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An investigation on citrus peel as the lignocellulosic feedstock for optimal reducing sugar synthesis with an additional scope for the production of hydrolytic enzymes from the aqueous extract waste

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ABSTRACT

In the current study, the use citrus peels as the lignocellulosic feedstock for the production of reducing sugars and hydrolytic enzymes was evaluated. Citrus peels were subjected to a two-step pre-treatment procedure to obtain two major fractions-the water soluble pectin rich fraction and the water insoluble solid fraction enriched with cellulose and hemicellulose. Three parameters (solid loading, acid concentration and time) that were found to be critically influencing the acid hydrolysis process were optimized by using Response surface methodology (RSM) - central composite design (CCD) for the improved synthesis of reducing sugars. A maximal total reducing sugar concentration of 13.34 g/L was predicted for the optimal processing conditions of solid loading = 3.87% w/w, acid concentration = 1% w/w and time = 48.4 min and at the processing temperature of 121 °C in a steam autoclave. Experimental validation studies carried out at these optimal conditions showed a slightly higher reducing sugar concentration of 13.65 ± 0.3 g/L than the predicted value. Further studies demonstrating the use of pectin-rich liquid fraction as the nutrient medium for hydrolytic enzymes production resulted in enzyme activities of 5.38 ± 0.2 IU/mL for pectinase and 1.17 ± 0.1 FPU (Filter Paper Units) for cellulase, without supplementation of any salts to the medium. These results suggest that the pre-treated citrus peels can serve as a potent feedstock for bioethanol production, while by-products resulting from pre-treatment can be employed to produce hydrolytic enzymes such as pectinases and cellulases as demonstrated through this work.

1. Introduction

Lignocellulosic feedstocks are generated as wastes from food/fruit processing industries. These wastes are rich in carbohydrate content and thus form potential source of raw materials for biofuel production. About 1.3 billion metric tons of solid and liquid fruit biomass wastes are produced every year in the form of peels, stalks, leaves, seeds, etc (Perlack et al., 2005). Although some fraction of these wastes is utilized as cattle feed and composts, major portion of these wastes are discarded directly into landfills where they decompose and promote breeding of several pathogens (Seidl and Goulart, 2016). Some major fruit biomass feedstocks used in bio-refineries includes banana peels, apple pomace, sugarcane bagasse citrus fruits, etc. Citrus peels among the fruit wastes have been extensively studied, owing to their high carbohydrate content accompanied by lower lignin content and they constitute 25–30% by mass the whole fruit (Boluda-Aguilar et al., 2010).

Citrus peel wastes are mainly composed of cellulose, hemicellulose, lignin, pectin and trace amounts of tannins, ash, etc., (Ravindran and Jaiswal, 2016). Citrus peel biomass is a rigid structure that cannot be directly processed for bioethanol production due to the presence of lignin, pectin and traces of tannins, etc. Therefore, a pre-treatment step is required to completely remove these components and liberate the carbohydrate fraction for synthesis of bioethanol. Furthermore, the pectin fraction also can be utilized for the production of hydrolytic enzymes. Upon depolymerisation, cellulose and hemicellulose liberate hexose and pentose sugars respectively (Vyas et al., 2018). Presence of lignin during hydrolysis may result in production of phenolic compounds, which is highly toxic to microorganisms (Park and Kim, 2012). Therefore, a pre-treatment step is necessary to remove all the toxic compounds and to effectively disrupt the lignocellulose matrix prior to hydrolysis. Apart from removal of lignin, an effective pre-treatment technique must produce maximum amount of carbohydrates and less inhibitory products such as furfurals (Negro et al., 2016). Biomass pre-treatment techniques can be classified into three main categories-physical, chemical and biological treatment. Physical pre-treatment includes mechanical attrition and ultrasonic treatment. Physical pre-

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treatment commendably increases the surface area and porosity for improved cellulose accessibility during enzymatic digestion. However, it is an energy intensive process and lignin cannot be removed effectively (Amin et al., 2017). Therefore, mechanical treatment in conjunction with thermal or chemical pre-treatment technique is important for the effective removal of lignin (Liu et al., 2018). Chemical treatment involves use of acids, alkalis or any organic solvent to degrade biomass. However, organic solvents are rarely preferred due to their highly volatile nature and high cost.

Acid treatment involves use of concentrated or dilute acids at high pressure and temperature to solubilize and degrade polysaccharides into fermentable sugars. Concentrated acids are seldom used since they are highly corrosive and also degrade the carbohydrates into toxic compounds (Kazi et al., 2010). Dilute acid treatment is a commonly used chemical pretreatment technique and it has shown efficient separation of carbohydrates from lignin. However, operating at extreme conditions promote formation of inhibitory products such as furfural and acetyl groups that are toxic to enzymes and microorganisms during fermentation (Avci et al., 2013). Another commonly used pretreatment technique is alkali pretreatment. During this process, lignocellulosic biomass swells and lignin partially solubilizes in the solution, while the cellulose remains intact in the solid residue. However, alkali treatment requires long retention time and solvent recovery can be expensive (Singh et al., 2015). Alkali pretreatment assisted by ultra-sonication can be incorporated to effectively disrupt lignin and lower the retention time (Li et al., 2016). Sonication treatment causes cavitation of bubbles at the biomass interface and promotes faster disruption of lignocellulosic matrix when combined with alkaline treatment.

The carbohydrate fraction that results from the pre-treated biomass is further subjected to acid/enzymatic hydrolysis. During this step, the polysaccharides (cellulose and hemicellulose) are further degraded to their hexose and pentose sugars. These reducing sugars act as carbon source in the production of ethanol by using an appropriate yeast species through anaerobic fermentation process. However, the optimal conversion of complex sugars to monomeric reducing sugars with minimal toxic or by-products, demands judicious selection of processing variables. Processing variables and their optimal values for acid hydrolysis of different feedstock biomasses are given in Table 1.

This study deals with the utilization of citrus peel biomass to produce bioethanol and hydrolytic enzymes, aiming to ensure maximal utilization of citrus peel wastes. Accordingly, the citrus biomass is subjected to a two-step pre-treatment process (aqueous extraction followed by alkaline treatment) and subsequently subjected to acid hydrolysis for maximum recovery of reducing sugars. To achieve maximal concentration of reducing sugars from pre-treated citrus peels, the critical parameters like solid loading, acid concentration and time are optimized by statistical RSM- CCD. The aqueous extract resulting from first pretreatment step is subsequently used as the liquid medium for the production of hydrolytic enzymes using *Aspergillus niger*.

2. Materials and methods

2.1. Feedstock biomass and chemicals

Raw citrus peel (*Citrus limetta*) wastes were collected from Ice n Spice food outlet, Bits Pilani Goa Campus and sundried for 1–2 days. The dry citrus peels were stored in an airtight plastic container until further use. All chemicals including standard-grade sugars used in this work were purchased from Merck, India.

Table 1
Levels of variables and experimental ranges used for central composite design.

Variables	Levels				
	−2	−1	0	1	2
Time (min)	15	30	45	60	75
Acid concentration, w/w%	0.5	1	1.5	2	2.5
Solid loading, w/w%	0.5	1.625	2.75	3.875	5

2.2. Fungus strain

The fungus *Aspergillus niger MTCC* 478 used in this study was procured from Institute of Microbial Technology, Chandigarh, India. The culture was sub-cultured and maintained as spores on potato dextrose agar plates at 4 °C.

2.3. Characterization of citrus peel

The sun-dried citrus peels containing 2.32% moisture were used for compositional analysis.

2.3.1. Moisture content

Dried citrus peels were pulverised using house-hold mixer grinder. The powder was then dried in hot air oven for 24 h at 45 °C. The moisture content of the sample was determined by the change in weight of the sample.

2.3.2. Ash content

For ash content, about1g of dried biomass was heated in a muffle furnace at 525 °C for 4 h. The difference in crucible weight plus sample before and after was taken to calculate the % ash in the sample.

2.3.3. Soluble fraction

About 25 g of sample was subjected to extraction using Milli-Q water for 4–5 h. After extraction, the liquid fraction was filtered out and collected separately, while the solid residue was dried and weighed. The change in weight of the solid was used to determine the % soluble fraction present in the biomass. The liquid fraction was further analysed for protein and traces of carbohydrates.

2.3.4. Total protein

The total protein content in the extract was analysed using Lowry's method (Lowry et al., 1951).

2.3.5. Lignin

Lignin content of citrus peel was analysed using Klason lignin method as stated in the NREL protocol (Sluiter et al., 2008). Klason lignin method involves two-step acid hydrolysis and is a destructive process; therefore, the carbohydrates liberated from this technique cannot be used for fermentation. Initially a known amount of biomass was mixed with 75% H_2SO_4 and incubated at room temperature for 30 min. Subsequently, the acid concentration was reduced to 4% H_2SO_4 using 84 mL Milli-Q water and autoclaved at 121 °C for 1 h. The hydrolysed sample was subsequently filtered using vacuum filtration and the lignin content was analysed by change in weight of the solid residue. The acid soluble lignin was determined using UV-Spectroscopy by the procedure given in NREL protocol.

2.3.6. Reducing sugar and total sugar

2.3.6.1. Colorimetric method. Reducing sugars were analysed by 3, 5-Dinitrosalicylic acid method (Miller, 1959) and total sugars were estimated by phenol sulfuric acid method (Dubois et al., 1956).

2.3.6.2. TLC method for qualitative analysis of reducing sugars. TLC of the hydrolysate mixtures was carried out using silica gel plates (TLC Silica gel 60 F_{254}, Merck Germany) as the stationary phase and a solvent system comprising 42.8% Chloroform, 50% Acetic acid and 7.2% water on v/v basis as the mobile phase. Standard sugars were spotted on the parallel lanes to help detect and identify unknown sugars spotted in the samples lanes. The air-dried TLC plates were sprayed with orcinol reagent to show the presence of carbohydrates indicated by purple

spots.

2.3.6.3. LC-MS analysis for final quantification of reducing sugars. Liquid hydrolysates obtained after acid hydrolysis were analysed by LC-MS (Agilent Technologies). The individual hexose sugar concentrations (glucose, fructose, mannose, galactose and galactronic acid) were determined using ZORBEX C8 Plus Column with Methanol (0.1% Formic acid v/v)-Milli-Q water (0.1% Formic acid v/v) mixture as an eluent in the gradient phase with a flowrate of 200μl/min and column temperature of 80 °C. Pentose sugar concentrations were determined using ZORBEX HILIC Column with Methanol (0.1% Formic acid v/v)-Milli-Q water (0.1% Formic acid v/v) mixture as an eluent at a flowrate of 200μl/min and column temperature of 22 °C. Mass spectrometric detection of individual sugars was performed in an Agilent Technology 6460 Triple Quad UPLC–MS, equipped with electrospray ionization (Agilent, Santa Clara, CA, USA) source maintained at 350 °C. The capillary voltage was set at 4000 V for all analyses. The instrumentation parameters, namely, nebulizer gas flow pressure, fragmented voltage and collision energy, were set at 35 psi, 135 V and 45 V respectively. Nitrogen was used as the drying gas at a flow rate of 10 L/min. Quantification of CIP was performed using multiple reactions monitoring (MRM) mode. A triple quad system was set at the unit resolution. The peak area of all the components was integrated using Mass Hunter software version B.08 (Agilent Technology). Standard charts were prepared for all sugars, and concentrations of pentose sugars and hexose sugars were calculated from the individual slopes obtained from the standard plots.

2.3.6.4. EDAX analysis of citrus peels. The energy dispersive analysis of X-ray (EDAX) (FEI Quanta 250) was carried out to obtain the elemental composition of the citrus peels. The dry citrus peel pellet was coated with a 10 nm Au–Pd coat using a sputter coater (LEICA EM ACE 200). The elements which were found to be deficient on citrus peels were supplemented to formulate an effective liquid medium for the production of hydrolytic enzymes.

2.3.7. Pectin content

The pectin content of citrus peel was analysed by the method of Simpson et al. (1984).

2.4. Pre-treatment of citrus peel

The pre-treatment of citrus peel was carried out in two steps. In the first step, dried citrus peels minced to approximately 1 cm × 1 cm sizes were subjected to aqueous extraction in Soxhlet extraction apparatus. About 50 g of citrus peels was loaded into the extraction thimble and about 500 mL of water was taken in the reflux flask for extraction. The extraction was carried out until the extract turned into a colourless solution. The final liquid extract mainly comprising of pectin and other soluble components were used for hydrolytic enzymes production. The pectin-free citrus peel biomass was further oven dried at 50 °C and powdered in a mixer. The 100-mesh pass-through fraction was further used for acid hydrolysis experiments. In the second-step, alkaline pre-treatment of citrus peel was carried out using 0.5 M sodium hydroxide in a bath ultra-sonicator maintained at 100 °C sonicated for 30 min. The mass fraction (%w/w) of aqueous phase to solid phase was maintained as 10: 1 The solid fraction obtained after pre-treatment was washed several times with deionized water, dried and stored in a desiccator until further use. A small amount of liquid fraction was stored in Eppendorf tubes for analysis of carbohydrate losses.

2.5. Production of reducing sugars and enzymes from citrus peels

2.5.1. Parameter optimization for reducing sugars synthesis by acid hydrolysis

The acid hydrolysis optimization of pre-treated citrus peel was carried out by adopting statistical approach, involving Central Composite Design- Response surface methodology (CCD-RSM) for three parameters at five levels using Design Expert software (Design Expert Version 7.0.0, Stat-Ease, Inc., Minneapolis, USA). The experiments involved 20 runs with 14 star points and 6 centre points at zero levels (i.e., replicates) were carried out at different acid concentrations (prepared in water), solid loadings and time. The total weight of aqueous phase used for hydrolysis experiments was in the range of 47–49 mL. The design table involving 5 levels and 3 parameters namely time, acid concentration and solid loading diligently chosen through literature review are shown in Table 1. All the experiments were conducted in duplicates in 100 mL conical flasks at constant temperature of 121 °C. The hydrolysates were vacuum filtered and the solid fraction was dried in hot air oven. The liquid fraction obtained was neutralized by slowly adding powdered Na_2CO_3 until the effervescence stopped. The pH of the samples was also monitored during the neutralization process to ensure that the pH of the final neutralized sample was maintained within 7 ± 0.1. The neutralized samples were further subjected to total sugar and reducing sugar analysis. The optimal hydrolysis condition yielding maximal total reducing sugars was predicted by the 3-D surface plots. Predicted conditions were validated by experimentally conducting hydrolysis experiments in triplicates under optimal processing conditions. An ANOVA was performed to understand the interaction between variables and their effects on the hydrolysis. Model coefficients and significance of the fitness of the model were determined by regression analysis.

2.5.2. Use of liquid extract of citrus peel for production of hydrolytic enzymes by submerged fermentation

Aspergillus niger spores were propagated by inoculating a loopful of spores onto potato dextrose agar plates and incubated at 28 °C for a week. About 5 mL of 0.2% v/w Triton-X was used to extract the spores from the solid media and was subsequently transferred to 2 mL Eppendorf tubes and stored at 5 °C until further use. Citrus peel solutions of different concentrations were evaluated for the optimal enzyme production by diluting the extract with water. Accordingly, 100 mL citrus solutions of different concentrations (10, 30, 50, 70 and 100 %v/v) in 250 mL flasks were used for the experiments. The optimal concentration that resulted in maximal activity of pectinase was used for further media optimization studies. Three salts comprising of elements, which were identified to be lacking in the citrus biomass as revealed by the EDX analysis, were supplemented to the medium for the optimization studies. Accordingly, three salts namely KH_2PO_4, NH_4Cl, $FeSO_4.7H_2O$ were added at concentrations of 400 mg/L, 2 g/L, and 0.001 mg/L respectively to the medium separately and the performance was compared with that of a medium supplemented with all salts and a control medium without any salts supplementation. About 400 μL of spore suspension (containing approximately 2×10^6 spores/mL) served as inoculum for all the studies. All the experiments were carried out by incubating the flasks in a shaker incubator maintained at 28 °C and 150 rpm for 5 days. Once the fermentation was complete, the fungal pellets were removed and the supernatant containing the enzymes was collected in 10 mL aliquots. The supernatant was analysed for pectinase activity (Rangarajan et al., 2010) and cellulase activity (Ghose, 1987).

3. Results and discussion

The various pre-treatment and processing steps involved in the production of biomass and hydrolytic enzymes from citrus peel are shown is Fig. 1.

3.1. Characterization of citrus peel biomass

EDX analysis was performed to map the composition of various elements in the biomass. Elemental mapping of citrus peel facilitated formulation of an optimal medium for production of hydrolytic

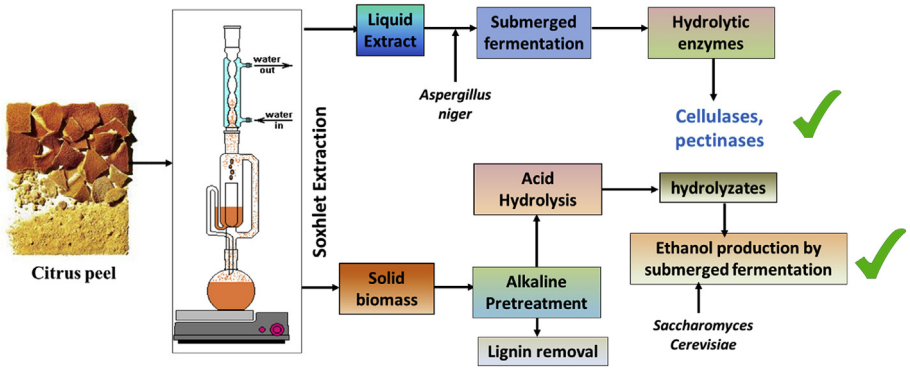

Fig. 1. Schematic representation of various steps involved in the use of citrus peel as ligno-cellulosic feedstock for the production of bioethanol and hydrolytic enzymes.

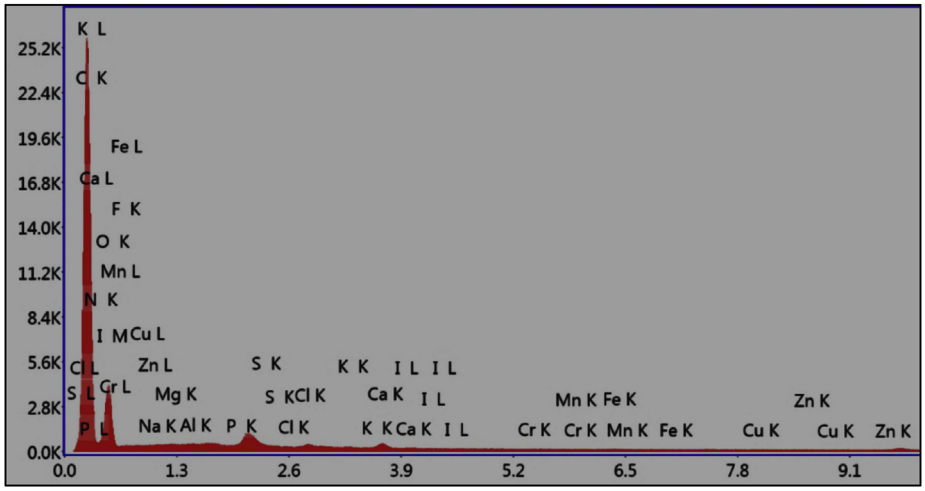

Fig. 2. EDX analysis of citrus peel for the identification of the lacking elements
(The analysis showed that the citrus peel biomass was deficient in Fe, N and P suggesting the need for formulating a nutrient medium with these elements supplemented for hydrolytic enzyme production).

enzymes by supplementing deficient elements in the form of their salts, as discussed later in section 3.3.2. The EDX analysis of citrus peel (Fig. 2) showed that it lacked most essential elements such as Iron, Potassium and phosphorus required for microbial activity and in turn for the optimal hydrolytic enzymes production.

Proximate analysis of citrus peel biomass revealed that the citrus peel biomass contained 2.32% embedded moisture and 5.57% of ash content. Compositional analysis of biomass is shown in Table 2. Citrus peel biomass contained 35% carbohydrates, 40.39% soluble fraction and 6.5% lignin. The high carbohydrate content and low lignin content of citrus peel suggests that it can serve as a potential feedstock for bioethanol production. As shown in Table 2, the percentage soluble content was 40.39% indicating that it can also be used as an effective substrate for the production of hydrolytic enzymes through a submerged fermentation process. The citrus peel possessed a good amount of pectin of about 6.6% w/w, most of which remained in the soluble form of the aqueous extract (see Table 2).

Table 2
Composition of citrus peel (dry weight basis).

Component	Percentage
Moisture content	2.32%
Ash	5.57%
Soluble content	40.39%
Lignin	6.5%
Protein	9.97%
Pectin	6.6%
Carbohydrates	35.25%

Table 3
Types of reducing sugars identified in the sample hydrolysate using thin layer chromatography.

Label No	Reducing sugar	Present/Absent
1	Xylose	Absent
2	Galacturonic acid	Absent
3	Arabinose	Present
4	Mannose	Absent
5	Fructose	Present
6	Cellobiose	Absent
7	Galactose	Present
8	Sucrose	Present
9	Glucose	Present

Thin layer chromatography of acid hydrolysates produced from pulverised citrus biomass showed prominent spots for the following reducing sugars: arabinose, glucose, fructose, galactose and sucrose (Fig. 3). However, presence of other sugars could not be ruled out, as there were a few more faint spots which disappeared during image correction process. A similar study on orange peel wastes using enzymatic hydrolysis corroborated with the current results confirming the presence of the above obtained reducing sugars(Talebnia et al., 2007). However, the spots that failed to resolve from each other indicating presence of oligosaccharides/polysaccharides in the hydrolysates even

Table 4
ANOVA for response surface quadratic model.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	225.36	9	25.04	34.12	< 0.0001	significant
A-Time	7.27	1	7.27	9.91	0.01	
B-Acid Concentration	0.86	1	0.86	1.17	0.30	
C-Solid loading	178.67	1	178.67	243.49	< 0.0001	
AB	6.07	1	6.07	8.27	0.02	
AC	2.43	1	2.43	3.31	0.10	
BC	3.99	1	3.99	5.44	0.04	
A^2	15.37	1	15.37	20.95	0.00	
B^2	0.08	1	0.08	0.11	0.75	
C^2	14.72	1	14.72	20.07	0.00	
Residual	7.34	10	0.73			
Lack of Fit	4.92	5	0.98	2.04	0.23	not significant
Pure Error	2.42	5	0.48			
Cor Total	232.70	19				

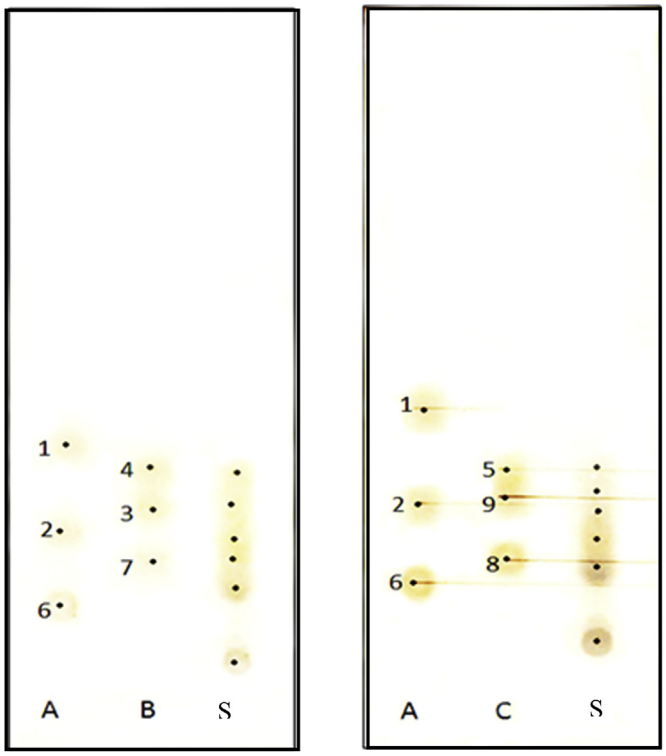

Fig. 3. TLC Analysis of acid hydrolysed sample.
(A, B and C are standard sugars lanes and S is the sample lane (the sugars corresponding to labelled spots in different lanes are given in Table 3) The original image is adjusted for brightness and contrast for proper visualisation of spots.

after hydrolysis.

3.2. Effect of pre-treatment on citrus peel biomass

Mechanical pre-treatment of biomass increased the surface area and porosity of the biomass. Subsequently extraction with water for citrus peel biomass yielded a soluble fraction of 40.39% w/w. This suggests that the extracted fraction would form a good substrate in production of hydrolytic enzymes. Subsequently the alkali pre-treatment of biomass using NaOH effectively produced lignin free solid fraction. The pre-treatment was conducted at mild operating conditions; therefore, there was no sign of inhibitory products formed during the process. A study conducted on alkaline pre-treatment of corn stover resulted in maximum solubilization of lignin leaving the carbohydrate fraction in the biomass intact(Chen et al., 2013).

3.3. Production of reducing sugars and enzymes from citrus peels

3.3.1. Parameter optimization for reducing sugars synthesis by acid hydrolysis

The objective of acid hydrolysis optimization is to obtain an optimal yield of reducing sugars with desirable requirements, namely; minimal amount of inhibitory products such as furfurals and acetyl groups, minimal energy consumption and minimal requirement of alkali for neutralization. The most significant parameters for acid hydrolysis optimization of citrus peel biomass were identified as time, acid concentration and solid loading.

In the ANOVA analysis of the model (Table 4), the p-values lower than 0.05 indicate that model terms are considered to be significant. In this case A, C, AB, BC, A^2, C^2 are significant model terms. As shown in Table 5, the predicted R-Squared value of 0.8163 is in reasonable agreement with the adjusted R-Squared value of 0.9401. The adequate precision of 22.067, a measure of signal to noise ratio (any value above 4 is desirable) further corroborates the robustness of the model.

The actual and predicted responses for the optimal conditions are shown in Table 6. The total reducing sugars can be also be obtained by employing the equation as given below predicted by the regression analysis:

$$R1 = -4.96330 + 0.093622A + 6.70864C + 0.11615AB + 0.032674AC - 1.25533BC - 0.00347A^2 - 0.60465C^2$$

where R1 is total reducing sugars (g/L), A = time; B = acid concentration (%w/w) and C = solid loading (%w/w).

The extent of influence of three individual parameters on the total

Table 5
Regression analysis.

Parameter	Value
Std. Dev.	0.8566
Mean	8.6594
C.V. %	9.8924
PRESS	42.7522
R-Squared	0.9685
Adj R-Squared	0.9401
Pred R-Squared	0.8163
Adeq Precision	22.0676

Table 6
Optimization statistics with actual and predicted responses.

Std Order	Factor 1	Factor 2	Factor 3	Response	
				Total reducing sugars (g/L)	
	A: Time	B: Acid Concentration	C: Solid Loading	Actual Value	Predicted Value
	Min	w/w %	w/w%	g/L	g/L
1	30	1	1.625	5.35	5.27
2	60	1	1.625	4.49	3.77
3	30	2	1.625	4.43	4.48
4	60	2	1.625	5.49	6.47
5	30	1	3.875	12.97	12.26
6	60	1	3.875	12.75	12.97
7	30	2	3.875	7.67	8.65
8	60	2	3.875	12.50	12.84
9	15	1.5	2.75	5.46	5.47
10	75	1.5	2.75	8.44	8.16
11	45	0.5	2.75	9.41	10.18
12	45	2.5	2.75	10.29	9.25
13	45	1.5	0.5	0.18	0.20
14	45	1.5	5	13.85	13.56
15	45	1.5	2.75	11.12	9.94
16	45	1.5	2.75	9.70	9.94
17	45	1.5	2.75	10.39	9.94
18	45	1.5	2.75	9.07	9.94
19	45	1.5	2.75	9.80	9.94
20	45	1.5	2.75	9.85	9.94

reducing sugar concentration can also be estimated from the above quadratic equation. The coefficient of acid concentration term falls to a negative value indicating that acid concentration has a negative effect on the reducing sugar concentration. The magnitude of the solid loading coefficient is higher, therefore, it has a positive effect on the reducing sugar concentration. A previous response surface optimization study on switch grass to produce fermentable sugars corroborates with this observation (Paniagua et al., 2016).

The 3D-surface plots (Fig. 4a–c) indicate that there are significant interactions between solid loading, acid concentration and time. Fig. 4a represents the total reducing sugar concentration as a function of acid concentration and solid loading corresponding to time at zero level i.e. 45 min. The increase in acid concentration showed a negative response on low solid loading. In addition, while the acid concentration was kept constant, the reducing sugar concentration significantly increased with increase in solid loading as well as time. It can also be observed that longer holding times and lesser solid loadings resulted in reduced conversion. The loss in reducing sugar synthesis at higher acid concentrations and prolonged time could be attributed to the formation of more inhibitory by-products (José C Martínez-Patiño et al., 2017a,b; Oberoi et al., 2010) The model F value = 34.12, implying that it is significant(Table 4).

The statistical optimization experiments for acid hydrolysis of pretreated citrus peels at 121 °C resulted in a predicted total reducing sugar concentration of 13.44 g/L (Fig. 5) at optimal processing conditions of solid loading = 3.87% w/w, acid concentration = 1% w/w and time = 48.4 min. Validation experiments in triplicates at predicted optimal conditions resulted in a reducing sugar concentration of 13.65 ± 0.35 g/L. The LC-MS studies conducted on the acid hydrolysate from optimal conditions revealed that pentose sugars which do not contribute to ethanol production were in negligible concentration (about 20 mg/L). This significant finding further strengthened our initial hypothesis that citrus peels can serve as a potent feedstock for bioethanol production.

3.3.2. Use of liquid extract of citrus peel for production of hydrolytic enzymes by submerged fermentation

Aqueous extract of citrus peel obtained from the first step of biomass pre-treatment was used for the submerged fermentative production of pectinase and cellulase. Firstly, the citrus peel extract of different dilutions (10, 30, 50, 70% v/v and without dilution, i.e., 100% extract) in water were tested for pectinase production. *Aspergillus niger* showed good growth in extracts of various dilutions, however, medium with 50% v/v extract resulted in maximal yield of pectinase with activity of 4.2 IU/mL as shown in Fig. 6. However, no significant cellulase activity was detected in all samples tested. In next stage of optimization studies, different salts namely $FeSO_4.7H_2O$, NH_4Cl and KH_2PO_4 added to the medium separately and in combination resulted in no significant difference in pectinase activity between them as shown in (Fig. 7). However, presence of all salts reduced the pectinase activity to 4.4 ± 0.1 IU/mL. To our surprise, extract without supplementation of any salts resulted in maximal pectinase activity of 5.38 ± 0.25 IU/mL.

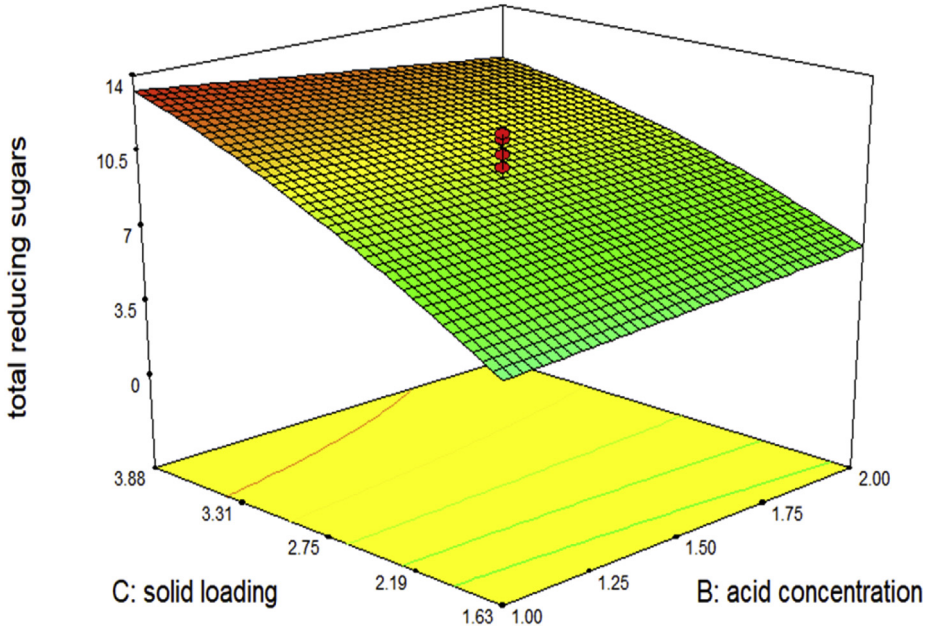

Fig. 4a. 3-D surface plot for interactions between solid loading and acid concentration.

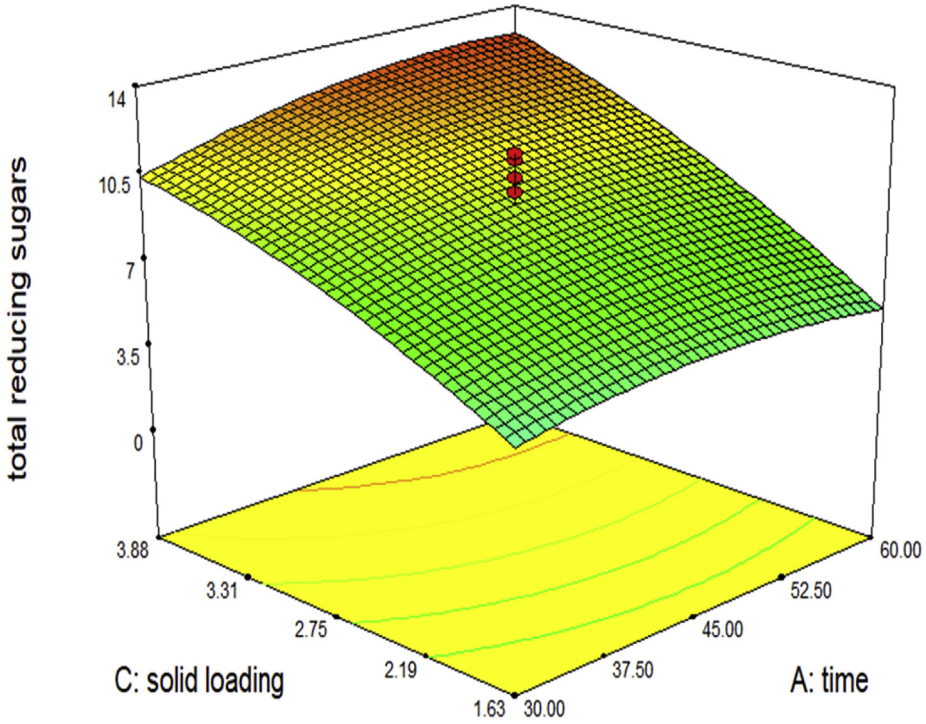

Fig. 4b. 3-D surface plot for interactions between solid loading and time.

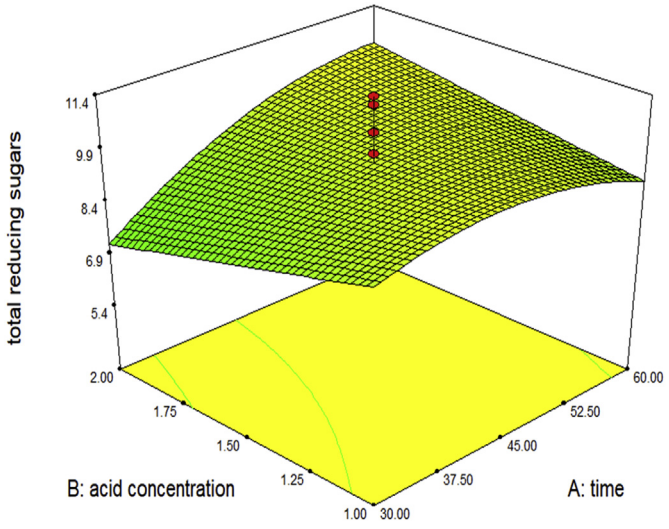

Fig. 4c. 3-D surface plot for interactions between acid concentration and time.

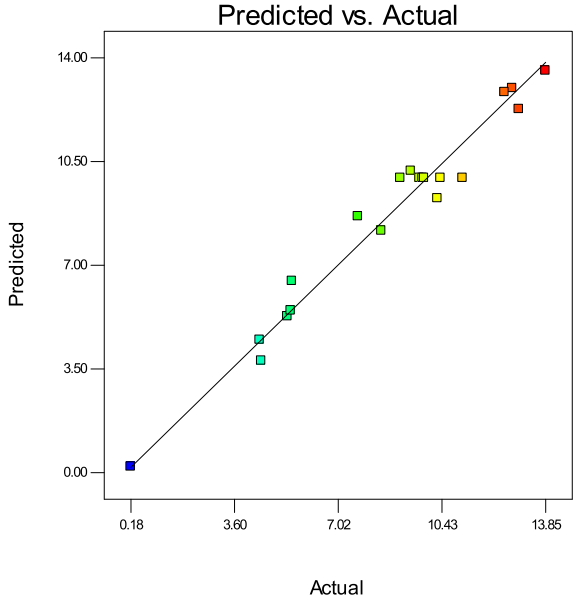

Fig. 5. Plot showing predicted vs Actual results.

This showed that pectin extract without any supplementation of salts would act as the potent medium for pectinase production. Also the amount of biomass generated (Fig. 7) without salts supplementation was comparatively higher (results not shown here) with dry fungal concentration of 8.5 g/L. It was also observed that the pectin extract supplemented with NH_4Cl showed significant quantity of cellulase production with activity expressed in filter paper units (FPU) of 1.17 ± 0.1, while no significant activity was detected in other media with/without salts added. However, further medium optimization studies are needed to evaluate the effect of other salts on the pectinase activity.

4. Conclusion

The characterization of citrus peel wastes revealed presence of 35% of carbohydrate, suggesting that it could be used as a potential feedstock in bioethanol production. Acid hydrolysis optimization of citrus peel resulted in a maximum reducing sugars concentration of 13.65 ± 0.3 g/L at optimal conditions of solid loading = 3.87 %w/w, acid concentration = 1 %w/w and hydrolysis time = 48.4 min. The citrus peel extract obtained from pre-treatment procedure was further subjected to submerged fermentation resulting in total pectinase activity of 5.38 ± 0.2 IU/mL, with co-production of cellulase with activity of 1.17 ± 0.1 FPU. Thus, this study on citrus peels strongly implies that citrus peel biomass can serve as a potential feed-stock for bioethanol production with greater scope to produce hydrolytic enzymes. The solid biomass after alkaline treatment yielded lignin residue which can also be used in various chemical application such as dispersants, protective UV-adsorbents, etc. (Agrawal et al., 2014) However

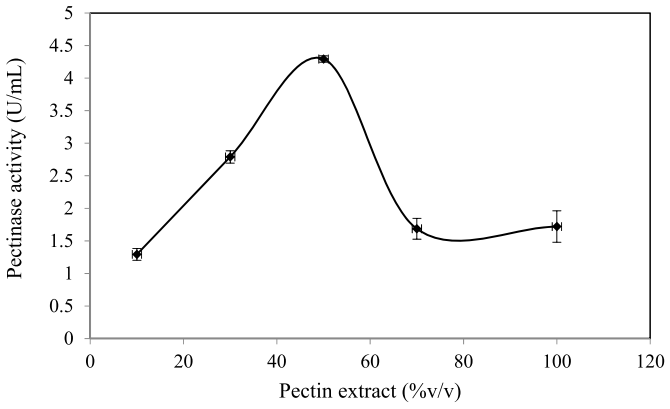

Fig. 6. Effect of dilution of extract on pectinase activity.

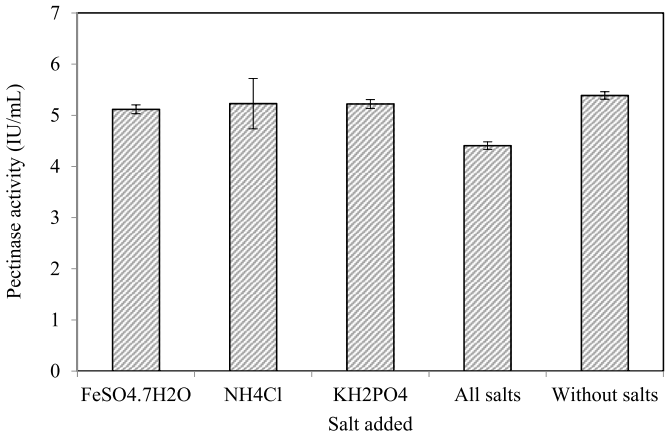

Fig. 7. Effect of supplementation of various salts to the pectin extract medium on pectinase activity.

further studies on scale-up of these techniques and life cycle analysis of process of bioethanol production for these feedstocks are highly recommended.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101259.

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