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An Economic & Ecological Exploration of Biovalorization Potential of Peels of *Citrus sinensis var mosambi* for Ethanol Production by *Pichia stipitis* NCIM 3498 & *Pachysolen tannophilus* MTCC 1077

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Abstract: *Pulverized peels of Citrus sinensis var mosambi consists of 32 ± 0.36% cellulose, 25 ± 0.18% hemicellulose and 18 ± 0.08% of lignin on dry solid (DS) basis. 1% NaOH delignified peels of C. sinensis var mosambi yielded 4.98% glucose, 4.46% raffinose and 7.67% fructose under steam explosion, with a hydrolytic efficiency of 90.32 ± 0.55%. Fourier transform infrared spectroscopy results indicated the penetration of NaOH in the amorphous region of the biomass and degradation of hemicelluloses The enzymes required for hydrolysis were prepared from culture supernatants of Trichoderma reesei NCIM 1052 using wheat bran as carbon source under submerged fermentation conditions. Enzyme activity (U/ml) of crude cellulase produced by T. reesei NCIM 1052 was 311.1 µmole/ml/min. Delignified C. sinensis var mosambi peel yielded 40.51 ± 0.42 g/l glucose when enzymatically hydrolysed by crude cellulase at the substrate enzyme ratio of 1:5. Simultaneous Saccharification and Fermentation (SSF) of peels of C. sinensis var mosambi by crude cellulase and separately entrapped Pichia stipitis NCIM 3498 and Pachysolen tannophilus MTCC 1077 cells in calcium alginate beads were also investigated in the present study. The fermentation experiments were carried out at flask level. The processing parameters setup for reaching a maximum response for ethanol production was obtained when applying the optimum values for temperature (30°C), inoculum level (6%) and fermentation medium (ammonium sulphate, KH_2PO_4, peptone and yeast extract) for P. tannophilus MTCC 1077 and P. stipitis NCIM 3498. Maximum ethanol concentration 9.6 g/l and 10.3 g/l was obtained from P. tannophilus MTCC 1077 and P. stipitis NCIM 3498 respectively at the optimized process conditions in anaerobic batch fermentation.*

Keywords: *Cell immobilization, Citrus sinensis var mosambi, FTIR, Enzymatic hydrolysis, HPLC, steam explosion.*

1. INTRODUCTION

With the inexorable depletion of world's energy supply, there has been an increasing interest worldwide in alternative sources of energy. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars and used as a partial gasoline substitute in a few countries of the world (Sharma et al. 2007). In India total ethanol demand is expected to rise from 3.8 billon liters in 2013 to 5.7 billion liters in 2017 (Bussiness standards, 2012). Lignocelluloses are the largest source of hexose and pentose sugars with prospective use for the production of fuel alcohol (Herrera 2004). The cellulosic and hemicellulosic fractions can be hydrolyzed to sugars, which eventually could be fermented to ethanol.

Bioethanol production from sugarcane and starch rich feed stocks such as corn, potato, etc., is considered first generation process and it has already been developed. Looking at the dwindling pace of food grains shortage and their skyrocketing prices globally, fuel ethanol production from value

added food and feedstock (maize grains, sorghum grains and sugarcane juice etc.) does not fit into the bill of suitable alternative energy sources (Krugman 2008). Therefore, second generation processes to produce bioethanol are gaining momentum. The second generation processes will use lignocellulosic materials for this purpose and biosphere clearly has sufficient supplies of lignocellulosic materials. The production of ethanol from lignocellulosic biomass has become one of the best alternatives, because these sources have widespread abundance and the cost of their procurement is relatively cheap. During *Citrus sinensis* juice production, about 50% weight of fruit is discarded as waste peels, membranes, juice vesicles and seeds (Braddock 1999). Currently, these solid wastes are spread on soil areas adjacent to the production locations (Castello et al. 2006). This way of waste handling leads to an uncontrolled leaching on soil and groundwater. These leachates contain organic compounds that severely threat the surrounding environment (Castello et al. 2010). However, alternatively bioethanol can be produced from this lignocellulosic waste.

Citrus sinensis (L.) Osbeck var. Pineapple (syn, C. *aurantium* L. var. *sinensis)* belongs to Rutaceae family and it is commonly known as sweet orange or *mosambi*. Its fruit is strengthening, cardiotonic, laxative, anthelmintic and removes fatigue. It possesses antiinflammatory and antioxidant properties (Rani et al. 2009). A survey of literature showed that this variety had not been subjected to chemical analysis so far. Until recently, only limited research has been undertaken to evaluate this common and exotic citrus i.e. sweet orange (*Citrus sinensis var mosambi*) (Dharmawan et al. 2006). Peels of sweet orange (*Citrus sinensis var mosambi*) are an important non edible lignocellulosic biomass, which are generated widely throughout India. But several important characteristics of peels of *C. sinensis var mosambi* such as easy availability, high cellulose content and no competition with the food chain makes it an ideal substrate for bioethanol production, and the present investigation had therefore been undertaken to explore the biovalorization potential of this waste.

Peels of *C. sinensis var mosambi* constitute an abundant and cheap lignocellulosic feedstock, but the processing techniques required for ethanol production are presently costly and extensive. The cost of ethanol produced from lignocellulosic materials with currently available technology and under the present economic conditions is not competitive with the cost of gasoline. To make the process economically viable, comprehensive process development and optimization are still required. The low enzymatic accessibility of the native cellulose is a key problem for biomass-to-ethanol processes because lignin forms a protective covering around cellulose and hemicellulose, protecting the polysaccharides from enzymatic degradation. To convert the biomass into ethanol, the cellulose must be readily available for cellulase enzymes. Thus, by removing the lignin, the cellulose becomes vulnerable to enzymes and allows the yeast to convert the glucose into ethanol during fermentation. Pretreatment with dilute acids and bases not only degrade the lignin, but also increases the surface area for enzymatic activity (Dawson and Boopathy 2007). After pretreatment, the enzymatic hydrolysis of substrate is a promising way for obtaining cellulose sugars (mostly glucose) from lignocellulosic materials [because it has the advantages of reduced sugar loss through side-reactions, is milder and more specific] (Adsul et al. 2005). The cellulose conversion option that many currently favor is the Simultaneous Saccharification and Fermentation (SSF) process (Sasikumar and Viruthagiri 2010). In this option, the cellulose hydrolysis and glucose fermentation steps are combined in a single vessel. Since cellulase is inhibited by glucose as it is formed, rapid conversion of the glucose into ethanol by yeast results in faster rates, higher yields, and greater ethanol concentrations than possible for Separate Hydrolysis and Fermentation (SHF). Furthermore, by combining the hydrolysis and fermentation steps in one vessel, the number of fermenters required is approximately one-half that for the SHF process. The presence of ethanol in the fermentation broth also makes the mixture less vulnerable to invasion by unwanted microorganisms (Wyman 1994).

The present study aimed at optimizing the conditions of physiochemical pretreatment as well as fermentation process using peels of *C. sinensis var mosambi* as substrate. Fourier transform infrared spectroscopy (FTIR) was used as an analytical tool for qualitative determination of the structural changes in peel of *A. cosmosus* after physiochemical pretreatment. The potential use of peel of *C. sinensis var mosambi* for ethanol fermentation (SSF) using crude cellulase and immobilized yeast *Pachysolen tannophilus* MTCC 1077 and *Pichia stipitis* NCIM 3498 was also investigated. The influence of process variables such as incubation temperature, inoculum concentration and different nutrients on ethanol production were studied to optimize the fermentation process. Economical and ecological aspects were considered in each step of this study.

2. MATERIAL AND METHODS

Raw material

Peels of pineapple (*C. sinensis var mosambi*) were collected from local fruit juice vendors of Gwalior (26°13′N 78°11′E / 26.22°N 78.18°E), Madhya Pradesh, India. Peels were then air dried for 4-5 days. Dried peels were ground into 100 mesh (0.15mm) fine powder by use of laboratory blender at 3000 rpm and were further preserved in sealed plastic bags at 4°C to prevent any possible degradation or spoilage.

Analysis of chemical composition of *C. sinensis var mosambi* peel

The cellulose, lignin and hemicellulose fractions of powdered peels were determined according to Technical Association of the Pulp and Paper Institute (TAPPI), test methods (1992).

Delignification

Various pretreatment techniques such as steam autoclaving in an autoclave at 15 psi (121°C) for 60 minutes; dilute sulphuric acid (1% v/v), concentrated sulphuric acid (10% v/v), sodium hydroxide (1% v/v), nitric acid (1% v/v) and calcium hydroxide (1% v/v) with steam autoclaving at 15 psi (121°C) for about 20 minutes, were adopted separately for the pretreatment of *C. sinensis var mosambi* peel. The pretreated peels were collected and filtered in crucibles followed by a wash with distilled water under suction. Finally it was dried at room temperature before enzymatic hydrolysis (Szczodrak and Fiedurek 1996, Kaar et al. 1998).

Hydrolytic enzymes production

Production of crude cellulase was done from *Trichoderma reesei* NCIM 1052. The medium for crude cellulase enzyme extraction was prepared by adding 45 g per l wheat bran, 15 g per l yeast extract, 10 g per l glucose, 2.5 g per l NH_4Cl, 0.5 g per l thiamine hydrochloride, 2.0 g per l K_2HPO_4, 0.5 g per l $MgSO_4.7H_2O$, 0.1 g per l $CaCl_2$ and 0.5 g per l KCl (Abadulla et al. 2000). This medium was inoculated with actively growing *T. reesei* NCIM 1052. The flasks were incubated for 10 days on a rotary shaker. After 10 days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to remove mycelia and spores. The supernatant was collected and used as the source of enzyme, which was stored at 4°C till use (Abadulla et al. 2000).

Enzyme assays

One ml of 0.05 M sodium citrate having pH 4.8 was added to a test tube. To this 0.5 ml of enzyme was added. One strip of Whatman No.1 filter paper (weighing 50 mg) was put into test tube (the filter paper strip has to be pushed down whenever it winds up the test tube). The tube along with blank was kept in a water bath at 50°C for 60 min. After 60 min, the tubes were taken out and dinitrosalicylic acid (DNSA) method of Miller (1959) was followed further to account for the amount of sugars released by the cellulase (Singhania 2006). One unit (U) of each enzyme activity is defined as the amount of enzyme, which produces 1 µmol reducing sugar as glucose in the reaction mixture per minute under the above-specified conditions.

Enzymatic hydrolysis

Enzymatic hydrolysis of 1% H_2SO_4 delignified peels of *C. sinensis var mosambi* (300 g) was carried out in a 5 L round bottom glass vessel (Borosil, Mumbai, India) (equipped with agitator for stirring and outer jacket for water circulation to maintain the required temperature) containing 3 L citrate buffer (pH 5.0 ± 0.2, 50 mM, 50 ± 0.5 °C) at 100 rpm. The cellulosic substrate was soaked in the citrate buffer for 2 h before adding the enzymes. Sodium azide was also added at a concentration of 0.005% to restrict any microbial growth during the course of enzymatic hydrolysis. The substrate soaked in citrate buffer was supplemented with cellulose 5 FPU/g at substrate to enzyme ratio of 1:5 (Singh et al. 1990). Samples were withdrawn after 48 hrs, centrifuged and supernatant analyzed for total reducing sugars released. The amount of reducing sugars was estimated by DNSA method as described by Miller (1959).

The extent of hydrolysis was calculated as:

Saccharification % = Reducing sugar concentration obtained X 0.98 X 100 / Potential sugar concentration in the pretreated substrate

Microorganism and maintenance

The wild type strain of *Pachysolen tannophilus* MTCC 1077 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, INDIA, and were used in the present investigation. The wild strain of *Pichia stipitis* NCIM 3498 (which is now known as *Scheffersomyces stipitis*) and *Trichoderma reesei* NCIM 1052 were procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. Yeast cultures were separately maintained on Malt Yeast Agar medium with following composition (g/l)-Malt extract, 3; Yeast extract, 3; Peptone, 5; Glucose, 10; Agar, 20, pH: 7.0 ± 0.2. The strain of *T. reesei* NCIM 1052 was maintained on PDA slants consisted of (g/l): potato, 200; dextrose, 20; agar, 25, pH: 4.8 ± 0.2. Stock cultures were stored at 4 °C. The liquid medium for the growth of inoculum for yeast was YEPD medium consisted of (g/l): yeast extract, 10; peptone, 20; dextrose, 20, pH: 5.00 ± 0.2 for 48 h at 28 ± 0.5 °C (Pasha et al. 2007). Inocula were grown aerobically in 250 ml Erlenmeyer flasks containing the above mentioned medium at 28°C in an Environmental Shaker (Remi Scientific) at 200 rpm for 24 h. Active cells were centrifuged in a clinical centrifuge (1200 rpm), washed with sterile water, and were used as inoculum.

Immobilization of yeast and SSF fermentation

Immobilization of yeast was done by sodium alginate method **(**Smidsrod and Skjak-Braek 1990). Batch experiments were conducted in a 500 ml capacity of Erlenmeyer flask. Other parameters, like temperature, inoculums level and nutrients were chosen as the most significant ones. The process was conducted at the initial substrate concentration of 20 g/l pretreated substrate (i.e. peel of *C. sinensis var mosambi*) and 200 ml citrate buffer (pH 5.0 ± 0.2, 50 mM) followed by sterilization for 15 min, at 15 psi (121°C). The substrate soaked in citrate buffer was supplemented with cellulase, at substrate to enzyme ratio of 1:5 (20g pretreated substrate: 100 ml crude cellulase) or 5 FPU of cellulase was used for hydrolyzing per gram of substrate. The saccharification was done for 24 hrs at 50°C after which simultaneous fermentation was conducted in same vessel by addition of 50 ml of sterilized hydrolysate (obtained after pretreatment) and different nutrients (nutrient parameter 1/2/3, as shown in table 1) at lower temperatures (30ºC / 32ºC / 34ºC). Immobilized yeast cells were used separately as inoculum at different concentrations viz. 2%, 4% or 6%. $MgSO_4$, 0.5 g/l; KCl, 0.5 g/l and $FeSO_4$ 0.01 g/l were used as common nutrients in all fermentation experiments other than different nutrient parameters mentioned above. Fermentation was carried out for 72 h after which samples were withdrawn and centrifuged in a laboratory centrifuge at 1200 rpm, and the supernatants were analyzed for ethanol concentration (Chandel et al. 2009).

Table 1. *Nutritional components used in various nutrient parameters*

Parameters	**1**	**2**	**3**
Nitrogen source	Ammonium sulphate (0.3%)	Sodium nitrate (0.3%)	Urea (0.3%)
Phosphorus source	Potassium Dihydrogen phosphate (0.15%)	Di potassium hydrogen phosphate (0.15%)	Sodium dihydrogen phosphate (0.15%)
Growth factors	Yeast extract (0.5%)	Malt extract (0.5%)	Meat extract (0.5%)
	Peptone (0.5%)	Soya Peptone (0.5%)	Tryptone (0.5%)

Analytical methods

Biochemical composition analysis

Total reducing sugars were estimated by dinitrosalicylic acid method of Miller (1959). The ethanol was estimated colorimetrically as described by Caputi et al. (1968).

The quantitative and qualitative analysis of sugars in the hydrolysates after pretreatment were analyzed using high performance liquid chromatography (HPLC, Waters) with sugar pak column (Waters USA) and a system composed of a 510 pump, a refraction index differential detector (RI 2414 USA) and a data processor with register (Waters, USA). The samples were filtered through membrane filters 0.45 µm (Millipore) before injection. The temperature of the column was maintained

at 70°C by column oven (Dyna, Mumbai) with injection valve of 20 µl. The RI detector was operated at 30°C and the solvent systems used were water as mobile phase at flow rate of 0.2 ml/min. Calculations and analysis were performed using Empower 2 software Build 2154 (Waters). All the experiments were carried out in duplicate and all the reported results were the mean value. The average standard deviation of the achieved results was less than 4%.

FTIR analysis of peel of *C. sinensis var mosambi*

The structural characteristics of polysaccharide sample were recorded on a Fourier- transform infrared spectrophotometer (IR Affinity- 1, Shimadzu, Japan). The sample was ground with KBr powder (spectroscopic grade) and then pressed into 1mm pellet for FT-IR measurement in the frequency range 4000-400 cm^{-1}, with a spectral resolution of 0.5 cm^{-1}. The spectra would be obtained with an average of 64 scans Analysis was performed in both the native and pretreated samples. The baselines of the spectra were adjusted and normalized with the IRsolution software, and the absorption bands at 1427 and 898 cm^{-1} were used to calculate the crystallinity index (Mirahmadi et al. 2010).

3. RESULT AND DISCUSSION

Chemical composition of peel of *C. sinensis var mosambi*

Peel of *C. sinensis var mosambi* was initially characterized with regard to its chemical composition. The pulverized material was found to contain 32 ± 0.36% cellulose, 25 ± 0.18% hemicellulose and 18 ± 0.08% of lignin on dry solid (DS) basis.

The holocellulosic (hemicellulose + cellulose) content of peels of *C. sinensis var mosambi* was found to be 57%. The presence of cellulose and hemicellulose together make the total carbohydrate content (TCC) of the substrate (53.0%). It can be fairly compared with the extensively explored lignocelluloses (corn stover, 58.29%; wheat straw, 54% and poplar 58.2%) for ethanol production (Chandel et al. 2007).

Delignification and enzymatic hydrolysis

Among the all chemicals used, steam explosion pretreatment of *C. sinensis var mosambi* peel with NaOH (1% v/v) resulted in maximum hydrolysis (Table 2). The hydrolysate obtained after this pretreatment consisted of 7.67% fructose, 4.98% glucose and 4.46% raffinose (combination of glucose, fructose and galactose). Peaks and area covered by different sugars representing the above percentages can be seen in fig. 1. 2.02% fructose and 1.51% glucose was present in hydrolysate when autoclaving was conducted using distilled water. The average volume of hydrolysate was 10.0 ml, making the sugar stay more concentrated. At larger scale, the concentration value would be lower because the volume of hydrolysate would be greater. A comprehensive account of the above result indicate that 90.32 ± 0.55% saccharification (maximum) was obtained when *C. sinensis var mosambi* peels were steam exploded with 1% NaOH. We collected a significant amount of pulp from *C. sinensis var mosambi* peel after this pretreatment step when the hydrolysate was centrifuged at 10,000 rpm for 10 min (fig. 2).

The presence of lignin in cellulosic substrates and the crystalline nature of cellulose make it inaccessible to cellulase and their coordinated action (Berlin et al. 2007). Lignin may hinder cellulose hydrolysis by inhibiting their function or by acting as an 'enzymatic trap', which leads to an unproductive adsorption of the cellulases. This may happen not only at middle lamellae but also within the cell walls. Besides, the lower mechanical resistance may also contribute to its enhanced digestibility (Rezende et al. 2011). The exposure of cellulose through structural alteration of the substrates is the crucial factor in hydrolysis of the remaining cellulosic fraction present in the cell wall.

It is required to remove lignin for altering the structure of cellulosic biomass aiding the amenability of cellulolytic enzymes which in turn release the fermentable sugars (Taherzadeh and Karimi 2007, Tu et al. 2007).Therefore lignocellulosic biomass is pretreated prior to enzymatic hydrolysis.

Alkaline treatment of NaOH (1% v/v) was found best suited for *C. sinensis var mosambi* peel which shows that the polymers are converted into monomer and trimers of sugars. Dilute NaOH treatment of lignocellulosic materials has been found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, separation of structural linkages between lignin and

carbohydrates, and disruption of the lignin structure (Fan et al. 1982). Hemicellulose fractions are also removed by alkaline treatments, reaching a maximum removal limit of approximately 96% using NaOH concentrations equal to 1% or higher. As a rule, considering the relative percentages of the three lignocellulosic components, pretreatment conditions using NaOH concentrations lower than 1% seem to be more efficient for the purposes of this work. NaOH concentrations higher than 1% do not result in further removal of any of the lignocellulosic components, and their use is thus unnecessary and uneconomic. Specifically, the pretreatment using NaOH 1% results in maximum removal of hemicellulose and lignin, considering the experimental conditions used in this work (Rezende et al. 2011). Alkaline pretreatment of lignocelluloses with NaOH can remove or modify its lignin by fracturing the ester bonds that form cross-links between xylan and lignin, thereby increasing the porosity of the biomass (Tarkow and Feist 1969). Several reactive and nonreactive phenomenon are involved which makes the above process very complicated. These phenomenon includes dissolution of nondegraded polysaccharides, peeling-off reaction (referred to as formation of alkali-stable in end-groups), hydrolysis of glycosidic bonds and acetyl groups, and decomposition of dissolved polysaccharides (Fengel and Wegener 1984). Therefore the efficiency of NaOH pretreatment depends greatly on the process conditions like temperature, concentration of NaOH, treatment time, as well as the inherent characteristics of the lignocelluloses used (Zhao et al. 2008, Sharma et al. 2007, Wanapat et al. 1985).

Low-NaOH concentration process was applied in this work, in which *C. sinensis var mosambi* peels were treated with 1% NaOH at 121°C at 15 psi for 20 min under steam explosion. It is a reactive destruction of lignocelluloses, while NaOH at high temperature disintegrates the lignin and hemicelluloses and removes them from the solid phase. This property of NaOH is used in pulping processes. Since the NaOH concentration is very less in this activity, its economy and environmental impact may not be so critical.

In contrast to the hydrolysates obtained after acid pretreatment, the hydrolysates obtained after alkali pretreatment does not require detoxification before fermentation further saving the time and money (Chandel et al. 2013). Hence we recommend the use of 1% NaOH for pretreating *C. sinensis var mosambi* peel.

Enzymatic hydrolysis of 1% NaOH pretreated *C. sinensis var mosambi* peel was carried out for depolymerization of cell wall carbohydrate fraction into fermentable sugars. Culture supernatant from *T. reesei* was used for enzymatic saccharification. Enzyme activity (U/ml) of crude cellulase produced by *T. reesei* NCIM 1052 was 311.1 µmole/ml/min and was loaded @5 FPU/g of substrate and then enzymatic hydrolysis was done at physical parameters (50 ± 0.5 °C, 100 rpm). During the course of enzymatic hydrolysis, a regular increase in released sugars was observed till 50 h and remained constant thereafter (data not shown). Enzymatic saccharification of 1% NaOH pretreated *C. sinensis var mosambi* peel yielded a maximum of 405.10 ± 0.45 mg/g sugars (40.51 ± 0.42 g/l) glucose with a hydrolysis efficiency of 71.65 ± 0.45% after 48 h of treatment.

It has been reported that the cell wall structure and components are significantly different in plants, which may influence the biomass digestibility (Hopkins 1999). Sunflower hulls hydrolyzed with *T. reesei* Rut C30 cellulase (25 FPU/g of substrate) showed 59.8% saccharification after pretreatment with sodium hydroxide 0.5% (w/v) at an autoclaving pressure of 15 psi for 1 h (Sharma et al. 2004). Chemical pretreatment not only removes lignin but also acts as a swelling agent, which enhances surface area of the substrate accessible for enzymatic action (Dien et al. 2006, Kim et al. 2008).

Substrate concentration is one of the main factors that affects the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheung and Anderson 1997). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis, and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme (Huang and Penner 1991, Penner and Liaw 1994). Huang and Penner (1991) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate Avicel pH 10.1 to the cellulase from *T. reesei* (grams of cellulase FPU [filter paper unit, defined as a micromole of reducing sugar as glucose produced by 1 ml of enzyme per minute] of enzyme) was greater than 5. Penner and Liaw

(1994) reported that the optimum substrate to enzyme ratio was 1.25 g of the microcrystalline substrate Avicel pH 10.5 per FPU of the cellulase from *T. reesei*.

The amounts of enzymes required for hydrolysis of pretreated raw material depend upon the pretreatment applied to the substrate and the availability of carbohydrate content in the substrate (Saha et al. 2005). Zheng et al. (2009) observed that high enzyme loadings did not alter saccharification and yields. Enzyme loading of 5 FPU/g substrate was found sufficient to hydrolyze the cellulose present in pretreated *C. sinensis var mosambi* peel. Our results indicate that hemicellulose removal and the possible relocalization of lignin moieties during pretreatment could yield the desired amount of sugar toward the goal of developing an intensified and simplified process for cellulose saccharification.

Fourier Transform Infrared (FTIR) Spectroscopy

It is observed that absorbance increased in the region between 3800–3000 cm^{-1} after the pretreatment from 1.251 in native to 1.41 in NaOH treated *C. sinensis var mosambi* peel [fig. 3 (a1)]. Absorption around 1463 cm^{-1}, 1733 cm^{-1} and 1515 cm^{-1} was seen in treated and native *C. sinensis var mosambi* peel [fig. 3(a2)]. In the range 1300–1000 cm^{-1}, the appearance of peek at 1058 cm^{-1} observed very prominently in treated *C. sinensis var mosambi* peel but absent in its native counterpart [fig. 3 (a3)]. This indicates penetration of these chemicals in the amorphous region of the biomass and degrading hemicellulose. Peek at 1164 cm^{-1} is due to asymmetrical stretching of C-O-C (Oh et al. 2005, Colom et al. 2003). Absorption at 1010 cm^{-1} is due to etheric bands, C-O groups (Elbeyli and Piskin 2004). There was a significant increase in these groups after pretreatment [fig. 3 (a3)]. The 1427 and 898 cm^{-1} absorption bands, which were assigned to the respective crystalline cellulose I and cellulose II, were used to study crystallinity changes. The absorbance ratio A_{1427}/A_{898} is called crystallinity index (CI). The peak ratio for the native peel was 1.019, while it was 1.037 for the pretreated peel. There is a great difference in CI of native and 1% NaOH treated peel (fig. 4)

The obtained data agree that the range 3800–3000 cm^{-1} comprises bands related to the crystalline structure of cellulose (Colom et al. 2003). The region is of great importance and is related to the sum of the valence vibrations of H-bonded OH and intramolecular and intermolecular hydrogen bonds.

The absorption in 1733 cm^{-1} is attributed to a C=O unconjugated stretching of hemicelluloses but also with the contribution of lignin. Absorption around 1733 cm^{-1}, indicates chemical changes in hemicellulose and/or lignin. The absorption around 1463 cm^{-1} refers to CH2 and CH3 deformation of lignin. Absorption around 1515 cm^{-1} is associated with C=C aromatic skeletal vibration (Colom et al. 2003, Pandey et al. 1999).

Since there is a great difference in CI of native and 1% NaOH treated peel, it appears that the pretreatment adversely affected the degree of cellulose crystallinity. For lignocellulosic biomass, crystallinity measures the relative amount of crystalline cellulose in the total solid. The crystallinity of the pretreated sample was increased due to removal of lignin and hemicellulose (both of which are amorphous). This result was consistent with another report (Dwivedi et al. 2010). These findings are also in agreement with the results of yield of glucose after the enzymatic hydrolysis of pretreated substrate with crude cellulase.

Ethanol Fermentation

The processing parameters setup for reaching a maximum response for ethanol production was obtained when applying the optimum values for temperature (30°C), inoculum level (6%) and fermentation medium (ammonium sulphate, KH_2PO_4, peptone and yeast extract) for *P. tannophilus* MTCC 1077 and *P. stipitis* NCIM 3498. Maximum ethanol concentration 9.6 g/l and 10.3 g/l was obtained after 72 h from *P. tannophilus* MTCC 1077 and *P. stipitis* NCIM 3498 respectively at the optimized process conditions in anaerobic batch fermentation (Table 3).

Hydrolysate generated after pretreatment of peels of *C. sinensis var mosambi* and enzymatic hydrolysates of pretreated substrate were used for ethanol production using yeasts *P. tannophilus* MTCC 1077 and *P. stipitis* NCIM 3498 separately under submerged batch culture cultivation. These microorganisms selected for bioconversion of ethanol have been established for the fermentation of glucose and xylose sugars for ethanol production (Agbogbo et al. 2006, Agbogbo and Coward-Kelly 2008). Among yeasts, *P. stipitis* and *P. tannophilus* resulted very interesting for their capacity to

ferment xylose. Yeasts metabolize xylose by means of the xylose reductase (XR) that converts xylose to xylitol and xylitol dehydrogenase (XDH) that convert xylitol to xylulose. After phosphorylation, xylulose is metabolized through the pentose phosphate pathway (PPP) (Zaldivar et al. 2001). Among the wild type yeasts fermenting xylose, *P. stipitis* was considered the most promising (Agbogbo and Coward-Kelly 2008) because it has a XR capable to use as cofactor both NADPH and NADH. For this reason, under anaerobic conditions, xylose fermentation in *Pichia stipitis* is carried out by using NADH.

It is worth noting, however, that fermenting D-xylose is practicable only when it is performed in conjunction with the overall utilization of lignocelluloses. Potential substrates envisioned for the application of pentose-fermenting technologies include hydrolysates of plant biomass or waste liquors of pulp- and paper-processing industries. Such materials often contain a variety of sugars such as D-glucose, D-mannose, D-galactose, D-xylose, L arabinose, L-rhamnose, fructose and cellobiose (du Preez et al. 1986, Neirinck et al. 1982). It is, therefore, desirable that microorganisms used for the fermentation of biomass-derived carbohydrates should be able to convert a mixture of hexose and pentose sugars to ethanol. However, yeasts that ferment D-xylose efficiently in batch culture may ferment the pentose poorly when other, more readily metabolized hexoses are present (as found in both the hydrolysates in this study). The preferential utilization of D-glucose over D-xylose has been noted in cultures of *P. tannophilus* (Slininger et al 1987), and *P. stipitis* (du Preez et al. 1986). This phenomenon has been postulated to arise from glucose repression of enzymes involved in xylose metabolism. It is known that xylose reductase (XR) and xylitol dehydrogenase (XDH) activities in *P. tannophilus* are induced in xylose-grown but not glucose-grown cells (Bolen and Detroy 1985). However, since these studies were done on single sugars, it was not certain whether low enzyme activities found in glucose grown cells resulted from catabolite repression or simply an inability of glucose to induce such activities.

Bicho et al. (1988) reported that in liquid batch culture, *P. tannophilus* utilized D-glucose and D-mannose rapidly and preferentially over D-xylose, while D-galactose consumption was poor and lagged behind that of the pentose sugar. In *P. stipitis*, all three hexoses were used preferentially over D-xylose. The results showed that the repressibility of xylose reductase and xylitol dehydrogenase may limit the potential of yeast fermentation of pentose sugars in hydrolysates of lignocellulosic substrates.

Our results are in agreement with some previous studies done on similar/ parallel substrates. 8.6 and 9.9 g/l ethanol production was reported from peels of sweet lemon using *Saccharomyces cerevisiae* in solid state fermentation after 24 and 72 hrs respectively (Mishra et al. 2012). In a parallel set of activity, same organism produced 10.3 g/l ethanol after 72 hrs of incubation in solid substrate fermentation. Wilkins et al. (2007) reported the work done with two ethanologenic yeasts, *S. cerevisiae* and *Kluyveromyces marxianus*, that were used to ferment hydrolyzed sugars extracted from Valencia orange peel waste. In these conditions *S. cerevisiae* produced more ethanol than *K. marxianus* at 24, 48, and 72 h of culture. With these results and for this reason, *S. cerevisiae* was preferred over *K. marxianus* to get more ethanol and higher growth rates than *K. marxianus*. The results reported showed that ethanol and cell mass yields were inhibited by the presence of limonene in orange peel waste. Ethanol production of 6.84 g/l was reported from citrus waste pulp using *S. cerevisiae* (Raposo et al. 2009). Bhatia and Paliwal (2011) reported that ammonium sulphate and yeast extract are best suited for growth of *P. tannophilus*. However in our studies, the production of ethanol from peels of *C. sinensis var mosambi* was found comparatively better than the studies reported earlier. Economical and ecological aspects considered in each step of our work further adds a value to data obtained.

Peel of *C. sinensis var mosambi* is a potential, renewable and low cost biomass for the production of ethanol by fermentation. 1% NaOH pretreated *C. sinensis var mosambi* peel showed the maximum saccharification. It is concluded that sweet orange waste might be used as a low-cost material for bioethanol production, thus representing the partial valorisation of sweet orange industrial residues. It can therefore be concluded that bioethanol can be extracted through Simultaneous Saccharification and Fermentation from the peelings of *C. sinensis var mosambi* using the yeast *P. tannophilus* MTCC 1077 *and P. stipitis* NCIM 3498 at the optimized process conditions in anaerobic batch fermentation.

The researchers would recommend the people to not throw away their sweet orange peelings whenever they consume the fruit, and also the sweet orange-made product manufacturers to store the peelings of the sweet orange they make use of. The researchers would also recommend local waste management committees to collect sweet orange peelings from citizens and submit them to laboratories.

Lastly, the researchers would like to recommend scientists to extract bioethanol from sweet orange peelings through the SSF process using *P. tannophilus* MTCC 1077 and *P. stipitis* NCIM 3498 and to conduct the experiment at optimum parameters for 72-hour fermentation period. Further research regarding other combinations and refinement of the different areas of this methodology for higher bioethanol yield, as well as the feasibility examinations and further studies about the economic technicalities of the study would as well be highly recommended.

Table 2. *Sugars liberated after steam explosion pretreatment of C. sinensis mosambi peel*

Chemicals used for pretreatment	***Sugars liberated after pretreatment***
1% HNO_3	2.16% Xylose, 3.04% Glucose
1% NaOH	**4.46% Raffinose, 4.98% Glucose, 7.67% Fructose**
1% H_2SO_4	4.11% Glucose, 1.30% Xylose
10% H_2SO_4	1.04% Glucose
1% $Ca(OH)_2$	0.53% Fructose
Distilled water (10 ml/g)	1.51% Glucose, 2.02% Fructose

Table 3. *Ethanol production (g/l) in various operational parameters*

Yeast	**Inoculum concentration**			**Nutrient Parameters**			**Temperature**		
***P. stipitis* NCIM 3498**	**2%**	**4 %**	**6 %**	**(1)**	**(2)**	**(3)**	**30°C**	**32°C**	**34°C**
	4.2	5.8	**10.3**	**10.3**	4.0	7.9	**10.3**	3.5	3.7
***P. tannophilus* MTCC 1077**	4.8	5.5	**9.6**	**9.6**	4.4	8.5	**9.6**	5.7	4.4

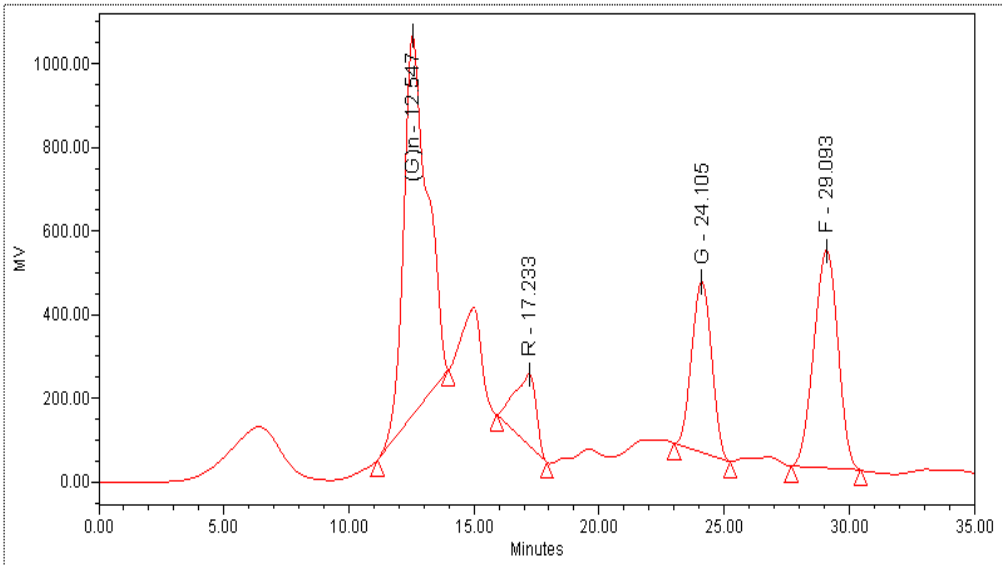
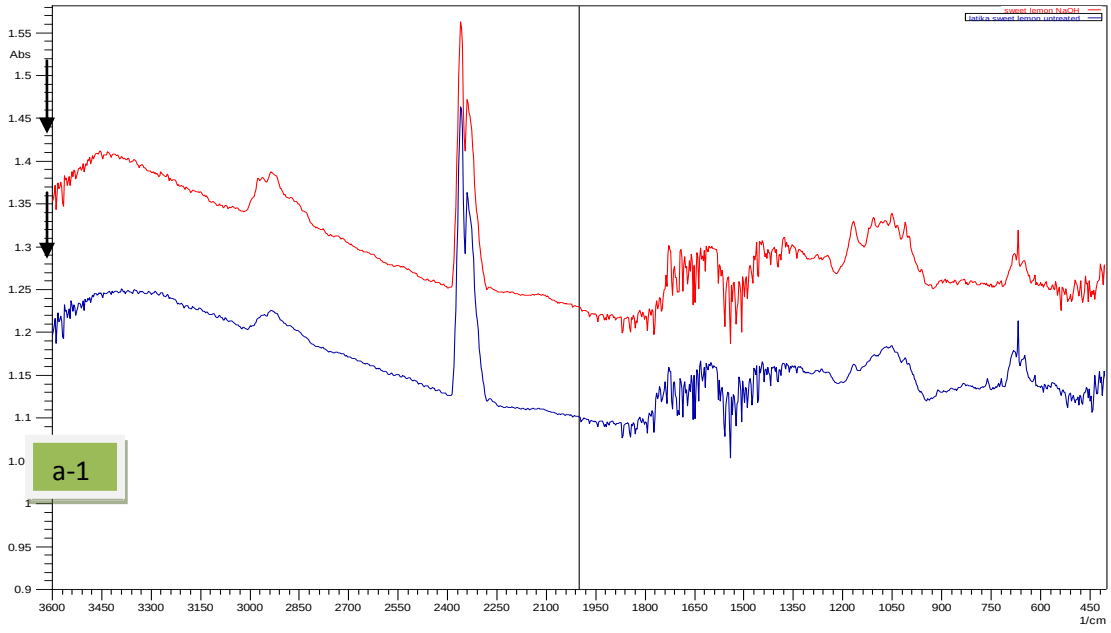
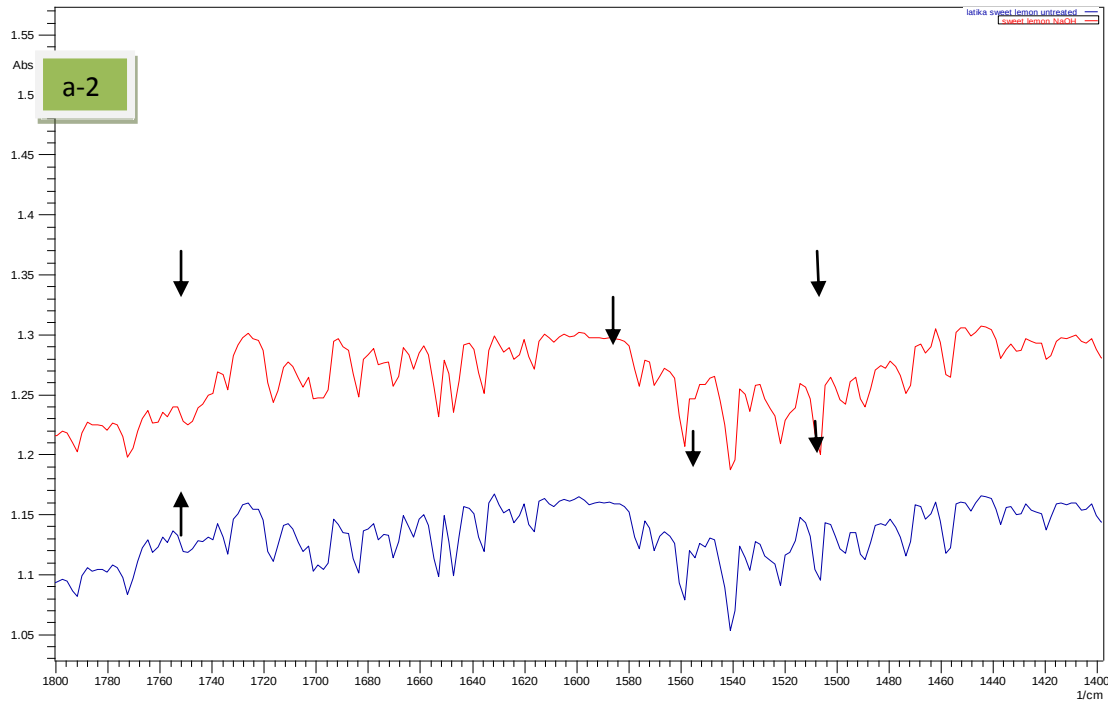


Figure 1. *HPLC chromatogram of sugars liberated from C. sinensis var mosambi peel after 1% NaOH steam explosion (G- glucose, R- Raffinose, F- Fructose, $(G)_n$- starch)*

Figure 2. *Pulp collected after the centrifugation of hydrolysate of pretreated peels of C. sinensis var mosambi*





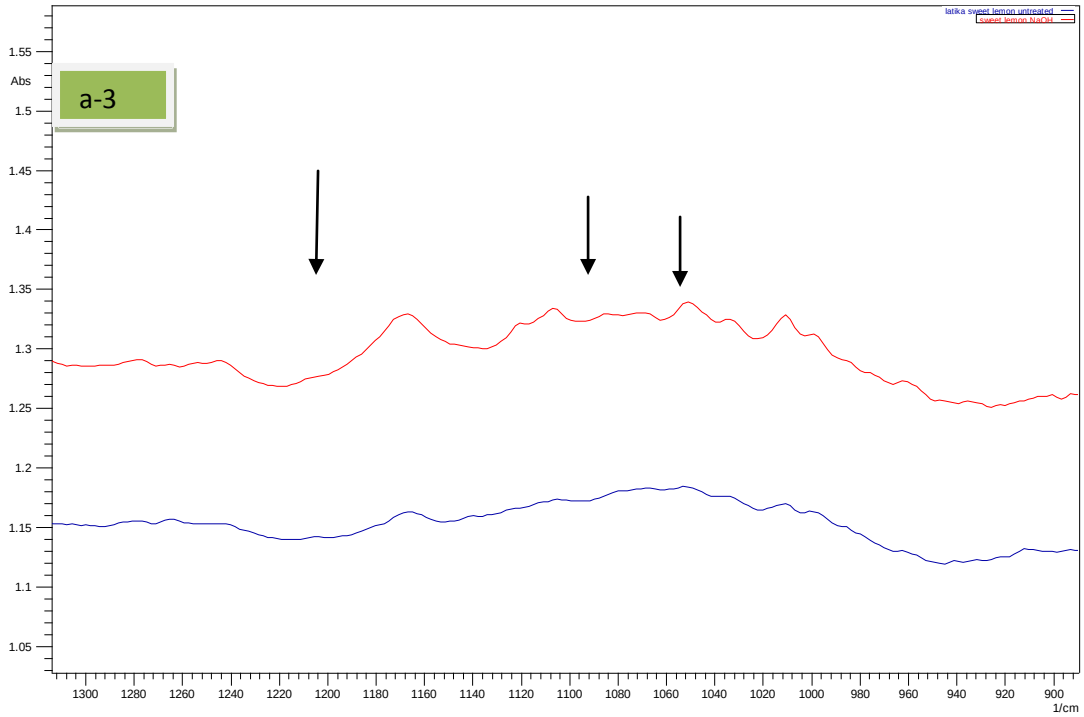


Figure 3. *(a1-a3) Overlapped view of FTIR spectra of native (blue online) and NaOH treated (red online) C. sinensis var mosambi peel*

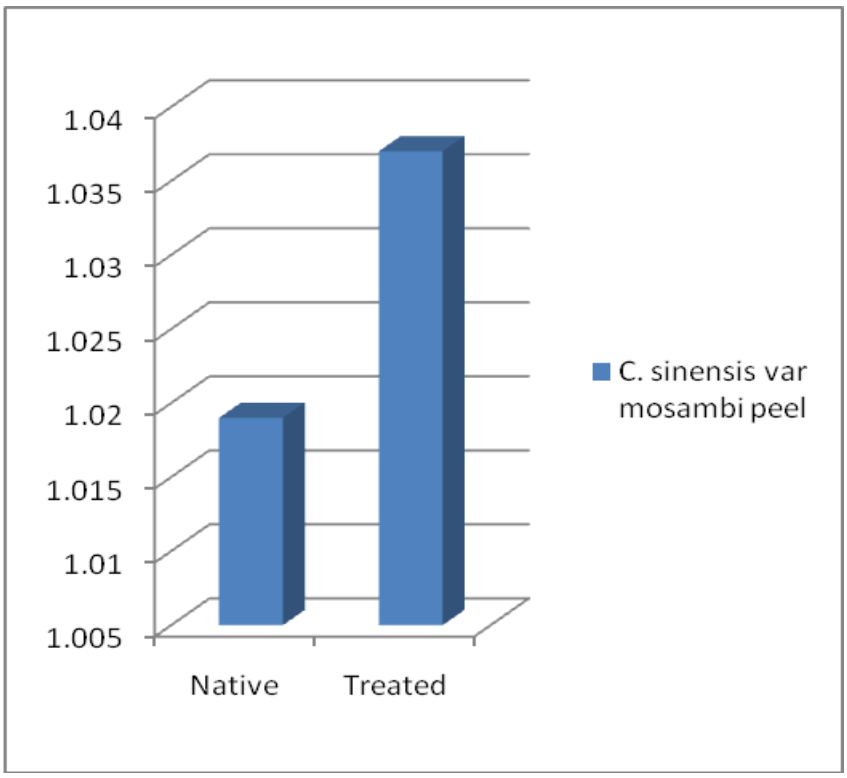


Figure 4. *Total crystallinity index of native and pretreated C. sinensis var mosambi peel*

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