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Impact of immobilized β-Glucosidase treatment on sugarcane juice

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Abstract

β-Glucosidase (BGL) from *Bacillus subtilis* was immobilized in polyacrylamide gel. Increasing the concentration of either acrylamide or bisacrylamide the yield of entrapped BGL was increased. The immobilized BGL showed 14.02% retention activity after storage for 25 days at 30ºC. However, after three times repeatedly use of immobilized enzyme exhibited retention of 8.5% residual activity. Change in physicochemical properties such as viscosity, density and reducing sugar was identified while sugarcane juice treated with free and immobilized BGL. A remarkable decrease in viscosity of the treated sugarcane juice was observed. Though, an increase in reducing sugar was affirmed in free BGL and immobilized BGL. The absorption spectrum of sugarcane juice was also recovered raised by delivering the phenolic. These bioactive phenolic compounds could be retrieved after passing by a suitable adsorbent like charcoal and used as dietary supplements or other medicinal purposes.

Introduction

India is the world's second highest producer of sugar followed by Brazil. Sugar manufacturing industries stand to a major hurdle in the production of sugar of high precision. Normally, the browning of sugarcane juice is due to the enzymatic and non-enzymatic reactions of phenolic compounds adversely deteriorate sugar quality, purity, and color. The phenolic compounds thus impart an undesirable colour of the sugarcane juice and its removal is an important problem associated with sugar manufacture (Vickers *et al*., 2005). Despite their negative effect on sugar quality, these phenolic 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 attention about the utilization of the natural bioactive compounds in raw sugarcane juice (Li *et al*., 2011). These phenolic compounds are attached to the sugar molecules with a glycosidic bond. β-glucosidase (BGL) (EC 3.2.1.21) widely distributed throughout the plant kingdom. They hydrolyze alkyl- and aryl-β-glucosides, as well as diglucosides and oligosaccharides, to release β-D-glucose residue and terminal aglycone. BGL also catalyzes transglycosylation reactions which have great importance in wine or beverage industry because of their abilities to improve the aroma (Jatinder *et al*., 2007). The synthetic activity of BGL can be employed in the preparation of a variety of compounds such as oligosaccharides and glycoconjugates that have a potential for use as agrochemicals or drugs. Phenolics are responsible for structural and protective functions in plants, contributing to flavours, colour, astringency and bitterness of fruits and vegetables (Soto *et al*., 2011). BGL have been proposed to increase the aromatic potential of juices from their glycoside forms (Iembo *et al*., 2002). Hence, processing of sugarcane juice with BGL can be useful for the extraction of the phenolic compounds. Immobilization of enzyme often confers considerable stability towards temperature and organic solvents, in addition to providing a convenient means to separate and reuse the biocatalyst to improve process economics (Kim *et al*., 2009). BGL has immobilized on clay minerals or mineral–organic substances complexes (Quiquampoix, 1987; Chang *et al*., 2008), adsorption via covalent binding to solid supports (Busto *et al*., 1995), agarose gels (Spagna *et al*., 2000), polymer carriers such as nylon (Isgrove *et al*., 2001), silica gel (Synowiecki *et al*., 2006), Eupergit C (Tu *et al*., 2006), carbon nanotubes (Pau *et al*., 2008), alginate gel (Keerti *et al*., 2014) and SiO_2 nanoparticle (Agarwal *et al*., 2016).Polyacrylamide is a well-known low cost, high molecular weight polymer which has the advantage of sufficient chemical stability, a uniform physical state, porosity and

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commercial availability (Kirk-othmer, 1986). In our laboratory thermostable BGL from *Bacillus subtilis* has purified and characterized (Agarwal *et al*., 2016). In the present work, the immobilization of BGL in polyacrylamide gel and its utilization for extraction of phenolics from sugarcane juice was investigated.

Materials and Methods

Chemicals and bacterial culture

All chemicals and components used were of analytical grade and procured from Sigma Chemicals Ltd., Himedia Laboratories Ltd., GeNei, SRL, and Merck Pvt. Ltd. Recombinant BGL from *Bacillus subtilis* strains PS (identified using 16S rDNA sequencing; GenBank Accession number JQ066263) cloned in *E. coli* DH5α in our laboratory was used in this study (Chamoli *et al*., 2016). BGL was purified from the culture as per the method by Keerti *et al.* (2014).

Immobilisation of β-BGL in polyacrylamide gel

The entrapment of BGL in polyacrylamide gel was achieved by copolymerization of 1.5 mL of 15% (w/v) acrylamide in varying concentration of 1.5 mL of N,N'-methylenebisacrylamide (BIS) in the presence of BGL enzyme. Similarly, copolymerization of 1.5 mL of 1.2% BIS was conducted in varying concentration of 1.5 mL of acrylamide solution in the presence of BGL enzyme. The polymerization was initiated by a redox system composed of 0.5 mL of a 0.5% N,N,N',N'-tetramethylethylene-diamine (TEMED) and 0.5 mL of a 2.5% ammonium persulfate, the mixture was cooled on ice bath. Once the polymerization was complete (after about 30 min), the gel was mechanically dispersed using a homogenizer. The gel particles were then thoroughly washed with the acetate buffer (pH-5.0, 0.1M) until no enzyme activity was perceived in the final washing (Ortega *et al*., 1998).

BGL activity

BGL activity was assessed spectrophotometrically using pNPG as substrate. The reaction mixture, containing 100 µL BGL enzyme extract in acetate buffer (pH 5.0, 100 mM) and 100 µL of pNPG in a similar buffer, was incubated for 30 min at 60°C. The reaction was terminated by adding 2 mL of a 1MNa_2CO_3 solution and the absorbance was recorded at $\lambda_{405\ nm}$ (Martino *et al.,* 1996). The activity of immobilized BGL was determined using the procedure given above except 0.2 g immobilized enzyme was used in place of 100 µL enzyme extract. The immobilized BGL was stored in acetate buffer (100mM, pH 5.0) at 0ºC and 30ºC and activities were recorded periodically over a duration of 30 days. The residual activity of the BGL at different time intervals was estimated.

Treatment of sugarcane juice by BGL

Sugarcane juice 5 mL was incubated at 60°C for 30 min in the presence of 10 U of free or immobilized BGL and the reducing sugar was determined by Somogyi's method (Somogyi, 1952). Determination of relative density and viscosity coefficient of treated sugarcane juice with free and immobilized enzyme was obtained by density bottle method and Ostwald's viscometer at room temperature (Agrawal *et al*., 2016). After five times dilution of the treated sugarcane samples were scanned between the wavelengths range 400-800 nm for absorption spectra.

Statistical analysis

The mean values and standard deviations of three experiments were calculated and exhibited in the Figures as error bars. One-way ANOVA at the significance level of 0.005 and 0.001 was performed using Microsoft Excel 2007 add-in module: Analysis ToolPak.

Results and Discussion

Effect of immobilization of BGL on polyacrylamide gel

An increased concentration of acrylamide the yield of trapped BGL was increased as expected by the decrease of the gel porosity. However, the activity of the retained BGL enzyme reached a maximum of 75.75% when 15% acrylamide was used (Figure 1a). Higher monomer concentrations decreased the percentage of immobilization, possibly because of an inactivation of the BGL occurred during the polymerisation process (Degani and Miron, 1970). Similarly, immobilization yield was estimated by varying the concentration of bisacrylamide at a constant concentration of 15% acrylamide (Ortega *et al.*, 1998). An increased concentration of bisacrylamide the yield of entrapped BGL was increased. However, the retained enzyme reached a maximum of 68.48%, while 1.6% bisacrylamide was used (Figure 1b). Storage stability is one of the most important criteria for the application of an enzyme on the commercial scale. Generally, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced (Tumturk *et al*., 2007). The residual activity of the BGL at different time intervals was estimated (Figure 2). The

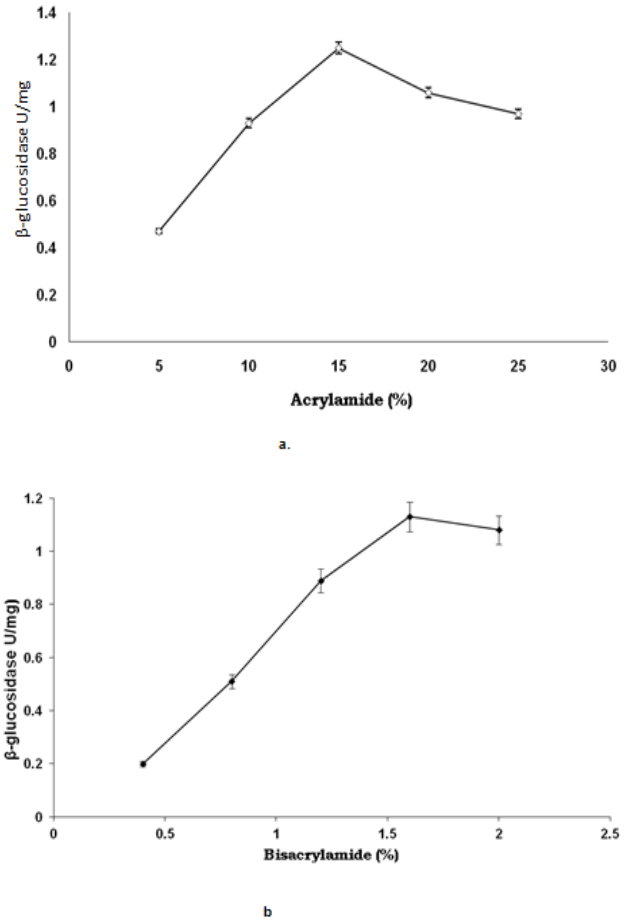

Figure 1. Effect of acrylamide and bisacrylamide concentration on activity of immobilized BGL
a. Different concentration of acrylamide b. Different concentration of bisacrylamide

residual activity of free BGL was observed gradually decreased from 0 to 4 days at 4ºC and no residual activity was observed after one day when the enzyme was stored at 30°C (Verma *et al.*, 2013). However, the retained immobilized BGL after 25 days was found to be 29.26% at 4°C and 14.02% at 30°C of its original activity. Tumturk *et al*., (2007) observed higher stability of the immobilized lipase, which could be attributed to the prevention of autodigestion and thermal denaturation as a result of multipoint attachment of the enzyme on the P (DMAm-co-AAm) and P (NIPA-co-AAM) /carragenan hydrogels (Yahsi *et al*., 2005; Sahin *et al*., 2005; Tumturk *et al.*, 2007; Yu *et al*., 2011). The number of reuse of immobilized enzymes is one of the most important aspects of industrial application. An increased stability could make the immobilized enzyme more advantageous than its free form (Su *et al*., 2009). The immobilized BGL was reused for 3 times and the residual activity decreased to 8.5%. Thus, immobilized enzyme activities decreased while reuse number increases. It exhibited that inactivation of the enzyme caused by the denaturation and the leakage of the enzyme from

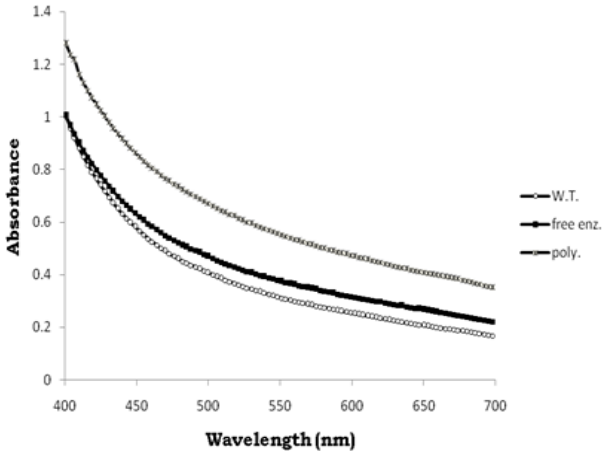

Figure 3. Absorption spectra of treated and untreated sugarcane juice by free and immobilized BGL. (W.T- juice without treatment, free enz– juice treated with free BGL, poly. – juice treated with immobilized BGL in polyacrylamide gel).

gels upon use and diffusion effects (Yan *et al.*, 2010). In polyacrylamide gel, the acrylamide pendant chains are capable of reacting with the components of the macro-environment to originate acrylic acid groups. Under these conditions, the enzyme is embedded in a highly negatively charged three-dimensional network formed by the many ionized carboxyl groups of the synthetic carrier. The enzyme is thus exposed to a strong electrostatic field, which may markedly affect its mode of action. Immobilization may cause changes in the structure of the enzyme and even steric impediments, depending on how the enzyme links to the support. These effects are exhibited in the activity and kinetics of the action of the enzyme and stability, and mainly affect its interactions with the substrate and, consequently its specific features of enzymatic action (Gonzalez-Saiz and Pizarro 2001).

Effect of BGL on sugarcane Juice

Effect of both free and immobilized BGL enzymes on sugarcane juice was analyzed. It was observed that after sugarcane juice treated with free and immobilized BGL for 30 min at 60ºC the physicochemical properties of juice changed such as the viscosity of the untreated juice was 2.009 centipoise decreased to 1.350 centipoises in free BGL and 1.499 centipoises in immobilized BGL. The reducing sugar was also increased from 6.348 g/L in untreated juice to 9.438 g/L in free BGL and 8.134g/L in immobilized BGL (Table 1). The BGL treated sugarcane juice passes through activated carbon increases the reducing sugar, reduces the density and change in absorbance was detected under visible spectrophotometric spectra analysis (data not shown). It showed that the juice passes

Table 1. Effect of free and immobilized enzyme on physio-chemical properties of sugarcane juice

Type of juice	Reducing sugar (g/L)	Density (g/ml)	Viscosity (Centipoise)
Untreated juice	6.348	1.065	2.009
Treated with Free BGL	9.438	1.066	1.351
Treated with immobilized BGL	8.134	1.06	1.449
BGL treated Juice Passes through activated charcoal	15.90	0.891	1.306

through the activated charcoal absorbs the phenolic compounds and the juice contains no colour. While the absorption spectra of BGL treated juice showed increased absorbance in the visible region (Figure 3). The increase of absorption spectra (hyperchromic shift) showed that the BGL hydrolysed the glycosidic linkage between the sugar and phenolic compounds present in the sugarcane juice and juice appear dark in colour. The similar results have also shown by immobilization of thermostable BGL on SiO_2 nanoparticle (Agrawal *et al*., 2016) and on alginate beads (Keerti *et al*., 2014) to elevate the phenolic content in sugarcane juice. These compounds also play an important role in growth and reproduction, protection against pathogens and predators (Bravo, 1998), besides contributing towards the colour and sensory characteristics of fruits and vegetables (Alasalvar *et al*., 2001). Elevation and extraction of the bioactive compounds from sugarcane juice by BGL treatment would provide high quality and high-activity phenolic extracts while precluding any toxicity associated with mineral acid hydrolysis. These bioactive phenolic compounds could be recovered after passing through a suitable adsorbent like charcoal and used as dietary supplements or other medicinal purposes (Agrarwal *et al*., 2016). Phenolic compounds are useful to prevent cardiovascular complications, diabetes, ulcers and cancer (Sachidanandam *et al*., 2005; Halliwell, 2007). These compounds are precipitated as press-mud during clarification of sugarcane juice in the sugar-making process.

Conclusion

Currently, there is not enough concern about the utilization of the natural bioactive compounds in raw sugarcane juice. This is a preliminary study to remove the phenolics those are linked by a glycosidic bond. This would be helpful in the extraction of phenolics which would otherwise be wasted during sugar making process. The phenolics may be released in ample amounts after treatment with BGL enzyme. In future, these phenolic compounds can be recovered by passing the BGL treated juice through activated charcoal, 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 with the organic solvents.

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