Ethanol Production from Vineplant Waste Hydrolysate Sugars by Native Yeast Strains

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Biomass from agricultural waste can be an excellent source of sustainable energy, the most notable of which is bioethanol. This study aimed to adapt and improve bioethanol production using a yeast strain that ferments the sugar content in undiluted and non-added nutrient vineplant bunch hydrolysates. Yeasts that were previously isolated and molecularly characterized were screened for their pentose fermenting capabilities, first in solid and then liquid mediums. Then, 10 native xylose fermenting yeast strains were tested for their ability to produce ethanol from acid hydrolysates from vineplant lignocellulosic waste. The five strains that exhibited the highest ethanol production underwent fermentation in the pure (non-detoxified) hydrolysate. The strain *Pichia kudriavzevii* D12 in the undiluted hydrolysate medium gave the highest ethanol concentrations and yields. Hence, *P. kudriavzevii* was selected for adaptation with sequential fermentations. As a result, a 59% increase in the ethanol production (g/L) was recorded for the D12 strain in the undiluted hydrolysate medium during the adaptation studies. A 2.9-fold increase in the yield (g/g) was obtained for this sample when compared with the reference medium. This study determined that a nondetoxified, organic waste medium prepared from vineplant bunches without added nutrients is a suitable substrate alternative for bioethanol production.

Keywords: Native yeast; Bioethanol; Lignocellulosic hydrolysate; Vineplants; Yeast fermentation

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INTRODUCTION

Bioethanol can be produced through the fermentation of sugars derived from a variety of sources. Lignocellulosic biomass, the most abundant, readily available, renewable organic material and source of energy on Earth, is composed mainly of lignin and carbohydrate polymers (cellulose and hemicellulose) (Ho *et al.* 2014). However, due to structural heterogeneity and chemical complexity, it is resistant to bioconversion (Hahn-Hägerdal *et al*. 2006; Ammar and Elsanat 2014; Saini *et al*. 2015).

Incomplete utilization of sugars increases production costs, and hence cost-effective bioethanol production from lignocellulosic biomass requires a highly efficient utilization of both cellulose and hemicellulose (Kumar *et al.* 2016). Therefore, some studies have focused on the selection of microbial strains that are capable of producing comparatively high yields of bioethanol at low costs. This can be achieved by fermenting both pentose and hexose sugars, of which lignocellulosic biomass is comprised (Hahn-Hägerdal *et al.*

2006; Navarro *et al*. 2010; Ammar and Elsanat 2014; Saini *et al*. 2015; Kumar *et al*. 2016). The conversion of carbohydrates to ethanol *via* yeasts, most commonly employing *Saccharomyces cerevisiae*, has been done for generations. However, the industrial process has always been challenged by the inability of *S. cerevisiae* strains to compete with other wild-type yeast strains, eventually leading to contamination. Additionally, only a few yeast strains have demonstrated the ability to convert pentoses. Studies have extensively investigated *Pachysolen tannophilus*, *Candida shehatae*, *Pichia stipitis*, and *Kluyveromyces marxianus* as xylose fermentators. Xylose fermentation has also been reported with the following species: *Brettanomyces*, *Clavispora*, *Schizosaccharomyces*, *Debaryomyces viz. D. nepalensis* and *D. polymorpha*, and *Candida viz. C. tenuis*, *C. tropicalis*, *C. utilis*, *C. blankii*, *C. friedrichii*, *C. solani*, and *C. parapsilosis* (Mussatto and Roberto 2004; Kuhad 2010).

In addition to being industrially stable, an ideal strain for the fermentation process should stably convert C5- and C6- sugars and exhibit resistance to inhibitory compounds, temperature, ethanol, and sugars. The application of genetically-modified microorganisms has been reported, but the stability of recombinant yeast strains is not guaranteed long term. Moreover, public concerns regarding the use of genetically-modified organisms have triggered the search for new approaches. This includes the production of industrially ideal yeast strains with the characteristics mentioned above using specific natural adaptation and systematic selection (Kahr *et al*. 2011).

As bioethanol fermentation progresses, the new complex properties detected in the fermentation medium challenges the yeasts as different carbon source-dependent inhibitors are formed. Therefore, exploring the natural diversity of yeasts to discover high yielding ethanol strains in stressful fermentation mixtures has been suggested to overcome high concentrations of glucose, mixtures of sugars (glucose and xylose), and/or a myriad of inhibitors (Ruyters *et al*. 2015). The development of robust microorganisms that are able to ferment hydrolysates to ethanol without detoxification would be economically favorable and highly important (Huang *et al*. 2009).

Mainly in the Western part of Turkey and some interior parts, viticulture is widely done. As compared to other vineplant growing countries, Turkey ranks 5th in production (FAO 2012). Following the harvest season, the vineyards are budded, and the useless vineplant bunches are collected and left in the land or burned.

This study aimed to improve bioethanol production using a native yeast strain that was adapted to ferment the sugar content in undiluted (UD) and non-added nutrient vineplant bunch hydrolysate medium (VBHM) in the presence of inhibitor compounds raising from dilute acid hydrolysis. As far as we know, such use of these residues has not been reported before.

EXPERIMENTAL

Yeast Strains

The 10 yeast strains used in this study were previously isolated from petroleumcontaminated soil samples and molecularly characterized (Tunalı-Boz *et al*. 2015). The list of strains is given in Table 1.

Table 1. Yeast Species Used in this Study

Isolate No.	Species	ITS2-5.8s rRNA-ITS2	D1/D2 Domain of 26 srRNA
		Accession No.	Accession No.
D3	*Candida parapsilosis*	KC182136.1	KC182121.1
D12	*Pichia kudriavzevii*	KC182124.1	JQ779970.1
D13	*Rhodotorula glutinis*	KC182125.1	JQ779971.1
D14	*Saccharomyces cerevisiae*	KC182126.1	JQ779972.1
D17	*R. mucilaginosa*	KC182128.1	JQ779974.1
D22	*Candida sinolaborantium*	KC182132.1	KC182119.1
D27	*Cryptococcus albidus*	KC182130.1	JQ277254.1
D44	*Cryptococcus diffuens*	KC182134.1	JQ277259.1
D54	*R. mucilaginosa*	KC182131.1	JQ779975.1
D88	*R. mucilaginosa*	KC182131.1	JQ779975.1

All of the cultures were maintained in yeast extract, peptone, and xylose agar (YPX) slants at -20 °C.

Inoculum Preparation

Agar plates and broths were inoculated from cultures that were inoculated in yeast nitrogen base (YNB Difco, 291940, Becton Dickinson France) broth to remove excess carbon after first being cultured in liquid YPX broth at 30 °C in a shaking incubator (WiseCube, witeg, Germany) at 120 rpm for 24 h.

Screening of the isolated yeast strains on xylose agar plates

Strains were streaked on xylose agar plates (YPX agar) (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, and 15 g/L agar). The ability of the strains to utilize xylose was evaluated after incubation at 30 °C for 2 d. Agar plate screening was done in triplicate.

Media and Fermentation Conditions

Fermentation on the defined YPX media

The isolates utilizing xylose were subjected to small-scale fermentation experiments performed on a YPX medium, which consisted of yeast extract (10 g/L), peptone (20 g/L), xylose (25 g/L), KH_2PO_4 (2.5 g/L), and $(NH_4)_2SO_4$ (1 g/L). The strains were pre-cultivated in a YPX medium, and 1 mL of yeast suspension was transferred to the YNB broth. Then, the suspension was inoculated in 25 mL of YPX broth dispersed in 125 mL flasks. The flasks, which were sealed with aluminum foil and parafilm, were incubated and shaken (100 rpm) at 30 °C for 4 d. Samples were taken daily to analyze the sugar and ethanol contents.

Preparation of the vineplant bunch hydrolysate by dilute acid treatment

Vineplant bunches were chopped to less than or equal to 2 cm by a shredder and soaked with 0.7 M hydrochloric acid at a 1:2 ratio (w:v, particle:acid). The conditions during pretreatment were as follows: room temperature for 30 min, and then 90 °C for 40

min. Following this, distilled hot water was added at a 1:2 ratio. The pH values of the suspensions were adjusted to 5.0, and then the suspensions were filtered (Olsson and Hahn-Hagerdal 1996). Hydrolysate fermentation medium was named as VBHM.

Determination of the sugar content in the hydrolysate

The reduced sugar content in the liquid hydrolysate was determined by the dinitrosalicylic acid method according to Miller (1959).

Determination of the pentose (xylose) sugar content in the hydrolysate

Pentose (xylose) sugar content in the hydrolysate was determined by xylose assay kit (Elabscience, BC0018, Dunwoody, GA, USA) according to the manufacturer's instructions.

Inoculation and fermentation on the hydrolysate medium

To enhance the ethanol production in the presence of potential inhibitory compounds in the hydrolysate, raising from the acid hydrolysis such as hidroxynmethylfurfural (HMF), phenolics, furan *etc.* , several yeast strains were adapted to grow in increasing concentrations in the hydrolysate media ranging from 30% to 100%. Fermentation proceeded at the conditions indicated above. The hydrolysate suspensions were prepared by adding sterilized distilled water. All of the suspensions were inoculated with 3% (10^8 cells/mL) inoculum that were grown on an YPX medium. Samples were taken every 24 h for analysis of the reduced sugar and ethanol contents.

Adaptation of the yeast strains on the VBHM

According to the fermentation results obtained from the diluted VBHM, the D12 strain was used for ongoing adaptation cycles with an UD hydrolysate. A total of 10 cycles were performed.

Fermentation Analysis

Fermentation samples (1 mL) were taken to evaluate the growth of the strains on the fermented medium. The samples were serially diluted, spread on a plate, and counted.

The ethanol concentration was measured daily throughout the fermentation process with an Ethanol Assay Kit (MAK076, Sigma-Aldrich, St. Louis, MO63103, USA) according to the instructions provided by the manufacturer. The ethanol yields were calculated based on 1 g of ethanol per 1 g of consumed substrate (hydrolysate). All of the theoretical yields were calculated from the ethanol yields based on the consumed sugar.

RESULTS AND DISCUSSION

Screening Yeasts on the YPX Agar and Fermentation in the YPX Broth

All of the screened strains were able to grow on the YPX agar. Based on the screening results and their growth rates, to confirm the xylose assimilation ability of these strains, five of them underwent fermentation in a liquid-defined medium. Each of the

strains were able to grow on the medium with xylose. The xylose consumption rates and ethanol concentrations are shown in Table 2. All of the strains were able to produce ethanol from xylose in the range of 4.3 g/L to 5.6 g/L. The highest period of xylose utilization was 96 h of fermentation. The highest ethanol production was observed with *P. kudriavzevii* D12 at 5.6 g/L, whose xylose assimilation was 25 g/L, which resulted in a yield of 0.22 g/g and the consumption of all of the sugar by 96 h.

Table 2. Xylose Consumption by the Yeasts during Aerobic Cultivation Using a Defined Medium

Yeast Strain	Xylose Consumption (%) / Time (h)	Maximum Ethanol Concentration (g/L)	Ethanol Yield (g/g)
***Pichia kudriavzevii* D12**	100 / 96	5.6 ± 0.83	0.22 ± 0.04
***Candida sinolaborantium* D22**	80 / 96	4.6 ± 0.83	0.23± 0.04
***Candida parapsilosis* D3**	84 / 96	4.6 ± 0.22	0.22± 0.02
***Cryptococcus diffuens* D44**	84 / 96	4.8± 0.48	0.23± 0.04
***Rhodotorula mucilaginosa* D88**	86 / 96	4.3 ± 0.48	0.2± 0.83
***R. mucilaginosa* D93**	80 / 96	4.3 ± 0.48	0.22± 0.04

Recently, most research has focused on isolating xylose-fermenting yeast strains from samples collected from natural sources (plants, leaves, roots, flowers, fruits, *etc*.) or various habitats, such as industrial, aquatic, and soil habitats (Cadete *et al*. 2012; Lorliam *et al*. 2013; Tikka *et al*. 2013; Chaudhary and Karita 2017).

Thirty native yeast strains have been evaluated for ethanol production from xylose as the sole carbon source. The ethanol produced by these strains was 3 g/L to 6 g/L. The highest ethanol production was observed with *Candida tropicalis* S4, which produced 6 g/L of ethanol from 56 g/L xylose under aerobic conditions (Martins *et al.* 2018). In general, naturally xylose fermenting yeasts are able to ferment xylose only when oxygen flow is tightly regulated (Hou 2012; Long *et al.* 2012; Su *et al.* 2014).

Natural xylose fermenting strains, such as *Scheffersomyces stipitis*, *Spathaspora passalidarum*, and *Spathaspora arborariae*, have been tested for ethanol production from xylose under aerobic and oxygen-limited conditions. The ethanol production equivalents of 8.05 g/L, 10.06 g/L, and 8.65 g/L, respectively, were recorded under aerobic conditions, while 16.48 g/L, 16.36 g/L, and 11.47 g/L, respectively, were recorded under oxygen-limited conditions. These results show that the conversion of xylose into ethanol is efficient under anaerobic conditions (Veras *et al.* 2017). Veras *et al.* (2017) also pointed out the low flow air during oxygen-limited fermentation and the usage of defined mineral medium for *C. tenuis* resulted in lower initial cell density, poor ethanol production, and significant xylitol formation.

Sugar Content of the VBHM

Lignocellulosic substrates are characterized as containing a variety of sugars, including hexoses (resulting mainly from cellulose degradation) and pentoses (resulting from hemicellulose degradation). Ferreira *et al.* (2011) reported that the xylose and glucose contents in the hydrolysate obtained from sugarcane bagasse were equivalent to 76.1 g/L and 10.1 g/L, respectively. The sugar contents of various raw materials have been reported as 33 g/L xylose and 65 g/L glucose in sweet sorghum bagasse; 20.7 g/L xylose and 47.8 g/L glucose in wheat straw; and 9 g/L xylose and 40 g/L glucose in corn stover, 255 g/kg xylose and 92.51 g/g glucose in corncob (Olofsson *et al*. 2008; Rudolf *et al*. 2008; Faga *et al*. 2010; Gupta *et al.* 2012). Pentose (xylose) and hexose sugars were measured in the liquid phase of the VB hydrolysate were lower than in literature and were 19 g/L and 11.5 g/L, respectively. However, since the xylose content is higher that the glucose it can be advantageous to adapt the strains to the hydrolysate for xylose consumption and avoiding from the glucose inactivation

Fermentation on the VBHM

All strains were able to grow in UD (100 %), non-detoxified, and non-nutrient added VBHM as recorded by the spectophotometer (OD_{600}). Thus, this was used as the ethanol fermentation medium for the yeast strains. The fermenting abilities of the strains on the UD VBHM are given in Table 3.

Table 3. Comparison of the Highest Fermentation Results for Five Yeast Strains in the VBHM

Yeast Strain	Sugar Utilization (%)	Maximum Ethanol Concentration (g/L)	Ethanol Yield (g/g) (per consumed g of sugar)
Pichia kudriavzevii* D12	62.8	5.1	0.42
Candida sinolaborantium* D22*	48.7	2.2	0.23
Cryptococcus diffuens* D44	40.6	3.3	0.43
Rhodotorula mucilaginosa* D88*	44.6	2.3	0.36
R. mucilaginosa* D93*	48.7	3.6	0.39
Table shows the average results of three runs; *after 48 h; **after 72 h			

The ethanol yield obtained using dilute acid hydrolysis and fermentation are reported as only 50 to 60% of theroretical values.

In dilute sulfuric acid process for the hydrolysis of biomass to form sugars, hemicellulose can be broken down at lower temperatures (around 160 ℃) than cellulose hydrolysis (200 to 240 ℃) (Wyman 1994). In our study, since lower temperatures were used during hydrolysis, the obtained sugars were mainly derived from hemicellulose breakdown. Therefore the yields obtained by fermentation of these sugars was somewhat

lower. Unfortunately, these conditions are severe enough to degrade glucose into undesirable coproducts such as HMF.

Most native xylose fermenting yeasts are subject to glucose repression and glucose inactivation. In repression, glucose inhibits the synthesis of xylose-metabolizing enzymes at the transcriptional level. During inactivation, glucose inhibits the activities of xylose transport and/or other xylose metabolizing enzymes. As a result, in a glucose-xylose mixture, once glucose is fermented before xylose and if the yeast is not sufficiently tolerant to ethanol, it does not complete the xylose fermentation. However, there are exceptions to this statement such as *Spathaspora passalidarum*, which utilizes both xylose and glucose and ferments simultaneously under oxygen limited conditions (Long *et al.* 2012).

When comparing the resulting fermenting abilities of the strains in the YPX broth and UD VBHM, lower ethanol concentrations were obtained from the UD VBHM. However, the ethanol yields in the UD VBHM were two times higher. It was previously reported that yeasts that convert xylose to ethanol efficiently in defined media often perform poorly in pretreated biomass hydrolysates or waste liquors from lignocellulosic material. The decreased fermentation efficiency was attributed to the inhibitory effect of hexoses on xylose utilization (Harner *et al.* 2015).

Ferreira *et al.* (2011) reported that *Scheffersomyces stipitis* UFMG-IMH 43.2 was able to simultaneously ferment and convert xylose and glucose to ethanol on hydrolysate media prepared from sugarcane bagasse supplemented with urea, $MgSO_4$, $7H_20$, and yeast extract. The highest ethanol concentration (9.1 g/L) was recorded when the culture medium was supplemented with 5 g/L yeast extract and contained an initial xylose concentration equivalent to 52.5 g/L. However, the addition of high-cost nutrients is not economically feasible.

Adaptation of Yeast Strain on the VBHM

The yeast strain was successfully adapted to the UD VBHM in this study. Higher ethanol yields were produced by the adapted strain on VBHM compared with the unadapted parent strain (Table 4).

Table 4. Comparison of the Fermentation Results for *P. kudriavzevii* on the VBHM during the Adaptation Studies

Yeast	Consumed Sugar (%)		Ethanol Concentration (g/L)		Ethanol Yield (g/g)	
	First Period	Last Period	First Period	Last Period	First Period	Last Period
Adapted *P. kudriavzevii*	62.8	81.6	5.1	8.63	0.42	0.58

Prior to ethanol fermentation by a microorganism, the feedstock needs to be processed by saccharification technology to release fermentable sugars. Dilute sulfuric acid hydrolysis, which is extensively employed in the industry, is thought to be a promising pretreatment method. Unfortunately, the fermentation and pretreatment processes are

always followed by the release of degradation products (weak acids and furan derivative), which leads to microbial growth inhibition. Therefore, a successful hydrolysate fermentation process requires either the detoxification of lignocellulosic hydrolysates or the use of inhibitor-tolerant microorganisms (Palmqvist and Hahn-Hägerdal 2000; Almeida *et al*. 2007).

Several studies have reported on the ability of *Pichia* strains to produce optimum yields after their successful adaptation to dilute acid pretreated hydrolysate with or without detoxification (Jeffries 1985; Nigam 2001; Hahn-Hägerdal *et al.* 2007; Ruyters *et al.* 2015). *Pichia stipitis* has been successfully adapted to grow in a medium with a 60% hydrolysate content and yield equivalent to 0.30 g ethanol/g sugar (Groves 2009). However, Huang *et al*. (2009) reported that a xylose fermenting strain of *P. stipitis* has a poor growth rate when inhibitors are present.

Mussatto *et al.* (2012) have reported that the ethanol production by three yeast strains from detoxified coffee silverskin (CS) hydrolysate that contain xylose sugar prepared by sulfuric acid solution. Among them *P. stipitis* consumed all the sugars in hydrolysate, but in longer fermentation time with the 0.11 g/g yield. They assumed that ethanol production did not cause any inhibition in yeast metabolism.

The strain *P. kudriavzevii* produced noticeably larger amounts of ethanol in acidic media with high salt concentrations compared with the high ethanol producing strain of *S. cerevisiae* (Isono *et al.* 2012).

Yuan *et al.* (2017) was the first to study the ethanol production from the multistress tolerant strain *P. kudriavzevii* when cultivated on various acid-treated lignocellulosic feedstocks without detoxification or added nutrients. The strain recorded a 39% increase in ethanol (33.4 g/L) compared with that produced by *S. cerevisiae* BCRC20270 at 30 °C.

Moon *et al.* (2012) determined the ability of *S. cerevisiae* to produce ethanol on both a rice hull hydrolysate (RHH) containing 19.8 g/L glucose without detoxification and a reference medium containing 20 g/L glucose. It was found that the ethanol yield (0.47 g/g glucose) recorded on the RHH medium was slightly lower than that of the reference medium (0.49 g/g).

Telli-Okur and Eken-Saraçoğlu (2008) studied the ethanol production using *P. stipitis* with detoxified sunflower seed hydrolysate. A maximum ethanol production equivalent to 11 g/L was recorded when the hydrolysate contained 48 g/L total fermentable sugars.

In studies where pentose-fermenting strains of recombinant *S. cerevisiae* were evaluated in undetoxified pentose-rich lignocellulosic hydrolysates, such as sweet sorghum bagasse, sugar cane bagasse, and wheat straw, the maximum ethanol concentrations ranged from 18 g/L to 43 g/L and the xylose conversion rates were 56% to 90% after 48 h (van Maris *et al.* 2007; Olofsson *et al*. 2008; Rudolf *et al*. 2008; Faga *et al*. 2010). The specific xylose consumption rates obtained from the hydrolysates were also clearly lower than those on synthetic media.

Kalhorinia *et al.* (2014) screened the ability of three different *Candida* strains to produce ethanol. The highest ethanol yielding strain, *C. intermedia* (MTCC-1404), was further tested for its ethanol production ability under different conditions. An optimum ethanol production equivalent of 9 g/L ethanol and a 0.4-g/g yield was obtained when the

strain was incubated at 30 °C for 48 h in a medium containing 5% D-xylose and 0.2% glucose.

In this study, the strains presented xylose assimilation on the defined medium and VBHM. The strains showed moderate sugar consumption and ethanol production on the VBHM compared with the reference defined medium. Kuhad *et al.* (2011) stated that various sources of lignocellulosic biomasses have been used by *P. stipitis* strains for ethanol production. The ethanol yield was between 0.3 g/g and 0.45 g/g, and the ethanolproduction was 5.16 g/L (on *Lantana camara* as a substrate) to 25 g/L (on corn stover as a substrate).

CONCLUSIONS

1. Utilizing the natural diversity of yeasts represents an opportunity to increase the number of strains that demonstrate the suitable characteristics for bioethanol fermentation from lignocellulosic wastes. Designing fermentation media with their corresponding strains is vital to improve the conversion of xylose to ethanol.
2. Fermentation on VBHM did not yield significant ethanol amounts, probably due to the low concentration of sugars. However, the hydrolysate acted as an efficient medium for the yeast's growth. Thus, concentrating the hydrolysate could be an alternative to improve ethanol production, as also stated in Mussatto *et al*. (2012). In addition, adapted natural microorganisms to specific hydrolyzed lignocellulosic wastes for ethanol production can be regarded as advantageous from the aspects of development of plants, energy security, biosecurity, and environmental safety related issues.

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