Evaluation of fermentation parameters and yeasts selection for ethanol production from sweet sorghum juice

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Abstract
There are several organisms able to ferment sugars and produce ethanol and parameters applied during fermentation process directly affect metabolic activity of the organisms. Selecting the proper strain of organism and optimal parameters for fermentation is essential for fermentation performance and ethanol production and yield. From twelve microbial strains tested (yeasts from Saccharomyces and Kluyveromyces genus and bacteria from Zymomonas genus), the yeasts strains of Saccharomyces cerevisiae performed better fermentation in sorghum juice and produced higher ethanol yields. Inoculation rate should not exceed 5% of well-developed vegetative culture or 0,5 g·l⁻¹ dry yeast. One yeast strain have been selected as having better performance and producing high yields of ethanol at 37°C in sweet sorghum juice (over 83% of theoretical yield). Supplementing sorghum juice with exogenous nitrogen and phosphorus sources the yields of ethanol slightly increases.

Keywords: fermenting organisms, fermentation parameters, Saccharomyces, selection, sweet sorghum juice

1. Introduction
Sorghum has been cultivated from ancient time as source of food and feed. Sorghum seeds are used today as staple food in some regions. Since it accumulates sugars in stalk, sweet sorghum is used as source of sugar for food and fermentation industry, or as edible syrup. Sorghum sweet juice usually contains enough fermentable sugars to be used directly as fermentation medium, without the need for enzymatic hydrolysis of polysaccharides, as in ethanol from starch technologies. In some cases, sugar content of sorghum sweet juice is similar with the sugar content of cane juice, the difference consists of the sugars composition: as in sugar cane juice predominanty sucrose is present, in sorghum juice is a mixture of sucrose, glucose and fructose (Worley & al, [1], Smith & al, [2], Vintila & al, [3]). This makes the sorghum juice to be inappropriate for crystallization to produce crystal sucrose, but can be concentrated to obtain syrup. Ethanol production from sorghum juice can be systematized in three steps: juice extraction, ethanol fermentation and ethanol separation. As the last two steps depend on the quality and concentration of the raw material, several researches have been made to evaluate the impact of juice concentration on ethanol yield in fermentation step (Sasaki & al, [4], Mazumbar & al, [5]). Besides concentration of sugars and content of other yeast nutrients in sorghum juice, nitrogen source addition, type of inoculums and performance of fermenting organism are main factors affecting ethanol yield, productivity and efficiency of the process. There are several organisms able to ferment sugars and produce...
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ethanol and parameters applied during fermentation process directly affect metabolic activity of the organisms. Selecting the proper strain of organism and optimal parameters for fermentation is essential for fermentation performance and ethanol production and yield. The aim of this research was to investigate capacity of several microorganisms to ferment sugars from sorghum juice, to evaluate the influence of inoculation rate and temperature, nitrogen phosphorus source addition on the productivity of ethanol fermentation process.

2. Materials and methods
2.1. Sorghum juice extraction
Romanian hybrid F135ST of sweet sorghum \textit{[Sorghum bicolor] (L) Moench} produced by INCDA Fundulea - Romania, was cultivated at the Experimental Didactic Station of USAMVB “King Mihai of Romania” from Timisoara in 2014. The plants were harvested at the early milk stage of maturity and chopped to 2 cm theoretical length of cut using a forage harvester. The juice was extracted from sorghum biomass using a stainless steel press (5.5 Litre Cross Beam Press – Ferrari Group Italy).

2.2. Selection of fermenting microorganisms
Twelve microorganisms were studied for their capacity to ferment sugars from sorghum juice: nine strains of \textit{Saccharomyces cerevisiae}: \textit{S. cerevisiae} CMIT 2.1, \textit{S. cerevisiae} CMIT 2.7, \textit{S. cerevisiae} SMR-4 (killer yeast), \textit{S. cerevisiae} K5-5A, \textit{S. cerevisiae} K5-5A rho-, \textit{S. cerevisiae} Hansen CBS 5926 (\textit{S. boulardii}), \textit{S. cerevisiae} Cara R581, \textit{S. cerevisiae} CMIT 2.24 and \textit{S. cerevisiae} CMIT 2.25, two strains of \textit{Kluyveromyces} sp. (\textit{K. marxianus} DSM 5418 and \textit{K. lactis} var. \textit{lactis} DSM70799), and one bacterial strain (\textit{Zymomonas mobilis} DSM 424). The strains noted CMIT are local strains and the number represents the position in the catalogue of the Collection of Industrial Microorganisms of USAMVB “King Mihai of Romania” – Timisoara. The microorganisms are preserved by deep-freezing at -70°C, in 19% glycerol solution. The cryotubes containing frozen suspension of microorganisms are thawed and transferred on solid culture media in plates to obtain single colonies. One colony is used to inoculate 20 mL of specific sterile liquid medium in 100 mL Erlenmeyer flasks covered with cotton plugs (culture media used to obtain inoculums are: YPD for \textit{Saccharomyces} sp., YM for \textit{Kluyveromyces} sp., and ZM for \textit{Zymomonas} sp.). The inoculated flasks were incubated for 18 hours in an aerated incubator with rotating shaker (GFL 3031) at 30°C and agitation speed of 150 r·min⁻¹. These cultures have been used to inoculate 15 ml. sterile tubes containing 12 mL sorghum juice. Part of the juice was sterilized and flocculation of proteins and other components have occurred, while other part of the juice was fermented unsterilized. Each strain of microorganisms has been inoculated (600 µL inoculums for each 12 mL sorghum juice) in three tubes of sterile and unsterile sorghum juice. The tube's covers were perforated with short injection needles to allow gas releasing. In the outer end of each needle flexible tubes were inserted and the other ends of the tubes were immersed in graduated tubes filled with water and immersed upside down in water bath. This way, the volume of released gas during fermentation was measured. The inoculated tubes together with gas collecting tubes were incubated at 32°C for the first 48 hours and at 30°C for other 48 hours. We applied this long period of fermentation (in total 96 hours) to evaluate the fermentation capacity of all tested strains – although \textit{Saccharomyces} yeasts are known to rapidly ferment sugars (under 48 hours), the \textit{Kluyveromyces} and \textit{Zymomonas} organisms may need longer time to ferment all sugars found in sorghum juice.
2.3. Establishing optimal inoculation rate

To evaluate the optimal inoculation rate of sorghum juice, we have chosen a regular bakery yeast. As the strain is available in dried form as well, we have used the dry form and the vegetative form to inoculate sorghum juice. The dried yeast was used in the following rates of inoculation: 0.5 g·l⁻¹, 1 g·l⁻¹, 2 g·l⁻¹ and 3 g·l⁻¹. Inoculums applied using vegetative form of the yeast strain were 24 hours cultures in YPD broth as follows: 5 % and 10 % inoculation rate of liquid YPD culture obtained from dried yeast (YPD was inoculated with 0.5 mg/ml dry yeast); and 10 % inoculation rate of liquid YPD culture obtained from single colony of the same yeast strain. These seven inoculation rates were applied to eight 500 mL flasks equipped with gas outlets containing 250 mL sorghum juice. The gas released during fermentation was collected in special gas collecting bags. Samples were harvested after 24 and 48 hours of fermentation and concentration of ethanol was measured.

2.4. Establishing optimal fermentation temperature

There is a variety of yeast strains able to ferment sugars at low temperatures (as yeasts used in brewery) and yeasts growing and fermenting faster at high temperatures. From the total of twelve microbial strains tested for the capacity to produce ethanol from sweet juice, six best producing ethanol strains where selected to test their optimal fermentation temperature (in an interval of 10°C). The strains where inoculated into 500 mL flasks equipped with gas outlets containing 250 mL sorghum juice. Four flasks where inoculated with each strain and where incubated at four temperatures: 27, 32, 35 and 37°C. Samples were harvested every four hours after inoculation and concentration of ethanol was measured.

2.5. Ethanol fermentation and ethanol yields

The yeast strain *S. cerevisiae Hansen* CBS 5926 (*S. boulardii*) was selected after previous tests to be used in ethanol fermentation of sorghum sweet juice. Four sets of fermentation batches were conducted. In the first set (F1), *S. cerevisiae* CBS 5926 was inoculated into sorghum juice without any supplements. The inoculation rate was 5% inoculums of 24 hours yeast cultures on YPD medium. In the other three sets of batches, the yeast was inoculated into sorghum juice with the following supplements: nitrogen source (by adding 2.2 g·l⁻¹ nitrogen from (NH₄)₂SO₄), phosphorus source (by adding 0.8 g·l⁻¹ phosphorus from KH₂PO₄) and combined nitrogen / phosphorus source, (by adding both minerals in the previous concentrations). These concentrations of nitrogen and phosphorus where established from previous research and indicated by Y. Jianliang & al, [6].

Ethanol yield (%) was calculated as the concentration of ethanol produced (g·l⁻¹) multiplied by 0.51 divided by the concentration of initial sugar minus the concentration of residual sugar, all multiplied by 100, where 0.538 indicates the theoretical ethanol yield (0.538 g ethanol / g sucrose; Sasaki & al [4], Andrzejewski & al [7]). Ethanol production rate, or ethanol productivity (reported in g·l⁻¹·h⁻¹) was calculated dividing the final concentration of ethanol (g·l⁻¹) by the fermentation time required to obtain this ethanol concentration.

2.6. Sample analysis

Total soluble solids were measured as degrees Brix using a refractometer (Hanna Instruments, Germany). To determine the total sugars, samples harvested from sorghum juice and fermentation media were centrifuged to remove solids and supernatant was analyzed by DNS method (T.K. Ghose [8]). Ethanol concentration was estimated by NIR methods [Near Infrared] using Alcolyzer M (Anton Paar - Austria). This device uses a patented method (US
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6,690,015; AT 406711) based on near infrared (NIR) spectroscopy to determine the alcohol content in a highly alcohol-specific range between 1150 nm and 1200 nm. The evaluation method uses the significant alcohol peak in this area and two spectral points very close to it for defining the baseline.

3. Results and discussions

Juice was extracted from sorghum stalks and the characteristics in table 1 where obtained. The concentration of reducing sugars is relatively low. For the next working phases, when fermenting microorganisms will be selected and optimal inoculation rate will be set, we have used as fermentation medium the raw harvested sweet juice, without any supplements or concentration. The freshly harvested juice was stored at -20°C until use.

![Table 1. Characteristics of sorghum juice extracted by pressing](attachment:table1)

<table>
<thead>
<tr>
<th>Extracted sweet juice / biomass (g/g)</th>
<th>Concentration of reducing sugars (g/l)</th>
<th>°Brix</th>
<th>Dry mater of resulted bagasse (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.22</td>
<td>84.52</td>
<td>15.8</td>
<td>30.50</td>
</tr>
</tbody>
</table>

To increase the concentration of fermentable sugars the juice can be concentrated or readily fermentable sugars can be added to the juice. For the next working phases, when yeasts will be selected and optimal fermenting parameters will be set, we have used as fermentation medium the harvested sweet juice additivated with sucrose to obtain 120 g·l⁻¹ reducing sugars. In this sucrose-added juice, Brix increased to 18.5.

In the phase of selection of fermenting organism, the twelve microbial strains have been cultivated at 32°C in sweet juice; the gas released during fermentation was collected and measured and the ethanol concentration was measured after 24 hours and at the end of the 48 hours of incubation. The fermentations where conducted in triplicate and sterilized / non-sterilized sorghum juice was used as fermentation medium. Regarding this aspect, coagulation and separation has occurred during the sterilization of sorghum juice in autoclave at 121°C for 20 minutes. Some components from sorghum juice (mainly proteins) decanted after sterilization and the juice was clarified. Still, we have found no differences between concentrations of ethanol produced in sterile and unsterile sorghum juice with the 12 strains of organisms used in our study. Results obtained and presented in figure 1 and 2 indicate high differences in the fermentation capacities of the tested organisms (average productions in triplicates in sterile and unsterile sorghum juice), with bacteria *Zymomonas mobilis* producing lowest concentration of ethanol during the period of 48 hours of incubation. There are slow fermenting strains, as *Z. mobilis, K. lactis*, or some of the *S. cerevisiae* strains, producing ethanol after 24 hours of incubation. The strains displaying the fastest fermenting metabolisms and highest ethanol concentrations have been further evaluated to establish the main fermentation parameters, such as inoculation rate, time of fermentation and temperature of incubation.
Applying different types of inoculums containing the same yeast stain to start the fermentation process, we have found that weak inoculums obtained by inseminating YPD broth with a single colony of yeast leads to a late start of growth and slow process of fermentation (table 2). All three cases of inoculation with vegetative cultures of yeast in YPD broth (table 2, lines 1-3) leads to lower concentration of ethanol produced in fermentation medium comparing with inoculums consisting on dry yeast (table 2, lines 4-7). If inoculation with dry yeast is available, 0.5 g·l⁻¹ appears to be optimal rate of inoculation, higher inoculation rates are not justified as higher rates led to lower ethanol concentration after 48 hours of fermentation and the differences between the ethanol productions at 24 hours of fermentation are insignificant. Still, in industrial scale dry yeast is too expensive to be used as inoculums. Hence, if vegetative yeast culture have to be applied, we recommend inoculation with 5% of reach inoculums, as data in table 2 indicate no improvement, or even lower concentration of ethanol in cultures where higher inoculation rate was applied (10%). Reach inoculums was obtained in our study by cultivating yeast in YPD inoculated with 0.5 g·l⁻¹ dry yeast.
Table 2. Influence of inoculation rate in ethanol production

<table>
<thead>
<tr>
<th>Type of inoculum and inoculation rate</th>
<th>Ethanol concentration (g%) in fermentation samples</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vegetative culture in YPD broth from single colony - 10%</td>
<td>1.51</td>
<td>4.18</td>
<td></td>
</tr>
<tr>
<td>2. Vegetative culture in YPD broth from dry yeast - 5%</td>
<td>2.72</td>
<td>4.65</td>
<td></td>
</tr>
<tr>
<td>3. Vegetative culture in YPD broth from dry yeast - 10%</td>
<td>2.44</td>
<td>4.65</td>
<td></td>
</tr>
<tr>
<td>4. Dry yeast - 0.5 g l⁻¹</td>
<td>3.36</td>
<td>4.98</td>
<td></td>
</tr>
<tr>
<td>5. Dry yeast - 1 g l⁻¹</td>
<td>3.43</td>
<td>4.71</td>
<td></td>
</tr>
<tr>
<td>6. Dry yeast - 2 g l⁻¹</td>
<td>3.46</td>
<td>4.74</td>
<td></td>
</tr>
<tr>
<td>7. Dry yeast - 3 g l⁻¹</td>
<td>3.55</td>
<td>4.24</td>
<td></td>
</tr>
</tbody>
</table>

Temperature of fermentation is an important parameter in any bioprocess and data in figure 3 demonstrate that in the bioprocess of ethanol production this parameter should be carefully chosen as the productivities of different yeast strains can be strongly affected by temperature. The strain *S. cerevisiae* CBS 5926 is an organism largely used in probiotics and related products in humans or animals. Consequently it is selected to tolerate higher temperatures compared with the other yeasts studied here. It grows better at 37°C and produces ethanol in high concentration at this temperature. On the contrary, *S. cerevisiae* Cara R581 is a yeast used in brewery, where the fermentation process is conducted at lower temperatures and our data demonstrate highly intolerance for temperature higher than 35°C. Regarding the other yeast strains studied here, temperatures between 32 and 35°C are optimal for ethanol production.

If we compare the productions of ethanol of the tested strains, our data indicate *S. cerevisiae* CBS 5926 as producing the highest concentrations of ethanol at the temperature of 37°C (last graph in figure 3). Consequently, we have selected this yeast strain to be used in our next research to ferment sweet sorghum juice at 37°C.

**Figure 3.** Temperature tolerance related to ethanol yields in some *Saccharomyces cerevisiae* strains

![Graphs showing temperature tolerance for different Saccharomyces cerevisiae strains](image_url)
In the case of batches where sorghum juice was supplemented with nitrogen source (by adding 2.2 g·l⁻¹ nitrogen from (NH₄)₂SO₄), phosphorus source (by adding 0.8 g·l⁻¹ phosphorus from KH₂PO₄) and combined nitrogen / phosphorus source, (by adding both minerals in the previous concentrations), the ethanol yields are presented in figure 4.

In the first batch, sweet sorghum juice containing 120 g·l⁻¹ reducing sugars, with no exogenous nitrogen or phosphorus sources added was fermented with *S. cerevisiae* CBS 5926. The maximum ethanol concentration of 54,1 ±0,4 g·l⁻¹ was reached after 48 h of fermentation. The ethanol productivity (maximum ethanol concentration divided by fermentation time) was 1,127 g·l⁻¹·h⁻¹. The ethanol yield (grams of ethanol produced per gram of reducing sugars) was 0,451±0,009, which represent an ethanol yield of 83,83% of theoretical yield of 0,538. In the second batch, where sweet sorghum juice was supplemented with nitrogen (NH₄)₂SO₄), the ethanol production is almost opposable with ethanol production in the batch with no exogenous nitrogen source and the maximum ethanol concentration of 54,0 ±0,45 g·l⁻¹ was reached after 48 h of fermentation. The ethanol...
productivity in this case was 1,125 g·l⁻¹·h⁻¹ and the ethanol yield was 0,450±0,01, which represent an ethanol yield of 83,64% of theoretical yield. These data indicates that adding ammonium sulphate to sorghum juice does not cause any increase of ethanol yield. In the third batch, where sweet sorghum juice was supplemented with phosphorus (KH₂PO₄), the ethanol production is slightly higher comparing with the first two batches. A comparable ethanol concentration of 54,9 ±0,35 g·l⁻¹ was reached after 48 h of fermentation. The ethanol productivity in this case was 1,144 g·l⁻¹·h⁻¹ and the ethanol yield was 0,457±0,009, which represent an ethanol yield of 85,04% of theoretical yield. These data indicates that by adding exogenous phosphorus source to sorghum juice, the ethanol yield can slightly increase. In the fourth batch, where sweet sorghum juice was supplemented with phosphorus and nitrogen, the ethanol production is the highest comparing with the other three batches. The highest ethanol concentration of 56,1 ±0,4 g·l⁻¹ was reached after 48 h of fermentation. The ethanol productivity in this case was 1,169 g·l⁻¹·h⁻¹ and the ethanol yield was 0,467±0,008, which represent an ethanol yield of 86,90% of theoretical yield. These yields are comparable with those obtained by Sasaki et al. [4] in concentrated sorghum juice.

4. Conclusions

➢ From twelve microbial strains tested for their capacity to ferment sugars from sweet sorghum juice and produce ethanol, six yeast strains of *Saccharomyces cerevisiae* were selected as organisms producing the highest yields of ethanol;

➢ Optimal inoculation rate and temperature for fermentation of sweet sorghum juice for ethanol production was established for the tested strains and one strain was selected for fermentation of sweet sorghum juice at 37°C;

➢ Adding exogenous nitrogen and phosphorus sources to sorghum sweet juice can slightly increase ethanol yield and productivity. The increasing in laboratory scale of ethanol yield with 3% by adding nitrogen and phosphorus sources need to be evaluated in large scale, as these additions need to be economically feasible.

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6. References