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ORIGINAL ARTICLE

Purification and biochemical characterization of an extracellular β-D-fructofuranosidase from *Aspergillus* sp.

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Abstract

This study focused on the purification and characterization of an extracellular β-D-fructofuranosidase or invertase from *Aspergillus sojae* JU12. The protein was purified by size exclusion chromatography with 5.41 fold and 10.87% recovery. The apparent molecular mass of the enzyme was estimated to be ~ 35 kDa using SDS-PAGE and confirmed by deconvoluted mass spectrometry. The fungal β-D-fructofuranosidase was suggested to be a monomer by native PAGE and zymography, and was found to be a glycoprotein possessing 68.92% carbohydrate content. The products of enzyme hydrolysis were detected by thin layer chromatography and revealed the monosaccharide units, D-glucose and D-fructose. β-D-fructofuranosidase showed enhanced activity at broad pH 4.0–9.0 and activity at a temperature range from 30 to 70 °C, while the enzyme was stable at pH 8.0 and 40 °C, respectively. The β-D-fructofuranosidase activity was lowered by metal ion inhibitors Ag^{2+} and Hg^{2+} whereas elevated by SDS and β-ME. The fungal β-D-fructofuranosidase was capable of hydrolyzing D-sucrose and the kinetics were determined by Lineweaver–Burk plot with K_m of 10.17 mM and V_{max} of 0.7801 µmol min^{-1}. Additionally, the extracellular β-D-fructofuranosidase demonstrated tolerance to high ethanol concentrations indicating its applicability in the production of alcoholic fermentation processes.

Keywords *Aspergillus* sojae · B-D-fructofuranosidase · Ethanol tolerant · Glycoprotein · Invertase · Purification

Introduction

β-D-fructofuranosidase (EC 3.2.1.26) is also known as invertase and catalyzes the hydrolysis of the disaccharide D-sucrose producing D-glucose and D-fructose. The hydrolytic enzyme produces the invert sugar mixture (1:1) of dextrorotatory and levorotatory monosaccharides, which possesses lower crystallinity than D-sucrose (Alberto et al. 2004). β-D-fructofuranosidase is required in numerous applications in the food industries. The breweries and baking industrial sectors demand β-D-fructofuranosidases due to the property of non-crystallization and hygroscopicity (Bayramoglu et al. 2003). The enzyme is capable to maintain moisture, freshness and softness in food products for longer hours, also for the production of artificial honey soluble β-D-fructofuranosidases are preferred. The sugar mixture obtained from the enzymatic hydrolysis by β-D-fructofuranosidase does not alter the colour, flavour, texture of the food stuffs when compared to acidic hydrolysis treatments (Arica et al. 2000; Shaheen et al. 2008).

β-D-fructofuranosidase are reported in plants (Roitsch and Gonza´lez 2004; Chaira et al. 2010), microbial diversity such as bacteria (Yoon et al. 2007; Awad et al. 2013), fungi (Kurakake et al. 2010; Rustiguel et al. 2011; Gracida-Rodríguez et al. 2014) and yeasts (Plascencia-Espinosa et al. 2014; Andjelković et al. 2015). β-D-fructofuranosidases are mostly studied in *Saccharomyces cerevisiae* strains (Rashad and Nooman 2009; Andjelković et al. 2010; Veneshkumar et al. 2011; Shankar et al. 2013). Comparatively, there are lesser findings on β-D-fructofuranosidases from molds which deserves attention (Alves et al. 2013). However, majority of the fungal β-D-fructofuranosidases reported so far are largely filamentous fungi especially from *Aspergillus* sp.

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(Lucca et al. 2013; Rustiguel et al. 2015), *Penicillium* sp. (Flores-Gallegoss et al. Flores-Gallegos et al. 2012), *Rhizopus* sp. (Goulart et al. 2003) and *Fusarium* sp. (Wolska-Mitaszko et al. 2007). There is a huge demand for β-D-fructofuranosidases from filamentous fungi with potential characteristic features due to their biotechnological applications for the production of invert sugar syrup, food and beverages. The production of β-D-fructofuranosidases by submerged fermentation (SmF) and solid-state fermentation (SSF) systems have been earlier reported (Alves et al. 2013; Oyedeji et al. 2017).

Extracellular β-D-fructofuranosidases are industrially desired for the ease in down-streaming processes. As per Andjelković et al. (2010), the search for stable extracellular β-D-fructofuranosidases for D-sucrose hydrolysis is ongoing. Thus, new microbial strains producing potential β-D-fructofuranosidases with biotechnological significance are to be identified from the largely unexplored fungal biodiversity. The purification and characterization of β-D-fructofuranosidase is crucial to understand the hydrolytic action and nature of the enzyme. Thus, the aim of the present study was, therefore, to purify and characterize an external β-D-fructofuranosidase from *Aspergillus sojae* JU12 to unravel the enzymic properties.

Materials and methods

Materials

Acrylamide, *N*,*N*′-bis-acrylamide, β-mercaptoethanol (β-ME), coomassie brilliant blue (CBB) R-250, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), iodoacetamide (IAA), *N*-acetyl imidazole (NAI), N-bromosuccinimide (NBS), *N*-ethylmaleimide (NEM), phenylmethylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS) and tosyl-L-phenylalanine chlormethyl ketone (TLCK) were purchased from Sigma-Aldrich, Co, St Louis, MO (USA). Bovine serum albumin (BSA), 3,5-dinitrosalicylic acid (DNS), potato dextrose agar (PDA), Schiff's reagent, periodic acid, D-sucrose, 2,3,5-triphenyl tetrazolium chloride (TTC) were procured from HiMedia Laboratories (Mumbai, India). Molecular weight marker (medium range) and silica gel 60 F_{254} plates (20 × 20 cm) were purchased from Merck Pvt. Ltd. (Mumbai, India). All the other chemicals used were of analytical grade.

Fungal strain and fermentation medium

β-D-fructofuranosidase producing fungus was isolated from black soil of sugarcane growing regions in Maddur (Karnataka, India). The microbial colony was identified by 18S rRNA molecular sequencing as *Aspergillus sojae* JU12 (GenBank accession number MG051335.1), was used in the present study. The strain was preserved in 40% (v/v) glycerol stocks and revived on PDA medium. The SSF medium consisted of orange peel substrate (20 g) moistened with 50% diluted molasses medium (50% total sugars), fortified with beef extract (1.5%, w/v) as the nitrogen source accompanied with salts and trace elements (w/v) KH_2PO_4 0.35%, $MgSO_4{\cdot}7H_20$ 0.075% and $FeSO_4{\cdot}7H_20$ 0001%. The solid-substrate medium was inoculated with 9% (v/w) fungal inocula (1×10^8 spores/ml) and incubated at 37 °C for 120 h for maximum productivity. The enzyme was obtained by mechanical agitation for 1 h at 3 g with 40 ml of extraction buffer and the contents were centrifuged for 10 min, 11, 200 g at 4 °C. The enzyme activity and protein content were assayed in the cell-free supernatant which served as the extracellular crude enzyme.

Determination of β-D-fructofuranosidase activity and protein content

β-D-fructofuranosidase activity was estimated in the reaction assay mixture consisting of 0.1 ml of appropriately diluted enzyme (about 150 U) added to 1% (w/v) D-sucrose in 0.5 ml Tris–HCl (0.1 mol l^{-1}, pH 8.0), and incubated at room temperature (28 ± 2 °C) for 30 min. The reducing sugars were measured by the addition of 1.0 ml DNS and incubated in a boiling water bath for colour development (Miller 1959). The enzyme activity was measured at 540 nm using D-glucose as the standard. One unit of β-D-fructofuranosidase activity was defined as amount of enzyme which released 1 µmol of reducing sugars per min under the assay conditions. The protein content (about 0.09 mg of total proteins) was determined by the method of Lowry et al. (1951) with BSA as the standard. The absorbance was recorded using UV–Visible Spectrophotometer (Systronics Double Beam 2202, India).

Purification of extracellular β-D-fructofuranosidase

The crude β-D-fructofuranosidase extract was precipitated with ammonium sulphate at 80% saturation and incubated at 4 °C overnight. The protein pellet was harvested by centrifugation at 11,200 g for 10 min at 4 °C, and resuspended in minimum aliquots of Tris–HCl buffer (0.1 mol l^{-1}, pH 8.0). The protein was dialysed against the same buffer (0.01 mol l^{-1}) and lyophilized (Freeze dryer, Model LY3TTE, Snijders Scientific, Tilburg Holland) for 7 h. The lyophilized powder served as the partially purified β-D-fructofuranosidase. The enzyme was appropriately diluted and loaded on to the automated size exclusion chromatography column using the fast protein liquid chromatography (FPLC) system (ÄKTAprime plus, GE Healthcare, UK). The Sephadex G-100 column (1.4 cm × 21 cm) equilibration was

performed with Tris–HCl (0.05 mol l^{-1}, pH 8.0), and the protein was eluted at a flow rate of 1.2 ml min^{-1}. The eluate of 3.0 ml was collected as a single fraction and the active fractions were pooled, lyophilized based on the protein content and enzyme activity.

Electrophoresis and spectrometry

PAGE and zymography

Native PAGE and zymography for β-D-fructofuranosidase was performed under non-reducing conditions at 4 °C and the samples were separated on a 15% gel. The electrophoresis was carried out at 50 V in the stacking gel (4%) and 100 V in the separating gel (15%) for a duration of about 3–4 h. The protein bands in native PAGE were stained with 0.25% (w/v) CBB R-250 and destained overnight with 5% (v/v) methanol and 7.5% (v/v) glacial acetic acid. The corresponding zymogram was recovered, washed twice in Tris–HCl buffer (0.1 mol l^{-1}, pH 8.0) and incubated at 40 °C for 1 h in the presence of 8% (w/v) D-sucrose solution. The gel was submerged in a developing solution containing 0.2% (w/v) TTC in 0.1 mol l^{-1} NaOH, and heated in a boiling water bath until appearance of bands (Chaira et al. 2010).

SDS-PAGE

The SDS-PAGE (15%) was performed under reducing conditions at room temperature and was set up similar to the native PAGE (Laemmli 1970). The standard molecular weight medium range markers were used to compare the protein migration. Likewise, post electrophoretic run the reducing gel was stained and destained as that of the native gel (Laemmli 1970; Warchol et al. 2002).

Mass spectrometry

The purified β-D-fructofuranosidase was passed through C18 column (Agilent Poroshell 120, 4.6 × 7.5 mm, 2.7 µm) of the Agilent 1290 Infinity LC–MS system coupled to the quadrupole time of flight (Q-TOF, Agilent 6530). The solvents used were solvent A (0.1% formic acid in water) and solvent B (90% acetonitrile, 0.1% formic acid in water). The chromatography gradient initiated at 3% of solvent B was peaking at 95% within 15 min and lowered at 3% of solvent A. The spectrum was visualized with the Agilent Mass Hunter Qualitative Analysis software.

Thin layer chromatography

The chromatographic plate was spotted with 10 µl of D-sucrose, D-glucose, D-fructose as the standards, and β-D-fructofuranosidase hydrolysate. The sample hydrolysate was prepared in a mixture of 1:1, enzyme and substrate (1%, w/v) in buffer and incubated overnight at 37 °C. The samples were loaded on the TLC plate and placed inside the pre-saturated chamber which consisted of butanol–ethanol–water (5:3:2). The plate was air dried, dipped in the developing solution [0.2% (w/v) orcinol in a mixture of 1:9 (v/v) of H_2SO_4 and methanol] and heated at 100 °C (Rustiguel et al. 2011).

Carbohydrate composition and glycoprotein analysis

To determine the carbohydrate content of the enzyme, the total phenol sulphuric acid method was conducted with D-glucose as standard (Dubois et al. 1956). A primary glycoprotein test was performed at room temperature (28 ± 2 °C), wherein 0.5 ml of β-D-fructofuranosidase (about 150 U) was added to 1.0 ml of concentrated sulfuric acid and absolute ethanol (1:1) and the control was devoid of the enzyme. Furthermore, the enzyme was electrophoresed on SDS-PAGE for glycoprotein detection by Periodic acid-Schiff (PAS) staining (Møller and Poulsen 2002).

Biochemical characterization of purified β-D-fructofuranosidase

Effects of pH and stability

To determine the effect of pH, β-D-fructofuranosidase activity (about 150 U) was measured in various buffers (0.1 mol l^{-1}) of glycine–HCl (pH 2.0–3.0), sodium acetate (pH 4.0–5.0), phosphate (pH 6.0–7.0), Tris–HCl (pH 8.0–9.0), carbonate (pH 10.0) and glycine–NaOH (pH 11.0–12.0) buffers. The enzyme stability with optimal pH buffers (pH 6.0–8.0) was performed from 0 to 6 h at room temperature (28 ± 2 °C) and the enzyme activity was estimated as mentioned previously. The control was considered with maximum activity as 100% (Warchol et al. 2002).

Effects of temperature and stability

The effect of temperature on β-D-fructofuranosidase activity (about 150 U) was investigated from 0 to 80 °C, by pre-incubation with the optimally active buffer (0.1 mol l^{-1} Tris–HCl, pH 8.0) at various temperatures. The enzyme thermostability was checked at the optimal temperatures (40–60 °C) with the same buffer (pH 8.0), and the activity was assayed as mentioned earlier. The reaction mixture without heat treatment served as the control (100%).

Effects of metal ions

The various metal ions of the concentrations 1 and 10 mM (Ag^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Mg^{2+},

Mn^{2+}, Zn^{2+}) were tested. The enzyme (about 150 U) was pre-incubated with the metal ions for 15 min at optimal pH (0.1 mol l^{-1} Tris–HCl, pH 8.0) and optimal temperature (40 °C), and the assay was performed as previously. The reaction mixture without the addition of metal ions was considered as control with 100% activity.

Effects of group specific reagents

The effect of group specific reagents was investigated with the concentrations 1 and 10 mM (NEM, NBS, DTT, PMSF, NAI, SDS, EDTA, TLCK, β-ME, IAA). The enzyme (about 150 U) was pre-incubated with the reagents for 15 min, at optimal conditions pH 8.0 and 40 °C and β-D-fructofuranosidase activity was estimated as before. The reaction mixture without any group specific reagents was observed as control (100%).

Determination of kinetics

Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) was evaluated by measuring the rate of $_D$-sucrose hydrolysis with the optimum buffer and temperature conditions. The kinetic parameters were determined using D-sucrose at 0–50 mM and was calculated using the Lineweaver–Burk plot (Hyper32 software).

Effect of ethanol on β-fructofuranosidase

The effect of ethanol (0–20%, v/v) was tested on enzyme, and the activity was assayed as earlier. The reaction mixture devoid of ethanol was observed as control (Gargel et al. 2014).

Statistical analysis

All the experiments were conducted in triplicates and the results were expressed as mean ± standard deviation (SD) using Microsoft Excel (2016). One way analysis of variance (ANOVA) and means for groups in homogeneous subsets were determined by Duncan's multiple range test (DMRT) at $P_{0.05}$, using the statistical software (IBM SPSS Statistics V20.0.0). The values indicated with different letters on the error bars were considered significant.

Results and discussion

Purification studies of β-D-fructofuranosidase

There are various reported species of the genus *Aspergillus* such as *A. niger* (L'Hocine et al. 2000; Nguyen et al. 2005; Nadeem et al. 2009), *A. nidulans* (Alves et al. 2013), *A. ochraceus* (Guimaraes et al. 2007), *A. parasiticus* (Lucca et al. 2013), *A. phoenicis* (Rustiguel et al. 2011), known to produce β-D-fructofuranosidases. The isolated microorganism was identified morphologically by lactophenol cotton blue staining as *Aspergillus* sp. Further, the isolate was molecular sequenced by 18S rRNA and confirmed to be *sojae* species, the strain was deposited in the GenBank with the accession number MG051335.1. The gene sequences were subjected to perform Basic Local Alignment Search Tool (BLAST), a tool of the National Center for Biotechnology Information (NCBI) Genbank database. The selected sequences were chosen to be aligned using Clustal Omega, a software which was used to construct the phylogenetic tree. β-D-fructofuranosidases occurs in both intracellular and extracellular forms. The process of purification of enzymes is necessary, to understand enzymic nature or behaviour (Lincoln and More 2017). The purification of secreted β-D-fructofuranosidases from the culture filtrate has low contaminating proteins and is less complex than cellular enzymes. Purification of extracellular β-D-fructofuranosidases from *Aspergillus* species yields important products crucial for various applications in fermentation industries (Chen et al. 1996). The results of the purification steps of *A. sojae* β-D-fructofuranosidase is summarized in the Table 1. Majority of the fungal β-D-fructofuranosidases have been partially purified by ammonium sulphate precipitation as the first step of purification (L'Hocine et al. 2000), however, only a few studies have demonstrated acetone or ethanol precipitation (Hayashi et al. 1991; Chávez et al. 1997; Ghosh et al. 2001). *A. sojae* JU12 β-D-fructofuranosidase (about 90 mg L^{-1} of activity) was eluted from the Sephadex G-100 column with 5.41 fold and recovery of 10.87%. *A. niger*

Table 1 Summary of purification profile of *Aspergillus sojae* β-D-fructofuranosidase

Purification step	Total activity (U)	Protein content (mg)	Specific activity (U mg^{-1})	Fold	Recovery (%)
Cell-free extract	1561.35	4.48	348.51	1.00	100.00
Ammonium sulphate fractionation (0-80%)	462.88	1.17	395.62	1.14	29.65
Size exclusion chromatography	169.77	0.09	1886.31	5.41	10.87

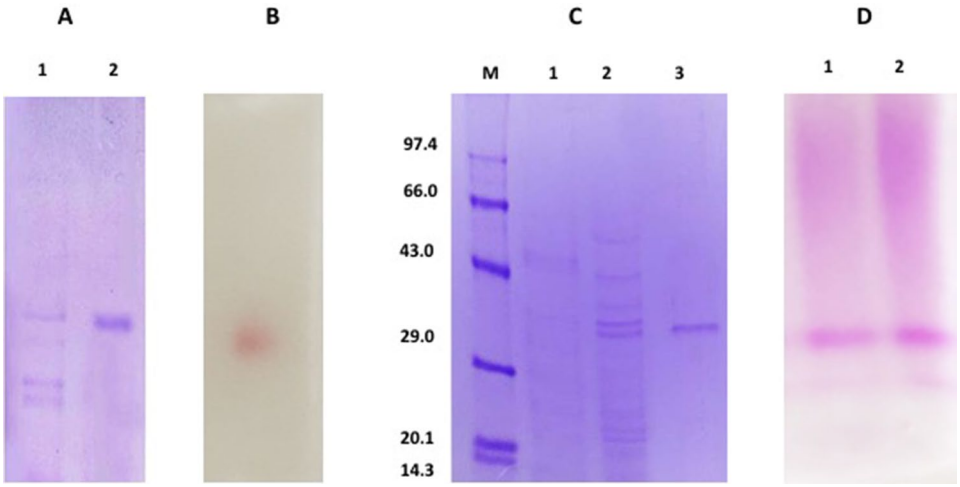

Fig. 1 Electrophoretic analysis of *Aspergillus sojae* JU12 β-D-fructofuranosidase. **a** Native-PAGE: lane 1: crude extract, lane 2: purified enzyme (10 µg). **b** Zymogram depicting a single pink band of enzyme activity: Purified enzyme (10 µg). **c** SDS-PAGE: (M) protein molecular weight medium range marker (kDa) phosphorylase **b** (97.4), bovine serum albumin (66.0), ovalbumin (43.0), carbonic anhydrase (29.0), soyabean trypsin inhibitor (20.1) and lysozyme (14.3); lane 1: crude extract, lane 2: ammonium sulphate fraction (80%) (20 µg), purified enzyme (10 µg). **d** Glycoprotein staining: lane 1: ammonium sulphate fraction (80%) (20 µg), lane 2: purified enzyme (10 µg)

β-D-fructofuranosidase was purified by gel filtration on Sephadex G-150 followed by DEAE Sephadex A-50 column and comparatively, a low purification fold of 8.65 and 0.84% yield was observed. Similarly, a purification fold of 3.11 and 9.33% of external β-D-fructofuranosidase was recovered from *Fusarium solani* (Bhatti et al. 2006). On the other hand, β-D-fructofuranosidase from *A. phoenicis* (Rustiguel et al. 2011) and *A. ochraceus* (Guimaraes et al. 2007) were produced by SSF and purified by two chromatographic steps, DEAE cellulose and Sephacryl S-200 to obtain 14.46% yield and purification fold of 18.77; 24% yield and 7.1 fold, respectively. Purification of fungal β-D-fructofuranosidases in multiple steps such as three-stages (Nguyen et al. 2002), four-stages (Ghosh et al. 2001) or as many as eight steps of purification (L'Hocine et al. 2000), reveled lesser than 30% yields and the numerous purifying stages are costly and time consuming.

Determination of molecular mass, homogeneity and activity of purified β-D-fructofuranosidase

A. sojae β-D-fructofuranosidase represented a single band under native conditions (Fig. 1a), and corresponding bands on β-D-fructofuranosidase zymogram were observed (Fig. 1b). Occurrence of single molecular form of β-D-fructofuranosidases is common in most *Aspergillus* strains (L'Hocine et al. 2000; Oyedeji et al. 2017) or other fungi (Bhatti et al. 2006). However, this feature is contrasting in few fungal strains depicting oligomeric forms of β-D-fructofuranosidases (Nguyen et al. 2005; Quiroga et al. 1995), as well as yeast β-D-fructofuranosidases that exists as multiple isomers (Andjelković et al. 2010). The purified β-D-fructofuranosidase from *A. sojae* yielded a single homogenous peak by size exclusion chromatography which corresponded to the apparent molecular mass of 35 kDa on reducing SDS-PAGE (Fig. 1c). Further, the mass of the protein was evaluated by mass spectrometry and found to be 33,251.00 (Fig. 2). The molecular weight of *A. sojae* β-D-fructofuranosidase was found to be slightly lower than the mass of 37 kDa of the reported fungal strains *A. japonicus* MU-2 (Hayashi et al. 1992) and *Clostridium perfringens* (Ishimoto and Nakamura 1997), whereas found to be higher than the filamentous fungus *Termitomyces clypeatus*

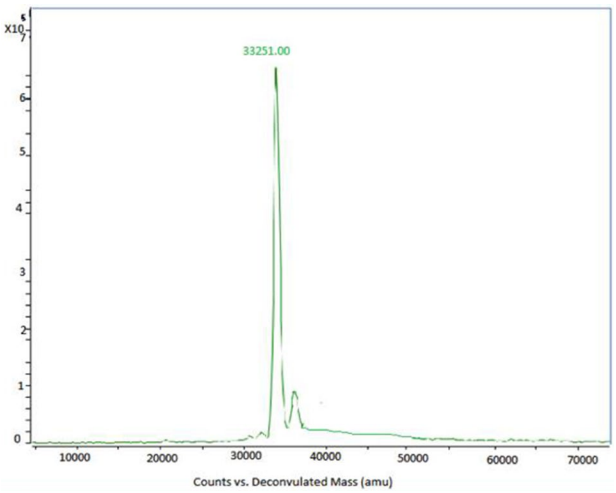

Fig. 2 Deconvoluted mass spectrum of β-D-fructofuranosidase from *Aspergillus sojae* JU12 strain

which showed a low-size protein of 13.5 kDa, single band in SDS-PAGE (Chowdhury et al. 2009). Molecular masses of some of the reported *Aspergillus* strains ranged from 66 to 430 kDa (Nadeem et al. 2015). Low molecular weight β-D-fructofuranosidases have been reported in bacterial and yeast β-D-fructofuranosidases, such as *Bacillus cereus* TA-11 of 26 kDa (Yoon et al. 2007) and *Rhodotorula glutinis* of 47 kDa, respectively (Rubio et al. 2002). In the present study, homogeneity of the bands in denaturating and non-denaturating electrophoretic gels confirmed the active protein to be a monomer. Comparatively, a low molecular weight monomeric fungal protein from *A. sojae* is reported, than β-D-fructofuranosidases of other *Aspergillus* strains. The enzyme hydrolysate revealed a mixture of D-glucose and D-fructose on the thin layer chromatogram (Fig. 3).

Glycoprotein analysis of β-fructofuranosidase

β-D-fructofuranosidase from *A. sojae* was found to be a glycoprotein containing about 68.92% of total sugar content. In the qualitative test, the appearance of brown colour indicated the presence of glycoproteins which was further confirmed by the development of pink coloured PAS stained bands on SDS-PAGE (Fig. 1d), that were coincident to the previously conducted electrophoretic experiments (Fig. 1c). The carbohydrate content of *A. sojae* β-D-fructofuranosidase was comparatively higher than the reported *Aspergillus* strains, *A. japonicus* with 20% (Hayashi et al. 1992) and *A. niger* with 17% (Nguyen et al. 2005), other fungal strains *Aureobasidium* sp. possessed 30–53% (Hayashi et al. 1991), *Pycnoporus sanguineus* with 24% (Quiroga et al. 1995), and bacterial β-D-fructofuranosidase *L. reuteri* strain showed 48% carbohydrate moiety (De Gines et al. 2000). The internal β-D-fructofuranosidases are found to be non-glycosylated and aggregated, whereas glycosylation in extracellular invertases are found to stabilize the enzyme and prevents thermal denaturation (Kern et al. 1992).

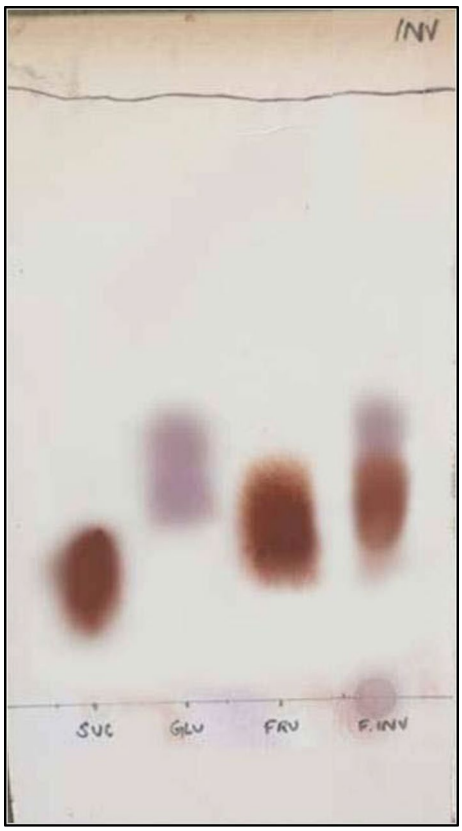

Fig. 3 Thin layer chromatogram of *Aspergillus sojae* β-D-fructofuranosidase loaded with the standards. Lane 1: D-sucrose, lane 2: D-glucose, lane 3: D-fructose, lane 4: enzyme hydrolysate spotted after an overnight incubation at 37 °C, representing D-glucose and D-fructose released from the disaccharide

Biochemical characterization of purified β-fructofuranosidase

Effects of pH and stability

β-D-fructofuranosidase showed high activity from pH 4.0 to 9.0 (Fig. 4a). The stability studies were carried from pH 6.0 to 8.0 and the enzyme was found to be optimally active at pH 8.0 up to 6 h, compared to the lower pH conditions (Fig. 4b). The enzyme activity was significantly influenced by any variation in pH. Mostly, β-D-fructofuranosidases known so far are active in acid ranges, especially from yeasts. There are also many reports on acidic β-D-fructofuranosidases from fungi such as *Aspergillus* strains which are stable at pH 3.0–6.2 (Nadeem et al. 2009; Lucca et al. 2013). Although the fungal β-D-fructofuranosidase has shown enhanced activity at broad pH ranges, the β-D-fructofuranosidases optimum at alkaline conditions are rarely observed in microorganisms, and this neutral/alkalophilic nature could be beneficial in the development of biosensors or enzyme electrode sensors, digestive aids and biotechnological applications.

Effects of temperature and stability

β-D-fructofuranosidase exhibited a thermostable nature and was active at a broad temperature range from 30 to 70 °C, however, a slight decline in activity beyond 60 °C was recorded (Fig. 4c). Thermostability of β-D-fructofuranosidase at 40 °C was found to be more stable than higher temperatures (Fig. 4d). Similar observations were seen in extracellular β-D-fructofuranosidases from *T. clypeatus* wherein 47 °C was optimally active (Chowdhury et al. 2009), and 38–56 °C was a suitable range for the enzyme from *A. parasiticus* strain (Lucca et al. 2013). Thermal stability of β-D-fructofuranosidases at physiological temperature ranges are advantageous for applications

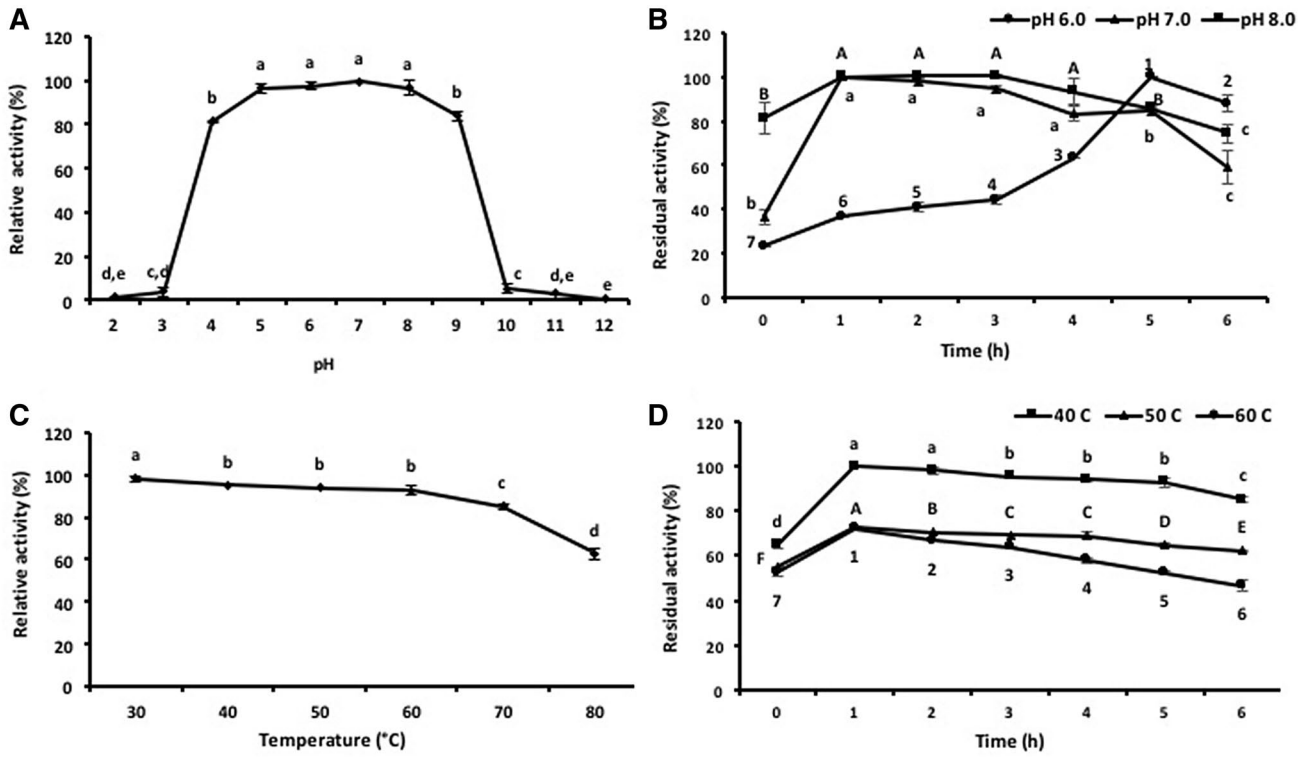

Fig. 4 Enzymic properties and characteristics of *Aspergillus sojae* JU12 β-D-fructofuranosidase. **a** pH-dependent activity profile. **b** Stability of the enzyme at pH 6.0, 7.0 and 8.0. **c** Temperature-dependent activity profile. **d** Thermostability of the enzyme at 40, 50 and 60 °C. Error bars represent the mean ± SD and different letters on the error bars shows the factors as statistically different at $P_{0.05}$

in production of cough syrups and digestive formulations, or food additives, also required for centre-filled chocolates, cookies, cakes or fondants which necessitate thermostable enzymes.

Effects of metal ions and group specific reagents

The metal ion inhibitors Ag^{2+} and Hg^{2+} lowered *A. sojae* β-D-fructofuranosidase activity (Table 2). Similar observations were recorded in *A. japonicas* (Hayashi et al. 1992), *A. niger* (L'Hocine et al. 2000; Nguyen et al. 2005) and

Table 2 Effect of metal ions and group specific reagents on *Aspergillus sojae* β-D-fructofuranosidase

Metal ions	Relative activity (%)		Reagents	Relative activity (%)	
	1 mM	10 mM		1 mM	10 mM
Control	100 ± 0.00^{c}	100 ± 0.001^{f}	Control	100 ± 0.001^{b}	100 ± 0.00^{c}
Mg^{2+}	$93 \pm 1.50^{d,e}$	136.25 ± 1.56^{a}	NEM	82.22 ± 2.14^{d}	83.33 ± 0.00^{e}
Ca^{2+}	105.75 ± 1.98^{b}	130.5 ± 0.75^{b}	NBS	15.11 ± 1.02^{g}	57.78 ± 1.02^{g}
Cu^{2+}	100.25 ± 1.73^{c}	95.25 ± 0.00^{g}	DTT	97.11 ± 1.39^{b}	90.67 ± 1.76^{d}
Zn^{2+}	91.5 ± 0.75^{e}	85.25 ± 1.89^{h}	PMSF	68 ± 1.33^{f}	85.11 ± 1.76^{e}
Ag^{2+}	2.01 ± 0.43^{j}	4.25 ± 1.15^{k}	NAI	70.6 ± 2.84^{f}	83.78 ± 1.54^{e}
Co^{2+}	63.5 ± 0.87^{h}	66.75 ± 0.001^{i}	SDS	86.22 ± 1.02^{c}	126.89 ± 5.05^{b}
Mn^{2+}	78.25 ± 0.43^{g}	116.5 ± 1.15^{d}	EDTA	77.11 ± 0.77^{e}	83.33 ± 1.33^{f}
K^{+}	78.25 ± 1.15^{f}	104 ± 0.001^{e}	TPTZ	78 ± 1.33^{e}	50.89 ± 1.54^{h}
Fe^{2+}	110.5 ± 1.56^{a}	103 ± 1.56^{e}	β-ME	103.11 ± 1.39^{a}	144 ± 1.76^{a}
Hg^{2+}	2.0 ± 0.43^{j}	9.25 ± 0.43^{j}	IAA	77.11 ± 2.34^{e}	74.89 ± 0.38^{f}
Na^{+}	94.5 ± 0.75^{d}	98.75 ± 0.001^{f}			
Fe^{3+}	25.75 ± 0.87^{i}	121.25 ± 1.15^{c}			

The mean ± SD values with different letters indicate significant differences at $P_{0.05}$

A. terreus strains, wherein β-D-fructofuranosidase was completely inhibited by Hg^{2+} as well as Ag^{2+} (Giraldo et al. 2014). Hg^{2+} at 2 mM concentration inhibited β-D-fructofuranosidases from *Fusarium oxysporum* (Gupta et al. 1989), and also at 5 mM of Hg^{2+} *A. niger* β-D-fructofuranosidase was entirely inhibited (Rubio and Maldonado 1995). These metal ions interact with the sulfhydryl groups in β-D-fructofuranosidases which are responsible for enzyme activity, thus leading to conformational changes and protein precipitation. Co^{2+} showed about 40% inhibition on *A. sojae* β-D-fructofuranosidases, whereas in case of *A. niger* β-D-fructofuranosidases 20% inhibition was reported (Nguyen et al. 2005), on the contrary the activity was promoted in *S. cerevisiae* β-D-fructofuranosidase (Rashad and Nooman 2009). From the Table 2, at both the tested concentrations there was stimulation of β-D-fructofuranosidase activity by Ca^{2+} and Fe^{2+}, whereas enhancement of activity was found to be higher at 10 mM of Mg^{2+}, Mn^{2+}, K^{+} and Fe^{3+} than 1 mM. The effect of other metal ions such as Cu^{2+}, Zn^{2+}, Na^{+} and few other ions at lower concentrations were associated mostly with enzyme stabilization, which clearly demonstrates the significant action of these ions at the β-D-fructofuranosidase active sites. Likewise, the activity of the purified β-D-fructofuranosidase was elevated by Ca^{2+} and Mg^{2+} (Hayashi et al. 1992; Giraldo et al. 2014). The metal ions K^{+}, Na^{+}, Ca^{2+} (Oyedeji et al. 2017), Mn^{2+}, Cu^{2+}, Fe^{2+}, K^{+} (Esawy et al. 2014) were found to be mostly β-D-fructofuranosidase activators, while Zn^{2+} (Nguyen et al. 2005) has also been reported as β-D-fructofuranosidase inhibitors. β-D-fructofuranosidase activity was inhibited in the presence NBS, 15–30% inhibition in case of NAI and PMSF a serine protease inhibitor, 20–50% inhibition by TPTZ. On the contrary, an elevation was observed by SDS and β-ME, while all the other group specific reagents did not exhibit much variation (Table 2). In some reports, the β-D-fructofuranosidase activity of filamentous basidiomycota was not affected by SDS and DTT, whereas completely inhibited by β-ME (Chowdhury et al. 2009). Thus, the influence of metal ions and reagents on enzyme activity is different in fungal β-D-fructofuranosidases and even among the *Aspergillus* strains.

Kinetics of *A. sojae* β-D-fructofuranosidases

The kinetics of the enzyme was determined by Lineweaver–Burk plot and K_m of 10.17 mM and V_{max} of 0.7801 µmol min^{-1} values were attained (Fig. 5). *Aspergillus* β-D-fructofuranosidases were found to have highest affinity for D-sucrose substrate (Guimaraes et al. 2007; Alves et al. 2013). *A. parasiticus* showed similar K_m of 10.0 (Lucca et al. 2013), whereas most of the reported β-D-fructofuranosidases demonstrated high K_m and V_{max} values 117 mM and 12,500 µmol min^{-1} (Nadeem et al. 2009),

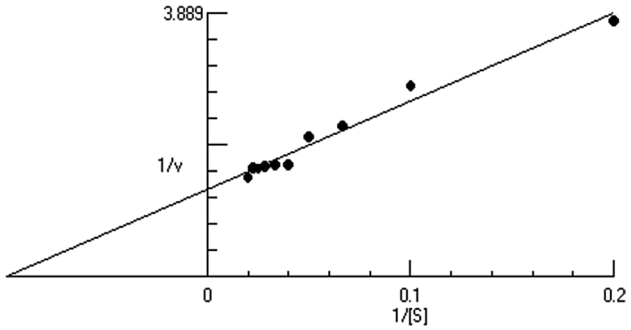

Fig. 5 Lineweaver-Burk plot of *Aspergillus sojae* JU12 β-D-fructofuranosidase for the substrate D-sucrose

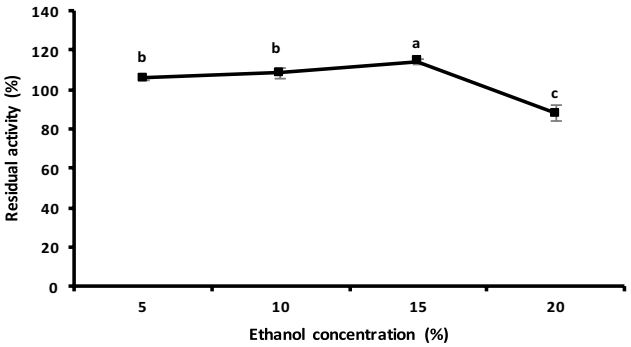

Fig. 6 Effect of ethanol on the activity of *Aspergillus sojae* β-D-fructofuranosidase. The mean ± SD values with different letters indicate significant differences at $P_{0.05}$

35.67 mM and 3.98 µmol min^{-1} (L'Hocine et al. 2000) and 35.5 mM and 60 µmol min^{-1} (Aslam et al. 2013), respectively. From the results, it can be suggested that *A. sojae* β-D-fructofuranosidase was found to have increased affinity for D-sucrose than the reported fungal and yeast strains.

Unravelling ethanol tolerance of *A. sojae* β-D-fructofuranosidase

A. sojae β-D-fructofuranosidase was found to be tolerant with maximum activity from 5 to 15% (v/v) of ethanol concentration, whereas at the maximum tested concentration of ethanol (20%, v/v) the activity dropped to 88.04% (Fig. 6). β-D-fructofuranosidase from *Candida stellata* strain demonstrated enzyme activities of 63 and 58% at 5 and 7.5% of ethanol concentrations, respectively (Gargel et al. 2014). As per Bai et al. (2008), the alcohol fermentation processes in industries usually requires ethanol tolerance up to 10–15% during the completion process. Therefore, the potential extracellular β-D-fructofuranosidase from *A. sojae* can be effectively utilized in the production of alcohol beverages or alcoholic fermentation as it exhibits good ethanol tolerance.

Conclusion

The present study focuses on the purification and characterization of β-D-fructofuranosidase from *Aspergillus sojae* JU12 which unravels the enzymic properties, that help in better understanding of its application in industries. *A. sojae* β-D-fructofuranosidase typically belongs to GH32 family of glycosidases demonstrated characteristics suitable for industrial requirements. The ethanol tolerance level of the fungal β-D-fructofuranosidase indicates its potentiality in the production of alcoholic beverages or fermentation processes. Thus, the extracellular β-D-fructofuranosidase produced from economical agro-wastes was identified to be thermostable at neutral/alkalophilic conditions possessing high affinity for D-sucrose and exhibited efficient ethanol tolerance. The investigation of the physico-chemical properties is crucial so as to further improve enzyme production or design genetic engineering studies. Although β-D-fructofuranosidases have been studied extensively from yeasts, the scope for finding new microbes with better prospects and properties triggers further research in this field.

Compliance with ethical standards

Conflict of interest There are no known conflicts of interest associated with this publication.

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