Immobilization of β -glucosidase onto silicon oxide nanoparticles and augment of phenolics in sugarcane juice

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Abstract Purified β -glucosidase was immobilized on SiO_2 nanoparticles with 52 % efficiency and 14.1 % yield. It had a temperature optima at 60 °C and pH optima of 5.0. Immobilized enzyme was fairly stable at 60–70 °C. After immobilization, the K_m value of β -glucosidase for *p*-nitrophenyl- β -D-glucopyranoside (pNPG) increased from 0.9 to 1.074 mM and $V_{\max}$ decreased from 3.5 to 1.513 U/mg. The immobilized enzyme showed improved storage stability at temperature 4 and 25 °C and was reusable for up to ten cycles with 70 % residual activity in pNPG and 60 % residual activity in sugarcane juice treatment. Sugarcane juice density, viscosity; surface tension etc. changed after treatment with immobilized β -glucosidase. β -Glucosidase treated sugarcane juice showed higher phenolics than untreated sugarcane juice. Caffeic acid which was absent in juice, was detected in β -glucosidase treated juice at a concentration of about 1 mg/L.

Keywords Immobilized enzymes · β -Glucosidase · Kinetic parameters · Silicon oxide nanoparticles · Sugarcane juice · Phenolics

Introduction

India is the world's second the largest producer of sugar followed by Brazil. Sugar manufacturing industries face a major challenge in the production of sugar of high clarity and low colour. Usually the so called 'browning' of sugarcane due to the action of PPO (polyphenol oxidase) enzyme on phenolic compounds adversely deteriorate sugar quality, clarity and colour. The phenolic compounds thus impart an undesirable colour to the sugarcane syrup and its removal is an important problem associated with sugar manufacture (Vickers et al. 2005).

Despite their negative effect on sugar quality, the phenolics helps in the prevention of oxidative stress induced diseases such as cardiovascular complications, diabetes, ulcers and cancer (Halliwell 2007). Thus, these phenolics are also called bioactive compounds. These bioactive constituents in sugarcane juice are wasted during the sugar-making process. Currently, there is not enough concern about the utilization of the natural bioactive compounds present in raw sugarcane juice (Li et al. 2011). In general, phenolic compounds, viz. the hydroxybenzoic acids (gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the $\text{C}_6\text{--C}_1$ structure) and hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric and sinapic acids, which are aromatic compounds with a ($\text{C}_6\text{--C}_3$) three-carbon side chain) accumulate in sugarcane juice as linked to mono- or di-glycosides and require enzymatic or acidic hydrolysis for their liberation (Bravo 1998).

β -Glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of alkyl- and aryl- β -glucosides, as well as diglucosides and oligosaccharides. β -Glucosidases can be classified, on the basis of substrate specificity, as (1) aryl- β -D glucosidases (having strong affinity for aryl- β -D-glucosides), (2) cellobiases (hydrolyze only disaccharides) and (3) broad

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specificity glucosidases and exhibit activity on many substrate types and are the most commonly found β -glucosidases (Rojas et al. 1995).

Elevation and extraction of the bioactive compounds from sugarcane juice by β -glucosidase treatment would be able to provide high quality and high active phenolic extracts while precluding any toxicity associated to the acidic hydrolysis. Also, these bioactive phenolic compounds can be recovered after passing through a suitable adsorbent like charcoal and used as dietary supplements or for other medicinal purposes. Further, the juice left after recovery of the phenolics would have a desirable quality and clarity. The changes in visco-elastic properties and reducing sugar content of juice would have also been considered.

Hence, an approach was aimed to covalently immobilization of purified β -glucosidase onto silicon oxide nanoparticles. Immobilized β -glucosidase was then tested for its efficacy in causing the release of glycosides-bound phenolic compounds in sugarcane juice.

Materials and methods

Source of β -glucosidase

The β -glucosidase was previously purified from developed mutant of *Bacillus subtilis* strain (PS) in our laboratory (Agrawal et al. 2012, 2013)

β -Glucosidase assay

β -Glucosidase activity was assayed by incubating the reaction mixture containing 10 mM *p*-nitrophenyl- β -D-glucopyranoside (pNPG) and purified β -glucosidase in 1:1 ratio for 1 h at 60 °C. The reaction was stopped by adding 2 ml of 1 M Na_2CO_3 . The *p*-nitrophenol release was monitored at $\lambda_{405\text{nm}}$ in UV-visible spectrophotometer (Jäger et al. 2001). One unit (U) of β -glucosidase was defined as the amount of the enzyme to produce one μmol *p*-nitrophenol per min under the standard assay conditions and the specific activity was defined as the number of units of β -glucosidase per mg of protein. Total protein content was determined by Bradford method (Bradford 1976).

Synthesis and TEM analysis of SiO_2 nanoparticles

Synthesis of silica nanoparticles was done as described earlier (Stober and Fink 1968). Silica nanoparticles were prepared by a simple one-step protocol which involves the condensation of tetraethyl orthosilicate (TEOS) in ethanol: water mixture under alkaline conditions at room temperature. Addition of ethanol (20ml) to 2 ml of TEOS was

added followed by 4 ml of concentrated NH_4OH and stirring for 8 h. The mixture obtained was centrifuged to get the silica nanoparticles. The SiO_2 nanoparticles were analyzed by TEM.

Functionalization of SiO_2 nanoparticles

The functionalization of silicon oxide nanoparticles was achieved by treating the nanoparticles with glutaraldehyde. The silicon oxide nanoparticles were washed twice with deionized water and recovered by centrifugation. The washed silicon oxide nanoparticles were suspended in 1 M glutaraldehyde (Libertino et al. 2008). Support activation was carried out at 25 °C in a shaker (150 rpm) for 4 h. The activated support was removed by centrifugation at 5700g and then washed thrice with 30 ml of distilled water to remove the glutaraldehyde and subsequently washed with 100 mM sodium acetate buffer (Stober and Fink 1968).

Immobilization of β -glucosidase on SiO_2 nanoparticles

Isolated β -glucosidases were immobilized on the functionalized silicon oxide nanoparticles by covalent binding method. Activated SiO_2 nanoparticles are supposed to contain $-\text{OH}$ group. The amino group of lysine present on the surface of the β -glucosidase reacts with the activated silicon oxide nanoparticles to form the covalent bond at multiple points (Singh et al. 2011). A high surface-to-volume ratio of these nanosized particles induces the increase of specific surface area available to biomolecules which allows easier enzyme anchoring and catalytic reaction.

β -Glucosidase (30 U per 50 mg of activated support) was mixed with the activated support in 100 mM sodium acetate buffer, pH 5.0. The immobilization process was performed at 25 °C in the shaker (150 rpm) for 36 h. Non-covalently adsorbed protein was removed thereafter by thorough washing of the nanoparticles with deionized water and sodium acetate buffer (100 mM, pH 5.0). The washed carrier was directly used for the determination of activity and stability. The supernatant was also checked for any remaining β -glucosidase activity (Singh et al. 2011).

The immobilization efficiency and immobilization yield were calculated as follows:

$$\text{Immobilization efficiency} = (\alpha_i/\alpha_f) \times 100$$

$$\text{Immobilization yield} = \{[\text{Pi} - (\text{Pw} + \text{Ps})]/\text{Pi}\} \times 100$$

where α_i is the total activity of the immobilized enzyme and α_f is the total activity of the free enzyme. Pi is the total protein content of the crude enzyme preparation; Pw and Ps

are the protein concentration of wash solution and supernatant after immobilization, respectively.

FTIR analysis

Binding of β -glucosidase to SiO_2 nanoparticles was confirmed by Fourier transform infrared (FTIR) spectroscopy (Thermoscientific Nicolet 6600).

Characterization of free and immobilized β -glucosidase

pH and temperature optima

The optimal pH was obtained by taking 50 μl of enzyme and added 50 μl of different buffers containing 10 mM pNPG. Reaction was allowed to take place for 30 min at 60 °C. Buffers used for different pH were 100 mM citrate buffer for pH 3.0, 4.0 and 6.0; 100 mM acetate buffer for pH 5.0 and 100 mM phosphate buffer for pH 7.0 and β -glucosidase activity was determined.

To test the optimum temperature of the enzyme, 50 μl of enzyme solution was added into 50 μl of 100 mM acetate buffer (pH 5.0) containing 10 mM pNPG. The reaction was allowed for 30 min at various temperatures i.e., 30, 40, 50, 60, 70 °C and β -glucosidase activity was determined.

pH and temperature stability

pH stability was determined by pre-incubating the purified β -glucosidase with different buffers such as citrate buffer (100 mM, pH 3.0), acetate buffer (100 mM, pH 5.0), phosphate buffer (100 mM, pH 7.0) at 37 °C for different time interval (0–60 min), in absence of pNPG. After incubation, pH was adjusted to 5.0 by adding acetate buffer (100 mM, pH 5.0). pNPG (10 mM, pH 5.0) was added to the reaction mixture and incubated at 60 °C for 30 min and the residual activity of the enzyme was calculated. The enzyme without incubation treatment was taken as control and its activity was considered to be 100 %.

To determine thermal stability of purified β -glucosidase was pre-incubated in acetate buffer (100 mM, pH 5.0) at 37, 60 and 70 °C for different time intervals (0–60 min) in absence of pNPG. After incubation, pNPG (10 mM, pH 5.0) was added in each test tube. Reaction was carried out at the optimum temperature (60 °C) for 30 min and the residual activity of the enzyme was calculated. The enzyme without incubation treatment was taken as control and its activity was considered to be 100 %.

Determination of kinetic parameters

β -Glucosidase activity was determined at different concentration of pNPG (0.1–1.1 mM). The reaction mixture was prepared and β -glucosidase activity was determined. The prepared reaction mixture was incubated at 60 °C for 30 min and the absorbance was recorded at 405 nm. Michaelis–Menten constant (K_m), maximum velocity (V_{max}), turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) for purified β -glucosidase were determined using Prism 5 (Graphpad Software, Inc., CA, USA). Michaelis–Menten plot and Lineweaver–Burk plot were also studied for the enzyme kinetics.

Storage stability

Free and immobilized β -glucosidase in acetate buffer (100 mM, pH 5.0) was stored at 4 and 25 °C for 12 days. The residual β -glucosidase activity was determined at an interval of 1 day at optimum temperature 60 °C and pH 5.0.

Reusability of immobilized β -glucosidase

The reusability of the immobilized β -glucosidase was assessed at 60 °C by carrying out the hydrolysis of pNPG and treatment of sugarcane juice under the standard assay condition. After each cycle of hydrolysis, the immobilized β -glucosidase was recovered by centrifugation at 4000g for 30 min and washed with deionized water and 100 mM acetate buffer (pH 5.0). In the second cycle, the immobilized enzyme was re-dissolved in fresh acetate buffer (100 mM, pH 5.0) and activity of β -glucosidase was measured. The activity of the immobilized β -glucosidase in the first cycle was considered as 100 %.

Preparation of sugarcane juice sample and enzyme treatment

Sugarcane juice was obtained cleaned sugarcane culms (*Saccharum officinarum*), purchased from Crop Research Center, G.B.P.U&T., Pantnagar, Uttarakhand, India). The juice samples were filtered using Whatman filter prior to the preparation of sugarcane juice samples. Four sugarcane juice samples were prepared as follow juice (5 ml) without any subsequent treatment (control), juice (5 ml) incubated with immobilized β -glucosidase enzyme (10U) for 30 min at 60 °C (JIE), juice (5 ml) boiled at boiling water bath for 30 min (JB) and juice (5 ml) passed through activated charcoal (JCT).

Physico-chemical properties and reducing sugar estimation of treated sugarcane juice samples

All the above four juice samples were tested for density, viscosity, surface tension, UV–absorption spectra and reducing sugar content. Water was taken as a reference. All the measurements were done at room temperature (Bahl et al. 2010). All the four sugarcane juice samples were scanned between the wavelength range 400–800 nm for absorption spectra. The total amount of reducing sugars was determined by the dinitrosalicylic acid (DNS) method (Miller 1959).

HPLC analysis of phenols in sugarcane juice

High Performance Liquid Chromatography (HPLC) (Ultimate 3000, Dionex) was performed for estimation of phenols in sugarcane juice samples to assess the effect of enzyme on release of phenols. Extraction of phenolics was carried in all four samples as described by Nayaka et al. (2009). Sugarcane juice samples (5 ml) were acidified to pH 2 with 6 M HCl and extracted six times with diethyl ether using separatory funnel. The ether extracts were then combined and washed twice with equal volumes of distilled water. Ether was evaporated in desiccators. The extracted phenolic acids were dissolved separately in 2 ml of water: methanol (75: 25, v/v).

Caffeic acid, *p*-hydroxy benzoic acid (PHBA) and gallic acid were used as standard for HPLC analysis. All solvents and distilled water were used HPLC grade. The stock solution of each standard was prepared in methanol (1 mg/ml i.e., 1000 ppm). Different concentration of each standard (i.e. 100, 10, 5, 2.5 and 1 ppm) was prepared in the mobile phase methanol: water (80: 20, v/v) and the standard curves were plotted.

Mobile phase, standards and samples were filtered through 0.22 µm flour pore filters (Millipore) before injecting into the HPLC column. Samples (20 µl) were injected into HPLC column for analysis using a micro Hamilton syringe. Absorbance was recorded at 280 nm with UV-detector. The column was C₁₈ reverse phase (250 mm long × 4.4 mm i.d.; Particle size 5 µm with mobile phase 2 % acetic acid in water: methanol (82:18, v/v) in isocratic mode for 30 min at a flow rate of 1 ml/min was used.

Statistical analysis

Graphs were plotted using Origin 6.0 software. Each value in graphs and tables represents the mean of triplicate measurements. The data obtained was analyzed by completely randomized design (CRD). Standard error of means (SEM±) and critical difference (CD) was calculated at 5 % level of significance.

Results and discussion

Characteristics of immobilized β-glucosidase

Size of synthesized silicon oxide nanoparticles were found to be 0.02–0.06 µm using TEM (Fig. 1a). After immobilization of purified β-glucosidase (isolated from *B. subtilis* mutant, PS-CM5-UM3) on SiO₂ nanoparticles, the size of nanoparticles were 0.06–0.1 µm (Fig. 1b).

The functionalized silica nanoparticles and β-glucosidase bound nanoparticles were revealed by FTIR spectroscopy (Fig. 2). FTIR analysis showed the characteristic SiO₂ peaks (444, 1059 and 1712 cm^{−1}) and shifts in these peaks were observed in the spectra of β-glucosidase-bound silica nanoparticles. Peaks between 3000 and 3500 cm^{−1} were due to vibration modes of O–H and –NH groups present in the β-glucosidase (Pathmamanoharan et al. 1996). The peaks observed at 3285 cm^{−1} are therefore due to –OH and –NH groups of β-glucosidase. Peaks at 2886 and 2942 cm^{−1} were depicted due to C–H stretching and the peaks observed at 1642 cm^{−1} were due to amide II of the enzyme while peaks between 1212 and 1414 cm^{−1} confirmed –CH in β-glucosidase. The shifting in peak from 1059 to 1038 and 798 to 790 cm^{−1} corresponded to –C=O binding of β-glucosidase on nanoparticles, which clearly demonstrated the binding of β-glucosidase to the surface of the silica nanoparticles. The results were similar to the

Fig. 1 TEM micrographs of SiO₂ nanoparticles studied by transmission electron microscope (JEM 1011) operated at an accelerating voltage of 80 kV. **a** SiO₂ nanoparticles, **b** β-glucosidase (isolated and purified from *Bacillus subtilis* mutant PS-CM5-UM3) immobilized on SiO₂ nanoparticles

Fig. 2 FTIR spectra of activated silicon dioxide nanoparticles and β -glucosidase bound SiO_2 nanoparticles. Activated SiO_2 nanoparticles (light line) and β -glucosidase bound SiO_2 nanoparticles (bold line)

FTIR spectra of free β -galactosidase from *K. lactis* and its immobilization onto the SiO_2 nanoparticle (Verma et al. 2012). It was found that the immobilization efficiency of the isolated β -glucosidase was 53.56 % with 81.81 % immobilization yield. Results signified that a proper association between enzyme and the support occurred without affecting the enzyme integrity (Cecchini et al. 2007; Guisán et al. 1996).

pH and temperature optima of free and immobilized β -glucosidase

Both free and immobilized β -glucosidase had the optimum pH 5.0. The immobilized β -glucosidase exhibited 6, 16, 38, 22 and 11 U/mg activities at pH 3.0, 4.0, 5.0, 6.0 and 7.0 respectively. While free β -glucosidase showed 3, 18 and 37, 21 and 4 U/mg activities at pH 3.0, 4.0, 5.0, 6.0 and 7.0 respectively (Fig. 3a).

The optimum temperature for the free β -glucosidase was 60 °C while for immobilized β -glucosidase it was 60–70 °C. Free β -glucosidase showed 2, 3, 10, 35 and 29 U/mg activity while immobilized β -glucosidase exhibited 6, 9, 11, 37 and 36 U/mg activity at 30, 40, 50 and 70 °C, respectively (Fig. 3b). The overall stability of β -glucosidase thus increased after covalent immobilization on SiO_2 nanoparticles. This increase of optimum temperature range may be due to conformational stability of the enzyme (Wang et al. 2011). Singh et al. (2011) also observed that the optimum temperature of the free β -glucosidase, isolated from *Agaricus arvensis* was 65 °C, but the β -glucosidase immobilized on SiO_2 nanoparticles had maximal activity at 70 °C. They also observed that for the free enzyme, increase in temperature resulted in a sharp decrease in the relative activity of the free enzyme. In contrast, the immobilized enzyme showed no such tendency and retained 82 % of its optimum activity even at 85 °C (Singh et al. 2011).

pH and thermal stability of free and immobilized β -glucosidase

The residual activity at pH 5 and pH 7 for free β -glucosidase was 69 and 60 % respectively while immobilized β -glucosidase respectively had 89 and 84 % of residual activity after 60 min of incubation time. Free β -glucosidase was highly inactive at pH 3 retaining only 24 % of residual activity after 30 min of incubation time while immobilized β -glucosidase could retain about 45 % of residual activity after 40 min of incubation time under same conditions (Fig. 4a).

After immobilization, temperature stability of β -glucosidase increased. Free β -glucosidase had 76, 72 and 53 % of residual activity while immobilized β -glucosidase had 86, 80 and 70 % of residual activity after 60 min of incubation at 37, 60 and 70 °C respectively (Fig. 4b). It has also reported about 288-fold increase in the thermal stability at 65 °C, after immobilization of β -glucosidase on SiO_2 nanoparticles (Singh et al. 2011). This transition in stability of immobilized β -glucosidase may be attributed to the multiple covalent bonds that alter the conformational flexibility and thermal vibrations, thus preventing the immobilized protein from unfolding and denaturing (Mateo et al. 2000; Wong et al. 2009).

Kinetic parameters of free and immobilized β -glucosidase

Kinetics of immobilized β -glucosidase was studied by plotting Michaelis–Menten and Lineweaver–Burk plot (Fig. 5a, b). The V_{max} and K_m values were 1.513 U/mg and 1.074 mM respectively for the immobilized β -glucosidase. For free β -glucosidase these values were 3.5 U/mg and 0.9 mM respectively. K_{cat} values for free and immobilized β -glucosidases were 87 and 37 s^{-1} . Catalytic efficiency (K_{cat}/K_m) for free and immobilized β -glucosidases were

Fig. 3 Optima of β -glucosidase: **a** pH and **b** temperature. Free enzyme (filled circle) and immobilized enzyme (filled triangle). pH—free: CD at 5 % = 2.36 and SEM $\pm$ = 0.75; immobilized: CD at 5 % = 3.64 and SEM $\pm$ = 1.16. Temperature—free: CD at 5 % = 3.76 and SEM $\pm$ = 1.19; immobilized: CD at 5 % = 2.73 and SEM $\pm$ = 0.86

81.0 and 3.5 mM/s respectively. Increased K_m indicated that immobilization has slightly altered the affinity of the β -glucosidase for its substrate. But since this change in apparent K_m is negligible it may be considered that covalent immobilization had no adverse effect on catalytic site of the enzyme. However, decrease in V_{max} , K_{cat} and catalytic efficiency (K_{cat}/K_m) of the enzyme suggests the influence of covalent immobilization of enzyme on its accessibility to its substrate. This may also be attributed to decreased flexibility of the enzyme. However in other reports, immobilization of β -glucosidase on SiO_2

nanoparticles caused an increase in both the K_m (2.5 mM for free and 3.8 mM for immobilized) and V_{max} (3028 U/mg protein to 3347 U/mg protein) of the enzyme (Singh et al. 2011). The K_m and V_{max} values were 27.1 mM and 3.41 mmol/mg/min for free glucose oxidase while 14.6 mM and 0.116 mmol/mg/min for glucose oxidase immobilized on aminated- $CoFe_2O_4/SiO_2$ magnetic nanoparticles (Wang et al. 2009). Similar results were found by Georgelin et al. (2010) with increase K_m (from 7.6 mM to 8.6 mM) and decreased k_{cat} (from 69 to 34 s $^{-1}$) after immobilization of β -glucosidase on functionalized γ - $Fe_2O_3 @ SiO_2$ core-shell magnetic nanoparticles.

Storage stability and reusability of immobilized β -glucosidase

Storage stability of immobilized β -glucosidase was increased as compared to the free β -glucosidase (Fig. 6). Immobilized β -glucosidase retained about 86 % residual activity on 12th day when stored at 4 °C and 10 % residual activity on 9th day at 25 °C. While the free β -glucosidase had 21 % residual activity on 12th day when stored at 4 °C and 4 % on 6th day at 25 °C. The immobilized β -glucosidase unveiled 70 % of its original activity with pNPG substrate and 60 % residual activity in sugarcane juice treatment, even after the 10th cycle of reuse (Fig. 7). *A. arvensis* β -glucosidase immobilized on SiO_2 nanoparticles exhibited 95 % of its original activity even after the 25th cycle of reuse (Singh et al. 2011). Thus, after immobilization β -glucosidase could be used for multiple cycles of hydrolysis without any significant loss in its activity (Su et al. 2010; Li et al. 2011; Chang and Juang 2007). Such improvement in overall performance i.e., high stability and reusability makes the immobilized β -glucosidase useful for food and flavoring industries.

Effects of β -glucosidase on sugarcane juice

Changes in physico-chemical properties of sugarcane juice after different treatments, was best obtained when juice was treated with immobilized β -glucosidase enzyme (JIE). It was observed that when sugarcane juice was treated with 10 U of immobilized β -glucosidase, there was about 10 % decrease in its density (from 1.113 to 1.011 g/ml) and about 13 % in its viscosity (from 1.789 to 1.556 centipoise) while reducing sugar content increased about 25 % (from 18.21 to 22.82 mg/ml (Table 1)). However, there was no significant change (only 1.7 %) in its surface tension (from 77.178 to 75.935 dynes/cm) after treatment with immobilized β -glucosidase. Boiled sugarcane juice (JB) also showed a decrease in density and viscosity but the change was poor as compared to that obtained after treatment with immobilized (JIE). Also, in case of the juice passed

Fig. 4 Stability of free purified and immobilized β -glucosidase: **a** pH stability: free purified, pH 3 (filled diamond), pH 5 (filled square), pH 7 (filled triangle) and Immobilized: pH 3 (times symbol), pH 5 (asterisk), pH 7 (filled circle); **b** temperature stability: free purified, 37 °C (filled diamond), 50 °C (filled square), 60 °C (filled triangle), immobilized 37 °C (times symbol), 50 °C (asterisk), 60 °C (filled circle). pH (free)—pH 3

through charcoal (JCT), a decrease in reducing sugar content (15.72 mg/ml), density (0.0891 g/ml) and viscosity (1.306) was observed. Decrease in surface tension was insignificant in all the three cases after different treatments.

Decrease in viscosity after treatment of juice with β -glucosidase enzyme and boiling occurred due to the hydrolysis of macromolecules to monomers. Liquids with large, elongated molecules show a high viscosity as they interact more strongly with one another, forming aggregates (Junk and Pancoast 1973). Hydrolysis of sugars and organic compounds may be the reason behind changed density of the juice samples. Reason for increased reducing sugar after treatment of juice with β -glucosidase and after boiling must be the hydrolysis of bonds between sugar and phenolic compounds causing release of sugars in the juice.

Decrease in reducing sugar in the juice passed through charcoal indicates the adsorption of sugars in activated charcoal.

Treatment of sugarcane juice with immobilized β -glucosidase (JIE) showed Hyperchromic shift (Fig. 8). This may be due to the breakdown of the bonds between glycosides and phenolics leading to release of the phenolic compounds in the juice. Little hyperchromic effect was also observed in juice boiled for 30 min (JB) due to hydrolysis of glycosidic bonds between phenolics and glucose residues. Sugarcane juice passed through activated charcoal showed hypochromic shift. Less absorbance of activated charcoal treated juice (JCT) could be due to adsorption of some phenolic compounds by the activated charcoal.

Fig. 5 Kinetics of free (filled circle) and immobilized (red triangle) β -glucosidase. **a** Michaelis–Menton plot, **b** Lineweaver–Burk plot. Free: CD at 5 % = 0.53 and SEM $\pm$ = 0.18; immobilized: CD at 5 % = 0.19 and SEM $\pm$ = 0.066 (color figure online)

HPLC of sugarcane juice

Sugarcane juice samples with four different treatments were analyzed for the phenolics (Table 2). Gallic acid, *p*-hydroxybenzoic acid and caffeic acid were used as phenolic standards. The phenolics of sugarcane juice samples undergone different treatments was analyzed by HPLC and the data is presented in Table 2.

Two phenolic compounds, phenolic *p*-hydroxy benzoic (PHBA) and gallic acid were identified in sugar juice. Juice treated with immobilized β -glucosidase (JIE) was recorded

to have all three phenolic caffeic acid, PHBA and gallic acid whereas one phenolic i.e. gallic acid was identified in boiled juice (JB) and juice passed through activated charcoal (JCT). PHBA and gallic acid were found to be the major phenolic whereas caffeic acid as minor. Control sugarcane juice had 4.37 ppm PHBA and 21.43 ppm gallic acid while caffeic acid was absent in it. In juice treated with immobilized β -glucosidase showed an increase in caffeic acid, PHBA and gallic acid 1.04, 15.77 and 51.33 ppm respectively. However, boiled juice and juice treated with activated charcoal contained only gallic acid at a concentration of 34.89 and 20.09 ppm, respectively.

Immobilized β -glucosidase treatment sugarcane juice PHBA and 2.4 folds gallic acid as compared to the control. Caffeic acid which was absent in control sample was released in the juice samples treated with immobilized β -glucosidase at a concentration of about 1.04 ppm. Thus, β -glucosidase treatment was very efficient in causing the release of phenolics possibly by hydrolyzing the bonds between the phenolics and their glycoside counterparts. Also, β -glucosidase treatment gave better results than the boiling of juice as after boiling release of only one acid was elevated while after β -glucosidase treatment, the concentration of all the three acids was increased. It was observed that PHBA was removed after boiling but the content of gallic acid increased up to 0.63 folds. Absence of PHBA in boiled juice may be due to its precipitation during boiling for 30 min while increase in gallic acid content signified the release of the phenolics after hydrolysis of its glycoside counterpart. Due to some unknown reasons, it was observed that activated charcoal could not adsorb gallic acid. While absence of PHBA in the juice passed through activated charcoal symbolized its adsorption in charcoal particles. Kai et al. (2012) reported the presence of gallic acid in sugarcane juice within a range of 20–100 ppm. They also observed that the content of gallic acid was 21.70 ppm in sugarcane juice. While, caffeic acid was found to be absent in raw sugarcane juice (Duarte-Almeida et al. 2011).

These phenolic acids are bioactive compounds as they prevent cardiovascular complications, diabetes, ulcers and cancer (Halliwell 2007; Sachidanandam et al. 2005). These components present in sugarcane juice are not utilized during the sugar-making process. Currently, there is not enough concern about the utilization of the natural bioactive compounds in raw sugarcane juice (Li et al. 2011). This study thus signified how enzyme-assisted processing of the sugarcane juice may increase the extractability of phenolic components. This would be helpful in extraction of those bound phenolics which would otherwise be wasted. The phenolics may be released in ample amounts after treatment with β -glucosidase enzyme. Finally, these

Fig. 6 Storage stability of free and immobilized β -glucosidase: Free: 4 °C (filled triangle), 25 °C (times symbol), immobilized: 4 °C (filled diamond), 25 °C (filled square). Free- 4 °C

Fig. 7 Reusability of immobilized β -glucosidase with substrate pNPG (square) and sugarcane juice (filled square). CD at 5 % = 0.57 and SEM $\pm$ = 0.19

bioactive compounds can be recovered by passing the juice treated with β -glucosidase through activated charcoal to processed and can be used as health-supplements in pill form. This method is able to provide high quality and high activity extracts while precluding any toxicity associated to the organic solvents.

Conclusion

Immobilization of purified β -glucosidase on SiO₂ nanoparticles is promising as it increased enzyme stability towards pH and temperature. Immobilization also improved enzyme reusability. Efficient release of the phenolic compounds occurred when sugarcane juice was treated with the immobilized β -glucosidase. This attracts attention for enzyme-assisted phenolics elevation and

Table 1 Physiochemical properties of the sugarcane juice

Treatments	Reducing sugar (mg/mL)	Density (g/mL)	Surface tension (dynes/cm)	Viscosity (centipoise)
Control	18.21	1.11	77.17	1.78
JIE	22.82	1.01	75.93	1.55
JB	18.21	1.02	76.45	1.56
JCT	15.90	0.89	75.83	1.30

Density, viscosity, surface tension and reducing sugar were determined for control: sugarcane juice before treatment, JIE: after treatment with immobilized β -glucosidase, JB: juice boiled for 30 min and JCT: sugarcane juice passed through charcoal

Reducing sugar: CD at 5 % = 14.10 and SEM $\pm$ = 4.48; density: CD at 5 % = 0.043 and SEM $\pm$ = 0.014; surface tension: CD at 5 % = 3.87 and SEM $\pm$ = 1.23; viscosity: CD at 5 % = 0.65 and SEM $\pm$ = 0.42

Fig. 8 Absorption spectra of different sugarcane juice samples: control, juice without any treatment (*orange diamond*); JIE, Juice treated with immobilized β -glucosidase (*green triangle*); JB, boiled juice (*purple times symbol*) and JCT, Juice passed through activated charcoal (*blue asterisk*) (color figure online)

Table 2 Phenolic acid content in different sugarcane samples as analyzed by HPLC

	Total number of acids identified	Caffeic acid (ppm)	PHBA (ppm)	Gallic acid (ppm)
Control	2	—	4.36	21.42
JIE	3	1.04	15.77	51.33
JB	1	—	—	34.88
JCT	1	—	—	20.09
Retention time	—	17.7	12.7	4.2

extraction of these phenolics for utilization as health-supplements.

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