Full Length Research Paper

Investigation of Bioethanol Production Potentials of *Kluyveromyces marxianus* and *Saccharomyces pastorianus* by Alternative Carbohydrate Sources

Akyol, I.¹, Cömertpay, S.² and Ebru, S.²

¹,²,³ Kahramanmaraş Sutcu İmam University, Research and Development Center for University-Industry -Public Relations (USKIM), Microbiology and Microbial Genetics Laboratory 46060/Avsar, Kahramanmaras, Turkey

Accepted 10 August, 2015

Bioethanol is considered as the most important renewable energy alternative to petroleum-derived fuels, and its production through microbial biconversion of carbohydrates presents remarkable economic and environmental benefits. Two distinct species of microorganisms, *Kluyveromyces marxianus* and *Saccharomyces pastorianus*, were examined in this study for their abilities to form ethanol through the fermentation of carbohydrates from various sources. Whey was used as the only carbon source for *Kluyveromyces marxianus* whereas *Saccharomyces pastorianus*’ potential was investigated with sugar beet, carrot and carob. The amounts of sugars present and the ethanol produced in each condition were determined by HPLC at the end of each day while the number of the cells was detected only on the last. When the plant based carbohydrate sources with *Saccharomyces pastorianus* were compared, the most rapid proliferation of cells (7.0X10⁶ CFU/mL) and the highest amount of ethanol production (7.06 µL/mL) were found in carob. Additionally, of all conditions examined, the highest number of cells proliferated (1.45X10⁷ CFU/mL) and the maximum amount of ethanol produced (26.62 µL/mL) were observed for *Kluyveromyces marxianus* in whey. Therefore, we concluded that carob had a good potential to be used in industrial bioethanol production and *Kluyveromyces marxianus* was an effective species to produce ethanol through fermentation.

Key words: Bioethanol; whey; carob; carrot; sugar beet; *Kluyveromyces marxianus*; *Saccharomyces pastorianus*.

INTRODUCTION

Bioethanol, which refers to biologically produced ethanol, is an attractive alternative fuel to supplement the depleting stores of fossil oils (Yi Zheng, 2009 #18) and to reduce the emissions of green house gases that might cause climate change (Rass-Hansen, 2007 #11). Bioethanol presents a sustainable energy source, and it can be generated from several different feedstocks by microbial bioconversion (fermentation) of carbohydrates (Balat, 2008 #12). There is a wide range of carbon sources that can be used to produce bioethanol, and different sources can be available in different places on earth depending on the climate and the soil conditions. (Villegas-Silva, 2014 #14). In USA, for instance, where the largest amount of ethanol is produced biologically, corn is the most preferred carbon source while the second most productive country, Brazil, generates its ethanol from sugar cane (Sanchez, 2008 #15).

Ethanol can simply be derived from any material that comprises carbohydrates. The raw materials commonly used in bioethanol production are roughly categorized in three groups as sugars (sugarcane, sugar beets, molasses and fruits), starches (corn, cassava, potatoes and root crops), and cellulose materials (from wood, agricultural residues, waste sulfite liquor from pulp, and paper mills) (Lin, 2006 #16). While sugars can be transformed into ethanol directly, starches and cellulotic
materials, which are generally found as lignocellulosic biomass in plants, must be converted to fermentable sugars before the actual ethanol-making process. In order to facilitate rapid and efficient hydrolysis of carbohydrates to obtain monomeric sugars, it is required to alter the submicroscopic composition of the biomass in addition to its macroscopic and microscopic structure (Chang, 2000 #17). These alterations are achieved in the first step of the biomass-to-fermentable sugar conversion process that is termed as pretreatment. Pretreatment methods can be physical, chemical, biological or a combination of any (Yi Zheng, 2009 #18). Despite being viewed as one of the most expensive steps in bioethanol production, these treatments have immense capacity to improve the efficiency and to lower the cost of the biological ethanol production (Mosier, 2005 #19).

Once simple sugars are formed, enzymes from microorganisms can readily produce ethanol through fermentation. However, microorganisms used to convert carbohydrates to ethanol must own certain characteristics such as the ability of fermenting the variety of sugars into ethanol as the sole fermentation product, tolerating the inhibitory compounds typically present in dilute-acid hydrolysates (ethanol, acedic acid, furfural and assorted phenolic compounds), fermenting sugar at low pHs that help inhibit the growth of most bacterial contaminants, and not requiring oxygen while being able to tolerate the incidental introduction of it during processing (Picataggio and Zhang, 1996). Although there is no single microorganism known to naturally possess all these traits, *Saccharomyces cerevisia* (*S. cerevisia*) is the most commonly used strain for its rapid fermentation rate under acidic conditions, ability to ferment glucose to ethanol as virtually the only product, and its tolerance to acedic acid and ethanol (Torbjörn Lindén, 1989 #20). However, *S. cerevisiae* lacks the ability of fermenting lactose because it does not have neither β-galactosidase nor a lactose permease system. This inability prevents *S. cerevisiae* from using whey as a carbon source in bioethanol production. Most of the Kluyveromyces species, on the other hand, are capable of using lactose in cheese whey for ethanol fermentation. Although Kluyveromyces species is similar to Saccharomyces, it cannot industrially compete with Saccharomyces due to certain technological aspects. Fermentation strategies of mixed culture of these two genus, however, were found to be considerably successful, especially in the production of ethanol, single-cell protein and vitamin, and disposing of waste water (Chen, J. Et al., 2007; Miyano et al., 2000; Mamma et al., 1996; Rodriguez and Gallardo, 1993). In a relatively recent study conducted by Guo et al. (2010), ethanol productions from cheese whey powder solution by using free or immobilized cells of *Kluyveromyces marxianus* (*K. Marxianus*) in monocultures or mixed with *S. cerevisiae* were investigated, and the maximum ethanol fermentation efficiency was reported to be achieved (79.9% of the theoretical value) by the mixture of immobilized cells.

Despite being the best known and the most widely used of the yeasts in bioethanol production, *S. cerevisiae* is not the only species of Saccharomyces that can be beneficial in this industry. There are several other Saccharomyces species with robust growth rates and high ethanol tolerance. *Saccharomy pastorianus* (*S. pastorianus*), for instance, has sufficient favorable growth- and ethanol-tolerance characteristics to be considered a fermentative candidate species for cellulosic ethanol production and may provide increase tolerance to biomass sugar hydrolysate inhibitors compared with *S. cerevisiae* (Miller et al., 2012). In point of fact, *S. pastorianus* is currently used in the lager brewing industry and is responsible for 90% of the beer market (Saerens, 2010).

The present work aimed to investigate the bioethanol producing abilities of two species of microorganisms, namely *K. marxianus* and *S. pastorianus*, through the fermentation of the sugars found in a variety of biosources. In the scope of this study, whey, a by product from the dairy industry, which represents a disposal problem due to its enormous production rate all over the world (9 kg of whey /1 kg of cheese) (Ozmihci, 2007), was fermented by *K. marxianus*. Sugar beet, carrot and carob, on the other hand, were exploited as the carbon sources for the fermentation proceeded by *S. pastorianus* due to their high production rate.

**RESULTS AND DISCUSSION**

**Results**

Four different combinations of microorganisms, *K. marxianus* and *S. pastorianus*, and carbon sources, whey, sugar beet, carrot and carob, have been examined in the scope of this study, and all were observed to be able to produce ethanol although the amount varied considerably. As shown in Table 1, the amount of two sugars were also measured along with the ethanol produced through fermentation in order to examine the ethanol producing capabilities of the microorganism-carbon source pairs studied. Additionally, proliferation rate of the microorganisms in each condition was determined by counting the cells at the end of the study and was reported in terms of Colony Forming Units per mL of the cell solution plated.

**K.marxianus and Whey**

*K. marxianus* and whey were the highest ethanol producing pair at the end of the fifth day (20.89 g/mL) despite the fact that they performed their maximum production on the third day of the fermentation (26.61 g/mL). In addition to ethanol, the amount of two sugars, lactose and glucose, were overlooked through the
Table 1. Ethanol and two types of sugar amount produced when each carbohydrate source was used. Metabolites were measured by HPLC. Whey was used with *K. marxianus* while sugar beet, carrot and carob were used with *S. pastorianus*.

<table>
<thead>
<tr>
<th>Days</th>
<th>Whey (K. marxianus)</th>
<th>Sugar Beet (S. pastorianus)</th>
<th>Carrot (S. pastorianus)</th>
<th>Carob (S. pastorianus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galactose</td>
<td>Glucose</td>
<td>Ethanol</td>
<td>Fructose</td>
</tr>
<tr>
<td>0</td>
<td>40.81±6.65</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>1</td>
<td>18.64±6.31</td>
<td>0.39±0.01</td>
<td>16.30±2.28</td>
<td>5.88±1.71</td>
</tr>
<tr>
<td>2</td>
<td>2.65±0.02</td>
<td>0.39±0.01</td>
<td>24.18±2.32</td>
<td>7.37±0.79</td>
</tr>
<tr>
<td>3</td>
<td>2.25±0.04</td>
<td>0.29±0.00</td>
<td>26.61±1.25</td>
<td>7.19±0.69</td>
</tr>
<tr>
<td>4</td>
<td>1.98±0.70</td>
<td>0.26±0.01</td>
<td>26.23±2.61</td>
<td>4.60±0.93</td>
</tr>
<tr>
<td>5</td>
<td>1.85±0.35</td>
<td>0.21±0.01</td>
<td>20.89±2.20</td>
<td>3.48±0.90</td>
</tr>
</tbody>
</table>

Fermentation and the amount of lactose was discovered to decrease from 40.81 g/mL to 1.85 g/mL till the end of the fifth day while the glucose levels increased from 0.00 to 0.39 g/mL on the first day then lowered around 0.21 g/mL towards the end of the fifth day. The slight increase in glucose levels whilst lactose levels dramatically decrease and the ethanol is generated at considerable levels indicate the intermediative role of glucose in the process of ethanol production from lactose. Besides, the number of *K. marxianus* cells was counted as 1.45X10⁶ at the end of the study.

**S. pastorianus and Sugar Beet**

When *S. pastorianus* was incubated with sugar beet for five days, the ethanol produced at the end of the fermentation was found to be 6.73 g/mL, which was not only the second largest amount measured, but was also the highest among those generated by *S. pastorianus* on the fifth day of the study. As sugar sources, glucose and fructose levels were measured in this study. Glucose levels were observed to decrease from 13.97 to 0.51 g/mL from the first day to the fifth. Fructose levels, on the other hand, started at 0.00 on the first day and increased till the third by reaching its maximum (7.37 g/mL), where it began to drop to 3.48 g/mL on the fifth. Finally, *S. pastorianus* cells in sugar beet were enumerated as 5.3X10⁶.

**S. pastorianus and Carob**

Ethanol production of *S. pastorianus* from carob followed a different pattern from the previous pairs. Here, instead of increasing by time, the amount of ethanol present in the solution on the third day reached its maximum (7.06 g/mL), and it slightly decreased to 6.69 g/mL towards the fifth. As the sugars of carob, fructose and glucose levels, however, were observed to decrease through the fermentation, from 8.66 to 2.28 g/mL and from 3.42 to 0.49 g/mL, respectively. At the end of the fermentation period, *S. pastorianus* cells in carob were counted and the number was determined as 5.3X10⁶ CFU/mL.

**DISCUSSION**

In this study, the measurements for the ethanol production and the sugar (fructose, glucose, lactose) consumption were performed at five different time points with 24 hour difference starting from the moment the experiment was initiated and continuing till the end of the fifth day. When the values acquired on the first and the fifth days were compared, ethanol producibilities of most sugar source-microorganism pairs were found to be higher on the first day than they were on the fifth day. This loss in ability could be explained by the inhibitive effect of ethanol in the media (Sofia et al., 2013).
On the contrary, in the experiments where carrot and carob were fermented by S. pastorianus, the actual concentrations of ethanol were found to decrease from the first day to the fifth as well as their ratio to the sugars used. Although this observation can be partly explained by the inhibitory effects of ethanol, the rapid usage of carbon sources by S. pastorianus on the first day, therefore not producing enough ethanol to compensate the amount lost by evaporation in later days also needs to be considered in order to understand the real nature of this outcome. As for sugar beet-S. pastorianus pair, however, a completely different pattern of ethanol production was monitored. The measurements revealed that both the actual amount of ethanol present in the media and its ratio to the amount of glucose that was used were higher on the fifth day compared to the first day. This difference could be resulted by the fructose produced by this plant. While glucose in sugar beet was observed to be fermented starting from the first day, fructose seemed to be generated till the third day of the incubation. After the third day, with the exhaustion of glucose to be used, fructose was begun to be fermented into ethanol. Consequently, an adequate amount of sugar remained present to compensate the ethanol lost by evaporation and the inhibition caused by ethanol itself.

The results obtained through the experiments presented in this study appeared to be compatible with the literature. In a previous study conducted by Christensen et al. (2011), 4 types of treatments were applied to whey before being fermented by K. marxianus. When the amounts of ethanol produced at the end of the second day were compared, it was found that whey with no treatment presented the highest yield by producing 0.51 g of ethanol per g of lactose used. In our experiments, we observed 0.63 g of ethanol production for each g of lactose consumed. Considering that the whey in our study was not treated in any way mentioned in the study above and the fact that the conditions for fermentation in our study were quite similar to theirs, it can be concluded that K. marxianus strain used in our study had a better potential to fermentate the whey provided.

In another study carried by Rodriguez et al (2010), where sugar beet pomace and juice were fermented by Saccharomyces cerevisiae, the yields of ethanol manufacture at the end of 48 hours of incubation were measured as 137.5% and 77.7%, respectively (Bioethanol production from grape and sugar beet pomaces by solid state fermentation). In our experiment, using S. pastorianus as the fermentative microorganism with the sugar beet pomace (homogenate), the ethanol production rate was calculated as 25.93 per 100 g of glucose depleted at 48 hours. This value was less than one fifth of what was produced by S. cerevisiae under similar experimetal conditions in their study. The fact that there is such a significant difference between the ethanol amounts produced by S. pastorianus and S. cerevisiae clearly demonstrated that S. pastorianus was not as efficient in bioethanol production as S. cerevisiae when the sugar pomace was used as the carbohydrate source.

On the other hand, when a Taiwanese group performed a study with Carrot pomace by adding a 12 h pretreatment with accellerase TM 100 (to produce fermentable sugars, such as glucose, fructose, sucrose), they found that there was no increase in ethanol production whether pretreatment was included or not. They also reported the maximum ethanol production yield as 18.5% at the 42nd hours of incubation of carrot pomace with K. marxianus (Yu et al., 2013). In our experiment, we did not measure the ethanol concentration at the 42nd hour. However, we did measure it on the 24th and the 48th hours, and we calculated %54.58 and 50.18% production yields, respectively. 42nd hour being between the 24th and 48th hours, we would reasonably expect to obtain a value lower than 54.58 and higher than 50.18 as the production yield at the time point. Therefore, by considering that S. cerevisiae was able to produce with 18.5% yield and any value between 50,18 and 54,58 will be approximaely three-fold of 18,5, we suggested that S. pastorianus might be better at fermenting carrot sugars into ethanol than S. cerevisae under similar experimental conditions.

In a recent study performed by a group of scientists in Iran, (Saharkhiz et al., 2013) two microorganisms, namely Z. Mobilis and S. cerevisiae, were investigated for their potentials to produce ethanol from carob pods without a pretreatment. These microorganisms were determined to produce 0.42 and 0.40 g of ethanol for a gram of carob used at the 40th hour of fermentation, respectively. In our experiment, however, 1.13 g of ethanol was produced per gram of carob used by S. pastorianus on the first day of the fermentation and it remains fairly the same till the end of the fifth day. Bearing in mind the fact that the temperature and the pH values were similar in our and their experiments, we were able to propose that S. pastorianus had nearly 2-fold higher ability to fermentate carob sugar into ethanol than the two microorganisms examined did. Besides, through the analyses of all carbon sources used by S. pastorianus, we were compelled to assume that not only carob may be the best sugar source for S.pastorianus in terms of ethanol producing abilities from carob.

Through the experiments presented here, we investigated ethanol manufacture potential of two microorganisms, K. marxianus and S. pastorianus. Whey was used as the only carbon source for K. marxianus while sugar beet, carrot and carob were inspected with S. pastorianus. All measurements were repeated three times and the mean values were used for plotting the graphs with the deviation bars. Of all measure ments, there was not a single value that needed to be excluded. Hence, all the data obtained were included in the evaluation of the results. Conclusively, whey fermented...
by *K. marxianus* was found to generate ethanol more efficiently than sugar beet and carrot fermented by *S. pastorianus*, fermentation of carob by *S. pastorianus*, on the other hand, was observed to result in producing ethanol with the highest yield. Although our results are consistent in its replications, we are aware that the se

For each condition, the process was repeated three times and the average value was presented as CFU/mL.

**Ethanol Collection**

A distillation system was set up as to collect the ethanol produced during fermentation. Distillation pipe was held by two support sticks. To avoid cracks on the distillation pipe, cold water was circulated through it all the time. Fermented sample was placed into pear-shaped bottle after the volume and the weight were determined. This bottle, which was connected to the distillation pipe, was place on a magnetic stirrer. The temperature was increased slowly and it was read on the thermometer placed. Once the temperature reached 78 °C, evaporated ethanol started to be condensed through the distillation pipe. A sudden increase in temperature indicated the end of the ethanol vaporization.

**HPLC Measurement**

HPLC (High Performance Liquid Chromatography) analyses were performed by UFLC Shimadzu Prominence HPLC equipment (Shimadzu, Kyoto, Japan) containing RID detectors, two-channel gradient pump (Shimadzu LC-10AD), autosampler (SIL 20A HT) and colon oven (CTO-20AC). The mobile phase used in sugar-alcohol analysis was sulphuric acid and water, and the separation time was picked as 1 minute. 2 ml of each sample stored at -20 °C was used for the analysis.

**Statistical Analysis**

The means and differences between means were tested by one-way ANOVA using SPSS software (SPSS Inc., Chicago, IL). The results were considered to be statistically significant when the p-value was lower than (or equal to) 0.001.

**Author Contributions**

E.S. performed all experiments and contributed to the interpretation of the data. S.C. and I.A. contributed to writing of the manuscript and the interpretation of the data.

**Conflicts of Interest:** We do not declare any conflicts of interest.

**REFERENCES**


Christensen, A.D., Kádár, Z., Oleskowicz-Popiel, P. and Thomsen, M.H.

**MATERIALS METHODS**

**Incubation of Yeast**

Yeast Malt Broth Medium (YMAM) was prepared by adding 1.5% bacto agar into Yeast Malt Broth Medium (YMBM), which was made by solving 3 g of yeast extract, 3 g of malt extract, 5 g of pepton and 5 g of glucose in 1000 mL of distilled water. The both media were autoclaved at 121 °C for 15 minutes before use. Lyophilized microorganisms (*Klyveromyces marxianus* and *Saccharomyces pastorianus*) were activated in 10 mL tube containing YMBM and proliferated on YMAM at 25 °C. Then they were inoculated into YMAM and continued to grow by shaking at 150 rpm at 30 °C for 48 hours.

**Preparation of Carbohydrate Sources**

**Whey** 300 mL of raw milk was heated to 68 °C for 10 minutes and cooled down to 42-45 °C. Yeast (Mayasan Chesee Yeast, Turkey) was added and waited until coagulation. Coagulates were collected and drained. After draining, the coagulates were pressed and the whey released under the pressure was collected. 100 mL of whey was transferred to a 250 mL bottle. The pH was adjusted to 4.5, and the media was autoclaved at 121 °C for 15 minutes.

**Sugar Beet, Carob, Carrot** 1 kg of each specimen was homogenized by blender. 5 g of the homogenized sample was placed into a 250 mL bottle and filled up to 100 mL with distilled water. After adjusting the pH to 4.5, the solution was autoclaved at 121 °C for 15 minutes.

**Cell Counting** Standard Spread Plate Technique was used to enumerate the viable cells grown in each sugar source. At the end of the fermentation, 100 µL of the media containing microorganisms was diluted 10^5 (Total dilution factor) times. 100 µL of the diluted microorganism solution was put on YMAM with Dragaski Loop and was incubated at 25 °C for 24 hours. Once the incubation was completed, visible colonies were counted and the number of the cells were determined as Colony Forming Units (CFU) per unit volume of cell solution calculated by the formula below.

\[ \text{CFU/mL} = \frac{\text{Number of colonies plated per mL}}{\text{Total dilution factor}} \]
Akyol et al., 225


