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Purification and Characterization of an Endoinulinase from *Xanthomonas campestris* pv. *phaseoli* KM 24 Mutant

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Summary

An extracellular endoinulinase from *Xanthomonas campestris* pv. *phaseoli* KM 24 mutant was purified to homogeneity by gel filtration chromatography and showed a specific activity of 119 U/mg. The optimum pH and temperature of the purified enzyme were found to be 6.0 and 50 °C, respectively. The enzyme was stable up to 60 °C, retaining 60 % of residual activity for 30 min, but inactivated rapidly above 60 °C. The enzyme was found to be stable at pH=6–9 when it retained 100 % of its residual activity. The Lineweaver-Burk plot showed that the apparent K_m and v_{max} values of the inulinase when using inulin as a substrate were 1.15 mg/mL and 0.15 μM/min, respectively, whereas the k_{cat} value was found to be 0.145 min^{-1}. The calculated catalytic efficiency of the enzyme was found to be 0.126 (mg·min)/mL. The purified inulinase can be used in the production of high fructose syrups.

Key words: Xanthomonas campestris pv. *phaseoli* KM 24 mutant, inulinases, endoinulinases, exoinulinases, fructooligosaccharides, inulin

Introduction

Inulin is considered as a renewable raw material in the production of fructose syrup and fructooligosaccharides (FOS) (*1*) and hence, enzyme inulinases are widely used in food and pharmaceutical industries (*2*). Microbial inulinases can be classified into exo- and endo-acting enzymes according to their modes of action on inulin. Endoinulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse the internal β-2,1-fructofuranosidic linkages to yield inulooligosaccharides such as inulotriose, inulotetraose and inulopentaose as their main products. Exoinulinases (β-D-fructan fructohydrolase; EC 3.2.1.80) successively cleave off terminal fructose units from the non-reducing end of inulin, and also hydrolyse sucrose and raffinose (*3,4*). Therefore, inulinases could be used for production of either high fructose syrups by exo-enzymatic hydrolysis of inulin with D-fructose content over 95 %, or for production of oligofructoside syrups by endo-enzymatic hydrolysis (*5*). Inulinases are produced by a few bacteria (*Xanthomonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Thermotoga* sp., *Bifidobacterium* sp., *Geobacillus* sp. and *Clostridium* sp.), fungi (*Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp.) and yeast (*Kluyveromyces* sp.) (*4*). The optimization of the nutritional and growth parameters of *X. campestris* pv. *phaseoli* for the production of endoinulinase using the submerged and solid-state cultivations has been reported earlier (*6*). The rate of endoinulinase and FOS production was further enhanced through ethylmethanesulphonate (EMS) mutagenesis of *X. campestris* pv. *phaseoli* and the mutant was named *X. campestris* pv. *phaseoli* KM 24 (*Xcp* KM 24) (*7*). The present study, therefore, focuses on the purification and characterization of endoinulinase from *Xcp* KM 24 using gel filtration chromatography.

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Materials and Methods

Bacterial strain

Strain *Xanthomonas campestris* pv. *phaseoli* was obtained from the culture collection of the Department of Microbiology at the University of KwaZulu-Natal, Durban, South Africa. The strain was improved by chemical mutagenesis using EMS as described earlier (*8*) with slight modifications. Optimized medium was used to test the mutants for their ability to produce inulinase and FOS. The strain was named *Xanthomonas campestris* pv. *phaseoli* KM 24 (*Xcp* KM 24) (*7*) and stored as 70 % glycerol stocks at –70 °C.

Endoinulinase production by Xanthomonas campestris pv. phaseoli KM 24

For the production of endoinulinase, the mutant *Xcp* KM 24 was inoculated into 50 mL of the inulinase production medium (IPM) containing (in g/L): inulin 20, yeast extract 20, $(NH_4)_2HPO_4$ 5, $NH_4H_2PO_4$ 2, $MnCl_2$ 0.5, KCl 0.5, $MgSO_4$ 0.5 and $FeSO_4$ 0.01 (pH=7.0) (*9*). Pure inulin prepared from chicory roots was obtained from Sigma-Aldrich, St. Louis, MO, USA. IPM was dispensed into 250-mL Erlenmeyer flasks and inoculated with 10 % (by volume) of a 16-hour-old *Xcp* KM 24 culture and incubated at 37 °C for 120 h with shaking at 150 rpm. Samples were withdrawn every 12 h, centrifuged at 10 000×*g* and the supernatant was used for determining inulinase activity (*6,7*).

Determination of inulinase activity

Inulinase activity was determined by quantifying the amount of reducing sugars released from inulin as described earlier (*10*). The reaction mixture containing 0.1 mL (10 μM) of crude enzyme extract and 0.9 mL of sodium acetate buffer (100 mM, pH=5.5) was incubated at 50 °C. The reaction was started by adding 1 mL of 2 % inulin and allowed to react for 20 min. One inulinase unit (IU) was defined as the amount of enzyme that produces one micromole of fructose equivalent per minute under standard assay conditions.

Concentration and purification of inulinase

One litre of inulinase production culture medium supernatant was sequentially subjected to precipitation with ammonium sulphate from 20 to 100 % at intervals of 20 % at 4 °C for 16 h. The pellets were collected by centrifugation, dissolved in 5 mL of sodium phosphate buffer (pH=7) and dialysed through the 12-kDa cut-off dialysis membrane from Sigma-Aldrich against the same buffer. The fractions of 40, 60 and 80 % showed inulinase activity and they were pooled together. The sample was concentrated using ultrafiltration spin column (Amicon Ultra-15 Centrifugal Filter Unit, molecular mass cut-off 30 kDa, cat. no. UFC903024, Merck KGaA, Darmstadt, Germany) and 1 mL of enzyme sample (1 mg/mL) was loaded into approx. 75-mL Sephadex G-100 column (40 cm×0.75 cm; GE Healthcare Life Sciences, Little Chalfont, UK) and eluted with 50 mM phosphate buffer at a flow rate of 1 mL/min. Fractions of 4 mL were collected and assayed for protein content (at 280 nm) and inulinase activity as described above (data not shown). The fractions showing inulinase activity were pooled together and concentrated again by passing the samples through ultrafiltration spin column in the centrifuge at the speed of 6500×*g* for 15 min. A small volume of fractions (200 μL) was precipitated with 800 μL of chilled acetone to check the purity and homogeneity of the protein by subjecting it to 12 % SDS-PAGE (*11*) run on constant potential difference of 100 V. The gel was stained with Coomassie Brilliant Blue R-250 (CBB R-250) and the protein content was determined as previously described by Bradford (*12*) using bovine serum albumin (BSA) as standard.

Determination of optimum pH and temperature of purified inulinase

In order to determine the optimum pH of the purified inulinase, 0.5 % (by mass per volume) inulin substrate solutions were prepared in the following buffers (100 mM): citrate buffer (pH=4–6), sodium phosphate buffer (pH=7), Tris-HCl (pH=8 and 9) and glycine-NaOH (pH=10). The enzyme (1 μM) was incubated with the substrate at 50 °C for 20 min. To determine the optimum temperature, the enzyme (1 μM) was incubated with the substrate prepared in the optimum pH buffer. The assay mixture containing 50 μL (1 μM) of the enzyme solution and 950 μL of the substrate solution in sodium phosphate buffer (100 mM, pH=7) was incubated at temperatures ranging from 25 to 90 °C.

Determination of pH and temperature stability

The pH stability of the enzyme was determined by incubating 1 mL (10 μM) of the enzyme at pH=4–9 at 50 °C. Aliquots of 100 μL were removed at time intervals of 0, 10, 20, 30, 60, 90, 120, 150 and 180 min, and assayed by incubating with 900 μL of inulin substrate as mentioned above. Temperature stability of the enzyme was determined by incubating inulinase (10 μM) at different temperatures *i.e.* 50–90 °C at the intervals of 10 °C. The aliquots of 100 μL were removed at time intervals of 0, 10, 20, 30, 60, 90, 120, 150 and 180 min, and assayed for inulinase activity. For determination of temperature stability of the purified enzyme, the substrate was prepared in sodium phosphate buffer (100 mM, pH=6). The final concentration of the enzyme in the assay reaction mixture was 1 μM.

Determination of K_m, v_{max} and k_{cat} values

The initial rate of enzymatic activity was measured to determine kinetic parameters for the substrate hydrolysis. The Michaelis-Menten constant (K_m) was determined from the Lineweaver-Burk plot by applying the Michaelis-Menten equation (Eq. 1). The activity of inulinase was measured using Lineweaver-Burk plot analysis, by incubating it at substrate concentrations from 0.2–15 μg/mL in sodium phosphate buffer (pH=6 and 50 °C). The reciprocal values of the rate of substrate hydrolysis ($1/v$) were plotted against the reciprocal values of the substrate concentrations (1/[S]), and the K_m values were determined by fitting the resulting data using ORIGIN v. 8 Pro software (OriginLab Corporation, Northampton, MA, USA). The v_{max} was also determined from the Lineweaver-Burk plot. The catalytic constant of the enzyme substrate reaction

(k_{cat}), also referred to as the turnover number, represents the number of reactions catalysed by each active site per unit time and was determined by Eq. 2, while the catalytic efficiency of the enzyme was calculated by using Eq. 3:

$$1/v_0=(K_m/v_{max})(1/[S])+1/v_{max} \qquad /1/$$

$$k_{cat}=v_{max}/[E]_t \qquad /2/$$

$$\text{Catalytic efficiency}=k_{cat}/K_m \qquad /3/$$

where [S] is the substrate concentration, v_0 is the initial velocity, v_{max} is the maximum velocity, K_m is the Michaelis-Menten constant and $[E]_t$ is the total enzyme concentration (1 μM).

Thin layer chromatography to determine the catalytic nature of the inulinase

The products of inulin hydrolysis with purified inulinase from *Xcp* KM 24 strain were determined by performing the thin layer chromatography (TLC). The enzyme reaction (0.5 mL of purified inulinase (1 μM) and 0.5 mL of inulin (0.5 %, by mass per volume)) was carried out at 50 °C and pH=6.0 for 24 h. Precoated TLC plates (Silica gel 60, Merck, Darmstadt, Germany) spotted with samples were developed with the solvent system, ethyl acetate/acetic acid/2-propanol/formic acid/water (25:10:5:1:15, by volume). Sucrose (Sigma-Aldrich), glucose (Sigma-Aldrich), fructose (Merck), 1-kestose, 1,1-kestotetraose and 1,1,1-kestopentaose (Megazyme, Wicklow, Ireland) were used as standards. The TLC plates were developed using detection reagent containing 1 % (by mass per volume) orcinol and 10 % (by volume) sulphuric acid in absolute ethanol and heating them at 100 °C for 5 min to detect sugars (*7*).

Statistical analysis

All the kinetic parameters were determined by fitting the data using ORIGIN v. 8 Pro software (OriginLab Corporation). The assays for the kinetic analysis and rate constant determinations were carried out in triplicate, and the average value was considered throughout. The p-value lower than 0.05 was considered statistically significant.

Results and Discussion

Purification of inulinase

The present study reports the purification and characterization of an endoinulinase from *Xcp* KM 24, a mutant strain of *Xanthomonas campestris* pv. *phaseoli*. The endoinulinase produced by *Xcp* KM 24 was purified to homogeneity in two steps, ammonium sulphate precipitation and Sephadex G-100 column chromatography with 77 % yield, and had a specific activity of 174.74 U/mg. Table 1 shows the summary of the enzyme purification and the total yield. The final enzyme preparation was homogeneous on SDS-PAGE, with a molecular mass of 55 kDa (Fig. 1). A considerable variation in molecular mass of inulinases has been reported earlier, *e.g. Arthrobacter* sp. (75 kDa), *Bacillus stearothermophilus* KP1289 (54 kDa), *Aspergillus candidus* (54 kDa), *Penicillium* sp. TN-88 (68 kDa), *Kluyveromyces marxianus* var. *bulgaricus* (57 kDa) and *Streptomyces* sp. (45 kDa) (*13–18*). Five exoinulinases from *Aspergillus ficuum* showed the same molecular mass of 74 kDa and three endoinulinases had a molecular mass of 64 kDa (*19*). A new thermophilic inulinase-producing strain *Bacillus smithii* T7, which grows optimally at 60 °C, was isolated from soil samples with a medium containing inulin as a sole carbon source. Maximum inulinase yield of 135.2 IU/mL was achieved with medium pH=7.0, containing 2.0 % inulin. The purified inulinase from the extracellular extract shows endoinulinolytic activity (*20*). *A. ficuum* JNSP5-06 produces five enzymes with molecular masses of 70, 40, 46, 34 and 31 kDa (*21*). *A. ficuum* JNSP5-06 endoinulinase expressed in *Escherichia coli* exhibited *M* of 60 kDa (*22*), which is in contrast to the above study. The crystal structural analysis of inulinase from *A. ficuum* JNSP5-06 at 1.5 Å and its comparison with other glycoside hydrolase family 32 (GH32) enzymes reveal the presence of an extra pocket in the INU2 catalytic site, formed by two loops and the conserved motif W-M(I)-N-D(E)P-N-G. This cavity could explain the endo-activity of the enzyme,

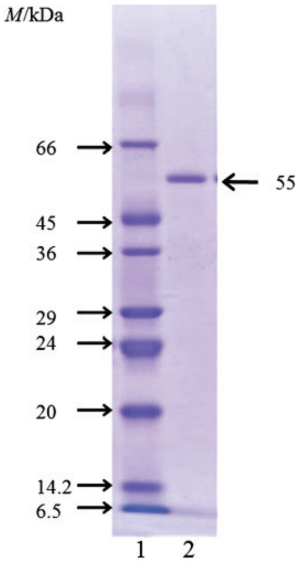

Fig. 1. SDS-PAGE of purified inulinase. Lane 1: molecular mass marker, lane 2: purified endoinulinase

Table 1. Summary of the purification and yield of endoinulinase from *Xanthomonas campestris* pv. *phaseoli* KM 24 supernatant

Purification step	γ(protein) / mg/mL	Endoinulinase activity / U/mL	Yield / %	Specific activity / U/mg	Purification factor
Crude culture filtrate	0.217	22.12	100	102	1
$(NH_4)_2SO_4$ precipitation	0.179	18.76	85.2	104.8	1.02
Sephadex G-100	0.097	16.95	77	174.74	1.74

the critical role of Trp40, and particularly the cleavage at the third unit of the inulin (-like) substrates (*23*). Most of the inulinases from fungi have been reported to have a molecular mass above 50 kDa (*24*). The molecular mass of the purified inulinase from the supernatant of the cell culture of the marine yeast *Cryptococcus aureus* G7a was estimated to be 60 kDa (*25*), while the molecular mass of the purified inulinase from *Pichia guilliermondii* strain 1 was estimated to be 50 kDa (*26*). However, it has been reported that the *M* of extracellular inulinase from the terrestrial yeast *Kluyveromyces fragilis* is 250 kDa. The molecular mass of the purified exoinulinase from bacteria was estimated to be approx. 54 kDa (*27,28*). This suggests that molecular mass of the exoinulinases from bacteria is almost the same as of the exoinulinases from yeasts. *K. marxianus* CBS 6556 inulinase (rKmINU) gene expressed in methylotrophic host *Pichia pastoris* showed a specific activity of 2714 U/mg (*29*), which is 12-fold higher than those of other inulinases described previously. It displayed excellent stability from 30 to 50 °C and pH=3.0–5.0, and its half-life was over 96 h under these conditions. Moreover, rKmINU saccharified Jerusalem artichoke tuber juice effectively (*29*). A 79.8-kDa endoinulinase gene (*enIA*) from *Arthrobacter* sp. S37 overexpressed in *Yarrowia lipolytica* Po1h showed endoinulinase and specific endoinulinase activities of 16.7 U/mL and 93.4 U/mg, respectively (*30*). From *Lactobacillus casei* IAM1045, *levH1* gene encoding an inulinase was cloned and sequenced, and structure-function relationship was investigated by site-directed mutagenesis (*31*). This gene product belongs to GH32 enzyme group, and is composed of four domains. From the catalytic domain of *levH1* gene, the 8th motif was newly found in the β-sandwich module, and the necessity of its D683 residue for catalysis was confirmed. *LevH1* was found to be an exo-type inulinase producing exclusively fructose, and the knockout of *levH1* resulted in the loss of the bacterial ability to catabolize inulin for growth (*31*). An inulinase of *M*=66 kDa from a marine bacterium *Bacillus cereus* MU-31 is also reported to have an activity of 96 U/mL (*32*).

Optimum temperature and thermostability of inulinase

The inulinase activity measured as a function of temperature from 30 to 90 °C shows that the activity of the enzyme was the highest at 50 °C (Fig. 2). The enzyme is stable at up to 60 °C, retaining over 60 % activity for 30 min, but inactivated rapidly at higher temperatures. At 90 °C, the enzyme lost its complete activity within 10 min (Fig. 3). A novel inulinolytic strain of *Xanthomonas* sp. has been reported that produces an endoinulinase optimally active at 45 °C and pH=6 (*33*). The optimal temperature of the purified enzyme from the marine yeast *C. aureus* G7a is reported to be 50 °C and the enzyme is very stable at up to 65 °C (*25*). Therefore, inulinase produced by *Xcp* KM 24 seems to have considerable thermostability as compared to others reported in literature. However, the inulinase activity produced by *P. guilliermondii* strain 1 is the highest at 60 °C and the enzyme is very stable at up to 60 °C (*26*). Inulinase from terrestrial microorganisms in general shows the highest activity below 50 °C, whereas optimum temperature is mostly between 30 and 55 °C (*16,24,34,35*). The optimum temperature for an endoinulinase from *B. smithii* T7 was 70 °C, the $t_{1/2}$ of the endoinulinase was 9 h and 2.5 h at 70 and 80 °C, respectively (*20*).

Optimum pH and pH stability of inulinase

The inulinase activity was measured in the pH range of 4–10 in the buffers with the same ionic concentrations. The results indicate the enzyme to be optimally active at pH=6.0 (Fig. 4). The activity of the purified enzyme was stable between pH=6.0–9.0 (Fig. 5). After 2 h at 50 °C, more than 40 and 45 % of the residual activity remained at pH=6.0 and 9.0, respectively. An extracellular endoinulinase purified from *X. oryzae* (*9*) was optimally active at pH=7.5 and 50 °C and stable over a pH range of 6.0–9.0. An exoinulinase of 83 kDa was purified from *Streptococcus salivarius* with optimum pH=7.0 (*36*). The inulin-inducible inulinase from *Clostridium acetobutylicum* was reported to be produced both extra- and intracellularly with the pH and temperature optima of 5.5 and 47 °C, respectively (*37*). The endoinulinase from *Clostridium thermoautotrophicum* was maximally active at 60 °C and neutral pH (*38*). The D-fructofuranosidase of *Bifidobacterium infantis* is a monomeric protein of 70 kDa and possesses both inulinase and invertase activities (*39*), and the purified endoinulinase showed the optimum pH and temperature of 6.0 and 37 °C, respectively. The optimum pH for

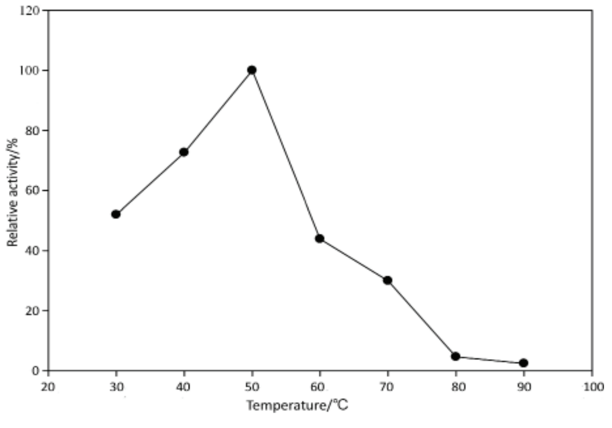

Fig. 2. Optimum temperature for the purified endoinulinase determined by performing the standard enzyme assays at different temperatures in 100 mM citrate buffer (pH=6)

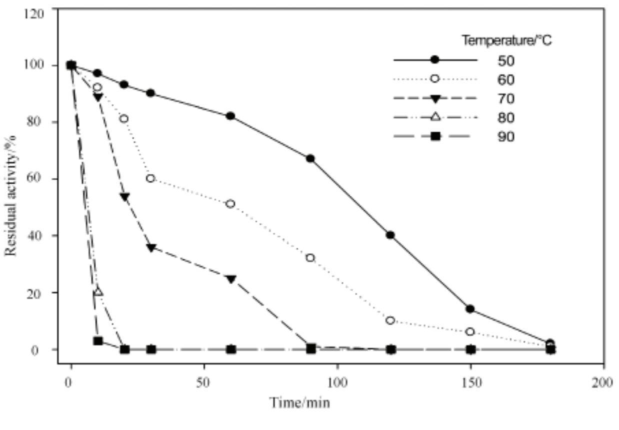

Fig. 3. Temperature stability of endoinulinase (1 μM) of *Xcp* KM 24 mutant determined by incubating the enzyme at different temperatures (50–90 °C)

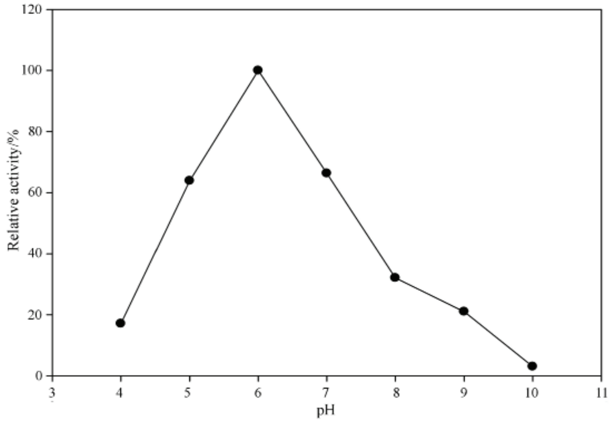

Fig. 4. Optimum pH for the purified endoinulinase determined by performing the standard enzyme assays at different pH values and 50 °C

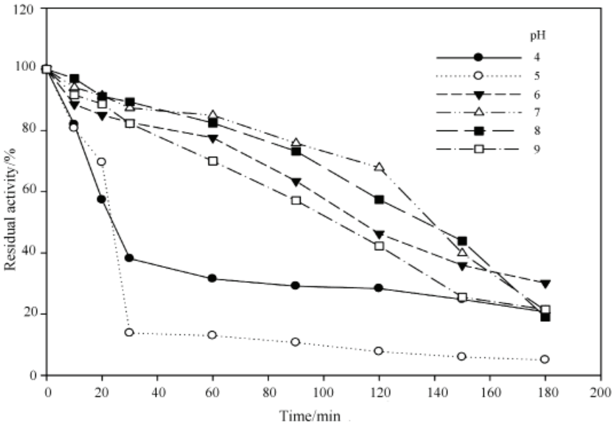

Fig. 5. pH stability of endoinulinase determined by incubating the enzyme at pH=4–9 and 50 °C and performing the enzyme assay at pH=6.0

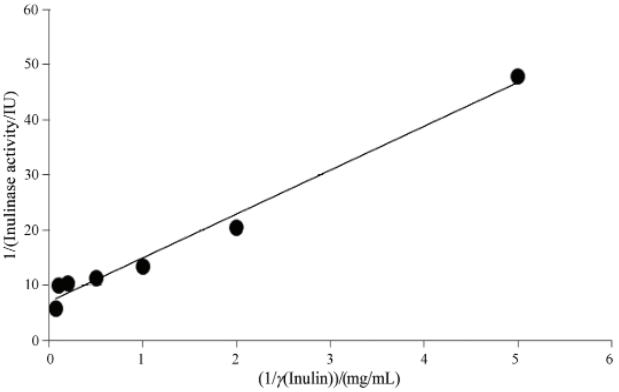

Fig. 6. Lineweaver-Burk plot for inulinase activity. Enzyme (1 μM) was incubated with inulin (0.2–15 μg/mL) at 50 °C and pH=6.0 for 20 min. The reciprocal values of enzyme units (1/*v*) were plotted against the reciprocal values of the substrate concentrations (1/[S]), and the K_m values were determined by fitting the resulting data using ORIGIN v. 8 Pro. IU=inulinase unit (the amount of enzyme that produces 1 μmol of fructose equivalent per min)

an endoinulinase from *B. smithii* T7 was 4.5 and the enzyme was stable at pH=4.0–8.0 (*20*). The gene encoding for one of the most thermostable bacterial inulinases, which retained 85 % of its initial activity after 5 h at 80 °C and pH=7.0, was cloned from *Thermotoga maritima* (*40*). The optimum pH values of the purified inulinases from fungi and yeast are in the range of 4.5–6.0 (*2,26,34,35*).

Kinetic properties of inulinase

Enzymes are characterized by measuring their reactions with regard to their substrate affinities and maximal velocity rates. By measuring the rate of substrate utilization (v) at different substrate concentrations [S], K_m and v_{max} can be calculated using Lineweaver-Burk, Eadie/Hofstee or Wilkinson methods (*41,42*). In this study, the Lineweaver–Burk plots showed that the apparent K_m and v_{max} values of the inulinase when using inulin were 1.15 mg/mL and 0.0000261 mg/(mL·min), respectively (Fig. 6). The k_{cat} value was found to be 0.145 min^{-1}. The calculated catalytic efficiency of the enzyme was found to be 0.126 mg/(mL·min). The K_m=1.15 mg/mL for this enzyme is lower than that of other reported inulinases, *e.g.* K_m values of the two endoinulinases, Endo-I and Endo-II, produced by *A. ficuum* JNSP5-06 were 14.8 and 25.6 mg/mL, respectively (*21*). *A. ficuum* JNSP5-06 endoinulinase was expressed in *E. coli* and the K_m and v_{max} values with inulin as the substrate were found to be (67.4±4.2) mg/mL and (349.2±13.7) mg/(mL·min), respectively (*22*). The K_m value of inulinase from *P. guilliermondii* strain 1 using inulin as substrate was 21.1 mg/mL (*43*). *Streptomyces* sp. ALKC4 endoinulinase showed K_m (1.63 mM) and v_{max} (450 IU/mg) using inulin as substrate, which is almost the same as the enzyme reported in this study (*18*). An endoinulinase from *B. smithii* T7 exhibited comparatively lower K_m (4.17 mM) and higher v_{max} (833 IU per mg of protein), which demonstrated that this endoinulinase has greater affinity for inulin substrate (*20*). *Debaryomyces cantarelli* (15 mM) (*44*), *Candida salmenticensis* (17 mM) (*45*) and *A. ficuum* (10–15 mM) (*19*) showed higher K_m than the inulinase reported here, which makes it a better candidate for inulin hydrolysis. Because of lower K_m value and high thermal stability, *Xcp* KM 24 endoinulinase can be used for commercial applications like for large scale FOS (alternative sweetener) production, and can help reducing the overall production cost.

Product of inulin hydrolysis by purified inulinase

Thin layer chromatography analysis of products of inulin hydrolysis showed that oligosaccharides were the predominant end product during hydrolysis for 10 min to 2 h (Fig. 7). Oligosaccharides with various degrees of polymerization were observed in the samples after hydrolysis for 10 and 30 min or 2 h and this hydrolysis pattern suggests the presence of an active endoinulinase. The prolonged enzyme hydrolysis (24 h) did not result in any kind of mono- (glucose and fructose) or disaccharides (sucrose) in the hydrolysates, which pointed to the absence of exoinulinase activity of the enzyme (data not shown). However, the prolonged enzyme hydrolysis (24 h) by the crude extract reported in our previous study with this organism (*7*) revealed the production of mono- (glucose and fructose) or disaccharides (sucrose) in the hydrolysates,

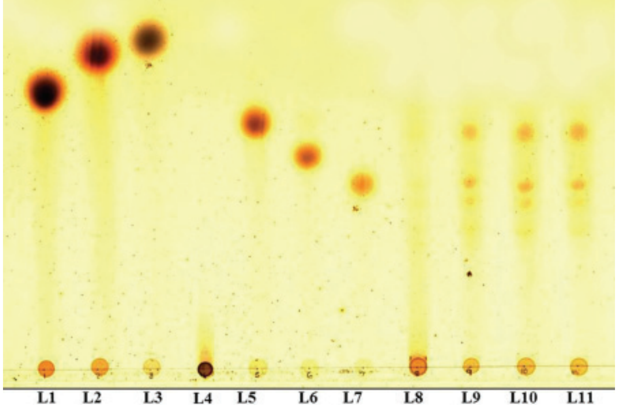

Fig. 7. Thin layer chromatography of reaction products formed during hydrolysis of inulin by purified inulinase from *X. campestris* KM 24. L1=sucrose, L2=glucose, L3=fructose, L4=inulin, L5=1-kestose, L6=1,1-kestotetraose, L7=1,1,1-kestopentaose, L8=enzyme+inulin (0 h), L9=enzyme+inulin (10 min), L10=enzyme+inulin (30 min), L11=enzyme+inulin (2 h)

which pointed to the presence of exoinulinase in the extract at low or undetectable levels, despite ammonium sulphate precipitation, with only endoinulinase activity observed. Inulin hydrolysis by an extracellular inulinase of *Rhizopus* sp. resulted in the production of fructose and oligosaccharides after 24 h of incubation (*46*). Fructose formation was completely absent when inulin was hydrolysed with crude endoinulinase of *X. oryzae* No. 5 (*47*). The native endoinulinase produced by *Arthrobacter* sp. S37 hydrolysed inulin at optimal pH=7.5 and 50 °C mainly into inulotriose (F3), inulotetraose (F4) and inulopentaose (F5) (*12*). Inulobiose was the major product of the activity of immobilized endoinulinase produced by *Pseudomonas* sp. No. 65 or immobilized recombinant *E. coli*, possessing endoinulinase gene (*48*). In soluble form, the endoinulinase produced by *Pseudomonas* sp. No. 65 gave two major components, inulobiose and DP3 oligosaccharides (*49*). When inulooligosaccharide (IOS) from chicory juice was hydrolysed by an endoinulinase from *Pseudomonas* sp, the major reaction products were DP2, DP3 and DP4 (*32*). After the endoinulinase gene (*inul*) of *Pseudomonas* sp. was expressed in *E. coli* HB101, the intact cells of the recombinant *E. coli* and the native enzyme from *Pseudomonas* sp. were used to produce IOS. It was found that higher levels of inulobiose (the smallest molecule in the product) were observed when intact cells were used (*49*).

Conclusions

Inulin-hydrolysing enzymes (inulinases) are widely used in food and pharmaceutical industries. Endoinulinases hydrolyse the internal β-2,1-fructofuranosidic linkages to yield inulooligosaccharides such as inulotriose, inulotetraose and inulopentaose as their main products. We have reported previously the optimization of the nutritional and growth parameters for *Xanthomonas campestris* pv. *phaseoli* to enhance the endoinulinase and fructooligosaccharide (FOS) production through ethylmethanesulphonate mutagenesis of the organism and named it *X. campestris* pv. *phaseoli*, mutant KM 24 (*Xcp* KM 24). The present study, therefore, focused on the purification and characterization of endoinulinase from *Xcp* KM 24 using gel filtration chromatography. Since the importance and potential applications of FOS is growing globally, this endoinulinase could be used for commercial applications like for large scale FOS (alternative sweetener) production.

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References

1. Yun JW. Fructooligosaccharides – occurrence, preparation and application. Enzyme Microb Technol. 1996;19:107–17. http://dx.doi.org/10.1016/0141-0229(95)00188-3
2. Singh RS, Singh RP. Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. Food Technol Biotechnol. 2010;48:435–50.
3. Deryeke DG, Vandamme EJ. Production and properties of Aspergillus niger inulinase. J Chem Technol Biotechnol. 1984; 34:45–51. http://dx.doi.org/10.1002/jctb.280340108
4. Singh P, Gill PK. Production of inulinases: Recent advances. Food Technol Biotechnol. 2006;44:151–62.
5. Mazutti M, Bender JP, Treichel H, Luccio MD. Optimization of inulinase production by solid-state fermentation using sugarcane bagasse as substrate. Enzyme Microb Technol. 2006;39:56–9. http://dx.doi.org/10.1002/jctb.2273
6. Ayyachamy M, Khelawan K, Pillay D, Permaul K, Singh S. Production of inulinase by Xanthomonas campestris pv. phaseoli using onion (Allium cepa) and garlic (Allium sativum) peels in solid state cultivation. Appl Microbiol Lett. 2007;45:439–44. http://dx.doi.org/10.1111/j.1472-765X.2007.02222.x
7. Naidoo K, Ayyachamy M, Permaul K, Singh S. Enhanced fructooligosaccharides and inulinase production by a Xanthomonas campestris pv. phaseoli KM 24 mutant. Bioprocess Biosyst Eng. 2009;32:689–95. http://dx.doi.org/10.1007/s00449-008-0293-6
8. Kamal FHM, Mahnaz MA, Seyed AM. Mutagenesis of Xanthomonas campestris and selection of strains with enhanced xanthan production. Iran Biomed J. 2003;7:91–8.
9. Cho YJ, Sinha J, Park JP, Yun JW. Production of inulooligosaccharides from chicory extract by endoinulinase from Xanthomonas oryzae No. 5. Enzyme Microb Technol. 2001;5:439–45. http://dx.doi.org/10.1016/S0141-0229(00)00341-0
10. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426–8. http://dx.doi.org/10.1021/ac60147a030
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5. http://dx.doi.org/10.1038/227680a0
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54. http://dx.doi.org/10.1016/0003-2697(76)90527-3
13. Kang SI, Chang YJ, Oh SJ, Kim SI. Purification and properties of an endo-inulinase from an Arthrobacter sp. Biotech-

nol Lett. 1998;20:983–6.
http://dx.doi.org/10.1023/A:1005440910352

14. Kato K, Araki T, Kitamura T, Morita N, Moori M, Suzuki Y. Purification and properties of a thermostable inulinase (β-D-fructan fructohydrolase) from Bacillus stearothermophilus KP1289. Starch-Stärke. 1999;51:253–8.
http://dx.doi.org/10.1002/(SICI)1521-379X(199907)51:7<253::AID-TAR253>3.0.CO;2-7
15. Kochhar A, Gupta AK, Kaur N. Purification and immobilization of inulinase from Aspergillus candidus for producing fructose. J Sci Food Agric. 1999;79:549–54.
http://dx.doi.org/10.1002/(SICI)1097-0010(19990315)79:4<549::AID-JSFA216>3.0.CO;2-0
16. Kushi RT, Monti R, Contiero J. Production, purification and characterization of an extracellular inulinase from Kluyveromyces marxianus var. bulgaricus. J Ind Microbiol Biotechnol. 2000;25:63–9.
http://dx.doi.org/10.1038/sj.jim.7000032
17. Nakamura T, Shitara A, Matsuda S, Matsuo T, Suiko M, Ohta K. Production, purification and properties of an endoinulinase of Penicillium sp. TN-88 that liberates inulotriose. J Ferment Bioeng. 1997;84:313–8.
http://dx.doi.org/10.1016/S0922-338X9789250-1
18. Sharma AD, Gill PK. Purification and characterisation of heat-stable exo-inulinase from Streptomyces sp. J Food Eng. 2007;79:1172–8.
http://dx.doi.org/10.1016/j.jfoodeng.2006.04.008
19. Ettalibi M, Baratti JC. Purification, properties and comparison of invertase, exoinulinases and endoinulinases of Aspergillus ficuum. Appl Microbiol Biotechnol. 1987;26:13–20.
http://dx.doi.org/10.1007/BF00282143
20. Gao W, Bao Y, Liu Y, Zhang X, Wang J, An L. Characterization of thermostable endoinulinase from a new strain Bacillus smithii T7. Appl Biochem Biotechnol. 2009;157: 498–506.
http://dx.doi.org/10.1007/s12010-008-8313-1
21. Chen H, Chen X, Li Y, Wang J, Jin Z, Xu X, et al. Purification and characterisation of exo- and endo-inulinase from Aspergillus ficuum JNSP5-06. Food Chem. 2009;115:1206–12.
http://dx.doi.org/10.1016/j.foodchem.2009.01.067
22. Chen X, Xu X, Jin Z, Chen H. Expression of an endoinulinase from Aspergillus ficuum JNSP5-06 in *Escherichia coli* and its characterization. Carbohydr Polym. 2012;88:748–53.
http://dx.doi.org/10.3109/1040841X.2012.694411
23. Pouyez J, Mayard A, Vandamme A, Roussel G, Wouters J, Housen I, et al. First crystal structure of an endo-inulinase, INU2, from Aspergillus ficuum: Discovery of an extra-pocket in the catalytic domain responsible for its endo-activity. Biochimie. 2012;94:2423–30.
http://dx.doi.org/10.1016/j.biochi.2012.06.020
24. Pandey A, Fontana JD, Krieger N, Selvakumar P, Soccol CR, Soccol VT. Recent developments in microbial inulinases: Its production, properties and industrial applications. Appl Biochem Biotechnol. 1999;81:35–52.
http://dx.doi.org/10.1385/ABAB:81:1:35
25. Sheng J, Chi ZM, Yan KR, Wang XJ, Gong F, Li J. Use of response surface methodology for optimizing process parameters for high inulinase production by the marine yeast Cryptococcus aureus G7a in solid-state fermentation and hydrolysis of inulin. Bioprocess Biosyst Eng. 2009;32:333–9.
http://dx.doi.org/10.1007/s00449-008-0252-2
26. Gong F, Chi M, Sheng J, Li J, Wang X. Purification and characterization of extracellular inulinase from a marine yeast Pichia guilliermondii and inulin hydrolysis by the purified inulinase. Biotechnol Bioprocess Eng. 2008;13:533–9.
http://dx.doi.org/10.1007/s12257-007-0177-7
27. Kwon HJ, Kim HY, Choi YJ. Cloning and characterisation of Pseudomonas mucidolens exoinulinase. J Microbiol Biotechnol. 2000;10:238–43.
28. Tsujimoto Y, Watanabe A, Nakano K, Watanabe K, K Matsui, K Tsuji. Gene cloning, expression and crystallization of a thermostable exoinulinase from Geobacillus stearothermophilus KP1289. Appl Microbiol Biotechnol. 2003;62:180–5.
http://dx.doi.org/10.1007/s00253-003-1261-3
29. Zhang S, Yang F, Wang Q, Hua Y, Zhao ZK. High-level secretory expression and characterization of the recombinant Kluyveromyces marxianus inulinase. Process Biochem. 2012;47: 151–5.
http://dx.doi.org/10.1016/j.procbio.2011.10.002
30. Li Y, Liu G, Wang K, Chi Z, Madzak C. Overexpression of the endo-inulinase gene from Arthrobacter sp. S37 in Yarrowia lipolytica and characterization of the recombinant endo-inulinase. J Mol Catal B: Enzyme. 2012;74:109–15.
http://dx.doi.org/10.1016/j.molcatb.2011.09.006
31. Kuzuwa S, Yokoi K, Kondo M, Kimoto H, Yamakawa A, Taketo A. Properties of the inulinase gene levH1 of Lactobacillus casei IAM 1045; cloning, mutational and biochemical characterization. Gene. 2012;495:154–62.
http://dx.doi.org/10.1016/j.gene.2011.12.004
32. Meenakshi S, Umayaparvathi S, Manivasagan P, Arumugam M, Balasubramanian T. Purification and characterization of inulinase from marine bacterium Bacillus cereus MU-31. Indian J Geo-Marine Sci. 2013;424:510–5.
33. Park JP, Bae JT, You DJ, Kim BW, Yun JW. Production of inulooligosaccharides from inulin by a novel endoinulinase from Xanthomonas sp. Biotechnol Lett. 1999;21:1043–46.
http://dx.doi.org/10.1023/A:1005632526442
34. Zhang L, Zhao C, Wang J, Ohta Y, Wang Y. Inhibition of glucose on an exoinulinase from Kluyveromyces marxianus expressed in Pichia pastoris. Process Biochem. 2005;40:1541–45.
http://dx.doi.org/10.1016/j.procbio.2004.01.057
35. Singh R, Sooch BS, Puri M. Optimisation of medium and process parameters for the production of inulinase from a newly isolated Kluyveromyces marxianus YS-1. Bioresour Technol. 2007;98:2518–22.
http://dx.doi.org/10.1007/s10295-007-0237-1
36. Takahashi N, Mizuno F, Takamori K. Purification and preliminary characterization of exo-β-D-fructosidase in Streptococcus salivarius KTA-19. Infect Immunol. 1985;47:271–6.
37. Looten P, Blanchet PD, Vandecasteele JP. The β-fructofuranosidase activities of a strain of Clostridium acetobutylicum grown on inulin. Appl Microbiol Biotechnol. 1987;25:419–25.
http://dx.doi.org/10.1007/BF00253311
38. Drent WJ, Gottschal JC. Fermentation of inulin by a new strain of Clostridium thermoautotrophicum isolated from Dahlia tubers. FEMS Microbiol Lett. 1991;78;285–92.
http://dx.doi.org/10.1111/j.1574-6968.1991.tb04457.x
39. Warchol M, Perrin S, Grill JP, Schneider F. Characterisation of a purified β-fructofuranosidase from Bifidobacterium infantus ATCC 15697. Lett. Appl Microbiol 2002;35:462–7.
http://dx.doi.org/10.1046/j.1472-765X.2002.01224.x
40. Liebl W, Brem D, Gotschlich A. Analysis of the gene for β-fructosidase invertase, inulinase of the hyperthermophilic bacterium Thermotoga maritima and characterization of the enzyme expressed in Escherichia coli. Appl Microbiol Biotechnol. 1998;50:55–64.
http://dx.doi.org/10.1007/s002530051256
41. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc. 1934;56:658–66.
http://dx.doi.org/10.1021/ja01318a036
42. Counotte GHM, Prins RA. Calculation of K_m and V_{max} from substrate concentration versus time plot. Appl Environ Microbiol. 1979;38:758–60.
43. Zhang T, Gong F, Chi Z, Sheng J, Li J, Wang X. Cloning and characterisation of the inulinase gene from a marine yeast

Pichia guilliermondii and its expression in Pichia pastoris. Anton Leeuw Int J G. 2009;95:13–22.
http://dx.doi.org/10.1007/s10482-008-9281-8

44. Beluche I, Guiraud JP, Galzy P. Inulinase activity of Debaromyces cantarelli, Folia Microbiol Praha. 1980;25:32–9.
http://dx.doi.org /10.1007/BF02876395

45. Guiraud JP, Viard-Gaudin C, Galzy P. Inulinase of Candida salmanticensis, Agric Biol Chem. 1980;44:1245–52.
http://dx.doi.org/10.1271/bbb1961.44.1245

46. Ohta K, Suetsugu N, Nakamura T. Purification and properties of an extracellular inulinase from Rhizopus sp. strain TN-96. J Biosci Bioeng. 2002;94:78–80.
http://dx.doi.org/10.1016/S1389-17230280120-7

47. Cho YJ, Yun JW. Purification and characterization of endoinulinase from Xanthomonas oryzae No 5, Process Biochem. 2002;5:1325–31.
http://dx.doi.org/10.1016/S0032-95920200018-3

48. Yun JW, Kim DH, Uhm TB, Song SK. Production of high-content inulo-oligosaccharides from inulin by a purified endoinulinase. Biotechnol Lett. 1997;19:935–8.
http://dx.doi.org/10.1023/A:1018366410586

49. Yun JW, Choi YJ, Song CH, Song SK. Microbial production of inulo-oligosaccharides by an endoinulinase from Pseudomonas sp. expressed in Escherichia coli. J Biosci Bioeng. 1999;81:291–5.
http://dx.doi.org/10.1016/S1389-17239980034-6