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Extraction of p-coumaric acid from agricultural residues and separation using 'sugaring out'

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Abstract−We investigated the extraction of para-coumaric acid (pCA) from different agriculture residues (corn stover, sugarcane bagasse, sorghum stalk, pearl millet stalk, green gram shell, groundnut shell, sesame shell) using sugarcane bagasse alkaline hydrolysis and separation of pCA using sugaring out - a new phase separation method. Primary screening of different feed stocks was by alkaline hydrolysis with 2 M NaOH for 6 h at room temperature. Sugarcane baggase resulted into significant amount of pCA (1.1 g/L) and small amount of ferulic acid (FA) (0.23 g/L). The optimized alkaline hydrolysis conditions (2 M NaOH and 16 h) resulted into maximum pCA release of 2.0 g/L. The pCA was separated from alkaline hydrolysate using sugaring out, a two phase separation method that results in aqueous phase and the organic solvent (acetonitrile) phase. Sugaring-out separated more than 90% of the pCA from the alkaline hydrolysate. Results of HPLC using standard pCA and FA showed that the main component of the separated top (organic solvent) phase was pCA rather than FA.

Keywords: p-Coumaric Acid, Alkaline Hydrolysis, Sugaring Out, Agriculture Residue, ATPS

INTRODUCTION

Para-coumaric acid (pCA) finds large number of applications in health, food, pharmaceutical and cosmetic industries due to its physiological functions such as antioxidant, anti-mutagenesis, anti-genotoxicity, antimicrobial [1-3], anti-inflammatory, and anti-thrombosis. pCA (Fig. 1) confirmed protective effect in doxorubicin-induced oxidative stress in rats [4] and also from ultra-violet B induced oxidative damage in SIRC cells [5]. pCA also plays a vital role in human immune regulations [6] and is known to restrain cellular melanogenesis [7]. Different aromatic products such 4-vinylphenols [8] and p-hydroxybenzoic acid [9,10] can be produced from pCA.

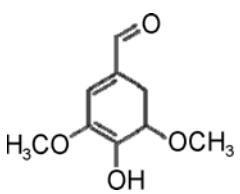

Fig. 1. Structure p-coumaric acid.

In lignocellulose material, coumaric acid is attached to lignocellulose forming lignin-phenolics and carbohydrate complexes. In this complex phenolic hydroxyl groups form ether linkages primarily linked with lignin and these patterns vary with raw material. A few lignocellulose materials (cereal bran, corn cobs etc.) have been identified as having ether links between hydroxycinnamic acid to lignin [11-13]. Also, pCA is ester-linked in material like sugarcane bagasse [14]. Thus, the pCA yield depends on type of raw material. Hence, there is a need to explore other lignocellulose materials which will yield high pCA.

Linkages with lignin suggest that alkaline treatment could release pCA from the material. However, there are other phenolic acids/compounds which could also be released during this treatment, making the separation process complex [15]. Attempts to separate pCA from hydrolysate (obtained after treatment of brewer's spent grain) were unsuccessful [16]. Different methods for separation of phenolic compounds such as chromatography [17], solvent extraction [18] and aqueous two phase separation [19-21] are reported in the literature. In some chromatographic processes the product was brown and problems arose during crystallization of pCA and regeneration and reuse of resin [17]. Zhao et al. [22] used activated charcoal to solve these problems. Solvent extraction is another method

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which can be used in separation of pCA; however, application of solvents like butyl acetate, hexanol, octanol, olyel alcohol etc. which have a high boiling point (>120 °C) makes the solvent recovery process energy intensive.

Sugaring-out - a relatively new and less explored two phase separation method invented in our laboratory at University of Illinois at Urbana-Champaign (USA) - uses acetonitrile (ACN) (a low boiling point solvent) [23-26]. Water and ACN are miscible in all proportions at room temperature. However, addition of sugar in the mixture forms two phases with upper phase being rich in ACN and the lower aqueous phase [23,24]. Sugaring out does not change the environment conditions (e.g. pH), and hence high quality material of construction is not needed. Also, pretreatment of biomass generates some sugars; hence, less amount of external sugar will be required for phase separation. Maximum amount of sugars is retained in aqueous phase after phase separation [23-26], which could be utilized for ethanol/butanol fermentation, and recovery and recycling of sugars will not be needed. Being a relatively new method, sugaring out is less studied for other applications [23,26]. Hence, this work was undertaken with an objective of producing pCA from various less explored lignocellulose materials and separating it using sugaring out. Primary screening of the biomass involved using alkaline hydrolysis, which was subsequently optimized for maximum pCA generation. Separation of pCA from alkaline hydrolysate was carried out using optimized conditions.

MATERIALS AND METHODS

1. Chemicals

Para-coumaric acid (AR grade), sucrose (pure) and HPLC-grade ACN (purity 99.8%) were obtained from SD Fine Chemicals (Mumbai, India). D-glucose (extrapure, AR), D-xylose (extrapure, AR), sucrose (pure), ferulic acid (FA) (98% pure) were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Deionized water from an in-house facility was used for all the experiments. Acetic acid (99.5%) was obtained from Lobachemie (Mumbai, India).

2. pCA Extraction

Primary screening of various agriculture residues was by alkaline hydrolysis with 2 M NaOH for 6 h at 120 RPM and room temperature (27-31 °C) [27]. Feed stocks were milled and particles smaller than 1 mm were treated using NaOH (as a catalyst) at a solid to liquid ratio of 0.084 g of solid/g of NaOH solution. Reaction was for 6 h at room temperature (27-31 °C) in shake flasks. After hydrolysis, the entire mixture was centrifuged (4,000 RPM for 20 min) and the supernatant was taken for further studies. For extraction of pCA from alkaline hydrolysate, pH was adjusted to 3.0 using 2 M HCl. Hydrolysate was analyzed for pCA and FA. The hydrolysis conditions, i.e. hydrolysis duration (4-24 h) and alkali concentration (0.5-4 M), were optimized further for maximum pCA generation.

3. Separation of pCA from Hydrolysate using Sugaring Out

pCA was separated from hydrolysate by sugaring out using optimized separation conditions. Optimization of phase separation conditions (type of sugar, sugar concentration, solvent amount and pH) was carried out for a model system containing 0.2 g/L of aqueous pCA solution. pCA solution (5 mL) containing required amount of sugar (mass fraction - 0.1 to 0.2 in aqueous sugar solution) was mixed with equal amount of ACN (varied as needed for ACN: aqueous solution ratio). Both ACN and aqueous phase were mixed thoroughly on a vortex (Remi Cyclomixer CM101) and incubated for 5 h at 4 °C in a water bath (ESCY IC1201) where the temperature was controlled to ±0.1 °C. The temperature inside the water bath was measured with a thermometer (Brannan, UK). Effect of pH on sugaring out was studied by adjusting the pH of pCA solution (3.0 to 13.0) with 2 M NaOH solution or 2 M H_2SO_4 solution as required. Samples were collected from the top phase and bottom phase with a syringe. Volumes of top and bottom phase were recorded for calculations. All the experiments were performed in duplicate. Optimized separation conditions were employed for separation of pCA from alkaline hydrolysate following the same protocol as mentioned earlier.

Results are expressed as % pCA separation

% pCA separation (w/w)=(Amount of pCA in the organic solvent phase)/(Total amount of pCA)×100

4. Analysis

The amount of pCA and FA was measured following the method reported earlier [28]. pCA and FA were analyzed with high performance liquid chromatography (HPLC) (DIONEX ultimate 3000) using reverse phase C-18 column (Acclaim 120) of size 4.6 mm× 250 mm (0.5 μm) and diode array detector at 320 nm. Prior to analysis, alkaline hydrolysate samples were neutralized using 2 N HCl and filtered through 0.22 micron filter. Eluent consisting of 80% (v/v) water (1% acetic acid) and 20% (v/v) acetonitrile was used at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

1. Extraction of pCA from Different Agriculture Residues (Feed Stocks)

Primary screening of feed stocks was carried out by conduct-

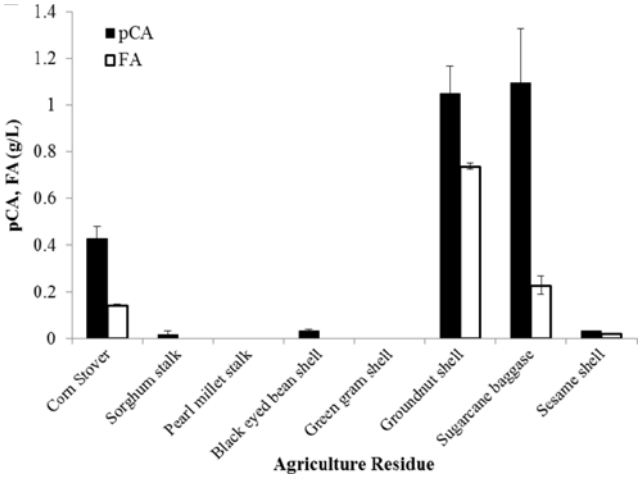

Fig. 2. Extraction of p-CA and FA from different agriculture residues obtained after alkaline treatment (Solid to liquid ratio= 0.084 g/g of NaOH solution 120 RPM for 6 h).

ing alkaline hydrolysis of the agriculture residue with 2 M NaOH for 6 h at room temperature and analyzing the concentrations of pCA and FA in the hydrolysate (Fig. 2). Feedstock showing high amount of pCA and lowest amount of FA was selected for further studies. It was observed that groundnut shell had significant amount of both FA (0.74 g/L) and pCA (1.05 g/L) and sugarcane bagasse had large amount of pCA (1.1 g/L) and very less FA (0.23 g/L). Corn stover also resulted into 0.43 g/L pCA and 0.14 g/L FA. Pearl millet stalk and green gram shell did not generate FA or pCA. Further, sorghum stalk, black-eyed bean shell, sesame shell showed less than 0.04 g/L of pCA. Ferulic acid was not generated during alkaline pretreatment of sorghum stalk, pearl millet stalk, black-eyed bean shell, green gram shell. Since the objective of the study was mainly generating pCA, sugarcane bagasse which produced significant amount of pCA and less amount of FA was selected for further studies.

Optimization of alkaline hydrolysis was carried out to generate maximum amount of pCA (Fig. 3). Effect of two parameters mainly concentration of alkali and duration of hydrolysis was varied and pCA and FA produced was monitored. Maximum pCA (2.0 g/L) was obtained with 2 M NaOH when hydrolysis was carried out for 16 h (Fig. 3(a)). 0.5 M and 4 M NaOH resulted into less pCA release. This could be because of extreme conditions (0.5 M is too mild whereas 4 M is extreme) during the hydrolysis. Solubility of pCA increases with increase in pH, i.e., with alkalinity (Fig. 3(a)). Hence, with 0.5 M alkali, less pCA was extracted from the biomass. It increased with increase in alkalinity and reached a saturation level. There was insignificant effect of duration of hydrolysis and NaOH concentration on FA generation (0.2 to 0.325 g/L) (Fig. 3(b)).

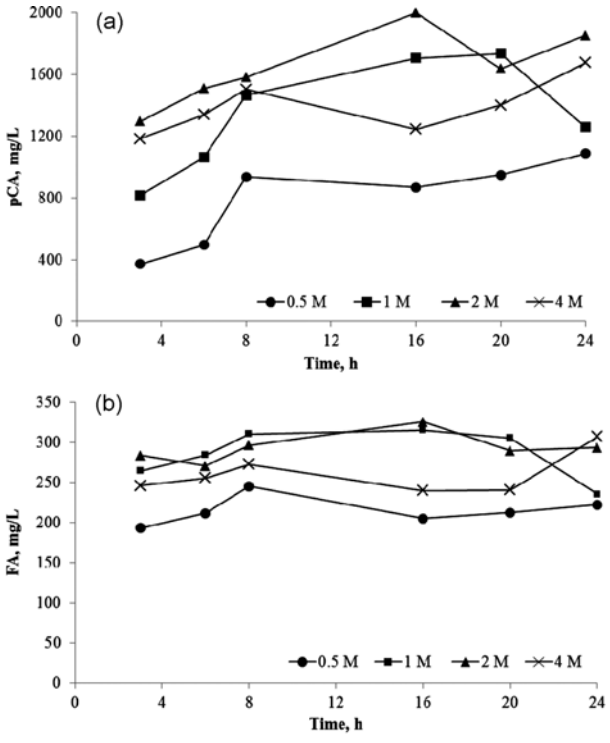

Fig. 3. Optimization of alkaline hydrolysis conditions for maximum pCA (a) and FA (b) production from sugarcane bagasse (Solid to liquid ratio=0.084 g/g of NaOH solution Room temperature and 120 RPM).

2. Separation of pCA using Sugaring Out: Optimization of Process Parameters

Separation of pCA from alkaline hydrolysate consisted of using sugaring out, a two phase separation method. Separation condi-

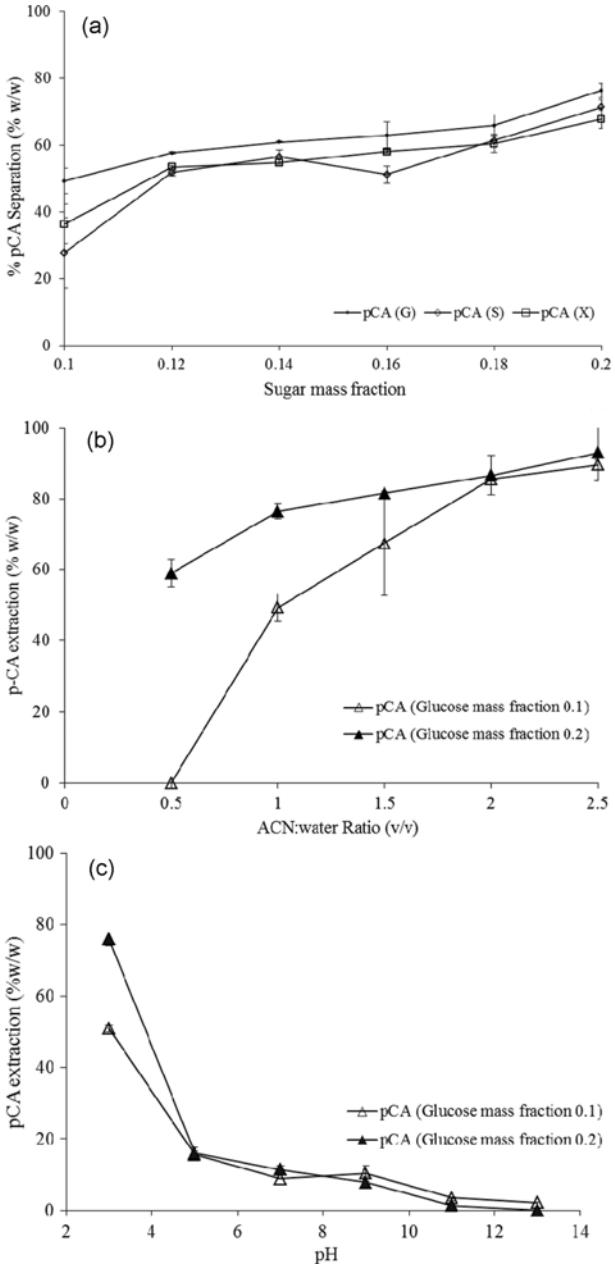

Fig. 4. (a) Effect of type of sugar (monosaccharide: G - glucose and X - xylose and disaccharide: S - sucrose) and sugar concentration on p-CA separation (ACN : Aqueous solution=1 : 1; temperature=4 °C, p-CA=0.2 g/L). (b) Effect of ACN: aqueous solution (containing pCA) ratio on pCA separation (Sugar - Glucose, temperature=4 °C, p-coumaric acid=0.2 g/L). (c) Effect of pH on pCA separation (Sugar - Glucose, ACN : Aqueous solution=1 : 1, temperature=4 °C, p-coumaric acid=0.2 g/L).

tions were optimized to achieve maximum separation efficiency with a model system consisting of aqueous solution of 0.2 g/L of pCA. Different parameters were optimized, which include type of sugar, concentration of sugar, amount of solvent and pH.

2-1. Effect of Type of Sugar and its Concentration

Separation of pCA from a model system consisting aqueous solution of 0.2 g/L of pCA used different sugars (glucose, xylose and sucrose) at 4 °C. Separation efficiency was higher in case of glucose (49 to 76% w/w) than other sugars (28% to 71% w/w) (Fig. 4(a)). The performance of the sugars as a phase separating agent can be ranked as: glucose>xylose>sucrose. The separation efficiency increased with increase in sugar concentration. It increased from 49% to 76% (w/w) when the glucose mass fraction was increased from 0.1 to 0.2 g/L. Hence, glucose (mass fraction 0.1 and 0.2) and 4 °C, were chosen as separating conditions for the experiments.

For the same mass of sugar (e.g. 200 g/L), the moles of glucose (1.11 mole) is more than sucrose (0.584 mole) and less than xylose (1.33 mole). Sucrose can form eight hydrogen bonds, whereas glucose and xylose can form, respectively, five and four hydrogen bonds. Thus, it is expected that number of hydrogen bonds formed by same mass of glucose, sucrose and xylose, will be more in case of glucose than sucrose and xylose. Therefore, pushing out more amount of acetonitrile and resulting into higher separation efficiency.

Increase in pCA separation efficiency with sugar concentration can be explained based on interaction between ACN and water molecules. Water surrounds the acetonitrile molecule (which forms three dimensional clusters) through hydrogen bond. ACN molecules have dipole-dipole interaction among themselves. It is likely that the hydrogen bond between the water and ACN molecule is replaced by phase separating agent (sugar) that leads to self-association of ACN molecules and formation of a separate phase. Thus, increase in sugar concentration would lead to increase in upper phase volume. And since ACN is relatively non-polar compared to water, ACN favors the extraction of relatively non-polar pCA. In addition, pCA has poor solubility in water due to less degree of hydrogen bonding and hence it is displaced easily from an aqueous phase.

2-2. Effect of ACN Volume

Volume of solvent is another parameter that affects the separation efficiency [22]. ACN: aqueous phase volume was increased from 0.5 to 2.5 (v/v) by increasing the volume of ACN alone. Sugaring out was carried out with glucose (mass fraction 0.1 to 0.2) and the separation of pCA was studied at 4 °C. With increase in the ratio of ACN to aqueous phase (from 0.5 to 2.5), separation efficiency increased drastically from 59% to 93% w/w (Fig. 4(b)). Significant increase in extraction efficiency was seen when the ratio was increased from 0.5 to 1.5. However, the increase was insignificant above the phase ratio of 2.0. Maximum extraction (93% w/w) was obtained at ACN to aqueous solution ratio of 2.5 at a glucose mass fraction of 0.2. Increase in ACN quantity led to increase in top phase volume. This increase in upper phase volume is exponential [23], but the decrease in concentration is not exponential; hence, it is expected that the separation efficiency would increase. It is assumed that additional ACN is displaced from the aqueous phase because the saturation is already achieved at a lower ACN concentration. No phase separation was observed at ACN: aqueous phase ratio of 0.5 (v/v) for glucose mass fraction of 0.1.

2-3. Effect of pH

Experiments were performed over a broad pH range (3.0 to 13.0). It was observed that pH had significant effect on separation of pCA (Fig. 4(c)). Separation efficiency decreased with increase in pH from 3.0 to 13.0. Maximum separation of pCA (76% w/w) was observed at acidic pH (3.0) and glucose mass fraction of 0.2. When pH was increased from 3.0 to 5.0, drastic decrease in separation efficiency occurred from 76% to 16% (w/w) and from 50.9% to 15.99% (w/w), respectively, for glucose mass fraction of 0.2 and 0.1, respectively. For pH higher than 11.0, no pCA was extracted into organic solvent phase. Glucose concentration had significant effect at very low pH (3.0). Better separation (76% w/w) was achieved at high glucose mass fraction (0.2) as compared to low glucose mass fraction (0.1) at pH of 3.0. Separation efficiencies were approximately same for pH>5.0 for both the glucose concentration (0.1 and 0.2).

pCA is relatively hydrophobic compound and has less solubility in water. Solubility in water is due to the presence of $COOH^-$ and OH^-. At low pH, it appears in non-ionized form, hence the interaction with surrounding water molecules is less, forcing it into organic solvent phase. By virtue of the presence of OH^- and $COOH^-$ group, ferulic acid is present in an ionized form at higher pH which increases the interaction with water molecules, thereby increasing its affinity towards aqueous phase. Since pCA is in non-ionized form, at low pH, glucose has maximum effect.

3. Separation of pCA from Alkaline Hydrolysate using Sugaring Out

Optimized conditions for sugaring out were used for separation of pCA from alkaline hydrolysate (Fig. 5). It was observed that 91.6% (w/w) of the pCA was separated from the sugarcane baggase hydrolysate. Loading capacity of ACN was very high for pCA than that would be encountered in this application. From Fig. 4 and 5, the loading capacity increased with increase in the amount of pCA in the solution. For extraction conditions ACN : water ratio=1.5, glucose mass fraction of 0.2 and pCA concentration of 0.2 g/L, the extraction efficiency was 81.5% (Fig. 4(b)), which remained con-

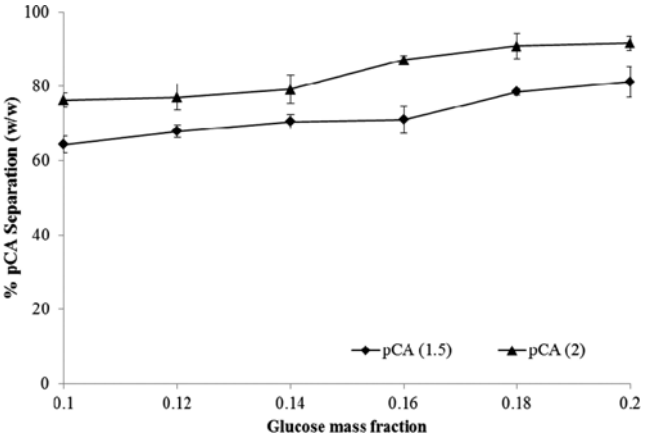

Fig. 5. Separation of pCA from sugarcane bagasse hydrolysate using sugaring out (pH=3.0, temperature=4 °C, ACN: Hydrolysate ratio=1.5 and 2.0).

stant even when the pCA concentration in the hydrolysate was 2 g/L. This indicates that ACN has very high loading capacity than the typical amount of pCA generated in hydrolysis of biomass.

The results of HPLC indicated that the main component of the top organic solvent phase (ACN rich phase) was pCA rather than FA. Low amount of FA in the top phase could be attributed to the small amount of FA in the hydrolysate itself. This is possible because FA and pCA associate with lignin and hemicelluloses in a different way, which depends on the raw material identity [27]. Also, the bond between pCA and hemicelluloses is weaker than the bond between FA and hemicelluloses, making the former easier to hydrolyze by alkali. Another reason for better separation of pCA, is the low distribution coefficient of FA than pCA. Wang et al. [25] obtained distribution coefficients of 4 and 6.5, respectively, for FA and pCA during sugaring out carried out at 1 °C with ACN: water ratio of 1 and glucose concentration of 50 g/L.

Separation of ACN from the sugaring out process is already discussed elsewhere [23]. Future scope of work includes investigation of other methods (pervaporation, azeotropic distillation, etc.) for removal of residual ACN from the aqueous phase and recovery of pCA from ACN-rich phase using vacuum distillation and recycling of recovered ACN for extraction.

CONCLUSIONS

Alkaline hydrolysis of various unexplored agriculture residues was investigated. Sugarcane bagasse produced the maximum amount of pCA (2.0 g/L) and less amount of FA (0.33 g/L). New application of sugaring out for recovery of pCA from sugarcane bagasse hydrolysate was studied. Separation of pCA from model system was optimized and the optimized separation conditions were used for separation of pCA from alkaline hydrolysate. 91.6% (w/w) separation of pCA was achieved (in the top phase) with sugaring out. HPLC analysis of the top phase (obtained after phase separation) rich in ACN showed pCA as main component rather than FA.

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