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ETHANOL FERMENTATION FROM MICROWAVE-ASSISTED ACID PRETREATED RAW MATERIALS BY *Scheffersomyces stipitis*

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Abstract

The production of value-added products from renewable resources have been studied by researchers for years and are still being studied due to the fact that there is a point of interest. In this study, ethanol production from microwave-assisted acid pretreated renewable resources including barley husk, wheat bran, and rye bran was performed. The hydrolysis of renewable resources were performed at 700 W of microwave power, 6.92 min of irradiation time, 1:18.26 w/v of solid-to-liquid ratio, and 3.67% v/v of acid ratio for barley husk, 600 W, 6.92 min, 1:16.69 w/v, and 1.85% for wheat bran, and 460 W, 6.15 min, 1:17.14 w/v, and 2.72% for rye bran. The hydrolysates were detoxified with 2% (w/v) activated charcoal at 30ºC and 150 rpm for 30 min. Then, the enriched hydrolysates were utilized to produce ethanol in shake flask fermentation by Scheffersomyces stipitis (ATCC 58784) that is a xylose fermenting yeast at 150 rpm and 30ºC with 5% (v/v) inoculum size. Results indicated that the highest ethanol production (6.15 g/L) was performed in shake flask fermentation with wheat bran medium. However, the lowest ethanol concentration (1.37 g/L) was obtained from barley husk medium. Also, 4.89 g/L of ethanol was produced in rye bran media. Nonetheless, while the highest ethanol yield was calculated to be 44.11% in wheat bran media, the lowest ethanol yield was 12.78% in barley husk medium. On the other hand, the sugar utilization yields in barley husk, wheat bran, and rye bran mediums were also computed to be 85.33, 94.09, and 94.74%, respectively. Consequently, raw materials used in this study can be utilized as good carbon sources for production of ethanol by fermentation.

Key words*: microwave-assisted, pretreatment, detoxification, Scheffersomyces stipitis, shake flask fermentation, ethanol.*

INTRODUCTION

Renewable resources such as agricultural residues, agro-industrial residues, food processing wastes, human and animal wastes etc., are the most abundant available and inexpensive materials on the earth. Therefore, these wastes can be evaluated for the production of value-added products/chemicals by biotechnological processes due to their high carbohydrate contents since they consisted of cellulose (40-50%), hemicellulose (25-30%), lignin (15-20%), and the other extractives components (Germec et al., 2016c; Menon & Rao, 2012).

Energy consumption has increased day by day coupled with the world population (Zhu et al., 2006). Fossil fuels have been utilized to meet the energy and organic chemical demand of the world for years. But fossil fuel reserved are reduced with each passing day and thus are becoming expensive. To overcome such problems, the attempts are increased for the production of biofuels from renewable resources. Therefore, alternative resources and approaches required to be investigated to meet the energy requirement of the world (Fatehi, 2013).

An important step for production of biofuels such as bioethanol by fermentation is pretreatment (Menon & Rao, 2012). The pretreatment types are mechanical, physical, chemical, physico-chemical, and biological, which are applied to renewable resources for production of value-added products/chemicals (Fatehi, 2013; Menon & Rao, 2012). Once is microwave-assisted dilute acid pretreatment, which is one of the physico-chemical pretreatment processes (Zhang et al., 2017). This pretreatment process is a promising technology for the production of fermentable sugars from the renewable resources and following by the production of value-added products such as ethanol by biotechnological

processes (Zhao et al., 2010). To our knowledge, there are no reports on ethanol production from microwave-assisted dilute acid pretreated barley husk, rye bran, and wheat bran. The objective of this study was to evaluate the suitability of ethanol production from microwave-assisted dilute acid pretreated barley husk, rye bran, and wheat bran by using *S. stipitis*.

MATERIALS AND METHODS

Raw material

Barley husk and rye branwere provided from Health Agricultural Products and Food Ind. Trade. Co. Ltd in Konya, Turkey. Wheat bran was obtained from a local feed factory in Osmancik (a district of Corum), Turkey. Among these, barley husk was milled to increase the hydrolysis efficiency by using a grinder (Bosch MKM6000, Ljubljana, Slovenia). Raw materials were stored at +4ºC until used.

Microwave-assisted dilute acid pretreatment of raw materials

Microwave-assisted dilute acid hydrolysis of the raw materials (10 g in the liquid phase) was performed in a microwave oven (Beko MD 1610, voltage 230-240 V, ~50Hz, frequency 2450 MHz, and maximum power 1200 W, Foshan, Guangdong, China). Optimum hydrolysis conditions were determined using Box-Behnken Response Surface Methodology by Germec et al. (2017). The optimum conditions were 700 W, 6.92 min, 1:18.26 w/v, and 3.67% for barley husk, 600 W, 6.92 min, 1:16.69 w/v, and 1.85% for wheat bran, and 460 W, 6.15 min, 1:17.14 w/v, and 2.72% for rye bran, respectively. After hydrolysis, the reaction mixtures were cooled to room temperature and then filtered. The hydrolysates were stored at +4ºC until used for fermentation (Germec et al., 2017).

Detoxification with activated charcoal

In order to decrease the concentration of inhibitors liberated during the pretreatment of raw materials, the hydrolysate (100 ml) was detoxified with activated charcoal detoxification method. Briefly, it was performed using a shaking incubator (CERTOMAT® IS, Gottingen, Germany) at 30ºC and 150 rpm with 2% (w/v) activated charcoal for 30 min. Following the detoxification, the activated charcoal was separated from the hydrolysates by using a centrifuge (4000 rpm, 20ºC, and 30 min) (VWR Mega Star 3.0R, Osterode am Harz, Germany) and then the supernatants were removed for the fermentation to ethanol (Germec et al., 2016a; Mateo et al., 2013).

Microorganism and medium

Scheffersomyces stipitis ATCC 58784 was obtained from American Type Culture Collection (Manassas, VA, USA). *S. stipitis* ATCC 58784 was grown at 30ºC for 48 h in a yeast extract-malt (YM) medium containing 10 g of glucose, 3 g of yeast extract, 3 g of malt extract, and 5 g of peptone per liter of deionized water. The medium pH was adjusted to 6.2 with 4 N NaOH and HCl. The culture was stored at 4ºC and sub-cultured bi-monthly in order to maintain viability. For a long-term storage, stock cultures were maintained in 20% glycerol at -80ºC. *S. stipites* was grown in 250 mL flasks containing 100 mL of YM at 30ºC and 150 rpm for 24 h for inoculation (Germec et al., 2016b; Zhu et al., 2011).

Ethanol fermentation medium

The base-line medium was composed of 10 g of glucose, 3 g of yeast extract, 3 g of malt extract, and 5 g of peptone per liter of deionized water. For fermentations, the detoxified hydrolysates were used as carbon source instead of glucose, but all other ingredients were added in the fermentation medium (Germec et al., 2016b; Germec et al., 2016d).

Shake flask fermentation

Shake flask fermentations were carried out in a shaking incubator (CERTOMAT® IS, Gottingen, Germany) with 250 ml flasks containing 100 ml of the prepared mediums from the detoxified raw material hydrolysates. All fermentation runs were performed in duplicate. The initial pH of mediums was adjusted to 6.2 by adding 8 N NaOH and 4 N HCI. Then, the flasks were autoclaved at 121.1ºC for 15 min. After autoclaving and cooling down to room temperature, 5% (v/v) of

prepared inoculum at 30ºC for 24 h was used to inoculate into the flasks and ethanol fermentations were performed for a period of 120 h. During ethanol fermentations, temperature was maintained at 30ºC and agitation speed was kept at 150 rpm. Samples (1 ml) were collected every 4 or 8 h for the first 12 h and every 12 or 24 h for the remainder of the fermentation and analyzed for residual sugar, ethanol production (*P*) as well as optical cell density for biomass concentration (*X*) in fermentation broth (Germec et al., 2016a).

Analysis

Ethanol

The ethanol was determined by using a HPLC (Thermo Scientific UltiMate 3000, Dreieich, Germany) equipped with a RefractoMax 520 refractive index detector, autosampler, column oven, and computer controller. Separations were performed on a Transgenomic ICSep ORH-801 column (Apple Valley, MN) at 70ºC using 0.01 N H_2SO_4 as the mobile phase with a 20 µL injection volume. The flow rates of 0.5 ml/min was used for analysis of ethanol.

Residual sugar concentration

The residual sugar concentration in the fermentation broth was analyzed by 3,5-dinitrosalicylic acid method (Miller, 1959). Briefly, a measurement of absorbance at 575 nm was recorded. A calibration curve for the spectrophotometric measurements (Thermo Scientific Evolution 201 UV-Vis, Shanghai, China) was created using a glucose solution. Deionized water was used as a blank. Absorbance values were converted to residual sugar concentration by using the obtained standard curve, which was $y=60.401\times Abs_{575}+0.5751$. Here y is glucose concentration, g/L (Germec et al., 2016a; Germec et al., 2015).

Biomass

The optical cell density was measured using a spectrophotometer (Thermo Scientific 201 UV-Visible Evolution, Shanghai, China) at 600 nm. Uninoculated media was used as a blank. Absorbance values were converted to biomass concentrations by using a standard curve, which was $y=0.3047\times Abs_{600}-0.2656$, where y is biomass concentration, g/L (Germec et al., 2016b).

Kinetic parameters

Following kinetics were calculated as follows:

- Sugar consumption (S, g/L) = $S_f - S_i$
- Ethanol production (P, g/L) = $P_f - P_i$
- Ethanol yield ($Y_{P/S}$, %)= $(P/S) \times 100$
- Biomass yield ($Y_{X/S}$, %) = $(X/S) \times 100$
- Product yield per biomass ($Y_{P/X}$, g/g) = P/X
- Maximum consumption rate (Q_S, g/L/h) = $(-ds/dt)_{max}$
- Maximum production rate (Q_P, g/L/h) = $(dp/dt)_{max}$
- Sugar utilization yield (SUY, %) = $(S/S_m)\times 100$
- Theoretical ethanol yield (TY, %) = $(Y_{P/S}/51.1)\times 100$

where, S is the sugar consumption (g/L);S_i and S_f are residual sugar concentrations at the beginning and at the end of the fermentation (g/L), respectively; P is the ethanol production (g/L); P_i and P_f are ethanol concentrations at the beginning and at the end of the fermentation (g/L), respectively (g/L); X is biomass production (g/L); Q_S is the slope of the steepest part of sugar consumption profiles (g/L/h); Q_P is the slope of the steepest part of ethanol production profile (g/L/h), the slopes were calculated using at least three points; and S_m is maximum sugar concentration (g/L).

RESULTS AND DISCUSSIONS

In this study, ethanol production from microwave-assisted dilute acid pretreated and detoxified raw material hydrolysates was performed and the results were evaluated in terms of kinetic parameters.

Ethanol production in shake flask fermentation with barley husk medium

Figure 1 depicts the sugar consumption, cell growth, ethanol production plots belong to ethanol fermentation in shake flask fermentation with barley husk medium. Figure 1 shown that the sugar consumption stopped at 96 h of fermentation. At this point, the yeast growth also entered into the stationary phase. However, it indicated that ethanol production is

still continuing up to 120 h cultivation. Because, the ethanol concentration produced was both quite low and not economical (Figure 1).

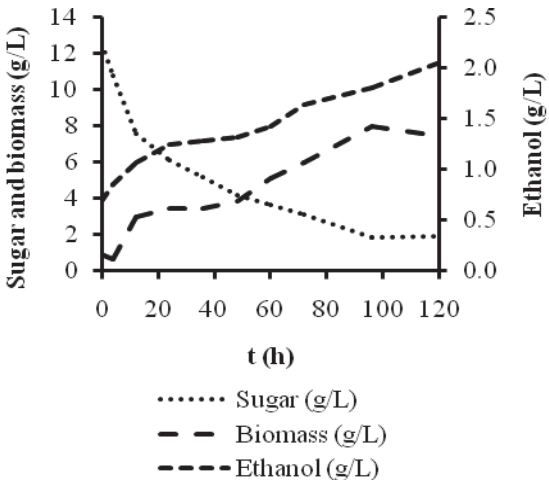

Figure 1. Diagram belong to ethanol production from the detoxified barley husk hydrolysate in shake flask fermentation

Table 1 shows the kinetic parameters for shake flask ethanol fermentation with barley husk medium. The sugar concentration consumed through fermentation was 10.72 g/L. However, 1.84 g/L of sugar was not consumed by the yeast *S. stipitis* (data not shown). On the other hand, although 85.33% of sugar was consumed by the yeast, the ethanol concentration produced was 1.37 g/L, fairly low. Therefore, while the ethanol yield was stayed at 12.78%, the theoretical ethanol efficiency was also 25.01%, which was about 4-times lower than the theoretical efficiency. Additionally, the biomass yield was rather high, which yielded as 68.39%, but the ethanol yield per biomass was 0.19 g/g. In addition, maximum consumption and maximum production rates were also calculated to be 0.41 and 0.03 g/L/h, respectively.

Table 1. The kinetic parameters for detoxified barley husk fermentation

Kinetics	Value	Unit
S	10.72	g/L
P	1.37	g/L
$Y_{P/S}$	12.78	%
$Y_{X/S}$	68.39	%
$Y_{P/X}$	0.19	g/g
Q_S	0.41	g/L/h
Q_P	0.03	g/L/h
SUY	85.33	%
TY	25.01	%

In the literature, no study was carried out the ethanol production from the microwave-assisted dilute acid pretreated and detoxified barley husk hydrolysate by using *S. stipitis*. However, Palmarola-Adrados et al. (2005) was performed the ethanol production from barley husk hydrolysate by using baker's yeast. Our results were not assisted their results. Indeed, they reported that the theoretical ethanol yield was achieved to be 92% and the hydrolysate samples were fermented within the first 5 h. Kim et al. (2008) investigated the bioethanol production from the SAA (soaking in aqueous ammonia)-pretreated barley hulls by using recombinant *Escherichia coli*. Results indicated that the ethanol produced was 24.1 g/L, which corresponded to 89.4% of the maximal theoretical yield depending on the glucan and xylan. Consequently, further researches should be taken place with regard to the ethanol production from microwave-assisted acid pretreated barley husk hydrolysate.

Ethanol production in shake flask fermentation with rye bran medium

Ethanol fermentation performed in shake flask fermentation with rye bran medium was demonstrated in Figure 2.

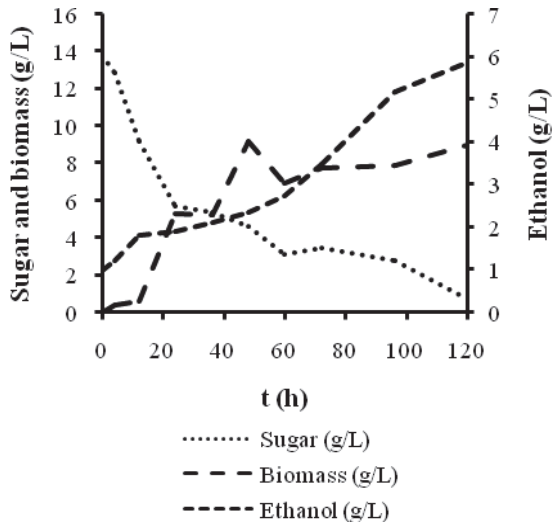

Figure 2. Diagram belong to ethanol production from the detoxified rye bran hydrolysate in shake flask fermentation

The sugar in the media was swiftly consumed in the first 24 h of fermentation and the ethanol was relatively increased. Within the same period, the cell growth also increased rapidly.

After the 24 h of fermentation, the sugar's consumption continued until the end of the fermentation, slowly. At the end of the fermentation, 0.73 g/L of sugar was not consumed by the yeasts. The maximum biomass concentration (9.16 g/L) was at 48 h of fermentation (data not shown). After this point, cell growth entered to the stationary phase. But, interestingly, the ethanol production was swiftly increased when the yeast enter to stationary phase (Figure 2).

According to data from in Table 2, the ethanol yield was 37.36% when the sugar consumption and ethanol production were 13.08 and 4.89 g/L, respectively. On the other hand, the biomass yield was 65.93% since the growing biomass concentration was 8.62 g/L. Therefore, the product yield per biomass was 0.57 g/g.

Table 2. The kinetic parameters for detoxified rye bran fermentation

Kinetics	Value	Unit
S	13.08	g/L
P	4.89	g/L
$Y_{P/S}$	37.36	%
$Y_{X/S}$	65.93	%
$Y_{P/X}$	0.57	g/g
Q_S	0.35	g/L/h
Q_P	0.07	g/L/h
SUY	94.74	%
TY	73.10	%

As the 13.08 g/L of the sugars in the media was consumed by the yeast and the initial sugar concentration was 13.80 g/L, the sugar utilization yield was calculated to be 94.74%, nearly whole sugar. Besides, theoretically, the maximal ethanol yield from the glucose is 51.1%. Since the ethanol yield was 37.36% in this part of the study, the theoretical ethanol yield was 73.10%, which was relatively close to maximal theoretical ethanol yield. In addition, maximum consumption rate and maximum production rate were also calculated, which were yielded to be 0.35 and 0.07 g/L/h, respectively.

In the literature, while there is no study related to ethanol production from microwave-assisted dilute acid pretreated and detoxified rye bran hydrolysate, however, the ethanol was produced from the rye bran. For instance, Wang et al. (1998) was taken place the fermentation of sugars to ethanol obtained from enzymatic hydrolysis of fall rye. The results indicated that the ethanol production was 409 L/tones, which was equivalent to 90.1% of theoretical ethanol yield. Vidmantiene et al. (2006) was performed the fermentation to ethanol using the baker's yeast of sugars releasing by enzymatic hydrolysis of rye bran and reported that the maximum ethanol concentration and ethanol yield were 44 g/L and 45.7% (89.4% of theoretical yield), respectively. The yields (90.1 and 89.4% of theoretical maximal ethanol yield) were relatively higher than our theoretical yield value.

Ethanol production in shake flask fermentation with wheat bran medium

Figure 3 displays the sugar consumption, cell growth, and ethanol production plots belong to the shake flask fermentation of the microwave-assisted dilute acid pretreated and detoxified wheat bran hydrolysate. According to Figure 3, the sugars in wheat bran hydrolysate was rapidly consumed until the 48 h of fermentation by the yeast *S. stipitis*.

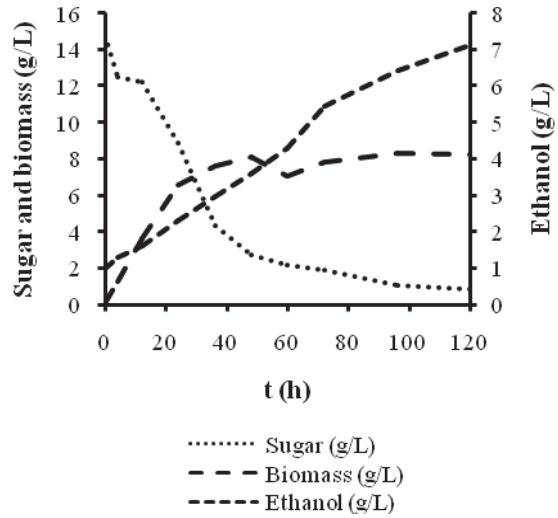

Figure 3. Diagram belong to ethanol production from the detoxified wheat bran hydrolysate in shake flask fermentation

Nonetheless, both ethanol production and cell growth was increased with decreasing of the sugars in the media. After the 48 h of fermentation, along with the sugar consumption was continued at a slow rate until the end of the fermentation, while the ethanol concentration was normally increased, the cell growth stopped. The highest biomass concentration

was observed at 96 h of fermentation while maximum ethanol production and the lowest sugar concentration were determined at the end of the fermentation (Figure 3).

The kinetic results calculated using the experimental data from the fermentation of wheat bran hydrolysate were given in Table 3. The ethanol concentration produced and the sugar concentration consumed were 6.15 and 13.95 g/L, respectively. Therefore, the results indicated that the ethanol yield was quite close to theoretical ethanol yield, which achieved to be 44.11% (86.32% of the theoretical value).

Table 3. The kinetic parameters for detoxified wheat bran fermentation

Kinetics	Value	Unit
S	13.95	g/L
P	6.15	g/L
$Y_{P/S}$	44.11	%
$Y_{X/S}$	59.09	%
$Y_{P/X}$	0.75	g/g
Q_S	0.33	g/L/h
Q_P	0.10	g/L/h
SUY	94.09	%
TY	86.32	%

However, the growing biomass concentration was also 8.25 g/L, thus, the biomass yield was calculated to be 59.09%. After calculating the ethanol yield and biomass yield, the product yield per biomass was also computed to be 0.75 g/g. Almost whole of the sugar was consumed by the yeast, therefore, the sugar utilization yield was 94.09%. On the other hand, maximum sugar consumption rate and maximum ethanol production rate were 0.33 and 0.10 g/L/h, respectively.

In the literature, many studies were performed with respect to the microwave-assisted extraction of wheat bran, but no study was taken place with regard to the fermentation of microwave-assisted dilute acid pretreated and detoxified wheat bran hydrolysate to ethanol by the yeasts. Okamoto et al. (2011) performed the ethanol production from wheat bran by using the white rot fungus *Trametes hirsuta* and reported that the highest ethanol concentration was 4.3 g/L, corresponding to 78.8% of the theoretical yield. Investigated the ethanol production from the unfiltered wheat bran hydrolysates by using *S. cerevisiae* and reported that the ethanol yield was 49%, corresponding to approximate 96% of theoretical ethanol yield.

CONCLUSIONS

In this study, the ethanol production from the microwave-assisted dilute acid pretreated and activated charcoal detoxified raw material hydrolysates was performed and the results were evaluated in terms of kinetics. Results indicated that, the low kinetic results were obtained from the fermentation of barley husk hydrolysate. Therefore, it should be investigated further why this is so. Good results were achieved from the fermentation of rye bran and wheat bran, especially the latter. Thus, these results can be further improved under the controlled fermentation conditions. In conclusion, the raw materials used in this study can be good, inexpensive, and alternative carbon sources for the production of value-added products by biotechnological processes.

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