<td>S. cerevisiae</td>
<td>24.8</td>
<td>68.9</td>
<td>[27]</td>
</tr>
<tr>
<td>Syrup dates (Ruzaiz variety)</td>
<td>48</td>
<td>-</td>
<td>S. cerevisiae</td>
<td>48.9</td>
<td>-</td>
<td>[39]</td>
</tr>
</tbody>
</table>

*: Nitrogen source addition, Ca: calcium source addition; -: Without nutrient addition

3.3. IR Analyses

The FTIR spectra were recorded for the synthesized and commercial ethanol. To control troll samples, only the bioethanol produced under optimal production conditions was analyzed (0.4 g/L CaCl$_2$ and 1 g/L NH$_4$Cl).

The FTIR spectra are presented in Figure 7. Compared to the commercial product, the typical absorption bands were observed for all the logged spectra. A large and strong band was observed at 3340 cm$^{-1}$, related to the O–H stretching frequency, indicating the
presence of the hydroxyl groups of alcohol. The bands detected at 2971 and 2881 cm\(^{-1}\) were attributed to the C-H stretching vibration. The additional band at 1386 cm\(^{-1}\) confirmed the existence of the C-OH band of ethanol. The two bands observed at 1087 and 1045 cm\(^{-1}\) were assigned to the stretching vibration of C-OH [3,40]. Moreover, a C-C stretching band was also detected at 880 cm\(^{-1}\). However, compared to the commercial spectrum, a weak peak observed at 1644 cm\(^{-1}\) was detected for the elaborated bioethanol samples. This latter peak was relative to the water bands, which correspond to the bending vibration of the H-O-H angle and the stretching vibration of the O-H groups, respectively [41]. This behavior was probably due to the azeotrope mixture (ethanol and water).

Figure 7. Infrared spectra of the pure commercial ethanol, the control, and the optimal bioethanol products.
3.4. 1 H NMR Analysis

Figure 8 displays the 1 H NMR spectrum of the optimal synthesized bioethanol samples. The first important point is the high purity of the two elaborated bioethanol samples. In fact, contrary to the FTIR analyses, which showed a slight trace of water, when bioethanol was examined with a 1 H NMR spectrometer, no peaks from impurities or water were observed, as shown in Figure 8. Furthermore, there was a great resemblance between the recorded 1H NMR spectrum and that quoted in the bibliography. Indeed, bioethanol exhibited a strong triplet signal at 1.00 ppm, conforming to the protons from the methyl group, -CH3--; a multiplet signal at 3.66 ppm, shaped by the protons of the methylene group, -CH2--; and a small peak cited at 4.2 ppm, representing the single H in the OH group [42].

![Figure 8. 1H NMR spectra of the optimally produced bioethanol: (A) 1 g/L NH₄Cl and (B) 0.4 g/L CaCl₂.](image)

As a general comment, the spectroscopy analysis (FTIR and NMR) of the manufactured bioethanol confirmed that the ethanol group (C₂H₅OH) was present. In addition, a superb agreement between the elaborated and commercial ethanol was confirmed.

3.5. Technical Design

The amount of higher alcohols formed during fermentation depends on several parameters, such as the yeast strain, the experimental conditions, and the type of fermenter (bioreactor) used. Due to the importance of technical materials as tools that help in process control, enhancement in substrate conversion, and improvement in the productivity and the quality of the fermentation product [43], a laboratory fermenter (bioreactor) was designed. Figure 9A shows the block flow diagram for bioethanol production, including the most important part, which is the fermenter. Generally, a successful fermenter displays a beneficial effect on the biological reaction. For this reason, we tried to take many considerations into account in our design. Particular care was given to several elements, such as temperature, pH-regulating devices, sufficient aeration, agitation, input (nutrients, yeast, etc.), and a drain or overflow vent to extract the waste biomass and its products from the cultured microorganisms. The designed fermenter/bioreactor was a stainless-steel vessel. The size was fixed to be 20 L. Figure 9A illustrates the different parts of the designed laboratory-scale fermenter.
Figure 9. Schematic representation of bioethanol production processes: (A) lab-scale and (B) scale production design of bioethanol produced from palm dates.

The scale-up of a bioreactor (the step from a small scale to a production scale) is a challenging task, since many different aspects of engineering (physical and metabolic processes) and economic considerations need to be taken into account. The final scale-up will essentially be a delicate compromise between inherently conflicting desirable characteristics. One might regard a scale-up as more an art than a science [44]. Recently, the major bioreactor scale challenge has been translating lab-scale product design into large-scale production. In this section, we tried to make a plant design for ethanol production...
with *Saccharomyces cerevisiae* yeast and nutrients added under anaerobic conditions. The proposed flow diagram of the designed plant is explained in Figure 9B.

This plant consists of three chief units, which include a hydrothermal unit, a fermentation unit, and a distillation unit. They are described as follows:

3.5.1. Hydrothermal Unit

The commercial method for extracting sugars from fruit is a simple hydrothermal treatment [45]. The hydrothermal unit is an integral section for sugar production from date fruits. In Reactor 1, the pretreatment dates (pitted and crushed) are converted to glucose by a hydrothermal extraction using water at a temperature of about 80 °C for 45 min. The juice from the hydrothermal extraction was filtered to separate cellulosic fibers (Separator 1) and then transferred into Vessel 1.

3.5.2. Fermentation Unit

Extracted sugar can be served as an essential carbon source for yeast growth and bioethanol production in anaerobic conditions [46]. In the fermentation unit, glucose from date feedstock is converted to carbon dioxide and ethanol using *S. cerevisiae* and supplemented nutrients in Reactor 2. For all that, environmental factors are known to significantly affect the growth and metabolism of microorganisms as well as ethanol yield production. Such environmental factors include pH and temperature. Therefore, during alcoholic fermentation, we tried to keep the optimal and suitable conditions for the activity and growth of the considered mesophilic bacteria in the simulation. Subsequently, the fermentation process is performed at a temperature of around 30 °C and a pH of 4–5, which are suitable conditions for alcoholic fermentation [21,47].

3.5.3. Distillation Unit

The major concern in the production of ethyl alcohol on an industrial scale is obtaining anhydrous ethanol whose concentration exceeds 98%. This objective is achieved by eliminating water. Distillation, as a simple and traditional method, could be performed to remove water content. The principle of this technique is based on the exploitation of differences in the boiling temperatures of substrates in a solution. Indeed, reaching the boiling temperature of ethanol (78.5 °C) leads to the separation of water. Hence, bioethanol with the intended purity was recuperated.

The output stream from Reactor 2 with a molar fraction of ethanol, about 9–10%, was transferred to Vessel 2 for the removal of the CO₂ gas generated from the process. The top stream of Vessel 2 conducts to a separation unit to recuperate the ethanol and the CO₂ gas moves to the scrubber unit. After that, impurities from the mixed stream will be removed inside the separation unit. At this time, clarified wine is introduced to Distillation Column 1 at a temperature of about 84 °C, referring to Salikandi et al. [48]. It should be noted that only a minimal amount of approximately 4% of water could be found in the final bioethanol produced by these standard-phase distillation techniques. The various non-fuel applications of bioethanol do not require the total elimination of water. However, for fuel applications, the purification and drying of bioethanol must be complete and this could be ensured by various techniques, including extractive distillation, molecular sieves or adsorption processes, chemical dehydration, azeotropic distillation, membrane processes, diffusion distillation, and vacuum distillation [49]. In this case, azeotropic distillation techniques were chosen to purify and dry the bioethanol. Thus, for ethanol dehydration, the azeotropic ethanol/water mixture is transferred to Distillation Column 2. Then, the azeotropic point is broken at 75 °C via the use of a separating agent. The agent stream is composed of a mixture of ethylene glycol (60 mol%) and glycerol (40 mol%), according to Gil et al. [50]. In order to increase the temperature of the glycol mixture to reach 75 °C, a shell and tube heat exchanger is used. The shell inlet is indeed the lower stream of Distillation Column 1 [51]. Table 3 is an overview of the different operating parameters of Distillation Column 2.
Table 3. Various operating parameters of Distillation Column 2.

<table>
<thead>
<tr>
<th>Operating Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature of alcoholic wine</td>
<td>75 °C</td>
</tr>
<tr>
<td>Feed/solvent ratio</td>
<td>1.25</td>
</tr>
<tr>
<td>Reflux ratio</td>
<td>1.1</td>
</tr>
<tr>
<td>Number of theoretical steps</td>
<td>16</td>
</tr>
<tr>
<td>Feed stage</td>
<td>7</td>
</tr>
<tr>
<td>Column pressure</td>
<td>1 atm</td>
</tr>
</tbody>
</table>

Finally, a highly pure bioethanol product with a concentration varying from 97 to 99 mol% is recuperated. The techno-economic investigation of the production of bioethanol from palm dates as a feedstock in Saudi Arabia will be examined in detail in the next publication.

4. Conclusions

In summary, the results obtained in the present study revealed the performance of “Khodhari” date by-products from the Saudi Qassim region as a promising low-cost feedstock for bioethanol production using *Saccharomyces cerevisiae*. This production could well be enhanced by adding different nutrient sources during the fermentation process. Indeed, the results confirmed the excellent effect of calcium and nitrogen sources added at different concentrations ranging from 0 to 1 g/L. Supplementation with calcium sources such as CaCl\(_2\) and CaCO\(_3\) moderately improved bioethanol production, and a more significant effect was detected with calcium chloride, with a recorded production increase of 31.3%. The addition of yeast extract and NH\(_4\)Cl to the date juice fermentation media led to a substantial enhancement in ethanol concentration and productivity. The addition of NH\(_4\)Cl exhibited comparatively higher productivity and ethanol concentration than yeast extract used as a nitrogen source (with a production increase of 106.6%). Spectroscopy analyses (FTIR and NMR) of the manufactured bioethanol confirmed the obvious presence of the ethanol group (C\(_2\)H\(_5\)OH). In addition, a superb concordance between elaborated and commercial ethanol was confirmed. Thus, “Khodhari” date palm fruit represents an appreciated natural source of bioethanol. It can be considered a renewable and sustainable alternative biofuel or medical alcohol.

To achieve a higher concentration of ethanol, a laboratory-scale fermenter was designed. For the industrialization of this fermentation process, an elaborated flow diagram of the designed plant was proposed. The simplicity of the developed process encourages the launch of a bioethanol production plant in Saudi Arabia. However, this phase will be completed after a thorough techno-economic analysis, which will be the focus of the next publication.


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References


2. Ghazanfar, M.; Nadeem, M.; Shakir, H.A.; Khan, M.; Ahmad, I.; Franco, M.; Chen, L.; Irlan, M. Valorization of *Bombax ceiba* Waste into Bioethanol Production through Separate Hydrolysis and Fermentation and Simultaneous Saccharification and Fermentation. *Fermentation* 2022, 8, 386. [CrossRef]


5. Almutawa, A.A. Date production in the Al-Hassa region, Saudi Arabia in the face of climate change. *J. Water Clim. Chang.* 2022, 13, 2627–2647. [CrossRef]


8. Pejin, J.D.; Mojović, L.V.; Pejin, D.J.; Kocić-Tanackov, S.D.; Savić, D.S.; Nikolić, S.B.; Djukić-Vuković, A.P. Bioethanol production from triticate by simultaneous saccharification and fermentation with magnesium or calcium ions addition. *Fuel* 2015, 142, 58–64. [CrossRef]


28. Jiménez-Martí, E.; Aranda, A.; Mendes-Ferreira, A.; Mendes-Faia, A.; del Olmo, M.L. The nature of the nitrogen source added to nitrogen depleted vinifications conducted by a Saccharomyces cerevisiae strain in synthetic must affects gene expression and the levels of several volatile compounds. Antonie Leeuwenhoek 2007, 92, 61–75. [CrossRef]
42. Zuriarrain, A.; Zuriarrain, J.; Villar, M.; Berregi, I. Quantitative determination of ethanol in cider by 1H NMR spectrometry. Food Control 2015, 50, 758–762. [CrossRef]