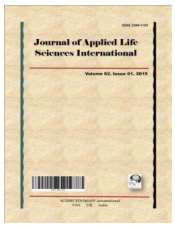

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Biomass Saccharification and Screening for Production of Pectinase from *Physocladia obscura* JEL 513

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aim: This study documents for the first time the production of pectinase by *Physocladia obscura* JEL 513 and the optimization of its production in solid-state fermentation.
Study Design: OFAT (One Factor At a Time).
Place and Duration of Study: Botany Department, Faculty of Biological Science, University of British Columbia, Vancouver BC, Canada between January and June 2016.
Methodology: *Physocladia obscura* JEL 513 was screened for its ability to grow on citrus peels and produce enzyme(s). After confirmation of pectinase production, several parameters of incubation time, pH and temperature on polygalacturonase activity were studied in screening and optimization investigations.
Results: The maximum yield of pectinase was 760.785 U/g at 25ºC in an orange waste peel medium of pH 6 after the fifth day of fermentation. High performance ion exchange liquid chromatography showed that, the concentration but not the relative monosaccharide composition changed in the investigated peels. Partial enzymatic degradation was evaluated by comparing the chemical thermal stability using a thermogravimetric technique of the fermented as well as the non-

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fermented citrus peels fibers.
Conclusion: These results suggest that *P. obscura* JEL 513 shows promise in transforming agro-waste into a substrate for commercial enzyme production with potential broad benefits for regional economies.

Keywords: Solid state fermentation; pectinase; Physocladia obscura JEL 513; citrus peels; saccharification.

1. INTRODUCTION

Solid state fermentation (SSF) is a technique that has been well known for centuries [1] and has lately become increasingly important. New interest in this technology derives from the fact that it is considered to be an appropriate approach for processes including the bioremediation or the biodegradation of toxic compounds, the detoxification of agricultural wastes, the biotransformation of crops and bio-pulping, etc. [2–4]. Moreover, SSF has been successfully applied in the preparation of new high value products, such as secondary metabolites, organic acids, pesticides, aromatic compounds, fuels and enzymes [5–9]. The advantages of SSF in comparison to traditional submerged fermentation are better yields, easier recovery of products, the absence of foam formation and smaller reactor volumes. Moreover, contamination risks are significantly reduced due to the low water contents and, consequently, the volume of effluents decreases [10]. However, SSF has some disadvantages 1) SSF is restricted to the microorganisms, which can grow at reduced moisture level and therefore the range of possible processes and products are limited 2) The removal of the metabolic heat generated during the growth may be a problem, especially at large-scale. 3) The solid nature of the substrate causes problems in the monitoring of the process parameters. 4) The mass transfer in the solid phase is limited to the diffusion. 5) The cultivation times are often longer. 6) The extracts containing the products obtained by leaching of the fermented solids are often viscous in nature. This high viscosity restricts vacuum concentration of the crude extract or further concentration leads to paste formation. Enzyme precipitates from such crude extracts are also gummy and resist drying, powdering, blending and reconstitution. 7) A major problem with SSF is that many important basic scientific and engineering aspects are yet to be characterized. Much of the work to-date is either qualitative or empirical due to the difficulties encountered in the quantification. 8) One of the major inherent problems is the difficulty in retrieving bacterial cells from the solid substrate after fermentation [11]. It has been shown that for some specific processes, particularly enzyme production, the costs of these techniques are lower and the production higher than submerged cultures [12]. Examples of this situation include cellulase production, which is estimated to be 100 times more economical with SSF [13], and lipase production, which is 78% cheaper with SSF [14]. For this reason, many researchers have recently focused on the production of industrial enzymes and, in particular, on reactor design [15–18], in the search for new solid supports [19–21] or process optimization [22–24]. In the field of enzyme production, several natural solids have been successfully employed: e.g., wheat, corn, rice, sugar cane and beet, banana waste, potato, tea, apple and citrus fruits, wheat flours and corn [9]. Of these supports, wheat fibers have been the most widely investigated. However, a very few researchers have published work on citrus peels, which today is a very significant waste product in agriculture industries. Citrus peels are eco-pollutants and inexpensive waste materials. They are the pectin-rich residues left after juice extraction by pressing orange. This under exploited material is generally disposed of in open areas, leading to serious environmental problems [9]. In contrast, the potential utilization of this waste to produce valuable products by SSF is promising. Several bioprocesses have been developed that use citrus peels as the raw material for the production of bulk chemicals and fine products by SSF.

The hydrolysis of pectin backbones can be achieved through the synergistic action of several enzymes, including depolymerases (polymethylgalacturonases, polygalacturonases, pectate lyases, and pectin lyases) and pectin methylesterases [25]. Several organisms are able to produce pectin-degrading enzymes, which are, in fact, widely used in a variety of food industries as a means to improve the yield and clarification of fruit juices [26]. Among fungi, ascomycetes, especially *Aspergillus niger* have been extensively investigated their value in

industrial production of pectinolytic enzymes is well established [27].

Evaluation of pectinases from other fungi in addition to ascomycetes may increase the range of options for enzymatic production. One group of fungi with potential for enzyme production that remains largely unexplored is Chytridiomycota. These fungi typically grow in water or damp soil and disperse using flagellated zoospores. Gleason et al. [28] discussed general patterns of carbohydrate use by Chytridiomycota, showing that many species can use cellulose as a sole carbon source. Many Chytridiomycota are cultured from plants or plant products, and the ability to digest plant cell walls is a general characteristic that is shared across many fungi that haven proven useful for the secretion of industrial enzymes for breakdown of lignocellulose or pectin. The Chytridiomycota currently best known for their industrial potential are probably the rumen fungi, which as symbionts in digestive systems of herbivores help their hosts to digest cellulose. Some of these fungi are active in secreting high and potentially commercially significant levels of celluloses [29-30]. While genome sequencing studies of Chytridiomycota have identified genes encoding pectinase enzymes and culture studies have shown the fungi can use pectin as a carbon source [31] there have been, to our knowledge, no direct demonstration of pectinase activity by any Chytridiomycota. The characterization of the pectinolytic activities is generally carried out in liquid media. For most enzymes, the type of cleavage is random (endo) or terminal (exo). These activities were routinely determined by spectrophotometer assays using dinitrosalicylic acid (DNS) (for exo-activity) [32] and thiobarbituric acid (for pectin and pectate lyase activities) [33] or by viscosimetry (for endo-activity) [34].

The aims of the current paper are to characterize the secreted enzymes of *P. obscura JEL 513* with particular attention to secreted exo-pectinases and to the microbial saccharification of citrus peels in a solid state fermentation media.

2. METHODS

2.1 Materials

The inducing substrate for exo-pectinolytic enzyme production was sterilized citrus peels (CP) with particle size of 1 to 2 mm. They were collected from local produce markets in Vancouver (British Columbia, Canada). The sterilization process was conducted (using autoclave) at 1.5 bar and 121°C for 40 min. Changes in CP were identified before and after the fungal growth by calculating carbohydrate, protein, lignin and ash content.

2.2 Microorganism

Physocladia obscura JEL 513 was isolated by Joyce Longcore in Orono, Penobscot County, Maine USA by collecting a wet soil along a trail with onion skin. Isolation involved microscopic examination of the onion skin to detect fungal colonies that had established on it after dispersing from the soil. Molecular phylogenetic analysis shows that the species is classified in Chytriomycetaceae, Chytridiales [35,36].

2.3 Fungal Spores Initiation

The stock culture of *P. obscura* JEL 513 was transferred to modified Peptonized milk Tryptone Glucose agar (mPmTG) where the composition of medium was as follow: 2 g glucose, 0.5 g peptonized milk, 0.5 g Tryptone and 10 g agar in 1 L of deionized water and the pH was adjusted to 6. Zoospore suspensions were prepared from 2-day-old culture agar plates incubated at room temperature ~20°C. Then the plates were flushed with 5 mL sterile distilled water (DW) to induce zoospore release. One ml ($1x10^6$ spores/ml) was used as an inoculum for the following experiments.

2.4 Solid State Fermentation

The solid-state fermentation (SSF) cultivations were carried out for 96 h at static conditions and 20°C in 250 ml Erlenmeyer flasks containing the sterilized citrus peels (CP) to which 15 ml nutrient medium per 5 g CP (dry weight basis) was added. One flask was left un-inoculated as a control. Enzyme was extracted at 20°C by adding fermented solids at 1:10 (w/v) sterile DW pH 6.0 and agitating at 350 rpm for 40 min. The extracts were vacuum filtered using microfiber filters (Whatman filter) and kept frozen at -20°C until analysis. Experiments were carried out in triplicate.

2.5 Screening for Extracellular Enzymes

After growing *P. obscura* JEL 513 on SSF medium, the remaining slurry was filtered

extracted and checked for the presence of extracellular enzymes as follow.

2.5.1 Screening for pectinase

The slurry extract was loaded in sterilized pectin agar medium [contained 1 g citrus pectin (Sigma) was added to 2 g agar in 100 mL de-ionized distilled water]. Three holes were made in the agar plates using a cork borer (0.9 mm) and 100 µL of slurry extract was loaded in these holes, then the plates were incubated for 24 h. (Three plates were used for each experiment). After incubation the plates were flooded with iodine solution and observed for the formation of clear zones around the holes indicative of pectinase activity [37].

2.5.2 Screening for amylase activity

The slurry extract was loaded in sterilized starch agar medium (contained 1 g soluble starch was added to 2 g agar in 100 mL de-ionized distilled water). Three holes were made in the agar plates using a cork borer (0.9 mm) and 100 µL of slurry extract was loaded in these holes, then the plates were incubated for 24 h. Again, three replicate plates were used for each experiment. After incubation the plates were flooded with iodine solution and observed for the formation of clear zones around the holes indicative of amylase activity [38].

2.5.3 Screening for cellulase activity

The slurry extract was loaded in sterilized carboxymethyl cellulose agar medium (contained 1 g carboxy-methyl cellulose was added to 2 g agar in 100 mL de-ionized distilled water). Three holes were made in the agar plates using a cork borer (0.9 mm) and 100 µL of slurry extract was loaded in these holes, then the plates were incubated for 24 h. Three replicate plates were used for each experiment. After incubation the plates were flooded with iodine solution and observed for the formation of clear zones around the holes indication of cellulase activity [39].

2.5.4 Screening for protease activity

The slurry extract was loaded in sterilized gelatin agar medium (contained 1 g gelatin was added to 2 g agar in 100 mL de-ionized distilled water). Three holes were made in the agar plates using a cork borer (0.9 mm) and 100 µL of slurry extract was loaded in these holes, then the plates were incubated for 24 h. Three replicate plates were used for each experiment. After incubation the plates were flooded with mercuric chloride solution and observed for the formation of clear zones around the holes indicative of protease activity [40].

2.6 Determination of Reducing Sugars and Proteins

Reducing sugars were determined using 3.5 DNS method of Miller [41] and expressed as galacturonic acid equivalents. The DNS reagent was prepared by dissolving 5 g of dinitrosalicylic acid (Sigma)) in 250 mL of distilled water at 90°C. When this solution reached room temperature, 100 mL of 2 N NaOH (Fisher) and 150 g of potassium sodium tartrate (Fisher) were added and the volume was brought to 500 mL with distilled water. Known concentrations of standard galacturonic acid were used for standard calibration curve. Then 500 µL of DNS reagent and either 500 µL of fermented broth, or (as a blank), 500 µL distilled water, were added to test tubes. The tubes were incubated in a water bath (100°C), for 5 minutes and then cooled on ice. Once on ice, stop the reaction immediately and to bring tubes up to their final volumes, 5 mls of water was added to each tube. When the tubes reached room temperature, 300 µL of the resulting reaction mixture of each test tube was transferred to a well of a 96 well microtiter plate. Then the absorbance was measured on a Biokinetics microtiter plate reader (EL340, Bio-Tek Instruments), at a wavelength of 540 nm. Protein content was estimated by the method of Bradford [42], using bovine serum albumin as a standard. Exo-pectinolytic activities were determined by measuring the amount of reducing sugars released [36] after 1 h of incubation of 1 ml of the slurry extract (or 1 ml distilled water as a control) at room temperature ~20°C with 1 ml citrus pectin (1%) as described by Aguilar and Huitron [43]. One unit (U) of exo-pectinolytic activity was defined as the amount of enzyme that liberated 1µmol of galacturonic acid per minute.

2.7 Media Optimization

To partially optimize the conditions for the production of pectinolytic enzyme(s) during SSF, alternative cultural conditions were tested in sequence. Incubation times from 1 to 10 days under static fermentation at 20°C were tested. Also, the effects of pH values (3, 4, 5, 6, 7 and 8) and incubation temperature range (20, 25, 30, 35 and 40°C) were measured.

2.8 Chemical Analyses of CP

2.8.1 Carbohydrate content

The carbohydrate content of the CP was determined before and after fungal growth determined according to Browning [44], with minor modifications. In brief, the CP material was ground using a Wiley mill to pass a 40-mesh screen, and then extracted overnight in hot acetone and allowed to dry over night at 50°C. An exact amount of the dried samples (~0.2 g) was hydrolyzed by adding 3 ml of 72% (w/w) H_2SO_4 and macerated for 2 hours. After 2 hours the contents were transferred to septa-sealed serum bottles and rinsed out with exactly 112 ml of deionized water. Standard controls (arabinose, galactose, glucose, xylose, mannose, and rhamnose) were prepared to estimate the exact concentration of the sugars in the processed samples. The hydrolyzed samples as well as the standard controls were autoclaved for 1 hour at 121°C. Then they were allowed to cool to room temperature and filtered using dry vacuum-filter sintered-glass crucibles (medium coarseness, previously weighed). One ml of the resulted filtrate was quantified by high-performance anion exchange liquid chromatography using a Dionex system (DX 600; Sunnyvale, CA, USA) equipped with a Carbo Pac PA1 column (Dionex).

2.8.2 Total lignin content

Insoluble lignin was determined [45] by weighing the glass crucible after filtration (dried overnight at 105°C). The percentage of insoluble lignin was calculated as follows:

$$\text{Insoluble lignin (\%)} = \frac{[(W_1(g) - W_0(g)]*100}{\text{Dried weigh of starting material (g)}}$$

$W_{1=}$ Weight of dried glass crucible after filtration
$W_{0=}$ Weight of dried empty glass crucible

Soluble lignin was measured by absorbance at 205 nm of a diluted sample (500 µL of filtrate to 1500 µL of 4% H_2SO_4) on UV-visible spectrophotometer (Thermo Scientific, Biomate-3S). Total lignin was calculated as follow:

Total Lignin (%) = Soluble lignin (%) + Insoluble lignin (%).

2.8.3 Ash content (Thermogravimetric analysis)

The samples were dried in the vacuum oven at 40°C for overnight before experiments. An exact amount(~ 20 mg) of sample was heated up to 650°C with 10°C /min heating rate in the air flow with TGA (Q500, TA instrument). Ash content was measured as weight percent at 525°C [46].

3. RESULTS AND DISCUSSION

3.1 Pectinase Production by *Physocladia obscura* JEL 513

Fungi are among the best pectin secretors known, which makes them attractive for industrial applications. In our study, the evaluation of both fermented (treated by the growth of *P. obscura* JEL 513) and unfermented citrus peels showed a production of pectinolytic enzyme under solid state fermentation. *P. obscura* JEL 513 has demonstrated its potential for pectinase production in fermentation systems and particularly in SSF. Results of the first screening investigation are presented in Fig. 1, exploring the screening of the strain for the production of different enzymes (protease, pecinase, amylase and cellulose). Of the enzymes tested, the pectinase activity was the highest.

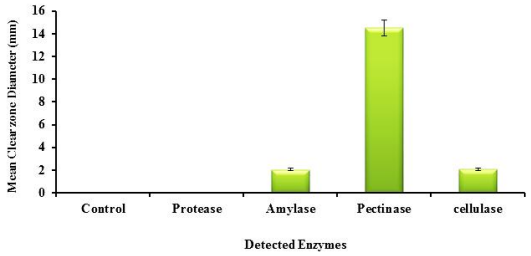

Fig. 1. Screening test for different enzymes production

3.2 Optimization of Pectinase Production by *P. obscura* JEL 513

The first optimization step focused on identification of optimal pH. A change in the pH of solid-state cultivation affects the enzyme production as well as enzyme concentration obtained after enzyme leaching. Therefore, this factor had to be optimized for pectinase production by *P. obscura* JEL 513 in the present SSF system. Maximum enzyme activity was achieved at pH 6.0 (Fig. 2A).

The second optimization investigation focused on the process parameters incubation time and temperature. The screening results demonstrated further increase of pectinase

activity at longer incubation times (7 days). The original experimental set-up included a time range from 1 to 10 days and a temperature range from 20 to 40°C. Maximal pectinase activity of 760.785 U/g was obtained after 5 days at 25°C (Fig. 2B and C).

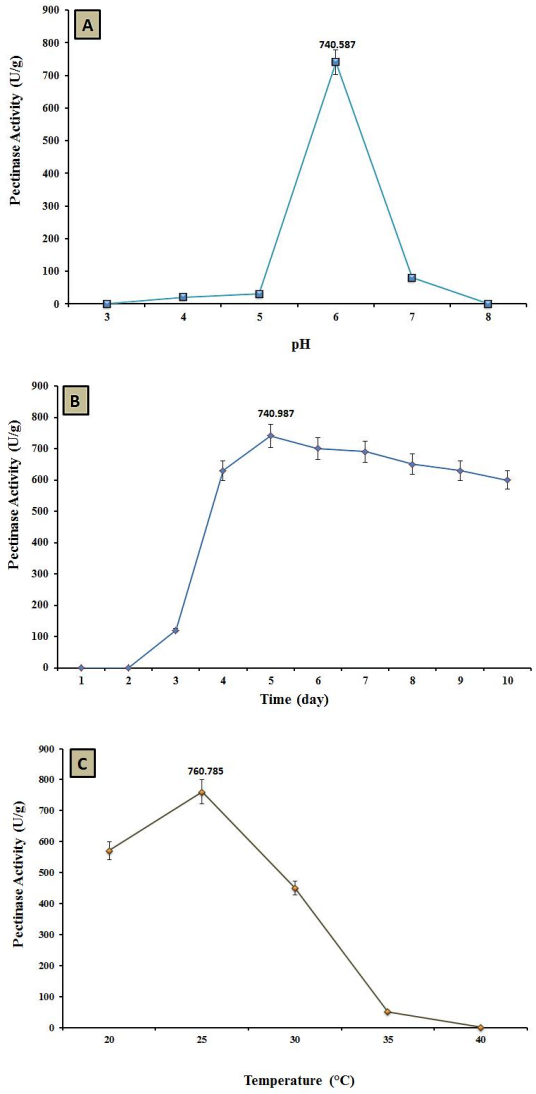

Fig. 2. Optimization of cultural conditions (A) pH, (B) incubation time, and (C) incubation temperature

Data analyzed showed that pectinase production was increased gradually during the fermentation period and reached to its maximum value (740.987 U/g) after 5th day of initial incubation under 25°C at pH 6. Production of pectinase increases with increase in fermentation period due to increase in activity of pectinase and it is also dependent on the type of fermentation including concentration of nutrients in the medium, organisms, and physiological conditions [47]. The effect of initial medium pH for pectinase enzyme production from 3 to 8 was investigated and maximum activity (740.587 U/g at pH 6) was found. The decline in enzyme activity was observed as the pH level increased from 6. According to Spagna et al. [48], maximum pectinase activity was under optimum pH 6.0 while, according to Pedrolli et al. [49] pH at value 5.5 was the optimum one.

To study the effect of temperature on the pectinase enzyme production, static flask experiments were run at various temperatures between 20 and 40ºC. Maximum enzyme activity (760.785 U/g) was observed in fermented culture at 25°C. Further increase in temperature changed the enzyme activity. The attained results are correlated to the data earlier reported by Pedrolli et al. [49] and Nighoskar et al. [50].

3.3 Physicochemical Analyses

3.3.1 Thermal gravimetric analysis (TGA)

The results of thermal gravimetric analysis (TGA) of treated (with fungal growth) as well as untreated (without fungal growth) CP are shown in Fig. 3A and B. The TGA curves and their first derivatives, the differential thermogravimetry (DTG) curves show initial limited weight loss at lower temperatures (1st process), which is attributable to the evolution of absorbed moisture. In the second process, rates of weight loss relative to temperature increases increased, possibly due to the decomposition of CP components, leading to the formation of carbonaceous char. This was followed by the last phase of weight loss, likely due to oxidation of charred product. Most of the mass loss occurred in the 2nd and 3rd processes. For the investigated biological treated CP, the start temperature of the 2nd degradation stages is higher than untreated CP (Fig. 3A and B).

The thermogravimetric analysis (TGA) charts showed the weight loss and its derivatives of untreated and treated CP, as a function of temperature. As can be possible, SSF leads to formation of carboxylic groups, which consequently enhances the formation of intra molecular hydrogen bonding with hydroxyl groups which need more temperature than untreated one.

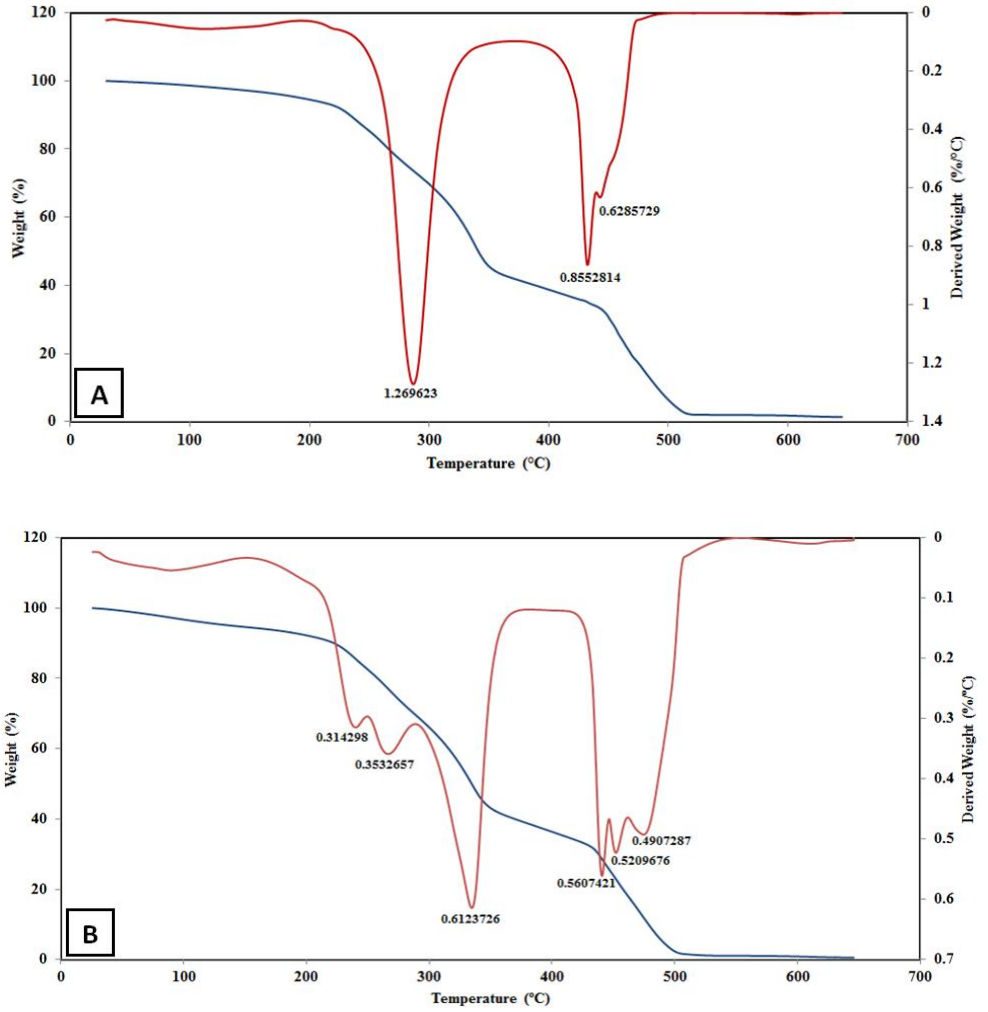

Fig. 3. Thermal gravimetric curve of the decay of treated (A) and untreated (B) CP with temperature. The blue line is the thermal gravimetric curve expressed as percent weight loss. The red line is the differential thermogravimetry and shows the patterns of acceleration of mass loss during heating

3.3.2 Chemical composition of substrates

The chemical compositions of untreated and treated CP are shown in Fig. 4A, B and Table 1. There were no differences observed between the monosaccharide compositions of the CP (Fig. 4), indicating that growth of *P. obscura* JEL 513on CP may not alter the chemical composition of the CP. HPLC analyzed the functional carbohydrate components in dissimilarly processed unfermented and fermented citrus peel samples. The fermented citrus peel is slightly higher in rhamnose, galactose, arabinose, glucose, xylose and mannose than unfermented citrus peel. This finding suggests the biochemical transformations occurred in the fermentation process with *P. obscura* JEL 513. Data shown in Table 1 indicating the relative percent lignin content (insoluble lignin plus acid-soluble lignin) of CP was increased 1.2-fold relative to untreated CP (from 8% to 9.6%), with a concomitant 1.1-fold increase in percent cellulose content (from 24.8% to 28.5%) and a significant 1.4-fold increase in hemicellulose content (from 14.4% to 20.7%).

Presumably, the relative increases in lignin, cellulose and hemicelluloses resulted from the loss of mass from the CP due to degradation of other chemical constituents.

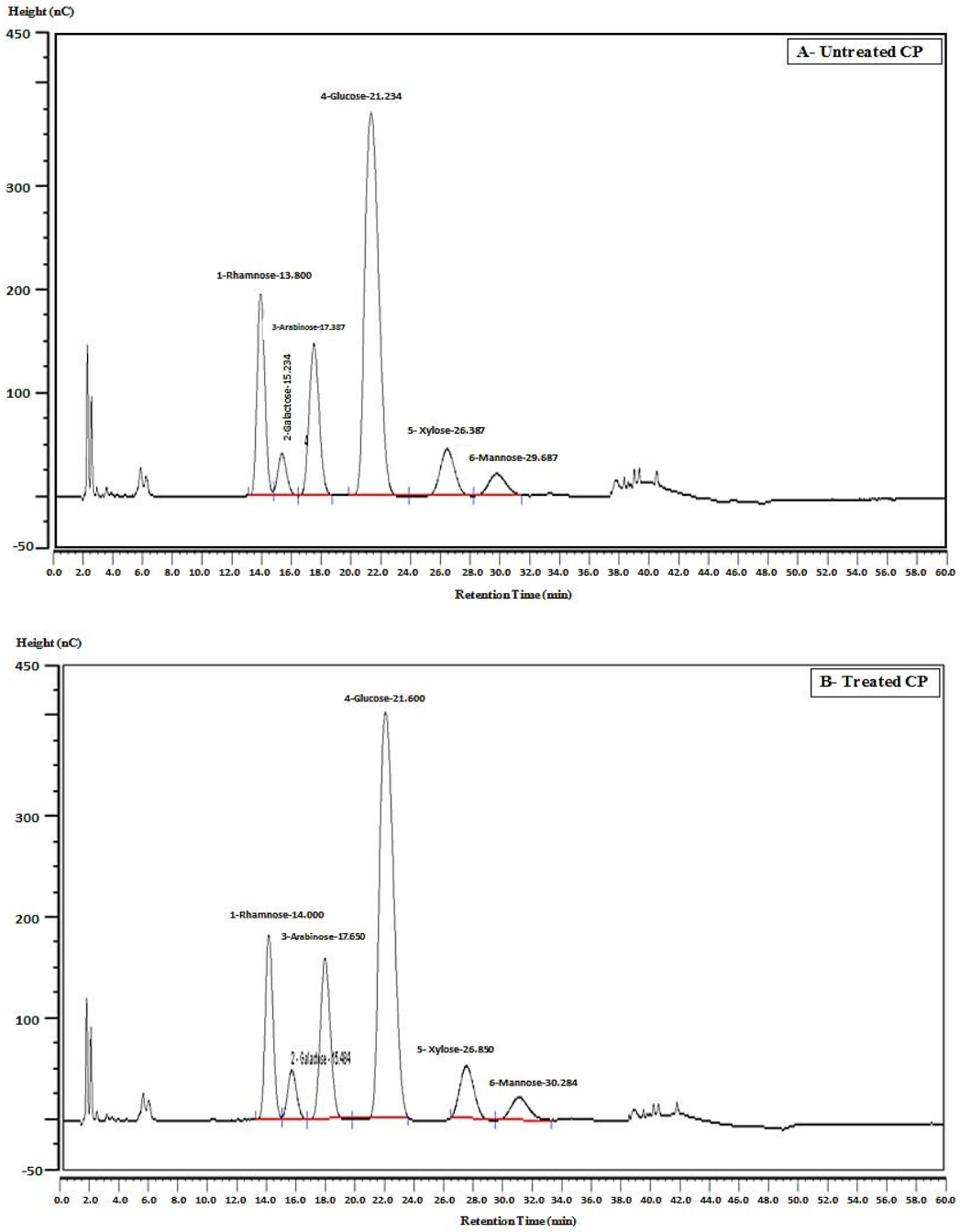

Fig. 4. Carbohydrate content of treated and untreated CP as measured by HPLC

Table 1. Total carbohydrate content of treated and untreated CP

Treatment	Carbohydrates					Total carbohydrate content (%)
	Lignin (%)			Celluloses (%)	Hemicelluloses (%)	
	Insoluble	Soluble	Total			
Untreated	2.4	5.6	8.0	24.8	14.4	47.2
Treated	3.7	5.9	9.6	28.5	20.7	58.8

4. CONCLUSION

In conclusion, the investigated indigenous strain *P. obscura* JEL 513 showed a satisfying potential for pectinase synthesis. Therefore, the results direct the scope of *P. obscura* JEL 513 for pectinase enzyme production in the presence of cheaper substrate without adding any other media constituents makes this biosynthesized enzyme useful in industrial sectors.

PG production by *P. obscura* JEL 513 was optimized on crude citrus peels, as a substrate in SSF. The present study demonstrated a promising potential for cost-efficient pectinolytic enzyme production by *P. obscura* JEL 513. Utilization of agricultural and agro industrial by-products developed an attractive sustainable bioprocess for enzyme production. High enzyme productivity obtained by *P. obscura* JEL 513 under optimized conditions will be a promising starting point for further future scale-up and polygalacturonase purification studies.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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