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FERMENTATION OF ACID-PRETREATED TEA PROCESSING WASTE FOR ETHANOL PRODUCTION USING *Saccharomyces cerevisiae*

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Abstract

Value-added products such as biofuels, enzymes, polysaccharides, pharmaceuticals, and organic acids can be produced from renewable resources such as carob pods, wheat straw, rice straw, rice husk, sugarcane bagasse, etc. by fermentation. Tea processing waste (TPW) is one of the renewable resources, which contains 13.60% cellulose, 32.16% hemicellulose, and 33.38% lignin. Due to its high carbohydrate content (total 45.76% wt), TPW can be used for production of value-added products. Therefore, the main objectives of this study were undertaken not only to determine the chemical composition of acid-pretreated tea processing waste hydrolysate (APTPWH) but also to investigate the effect of pH, inoculum size, agitation, and nitrogen sources on ethanol production from APTPWH without detoxification by using *Saccharomyces cerevisiae* in a stirred tank bioreactor. Results showed that it contains no HMF and lactic acid, 16.03 g/L fermentable sugar (FS), 0.43 g/L acetic acid, 2.61 g/L glucose, 4.14 g/L fructose+xylose, and 2.27 g/L phenolics. For batch ethanol fermentations, optimum conditions were found to be pH controlled at 5.5, 5% inoculum size (v/v), 150 rpm agitation speed, and yeast extract, which achieved as 25.55% yield ($Y_{P/S}$), 1.75 g/L ethanol production (P), and 0.38 g/L/h productivity (Q_P). Consequently, ethanol could be relatively produced from TPW without detoxification.

Key words: *tea processing waste, hydrolysis, chemical composition, ethanol fermentation.*

INTRODUCTION

Fossil fuels have been used to supply primarily the energy and also the organic chemicals requirements in the world for years. However, energy sector is confronted a problem such as reduction of petroleum fuel reserves. On the other hand, the world is face to face with some threats with respect to the large-scale utilization of petroleum fuels such as global warming, environmental pollution, and greenhouse effect. In 2050, the world population is expected to increase up to 10 billion, and thus this will increase the necessity to fuels of the world. Therefore, the attempts are enhanced for the production of biofuels from biomass due to increasing petroleum price and depletion of fossil fuel reserves. Accordingly, alternative sources need to be investigated in order to decrease the level of greenhouse gases released to environment and to supply the energy requirement of the world (Fatehi, 2013). Renewable resources are the most abundant and low-cost materials in nature, which generally exist in the form of pre- and post-harvest agricultural losses, agro-industrial wastes, wastes of food processing industries etc. (Galbe & Zacchi, 2012). TPW is also a kind of renewable resource released as a post-harvest waste of tea factory. Tea is a major commercial herbal crop in the tropical and subtropical regions. According to FAO data in 2013, the production amount of tea in the worldwide and Turkey are 5345523 and 212400 tons per year, respectively (FAOSTAT, 2015; Malkoc & Nuhoglu, 2007). Tea plant is grown in the Eastern Black Sea Region of Turkey, which is harvested three or four times a year in Turkey (Malkoc & Nuhoglu, 2007). In order to produce high quality tea, two and a half top leaves of the shoot on tea plant are harvested. But while tea producer cut the top leaves with special tea shears some overgrown woody shoots are mixed in the tea harvest. Therefore, these were untreated by tea factory

during the tea production process and thus formed into TPW, which is generated about 30-50 thousand tons per year in Turkey. Consequently, TPW is one of the most abundant renewable resources in tea growing countries such as China, Iran, Turkey, etc., which has not been evaluated for ethanol production (Malkoc & Nuhoglu, 2007).

Yeasts and bacteria have been used for ethanol production from pure carbon sources (glucose, sucrose, xylose, etc.), industrial plants (sugar cane, sugar beet, etc.) or by-products of food industry (whey, molasses, etc.) for many years. Among these, *S. cerevisiae* is the most used yeast from past to present as well as *Zymomonas mobilis* and *Pichia stipitis* (Atiyeh & Duvnjak, 2003). Therefore, the main objectives of this study are to determine the chemical composition of APTPWH and to examine the effect of pH, inoculation rate, agitation speed, and nitrogen sources on ethanol production from APTPWH by using *S. cerevisiae* in a stirred tank bioreactor.

MATERIALS AND METHODS

Raw material

TPW was provided from Çaykur Tea Company in Rize, a province of Turkey. It was milled to increase the hydrolysis efficiency by using a grinder (Bosch MKM6000, Ljubljana, Slovenia) and stored at room temperature until used. Besides, TPW composition analysis were studied before, which is consisted of 13.60% cellulose, 32.16% hemicellulose, 33.38% lignin, 20.86% extractives, and 0.10% moisture (Germec et al., 2016).

Dilute acid hydrolysis of TPW

Dilute acid hydrolysis of the milled TPW was performed using an autoclave (Hirayama HG-50, Saitama, Japan). Optimum hydrolysis conditions for TPW were studied by Germec et al. (2016). So the hydrolysis conditions of TPW were determined to be 120ºC, 12.5% solid loading (w/v), 1% dilute H_2SO_4 rate (w/v), and 15 min. After hydrolysis, the reaction mixture was cooled to room temperature and then filtered. The hydrolysate was stored at +4ºC until used for fermentation (Germec et al., 2016).

Microorganism and medium

S. cerevisiae ATCC 36858 was used for ethanol production from APTPWH, which was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The stock culture was grown at 30ºC for 48 h in medium including 50 g of glucose, 6 g of yeast extract, 4 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 1 g of $MgSO_4.7H_2O$, and 0.3 g of $CaCI_2.2H_2O$ per liter of deionized water. The stock culture was stored at 4ºC and sub-cultured bimonthly in order to maintain viability. For a long-term storage, cultures were preserved at -80ºC in 20% glycerol (Turhan et al., 2010).

Ethanol fermentation medium

The reference and stock culture medium was composed of 50 g of glucose, 6 g of yeast extract, 4 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 1 g of $MgSO_4.7H_2O$, and 0.3 g of $CaCI_2.2H_2O$ per liter of deionized water. For fermentations, APTPWH was used as carbon source instead of glucose, but all other ingredients were added in the fermentation environment (Turhan et al., 2010).

Batch ethanol fermentation

Batch ethanol fermentations were carried out in a stirred tank bioreactor (Sartorius Biostad B Plus, Goettingen, Germany) with a 5-L vessel (working volume of 2.5 L). The reactor vessel was autoclaved at 121.1ºC for 15 min. After autoclaving and cooling down to room temperature, prepared inoculum at 30ºC for 24 h was used to inoculate the reactor and ethanol fermentations were performed for a period of 48 h. During ethanol fermentations, temperature was maintained at 30ºC and pH was controlled by using automatic addition of 4N NaOH. Samples were collected every 2 or 4 h for the first 12 h and every 6 or 12 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 (Turhan et al., 2010).

Experimental design for ethanol fermentation

Five different ethanol production designs described as follows were evaluated in a stirred tank bioreactor.

- ***Effect of pH:*** Ethanol fermentation was carried out in enriched APTPWH with 3% inoculum at 30ºC, 150 rpm, and pH 5.5 or pH uncontrolled.
- ***Effect of inoculation rate:*** Three inoculation rates (1, 3, and 5%, v/v) were used to determine the effect of inoculation rate on the production of ethanol from APTPWH. Fermentations were performed at 30ºC, 150 rpm, and pH 5.5.
- ***Effect of agitation speed:*** In order to determine the effect of agitation speed on ethanol production from APTPWH, three different agitation speeds (100, 150, and 200 rpm) were used with the best inoculation rate.
- ***Effect of nitrogen source:*** Effect of three different nitrogen sources (yeast extract, beef extract, and ammonium nitrate) on the production of ethanol from APTPWH was examined with the best inoculation rate and agitation speed.
- ***Control:*** Non-enriched APTPWH was used for ethanol production with the best inoculation rate and agitation speed at pH 5.5 as control.

Analysis

Ethanol

Ethanol concentration was measured by using a bioanalyzer (Model YSI 2700, Yellow Springs, OH, USA) (Izmirlioglu & Demirci, 2012).

Sugars, organic acids, and total phenolics

Glucose, fructose, xylose, lactic acid, and acetic acid concentrations in APTPWH were determined by a HPLC system (LC-20 AD model, Shimadzu Kyoto, Japan) equipped with a refractive index detector. Separations were performed on a Transgenomics ORH-801 column (Apple Valley, MN, USA) at 65ºC using 0.0025 N H_2SO_4 as the mobile phase (20 µL injection volume, 0.6 ml/min). APTPWH was diluted with HPLC-grade water and filtered 0.45 µm membrane filters (Macharey-Nagel, Duren, Germany) prior to analysis (Kelebek et al., 2009).

The concentration of total FSs were analyzed by 3,5-dinitrosalicylic acid method (Miller, 1959). Absorbance values that measured at 575 nm were converted to FS concentration by using glucose standard curve. On the other hand, the analysis of total phenolic compounds were performed to Folin-Ciocalteu method (Singleton et al., 1999).

Hydroxymethyl furfural (HMF)

HMF concentration in the APTPWH was determined by a ThermoScientific HPLC system with a 20 µL sample loop and a Series 200 UV-Vis variable wavelength detector. Separation was performed on an Altima C18 column, 250 mm×4.6 mm, 5 µm particle size (Alltech, Sedriano, Italy) using 0.1 M H_2SO_4 solution in HPLC-grade water (resistance higher than 18 MΩ and methanol (Merc, Darmstadt, Germany) as the mobile phase (1.2 ml/min). The duration of HMF analysis was 23 min. The gradient mobile phase was used to be 90% of 0.1 M H_2SO_4 and 10% of methanol for 0-2 min, 70% of 0.1 M H_2SO_4 and 30% of methanol for 2-9 min, 70% of 0.1 M H_2SO_4 and 30% of methanol for 9-11 min, 40% of 0.1 M H_2SO_4 and 60% of methanol for 11-16 min, 40% of 0.1 M H_2SO_4 and 60% of methanol for 16-21 min, 90% of 0.1 M H_2SO_4 and 10% of methanol for 21-22 min, and 90% of 0.1 M H_2SO_4 and 10% of methanol for 22-23 min (Spano et al., 2009).

Biomass

The optical cell density was measured using a spectrophotometer (ThermoScientific 201 UV-Visible Evolution, Shanghai, China) at 620 nm. Uninoculated media was used as a blank. Absorbance values were converted to biomass concentrations by using a standard curve (Turhan et al., 2010).

Statistical analysis

The data were evaluated by using SAS statistical program (Version 9.00, SAS Institute INC., Cary, NC, USA). Duncan's multiple comparison test was used at significance level (P=0.05). All values were the average of two replicates and expressed in table as mean ± standard deviation.

RESULTS AND DISCUSSIONS

This study was undertaken not only to determine the chemical composition of APTPWH, but also to investigate separately the effect of fermentation parameters on ethanol

production from APTPWH without detoxification in stirred tank bioreactor.

Chemical composition of APTPWH

The inhibitors such as HMF, acetic acid, and phenolics are formed during the biomass pretreatment depending on the pretreatment severity, which are formed as a result of the degradation of cellulose, hemicellulose, and lignin in raw material (Uzuner & Cekmecelioglu, 2014). In this research, the acidic hydrolysis of TPW were performed at 120ºC, 12.5% solid loading (w/v), 1% (w/v) dilute H_2SO_4 rate, and 15 min (Germec et al., 2016). HMF and lactic acid were not detected, 16.03 g/L total FS, 0.43 g/L acetic acid, 2.61 g/L glucose, 4.14 g/L fructose+xylose, and 2.27 g/L total phenolic compounds were determined in APTPWH. The acid hydrolysis results showed that 16.03 g/L carbon source for ethanol fermentation was produced.

Similarly, Uzuner and Cekmecelioglu (2014) analyzed the level of chemical compounds in acid-pretreated hazelnut shell and reported that 2.59 g/L acetic acid, 0.0145 g/L HMF, 0.15 g/L phenolics, and no furfural was detected in hydrolysate. In conclusion, inhibitors such as HMF, acetic acid, furfural, and phenolics could be produced to be by-product during pretreatment of lignocellulosic materials.

Ethanol fermentation

Ethanol fermentation from APTPWH by using *S. cerevisiae* in a stirred tank bioreactor was evaluated in point of pH, inoculation rate, agitation speed, and nitrogen source.

Effect of pH on ethanol production

Effect of pH on ethanol production was initially evaluated at pH 5.5 and pH uncontrolled (Table 1, A and B). According to results, while $Y_{P/S}$, P, and Q_P values were calculated to be 20.07%, 1.61 g/L, and 0.31 g/L/h at pH 5.5, they were determined to be 10.17%, 0.89 g/L, and 0.13 g/L/h at pH uncontrolled, respectively. There was not significant difference between P values for pH controlled and uncontrolled fermentations but the $Y_{P/S}$ and Q_P values were significantly different ($p<0.05$). Therefore, the pH value for ethanol fermentation from APTPWH by *S. cerevisiae* was chosen to be 5.5.

The pH value of *S. cerevisiae* ethanol fermentations are generally ranged from 5 to 5.5 (Germec et al., 2015; Turhan et al., 2010; Yatmaz et al., 2013). The pH value of the fermentation medium is important. Ethanol production is completely inhibited if pH level drops to below 4 (Graves et al., 2006). Turhan et al. (2010), Yatmaz et al. (2013), and Germec et al. (2015) reported that the effect of pH on ethanol production from carob extract was statistically important and the optimal pH values were determined to be 5.5, 5.5, and 5.18, respectively.

Effect of inoculation rate on ethanol production

Three different inoculation rates (1, 3, and 5%, v/v) were tested to determine the effect on ethanol production from APTPWH. Agitation speed was fixed at 150 rpm while nitrogen source used in the media was yeast extract. According to inoculation rates fermentation results, the highest $Y_{P/S}$ (25.55%), P (1.75 g/L), and Q_P (0.38 g/L/h) was determined with 5% inoculation rate. In addition, lowest doubling time (t_d) was calculated for 5% inoculation rate. It means that conversion of sugar to ethanol is preformed faster and the microorganism with 5% inoculation rate was used sugar better than 1 and 3% inoculation rate (Table 1, D).

The effect of inoculation rate on ethanol production from carob extract by using immobilized *S. cerevisiae* cells in Ca-alginate in a stirred tank bioreactor was also studied. They reported that the best inoculation rate was found to be 5% v/v (Yatmaz et al., 2013). Results showed that inoculation rate had a significant effect on consumption rate (Q_S), growth rate (Q_X), specific growth rate (μ), and t_d values ($P<0.05$) while it had no significant effect on P, Q_P, and $Y_{P/S}$ values ($P>0.05$) (Table 1B-D). Besides P, Q_S, Q_P, Q_X, μ, and $Y_{P/S}$ values were generally enhanced with increasing of inoculation rate. Accordingly, the optimum inoculation rate for highest P, Q_P, and $Y_{P/S}$ was chosen to be 5% v/v, which was used for all following fermentations.

Effect of agitation speed on ethanol production

In order to determine the best agitation speed, three different agitation speeds (100, 150, and

200 rpm) were used for ethanol production from APTPWH. Nitrogen source was yeast extract. Results indicated that agitation speed had a significant effect on P, Q_S, μ, and t_d (P<0.05) (Table 1, D-F). The highest P, $Y_{P/S}$, Q_S, Q_P, Q_X, μ, and t_d values were obtained with 150 rpm agitation speed (Table 1, D). Yatmaz et al. (2013) studied the effect of agitation speed on P from carob extract by immobilized *S. cerevisiae* cells and reported the optimum agitation speed was 150 rpm. Consequently, kinetic parameter values were reduced at 100 and 200 rpm agitation speed, and thus the best agitation speed was determined to be 150 rpm, which was used for all following fermentations (Table 1, D-F).

Effect of nitrogen source on ethanol production

In order to investigate the effect of different nitrogen sources instead of yeast extract on ethanol production from APTPWH, beef extract and ammonium nitrate (6 g/L) were used individually and results were given in Table 1D, G, and H. The highest P and $Y_{P/S}$ were found to be 1.95 g/L and 28.72% by addition of ammonium nitrate in the media. However, there was no statistically an important effect between nitrogen sources in point of P and $Y_{P/S}$ (P>0.05). Besides, when yeast extract was used in the media; Q_X, μ, and t_d values were statistically significant according to the usage of ammonium nitrate in the media (P<0.05), but this was not valid for Q_X when beef extract was utilized in the media (P>0.05). On the other hand, Q_S and Q_P were not statistically important depending on the usage of yeast extract and ammonium nitrate in the media (P>0.05). Also, Q_S was decreased by addition of beef extract in the media compared to yeast extract and ammonium nitrate. Accordingly, nitrogen source used in the media had a statistically significant effect on P from APTPWH (P<0.05). Consequently, although higher P and $Y_{P/S}$ were obtained when ammonium nitrate was used in the media; higher Q_S, Q_P, Q_X, μ, and lower t_d were achieved by addition of yeast extract in the media. Therefore, the best results were obtained by using yeast extract in the fermentation environment. In conclusion, the best fermentation conditions were chosen to be pH 5.5, 5% inoculation rate (v/v), 150 rpm agitation speed, and yeast extract (Table 1, D).

Table 1. Summary of fermentation results.*

Kinetic parameters / Fermentation conditions	P (g/L)	$Y_{P/S}$ (%)	Q_S (g/L/h)	Q_P (g/L/h)	Q_X (g/L/h)	μ (h^{-1})	t_d (h)
A	$0.89^{abc} \pm 0.03$	$10.17^{b} \pm 1.21$	$0.35^{ab} \pm 0.10$	$0.13^{b} \pm 0.02$	$0.13^{bc} \pm 0.01$	$0.05^{de} \pm 0.01$	$13.77^{b} \pm 2.33$
B	$1.61^{abc} \pm 0.47$	$20.07^{ab} \pm 3.99$	$0.15^{b} \pm 0.02$	$0.31^{ab} \pm 0.06$	$0.11^{c} \pm 0.00$	$0.03^{e} \pm 0.00$	$25.26^{a} \pm 2.37$
C	$1.29^{abc} \pm 0.79$	$22.32^{ab} \pm 1.25$	$0.68^{a} \pm 0.09$	$0.21^{ab} \pm 0.10$	$0.19^{abc} \pm 0.05$	$0.07^{bcd} \pm 0.01$	$9.97^{c} \pm 1.4$
D	$1.75^{ab} \pm 0.05$	$25.55^{ab} \pm 0.88$	$0.68^{a} \pm 0.01$	$0.38^{a} \pm 0.03$	$0.24^{a} \pm 0.01$	$0.21^{a} \pm 0.02$	$3.35^{d} \pm 0.35$
E	$0.65^{c} \pm 0.02$	$10.66^{b} \pm 1.01$	$0.08^{b} \pm 0.00$	$0.21^{ab} \pm 0.12$	$0.15^{abc} \pm 0.04$	$0.07^{bcd} \pm 0.01$	$9.48^{bc} \pm 1.08$
F	$1.05^{abc} \pm 0.04$	$14.81^{ab} \pm 1.51$	$0.62^{a} \pm 0.15$	$0.30^{ab} \pm 0.06$	$0.21^{ab} \pm 0.03$	$0.11^{b} \pm 0.01$	$6.54^{cd} \pm 0.67$
G	$1.45^{abc} \pm 0.12$	$20.45^{ab} \pm 5.07$	$0.22^{b} \pm 0.00$	$0.27^{ab} \pm 0.03$	$0.16^{abc} \pm 0.00$	$0.10^{bc} \pm 0.00$	$7.04^{cd} \pm 0.27$
H	$1.95^{a} \pm 0.06$	$28.72^{a} \pm 2.65$	$0.66^{a} \pm 0.28$	$0.20^{ab} \pm 0.05$	$0.12^{bc} \pm 0.03$	$0.08^{bcd} \pm 0.01$	$9.33^{bc} \pm 1.71$
I	$0.81^{bc} \pm 0.03$	$9.51^{b} \pm 0.05$	$0.21^{b} \pm 0.04$	$0.20^{ab} \pm 0.01$	$0.14^{bc} \pm 0.01$	$0.06^{cde} \pm 0.00$	$11.04^{bc} \pm 0.07$

*All fermentation experiments were performed at 30ºC. **A:** 3% (v/v) inoculation rate, 150 rpm agitation speed, yeast extract, *pH uncontrolled*. **B:** 3% (v/v) inoculation rate, 150 rpm agitation speed, yeast extract, *pH 5.5*. **C:** *1% (v/v) inoculation rate*, 150 rpm agitation speed, yeast extract, pH 5.5. **D:** *5% (v/v) inoculation rate*, 150 rpm agitation speed, yeast extract, pH 5.5. **E:** 5% (v/v) inoculation rate, *100 rpm agitation speed*, yeast extract, pH 5.5. **F:** 5% (v/v) inoculation rate, *200 rpm agitation speed,* yeast extract, pH 5.5. **G:** 5% (v/v) inoculation rate, 150 rpm agitation speed, *beef extract*, pH 5.5. **H:** 5% (v/v) inoculation rate, 150 rpm agitation speed, *ammonium nitrate*, pH 5.5. **I:** 5% (v/v) inoculation rate, 150 rpm agitation speed, pH 5.5 (*non-enriched medium*). Values are given as the mean ± standard deviation of two replicates. Different letters in the same column indicate statistically significance between mean values (P<0.05).

Batch fermentation with using non-enriched APTPWH

To produce the ethanol from non-enriched APTPWH, fermentation conditions were set to be pH 5.5, 5% inoculation rate (v/v), and 150 rpm. The results were given in Table 1I, which were significantly low compared to the best fermentation conditions (Table 1, D). The P and $Y_{P/S}$ values for enriched medium at optimum conditions were 1.75 g/L and 25.55% while they were found to be 0.81 g/L and 9.51% for non-enriched medium, respectively (Table 1, D and I). The results for enriched and non-enriched medium demonstrated that using enriched medium for P from APTPWH was better than using non-enriched medium because of higher kinetic parameter values and ethanol concentration.

CONCLUSIONS

In this study, chemical composition of APTPWH was evaluated. According to results, no HMF and lactic acid, 16.03 g/L total FS, 0.43 g/L acetic acid, 2.61 g/L glucose, 4.14 g/L fructose+xylose, and 2.27 g/L phenolics were determined in APTPWH. *S. cerevisiae* was also used for *P* from APTPWH in a stirred tank bioreactor. The effect of pH, inoculation rate, agitation speed, nitrogen source as well as enrichment on *P* was investigated. The *P*, $Y_{P/S}$, Q_P, and Q_S were 1.75 g/L, 25.55%, 0.38 g/L/h, and 0.68 g/L/h respectively at optimized conditions for batch fermentations, respectively. Enrichment of medium had a statistically effect on ethanol production from APTPWH ($P<0.05$). In conclusion, TPW could be used as a potential substrate source for production of value-added products by fermentation.

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