# Process Optimization for Citrus Waste Biorefinery *via* Simultaneous Pectin Extraction and Pretreatment

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In a novel valorization approach for simultaneous pectin extraction and pretreatment (SPEP) of citrus waste (CW) by dilute nitric acid and ethanol, almost all of the CW was converted to bio-derived chemicals in a single-step process at a low/moderate temperature. The SPEP was performed at different temperatures (70 °C and 80 °C), pH (1.8, 3.0, and 4.3), and extraction times (2 h and 3 h) with a full factorial design. The maximum pectin yield of 45.5% was obtained at pH 1.8, 80 °C, and 2 h. The pectin yields at pH 1.8 were much higher than at pH 4.3 and 3. Also, the degree of methyl-esterification at pH 1.8 was higher than 50%, whereas at the higher pH, low methoxyl pectins were extracted. The treated CW obtained after the SPEP, free from limonene, was subjected to separate cellulolytic enzymatic hydrolysis and ethanolic fermentation. The glucose yields in the enzymatic hydrolysates were higher for the CW treated at pH 1.8. The fermentation of the enzymatic hydrolysates by *Mucor indicus* resulted in fungal biomass yields in the range of 355 to 687 mg per g of consumed sugars. The optimum conditions for obtaining the maximum SPEP yield (glucose + pectin (g) / raw material (g))*100) were pH 1.8, 80 °C, and 2 h, which resulted in a yield of 58.7% (g/g CW).

*Keywords: Citrus waste; Dilute-acid; Enzymatic hydrolysis; Ethanol; Fermentation; Full factorial design; Fungal biomass; Limonene; Mucor indicus; Pectin; Pretreatment*

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## INTRODUCTION

The annual world production of oranges in 2014 and 2015 was estimated to be 48.8 million metric tons (USDA 2015). Of the annual amount produced, 40% to 60% is used in the juice production industry (Wikandari *et al.* 2015). Approximately a quarter of the oranges produced in the European Union is consumed as processed fruit (USDA 2015), and 50% to 60% ends up as organic waste, which includes the seeds, peels, and segment membranes (USDA 2015). The production of such a large quantity of waste has created a lot of environmental concerns that deal with waste exploitation and management. This underutilized bio-waste residue has been subjected to different valorization strategies for individual products, which have been only marginally profitable. Incineration is a possible solution for the waste, but a drying process is necessary, which is a high-energy demand process. Other than waste reduction strategies, several studies have recently focused on finding ways to obtain value-added biological products, *e.g.*, pectin and biofuels, using the waste as a renewable source of pectin-rich and low lignin content cellulosic biomass (Lohrasbi *et al.* 2010; Pourbafrani *et al.* 2010; Rivas-Cantu *et al.* 2013; Boukroufa *et al.* 2015; Casas-Orozco *et al.* 2015).

Pectin is a heterogeneous polysaccharide with a dominantly esterified methyl group or de-esterified homogalacturonan (HG) backbone. Located in the cell wall and middle lamella of plants, pectin comprises the major component of the primary walls of non-woody plant cells. After cellulose, pectin acts as a major plant load-bearing component and plays a "glue" role to hold the cell wall components together (Himmel *et al.* 2007; Xiao and Anderson 2013). Therefore, pectin extraction from pectic substances could open up the recalcitrant cellulosic structure and make it amenable for enzymatic hydrolysis, and subsequently biofuel production.

Orange peel, a by-product of fruit-juice production, has been widely used as a raw material for pectin production by different extraction methods (Guo *et al.* 2012; Galant *et al.* 2014; Wang *et al.* 2014). Commercial citrus pectins are mainly extracted by hot dilute mineral acid (HCl, $HNO_3$, or $H_2SO_4$) extraction and recovered from the acid with an anti-solvent, *e.g.*, ethanol (Guo *et al.* 2012). The temperature (50 °C to 90 °C), pH (1 to 6), and time of extraction are the main parameters affecting the quantity and quality of the obtained pectic polysaccharides (Alba *et al.* 2015).

As reviewed by López *et al.* (2010), the majority of the recent studies on citrus waste (CW) valorization have been focused on the production of a single component, *i.e.*, pectin, biofuel, or essential oil (Rivas-Cantu *et al.* 2013). The depectinized peel in pectin extraction processes, for example, is considered a waste in current commercial plants and is mainly used as a low value cattle feed. The alternative potential application for the residue is the sustainable production of chemicals and biopolymers by taking an integrated biorefinery approach. A comprehensive example of integrated valorization of CW, with respect to all of the chemicals produced, was developed by Pourbafrani *et al.* (2010). The focus of that study was maximizing the sugar production by dilute sulfuric acid at a high temperature/pressure to obtain pectin and limonene, according to the optimum conditions of sugar yield. In another study, Boukroufa *et al.* (2015) developed an integrated green process for CW biorefining. However, carbohydrate polymers, a major class of orange peel constituents, were not considered in the biorefinery. Fidalgo *et al.* (2016) also used an eco-friendly and efficient process *via* microwave heating for extraction of pectin and essential oils from some citrus peels in laboratory and semi-industrial scales, while, the peels, after the extraction process, produced considerable amounts of organic waste.

In this study, a novel process called simultaneous pectin extraction and pretreatment (SPEP), which was aimed at enhancing both pectin extraction and sugar production from CW with dilute nitric acid at a low/moderate temperature, was investigated. The SPEP was aimed to enhance both pectin and enzymatic hydrolysis yields of CW compared with the recent studies. The cellulose and hemicellulose were enzymatically hydrolyzed and fermented to ethanol by a filamentous fungus, *Mucor indicus*. Finally, an integrated process optimization with a full factorial experimental design for temperature, time, and acid concentration for the SPEP parameters was performed.

## EXPERIMENTAL

### Raw Materials and Enzymes

The CW, a mixture of orange and grapefruit peels, seeds, and leaf residues after juice extraction, was obtained from Brämhults Juice AB (Borås, Sweden) and stored frozen

at -20 °C until use. An exhaustive extraction process with water in a Soxhlet apparatus for 12 h (4 cycles/h) was used to extract CW free sugars. The water soluble extracts were diluted 50 times, filtered through a 0.2 µm syringe filter, and analyzed for sucrose, glucose, and fructose concentration by a Megazyme K-SUFRG assay kit (Megazyme, Bray, Ireland). The extractive-free biomass was lyophilized at -50 °C, ground, and sieved to obtain particles with a size of less than 0.85 mm (20 mesh) and greater than 0.18 mm (80 mesh). The structural carbohydrate contents were determined using the standard method provided by the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (Sluiter *et al.* 2012). Briefly, the substrates were subjected to one step concentrated (72%) sulfuric acid (90 min, 30 °C), which was followed by one step dilute acid (120 °C, 60 min) to cleave all of the carbohydrate polymers to monomeric sugars. The remaining solids were considered lignin and were analyzed gravimetrically. The ash content was measured according to Sluiter *et al.* (2008). The remaining inorganic materials after burning the substrate at 575 °C were considered ash (Sluiter *et al.* 2008).

A commercial cellulase enzyme, Cellic® CTec2 (Novozymes A/S, Bagsvaerd, Denmark), was used for the hydrolysis of glucan. The activity of cellulase was measured according to the standard method LAP-005 (Adney and Baker 2008), which was 122 filter paper units (FPU) per mL of the crude enzyme. The β-glucosidase activity of the Cellic® CTec2 was 147 IU/mL. One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 µmol of p-nitrophenol (p-NP) from p-nitrophenyl-b-Dglucopyranoside (p-NPG) per min (Ximenes *et al.* 1996). The protein content of the enzyme was 87 ± 2 mg/g crude enzyme, which was quantified by the Bradford protein assay (Bradford 1976) with bovine serum albumin (BSA), and was used as a standard.

## Simultaneous Pectin Extraction and Pretreatment (SPEP) of CW

A SPEP process was performed according to Fig. 1. The process consisted of pectin extraction by hot dilute nitric acid and pectin precipitation by ethanol. Ethanol (95%) was first used as an organic solvent for leaching limonene from the depectinized substrate (Gironi *et al.* 1995), and then as an anti-solvent for the precipitation of pectin from the acid solution. The treated CW, which was depectinized and limonene-free substrates, was subjected to separate enzymatic hydrolysis and fermentation after soaking in distilled water overnight to eliminate the remaining ethanol. Any remaining impurities in the precipitated pectin were removed by washing with absolute ethanol (Fig. 1). The obtained pectin was dried overnight at ambient temperature under a laminar flow fume hood, and the pectin yields were analyzed gravimetrically.

A full factorial experimental design, 3 * 2 * 2 for pH, time, and temperature, was applied for the pretreatment and pectin extraction step. Careful processing conditions in regards to the temperature, pH, and time were applied during the extraction in order to prevent pectin degradation. Therefore, two levels of temperature (70 °C and 80 °C) and time (2 h and 3 h), and three levels of pH (1.8, 3.0, and 4.3) were established. The factor levels were selected based on previous studies (Galant *et al.* 2014). The pH of the CW and water mixture was 4.3. The solution pH was adjusted with nitric acid.

## Separate Enzymatic Hydrolysis and Fermentation of the Treated CW

The enzymatic hydrolysis of the treated CW was performed in 500 mL bottles at 1% initial solid loading. The samples were mixed with sodium citrate buffer (50 mM, pH 4.8) and autoclaved at 121 °C for 20 min. After cooling to room temperature, the hydrolysis

was conducted by adding 4 FPU/g-substrate of Cellic® CTec2 and incubating in a water bath shaker (OLS200, Grant Instruments, Ltd., Cambridge, UK) at 50 °C and 120 rpm for 72 h. One mL samples of the enzymatic hydrolysates was taken every 24 h, boiled briefly to deactivate the enzyme, and then stored at -20 °C for further analysis (Satari Baboukani *et al.* 2012).

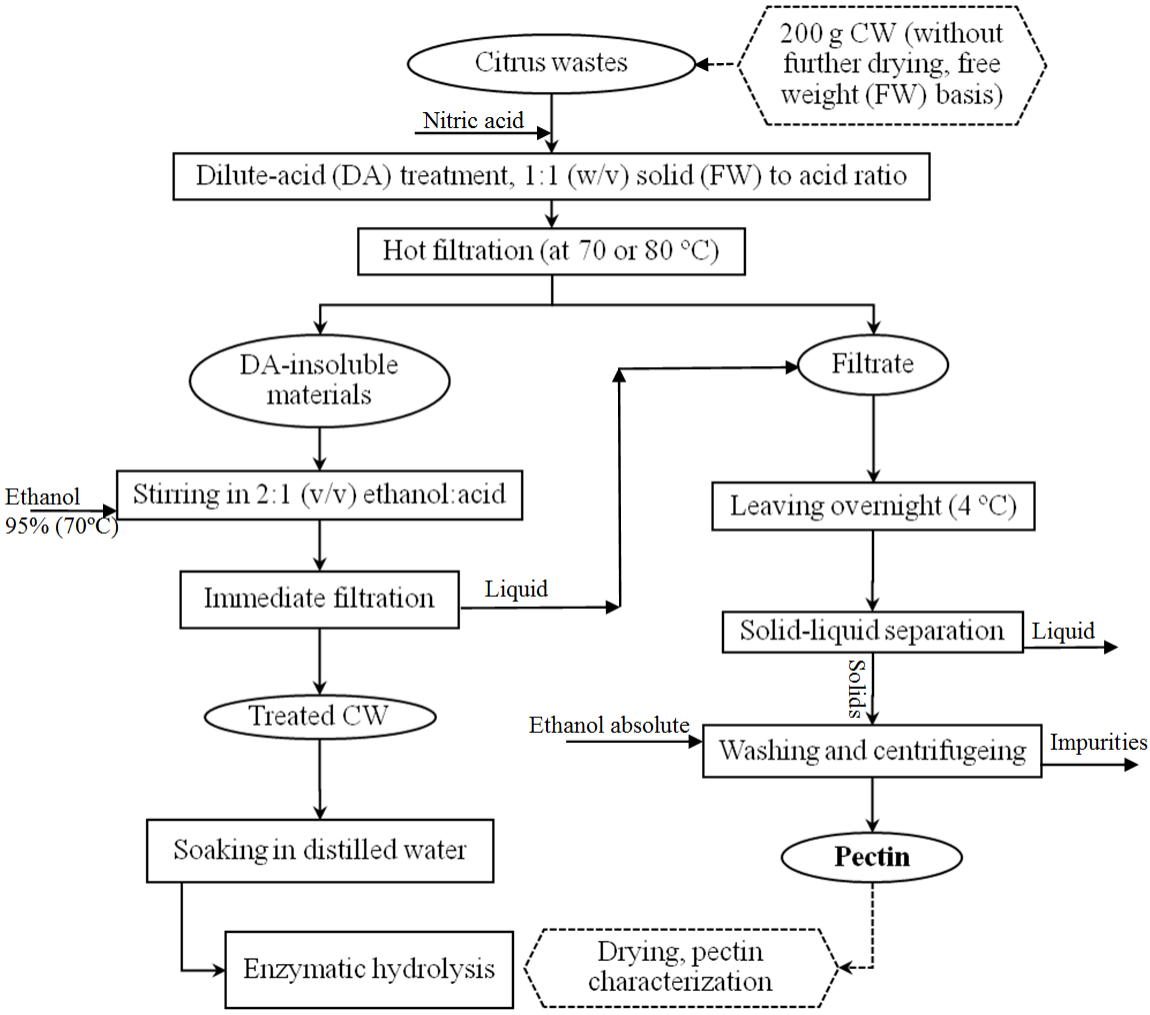


**Fig. 1.** Simultaneous pectin extraction and pretreatment (SPEP) process for the CW. The conditions applied for dilute acid treatment were pH 1.8, 3.0, and 4.3, at 70 and 80 ºC for 2 and 3 h using a full factorial experimental design.

After the hydrolysis, the enzymatic hydrolysates were aseptically transferred to 250 mL baffled Erlenmeyer flasks with 100 mL working volume, sealed with cotton plug stoppers, and supplemented with nutrients and salts to a final concentration of 5 g/L yeast extract, 7.5 g/L $(NH_4)_2SO_4$, 3.5 g/L $KH_2PO_4$, 0.75 g/L $MgSO_4 \cdot 7H_2O$, and 1.0 g/L $CaCl_2 \cdot 2H_2O$. The zygomycetes fungus *M. indicus* CCUG 22424 was obtained from Culture Collection University of Gothenburg, Sweden, and cultivated on agar plates, which contained 40 g/L glucose, 10 g/L soy peptone, and 20 g/L agar (pH 5.5), at 32 °C for 5 d (Satari *et al.* 2016a, b). The fungal spores were aseptically collected by pouring 10 mL of sterile water into the petri dishes, and then the contents were transferred to the Erlenmeyer flasks (2% v/v). The fermentation was conducted at 30 °C and 120 rpm for 3 d. After

fermentation, the medium was filtered using filter paper (Whatman no. 1), and the fungal mycelium was washed and lyophilized.

## Analytical Methods

*High performance liquid chromatography (HPLC) analysis*

The concentrations of sugars and metabolites were measured by high performance liquid chromatography (HPLC; Waters-0220, Milford, MA). For glucose, cellobiose, arabinose, mannose, xylose, and galactose analysis, a lead (II)-based column (300 * 7.8mm Aminex HPX-87P, Bio-Rad, Hercules, USA) was used at 85 °C with 0.6 mL/min ultra-pure water as the mobile phase. For the metabolite analysis, *i.e.* ethanol, and galacturonic acid, a hydrogen-based column (HPX-87H, Bio-Rad) was employed at 60 °C with 0.005 M $H_2SO_4$ at a flow rate of 0.6 mL/min as eluent. Authentic mixed sugars, galacturonic acid, and ethanol, in water with known concentrations were used as HPLC standards. A refractive index (RI) detector (Waters 2414 or 410) was used for the detection of sugars and metabolites (Lennartsson *et al.* 2012).

*Attenuated total reflection-Fourier transform infrared spectroscopy (ATR–FTIR)*

The attenuated total reflection–Fourier transform infrared spectroscopy (ATR–FTIR, Impact 410 iS10, Nicolet Instrument Corp., Madison, WI, USA) was conducted using a Bruker Optics Vertex system (Billerica, MA, USA) with a built-in diamond-germanium ATR single reflection crystal. The samples were pressed uniformly against the diamond surface using a spring-loaded anvil, and the spectra were collected from 4500 to 700 $cm^{-1}$ with an average of 32 scans and 4 $cm^{-1}$ resolution. The data were collected using Nicolet OMNIC 4.1 (Nicolet Instrument Corp., Madison, WI, USA) software and analyzed by eFTIR® (EssentialFTIR, Operant LLC, Sarasota, FL, USA). The FTIR spectra were used to determine both the degree of methyl-esterification (DE) and the galacturonic acid content (GAC) of the extracted CW pectin.

The DE of the pectin extracted at different conditions was calculated according Eq. 1 (Chatjigakis *et al.* 1998; Kyomugasho *et al.* 2015),

$$DE = (A_{1749} / (A_{1749} + A_{1630})) * 100 \quad (1)$$

where $DE$ is the degree of methyl-esterification, and $A_{1749}$ and $A_{1630}$ are the absorbance intensities at 1749 $cm^{-1}$ and 1630 $cm^{-1}$, respectively. Esterified pectin potassium salt with a known DE from Sigma-Aldrich (Darmstadt, Germany) was used for the peak calibration. A linear regression analysis was performed to obtain a relationship between the DE calculated from the absorption ratio and the real DE amounts from pectin with a known DE.

The GAC of the extracted CW pectin was determined by the method of Pereira *et al.* (2016). A linear relationship between the GAC and total carbonyl absorption band area was established by using commercial pectins with known GACs. This relationship was then used to determine the GACs of the pectin samples. The area above the baseline of the FTIR spectra between 1840 $cm^{-1}$ and 1550 $cm^{-1}$ was the total carbonyl peak area. Esterified pectins potassium salt from Sigma-Aldrich with a known GAC was used for the calibration.

## Statistical Analysis

The statistical analysis was performed using the software package MINITAB® version 17 (Minitab Inc., State College, PA, USA). The three variables, from the 3 * 2 * 2

factorial design with two replicates, were analyzed to investigate the main effects and all possible interactions of the studied factors. The analysis of variance (ANOVA) at a p-level of 0.05 was used to determine the significance of the main factors. The responses were modeled according to the general linear model of Eq. 2,

$$y = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + \varepsilon_{ijk} \quad (2)$$

where $y$ is the model response, $\mu$ is the model mean constant value, $\alpha$, $\beta$, and $\gamma$ are the effects of time, temperature, and pH on the treatment at the $i$, $j$, and $k$ level, respectively, and $\varepsilon$ is the model error (Dean and Voss 1999). The model responses were pectin yield, DE of the obtained pectin, and glucose yield after 72 h enzymatic hydrolysis of the treated CW. Including the significant two factor interactions gave a better model than using only the main effects.

## RESULTS AND DISCUSION

CW has a chemically complex and heterogeneous structure, and a high organic content that is highly biodegradable. CW typically consists of soluble sugars (mainly fructose, glucose, and sucrose), structural polysaccharides (cellulose, hemicelluloses, and pectin), lignin-like compounds (flavonoids), and essential oils. The CW used in this study contained 79.5% water, and its composition based on the dry weight is shown in Table 1.

**Table 1.** Composition of the Raw CW Used in this Study

| Components | Content (%) |
|---|---|
| Glucose¤ | 16.45 ± 0.92 |
| Sucrose¤ | 0.87 ± 0.29 |
| Fructose¤ | 15.77 ± 0.79 |
| Water soluble extractives¤¤ | 15.96 ± 5.04 |
| Total water extractives | 49.04 ± 3.04 |
| Galacturonan | 15.30 ± 3.10 |
| Glucan | 8.82 ± 1.14 |
| Arabinan | 2.14 ± 0.54 |
| Mannan | 0.82 ± 0.23 |
| Xylan | 1.94 ± 1.01 |
| Galactan | 3.06 ± 0.94 |
| Lignin | 1.95 ± 0.15 |
| Ash¤¤¤ | 4.75 ± 0.20 |
| ¤By Soxhlet extraction determined by the kit<br>¤¤ Other than sugars<br>¤¤¤ Of extractive-free biomass | |

A considerable fraction of the CW consisted of free sugars (33% total), which included glucose, fructose, and sucrose. These sugars can be easily extracted by water and used as a carbon- and nutrient-rich medium for the cultivation of fungi (Satari *et al.* 2016a). The carbohydrate polymers were the second most abundant constituent, and accounted for over 32% of the CW dry weight (Table 1). Moreover, the CW contained 1.95% lignin-like compounds (determined by Sluiter *et al.* 2012), 4.75% ash (determined by Sluiter *et al.* 2008). A limonene content of 3.78% and 6.07% protein have been previously reported for

this type of CW (Pourbafrani *et al.* 2010).

The chemical composition of the CW indicated the high potential of this type of waste for the production of value-added derived products that can be used in pharmaceutical and nutraceutical industries, as well as in biofuel production. Furthermore, the concentration of lignin-like compounds, which are undesired components in most biomass applications, was very low in the CW compared to the lignocellulosic biomass from agricultural wastes.

## Effects of Extraction Variables on the CW Pectin Yield

Different acids have been used for pectin extraction, *e.g.* sulfuric acid, citric acid, hydrochloric acid, and nitric acid (Galant *et al.* 2014; Methacanon *et al.* 2014). Citric and nitric acid were chosen for the present study, and the preliminary experiments showed that at pH 3, the same pectin yield was achieved for these two acids. However, the enzymatic digestibility was higher for the CW treated with nitric acid extraction (data not shown). Moreover, Methacanon *et al.* (2014) showed that higher pectin yields were obtained from pomelo peel with nitric acid extraction (at pH 2 and 3) compared to the yields from hydrochloric acid extraction. Thus, the SPEP was performed using nitric acid.

The presence of fruit flavor and peel oils, mainly D-limonene, has been reported to severely inhibit the growth of ethanol and biogas-producing microorganisms (Wikandari *et al.* 2013). As a solution, limonene removal by leaching using an organic solvent, such as hexane and ethanol, was suggested as a pretreatment step to improve the biofuel production yield (Wikandari *et al.* 2015). Ethanol, which was necessary for the pectin separation from the dilute-acid solution, was first used for limonene leaching from the depectinized CW. The final concentration of ethanol for precipitation of pectin from the acid solution was calculated, *i.e.,* ~67%, which is quite near the range of optimum concentration for pectin precipitation suggested by other researchers (*i.e.,* 70 to 75% (Yapo 2009)). The data from the ternary diagram of limonene-water-ethanol (Gironi *et al.* 1995) confirmed that extracted limonene is soluble after adding the limonene-rich ethanol solution to the pectin solution, since the specific ratio was mixed. Also, the CW extractable free sugars are also soluble in the liquid phase, and only the pectin can be precipitated. Another advantage of cellulosic ethanol production from CW *via* a biorefinery concept is that, unlike typical lignocelluloses, CW has a low lignin content. Although the hydrolysis of the CW was not too problematic in this study, the established pretreatment methods for the improvement of ethanol production from lignocelluloses may not be suitable for CW.

Table 2 shows the pectin yields based on the extractive-free CW, extracted at different conditions. The extraction at pH 1.8 resulted in considerably higher pectin yields compared to the yields obtained at higher pHs. Significantly higher pectin yields were also reported from *Cucumis melo* Inodorus at pH 1.0 in comparison to the yields at higher pHs (Denman and Morris 2015). The ash content of the treated CW, however, was not considerably dependent on the SPEP parameters and remained almost unchanged in the treated CW (Table 2).

The pectin yields were modeled as a function of temperature, pH, and extraction time, according to the general linear model. ANOVA was used to examine the statistical significance of the model terms (Table 3). The main effects of temperature, time, and pH, and the time*pH interaction were significant (p-value < 0.05). Therefore, the other interactions were omitted from the model. The model coefficients for the significant effects are summarized in Table 4. The time*pH interaction was significant only in the case of pH

1.8 (p-value = 0.001), and therefore, it was mentioned as a model product term in Table 4. The positive effect on the pectin yield from lowering the pH to 1.8 was highly significant (coefficient 19 and p-value = 0.000). Considerably higher pectin yields were also reported from pomelo peel when the pH was decreased from 3 to 2 for both hydrochloric and nitric acids (Methacanon *et al.* 2014).

**Table 2.** Experimental Design for Pectin Extraction, Pectin Yields, Ash Content of the Treated CW, and Degree of Methyl-Esterification

| Run number | Temperature (°C) | Time (h) | pH | Pectin yield from extractive-free CW (%) | GAC¤ (%) | Degree of methyl-esterification (%) | Ash content¤¤ |
|---|---|---|---|---|---|---|---|
| 1 | 70(-1) | 3(+1) | 1.8(-1) | 36.1 ± 1.5 | ≥ 80 | 60.41 ± 6.33 | 4.5 ± 0.5 |
| 2 | 70(-1) | 2(-1) | 1.8(-1) | 43.0 ± 0.6 | ≥ 80 | 69.67 ± 1.53 | 4.2 ± 0.3 |
| 3 | 80(+1) | 3(+1) | 1.8(-1) | 41.4 ± 3.3 | 70 | 64.93 ± 0.10 | 4.2 ± 0.4 |
| 4 | 80(+1) | 2(-1) | 1.8(-1) | 45.5 ± 3.5 | 72 | 68.09 ± 0.46 | 4.3 ± 0.3 |
| 5 | 70(-1) | 3(+1) | 4.3(+1) | 11.3 ± 0.1 | ≥ 80 | 34.18 ± 1.80 | 5.3 ± 0.5 |
| 6 | 70(-1) | 2(-1) | 4.3(+1) | 10.9 ± 0.6 | ≥ 80 | 35.74 ± 1.71 | 4.7 ± 0.2 |
| 7 | 80(+1) | 3(+1) | 4.3(+1) | 12.9 ± 1.8 | 77 | 38.42 ± 0.50 | 4.1 ± 0.3 |
| 8 | 80(+1) | 2(-1) | 4.3(+1) | 13.1 ± 1.2 | 72 | 47.07 ± 1.23 | 4.3 ± 0.3 |
| 9 | 70(-1) | 3(+1) | 3.0(0) | 11.2 ± 0.2 | 73 | 40.79 ± 2.89 | 4.2 ± 0.1 |
| 10 | 70(-1) | 2(-1) | 3.0(0) | 10.9 ± 1.2 | ≥ 80 | 44.21 ± 7.66 | 4.8 ± 0.2 |
| 11 | 80(+1) | 3(+1) | 3.0(0) | 12.8 ± 0.0 | 68 | 40.77 ± 0.21 | 4.9 ± 0.2 |
| 12 | 80(+1) | 2(-1) | 3.0(0) | 12.8 ± 0.3 | 67 | 40.46 ± 0.02 | 4.7 ± 0.1 |

¤Galacturonic acid content
¤¤Ash content (percent of dry weight) of the treated CW
The actual and coded values corresponded to low level (-1), middle level (0), and high level (+1). The results were the averages of two replicates ± standard deviation (SD).

Based on the statistical analysis, increasing the extraction time from 2 h to 3 h resulted in a significantly negative effect on the pectin yield. However, it was much less than the pH effect. The same pattern has been reported and proposed related to pectin hydrolysis, which can happen with longer extraction times (Denman and Morris 2015). The effect of the temperature on the pectin yield was less than that of the pH. However, a positive effect was observed when the temperature was increased from 70 °C to 80 °C. The highest predicted pectin yield of 46.0 ± 2.2% was obtained from the extraction at 80 °C, pH 1.8, and 2 h, which was comparable with the experimental data, *i.e.* 45.5 ± 3.5%.

**Table 3.** Statistical Parameters from ANOVA Table for the Pectin Yield

| Source | DF | Seq. SS | Adj. SS | Adj. MS | F-Value | p-value * |
|---|---|---|---|---|---|---|
| Temperature | 1 | 37.67 | 37.67 | 37.67 | 14.73 | 0.002 ** |
| Time | 1 | 18.64 | 18.64 | 18.64 | 7.29 | 0.017 * |
| pH | 2 | 4648.99 | 4648.99 | 2324.49 | 908.98 | < 0.001 *** |
| temp*time | 1 | 0.52 | 0.52 | 0.52 | 0.20 | 0.660 |
| temp*pH | 2 | 5.41 | 5.41 | 2.70 | 1.06 | 0.373 |
| time*pH | 2 | 42.16 | 42.16 | 21.08 | 8.24 | 0.004 ** |
| Error | 14 | 35.80 | 35.80 | 2.56 | | |
| Lack-of-Fit | 2 | 3.48 | 3.48 | 1.74 | 0.65 | 0.541 |
| Pure Error | 12 | 32.32 | 32.32 | 2.69 | | |
| Total | 23 | 4789.19 | | | | |

* The number of superscript stars represent the level of importance of p-values.

## Effect of Pectin Extraction Process on the Cellulosic Structure

The biorefinery process, which was applied for the extraction of pectin and pretreatment of the CW, involved some physico-chemical changes to improve the enzymatic accessibility of the cellulosic fraction. Pectin is mainly present in the secondary cell wall of dicots and, to a lesser extent, in the primary cell wall and middle lamella (Cheng *et al.* 2015). It acts like a glue to crosslink cell components together and form a hydrated gel that can restrict the access of enzymes to the substrate (Himmel *et al.* 2007). After pectin extraction, the recalcitrance network structure of the low lignin content cellulosic biomass was reorganized, and therefore, the substrate became susceptible to enzymatic attack. Moreover, the presence of pectin can increase the viscosity of the substrate slurry and limit the mass transfer during hydrolysis and fermentation.

**Table 4.** Summary of the Model Coefficients and Statistical Values for the Pectin Yield

| Term | Amount | Coef. | SE Coef. | 95% CI | T-value | P-value |
|---|---|---|---|---|---|---|
| Constant | - | 21.828 | 0.326 | ( 21.127; 22.528) | 66.87 | 0.000 |
| Temperature | 70 | -1.253 | 0.326 | ( -1.953; -0.553) | -3.84 | 0.002 |
| | 80 | 1.253 | 0.326 | ( 0.553; 1.953) | 3.84 | 0.002 |
| Time | 2 | 0.881 | 0.326 | ( 0.181; 1.581) | 2.70 | 0.017 |
| | 3 | -0.881 | 0.326 | ( -1.581; -0.181) | -2.70 | 0.017 |
| pH | 1.8 | 19.683 | 0.462 | ( 18.693; 20.673) | 42.64 | 0.000 |
| | 3.0 | -9.903 | 0.462 | (-10.893; -8.913) | -21.45 | 0.000 |
| | 4.3 | -9.780 | 0.462 | (-10.770; -8.790) | -21.45 | 0.000 |
| Time*pH | 2, 1.8 | 1.874 | 0.462 | ( 0.884; 2.865) | 4.06 | 0.001 |
| | 2, 3.0 | -0.941 | 0.462 | ( -1.931; 0.049) | -2.04 | 0.061 |
| | 2, 4.3 | -0.933 | 0.462 | ( -1.923; 0.057) | -2.02 | 0.063 |
| | 3, 1.8 | -1.874 | 0.462 | ( -2.865; -0.884) | -4.06 | 0.001 |
| | 3, 3.0 | 0.941 | 0.462 | ( -0.049; 1.931) | 2.04 | 0.061 |
| | 3, 4.3 | 0.933 | 0.462 | ( -0.057; 1.923) | 2.02 | 0.063 |

## FTIR Analysis of the Extracted CW Pectin

The most important characteristic of pectin is the DE. Commercial pectin is classified as either low DE (< 50%) or high DE (> 50%). The most important difference in these pectins is their gelation behavior. Gelation can occur in acidic conditions and high sugar concentrations through hydrogen bonding and hydrophobic interactions for high DE or according to the egg-box model in the presence of divalent ions for low DE (Gamonpilas *et al.* 2015).

The FTIR spectra were collected from the extracted pectin and used for the determination of DE by measuring the absorption intensity bands at 1740 $cm^{-1}$ (related to carbonyl group (C=O)) and 1630 to 1600 $cm^{-1}$ (related to carboxylate group ($COO^-$)). The FTIR spectrum of the pectin extracted at 70 °C after 2 h and the commercial esterified potassium salt pectin (for comparison) are presented in Fig. 2. The presented FTIR spectrum (Fig. 2) can be divided into two regions. The first region, between 3500 and 1800 $cm^{-1}$, exhibited two identical peaks. One broad band was at 3500 to 3000 $cm^{-1}$ due to the inter- and intra-molecular hydrogen-bonding stretching of the galacturonic acid backbone, and the other peak, C–H absorption from the methyl ester, was at 2940 $cm^{-1}$. The second region (below 1800 $cm^{-1}$) indicated the 'fingerprint' region of the carbohydrates, and represented the constituent monosaccharides in the pectin (Wang *et al.* 2014; Alba *et al.*

2015; Kyomugasho *et al.* 2015). Specifically, the peaks at around 1044, 1072, and 1147 $cm^{-1}$, which were assigned to pyranose ring vibrations (Guo *et al.* 2014), were present for both the commercial pectin and the extracted pectin (Fig. 2). For the pectin extracted at pH 1.8, relatively higher abundances of each band were observed in this region.

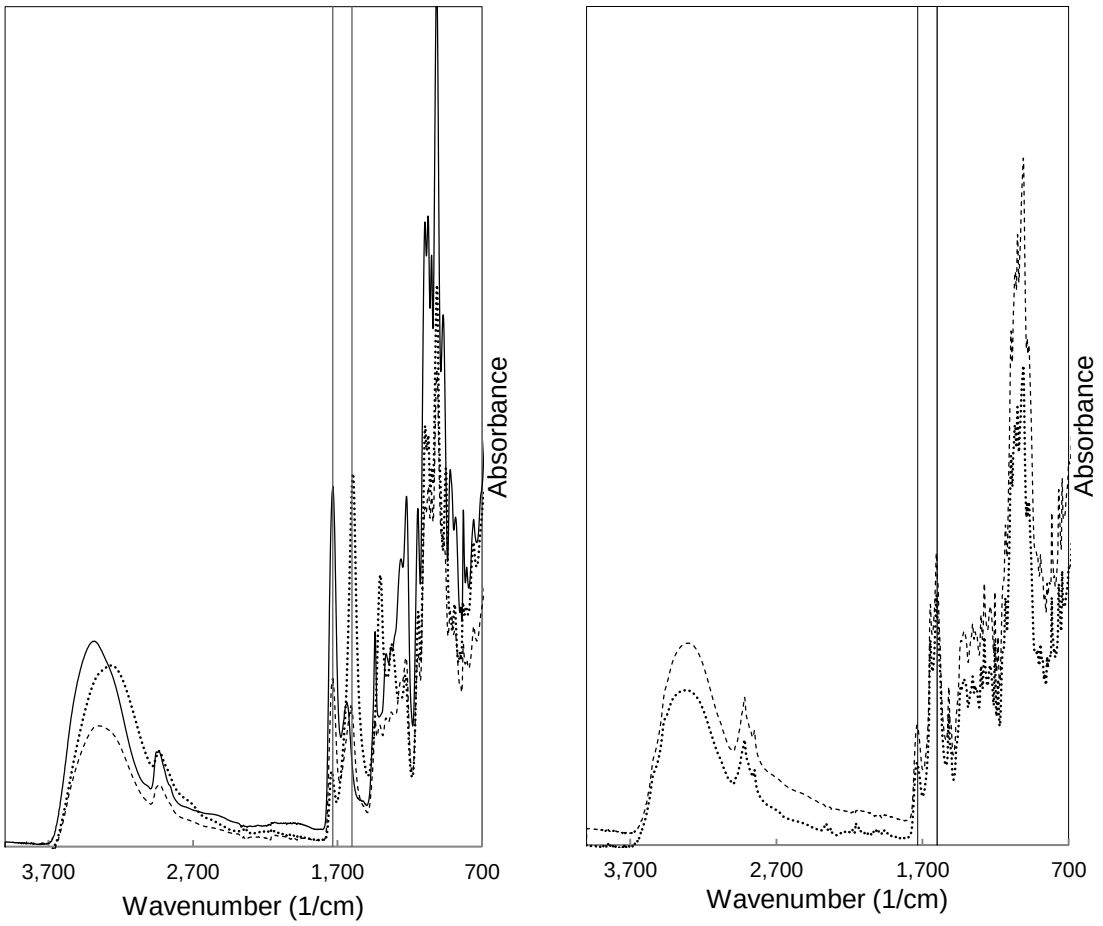


**Fig. 2.** FTIR spectra of the commercial and extracted pectin. The left three spectra were collected from the pectin with a DE of 30% (continuous line), 60% (dotted line), and 90% (dash line). The right three spectra belonged to the extracted pectin at 70 °C for 2 h at pH of 1.8 (continuous line), 3.0 (dotted line), and 4.3 (dash line). For comparison purposes, the FTIR spectra of the extracted pectins are in the same scale with those depicted for commercial pectins.

The presence of several peaks at 1650 $cm^{-1}$ and 1543 $cm^{-1}$ can affect the intensity of the characteristic peak at 1630 $cm^{-1}$, which was used for the DE calculation (Kyomugasho *et al.* 2015). This was caused by the interference of water (OH bending vibration of water at 1643 $cm^{-1}$) or protein amide bands (amide I: 1670 $cm^{-1}$ and amide II: 1588 $cm^{-1}$) in the pectin samples (Wang *et al.* 2014). In order to remove the water, the samples were kept over $P_2O_5$ before FTIR analysis. The peak deconvolution was suggested to remove the effect of the substances interference. However, this is only urgent in the case of high DE and high amounts of protein (Kyomugasho *et al.* 2015).

Table 2 shows the results of the DE calculation for all of the experiments. The statistical analysis revealed that the DE was highly dependent on the pH (p-value < 0.05), similar to the pectin yields. Moreover, the lower pH resulted in a higher DE. Although this

finding was in contrast with generally accepted trend of obtaining lower DE from the lower pH extraction as reported by other researchers (*e.g.*, Garna *et al.* (2007) and Yapo *et al.* (2009)), there are some new studies which reported a different trend. For example, Denman *et al.* (2015) and Pereira *et al.* (2016) obtained high DE pectin from *Cucumis melo* Inodorus and pomegranate peels at pH extraction of 1 and 2.4, respectively. Other than the pH, the extraction time and temp*pH interaction had significant effects on the DE (p-value < 0.05). The extraction temperature had no significant effect on the DE (p-value = 0.09). Only for pectin extracted at pH 1.8 was high DE pectin obtained. At higher pHs, low DE pectin was obtained (Table 2).

The purity of the extracted CW pectin, reported as GAC, was also determined from the FTIR spectra (Table 2). As reported by Monsoor *et al.* (2001), the linear relationship between the GAC and total carbonyl absorption band area was up to 80% GAC, and there was no significant difference in the peak areas of 80% and greater (Monsoor *et al.* 2001). Therefore, the calculation of GACs higher than 80% was not exact (Table 2). The extracted CW pectin had high GACs of at least 67%.

## Enzymatic Hydrolysis of the Treated CW

The limonene removal from the CW, apart from the pectin extraction, was beneficial to the enzymatic hydrolysis and subsequent fermentation by increasing the surface area and making the substrate more accessible to the enzymes. The limonene (boiling point = 178 °C) removal occurred at 120 °C to 160 °C, which was followed by a rapid decompression (Ciriminna *et al.* 2014). Leaching of the limonene from the CW by a solvent, such as hexane, under mild conditions was first proposed by Wikandari *et al.* (2015) as a pretreatment method for improving biogas production.

In this study, the treated CW was thoroughly washed and subjected to enzymatic hydrolysis without further drying. Figure 3 shows the glucose release profile during the enzymatic hydrolysis of the treated CW. At least 70% of the total glucose liberation occurred in the first 24 h of the hydrolysis in all of the experiments. It was more convenient to divide the behavior of the treated samples into two groups, samples extracted at pH 1.8 and samples extracted at higher pHs. The hydrolysis performance was significantly improved for the substrates treated at pH 1.8 compared to the samples at the other pH levels, where approximately similar performances with far lower hydrolysis yields were obtained. The statistical analysis revealed that other than the pH (p-value = 0.005), only the temperature (p-value = 0.03) and temp*pH interaction (p-value = 0.037) had significant effects on the glucose liberation after 72 h enzymatic hydrolysis. After excluding the insignificant terms from the model, the statistical results showed that only pH had a significant effect on the response (p-value = 0.002). The maximum yield was obtained from the sample with extraction conditions at 80 °C, pH 1.8, and 3 h (Fig. 4).

The HPLC analyses showed that glucose was the dominant sugar in the enzymatic hydrolysates, and the other sugars appeared in low concentrations. For the samples extracted at pH 1.8, maximum concentrations of 2.5%, 1%, and 3% (g/g treated CW) for arabinose, galactose, and xylose, respectively, were obtained after 72 h enzymatic hydrolysis. At the other pH levels, maximum values of 2.7%, 2.8%, and 1.5% (w/w) were detected after 72 h enzymatic hydrolysis for arabinose, cellubiose, and xylose, respectively. Moreover, mannose was not detected in the HPLC chromatograms.

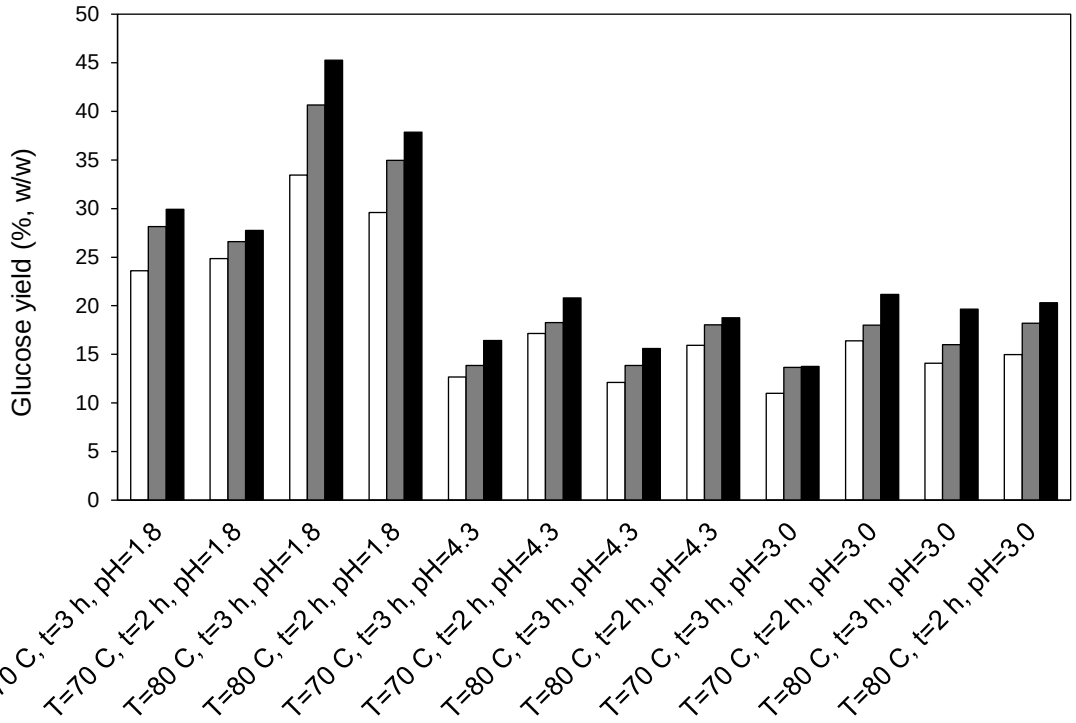


**Fig. 3.** Glucose weight percentages obtained from the treated CW (dry basis) during enzymatic hydrolysis with an enzyme loading of 4 FPU/g-substrate (all standard deviation (SDs) were less than 5%). The white, gray, and black columns represent samples after 24, 48, and 72 h hydrolysis, respectively. The descriptions in the x-axis represent the conditions applied for treating CW.

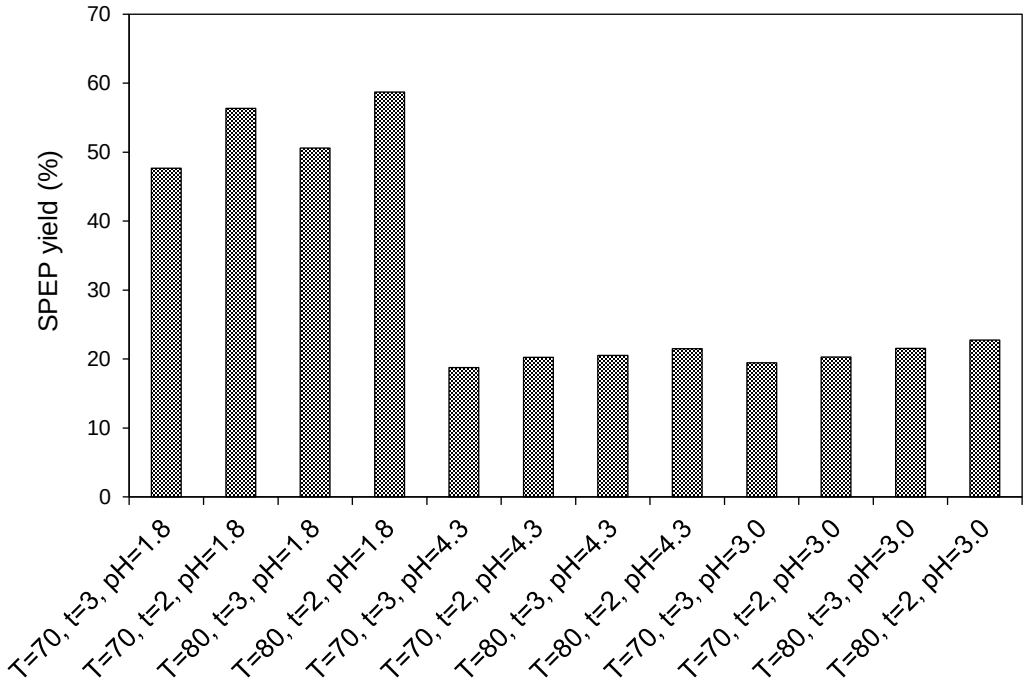


**Fig. 4.** Pectin and glucose obtained after 72 h enzymatic hydrolysis based on the dry raw materials (SDs were less than 5%). The descriptions in the x-axis represent the conditions applied for treating CW.

## A Mass Balance Approach for Optimization of the SPEP Process

The sum of the pectin and final glucose concentrations after enzymatic hydrolysis was considered for the optimization of the SPEP process. The yield of the SPEP process was defined according to the following formula,

$$Y_{SPEP} = (W_P + W_G) / W_{CW} \quad (3)$$

where $W_P$ is the weight of the extracted pectin, $W_G$ is the weight of the glucose obtained from the treated CW after 72 h, and $W_{CW}$ is the weight of the untreated CW used for the SPEP.

Considerable differences between the results of treatments at pH 1.8 and the other pH levels were observed (Fig. 4). The total glucose and pectin recovered at pH 3 and 4.3 were 18.8% and 22.7%, respectively, which were not significantly different. In contrast, the optimization approach for the first four experiments (pH 1.8) yielded almost 50% (Fig. 4).

In these cases, the temperature dependence on the yields was negligible. However, the yields were slightly higher for the extraction after 2 h compared to the yields for the extraction after 3 h. The optimal experimental conditions that were chosen corresponded to experiment numbers 2 and 4 (pH 1.8, 2 h, and 70 °C, and pH 1.8, 2 h, and 80 °C, respectively), which resulted in total yields of 56.4% and 58.7%, respectively.

## Fermentation of Enzymatic Hydrolysates for Fungal Biomass Production

After 24 h fermentation, all of the glucose was consumed by the fungus for all of the experiments. Besides the glucose, other sugars were also consumed during this period (data not shown). The authors' previous study showed that under the aeration condition with little amounts of spore inoculation (1 to $2*10^5$ spores/mL), the dominant morphology for the fungus was a filamentous-like form (Satari *et al.* 2016b). Under these conditions, the main product of fermentation was fungal biomass. The fungal biomass was enriched by the poly unsaturated fatty acids and single cell protein, which made it a nutritionally valuable food for animal feeding.

After 72 h fermentation, the fungal biomass was harvested, and the yields are presented in Table 5. The biomass yields were higher than those reported previously by Satari *et al.* (2016a) and Satari *et al.* (2016b). Ethanol was the dominant metabolite, which was detected during fermentation. The maximum ethanol concentration was produced after 24 h fermentation. Afterwards, it was either consumed by the fungi or evaporated, and was not detected after 48 to 72 h. Consumption of ethanol by the fungus as a carbon source in carbon exhaustion conditions might have been a reason for the high biomass yield obtained. However, the maximum ethanol concentration reached was below 20% of the theoretical yields (data not shown).

Two recent studies on the conversion of CW into ethanol based on the pretreatment of CW by a steam explosion process and dilute sulfuric acid hydrolysis process have been published by Lohrasbi *et al.* (2010) and Pourbafrani *et al.* (2010). Although the latter was similar to the process developed by Grohmann *et al.* (1995) and Stewart *et al.* (2013), their processes for the recovery of D-limonene have been proposed as a part of the latest development to improve the commercial potential. However, the processes are energy-intensive and deal with problems associated with working at high-pressure.

**Table 5.** *M. indicus* Fungal Biomass Production from the Enzymatic Hydrolysates of Treated CW

| SPEP conditions | | | Biomass yield¤ (mg/g sugar consumed) (SD ≤5%) |
|---|---|---|---|
| pH | *T* (°C) | Time (h) | |
| 1.8 | 70 | 3 | 497 |
| 1.8 | 70 | 2 | 584 |
| 1.8 | 80 | 3 | 355 |
| 1.8 | 80 | 2 | 454 |
| 4.3 | 70 | 3 | 677 |
| 4.3 | 70 | 2 | 534 |
| 4.3 | 80 | 3 | 687 |
| 4.3 | 80 | 2 | 498 |
| 3.0 | 70 | 3 | 597 |
| 3.0 | 70 | 2 | 440 |
| 3.0 | 80 | 3 | 664 |
| 3.0 | 80 | 2 | 630 |
| ¤ The medium was enriched with salts and nutrients, and the fungal biomass was harvested after 72 h cultivation. The SD was less than 5%. | | | |

## CONCLUSIONS

This study determined the potential of an abundant biowaste, CW, to produce value-added chemicals, especially, those that re-enter the food chain, *via* chemical and biochemical pathways. A full factorial experimental design with pH, time, and temperature as the independent variables was applied to the CW with the purpose of extracting pectin and pretreatment of the low lignin content cellulosic biomass. The most important conclusions were as follows:

1. The optimum conditions for higher pectin extraction together with glucose production were extraction at pH 1.8 and 80 °C after 2 h. At these conditions, 58.7% glucose and pectin was obtained from the CW (dry weight basis).
2. Lowering the pH to 1.8 considerably enhanced the pectin yield, DE, and enzymatic hydrolysis yield, compared with the higher applied pH.
3. Enzymatic hydrolysates fermentation by the filamentous fungus *M. indicus* yielded considerable amounts of fungal biomass, in the range of 355 to 687 mg per g of consumed sugars.

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## REFERENCES CITED

Adney, B., and Baker, J. (2008). *Measurement of Cellulase Activities* (NREL/TP-510-42628), National Renewable Energy Laboratory, Golden, CO.

Alba, K., Laws, A. P., and Kontogiorgos, V. (2015). "Isolation and characterization of acetylated LM-pectins extracted from okra pods," *Food Hydrocolloid.* 43, 726-735. DOI: 10.1016/j.foodhyd.2014.08.003

Boukroufa, M., Boutekedjiret, C., Petigny, L., Rakotomanomana, N., and Chemat, F. (2015). "Bio-refinery of orange peels waste: A new concept based on integrated green and solvent free extraction processes using ultrasound and microwave techniques to obtain essential oil, polyphenols and pectin," *Ultrason. Sonochem.* 24, 72-79. DOI: 10.1016/j.ultsonch.2014.11.015

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.* 72(1-2), 248-254. DOI: 10.1016/0003-2697(76)90527-3

Casas-Orozco, D., Villa, A. L., Bustamante, F., and González, L.-M. (2015). "Process development and simulation of pectin extraction from orange peels," *Food Bioprod. Process.* 96, 86-98. DOI: 10.1016/j.fbp.2015.06.006

Chatjigakis, A. K., Pappas, C., Proxenia, N., Kalantzi, O., Rodis, P., and Polissiou, M. (1998). "FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes," *Carbohydr. Polym.* 37(4), 395-408. DOI: 10.1016/S0144-8617(98)00057-5

Cheng, G., Zhang, X., Simmons, B., and Singh, S. (2015). "Theory, practice and prospects of X-ray and neutron scattering for lignocellulosic biomass characterization: Towards understanding biomass pretreatment," *Energy & Environmental Science* 8(2), 436-455. DOI: 10.1039/C4EE03147D

Ciriminna, R., Lomeli-Rodriguez, M., Carà, P. D., Lopez-Sanchez, J. A., and Pagliaro, M. (2014). "Limonene: A versatile chemical of the bioeconomy," *Chem. Commun.* 50(97), 15288-15296. DOI: 10.1039/C4CC06147K

Dean, A., and Voss, D. (1999). "Inferences for contrasts and treatment means," in: *Design and Analysis of Experiments*, Springer, New York, NY, pp. 67-101.

Denman, L. J., and Morris, G. A. (2015). "An experimental design approach to the chemical characterisation of pectin polysaccharides extracted from *Cucumis melo* Inodorus," *Carbohydr. Polym.* 117, 364-369. DOI: 10.1016/j.carbpol.2014.09.081

Fidalgo, A., Ciriminna, R., Carnaroglio, D., Tamburino, A., Cravotto, G., Grillo, G., Ilharco, L. M., and Pagliaro, M. (2016). "Eco-friendly extraction of pectin and essential oils from orange and lemon peels," *ACS Sustainable Chem. Eng.* 4(4), 2243-2251. DOI: 10.1021/acssuschemeng.5b01716

Galant, A. L., Luzio, G. A., Widmer, W. W., and Cameron, R. G. (2014). "Compositional and structural characterization of pectic material from frozen concentrated orange juice," *Food Hydrocolloid.* 35, 661-669. DOI: 10.1016/j.foodhyd.2013.08.012

Gamonpilas, C., Krongsin, J., Methacanon, P., and Goh, S. M. (2015). "Gelation of pomelo (*Citrus maxima*) pectin as induced by divalent ions or acidification," *Journal of Food Engineering* 152, 17-23. DOI: 10.1016/j.jfoodeng.2014.11.024

Garna, H., Mabon, N., Robert, C., Cornet, C., Nott, K., Legros, H., Wathelet, B., Paquot, M. (2007). "Effect of extraction conditions on the yield and purity of apple pomace pectin precipitated but Not washed by alcohol," *J. Food Sci.* 72(1), C001-C009. DOI:

10.1111/j.1750-3841.2006.00227.x

Gironi, F., Farias, I. G., and Lamberti, L. (1995). "Liquid-liquid equilibria for the water + ethanol + citral and water + ethanol + limonene systems at 293 K," *Journal of Chemical & Engineering Data* 40(3), 578-581. DOI:10.1021/je00019a007

Grohmann, K., Cameron, R. G., and Buslig, B. S. (1995). "Fractionation and pretreatment of orange peel by dilute acid hydrolysis," *Bioresource Technol.* 54(2), 129-141. DOI: 10.1016/0960-8524(95)00121-2

Guo, X.-M., Zhu, S.-M., Tang, Q., Yu, S. S.-j., (2014). "Characterisation of the turbid particles in the extraction of sugar beet pectins," *Food Chem.* 162, 99-103. DOI: 10.1016/j.foodchem.2014.04.045

Guo, X., Han, D., Xi, H., Rao, L., Liao, X., Hu, X., and Wu, J. (2012). "Extraction of pectin from navel orange peel assisted by ultra-high pressure, microwave or traditional heating: A comparison," *Carbohydr. Polym.* 88(2), 441-448. DOI: 10.1016/j.carbpol.2011.12.026

Himmel, M. E., Ding, S.-Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007). "Biomass recalcitrance: Engineering plants and enzymes for biofuels production," *Science* 315(5813), 804-807. DOI: 10.1126/science.1137016

Kyomugasho, C., Christiaens, S., Shpigelman, A., Van Loey, A. M., and Hendrickx, M. E. (2015). "FT-IR spectroscopy, a reliable method for routine analysis of the degree of methylesterification of pectin in different fruit- and vegetable-based matrices," *Food Chem.* 176, 82-90. DOI: 10.1016/j.foodchem.2014.12.033

Lennartsson, P. R., Ylitervo, P., Larsson, C., Edebo, L., and Taherzadeh, M. J. (2012). "Growth tolerance of Zygomycetes *Mucor indicus* in orange peel hydrolysate without detoxification," *Process Biochem.* 47(5), 836-842. DOI: 10.1016/j.procbio.2012.02.019

Lohrasbi, M., Pourbafrani, M., Niklasson, C., and Taherzadeh, M. J. (2010). "Process design and economic analysis of a citrus waste biorefinery with biofuels and limonene as products," *Bioresource Technol.* 101(19), 7382-7388. DOI: 10.1016/j.biortech.2010.04.078

López, J. A. S., Li, Q., and Thompson, I. P. (2010). "Biorefinery of waste orange peel," *Crit. Rev. Biotechnol.* 30(1), 63-69. DOI: 10.3109/07388550903425201

Methacanon, P., Krongsin, J., and Gamonpilas, C. (2014). "Pomelo (*Citrus maxima*) pectin: Effects of extraction parameters and its properties," *Food Hydrocolloid.* 35, 383-391. DOI: 10.1016/j.foodhyd.2013.06.018

Monsoor, M. A., Kalapathy, U., and Proctor, A. (2001). "Determination of polygalacturonic acid content in pectin extracts by diffuse reflectance Fourier transform infrared spectroscopy," *Food Chem.* 74(2), 233-238. DOI: 10.1016/S0308-8146(01)00100-5

Pereira, P. H. F., Oliveira, T. Í. S., Rosa, M. F., Cavalcante, F. L., Moates, G. K., Wellner, N., Waldron, K. W., and Azeredo, H. M. C. (2016). "Pectin extraction from pomegranate peels with citric acid," *Int. J. Biol. Macromol.* 88, 373-379. DOI: 10.1016/j.ijbiomac.2016.03.074

Pourbafrani, M., Forgács, G., Horváth, I. S., Niklasson, C., and Taherzadeh, M. J. (2010). "Production of biofuels, limonene and pectin from citrus wastes," *Bioresource Technol.* 101(11), 4246-4250. DOI: 10.1016/j.biortech.2010.01.077

Rivas-Cantu, R. C., Jones, K. D., and Mills, P. L. (2013). "A citrus waste-based biorefinery as a source of renewable energy: Technical advances and analysis of

engineering challenges," *Waste Manage. Res.* 31(4), 413-420. DOI: 10.1177/0734242X13479432

Satari, B., Karimi, K., Taherzadeh, M., and Zamani, A. (2016a). "Co-production of fungal biomass derived constituents and ethanol from citrus wastes free sugars without auxiliary nutrients in airlift bioreactor," *International Journal of Molecular Science* 17(3), 302. DOI: 10.3390/ijms17030302

Satari, B., Karimi, K., and Zamani, A. (2016b). "Oil, chitosan, and ethanol production by dimorphic fungus *Mucor indicus* from different lignocelluloses," *J. Chem. Technol. Biot.* 91(6), 1835-1843. DOI: 10.1002/jctb.4776

Satari Baboukani, B., Vossoughi, M., and Alemzadeh, I. (2012). "Optimisation of dilute-acid pretreatment conditions for enhancement sugar recovery and enzymatic hydrolysis of wheat straw," *Biosystems Engineering* 111(2), 166-174. DOI: 10.1016/j.biosystemseng.2011.11.009

Sluiter, A. D., Hames, B., Ruiz, R. O., Scarlata, C. J., Sluiter, J. B., and Templeton, D. W. (2008). *Determination of Ash in Biomass* (NREL/TP-510-42622), National Renewable Energy Laboratory, Golden, CO.

Sluiter, A. D., Hames, B., Ruiz, R. O., Scarlata, C. J., Sluiter, J. B., Templeton, D. W., and Crocker, D. (2012). *Determination of Structural Carbohydrates and Lignin in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO.

Stewart, D., Widmer, W. W., Grohmann, K., and Wilkins, M. (2013). "Ethanol production from solid citrus processing waste," U.S. Patent No. 20080213849.

USDA (2015). *Citrus: World Markets and Trade*, United States Department of Agriculture, Foreign Agricultural Service, Washington, DC.

Wang, X., Chen, Q., and Lü, X. (2014). "Pectin extracted from apple pomace and citrus peel by subcritical water," *Food Hydrocolloid.* 38, 129-137. DOI: 10.1016/j.foodhyd.2013.12.003

Wikandari, R., Gudipudi, S., Pandiyan, I., Millati, R., and Taherzadeh, M. J. (2013). "Inhibitory effects of fruit flavors on methane production during anaerobic digestion," *Bioresource Technol.* 145, 188-192. DOI: 10.1016/j.biortech.2013.01.041

Wikandari, R., Nguyen, H., Millati, R., Niklasson, C., and Taherzadeh, M. J. (2015). "Improvement of biogas production from orange peel waste by leaching of limonene," *BioMed Research International* 2015, 6. DOI: 10.1155/2015/494182

Xiao, C., and Anderson, C. T. (2013). "Roles of pectin in biomass yield and processing for biofuels," *Frontiers in Plant Science* 4, 67. DOI: 10.3389/fpls.2013.00067

Ximenes, E. A., Felix, C. R., and Ulhoa, C. J. (1996). "Production of cellulases by *Aspergillus fumigatus* and characterization of one β-glucosidase," *Curr. Microbiol.* 32(3), 119-123. DOI: 10.1007/s002849900021

Yapo, B. M. (2009). "Pectin quantity, composition and physicochemical behaviour as influenced by the purification process," *Food Res. Int.* 42(8), 1197-1202. DOI: 10.1016/j.foodres.2009.06.002

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