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A Comparative Study Concerned with the Effect of Different Pretreatments on Ethanol Production from Lemon Peel (*Citrus latifolia*)

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ABSTRACT: In this study the effects of autoclave, microwave and ultrasonic pretreatments with or without acid addition were investigated on the amount of glucose, soluble and insoluble lignin, furfural, yeast viability and bioethanol. The findings showed that autoclave-acid impregnated sample, has the highest glucose release from lignocellulose materials (14.61 and 14.95 g/l for solvent exposed and untreated sample, respectively) whereas for the control sample glucose content was at its minimal level. Pretreatments caused decrease in soluble and insoluble lignin and the highest decrease was caused by autoclave followed by microwave and ultrasonic pretreatments ($p \leq 0.05$). Moderate increase on furfural was observed for pretreated samples than the control. The most yeast viability and bioethanol content belonged to the autoclave samples especially the acid-impregnated ones (40.33%). Comparison between solvent treated and untreated samples indicated that significant differences were observed between the two tested groups ($p \leq 0.01$) in terms of lignin, cell viability and ethanol content but glucose and furfural didn't show a significant difference.

Keywords: *Autoclave, Lemon Peel, Microwave, Ultrasonic.*

Introduction

Ethanol is nowadays a substitute as well as additive to the traditional fossil fuels. Among different resources used for ethanol production, lignocellulose materials are the most abundant ones, and they are usually available at low cost. These materials could be processed to the sugar monomers by acid and/or enzymatic hydrolysis followed by fermentation of the sugars to ethanol. Acid hydrolysis is a fast and relatively inexpensive method for acquiring sugars from lignocelluloses, while enzymatic hydrolysis as well as fermentation usually takes longer times.

Persian lime (*Citrus latifolia)* is a citrus fruit that often is used as lemon juice in Persian cooking and its wastes is partly used for cattle feed. The peel of citrus fruit contains various carbohydrate polymers which make them an interesting choice for the production of metabolites such as ethanol by appropriate microorganisms. An individual or combination of mechanical, chemical, and biological pretreatment is required to break down cellulose, hemicellulose and pectin polymers present in the cell walls of citrus peels and convert them to their sugars' monomers (Gerchman *et al*., 2012). Some researches paid attention on citrus peel as a potential source for bioethanol production (Talebnia, 2008). According to our knowledge, research has not been carried out on bioethanol production from *Citrus latifolia*, thus the current work deals with ethanol production yield from Persian lime at different pretreatment conditions. Also the effect of

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solvent extraction on ethanol productivity has specifically been noticed.

Materials and Methods

Persian lime (*Citrus latifolia*) was kindly collected from lemon juicers at Neyshabur and then air dried. The moisture content was 8% (w/w) and the particles sizes were less than 1 cm.

- *Different trial configurations*

In this study, two groups of lemon peel samples were assessed. Group 1 was treated with n- hexane in order to reduce limonene content and group 2 without solvent extraction.

- *Pretreatment of lemon Peel with n-hexane*

Lemon peel was chopped into small pieces. The chopped lemon peel (40 g) was placed into an Erlenmeyer flask containing a known volume of n-hexane (150 ml). The Erlenmeyer flasks were shaken vigorously for 10 minutes and then kept for an additional period of time (20 minutes). After completing the extraction time, extracts were removed from residuals by vacuum filtration (Nguyen, 2012).

- *Dilute-acid pretreatment*

Lemon peels was submitted to a dilute-acid pretreatment (0.2M H_2SO_4 solution in a solid: liquid ratio of 1:5 w: w, at 120 °C for 40 min), which was performed in 100-ml flasks in uncovered state in a laboratory autoclave. After the reaction, the obtained solid residue was separated by filtration, washed with water until neutral pH and dried at 40°C to attain around 5% moisture content (Mesa *et al*., 2011). The original material and the acid pretreated lemon peels were chemically characterized to determine the glucose and lignin contents and later be used as a substrate for fermentation.

- *Microwave pretreatment*

A LG laboratory microwave with output power 800W was used in this experiment. Pretreatment was carried out as follows: 5 g of lemon peel was first placed in a beaker with 0.2M H_2SO_4 solution in a solid: liquid ratio of 1:5 w:w, The beaker was irradiated for 8 minutes in the microwave oven. Finally the volume was adjusted at 100 mL with distilled water if it's necessary (Beszedes *et al*., 2012).

- *Ultrasonic pretreatment*

Samples of lemon peel and water at the ratio of 1:5 (w/w) were placed in glass flasks and were subjected to ultrasonic pretreatment. The ultrasonic pretreatment was carried out in a sonicator (Model: Eurosonic 4D) at temperature of 60°C and frequency of 40 kHZ and duration of 10 min (Nikoloc *et al*., 2011).

- *Ethanol production*

In order to carry out the fermentation, 30 g of sample was mixed with 600 mL of distilled water and 0.6 g of *S. cerevisiae* and subjected to fermentation at 35 °C, pH of 4.5 with 120 rpm for the duration of 7 days.

- *Chemical analysis*

Following each pretreatment, the content of glucose, acid soluble lignin, insoluble lignin and furfural were determined and after the fermentation step, cell viability and bioethanol content were measured. All the measurements were conducted in triplicate order.

Glucose concentration was determined using glucose oxidase. This assay is specific for glucose. Standard glucose solutions in the range of 0.22 to 1.8 mg/ml were prepared. Samples were filtered prior to analysis. All the samples were analyzed in triplicate order by combining 100 µl of sample with 3.0 ml of assay solution containing glucose oxidase, horseradish peroxidase and o-dianisidine (all the reagents were obtained from Sigma) in 0.1 M sodium phosphate buffer at pH of 7.0. After incubating the samples at 37°C for 30

minutes, the absorbance was measured at 450 nm.

Lignin was analyzed according to description of Santi (2009).

Insoluble lignin was determined according to Nguyen (2012).

Furfural in the samples was analyzed according to Guang *et al*. (2010) using UV-Spectrophotometric method.

Viability of the yeast cells was investigated using the colony-forming unit method.

Analysis of ethanol was conducted using description of Gerchman *et al*. (2012). A YOUNG LIN Acme 6000 Gas Chromatograph equipped with a 100 m long capillary column with a 0.25 mm inside diameter and 0.2 µ film thickness and flame ionization detector (FID) was employed.

- *Statistical analysis*

All the statistical analysis was carried out using SAS version 8. Data were analyzed by analysis of variance (ANOVA) followed by Fisher LSD test to determine the significant differences ($p \leq 0.05$) between the means. Orthogonal contrast was used for comparison of the solvent treated samples vs. untreated samples.

Results and Discussion

- *Results from the pretreatment*

Tables 1 and 2 show the conditions and the pretreatments carried out on the peels. Different pretreatments caused different influences on analyzed properties. In general, autoclaved samples (especially at presence of sulfuric acid) have the most glucose content and cell viability and additionally, ethanol production is substantially higher. All the pretreatments show better results in glucose yield and viability than the control samples in both solvent treated and untreated forms.

Table 1. Different experimental configuration (Hexane- treated)

A_1	Hexane- treated lemon peel, autoclaved at 120 °C for 40 min.
A_2	Hexane- treated lemon peel, 0.2M H_2SO_4 solution in a solid : liquid ratio of 1:5 w:w, autoclaved at 120 °C for 40 min.
B_1	Hexane- treated lemon peel, microwave 800W, 8min.
B_2	Hexane- treated lemon peel, 0.2M H_2SO_4 solution in a solid : liquid ratio of 1:5 w:w, microwave 800W, 8min.
C_1	Hexane- treated lemon peel, ultrasonic at 60°C and frequency 40kHZ and 10min.
C_2	Hexane- treated lemon peel, 0.2M H_2SO_4 solution in a solid : liquid ratio of 1:5 w:w, ultrasonic at 60°C and frequency 40kHZ and 10min.
E	Hexane- treated

Table 2. Different experimental configuration (untreated)

a_1	Lemon peel, autoclaved at 120 °C for 40 min.
a_2	Lemon peel, 0.2M H_2SO_4 solution in a solid : liquid ratio of 1:5 w:w, autoclaved at 120 °C for 40 min
b_1	Lemon peel, microwave 800W, 8min.
b_2	Lemon peel, 0.2M H_2SO_4 solution in a solid : liquid ratio of 1:5 w:w, microwave 800W, 8min.
c_1	Lemon peel, ultrasonic at 60°C and frequency 40kHZ and 10min.
c_2	Lemon peel, 0.2M H_2SO_4 solution in a solid : liquid ratio of 1:5 w:w, ultrasonic at 60°C and frequency 40kHZ and 10min.
e	Without any pretreatments

Table3. Chemical characteristics of lemon peel after different pretreatments (n-Hexane treated).

Treatment	Glucose(g/l)	Soluble lignin (%)	Insoluble lignin (%)	Furfural(g/l)	Viability of *Sacharomyces cerevisiae*(%)
A_1	10.58667^{c}	2.613333^{d}	22.68^{c}	0.21^{a}	38.66667^{b}
A_2	14.61667^{a}	2.226667^{e}	18.26667^{d}	0.18^{cd}	40.33333^{a}
B_1	10.25333^{cd}	2.68^{cd}	18.34^{d}	0.22^{a}	31.66667^{d}
B_2	11.53333^{b}	2.253333^{e}	24.02^{b}	0.186^{c}	35.66667^{c}
C_1	9.673333^{d}	3.286667^{b}	16.06667^{e}	0.22^{a}	28^{e}
C_2	10.52667^{c}	2.836667^{c}	12.9^{f}	0.17^{d}	31^{d}
E	6.543333^{e}	3.576667^{a}	29.1^{a}	0.14^{e}	8.666667^{f}

Means, within each column, followed by the same letter (s) are not significantly different at the 0.05 probability level using Duncan's Multiple Range Test.

Table 4. Chemical characteristics of lemon peel after different pretreatments (Untreated).

Treatment	Glucose(g/l)	Soluble lignin (%)	Insoluble lignin(%)	Furfural(g/l)	Viability of *Sacharomyces cerevisiae*(%)
a_1	10.55333^{c}	3.68^{de}	23.33333^{b}	0.21^{a}	21.33333^{a}
a_2	14.95^{a}	3.503333^{f}	21.33333^{b}	0.18^{b}	22^{a}
b_1	10.25667^{cd}	3.856667^{bc}	26.96667^{ab}	0.22^{a}	15.33333^{c}
b_2	11.58667^{b}	3.58^{ef}	21.2^{b}	0.19^{b}	20.33333^{ab}
c_1	9.74^{d}	3.936667^{b}	22.96667^{b}	0.22^{a}	15^{c}
c_2	10.59333^{c}	3.74^{cd}	24.81667^{b}	0.18^{b}	18.33333^{b}
e	6.573333^{e}	4.416667^{a}	30.83333^{a}	0.14^{c}	3.013333^{d}

Means, within each column, followed by the same letter (s) are not significantly different at the 0.05 probability level using Duncan's Multiple Range Test.

- *Glucose content*

All of the pretreatments caused an increase in glucose content as compared to the control sample. In all the cases, acid addition has dramatic effect on glucose release from lignocellulose materials (Tables 3 and 4). Glucose content is the highest in autoclaved- acid-impregnated samples. This finding is in agreements with Talebnia (2008) that reported dilute acid hydrolysis caused glucose release from orange peel (about 6.38 and19.86g/L in two and one step acid hydrolysis). Nikoloc *et al*., (2011) pointed that ultrasonic and microwave pretreatments effectively increased the glucose concentration obtained after liquefaction by 6.82 and 8.48% respectively as compared to the untreated control sample.

- *Lignin content*

The results of lignin content are presented in Tables 3 and 4. In all the conditions that were tested, lignin concentration reduced in relation to the initial raw material. In all pretreatments, addition of acid led to less lignin content than control, this is due to more extraction of lignin in the presence of acids (Jedvert *et al*., 2012), therefore, lower amount of lignin remained in lemon peel after this step. As can be seen in Table 3, samples that treated under microwave and ultrasonic condition also show low lignin content, that is the outcome of the ability of these processes to extract lignin (Lai *et al*., 2012). The highest lignin content belonged to the control samples (3.5766 and 4.4166% solvent treated and untreated, respectively). It has been shown that insoluble lignin decreased in all the pretreated samples as compared to the control (Tables 3 and 4). This finding is fitted with Lai *et al*. (2012) that claimed pretreatments caused reduction

in lignin content of oil palm trunk. However, these data indicated that in the conditions tested in the present work, samples that exposed to n-hexane extraction showed less lignin content than those that were untreated and marked differences between these two groups (Table 5). This was presumably due to lignin fractionation by solvent under hexane treatment (Wang *et al*., 2010).

- *Furfural content*

Furfural is known as a strong inhibitor compound for yeast growth. Higher concentrations of furfural resulted in less fermentability of the hydrolyzates. The presence of furfural in the cultivation medium could result in reduced biomass yield, decrease in specific growth rate and ethanol productivity (Taherzadeh *et al*., 1999) that is the consequence of inhabitation of furfural on different enzymes such as hexokinase, phosphofructokinase, triose phosphate dehydrogenase, aldolase and alcohol dehydrogenase (Banerjee *et al*., 1981). At the present work, the furfural concentration was measured in the range of 0.14-0.22 g/l that is close to the furfural content that was reported by Talebnia (2008). No significant difference was observed in furfural content between the solvent treated and untreated groups (Table5). Bioethanol production was not affected by furfural concentration. In this respect, it might be stated that furfural in the samples are lower to alleviate bioethanol production. Banerjee *et al*. (1981) reported that furfural caused inhabitation on glycolytic enzymes.

- *Cell viability*

Viability of *Sacharomyces cerevisiae* at different examined conditions is presented in Tables 3 and 4. Obtained data showed that utilization of hexane dramatically caused higher viability and this could be attributed to the removal of limonene (essential oil of lemon). Limonene can hinder the yeast growth (Talebnia, 2008). In each pretreatment, autoclaving indicates higher viability (40.33% and 22% for hexane treated and untreated samples, respectively) that is likely due to the higher glucose yield and higher limonene removal under the mentioned circumstances. Other pretreatments also show more viability than the control that is probably due to more limonene removal. This finding is in agreement with Talebnia (2008) that showed negative effect of limonene on yeast growth.

- *Bioethanol production*

Figure 1 shows the bioethanol content at different tested conditions.

Bioethanol content does not reach more than 5.68% that is 76% of the theoretical bioethanol content. This can be explained partly to the presence of inhibitors such as furfural, limonene and other possible inhibitors that prevent the complete assimilation of glucose by yeast and complete conversion of glucose to bioethanol (Talebnia, 2008; Azhar *et al*., 1981). Microwave treatment also showed more positive effect on bioethanol content than ultrasonic. This effect has been distinguished by other researches. Boluda-Aguilar & López-Gómez. (2013) reported 60 L/1000 kg ethanol production from fresh lemon peel biomass. Among all the tested samples, solvent extraction followed with autoclave presented the highest bioethanol efficiency (Figure 1).

- *The effects of solvent extraction*

Essential oil extracted from orange peel contains approximately 90% D-limonene which is known as anti-microbial agent (Martin *et al*., 2010) and its negative effect on yeast growth is one important obstacle in bioethanol production from lemon peel. The effect of solvent extraction on glucose, lignin, furfural, viability and bioethanol is summarized in Table 5. Observation has been made that significant differences were

observed between the two tested groups ($p \leq 0.01$) in terms of lignin, cell viability and ethanol contents but glucose and furfural content didn't show a significant difference. It, therefore, indicates that solvent extraction does not have influence on glucose release from lignocellulose material of lemon peel but causes the enhancement of yeast viability and bioethanol production. In addition, solvent extracted samples contain less lignin contents than untreated ones that might be due to dissolution, leaching and fractionation of lignin (Wang *et al*., 2010).

The effect of solvent extraction is indicated by other researches such as Nguyen (2012) who reported that n-hexane was the most interesting solvent for limonene extraction from orange peel and orange peel treated by n-hexane gave higher methane production than the peels treated by other solvents.

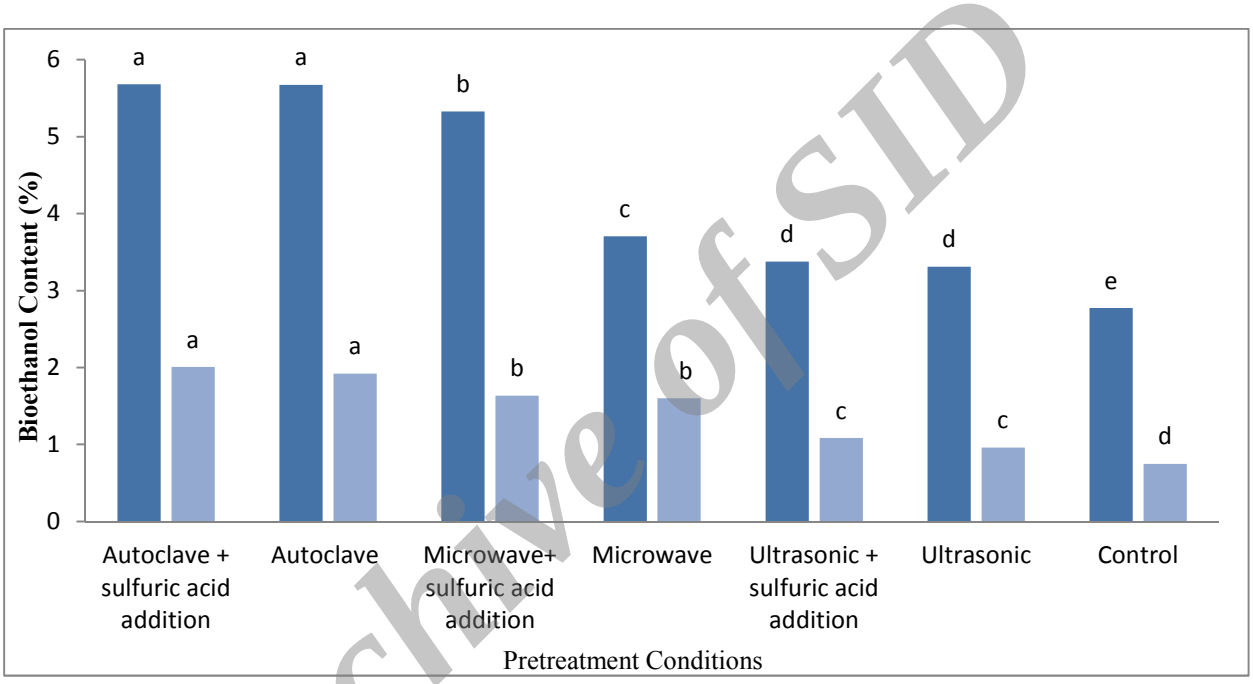

Fig. 1. Bioethanol content of lemon peel after different pretreatments (%.). Dark bars, solvent extracted samples., gray bars, no solvent extracted samples;. Common letters signify no significant differences according to Duncan's Multiple Range Test.

Table 5. Orthogonal contrast of the effect of solvent extraction on glucose, soluble lignin, insoluble lignin, viability and ethanol content of lemon peel

		MS					
SOV	**DF**	**Glucose(g/l)**	**Soluble lignin(%)**	**Insoluble lignin(%)**	**Viability(%)**	**Bioethanol(%)**	**Furfural(g/l)**
Treat	13	16.47767**	1.3419**	72.2457**	363.326**	9.01479**	0.00226**
Contrast	1	0.05794^{ns}	11.2323**	193.8441**	2030.0952**	84.80243810**	0.00005^{ns}
Error	28	0.1052**	0.0009	5.7257	0.4524	0.005	0.00003**
%CV	-	3.06840	2.81	10.70	2.85	2.59	3.11

* and **: Signficant at 0.05% and 0.01 probability levels, respectively.
ns: non- significant

Conclusion

Autoclave pretreatment process could be considered as an alternative process in bioethanol production from lemon peel. This is because mild autoclave heating is relatively simple, easy and rapid process. By using this approach, treated lemon peel would yield more bioethanol in later process. Microwave and ultrasonic pretreatments also cause the enhancement of bioethanol efficiency, but to a lesser extent than autoclave process. In addition, removal of limonene by solvent extraction causes an increase in the fermentation step and resulted in higher bioethanol production.

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References

Azhar, A. F., Bery, M. K., Colcord, A. R., Roberts, R. S. & Corbitt, G. V. (1981). Factors affecting alcohol fermentation of wood acid hydrolyzate. Biotechnol. Bioeng. Symp, 11, 293-300.

Banerjee, N., Bhatnagar, R. & Viswanathan, L. (1981). Inhibition of glycolysis by furfural in Saccharomyces cerevisiae. Eur. J. Appl. Microbiol. Biotechnol., 11, 224-228.

Beszedes, S., Tachon, A., Lemmer Ábel, M., Szab, G. & Hodur, C. (2012). Bio fuel from Cellulose by Microwave Irradation. International Journal of Engineering. Available at: http://annals.fih.upt.ro/pdf-full/2012/ANNALS-2012-2-04.pdf.

Boluda-Aguilar, M. & López-Gómez, A. (2013). Production of bioethanol by fermentation of lemon (*Citrus limon L.*) peel wastes pretreated with steam explosion. Industrial Crops and Products, 41, 188-197.

Gerchman, Y., Schnitzer, A., Gal, R., Mirsky, N. & Chinkov, N. (2012). A simple rapid gas-chromatography flame-ionizationdetector (GC-FID) method for the determination of ethanol from fermentation processes. African Journal of Biotechnology, 11(15), 3612-3616.

Guang, P., Xue, Y., Guang, P. & Fen, X. (2010). Rapid method for determination of furfural and 5-hydroxymethyl furfural in pre-extraction stream of biomass using UV spectroscopy. Available at: http://ww.ncbi.nlm.nih. goa/ pubmed/20302124.

Jedvert, K., Saltberg, A., Lindstrom, M. E. & Theliander, H. (2012). Mild Steam Explosion and Chemical Pre- treatment of Norway Spruce. BioResources, 7(2), 2051-2074.

Lai, L. W., Idris, A. & MohdYusof, N. M. (2012). Lignin extraction from oil palm trunk by microwave-alkali technique. Regional Annual Fundamental Science Symposium 2012 (RAFSS 2012).

Martin, M. A., Siles, J. A., Chica A. F. & Martin, A. (2010). Biomethanization of orange peel waste. Bioresource Technology, 101, 8993-8999.

Mesa, L., Gonzalez, E., Cara, C., Gonzalez, M., Castro, E. & Mussatto, S. I. (2011). The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse. Chemical Engineering Journal, 168, 1157–1162.

Nikoloc, S., Mojovic, L., Rakin, M., Pejin, D. & Pejin, J. (2011). Utilization of microwave and ultrasonic pretreatments in the production of bioethanol from corn. Clean Teehn Environ Policy, 13, 587-594.

Nguyen, H. (2012). Biogas Production from Solvent Pretreated Orange Peel. Master of Science Thesis in the Master's Program MPISC. Available at: http://publications.lib.chalmers.se/ records/fulltext/157557.pdf.

Santi, G. (2009). Second generation bioethanol production from orange peel waste. Available at:http://dspace.unitus.it/bitstream /2067/2566/1/gsanti_tesid.pdf.

Talebnia, F. (2008). Ethanol production from cellulosic biomass by encapsulated *Saccharomyces cerevisiae*. Available at: http://bada.hb.se/bitstream/2320/3525/2/Thesis% 20-%20Final- talebnia. pdf.

Taherzadeh, M. J., Gustafsson, L., Niklasson, C. & Liden, G. (1999a). Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering, 87, 169-174.

Wang, K., Xu, F. & Sun, R. (2010). Molecular characteristics of kraft-AQ pulping

lignin fractionated by Sequential organic solvent extraction. International Journal of Molecular Sciences. 11, 2988-3001.