

FTIR mapping of peels of *Litchi chinensis* after acid treatment and its SSF for ethanol production by *Pachysolen tannophilus* MTCC 1077

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Pulverized peels of *L. chinensis* consists of $40 \pm 0.38\%$ cellulose, $28 \pm 0.18\%$ hemicellulose and $25 \pm 0.31\%$ of lignin on dry solid basis. About 1% HNO₃ delignified peels of *L. chinensis* yielded 46.71% xylose and 12.42% arabinose under steam explosion, with a hydrolytic efficiency of $78.2 \pm 0.45\%$. Fourier transform infrared (FTIR) spectroscopy results not only indicated the penetration of HNO₃ in the amorphous region of the biomass and degradation of hemicelluloses but also revealed the structural differences before and after pretreatment. Simultaneous saccharification and fermentation (SSF) of pretreated peels of *L. chinensis* by crude cellulase and immobilized yeast *Pachysolen tannophilus* MTCC 1077 were investigated in the present study. Important process variables for ethanol production from pretreated peels of *L. chinensis* were optimized using response surface methodology (RSM) based on central composite design (CCD) experiments. A three level CCD experiments with central and axial points was used to develop a statistical model for the optimization of process variables such as incubation temperature (30°C, 32°C and 34°C) X1, inoculum level (2%, 4% and 6%) X2 and nutrients (1/2/3) X3. Data obtained from RSM on ethanol production were subjected to the analysis of variance (ANOVA) and analyzed using a second order polynomial equation and contour plots were used to study the interactions among three relevant variables of the fermentation process. 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, KH₂PO₄, peptone and yeast extract) for *P. tannophilus* MTCC 1077. Maximum ethanol concentration 10.5 g/l was obtained after 72 h from *P. tannophilus* MTCC 1077 at the optimized process conditions in anaerobic batch fermentation.

Keywords: *Litchi chinensis* peel, steam explosion, FTIR, enzymatic hydrolysis, RSM, HPLC.

Introduction

Soaring oil prices associated with concerns of climate change, inevitable depletion of world's energy supply and national energy security are driving us to utilize sustainable alternative energy sources, such as solar energy, nuclear energy, wind energy, hydropower, tidal energy, and so on¹. Unlike fossil fuels, ethanol is an oxygenated fuel and a renewable energy source with high octane value produced through fermentation of sugars. Further, ethanol can be a safer alternative to the common additive, methyl tertiary butyl ether (MTBE), in gasoline. The MTBE is toxic and is a known contaminant in ground water. Thus, ethanol can be a substitute to mitigate the problems associated with the rising energy demands across the world as well as a way to reduce green house gas emissions to an extent of 85%².

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 and sorghum grains, and sugarcane juice etc.) does not fit into the bill of suitable alternative energy sources³. 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 *Litchi chinensis* juice production, about 15% weight of fruit is discarded as waste peels. Rodriguez *et al.*⁴ investigated the use of

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Litchi chinensis peel for the biosorption of chromium (VI) in aqueous solution. However, alternatively bioethanol production can also be expected from this lignocellulosic waste.

Litchi (*Litchi chinensis*) is an evergreen sub-tropical fruit crop belonging to the family Sapindaceae, which is indigenous to Southern China and Northern Vietnam⁵. This tree is highly specific to climatic requirements and probably due to this reason its cultivation is restricted to a few countries in the world. India and China account for 91% of the world litchi production⁶. India produced 429,600 metric tones of litchi annually from 60,200 ha (National Horticulture Board, New Delhi, 2005). Huge amount of peel is generated by litchi juice manufacturers. But several important characteristics of peels of *L. chinensis* such as 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 valorization potential of this waste.

Peels of *L. chinensis* 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 and liberates hemicelluloses sugars usually arabinose and/or xylose in filtrates⁷. Due to highly acidic nature of hemicellulosic, hydrolysate, its neutralization is unavoidable step before using it for fermentation. Alkali most preferably calcium hydroxide is used for overliming of hydrolysate (pH 6.0 - 7.0). Overliming followed by neutralization leads to the removal of furfurals

and phenolics (toxic compounds liberated during pretreatment) by precipitation to the some extent. Further treatment of hydrolysate with an activated charcoal is an efficient and economical method of removing phenolic compounds, acetic acid, aromatic compounds, furfural, and hydroxymethyl furfural (HMF) toxic compounds inhibiting the yeast growth by adsorption without affecting levels of sugar in hydrolysate⁸. 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 specific⁹. The cellulose conversion option that many currently favor is the simultaneous saccharification and fermentation (SSF) process¹⁰. 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)¹⁰.

The present study aimed at optimizing the conditions of physiochemical pretreatment using peels of *L. chinensis* as substrate. The FTIR spectroscopy was used as an analytical tool for qualitative determination of the structural changes in peel of *L. chinensis* after physiochemical pretreatment. The potential use of peel of *L. chinensis* for ethanol fermentation (SSF) using crude cellulase and immobilized yeast *Pachysolen tannophilus* MTCC 1077 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.

Material and Methods

Raw Material

Peels of litchi (*L. chinensis*) 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 (0.15 mm) 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 *L. chinensis* Peel

The cellulose, lignin and hemicellulose fractions of powdered peels were determined according to Technical Association of the Pulp and Paper Institute (TAPPI), test methods¹¹.

Delignification & Detoxification

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 *L. chinensis* 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 hydrolysis¹².

Detoxification followed the standard methods of overliming (calcium oxide), neutralization with HCl and treatment with activated charcoal as described Chandel *et al.*¹³. These detoxifying agents were used directly without any preparation. After neutralization the mixture was left for 30 min under moderate mixing followed by vacuum filtration to remove precipitates and other salts. About 2.5% activated charcoal was then added and continued to mixing for 30 min in orbital shaker. The reaction mixture was filtered twice to remove charcoal and pH was cross checked. The pH was adjusted to 6.0-6.5¹³.

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 (per litre) 45 g wheat bran, 15 g yeast extract, 10 g glucose, 2.5 g NH₄Cl, 0.5 g thiamine hydrochloride, 2.0 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.1 g CaCl₂ and 0.5 g KCl. 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 use¹⁴.

Enzyme Assay

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 Miller¹⁵ was followed further to account for the amount of sugars released by the cellulase¹⁶. 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% HNO₃ delignified peels of *L. chinensis* (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 cellulase 5 FPU/g at substrate to enzyme ratio of 1:5 as described by Singh *et al.*¹⁷. 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 Miller¹⁵.

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 yeast *Pachysolen tannophilus* MTCC 1077 were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India, and were used in the present investigation. The wild strain of *Trichoderma reesei* NCIM 1052 were obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. Yeast culture was 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 medium¹⁸ consisted of (g/l): yeast extract, 10; peptone, 20; dextrose, 20, pH: 5.00 ± 0.2 for 48 h at $28 \pm 0.5^\circ\text{C}$. 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

Immobilization of yeast was done by sodium alginate method¹⁹. Batch experiment was conducted as per the central composite experimental design for ethanol production in a 500 ml capacity of Erlenmeyer flask. Parameters, like temperature, inoculum concentration and nutrient factor, were chosen as the most significant ones, considering the experimental design. The process was conducted at the initial substrate concentration of 20 g/l pretreated substrate (i.e. peel of *L. chinensis*) 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 (20 g 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 detoxified hydrolysate (obtained after pretreatment) and different nutrients (nutrient parameter 1/2/3, as shown in Table 1) at lower temperatures ($30^\circ\text{C}/32^\circ\text{C}/34^\circ\text{C}$). Immobilized yeast cells were used 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 concentration²⁰.

Analytical methods

Biochemical Composition Analysis

Total reducing sugars were estimated by DNSA method of Miller¹⁵. The ethanol was estimated colorimetrically as described by Caputi *et al.*²¹.

The quantitative and qualitative analysis of sugars in the hydrolysates after pretreatment were analyzed using high performance liquid chromatography (HPLC, Waters USA) with sugar pak column (Waters USA) and a system composed of a 510 pump, a refraction index (RI) differential detector (RI 2414 USA) and a data processor with register (Waters, USA). The samples were filtered through membrane filters $0.45 \mu\text{m}$ (Millipore) before injection. The temperature of the column was maintained at 70°C by column oven (Dyna, Mumbai) with injection valve of $20 \mu\text{l}$. 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 USA). All experiments were carried out in duplicate, and all the reported results are the mean values. The average standard deviation of the achieved results was less than 4%.

FTIR Analysis of Peel of *L. chinensis*

The structural characteristics of polysaccharide sample were recorded on a FTIR spectrophotometer (IR Affinity-1, Shimadzu, Japan). The sample was ground with KBr powder (spectroscopic grade) and then pressed into 1 mm pellet for FTIR measurement in the frequency range $4000\text{--}400 \text{ cm}^{-1}$, 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 IR solution software, and the absorption bands at 1427 and 898 cm^{-1} were used to calculate the crystallinity index²².

Experimental Design and Statistical Analysis

In the central composite design (CCD), the total number of experimental combinations was $2^K + 2K +$

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%) Peptone (0.5%)	Malt extract (0.5%) Soya Peptone (0.5%)	Meat extract (0.5%) Tryptone (0.5%)

n_0 , where K is the number of independent variables and n_0 is the number of repetitions of the experiments at the central point, which indicated that 20 experiments were required for this procedure. The dependent variable selected for this study was ethanol concentration, Y (g/l). The independent variables chosen were incubation temperature (30°C, 32°C and 34°C) X_1 , inoculum level (2%, 4% and 6%) X_2 and nutrients (1/2/3) X_3 . A mathematical model, describing the relationships among the process dependent variable and the independent variables in a second-order equation, was developed²³. Design-based experimental data were matched according to the following second-order polynomial equation (1).

$$Y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i < j}^k \sum_j b_{ij} x_i x_j + e$$

Where, i, j are linear, quadratic coefficients, respectively, while 'b' is regression coefficient, k the number of factors studied and optimized in the experiment and 'e' is random error. The quality of fit of the second order equation was expressed by the coefficient of determination R^2 , and its statistical significance was determined by F -test. The significance of each coefficient was determined using Student's t -test. The student t -test was used to determine the significance of the parameters regression coefficients. The P values (probability value) were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables. In general, larger magnitudes of t and smaller of P , indicates that the corresponding coefficient term is significant. The response surface equation was optimized for maximum yield in the range of process variables using Design Expert software version 9.0.2. Analysis of variance (ANOVA) for the final predictive equation was done using the same software package. Isoresponse contour plots were obtained based on the effect of the levels of three parameters and their interactions on the yield of ethanol by keeping the other parameters at their optimal concentrations. From these contour plots, the interaction of one parameter with another parameter was studied. The optimum concentration of each parameter was identified based on the hump in the contour plots¹⁰.

Results

Chemical Composition of Peel of *L. chinensis*

Peels of *L. chinensis* were initially characterized with regard to its chemical composition. Pulverized

peels of *L. chinensis* consists of $40 \pm 0.38\%$ cellulose, $28 \pm 0.18\%$ hemicellulose and $25 \pm 0.31\%$ of lignin on dry solid (DS) basis. The holocellulosic (hemicellulose + cellulose) content of peels of *L. chinensis* was found to be 68%.

Delignification and Enzymatic Hydrolysis

Among the all chemicals, steam explosion pretreatment of *L. chinensis* peel with HNO_3 (1% v/v) resulted in maximum hydrolysis (Table 2). The hydrolysate obtained after this pretreatment consisted of 46.71% xylose and 12.42% arabinose. A comprehensive account of the above result indicate that 78.2% saccharification (maximum) was obtained when *L. chinensis* peels were steam exploded with 1% HNO_3 .

The decrease of sugar content in acid treated samples with increasing of acid concentration (10% H_2SO_4) may be because of degradation of monomeric sugars (xylose, glucose) to furfural and hydroxymethyl furfural. These substances are toxic substances for yeast and can inhibit the yeast growth¹³. Insignificant amount of arabinose and xylose was liberated after pretreatment with 1% H_2SO_4 . Hence, we recommend the use of dilute acid i.e. 1% HNO_3 for pretreating *L. chinensis* peels.

Enzymatic hydrolysis of 1% HNO_3 pretreated *L. chinensis* 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 $\mu\text{mole/ml/min}$ and was loaded @5 FPU/g of substrate and then enzymatic hydrolysis was done at physical parameters ($50 \pm 0.5^\circ\text{C}$, 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% HNO_3 pretreated *L. chinensis* peel yielded a maximum of 150.10 ± 0.15 mg/g (1.50 ± 0.24 g/l) glucose with a hydrolysis efficiency of $30 \pm 0.22\%$ after 48 h of treatment.

Table 2 — Sugars liberated after steam explosion pretreatment of *L. chinensis* peel

Chemicals used for pretreatment	Sugars liberated after pretreatment
1% HNO_3	Xylose 46.71%, Arabinose 12.42%
1% NaOH	Xylose 0.56%
1% H_2SO_4	Xylose 8.42%, Arabinose 15.71%
10% H_2SO_4	Arabinose 29.6%
1% Ca(OH)_2	Glucose 0.02%, Xylose 0.76%

Enzyme loading of 5 FPU/g substrate was found sufficient to hydrolyze the cellulose present in pretreated *L. chinensis* peel. Our results indicate that hemicellulose removal and the possible relocation 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

The obtained data agree that the range 3800–3000 cm^{-1} comprises bands related to the crystalline structure of cellulose²⁴. 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. It is observed that absorbance increased in these regions after the pretreatment from 1.108 in native to 1.2135 in HNO_3 treated *Litchi chinensis* peel shown in Fig. 2 (a1). In the range 1300–1000 cm^{-1} , the appearance of peak at 1058 cm^{-1} observed very prominently in treated *L. chinensis* peel but absent in its native counterpart shown in figure 2 (a1). This indicates penetration of these chemicals in the amorphous region of the biomass and degrading hemicelluloses²⁴⁻²⁵.

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 CH_2 and CH_3 deformation of lignin^{24, 26}. These peaks were less prominent after the pretreatment. Apparent changes were also observed in the lignin-characteristic bands around 1606 cm^{-1} , therefore, it is reasonable to state that the lignin was also degraded by the action of the HNO_3 . These

observations could reflect that the lignin is not composed of percentages of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units which makes it highly condensed and very resistant to degradation²⁷ [Fig 2 (a2)].

The small peaks at 2850 cm^{-1} and 2918 cm^{-1} come from CH_2 and CH symmetric and asymmetric stretching, respectively [Figure 2 (a3)]. Both are characteristic of cellulose²⁵. These peaks were seen in treated and native *L. chinensis* peel. This result is in agreement with our pretreatment studies in which hydrolysate obtained after pretreatment was rich in only hemicellulose sugars, and cellulose was not degraded.

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 difference in CI of native and 1% HNO_3 treated peel (Fig. 3).

Optimization of Process Variables in Ethanol Fermentation

Important process variables for ethanol production from pretreated substrates were optimized using response surface methodology (RSM) based on central composite design (CCD) experiments. The experimental results associated to the processing set up of each independent variable are listed in Table 3.

Three level CCD matrix and the experimental responses of the dependent variable (ethanol conc.) are listed in Table 4.

Besides the linear effect of the ethanol concentration, Y g/l, the RSM also gives an insight about the parameters quadratic and combined effects. The analyses were done by using both Fisher's *F*-test and Student *t*-test statistical tools. The regression coefficient, *t* and *P* values for all the linear, quadratic and combined effects with a 95% significance level are given in the Table 5.

Figures 4 (A-C) show the isoresponse contour plots of the interactive effect of incubation temperature, inoculum concentration and nutrient factor on ethanol production. The response values for the variables can be predicted from these plots. The effect of inoculum concentration and nutrient on ethanol production, while other variable (temperature) was fixed at central level, is shown in Figure 4 (A). The effect of inoculum concentration and temperature on ethanol production, while other variable (nutrient)

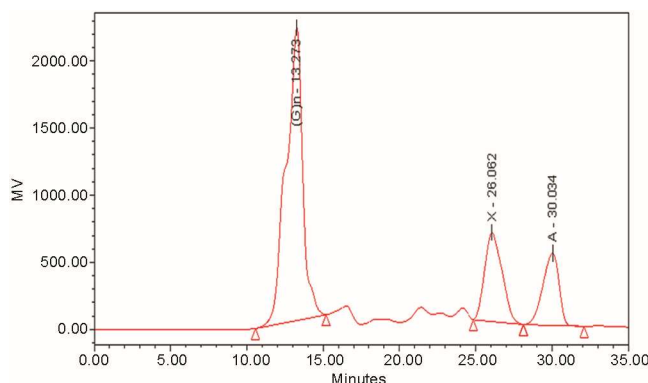


Fig. 1 — HPLC chromatogram of sugars liberated from *L. chinensis* peel after 1% HNO_3 steam explosion (G)- starch, X- xylose, A- arabinose

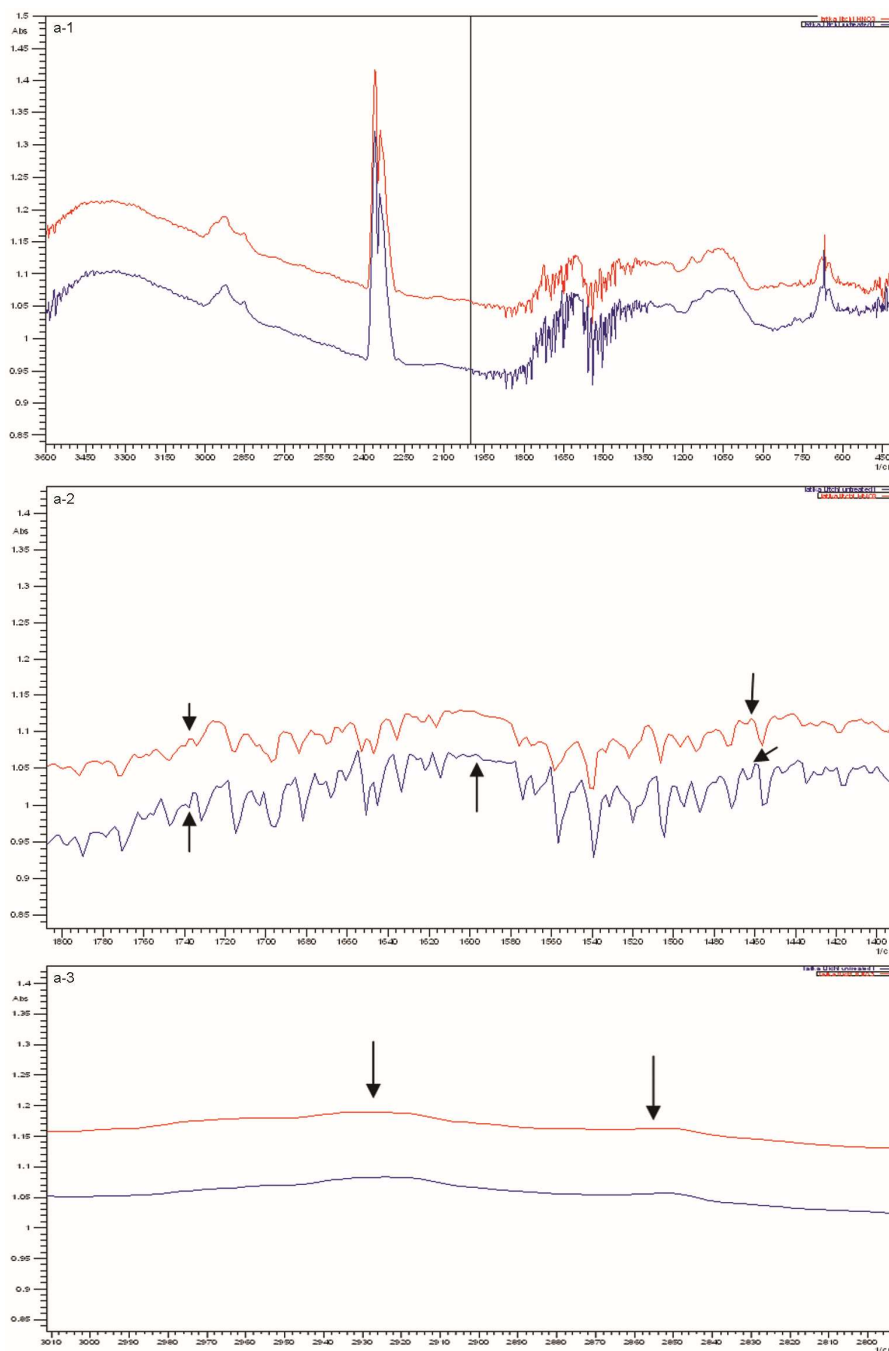


Fig. 2 (a1-a3) — Overlapped view of FTIR spectra of native (blue online) and HNO₃ treated (red online) *Litchi chinensis* peel.

was fixed at central level, is shown in Figure 4 (B). The effect of nutrient and temperature on ethanol production, while other variable (inoculum concentration) was fixed at central level, is shown in Figure 4 (C).

The Model F-value of 4.21 implies the model is significant (Table 5). There is only a 1.74% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case A, B, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The regression equation obtained from the ANOVA shows that the R^2 (coefficient of determination) was 0.7913 (value > 0.75 indicates fitness of the model). This is an estimate of the fraction of overall variation

in the data accounted by the model, and thus the model is capable of explaining 79.13% of the variation in the response. The 'adjusted R^2 ' is 0.6034, which indicates that the model is good (for a good statistical model, the R^2 value should be in the range of 0 to 1.0, and the

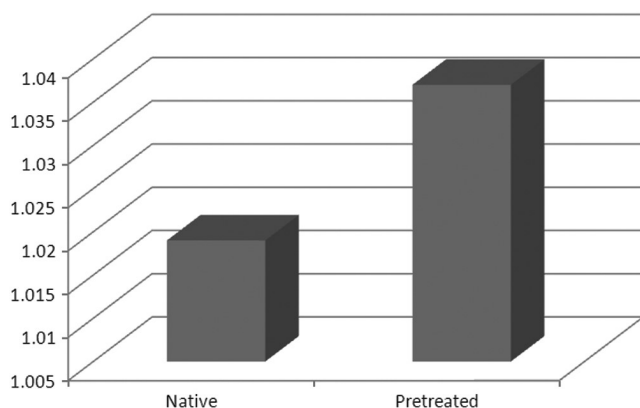


Fig. 3 — Total crystallinity index of native and pretreated *Litchi chinensis* peel.

Table 3 — Codes and actual levels of the independent variables for design of experiment

Name	Low	High	- alpha	+ alpha
Inoculum conc	2	6	2	6
Nutrient	1	3	1	3
Temperature	30	34	30	34

nearer to 1.0 the value is, the more fit the model is deemed to be). A negative "Pred R-Squared" implies that the overall mean is a better predictor of response than the current model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 9.994 indicates an adequate signal. Hence this model can be used to navigate the design space.

From equations derived by differentiating equation 1, the optimum values for the independent variables obtained were incubation temperature 30°C, inoculum concentration 6% and nutrient factor 1. Based on the model, the optimal working conditions were obtained to attain high ethanol yield. Response analysis revealed the maximum ethanol concentration (9.25864 g/l) by *P. tannophilus* could be achieved at the optimum process conditions [Figures 4 (A-C)].

Ethanol Fermentation

Detoxified hemicellulosic hydrolysate and enzymatic hydrolysates were used for ethanol production using yeasts *P. tannophilus* MTCC 1077 under submerged culture cultivation. This microorganism selected for bioconversion of ethanol has been established for the fermentation of pentose sugars for ethanol production²⁸⁻²⁹. The processing parameters setup for reaching a maximum response for ethanol production was obtained when applying

Table 4 — Three level CCD and the experimental responses of dependent variable, Y

Std	Run	Factor 1 A: Inoculum conc	Factor 2 B: Nutrient	Factor 3 C: Temperature	Response 1 Ethanol yield mg/ml	Predicted Values
16	1	4	2	32	4.3	4.62
6	2	6	1	34	5.3	6.12
5	3	2	1	34	4.5	3.07
1	4	2	1	30	3.8	4.57
2	5	6	1	30	10.5	9.02
10	6	6	2	32	5.8	5.72
20	7	4	2	32	4.3	4.62
7	8	2	3	34	3.7	4.76
19	9	4	2	32	4.3	4.62
17	10	4	2	32	4.3	4.62
15	11	4	2	32	4.3	4.62
18	12	4	2	32	4.3	4.62
11	13	4	1	32	5.4	5.70
3	14	2	3	30	2.9	1.66
12	15	4	3	32	4.2	3.54
9	16	2	2	32	3.6	3.52
14	17	4	2	34	4.6	4.67
8	18	6	3	34	5.9	4.71
13	19	4	2	30	4.3	4.57
4	20	6	3	30	2	3.01

Table 5 — ANOVA for the quadratic polynomial model for ethanol production

Analysis of variance table (Partial sum of squares - Type III)						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	41.12	9	4.57	4.21	0.0174	significant
<i>A</i> -Inoculum conc	12.10	1	12.10	11.16	0.0075	
<i>B</i> -Nutrient	11.66	1	11.66	10.75	0.0083	
<i>C</i> -Temperature	0.025	1	0.025	0.023	0.8823	
<i>AB</i>	4.80	1	4.80	4.43	0.0616	
<i>AC</i>	0.98	1	0.98	0.90	0.3642	
<i>BC</i>	10.58	1	10.58	9.75	0.0108	
<i>A</i> ²	0.12	1	0.12	0.11	0.7461	
<i>B</i> ²	0.26	1	0.26	0.24	0.6332	
<i>C</i> ²	4.602E-003	1	4.602E-003	4.243E-003	0.9493	
Residual	10.85	10	1.08			
Lack of Fit	10.85	5	2.17			
Pure Error	0.000	5	0.000			
Total	51.97	19				

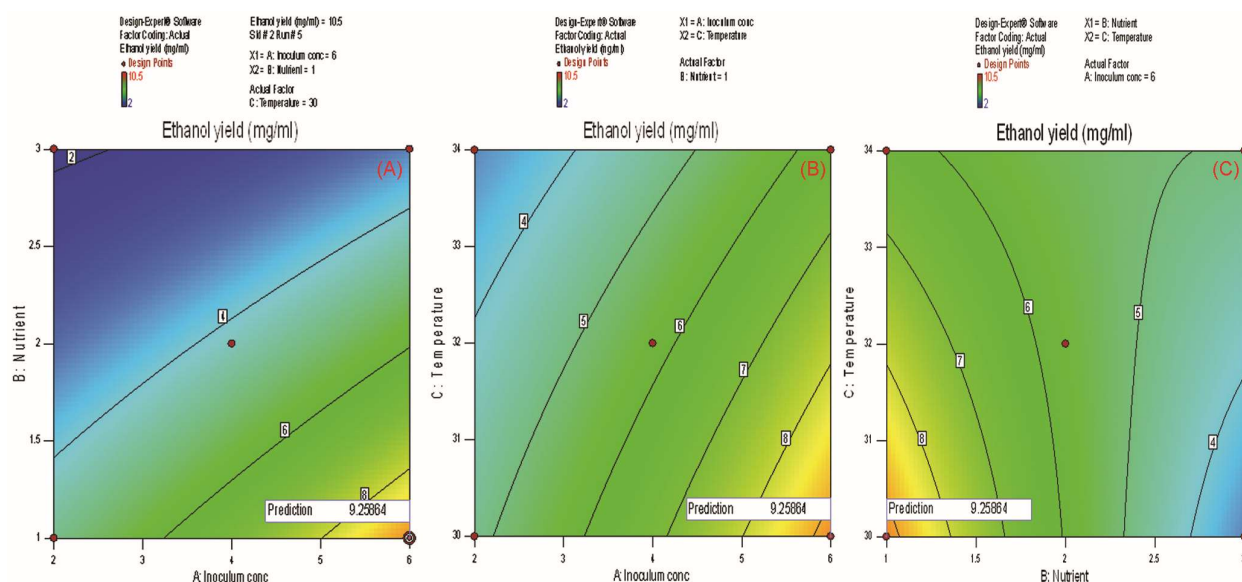


Fig. 4 A — Isoresponse contour plot for the effect of inoculum concentration versus nutrient on ethanol production.

Fig. 4 B — Isoresponse contour plot for the effect of inoculum concentration versus incubation temperature on ethanol production.

Fig. 4 C — Isoresponse contour plot for the effect of nutrient versus incubation temperature on ethanol production.

the optimum values for temperature (30°C), inoculum level (6%) and fermentation medium (composed of ammonium sulphate, KH_2PO_4 , peptone and yeast extract) for *P. tannophilus* MTCC 1077. Maximum ethanol concentration 10.5 g/l was obtained after 72 h from *P. tannophilus* MTCC 1077 at the optimized process conditions in anaerobic batch fermentation.

Discussion

The presence of cellulose and hemicellulose together make the total carbohydrate content of the

substrate (68.0%). It can be fairly compared with the extensively explored lignocelluloses (corn stover, 58.29%; wheat straw, 54%; poplar, 58.2%; birch 73%; spruce, 63.2%; sugarcane bagasse, 70%) for ethanol production²⁷.

The presence of lignin in cellulosic substrates and the crystalline nature of cellulose make it inaccessible to cellulase and their coordinated action³⁰. 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 digestibility³¹. 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 sugars³². Therefore, lignocellulosic biomass is pretreated with either chemically or biologically prior to enzymatic hydrolysis. Pretreatment of lignocellulose with acids primarily reduces lignin content along with some fractions of hemicellulose while decrystallizing cellulose. Thus, it affects both micro- and macro-accessibility of the cellulases to the cellulose³³.

The sugars liberated in the filtrate are hemicelluloses sugars. It is because of amorphous nature of hemicelluloses that they are easily degraded. The cellulose has a majority crystalline character and its structure is not so easily broken by a mineral acid, especially when it's at low concentration, as in this work. One percent HNO₃ steam explosion pretreatment has been interpreted as representing the hydrolysis of the easy to hydrolysis fraction of the xylan. Peaks and area covered by different sugars representing the above percentages can be seen in Figure 1. One aspect that should be emphasized is that high concentration achieved, especially for xylose was due to the scale used. The average volume of hydrolysate was 10ml, making the sugar stay more concentrated. At larger scale, the concentration value would be lower because the volume of hydrolysate would be greater.

Pretreatment of lignocellulose with either alkali or lime is rather similar to the kraft paper pulping technology. These chemicals remove a considerable fraction of carbohydrate in addition to lignin that lowers the recovery of fermentable sugars³⁴. It has been reported that the cell wall structure and components are significantly different in plants, which may influence the biomass digestibility³⁵. 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 h³⁶. Chemical pretreatment not only removes lignin but also acts as a swelling agent, which enhances surface area of the substrate accessible for enzymatic action³⁷.

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 hydrolysis³⁸. 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^{39, 40}. Huang and Penner³⁹ 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 Liaw⁴⁰ 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 substrate⁴¹. Zheng *et al.*⁴² observed that high enzyme loadings did not alter saccharification and yields. Rezende *et al.*³¹ reported 72% cellulose conversion from consecutive acid-base pretreated bagasse. Approximately 65% cellulose conversion was obtained after the enzymatic hydrolysis (1.91% w/w pretreated bagasse, 20 FPU/g enzyme loading, 0.05 g/g surfactant) of bagasse pretreated with dilute sulfuric acid (1.75% w/w bagasse content, 1.7% w/w HNO₃ loading, 150°C, and 30 min pretreatment time)⁴³. Kristensen *et al.*⁴⁴ also observed similar effect on wheat straw cell walls after hydrothermal pretreatment. It is possible that the acid pretreatment makes the lignin more receptive to cellulase adsorption through a change of surface properties, e.g. increased hydrophobicity or hydrogen bonding capacity. It is also possible that the treatment dissolves hemicelluloses associated with or covering lignin, thereby increasing the accessibility of lignin and hence the adsorption⁴⁵.

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 report⁴⁶. These findings are also in agreement with the results of yield of glucose after the enzymatic hydrolysis of pretreated substrate with crude cellulase.

The pair of the independent variables inoculum concentration and nutrient shows a less interactive effect (Figure 4 A) while keeping the third independent variable, temperature at 30°C. From Figure 4 A, it was evident that the interactive effects between the test variables were less significant not only from the circular nature of the contour plot and also from the probability value $P > 0.0616$ shown in Table 5.

The other pair of the independent variables inoculum concentration and temperature also shows a less interactive effect (Figure 4 B) while keeping the third independent variable, nutrient at 1. From Figure 4 B, it was evident that the interactive effects between the test variables were less significant not only from the circular nature of the contour plot and also from the probability value $P > 0.3642$ shown in Table 5.

The significant interaction between nutrient and temperature was apparent not only from the elliptical nature of the contour plot (Figure 4 C), but also from the low probability value ($P > 0.0108$) Table 5. The contours around the stationary point were elliptical and it became elongated more and more along the inoculum concentration axis, which meant that a small change of the response value would require a small move along the inoculum concentration axis. It was evident that the ethanol concentration steadily increased with increasing inoculum concentration upto 6%. So a higher inoculum concentration enhance the ethanol yield.

The response surfaces can be used to predict the optimum range for different values of the test variables and the major interactions between the test variables can be identified from the circular or elliptical nature of the contours. The circular nature of the contours signify that the interactive effects between the test variables are not significant and optimum values of the test variables can be easily obtained.

Among yeasts, *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)⁴⁷.

To the best of our knowledge this is first report on *Litchi chinensis* peels for ethanol production, hence we can only take a comparative account of few parallel researches done on the other fruit peels as far as ethanol production is concerned. About 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 respectively⁴⁸. Wilkins *et al.*⁴⁹ 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. Ethanol production of 6.84 g/l was reported from citrus waste pulp using *S. cerevisiae*⁵⁰.

A group of researchers studied ethanol production using the wastes abandoned from pineapple canning manufactures in anaerobic cultures of several yeasts⁵¹. This group reported that ethanol yield from sugar, 0.33 to 0.35 g ethanol/g sugar was remarkably lower compared with theoretical maximum, 0.50. This was caused by a high temperature cultivation at 40°C due to unavoidable ethanol evaporation during the cultivation. We found that temperature above 30°C was not conducive for ethanol production. Jongsomchai *et al.*⁵² suggested that the batch fermentation was more attractive to be used for ethanol production from squeezed juice of pineapple peels and stems than fed-batch fermentation. Bhatia and Paliwal⁵³ also found ammonium sulphate and yeast extract best suited for growth of *P. tannophilus*.

Peel of *L. chinensis* is a potential, renewable and low cost biomass for the production of ethanol by fermentation. One percent HNO₃ pretreated *L. chinensis* peel showed the maximum saccharification. litchi peel waste might be used as a low-cost material for bioethanol production, thus representing the partial valorisation of litchi peel industrial residues. It can therefore be concluded that bioethanol can be extracted through simultaneous saccharification and fermentation from the peelings of *L. chinensis* using the yeast *P. tannophilus* MTCC 1077 at the optimized process conditions in anaerobic batch fermentation.

The researchers would recommend the litchi-made product manufacturers to store the peelings of the litchi they make use of. Lastly, the researchers would like to recommend scientists to extract bioethanol from litchi peelings through the SSF process using *P. tannophilus* MTCC 1077 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.

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