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Purification and Characterization of Exo-Inulinase from *Paenibacillus* sp. d9 Strain

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Abstract
This study intended to purify and characterise exo-inulinase of diesel-degrading *Paenibacillus* sp. D9. The whole genome sequencing of *Paenibacillus* sp. D9 revealed to possess the *sacC* gene that is encoded as exo-inulinase/levanase. This isolate was capable of producing a maximum of 50.9 IU/mL of exo-inulinase activity within 3 days at 30 °C, 200 rpm and pH of 7.0 on minimal salt medium agar supplemented with 1% (w/v) inulin. An exo-inulinase of 58.5 kDa was purified using ammonium sulphate precipitation, HiTrap QFF column and MMC column chromatographies with a specific activity of 4333 IU/mg, 7.1% recovery and a 4.3-fold increase in purity. The purified D9 exo-inulinase had temperature and pH optimum at 40 °C and pH 4.0, respectively, with the Michaelis constant of 5.5 mM and a maximal velocity of 476.2 IU/mg, respectively. Catalytic constant, k_{cat} was calculated to be 42.6 s^{-1} with a catalytic efficiency (k_{cat}/K_m) of 7.6 s^{-1} mM^{-1}. The presence of Ca^{2+} enhanced the activity of D9 exo-inulinase while Hg^{2+} completely inhibited the activity, other compounds such as Fe^{3+} and Cu^{2+} had an inhibitory effect. The results of amino acid alignment and the complete degradation of inulin into fructose by the purified enzyme confirmed that inulinase from *Paenibacillus* sp. D9 is an exo-form. The phylogenetic tree based on the protein sequences indicates that bacterial exo-inulinases possess a common ancestry.

Keywords Exo-inulinase · *Paenibacillus* sp. · Enzyme purification · Characterization · Phylogenetic tree

Abbreviations

bp	Base pair
k_{cat}	Catalytic constant
K_m	Michaelis Constant
MSM	Minimal salt medium
PCR	Polymerase chain reaction
QFF	Q Sepharose® Fast Flow
SDS–PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
V_{max}	Maximum rate of reaction

1 Introduction

Inulin is a reserve polysaccharide naturally found in plant species. It is a linear chain of fructose polymers (β-1, 2 linkage) ranging between 2 and 60 residues, while short chains (< 10) are considered as fructo-oligosaccharides (FOS). Hydrolysis products of inulin *viz*. fructose and FOS are of high importance in the biotechnology industry [1–4]. The former has applications in the food and energy industries, whereas the latter is commonly used in the pharmaceutical industry. Fructose is said to form iron-fructose complex thus increasing iron absorption in the body [5] and has a high sweetening power compared to sucrose of up to 1.8-fold. Since fructose bypasses the Krebs cycle for glucose metabolism, it does not require insulin regulation thus is safe for people with diabetes [6]. FOS are currently used as food additives with many health benefits including promoting the growth of bifidobacteria (prebiotics) and maintaining a good balance in the intestinal microflora [7–12]. Food and Drug Administration (FDA) USA has given FOS the generally recognized as safe (GRAS) status as food additives [7].

Acid hydrolysis of inulin for fructose production is the most commonly used method and is unfavourable owing to the formation of by-products, undesired degradations and colour and high operational costs [13–15]. In comparison to acid catalysts, microbial inulinases possess more advantages for production of fructose and FOS. Substrate specificity and selectivity of microbial enzymes allow for high product

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yield of up to 95% pure fructose in a one-step enzymatic process [16, 17].

Microbial inulinases are classified into two categories, *viz* endo-acting and exo-acting inulinases. Endo-inulinase (1, 2-β-D-fructan fructanohydrolase, EC 3.2.1.7) randomly hydrolyse inulin internally producing fructooligosaccharides of different carbon lengths, whereas exo-inulinase (β-D-fructan-fructanohydrolase, EC 3.2.1.80) yields fructose by splitting off terminal fructofuranosidic bond of the non-reducing end of inulin [9]. Microorganisms are the apt candidates for inulinase production at a commercial scale owing to their high yield and easy cultivation. High inulinase producers include *Aspergillus* sp., *Streptomyces* sp. *Staphylococcus* sp., *Kluveromyces* sp. *Cryptococcus* sp. *Penicillium* sp. and *Rhizopus* sp [18, 19]. Bacterial inulinases are less studied compared to fungal and yeast inulinases.

Paenibacillus sp. D9 (assession number GQ368737), which was previously isolated from diesel contaminated site at the University of KwaZulu-Natal [20], possesses excellent diesel degradation ability. The whole genome of *Paenibacillus* sp. D9 has been sequenced and deposited at DDBJ/EMBL/GenBank under the accession JZEJ00000000. This bacterium was found to possess various industrially important extracellular enzymes such as xylanases, chitinases, amylases, and proteases. D9 strain was found to possess the *sacC* gene that is encoded as exo-inulinase/levanase. Therefore, this study reports the purification and characterization of exo-inulinase from *Paenibacillus* sp. D9.

2 Materials and Methods

2.1 Bacterial Strain

A glycerol stock of Gram-positive *Paenibacillus* sp. strain D9 (GQ368737), which was previously isolated from diesel contaminated site [20], was obtained from the University of KwaZulu-Natal. *Paenibacillus* sp. D9 was maintained on minimal salt medium (MSM) agar supplemented with 1% (w/v) inulin and stored at 4 °C. The plates were sub-cultured every 2 weeks and original stocks used every 3 months. The MSM medium was composed of 2.0 g, yeast extract; 3.0 g, peptone; 1.0 g, K_2HPO_4; 0.5 g, $MgSO_4{\cdot}7H_2O$; 0.15 g, $CaCl_2{\cdot}2H_2O$; 0.5 g, NaCl and 15.0 g bacteriological agar per litre according to the modified method of Nakamura [21].

2.2 Inulinase Production of *Paenibacillus* sp. D9

An overnight culture of *Paenibacillus* sp. D9 was prepared by inoculating a colony into sterile 5 mL MSM (containing 1% [w/v] inulin) and incubated at 30 °C, shaking at 200 rpm. The culture was subsequently standardised to an $OD_{600\ nm}$ of 1.0 using MSM. One millilitre of standardised culture was inoculated into 250 mL Erlenmeyer flasks containing 100 mL MSM with 1% (w/v) inulin and the mixture was incubated at 30 °C, 200 rpm for 4 days. An aliquot of 1 mL was collected each day, centrifuged at 13,000×*g* for 5 min and the supernatant was assayed for the exo-inulinase activity, protein concentration and protein profiling as described below.

2.3 Purification of Exo-Inulinase from *Paenibacillus* sp. D9

Culture medium containing the optimal crude exo-inulinase at day 3 was centrifuged at 13,000 × *g* for 20 min at 4 °C. The supernatant was subjected to 80% ammonium sulphate precipitation according to the modified method of Abu Elsouod et al. [22]. The pellet was collected by centrifugation (13,000×*g*, 20 min, 4 °C) and re-suspended in 10 mL Tris buffer (50 mM, pH 8.0). The suspension was dialyzed using a dialysis tubing (10 kDa molecular weight cut off [MWCO]) against 100 × volumes of 50 mM Tris buffer for 3 h, and dialyzed against the second batch of fresh buffer at 4 °C overnight. After dialysis, the crude enzyme was filtered through 0.2 micron filters (Whatman) to prevent column clogging in further purification steps. The crude enzymes were then concentrated by centrifugation at 5000×*g* for 10 min at 4 °C using spin column (EMD Millipore Amicon™) with 10 kDa MWCO. The concentrated enzyme eluent was loaded into a 5 mL HiTrap QFF column (GE Healthcare Life Sciences) which was pre-equilibrated with 50 mM Tris–HCl buffer, pH 8, at a flow rate of 1 mL/min using the AKTA purifier 100-950 system. The column was washed with five column volumes with 50 mM Tris–HCl. Bound proteins was eluted from the column using the gradient of 0–1.0 M NaCl at 1 mL/min in the same buffer [23]. Fractions of 4 mL each were collected and assayed for inulinase activity and protein concentration as described below.

Fractions containing inulinase activity were combined and concentrated using the spin column (EMD Millipore Amicon™10 kDa MWCO) as above. The partially purified protein sample was then subjected to the HiPrep 26/10 desalting column (GE Healthcare Life Sciences) to remove excess NaCl following the instruction of the manufacturer. Inulinase of D9 was further purified by loading the active fractions into a Capto-MMC column (GE Healthcare Life Sciences) at 1 mL/min after dialysis against 50 mM citrate buffer, pH 3.6 at 4 °C overnight. Bound protein was eluted gradually using the same buffer with 0–1.0 M NaCl gradient at 1 mL/min. Fractions of 2 mL each were collected and assayed for inulinase activity and protein concentration. The protein profiles of each fraction during the enzyme purification steps were used to monitor the purity of exo-inulinase using SDS–PAGE as described below.

3 Biochemical Characterizations of Crude and Purified Exo-Inulinase from *Paenibacillus* sp. d9

3.1 Determination of Exo-Inulinase Activity and Protein Concentration

The exo-inulinase activity was measured based on the modified method of Singh et al. [24]. Two hundred microlitres of each sample were added onto 1.8 mL of 1% (w/v) inulin dissolved in 50 mM citrate buffer, pH 5.0 (previously equilibrated at 50 °C for 5 min). The reaction mixture was incubated for 20 min at the same temperature, thereafter, stopped by the addition of 3 mL DNS reagent (10 g/L, 3,5-dinitrosalycylic acid; 0.2% [v/v], phenol; 0.5 g/L, sodium sulphite; 10 g/L, NaOH and 200 g/L, sodium tartrate) and boiled for 15 min according to Miller et al. [25]. The amount of fructose liberated from inulin was thus measured spectrophotometrically at 540 nm using a Varian UV spectrometer and estimated using pure fructose as a standard. Citrate buffer (+inulin) without the enzyme was used as a blank, while citrate buffer (−inulin) with and without the enzyme was used for enzyme correction. One enzyme activity (IU) was defined as the amount of enzyme liberating one micromole of fructose per min under assay condition. Different heavy metals or chemicals (Fig. 9) were added (final concentration of 5 mM) under optimal pH and temperature to study their effect on the enzyme activity. The protein concentration was performed according to the Bradford assay in the 96 wells microtiter plate [26] using bovine serum albumin as a standard (0–0.6 mg/mL). All experiments were performed in triplicate and the results were presented as the mean ± standard deviation.

3.2 Molecular Weight Determination and Protein Profile by SDS–PAGE

Protein profile was obtained according to the method described by Laemmli [27]. The 12% SDS–PAGE gel after electrophoresis was subsequently stained in Coomassie staining R250, destained and the image was captured using a UV transilluminator (Bio SYNGENE). The molecular weight of the protein band was calculated based on the molecular weight marker.

3.3 Optimum pH, Temperature, pH Stability and Thermo-Stability of Exo-Inulinase

Effect of pH and temperature was conducted according to a modified method Yuan et al. [23]. The crude inulinase from *Paenibacillus* sp. D9 was assayed at 50 °C with varying pH conditions from 3.0 to 9.0. The buffers used were: pH 3.0–5.0 (50 mM citric acid/sodium acetate), pH 6.0–7.0 (mono-/dibasic sodium phosphate), pH 8.0–9.0 (Tris[hydroxymethyl] aminomethane/hydrochloric acid). The effect of temperature on the crude enzyme was assayed at the optimum pH under varying temperature conditions (30–80 °C).

Determination of pH- and thermo-stability was conducted according to a modified method of Coghetto et al. [28]. Thermal stability of crude and purified exo-inulinase was investigated by pre-incubating the enzyme (20.0 nM final concentration) under 50 mM citrate buffer, pH 5.0, but at temperature (40–60 °C) respectively for 1 h. To determine pH stability of exo-inulinase, the enzyme was pre-incubated at different pH values from 3.0 to 9.0 respectively up to 1 h. The exo-inulinase activity was assayed for every 20 min intervals at the optimum pH and temperature. All experiments were conducted in triplicate.

3.4 Determination of K_m and V_{max} for the Purified Exo-Inulinase

The K_m and V_{max} values were determined using the Lineweaver–Burk plot method [29]. To study reaction rates, exo-inulinase activity was assayed under the standard conditions at different inulin concentrations (2.5–10.0 mM). Average M_r for inulin was calculated to be 5800 from the average DP of 36 [30]. k_{cat} was determined by measuring the reaction rate at different enzyme concentrations (20.0–80.0 nmol). The slope of the graph yielded the enzyme turnover number.

3.5 Determination of Hydrolysis Products from Inulin by Exo-Inulinase

Products of inulin hydrolysis by purified exo-Inulinase were determined using thin-layer chromatography (TLC) on silica gel according to the modified method of Gill et al. [31]. One percent (w/v) inulin was dissolved in 50 mM citrate buffer, pH 4.0, and incubated with 20.0 nM exo-inulinase for 1 h at 40 °C. Two negative controls were set up: one did not contain any enzyme and the other contained heat inactivated exo-inulinase (pre-incubated at 100 °C for 1 h). Glucose, sucrose and fructose standards (1 mg/mL) were used to determine hydrolysis products. The sugars were spotted on TLC silica gel and resolved using a solvent system of 20:20:5 (v/v/v) acetic acid-butanol-water. The plate was sprayed with 2% (v/v) 5-nitrosalycylic acid dissolved in 1:25 sulphuric acid-acetic acid and subsequently heated at 115 °C (10 min) for visualization of spotted sugars.

4 Bioinformatical Analysis

Protein blast analysis was performed using a program in National Center for Biotechnology Information (NCBI). Deduced exo-inulinase amino acid sequence from *Paenibacillus* sp. D9 was blasted against a database of known exo-inulinases from different organisms (*K. marxianus, Spirosoma liguale* DSM74, *Bacillus* sp. J13, *P. Polyxyma, P. amylolyticus, Cohnella* sp. and *P. graminis*). Amino acid alignment was carried out using Clustal Omega version 1.2.4 [32]. A phylogenetic tree was constructed using constraint-based alignment tool (COBALT) [33], the neighbour-joining method (algorithm) and the Jukers-Cantor method (distance measure). The branching confidence was estimated by bootstrapping with 1000 replicates.

5 Results

5.1 Exo-inulinase Production by *Paenibacillus* sp. D9

Production of exo-inulinase from *Paenibacillus* sp. D9 gradually increased from day 1 to day 3 with a maximum of 50.9 IU/mL and decreased at day 4 (42.8 IU/mL) when 1% (w/v) inulin was used as a sole carbon source (Fig. 1). The SDS–PAGE results showed that extra protein band with 58.5 kDa was induced by inulin since day 1 (Data not shown) that is corresponding with encoded exo-inulinase/levanase of *sacC* gene of *Paenibacillus* sp. D9.

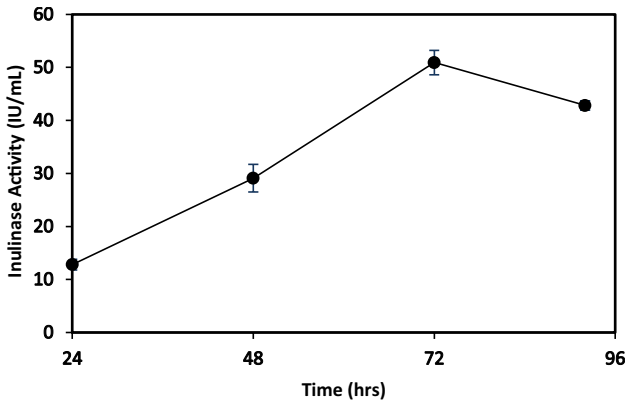


Fig. 1 Time course for exo-inulinase production by *Paenibacillus* sp. D9 when 1% (w/v) inulin was used as a sole carbon source. Error bars represent the mean ± SD of experiments in triplicate

5.2 Purification of *Paenibacillus* sp. D9 Exo-Inulinase

Exo-inulinase of *Paenibacillus* sp. D9 was purified to electrophoretic homogeneity (Fig. 2) using ammonium sulphate precipitation, QFF ion-exchange, and MMC ion-exchange chromatographies (Table 1). A 32.5% of total exo-inulinase activity in the crude extract was recovered through 80% saturated ammonium sulphate precipitation and the specific activity (SA) of inulinase was increased by 1.1 folds (Table 1). The active D9 inulinase activity was eluted out from the HiTrap QFF column when the NaCl concentration reached 0.25 mM. SA of inulinase was further increased to 2038.0 IU/mg with the purification fold of 2.0. Additional purification using HiPrep desalting column further enhanced the SA by 2.6 folds. Finally, D9 exo-inulinase was purified up to 4.3 folds using Capto-MMC ion-exchange column (Fig. 3), recovering 7.1% total inulinase activity with a SA of 4333.1 IU/mg (Table 1). From Fig. 2, the results in SDS–PAGE chromatography (Lane 5) shows the purified exo-inulinase with a MW of 58.5 kDa.

5.3 Biochemical Characterization of Crude and Purified Exo-Inulinase from *Paenibacillus* sp. D9

Both crude and purified D9 exo-inulinases were found to have a pH optima of 4.0, Fig. 4a, with highest activities of 55.0 and 60.4 IU/mL for purified and crude enzyme, respectively. The crude and purified exo-inulinase had similar levels of enzyme activities between pH 3.0 and 6.0 (Fig. 4a). D9 exo-inulinase showed lower enzyme activities towards alkalinity (pH > 7.0). The optimal temperature of the purified and crude D9-inulinase was found to be 40 °C with highest activities of 59.8 and 62.2 IU/mL, respectively, Fig. 4b. The enzyme activity of D9 exo-inulinase, both crude and purified forms, at 30 and 50 °C had at least 84.0% of the optimal

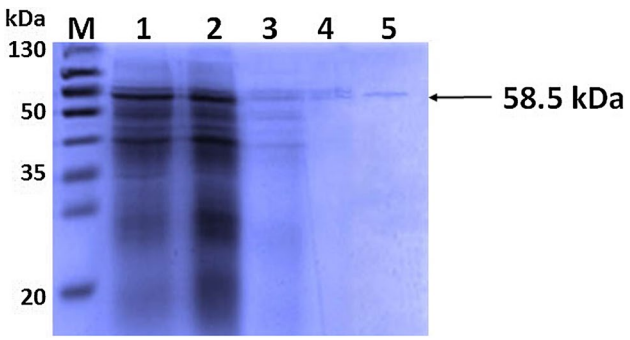


Fig. 2 SDS–PAGE showing protein profiling of each D9 exo-inulinase purification step. M: protein marker, Lane 1–5: crude enzymes, ammonium sulphate precipitation, cation-exchange, size exclusion and anion-exchange chromatography respectively

Table 1 Summary of the results in purification steps of D9 exo-inulinase

Purification step	Protein conc. (mg/mL)	Volume (mL)	Activity (IU/mL)	Total activity (IU)	SA (IU/mg)	Yield (%)	Purification fold
Crude	0.058	50	59	2950	1017	100	1.0
Ammonium sulphate[a]	0.043	20	48	960	1116	32.5	1.1
HiTrap QFF anion-exchange	0.026	15	53	795	2038	26.9	2.0
HiPrep 26/10 column	0.019	8	51	408	2684	13.8	2.6
Capto MMC cation-exchange	0.012	4	52	208	4333	7.1	4.3

Results represent the mean ± SD of experiments in triplicate

[a]Dialyzed proteins after 80% ammonium sulphate precipitation

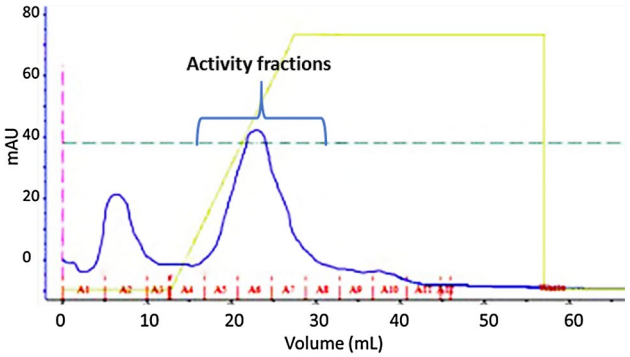


Fig. 3 Ion-exchange (MMC) chromatogram for final purification step of D9 exo-inulinase. Bound enzyme was eluted at NaCl gradient (0–1.0 M). mAU = milli absorbance units at 280 nm

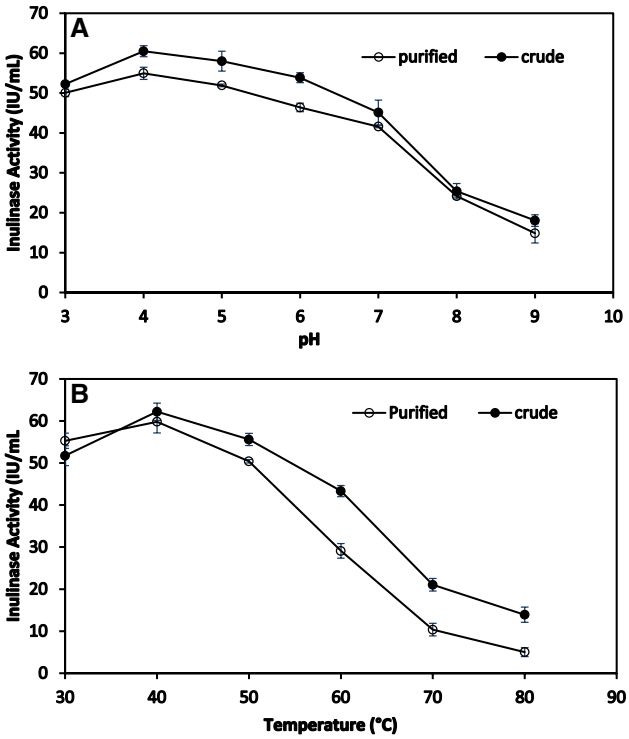


Fig. 4 Optimum conditions **a** pH; **b** temperature for crude and purified exo-inulinase activity of *Paenibacillus* sp. D9. Error bars represent the mean ± SD of experiments in triplicate

activity. Thereafter, enzyme activities decreased immensely with increases in temperature, reaching 5.0 IU/mL (8.4% residual activity) and 13.9 IU/mL (22.3% residual activity) at 80 °C for purified and crude enzyme, respectively.

The crude exo-inulinase was stable between pH 4.0–6.0 retaining up to 95.0% (pH 4.0), 87.9% (pH 5.0) and 65.6% (pH 6.0) residual activity after 1 h pre-incubation, Fig. 5a. More than 50.0% residual activity was retained after 40 min pre-incubation at pH 3.0 and at pH 7.0 while the enzyme lost 67.2 and 77.2% activity at pH 8.0 and 9.0, respectively. The purified exo-inulinase was also stable between pH 4.0–6.0, Fig. 5b. The enzyme retained 96.9, 80.2 and 67.6% residual activity after 1 h pre-incubation at pH 4.0, 5.0 and 6.0, respectively. More than 50.0% residual activity was retained after 20 min pre-incubation at pH 3.0 and at pH 7.0, but the enzyme lost 51.4 and 73.9% activity at pH 8.0 and 9.0, respectively.

The crude exo-inulinase was stable at 40 °C and relatively stable at 50 °C, retaining 65.4% residual activity after 1 h pre-incubation, Fig. 6a. Up to 79.9% relative activity was lost after 1 h pre-incubation at 60 °C. However, the purified exo-inulinase was less stable at 50 °C (Fig. 6b) compared to the crude enzyme. The purified inulinase was only stable for 20 min at 50 °C and lost 56.4% activity after 1 h pre-incubation. Additionally, at 60 °C, up to 88.8% residual activity was lost after an hour of pre-incubation.

The effect of different heavy metals and chemicals (5 mM) on purified exo-inulinase activity of *Paenibacillus* sp. D9 is shown in Fig. 7. The purified exo-inulinase lost total activity in the presence of 5 mM Hg^{2+}. Additionally, the purified exo-inulinase activity was strongly inhibited by the presence of Fe^{3+} and Cu^{2+} with residual activity of 52.8 and 69.5%, respectively. Exo-inulinase activity was enhanced in the presence of Ca^{2+} with a residual activity of 108.9%. Interestingly, the presence of EDTA showed no effect on the inulinase activity of *Paenibacillus* sp. D9.

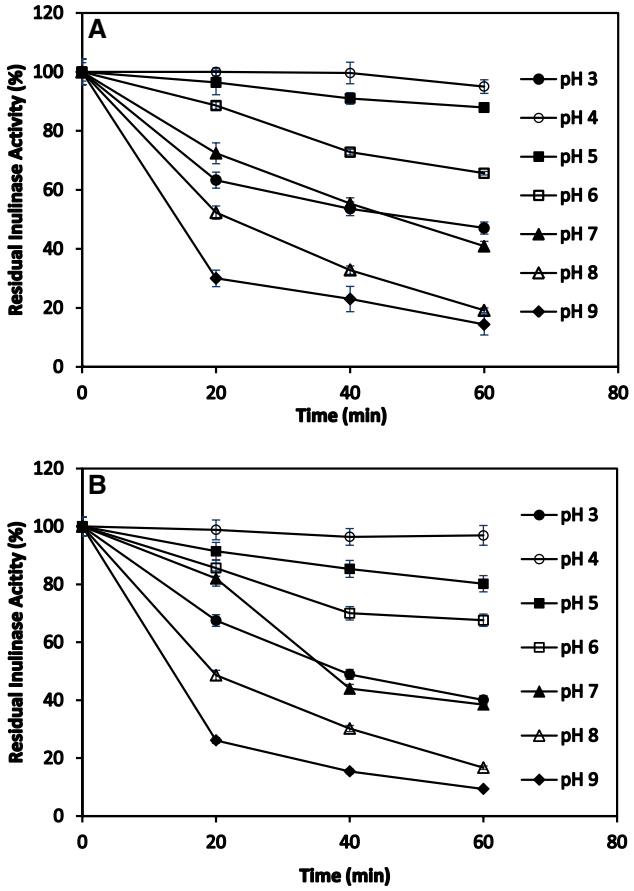


Fig. 5 pH stability of the crude (**a**) and purified (**b**) exo-inulinase of *Paenibacillus* sp. D9 determined by pre-incubating the enzyme at different pH. Results taken after 20 min incubation intervals. Enzyme activity was assayed at optimum pH and temperature. Error bars represent the mean ± SD of experiments in triplicate

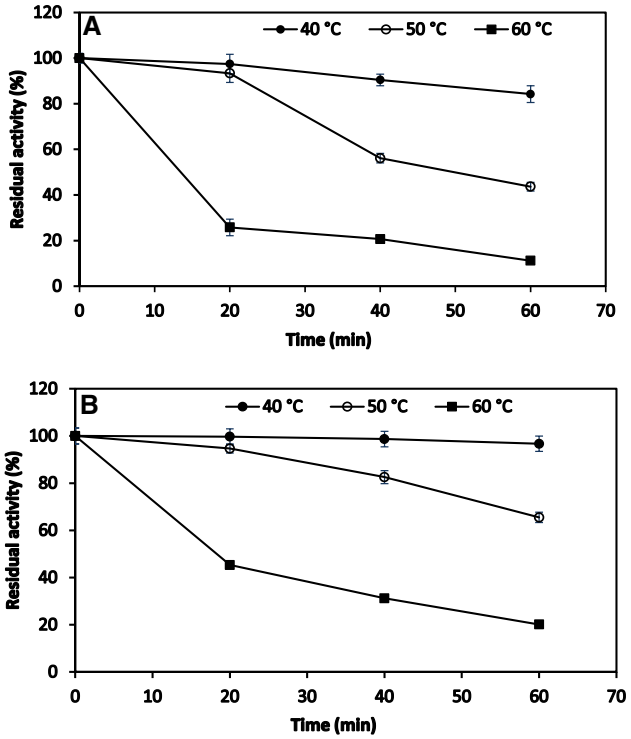


Fig. 6 Thermo-stability of the crude (**a**) and purified (**b**) exo-inulinase of *Paenibacillus* sp. D9 determined by pre-incubating the enzyme at different temperatures. Results taken after 20 min incubation intervals. Enzyme activity was assayed at optimum pH and temperature. Error bars represent the mean ± SD of experiments in triplicate

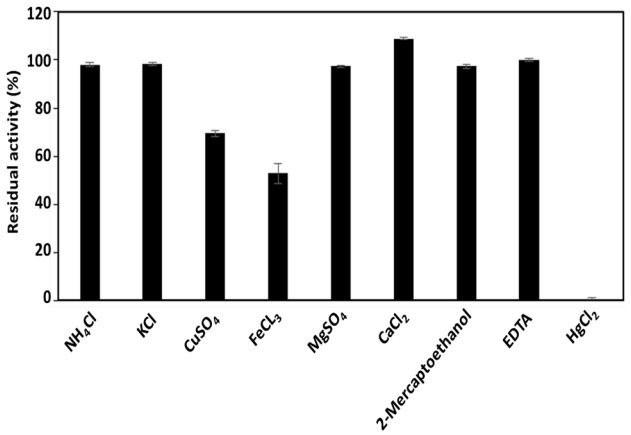


Fig. 7 The effect of different heavy metals and chemicals (5 mM) on purified exo-inulinase activity of *Paenibacillus* sp. D9. Enzyme activity was assayed at optimum pH and temperature. Error bars represent the mean ± SD of experiments in triplicate

5.4 Kinetic Study of Purified Exo-Inulianase from *Paenibacillus* sp. D9

The purified exo-inulinase has a K_m of 5.5 mM and V_{max} of 476.2 IU/mL, calculated based on the Lineweaver–Burk plot in Fig. 8. Catalytic constant, k_{cat} was calculated to be 42.6 s^{-1} with a catalytic efficiency (k_{cat}/K_m) of 7.6 s^{-1} mM^{-1}. Enzymatic hydrolysis of inulin by exo-inulinase yielded fructose as an end-product, Fig. 9. The TLC results show there was no inulin hydrolysis without exo-inulinase (IO) or when heat-denatured exo-inulinase (IE) was added. The results also indicate that almost all the inulin molecules were converted into fructose by adding D9 inulinase activity (E).

5.5 Bioinformatical Analysis

The phylogenetic tree (Fig. 10) shows that the deduced amino acid sequence of exo-inulinase from *Paenibacillus* sp. D9 has a close ancestor with other bacteria than with fungi such as *K. marxianus*. Figure 11 shows the multiple sequence alignments of exo-inulinases of the above-mentioned microorganisms. The amino acid sequence of D9 exo-inulinase was highly similar with that of *Paenibacillus*

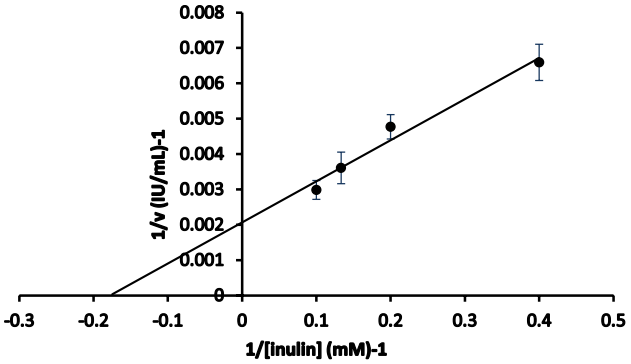


Fig. 8 Lineweaver–Burk plot for D9 exo-inulinase. The average M_r of inulin was estimated to be 5800 from the average DP of 36

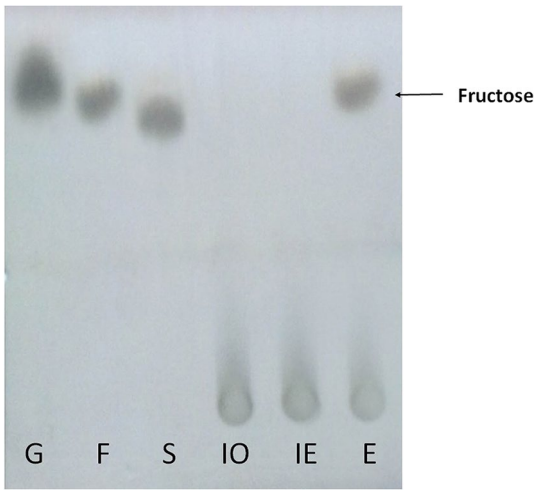


Fig. 9 Enzymatic hydrolysis of inulin by purified D9 exo-inulinase on Thin Layer Chromatography. *G* glucose, *F* fructose, *S* sucrose, *IO* inulin without enzyme, IE *inulin + inactivated exo-inulinase* and *E* inulin incubated with purified D9 exo-inulinase

polyxyma (63.0% identities) than with *S. linguale* DSM74 (54.1% identities). The amino acid sequence of *Paenibacillus* sp. D9 has conserved regions which are characteristic of exo-inulinases, WMNDPNG, RDP, ECP, SVEVF, FS and Q, red in Fig. 11. The conserved motifs accentuate the exo activity of the enzyme in inulin hydrolysis.

6 Discussion

In the present study, exo-inulinase was produced from *Paenibacillus* sp. D9 using inulin as a sole carbon source. Alignment of amino acid sequences of D9 inulinase with other bacterial inulinases and one fungal inulinase (Fig. 11) demonstrate that D9 exo-inulinase possesses conserved regions of WMNDPNG, Q, FS, RDP, ECP and SV(I)EV(L)F, which is consistent with bacterial

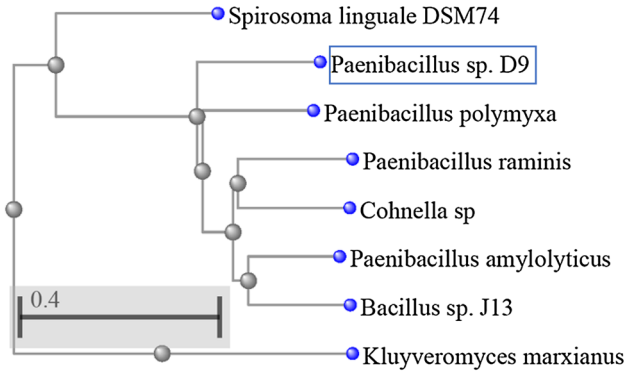
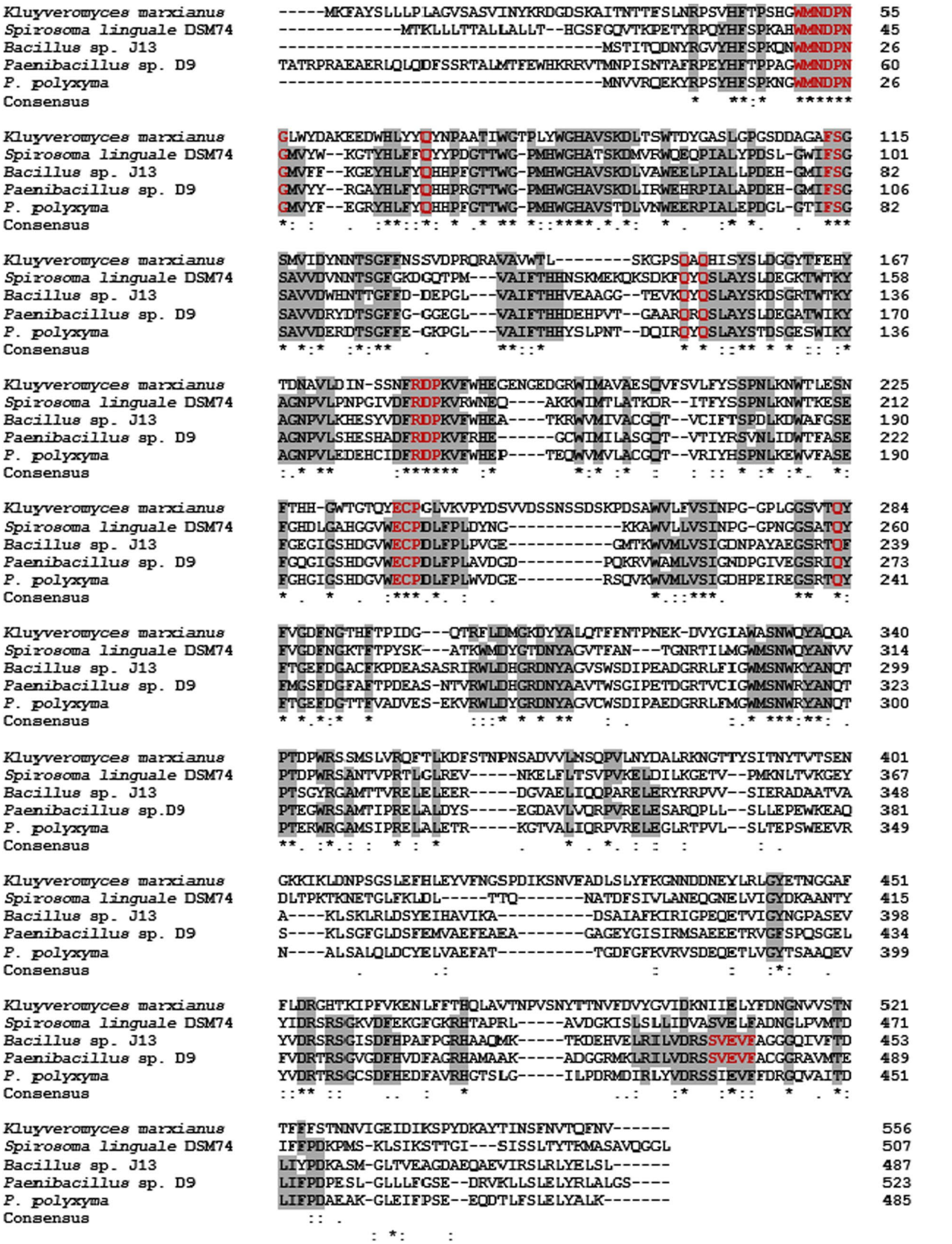


Fig. 10 Cladogram showing relatedness of exo-inulinases from different organisms. Constructed from COBALT. A phylogenetic tree was constructed using the neighbour-joining method (algorithm) and the Jukers-Cantor method (distance measure). The branching confidence was estimated by bootstrapping with 1000 replicates. The scale bar corresponds to 0.4 amino acid substitution per site. Highlighted in blue box is *Paenibacillus* sp. D9 taxon. (Color figure online)

exo-inulinases [34–36]. The conserved motifs of D9 exoinulinase accentuates the exo-activity of the enzyme in inulin hydrolysis as almost all the inulin molecules were converted into fructose by the action of D9 inulinase (Fig. 9). The results of the amino acid sequence alignment and phylogenetic tree suggest bacterial inulinases have evolved independently of fungal inulinases [37].

Highest inulinase activity of 59.6 IU/mL was obtained after 72-h incubation (Fig. 1). Results obtained in this study show that *Paenibacillus* sp. D9 produced higher exo-inulinase activity compared to *X. campestris* mutant (22.1 IU/mL) [38], *Bacillus* sp. SG113 (8.6 IU/mL) [39] and *Acinetobacter baumannii* (4.3 IU/mL) [40]. Among bacteria, *Paenibacillus* sp. D9 can be considered as a good inulinase producer under submerged fermentation compared to those reported by Singh et al. [41] Considerable inulinase production of *Lactobacillus pentosus* B235 of 315.7 IU/mL have been documented [42]. Recombinant exo-inulinase production of up to 722 IU/mL from *Lactobacillus paracasei* have been documented [43]. Literature reports that bacterial inulinase production is low compared to those of fungi and yeasts [22]. *Aspergillus ficuum* JNSP5-06, *A. sclerotiorum, A. tubingensis* CR16, *Kluveromyces marxianus* NRRL Y-7571 and *Pichia guilliermodii* mutant possessed high inulinase productions of 205.1, 114 586, 257, 586; 127.7 IU/mL, respectively [18, 44–47]. Nevertheless, exo-inulinase production by *Paenibacillus* sp. D9 was comparable to those fungal isolates such as *A. oryzae* (72.3 IU/mL) [44] and *Geotrichum candidum* (71.9 IU/mL) [48] and better than those of *A. terreus* (21.1 IU/mL) [16], *Ulocladium atrum* (36 IU/mL) [22] and many other thermostable fungal strains (up to 10.7 IU/mL) [8].


Kluyveromyces marxianus
Spirosoma linguale DSM74
Bacillus sp. J13
Paenibacillus sp. D9
P. polyxyma
Consensus

◂**Fig. 11** Multiple sequence alignment of amino acid sequences of exo-inulinases from different microorganisms, *viz. K. marxianus, S. liguale* DSM74, *Bacillus* sp. J13, *Paenibacillus* sp. D9 and *P. polyxyma*, respectively. Highlighted in grey and red are conserved amino acid sequences and amino acids characteristic to only exo-inulinases, respectively. Symbols "*, : and ." represent conserved amino acid, amino acid group with strongly and weakly similar properties, respectively. The alignment was constructed using Clustal Omega version 1.2.4. (Color figure online)

Exo-inulinase of *Paenibacillus* sp. D9 was purified to homogeneity (Fig. 2) with the yield of 7.1% and the purification fold of 4.3 (Table 1). The molecular weight (M_r) of purified D9 exo-inulinase was 58.5 kDa (Fig. 2) which is close to 56 kDa of *Paenibacillus polymyxa* ZJ-9 [49]. Majority of bacterial exo-inulinases have the M_r in the range of 47–73 kDa [50–56]. Interestingly, a multimeric structure with a 600 kDa relative M_r of β-fructosidase from *Bacillus stearothermophilus* that is composed of 60 kDa subunits has been reported [57]. Meanwhile most of fungal inulinases also have the molecular weight in a similar range (30–80 kDa) [6, 58, 59].

The properties of exo-inulinases from different bacteria vary significantly even though most of inulinases contain similar structures and molecular weights. A His-tagged exo-inulinase from *Bacillus polymyxa* MGL21 possesses a SA of 134 000 IU/mg with the K_m and V_{max} values of 0.7 mg/mL and 2500 µmol/min/mg protein, respectively [52] and an exo-inulinase of *Pseudomonas mucidolens* has a specific activity, K_m and V_{max} values of 9400 IU/mg, 11.5 mM and 1.1 µM/min/mg protein, respectively [34]. However, high SAs of extracellular inulinases from some fungal isolates such as *Alternaria alternata* (Fr.) keissler (80,700 IU/mg) [60], *Cryptococcus aureus* (15,826 IU/mg) [61] and *Pichia guilliermondii* (9697 IU/mg) [62] have also been reported in the literature. D9 exo -nulinase possessed a SA of 4333.2 IU/mg (Table 1), which was comparable to that of 4000 isolated from *B. stearothermophilus* [57]. The SAs of the above mentioned inulinases including D9 exo-inulinase from this study can be considered excellent inulin degraders that are higher than inulinases from other sources [6, 41].

This study found that both crude and purified D9 exo-inulinase had pH and temperature optima of 4.0 and 40 °C (Fig. 4). Many authors have reported on different pH and temperature optima, the ranges of pH from 4.0 to 6.0 and of temperature from 35 to 60 °C are considered optimal range for bacterial and fungal inulinases [6, 41, 60, 63]. Temperature optima at 75 °C of fungal inulinase from *Thielavia terrestris* [64] and at 70 °C of bacterial inulinases from *Bacillus smithii* have been reported [50]. Temperature optima of bacterial inulinase from *Lactobacillus pentosus* B235 was only at 25 °C [42]. Optimal activity of the exo-inulinase of *Bacillus* sp. snu-7 occurred at pH 7.0 and 50 °C [65]. Interestingly, a bacterial strain, *Marinimicrobium* sp. LS-A18 showed maximum inulinase activity at 55 °C and pH 9.0, respectively [66].

The D9 exo-inulinase was found to be stable between pH 4.0–6.0 and up to 50 °C. These results are in accordance with bacterial exo-inulinases from *L. casei, B. subtilis, Pseudomonas aeruginosa* and *Achromobacter* isolated from sugarcane soil [67] and *Sphingomonas* sp. GN25 [36]. This suggests that D9 exo-inulinase has a low pH spectrum thus potential for inulin hydrolysis in low pH conditions. Literature reports that most of inulinases are stable between pH 4.0–8.0 [6, 19, 41]. Bacterial inulinase from *P. mucidolens* was stable in the pH range 5.0–9.0 and it retained about 70% of residual activity after 4 h incubation at pH 6.0 and 55 °C [53] and those from *Arthrobacter* were stable in the pH range 6.0–9.0 or 6.0–10.0 [68, 69]. *Nocardiopsis* sp. produced thermostable inulinase that is stable within a wide pH range (5.0–11.0) [70]. Interestingly, more thermostable bacterial inulinases have been reported than the fungal counterpart. Bacterial inulinases from *B. smithii, B. stearothermophilus, Geobacillus stearothermophilus* and *Sphingomonas* sp. were stable up to 70–80 °C [50, 54, 56, 57]. Only inulinases from *A. fumigatus, A. niger* and *Rhizopus oligosporus* demonstrated their stability of up to 75 °C [71–73]. In the industry, enzyme thermo-stability that is > 60 °C is recommended for minimal microbial contamination [74]. In attempts to improve the inulinase thermal stability, immobilisation of inulinase from *A. niger* NCIM 945 on chitosan beads improved its thermo-stability from 80 to 100% residual activity after 2 h pre-incubation at 50 and 60 °C [75, 76]. Immobilisation of endo-inulinase with poly-D-lysine shifted the optimum temperature from 50 to 55 °C [77].

The effect of heavy metals and some known enzyme inhibitors on purified D9 exo-inulinase was also studied as shown in Fig. 9. Total inhibition by Hg^{2+} suggests an active role of—SH groups within the protein structure in the hydrolysis of inulin [78, 79]. Total inhibition of D9 exo-inulinase by $HgCl_2$ is in agreement with many studies [23, 53, 58, 69, 80]. Partial inhibition of D9 exo-inulinase by Cu^{2+} and Fe^{3+} was also found. The results in this study are in accordance with exo-inulinase from *U. atrum* [22]. The study also found that Ca^{2+} enhanced D9 exo-inulinase activity. Other studies also report that calcium ion can act as an enhancer/stabilizer for exo-inulinases [24, 60, 81, 82]. Interestingly, EDTA and β-mercaptoethanol had no effect on the D9 inulinase activity. Zhou et al. [56] reported that *Sphingomonas* sp. JB13 produced a novel detergent-, salt-, and protease-tolerant exo-inulinase.

Kinetic studies reveal the efficiency of enzymes in carrying out catalytic activities thus very useful for selection of enzymes for industrial purposes [83]. Exo-inulinase from this study was found to have a K_m and V_{max} of 5.5 mM and 476.2 IU/mL, respectively. The K_m value of

D9 exo-inulinase was comparable to those from *A. ficuum*, 4.8 mM and *B. subtilis*, 6.8 mM [84]. Lower K_m values of inulinases from different organisms have been reported from *K. marxianus* NRRL Y-7571, 1,5 mM [28]; *Arthrobacter* sp. S37, 0.3 mM [34]; and *R. oligosporus*, 0.9 mM [73]. Also, higher K_m values have been reported from *A. wentii*, 10 mM [83]; *Kluveromyces* sp., 13.3 mM [63]; and *A. ficuum*, 15 mM [85]. Further kinetic studies on exo-inulinase revealed that the enzyme has a turn-over number of 42.6 s^{-1} and a catalytic coefficiency of 7.6 s^{-1} mM^{-1}. Higher k_{cat} (s^{-1}) values were often reported from fungal exo-inulinases: 82.9 of *K. marxianus* [86], 130 of *A. niger* AF10 [82] and 587 of *K. marxianus* [87]. *Sphingobacterium* sp. GN25 produced exo-inulinase with a k_{cat} value of 261 [36]. These findings, together with the enzyme's high affinity for inulin, suggest exo-inulinase potential for high fructose production. As mentioned previously, fructose has many health benefits over sucrose. Fructose is an important ingredient in food industry [16]. Many studies have produced fructose syrups from inulin using inulinase [3, 30, 69, 88]. However, there is a long debate on whether fructose has health benefits or is a health threat [89]. Recent studies have not supported a correlation between fructose consumption and development of type 2 diabetes [90, 91].

7 Conclusion

In this study, exo-inulinase from *Paenibacillus* sp. D9 has been purified to electrophoretic homogeneity and was characterised. Amino acid alignment and the complete degradation of inulin into fructose confirmed that inulinase from *Paenibacillus* sp. D9 is an exo-form. The enzyme's low K_m suggests high affinity for inulin thus a potential in fructose syrup production. In addition, D9 exo-inulinase also has a high SA (4333.2 IU/mg) using inulin as the substrate. Optimum exo-inulinase activity of pH 4.0 and its stability at pH 4.0–6.0 suggests a potential for applications in low pH conditions. However, the enzyme's thermal stability needs to be improved to meet industrial needs. Molecular cloning and expression of D9 inulinase using other hosts, as well as immobilisation to improve its thermo-stability are recommended for the future studies. The purified D9 exo-inulinase or its immobilised form can also be explored for its industrial potentials.

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