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ORIGINAL ARTICLE

Ethanol production from Kinnow mandarin (*Citrus reticulata*) peels via simultaneous saccharification and fermentation using crude enzyme produced by *Aspergillus oryzae* and the thermotolerant *Pichia kudriavzevii* strain

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Abstract The aim of this study was to assess the potential of using the crude filtrate extract (CFE) produced by a newly isolated strain of *Aspergillus oryzae* and fermentation with a novel thermotolerant strain of *Pichia kudriavzevii* for the production of ethanol from kinnow peel waste (KP) via simultaneous saccharification and fermentation (SSF). High-performance liquid chromatography determination showed that pre-hydrolysis of KP with CFE at 3 cellulase filter paper units/g dry substrate (FPU/g-ds) at 50°C resulted in 24.87±0.75 g l^{-1} glucose, 21.98±0.53 g l^{-1} fructose, 10.86±0.34 g l^{-1} sucrose and 6.56±0.29 g l^{-1} galacturonic acid (GA) along with insignificant amounts of arabinose, galactose and xylose. Simultaneous saccharification and fermentation of hydrothermally pretreated KP at a substrate concentration of 15% (w v^{-1}) was conducted in a 2.5-l laboratory fermentor with *P. kudriavzevii* at 40°C after a 3-h pre-hydrolysis. Oligosaccharides were not produced during the SSF process. Ethanol production leveled off after 12 h, resulting in an ethanol concentration and productivity of 33.87 g l^{-1} and 2.82 g l^{-1} h^{-1}, respectively. These results demonstrate the potentiality of SSF using crude enzymes and *P. kudriavzevii* for the scale-up production of ethanol from KP.

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Introduction

Demand for fuels produced from renewable sources has increased in recent years due to increased oil prices, concerns about greenhouse gas production and increasing dependence on foreign sources of energy (Hill et al. 2006). The development of energy from renewable sources has the potential to provide domestic energy supplies, while reducing net greenhouse gas emission and developing a more favorable energy balance than traditional petroleum production (Farrell et al. 2006). Unlike fossil fuels, ethanol is a renewable energy source produced through the fermentation of sugars. It is currently used as a partial gasoline replacement in a number of countries in the world. Kinnow mandarin (*Citrus reticulata*) belongs to the citrus family of fruits and is an economically important fruit grown in India and Pakistan. According to Food and Agriculture Organization of the United Nations statistics, India produces around 7.13 million tonnes of citrus fruits annually, of which kinnow mandarin accounts for about 15%. Because of its high juice content and sweet taste, there has been an upsurge in kinnow production in India during the past few years. Kalra et al. (1989) reported that kinnow mandarin processing residues, composed of peels, pulp and seeds, are rich in carbohydrates and account for about 55–60% of the weight of the raw fruit. Dhillon et al. (2004) proposed that citrus peel waste could be used to produce important commercial products, such as enzymes,

ethanol, microbial biomass, volatile flavoring compounds, organic acids and antioxidants. The residues obtained from citrus fruits are rich in fermentable sugars, such as glucose, fructose, sucrose and galactose, as well as in insoluble polysaccharides, such as cellulose, hemicellulose and pectin (Grohmann et al. 1995; Oberoi et al. 2011). Despite this richness in sugars, proteins and minerals, fruit residues do not have any significant commercial use or value in India and are disposed off in municipal bins, leading to environmental pollution problems.

The low lignin content in citrus fruit residues relative to other lignocellulosic substrates renders the former attractive substrates for ethanol production as there is no need for expensive and energy-intensive pretreatment. Fruit residues may also offer substrate flexibility in the biomass-to-ethanol conversion process for use as biofuel in countries producing substantial quantities of fruits. The successful hydrolysis of citrus peel waste into sugars and their subsequent conversion into ethanol have been reported (Grohmann et al. 1994; Oberoi et al. 2011; Wilkins et al. 2007a, b). Previous studies conducted on the production of ethanol from citrus fruit residues used commercial enzymes and wild-type strains of *Saccharomyces cerevisiae* or recombinant *Escherichia coli* (Grohmann et al. 1994; Wilkins et al. 2007a). The total cost of cellulosic ethanol production is very high, but the cost can be drastically reduced if in-house produced enzymes are used for saccharification (Kadam 1996). According to Limtong et al. (2007), an ideal microorganism used for ethanol production must have rapid fermentation potential, appreciable thermotolerance, ethanol tolerance and high osmotolerance. Simultaneous saccharification and fermentation (SSF) increases the hydrolysis rate by reducing the product inhibition of enzymes, reduces tank usage by combining the saccharification and fermentation tanks into one tank, simplifies the operational process and decreases the processing time, thereby improving process economics (Faga et al. 2010; Shen et al. 2008). Different optimum temperature requirements for enzymes and the microbial strain is a major limitation in the SSF process. Thus, there is an urgent need to have thermotolerant yeast strains which are able to ferment sugars effectively at higher temperatures than is possible with conventional *S. cerevisiae* strains. In the recent past, there has been an upsurge in interest in thermophilic microorganisms, mainly because of their faster reaction rates, higher product yield and higher product resistance to degeneration at higher temperatures (Banat et al. 1998). However, we have not come across any report or study in which the crude enzyme produced by *Aspergillus oryzae* has been used for hydrolyzing a fruit residue. Similarly, the use of *Pichia kudriavzevii* in ethanol production from any fruit residue or lignocellulosic biomass is as yet undocumented. The aim of this study, therefore, was to assess the potential of using crude enzymes obtained from a newly isolated *A. oryzae* strain and a novel thermotolerant *P. kudriavzevii* isolate in a SSF process for ethanol production using kinnow peel waste (KP) in a 2.5-l laboratory fermentor.

Materials and methods

Materials

Kinnow mandarin (*Citrus reticulata*) residues composed of peels, pulp and seeds were procured from a fruit processing unit located in Ludhiana, Punjab, India. The peels were manually separated and washed with distilled water to remove any extraneous material, cut into small pieces and dried at 70°C in a hot-air oven until completely dry. The dried peels were ground to a fine powder using an electric mill. Celluclast (C-2730), Novozyme 188 (C-6105), and pectinase (P-2611) were procured from Sigma-Aldrich (St. Louis, MO). The fungal isolate used for cellulase production was isolated from rice straw, while the yeast strain used for fermentation was isolated from sugarcane juice. Details on the isolation of fungal and yeast strains are presented in the following subsections. The dehydrated media and media ingredients and analytical grade chemicals were purchased from Hi-Media Laboratories Pvt Ltd (Mumbai, India) and Fisher Scientific, India, respectively. The standards used for high-performance liquid chromatography (HPLC) determinations, such as those for glucose, fructose, sucrose, xylose, arabinose, galactose and galacturonic acid (GA), were procured from Sigma-Aldrich, and those for oligosaccharides, such as cellobiose, cellotriose, xylobiose and xylotriose, were procured from Megazyme International (Bray, County Wicklow, Ireland).

Isolation of cellulase-producing fungal strains

Rice straw with particle size of about 1 cm was impregnated with a nutrient solution composed of 2 g l^{-1} each of yeast extract, peptone and ammonium sulfate. The biomass was mixed with nutrient solution to a moisture content of 70% and exposed to ambient conditions for about 2 weeks. Fungi were isolated by the serial dilution method using sterilized Rose Bengal Chloramphenicol (RBC) agar. The plates were incubated at 30°C for 4–6 days and observed regularly for fungal colonies. Different characteristic colonies based on their morphology, color and shape were picked and purified by separate inoculation on sterilized RBC agar plates. The structures bearing spores and the arrangement of spores were examined microscopically. The cellulose hydrolytic capability of the isolated colonies was examined by observing the clear zone on inoculated culture

plates containing potato dextrose agar (PDA) medium supplemented with 1% carboxymethylcellulose (CMC) and congo red dye. The cellulase production capability of the isolates showing clear zones was further confirmed by the filter paper cellulase (FP) assay. The isolate which showed the highest FP activity was used for enzyme production. The isolate was identified through molecular characterization, as described in a subsequent section.

Enzyme production and extraction

The isolated fungal culture which showed highest FP activity among those isolates showing clear zones on the PDA medium plates containing CMC and congo red dye was selected for the assays of cellulase production. Spores from this isolate were harvested following inoculation on PDA plates and incubation at 30°C for 72 h. The plates were removed from the incubator, and spores were harvested using sterile water. The spore count was determined with a hemocytometer, and the spore concentration of 1×10^8 spores ml^{-1} was used for enzyme production. Solid state fermentation using rice straw and wheat bran at a ratio of 4:1 was employed for cellulase production. All of the enzyme production experiments were conducted in 250-ml Erlenmeyer flasks, with each flask containing 10 g substrate. The initial moisture content of 75% was made up with Mandel Weber medium, and the pH of the initial fermentation medium was set at 5.0 with 5N NaOH before sterilization. The flasks sterilized in the autoclave for 15 min, were cooled, inoculated with 1 ml fungal spore suspension and incubated at 30°C for 96 h. A set of three flasks was removed at 24-h intervals from the incubator and analyzed for cellulase production. Enzyme extraction from the flasks was done by the addition of a suitable volume of citrate buffer (0.1 M, pH 4.8) followed by vortexing, filtration and centrifugation. The supernatant was collected and analyzed for FP, endogluconase (CMCase), pectinase (exopolygalacturonase), xylanase and β-glucosidase activities. It was also used for enzymatic hydrolysis and for SSF, for which it was filtered through 0.2-μm PVDF membrane filter into a sterile bottle. Crude filtrate extract (CFE) was used immediately for the SSF process or stored refrigerated for 72 h prior to use. The CFE was analyzed for concentration of sugars, such as glucose, fructose, galactose, xylose and arabinose, as per the method described in the Analytical methods section.

Propagation of yeast cells

The yeast strain used during fermentation in our study was isolated from freshly extracted sugarcane juice (Dhaliwal et al. 2011). The isolated yeast culture was aseptically inoculated into 150-ml Erlenmeyer flasks containing 50 ml sterilized yeast extract peptone dextrose (YPD) broth. The pH of the medium was adjusted to 5.2, and the flasks were incubated at 40°C for 48 h and 120 rpm in an incubator shaker. The physiological growth pattern was studied using the culture density (OD_{600}), cell concentration and cell biomass for the isolated yeast strain grown in YPD broth under the incubation conditions described previously. The results revealed that the cells entered the log phase around 3 h and the stationary phase around 15 h, and that the cell concentration and cell biomass declined after 18 h (data not shown). Based on these data, 10 ml of the inoculum was aseptically transferred to 250-ml Erlenmeyer flasks containing 100 ml sterilized YPD broth and the flasks were incubated at 40°C for 15 h at 100 rpm in an incubator shaker. Subsequently, 50 ml inoculum from these flasks was aseptically transferred to 1-l flasks containing 500 ml sterilized YPD broth, and the flasks were then incubated under the same conditions as mentioned above. Fresh YPD medium was prepared just before each fermentation experiment. The cells were concentrated to a level of 2×10^9 cells ml^{-1} by centrifugation at 10,000 *g*, 4°C for 10 min.

Molecular characterization of identified fungal and yeast isolates

Genomic DNA was extracted from overnight cultures of the fungal and yeast isolates using a fungal genomic DNA isolation kit (Chromous Biotech Pvt Ltd, Bangalore, India). For molecular characterization of the fungal strain, the D1, D2 and D3 domains of the 26S rRNA gene were amplified using the primer set LROR (5′-ACCCGCTGAACTTAAGC-3′) and LR7 (5′-TACTACCACCAAGATCT-3′). The ITS1 and ITS4 rDNA region of the yeast isolate was amplified by PCR using primer set pITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and pITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The PCR analysis was performed in 25 μl of reaction volume containing 50–100 ng of genomic DNA, 1× Taq buffer, 1.5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} of each dNTP, 50 ng of each primer, and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India). The cycling conditions were 95°C for 4.5 min, followed by 30 cycles at 95°C for 30 s, 40°C for 30 s and 72°C for 1 min, with a final extension of 10 min at 72°C on a MiniOpticon thermal cycler (Bio-Rad, Hercules, CA). Amplification was verified by electrophoresis on a 1.5% (w v^{-1}) agarose gel in 1× TAE buffer using a 100-bp ladder (Bangalore Genei) as a molecular-weight marker and was visualized using ethidium bromide (1 mg ml^{-1}) stain in an Alphaimager EP Geldoc system (Alpha Innotech, San Leandro, CA). The 918- and 485-bp amplicons thus obtained for fungal and yeast isolates, respectively, were sequenced with an ABI 3130 genetic analyzer (Applied

Biosystems, Foster City, CA), and the data obtained were analyzed using BioEdit sequence analysis program (Hall 1999).

Effect of pretreatment and optimization of enzyme concentrations

Kinnow peel waste powder was suspended in distilled water at 15% (w v^{-1}) substrate concentration and supplemented with 2 g l^{-1}, each of yeast extract, $MgSO_4$ and peptone in polycarbonate capped flasks. A substrate concentration >15% (w v^{-1}) results in a solid viscous mass not easily amenable to enzymatic hydrolysis. The flasks were sterilized in an autoclave for 15 min, removed from the autoclave while hot and opened in the laminar flow bench under sterile air to allow the release of volatile compounds, such as D-limonene, which are considered inhibitory to yeasts (Wilkins et al. 2007b). In a previous study, we observed a drastic reduction in D-limonene concentration in kinnow waste obtained after the hydrothermal pretreatment of the dried biomass (Oberoi et al. 2011). The pH of the medium was adjusted to 5.5 using sterilized 5 N sodium hydroxide solution. In a previous study, we had optimized the enzyme concentrations using a statistical design for ethanol production from kinnow waste (Oberoi et al. 2011). It is noteworthy to mention here that the composition of kinnow peel and kinnow waste is different and since this study involved the use of crude enzymes, we decided to use commercially available enzymes at different concentrations before finalizing the crude enzyme concentration to be used during SSF. The enzyme concentration range was selected on the basis of previous studies (Oberoi et al. 2011; Wilkins et al. 2007a, b). The enzyme levels selected for commercial enzymes were 0–100 IU per gram dry substrate (g-ds) for pectinase, a cellulase loading of 0–8 filter paper units (FPU) g-ds^{-1} and β-glucosidase at 0–16 IU g-ds^{-1}. The ratio of FPU to β-glucosidase units was maintained at 1:2 as no cellobiose was detected during the hydrolysis of kinnow waste using cellulase and β-glucosidase in the ratio 1:2 (Oberoi et al. 2011). Commercial enzymes were appropriately diluted with citrate buffer (0.1 M, pH 4.8), filtered through 0.45-μm PVDF membrane (Millipore India Pvt Ltd, Bangalore, India) and added at the various selected concentrations to the flasks containing KP and the other nutrients mentioned previously. The flasks were subsequently inoculated with 10% yeast inoculum having a cell concentration of 2×10^9 cells ml^{-1} and incubated at 40°C, 100 rpm for 15 h in an incubator shaker. The experiment was conducted in triplicate, and the supernatant was separated after centrifugation. Ethanol was determined in the supernatant using ethanol membranes described elsewhere in the paper.

Enzymatic hydrolysis using crude enzyme

A separate experiment was conducted to analyze the effect of enzymatic hydrolysis on the biomass constituents in the residual biomass. KP powder was suspended in distilled water in the capped polycarbonate flasks. Water was added during the pretreatment in such a way so as to maintain the substrate concentration at 15% (w v^{-1}) after the addition of the enzyme. The flasks were sterilized in an autoclave for 15 min, cooled and the filtered CFE was added to the flasks containing pretreated substrate. Hydrolysis was performed at 50°C for 24 h at 120 rpm in an incubator shaker. A set of three flasks was removed at 6-h intervals, and the contents were filtered under vacuum using a Buchner funnel lined with Whatman filter paper (Whatman, New York, NY). The solid residue was dried in a hot-air oven at 70°C, and the dried biomass was analyzed for cellulose, hemicellulose, lignin and ash contents. All experiments were conducted in triplicate, and the statistical analysis was performed to analyze the difference in treatment means.

Ethanol production in a laboratory batch fermentor

On the basis of the results obtained from the initial experiments, in the batch reactor experiments, we used crude enzymes at a concentration of 3 FPU g-ds^{-1}. The batch fermentation experiment was performed in a 2.5-l batch reactor (Minifors, Infors HT, Switzerland). A 200-g sample of KP (moisture content 92%) was suspended in 600 ml water and supplemented with 2.0 g l^{-1} yeast extract, 2.0 g l^{-1} peptone and 1.0 g l^{-1} $MgSO_4{\cdot}H_2O$. The fermentor along with a calibrated pH probe, temperature probe, inlet air/gas provisions, condensate removal system, agitator, sample collection system, NaOH (5 N) solution and exhaust system was sterilized in an autoclave for 15 min. The fermentor was removed from the autoclave, sparged with sterile air to remove the volatile compounds and then cooled to 50°C by immersion in cold water. The pH of the medium was adjusted to 5.0 with the sterilized 5 N NaOH solution, and 567 ml of filtered CFE was added to the fermentor, which accounted for an FP activity of 3 FPU g-ds^{-1}. Hydrolysis of KP was performed at 50°C for 3 h at 120 rpm and a pH of 5.0. After a 3-h pre-hydrolysis, the temperature of the medium was brought down to 40°C, and the medium was inoculated with 10% (v v^{-1}) yeast cells at a cell concentration of 2×10^9 cells ml^{-1} in the inoculum. Agitation, pH and temperature were maintained at 120 rpm, 5.0 and 40°C, respectively, throughout the fermentation process. The temperature was selected to accommodate the enzymes as well as the fermenting microbial strain in the same vessel at the same time. Samples were drawn at 6-h intervals up to 24 h and analyzed for sugars, ethanol and GA concentrations. The

experiment was conducted three times in the same fermentor, and the results were statistically analyzed.

Analytical methods

Arabinose, fructose, glucose, sucrose, xylose and oligosaccharides, such as cellobiose, cellotriose, xylobiose and xylotriose, were analyzed with HPLC (Dionex Corp, Sunnyvale, CA) using a Shodex SP-0810 column (300× 7.8 mm) fitted with a SP-G guard column (Waters , Milford, MA). Degassed HPLC grade water was used as a mobile phase at a flow rate of 1.0 ml min^{-1}. The column oven and refractive index (RI) detector were maintained at 80°C and 50°C, respectively. Samples were diluted, centrifuged, and filtered through 0.45-μm RC membranes (Phenomenex Corp, Santa Clara, CA). Peaks were detected by the RI detector and quantified on the basis of area and retention time of the standards. Galacturonic acid concentration was determined using the modified dinitrosalicylic acid method described previously (Wang et al. 1997). Ethanol was determined with YSI 2786 ethanol membrane kits using YSI 2700 Select biochemical analyzer (YSI Inc, Buffalo, NY). The instrument was calibrated using the ethanol standards of 2.0 g l^{-1} and 3.2 g l^{-1} provided by the manufacturers. Samples were adequately diluted, centrifuged and injected into the instrument for analysis following the manufacturers' specified procedure.

Cellulose content was analyzed using a gravimetric method employing acetic nitrate reagent (Pereira et al. 1998), while hemicellulose was determined by subtracting acid detergent fiber (ADF) from neutral detergent fiber (NDF) using the method previously described by Goering and Vansoest (1970). Moisture and ash contents in the samples were determined by the AOAC method (2000). Sugars were extracted with ethanol and analyzed by the method described previously by Oberoi et al. (2011). Enzyme assays for FP activity, CMCase and xylanase were performed using previously described methods (Bailey et al. 1992; Ghose 1987; Wood and Bhat 1988). The concentration of reducing sugars (RS) was determined using the DNS reagent with glucose as a standard (Miller 1959). β-Glucosidase activity was estimated using *p*-nitrophenyl-β-*D*-glucopyranoside (pNPG) as a substrate (Oberoi et al. 2010b). Pectinase was determined in terms of exopolygalacturonase activity as per the previously described procedure of Oberoi et al. (2010a).

Statistical analysis

All experiments were carried out in triplicate, and the mean and standard deviation (SD) values were calculated using the MS Excel program. The significance for the treatment means was determined with JMP software (SAS, Cary, NC)

Results and discussion

Isolation and screening of cellulolytic microbial strains

Among the 12 fungal isolates which showed characteristic diversity in terms of colony morphology, spore color and microscopic spore characteristics, only four isolates showed clear zones on the PDA plates containing CMC and congo red. These four isolates were identified on the basis of morphological characterization and microscopic examinations. All four isolates belong to the genus *Aspergillus* and were screened for FP activity. Filter paper cellulase activity is a relative measure of the overall cellulose-hydrolyzing capacity of microbial cellulase preparations (Urbanszki et al. 2000). Isolate I showed the highest FP activity among the four strains and it also produced a higher enzyme concentration in a shorter time (Fig. 1). Thus, this isolate was used for the assays on enzyme production. Isolate I was identified and characterized on the basis of sequencing of the 26S rDNA region, which are described in detail later in this paper.

FP activity and CMCase, β-glucosidase and xylanase production of the selected isolate increased until 72 h, levelling off thereafter, whereas exopolygalacturonase activity levelled off after 96 h (Fig. 2). Fungi initially consumed the readily available sugars and produced hydrolytic enzymes; following depletion of the sugar concentration, particularly when the glucose concentration was low, the fungi began to use these hydrolytic enzymes for the production of sugars, resulting in a decrease in enzyme activity. In a previous study, we observed a similar trend in enzyme production using mixed-culture solid-state fermentation (Oberoi et al. 2010a). The major factors which determine the enzyme production capability of a culture are the C:N ratio, amount of available sugars in the fermentation medium, concentration of insoluble polysaccharides, such as cellulose, hemicellulose and pectin, and cultural

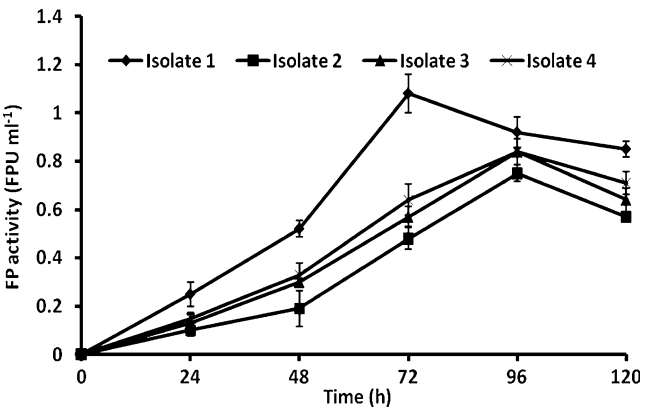

Fig. 1 Screening of four different isolates for filter paper cellulase (FP) activity

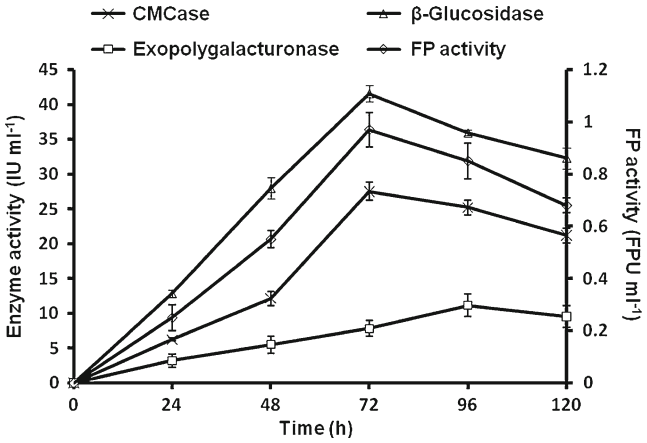

Fig. 2 Effect of incubation time on enzyme production by the newly isolated strain of *Aspergillus oryzae*

conditions. On the basis of our results, we decided to harvest the enzyme after 72 h for use during SSF.

Identification and characterization of fungal and yeast strains

Sequencing and analysis of the 26S rDNA region of the isolated fungal strain and of the ITS rDNA region of the isolated yeast strain revealed that these regions had the highest identity with *Aspergillus oryzae* and *Pichia kudriavzevii* (*Issatchenkia orientalis*), respectively. Phylogenetic relationships were inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known microorganisms. The isolated fungal and yeast strains and *A. oryzae* (NCBI accession no: AP007172) and *I. orientalis* (NCBI accession nos EF568018 and EF568014), respectively, belonged to the same branch. *I. orientalis* is a National Center for Biotechnology Information (NCBI) synonym for *P. kudriavzevii*. As per the molecular phylogenetic work of Kurtzman et al. (2008), the species ascribed to the genus *Issatchenkia* has been clustered within *Pichia*, and thus *Issatchenkia orientalis* has been replaced by *Pichia kudriavzevii* as the taxonomically valid entity. Based on morphology and the comparison of 26S rRNA and ITS rDNA gene sequences, the isolated fungal and yeast strains were identified as strains of *A. oryzae* and *P. kudriavzevii*, respectively. The 26S rRNA gene sequences and ITS sequences for the newly isolated strains of *A. oryzae* and *P. kudriavzevii* were submitted to GenBank under accession numbers HQ 122940 and HQ 122942, respectively. The isolates of *A. oryzae* and *P. kudriavzevii* have been deposited with the National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau Nath Bhanjan, India. There are a number of published reports on ethanol production using *I. orientalis*, whereas we have not come across any report on ethanol production using *P. kudriavzevii* from any fruit residue or cellulosic biomass, although both designations are synonyms for the same organism. We have recently reported ethanol production from sugarcane juice using *P. kudriavzevii* (Dhaliwal et al. 2011). Therefore, the literature reporting use of *I. orientalis* for ethanol production is cited elsewhere in this paper.

Effect of pretreatment on the composition of kinnow mandarin peel

Kinnow mandarin peel waste contains sugars, cellulose, hemicellulose and pectin (Table 1). Cellulose, hemicellulose and pectin can be enzymatically hydrolyzed to yield fermentable sugars. The presence of lignin in low concentrations (Table 1) renders KP an ideal substrate for ethanol production. Lignin in the lignocellulosic biomass binds to the cellulose and hemicellulose fractions, thereby reducing their accessibility to enzymes. Because of its composition, KP can potentially serve as a good substrate for ethanol production, especially for use as a biofuel. The increase in sugar concentration in ethanol extracts in pretreated KP accounted for the increase in total content when compared with KP (Table 1). The sterilization pretreatment facilitated the solubilization and subsequent extraction with ethanol of some of the sugars in KP that were strongly bonded to the insoluble polysaccharide fractions (Oberoi et al. 2011). The observed significant increase in cellulose concentration in pretreated KP (Table 1) indicates that the sterilization pretreatment did not hydrolyze cellulose.

Because of the sterilization hydrothermal pretreatment, sugars bound to the polysaccharide fractions solubilized, leading to a higher concentration of cellulose, hemicellulose and lignin in the pretreated KP (Oberoi et al. 2011). However, pretreatment led to partial solubilization of pectin (Table 1). Cellulose forms intra-molecular hydrogen bonds between adjacent glucose molecules. Pectin forms a matrix in which the cellulose microfibrils are embedded and bind adjacent cell walls together making it a fairly resistant

Table 1 Compositional analysis of Kinnow mandarin peels

Composition (%)	Kinnow peel	Pretreated kinnow peel
Cellulose	10.72±0.36	12.85±0.41
Hemicellulose	3.88±0.27	4.36±0.23
Pectin	22.88±1.24	16.45±0.95
Sugars in ethanol extract	29.66±1.48	37.12±1.74
Ash	3.52±0.19	3.89±0.20
Lignin	1.91±0.15	2.5±0.17
Protein	5.65±0.34	6.12±0.40

Data are presented as the mean ± standard deviation (SD) of $n=3$ trials

Phenolic compounds and fat account for the remainder of the composition

complex. We observed a similar trend in the profile of constituents after sterilization pretreatment of kinnow waste (Oberoi et al. 2011). Steric hindrance of cellulose and hemicellulose hydrolysis by pectin is supported by the cell wall model for flowering plants which states that a pectin matrix surrounds cellulose fibers coated with xyloglucan (Carpita and Gibeaut 1993). Mild pretreatment is thus essential for partial solubilization of hemicellulose and pectin, which otherwise are strongly bonded to each other. Pretreatment increases the surface area allowing the hydrolyzing enzymes to work more efficiently on pectin and cellulose (Wilkins et al. 2007b).

Simultaneous saccharification and fermentation using commercial enzymes

Ethanol concentration increased by about 80% with the use of cellulolytic and pectinolytic enzymes relative to the control for which no enzymes were used (Table 2). The absence of either cellulase or pectinase during SSF adversely affected ethanol yield (Table 2). Ethanol production with cellulase (3 FPU g-ds^{-1}) and pectinase (50 IU g-ds^{-1}) did not differ significantly with higher cellulase and pectinase concentrations (Table 2). Wilkins et al. (2007b) made similar observations during hydrolysis studies of citrus peel waste with cellulolytic and pectinolytic enzymes. Therefore, we decided to use the crude enzyme at 3 FPU g-ds^{-1} during SSF for ethanol production, which means that the concentrations of β-glucosidase, endoglucanase, exo-polygalacturonase and xylanase in the CFE were 261, 210, 69, and 1560 IU g-ds^{-1}, respectively.

Hydrolysis with the crude enzyme consortium

No sugars were detected in the CFE used for the hydrolysis and ethanol production experiments. Hydrolysis of pre-treated KP with CFE resulted in the solubilization of cellulose and hemicellulose (Table 3), which in turn led to an increase in ash and lignin concentrations. Cellulose, hemicellulose or residual biomass concentrations did not show a significant decline after 18 h (Table 3). The solubilization of cellulose and hemicellulose by the hydrolytic enzymes led to an increase in ash and lignin contents. The observed decline in hydrolysis rate after 12 h (Table 3) may be mainly due to the increased ash and lignin contents, which may envelope the cellulose and hemicellulose fractions, thereby reducing their accessibility to enzymes. The limited availability of cellulose and hemicellulose after 18 h could also have led to a decline in the hydrolytic ability of the enzymes. It is possible that some of the catalytic sites in the crude enzyme were blocked by salts or small-molecular-weight polypeptides. No cellobiose, cellotriose, xylobiose or xylotriose were formed during hydrolysis of the pretreated KP with crude enzymes, indicating that the enzymes, such as β-xylosidase and xylan esterase, were present in the CFE. However, enzyme assays for β-xylosidase, α-L-arabinofuranosidase and xylan esterase were not performed in our study. In a previous study, we reported that the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of CFE produced by mixed-cultures of *Trichoderma reesei* and *Aspergillus oryzae* produced many characteristic bands (Brijwani et al. 2010). The significant drop in pH during the enzymatic hydrolysis of KP is caused by the increase in GA concentration. Pectin in the cell walls of fruit residues is composed of GA units linked to the sugar moiety composed of rhamnose, galactose, arabinose and glucose. Several weak organic acids, such as acetic, malic, malonic, lactic and citric acid, are known to be present in citrus peels (Grohmann et al. 1999), and these inhibit *S. cerevisiae* growth at a lower pH due to an increase in undissociated acids (Wilkins et al. 2007b). A continuous decline in pH

Table 2 Ethanol production by simultaneous saccharification and fermentation using different concentrations of commercial enzymes

Cellulase (FPU g-ds^{-1})	β-glucosidase (IU g-ds^{-1})	Pectinase (IU g-ds^{-1})	Ethanol (g l^{-1})
0	0	0	18.92±0.37
5	10	20	30.12±0.28
5	10	50	32.11±0.35
5	10	80	32.54±0.46
5	10	100	32.23±0.30
5	10	0	24.56±0.39
3	6	50	31.89±0.28
8	16	50	31.69±0.26
0	0	50	21.13±0.17
3	6	100	32.01±0.22
8	16	100	31.77±0.41
0	0	100	22.16±0.21

Data are given as the mean ± SD of n=3 trials

Table 3 Compositional changes during enzymatic hydrolysis of kinnow peel waste with the crude filtrate extract

Time (h)	Cellulose (%)	Hemicellulose (%)	Ash (%)	Lignin (%)	pH
0	12.85±0.41 a	4.36±0.23 a	3.89±0.20 a	2.50±0.1 7 a	5.0
6	6.90±0.37 b	2.77±0.24 b	5.15±0.34 b	2.76±0.80 a	4.2
12	4.24±0.28 c	1.70±0.27 b	5.35±0.19 b	2.95±0.11 b	3.9
18	2.86±0.20 d	0.89±0.06 c	5.89±0.18 c	3.25±0.08 c	3.7
24	1.83±0.09 e	0.88±0.16 c	6.25±0.19 c	4.02±0.08 d	3.5
Least significant difference ($p<0.05$)	0.57	0.38	0.41	0.69	

Data are given as the mean ± SD for $n=3$ trials. The SD for pH values was within 5% of the mean value

Mean values followed by the same lowercase letter do not differ significantly.

The calculations for constituents were made on the basis of the initial substrate concentration

might adversely affect the hydrolytic ability of the enzymes present in the CFE.

Ethanol production in a batch fermentor

Hydrolysis of pretreated KP with the CFE at concentrations described previously resulted in a significant increase in glucose and fructose concentrations during the 3-h pre-hydrolysis. Arabinose, xylose and GA together with the hexose sugars were also formed during the 3-h pre-hydrolysis (Table 4). No glucose, fructose or sucrose was detected after 12 h SSF, and their concentrations even at 6 h were low compared to their initial concentrations (Table 4), indicating rapid fermentation of these sugars by yeast cells. Sucrose is converted to glucose and fructose by the invertase present in the yeast cells, and such monomers are subsequently fermented to ethanol. Some of the sugars, such as glucose, fructose and galactose, produced by enzymatic hydrolysis of insoluble polysaccharides during SSF were fermented to ethanol. Ethanol concentration levelled off after 12 h (Fig. 3), which could largely be due to the non-availability of hexose sugars for fermentation. Fermentation beyond 12 h led to a significant reduction in volumetric productivity because of the drop in fermentation rate. Ethanol concentration and productivity obtained in our study are comparable with or higher than those reported in previous studies (Table 5). Although, higher ethanol concentrations were reported in some of the previous studies (Table 5), it should be noted that crude enzymes were used in the present study for the hydrolysis of KP.

Glucose and fructose obtained at high concentrations after a 3-h pre-hydrolysis of KP in the laboratory fermenter are the preferred substrates for yeasts and are consumed before other substrates (Gancedo 1998). A high concentration of inoculum, the harvesting of cells during the log phase and the availability of fresh medium may have reduced the lag phase for the yeast cells. An increase in xylose, arabinose and GA concentrations indicates that the *P. kudriavzevii* strain was not able to metabolize and ferment these compounds. *Issatchenkia orientalis* strains are not capable of metabolizing pentose sugars, such as xylose and arabinose (Kurtzman et al. 1980). Arabinose is found in pectin side chains, whereas polymers of GA form the backbone of pectin molecules (Carpita and Gibeaut

Table 4 Sugar consumption and galacturonic acid production during simultaneous saccharification and fermentation in a batch fermentor

Time (h)	Glucose ($g\ l^{-1}$)	Fructose ($g\ l^{-1}$)	Galactose ($g\ l^{-1}$)	Arabinose ($g\ l^{-1}$)	Sucrose ($g\ l^{-1}$)	Xylose ($g\ l^{-1}$)	Galacturonic acid ($g\ l^{-1}$)
0[a]	24.87±0.75 a	21.98 ±0.53 a	1.25 ±0.12 a	1.48±0.14 a	10.86±0.34 a	0.23±0.03 a	6.56±0.29 a
6	3.33±0.15 b	4.9±0.29 b	1.04±0.09 a	2.56±0.13 a	2.5±0.17 b	0.25±0.05 b	7.12±0.25 b
12	ND	ND	1.14 ±0.10 a	2.68±0.19 b	ND	0.36±0.06 b	8.25±0.27 c
18	ND	ND	1.04±0.15 a,b	3.25±0.09 b	ND	0.39±0.31 c	9.01±0.52 c,d
24	ND	ND	0.85±0.09 b	3.57±0.16 c	ND	0.48±0.08 d	9.84±0.34 d
Least significant difference ($p<0.05$)	0.63	0.50	0.21	0.26	0.30	0..27	0.63

[a] After a 3-h hydrolysis

Data are given as the mean ± SD for $n=3$ trials

Mean values followed by the same lowercase letter do not differ significantly

1993). Arabinose and GA might have solubilized because of the hydrolysis of KP by the pectinase present in the CFE. An increase in xylose concentration during hydrolysis may be due to the solubilization of hemicellulose by the xylanase and β-xylosidase present in the CFE. No glucose was left after 6 h during SSF, whereas the RS concentration increased after 12 h. This result could primarily be due to the increase in the concentrations of xylose, arabinose and GA, which are not fermented by *P. kudriavzevii* cells, as mentioned previously (Fig. 3). Grohmann et al. (1994) reported that ethanol production from enzymatically hydrolyzed orange peel using *S. cerevisiae* at 35°C and pH 5.0 did not increase beyond 12 h. As noted earlier, commercial enzymes are expensive and enzymes such as cellulase, β-glucosidase, xylanase and pectinase are added separately to the fermentation medium, thereby further increasing the production cost.

The concentration of ethanol produced in the batch fermenter was higher than that produced in the shake flask, mainly because the pH was controlled during the entire process in the batch fermentor, which means that the enzyme and yeast cells did not have to adapt to continuously decreasing pH conditions. In addition, the batch fermentor provided better operational conditions, such as agitation and contact between the cells and medium (Oberoi et al. 2011). We also believe that the 3-h pre-hydrolysis at 50°C helped to effectively release soluble sugars bound to the insoluble polysaccharide matrix and also to have partially hydrolyzed the insoluble fractions, as 50°C is considered to be the optimal temperature for effective hydrolysis by cellulase and pectinase. The residual biomass at the end of the SSF process could be exploited for use as cattle feed because of impregnation of biomass with yeast cells. In our study, we also used a newly isolated thermotolerant strain of *P. kudriavzevii*, which to the best of our knowledge has not been used for ethanol production from any lignocellulosic biomass or fruit residue. Traditional yeasts used for ethanol production, such as *S. cerevisiae*, cannot be used at temperatures in the vicinity of 45–50°C that are optimum for cellulases (Oberoi et al. 2011).The traditional yeasts used in industrial fermentations perform well within the temperature range 30–35°C, and their fermentative ability is compromised at temperatures >35°C (Sa-Correia and van Uden 1982; Wilkins et al. 2007b). Abdel-Banat et al. (2009) reported that a 5°C increase in fermentation temperature greatly affects the cost of fuel ethanol production.

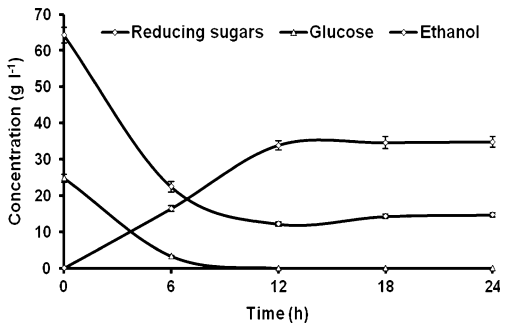

Fig. 3 Effect of fermentation time on sugar consumption and ethanol production during the simultaneous saccharification and fermentation (SSF) process in a laboratory fermentor

To maximize ethanol production, it is thus important to conduct SSF at temperatures close to optimum for cellulase, use thermotolerant yeasts for faster cellulose hydrolysis and a shorter fermentation time. Although the temperature during SSF was maintained at 40°C, we are now attempting to partially purify the crude enzyme obtained from the strain of *A. oryzae* used in this study and conduct SSF at temperatures ranging from 45 to 50°C with thermotolerant yeast strains in an attempt to produce ethanol from lignocellulosic biomass. A higher ethanol concentration has been reported from citrus peel waste by previous researchers using a combination of commercial enzymes, such as cellulase, β-glucosidase and pectinase, but the use of a CFE obtained from a strain of *A. oryzae* for ethanol production from any fruit residue is unprecedented. In addition, the use of a thermotolerant strain of newly isolated yeast strain of *P. kudriavzevii* holds promise for further evaluations. our results in terms of product yield and volumetric productivity are encouraging for future scale-up studies.

Conclusions

Hydrothermal pretreatment helped in partial solubilization of cellulose and pectin and also helped release sugars bonded to the insoluble fractions. A 3-h pre-hydrolysis with the crude enzyme produced by a newly isolated strain of *Aspergillus oryzae* prior to SSF resulted in production of glucose, fructose, galactose, arabinose, xylose, sucrose and galacturonic acid. The newly isolated thermotolerant strain of *Pichia kudriavzevii* fermented glucose, fructose, sucrose and galactose to ethanol whereas it could not metabolize arabinose, xylose and galacturonic acid produced by enzymatic hydrolysis during SSF. This study demonstrated that SSF with crude enzyme consortium obtained from *A. oryzae* and fermentation with *P. kudriavzevii* strain resulted in 33.87 g l^{-1} ethanol from kinnow peel in 12 h in a laboratory fermenter. Ethanol concentration during SSF leveled off after 12 h, suggesting high ethanol productivity for the process which means that a large number of batches could be completed in a short time. Hydrolysis with crude enzyme and ethanol productivity of 2.8 g l^{-1} h^{-1} in a SSF process indicates a good potential for scale-up studies.

Table 5 Comparative study of results obtained in the present study with those of previous investigations

Substrate	Pretreatment process		Microorganism used	Fermentation temperature (°C)	Ethanol concentration (g l^{-1})	Volumetric productivity (g l^{-1} h^{-1})	Reference
	Primary	Secondary					
Kinnow waste	Autoclave–sterilization at 15 psi for 15 min	SSF using commercial enzymes and galactose adapted cells	*Saccharomyces cerevisiae*	37	43.0	3.50	Oberoi et al. 2011
Orange peel hydrolysate	Separate hydrolysis and fermentation (SHF)		*S. cerevisiae* and *Kluveromyces marxianus*	35	37-40	0.50–0.55	Wilkins et al. 2007a
Citrus peel waste	Live steam 150–160°C for 2–4 min	SSF with commercial enzymes	*S. cerevisiae*	37	42	1.75	Wilkins et al. 2007b
Orange peel	0.5% (w/v) H_2SO_4 at 121°C for 15 min	Dilute acid hydrolysis	*S. cerevisiae*	30	30.3	3.37	Oberoi et al. 2010c
Kinnow:banana peel (4:6)	Autoclave–sterilization at 15 psi for 1 h	Enzymatic hydrolysis with crude enzymes	*Pachysolen tannophillus* and *S. cerevisiae*	30	26.84	0.74	Sharma et al. 2007
Citrus peel waste	High pressure steam at 70 psi	Enzymatic hydrolysis with commercial enzymes	*S. cerevisiae*	38	32.4	1.8	Zhou et al. 2008
Kinnow peel	Autoclave– sterilization at 15 psi for 15 min	Enzymatic hydrolysis with crude enzymes	*Pichia kudriavzevii*	40	33.87	2.82	This study

SSF Simultaneous saccharification and fermentation

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