

**Purification and kinetic characterization of a
fructosyltransferase from *Aspergillus aculeatus***

Iraj Ghazi, Lucia Fernández-Arrojo, Humberto Garcia-Arellano, Manuel Ferrer,
Antonio Ballesteros and Francisco J. Plou *

*Departamento de Biocatalisis, Instituto de Catalisis y Petroleoquimica, CSIC, Cantoblanco,
28049 Madrid, Spain*

* Corresponding author. Tel.: +34-91-5854869; Fax: + 34-91-5854760. *E-mail*: fplou@icp.csic.es.

URL: <http://www.icp.csic.es/abg>

ABSTRACT

A fructosyltransferase present in Pectinex Ultra SP-L, a commercial enzyme preparation from *Aspergillus aculeatus*, was purified to 107-fold and further characterised. The enzyme was a dimeric glycoprotein (20% w/w carbohydrate content) with a molecular mass of around 135 kDa for the dimer. Optimal activity/stability was found in the pH range 5.0-7.0 and at 60°C. It was stable or slightly activated (up to 1.4-fold) in the presence of reducing agents such as dithiothreitol and 2-mercaptoethanol, and detergents such as sodium dodecylsulphate and Tween 80. The enzyme was able to transfer fructosyl groups from sucrose as donor producing the corresponding series of fructooligosaccharides: 1-kestose, nystose and fructosylnystose. Using sucrose as substrate, the k_{cat} and K_m values for transfructosylating activity were $1.62 \pm 0.09 \cdot 10^4 \text{ s}^{-1}$ and $0.53 \pm 0.05 \text{ M}$, whereas for hydrolytic activity the corresponding values were $775 \pm 25 \text{ s}^{-1}$ and $27 \pm 3 \text{ mM}$. At elevated sucrose concentrations, the fructosyltransferase from *A. aculeatus* showed a high transferase/hydrolase ratio that confers it a great potential for the industrial production of prebiotic fructooligosaccharides.

Keywords: *Aspergillus aculeatus*, fructosyltransferase, β -fructofuranosidase, fructooligosaccharides, transfructosidase.

1. INTRODUCTION

Fructooligosaccharides (FOSs) possess extraordinary importance as functional food ingredients owing to their prebiotic properties (Simmering and Blaut, 2001). Two classes of enzymes are particularly useful for FOSs production at industrial scale: fructosyltransferases (EC 2.4.1.9) and β -fructofuranosidases, also called invertases (EC 3.2.1.26). Fructosyltransferases possess a higher transferring activity than β -fructofuranosidases (Antosova and Polakovic, 2001). Fructosyl-transferring enzymes have been purified and characterised from higher plants such as asparagus (Shiomi, 1982), onion (Fujishima et al., 2005), Jerusalem artichoke (Koops and Jonker, 1994), and from different microorganisms (bacteria and fungi) such as *Aspergillus niger* (L'Hocine et al., 2000), *A. japonicus* (Hayashi et al., 1992), *Bacillus macerans* (Park et al., 2001), *Schwanniomyces occidentalis* (Klein et al., 1989) and *Candida utilis* (Chavez et al., 1997). Although these proteins differ in their subunit structure, molecular weight, degree of glycosylation, chemical susceptibility and substrate specificity, they all display both hydrolytic and transfer activities which limits the FOSs production to the use of high sucrose concentrations.

Pectinex Ultra SP-L (from Novozymes A/S) is a liquid commercial enzymatic preparation from *Aspergillus aculeatus* that is currently utilised as a pectinolytic and cellulolytic preparation in fruit juice processing (Okai and Gierschner, 1991). Recently, it has been described the immobilization on different polymers of a fructosyltransferase activity existing in Pectinex Ultra SP-L, using the crude extract, and its application for FOSs synthesis (Ghazi et al., 2005; Tanriseven and Aslan, 2005). Herein we report for the first time the purification and characterization of a fructosyltransferase from *Aspergillus aculeatus*, which showed a notable thermal and pH stability but, more importantly, a very significant transfer to hydrolysis activity ratio.

2. MATERIALS AND METHODS

2.1. Materials

Pectinex Ultra SP-L (batch no. KRN05409), a commercial enzyme preparation from *A. aculeatus*, was kindly donated by Novozymes A/S (Denmark). All chromatographic materials were from Amersham Biosciences. Molecular weight markers for native polyacrylamide gel (PAGE), PNGase F and Ampholite (pH 3-7) for isoelectric focusing were from Sigma-Aldrich. Sucrose, glucose and fructose were from Merck. 1-Kestose and nystose were from TCI Europe. Fructosylnystose was from Megazyme (Ireland). All other chemicals were of analytical grade and obtained from readily available commercial sources.

2.2. Enzyme purification

The purification of fructosyltransferase from *A. aculeatus* was performed at room temperature on a Fast Protein Liquid Chromatography System (FPLC, Model LCC-500, Pharmacia, Sweden). Pectinex Ultra SP-L (10 ml) was filtered through a membrane (0.45 μm), dialysed overnight at 4°C against 20 mM sodium phosphate buffer (pH 5.8) (buffer A), and purified as follows. The sample was applied to a DEAE-Sepharose Fast Flow column (1.6 x 3 cm), previously equilibrated with the same buffer. The column was washed with the equilibrium buffer (50 ml), then with buffer A containing 0.15 M NaCl (75 ml), and finally by a linear gradient of NaCl (total volume of 125 ml, 0.15 to 0.45 M) at a flow rate of 1.5 ml·min⁻¹. Fractions of 1.5 ml were collected and assayed for activity towards sucrose. Active fractions were pooled, desalted and concentrated by ultrafiltration on a Centricon YM-10 (Amicon, Millipore), to a total volume of 4.5 ml. The sample was further purified on a Mono-Q HR 5/5 column (Pharmacia) pre-equilibrated with buffer A. Bound proteins were eluted with buffer A containing 0.15 M NaCl (15 ml) and then applying a linear gradient of NaCl (0.15-0.50 M, 45 ml) at 0.5 ml·min⁻¹. Active fractions were pooled and concentrated by ultracentrifugation on a Centricon YM-10. The concentrated solution was loaded onto two Sephacryl S-100 columns put in series (1.0 x 115 cm; 1.0 x 28 cm) equilibrated with 20 mM sodium phosphate buffer (pH 5.8) containing 0.15 M

NaCl. Separation was performed at 0.3 ml·min⁻¹. The active fractions were combined and used for the characterization of the purified enzyme.

2.3. Enzyme characterization

2.3.1. Standard activity assay: The activity towards sucrose, 1-kestose, nystose, raffinose, methyl- α -D-glucoside, levan, palatinose, melibiose, stachyose, and leucrose was determined at 60°C in 0.2 M sodium acetate buffer (pH 5.5) by measuring the release of reducing sugars (glucose or fructose) from 100 g l⁻¹ substrate solutions using the dinitrosalicylic acid (DNS) method (Sumner and Howell, 1935). The assay was adapted to 96-well microplate as described in our previous work (Ghazi et al., 2005). One enzyme unit was defined as that liberating 1 μ mol of reducing sugar (glucose or fructose) per minute.

2.3.2. Determination of kinetic parameters: Kinetic parameters (K_m and k_{cat}) were determined at 60°C measuring initial reaction rates. Sucrose (0.035–1.750 M, 12–600 g l⁻¹) and enzyme were incubated 60 min in 0.2 M sodium acetate buffer (pH 5.5). The reactions were terminated by heating the tubes in boiling water for 20 min and the reactions were analysed by HPLC to determine the amount of fructose and glucose liberated (Ghazi et al., 2005). The amount of free fructose is a direct measurement of hydrolytic activity whereas the amount of glucose minus the amount of free fructose corresponds to the transferase activity (van Hijum et al., 2004). Accordingly, one unit of hydrolytic activity was defined as that catalysing the release of 1 μ mol fructose per minute, whereas one unit of transferase activity was defined as that catalysing the transfer of 1 μ mol of fructose per minute. Kinetic parameters were calculated fitting the initial rate values to the Hill transformation of the Michaelis-Menten equation $V = V_{max} [S]^h (K_m^h + [S]^h)^{-1}$, where h is the Hill coefficient.

2.3.3. FOSs production: The purified fructosyltransferase was used for batch production of FOSs. The assay was carried out using 600 g l⁻¹ sucrose at 60°C in 0.2 M sodium acetate buffer, pH 5.5, and 10 U/ml pure enzyme (measured in the standard assay). Aliquots were taken at intervals, the enzyme inactivated by heating 20 min in boiling water, and then analysed by HPLC as previously described (Ghazi et al., 2005).

2.3.4. pH and temperature optima: The activity:pH and activity:temperature relationships were determined by the standard activity assay incubating the purified enzyme with sucrose, at different pH values and constant temperature (50°C), and at different temperatures (40-70°C) and constant pH (5.5), respectively. The assay was performed at 600 g l⁻¹ sucrose to mimic FOSs production conditions. The buffers (0.2 M) used were: citrate (pH 3.5 and 4.0), acetate (pH 4.0-6.0), phosphate (pH 6.0-7.0), HEPES (pH 7.0-8.0), and Tris-HCl (pH 8.0-9.0).

2.3.5. pH and thermal stability: The pH and thermal stability was assayed incubating the enzyme at pH and temperatures in the range 3.5-9.0 and 35-75°C, respectively. Incubation was carried out in the presence of 600 g l⁻¹ α-D-methyl-glucopyranoside. Aliquots (100 μl) were taken at intervals up to 24 h and the remaining activity was measured using the standard activity assay with sucrose.

2.3.6. Chemical stability: Chemical susceptibility was assessed incubating 60 min the enzyme with cations added as chloride salts (1 mM), detergents (0-0.5% w/v), reducing agents (10 mM) and inhibitors (10 mM) in 0.2 M sodium acetate buffer, pH 5.5. Upon incubation, an aliquot was withdrawn, chilled on ice, and hydrolytic activity was monitored by the standard assay with sucrose. Residual activity was expressed as the percent referred to the control value obtained without addition of chemical.

2.3.7. Deglycosylation assay: Deglycosylation of the enzyme was carried out with peptide-N-glycosidase F (PNGase F), according to the recommendations of the supplier. The glycosylation degree was calculated from the ratio of the determined molecular weights of the native and deglycosylated proteins (assessed by denaturing polyacrylamide gel electrophoresis).

2.4. Other methods

Polyacrylamide gel electrophoresis under denaturing (SDS-PAGE: 10% v/v) and native (8% v/v) conditions was performed according to Laemmli using a Mini-PROTEAN cell apparatus (Bio-Rad). On-gel activity assay for the native gel was performed according to Mukasa et al. (1982) method for β-fructofuranosidase activity. Isoelectric focusing (*pI* range 3-7) was carried out on a 4% (v/v) polyacrylamide slab gel under denaturing conditions. Bands were visualised by silver staining. The *pI* of the enzyme was estimated by comparison of its mobility to that of a Bio-Rad *pI* calibration

kit (pH 3.6-6.6) included on the gel. The protein concentration was determined according to Bradford method with BSA as the standard. For N-terminal amino acid sequencing, the purified enzyme was subjected to SDS-PAGE, and protein band were blotted to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was stained with Coomassie Brilliant Blue R-250, after which the bands of the enzyme were cut out and processed for N-terminal amino acid sequence using a precise sequencer.

3. RESULTS AND DISCUSSION

3.1. Pectinex Ultra SP-L yields a new fructosyltransferase

The existence of fructosyltransferase activity in the commercial Pectinex Ultra SP-L from *A. aculeatus* has been reported by several authors (Hang and Woodams, 1995; Tanriseven and Gokmen, 1999, Ghazi et al., 2005). In the present work, the enzyme has been purified for the first time, and its biochemical properties investigated. Homogeneous protein was isolated under conditions described in Experimental section, yielding 0.6 mg of pure enzyme per 170 mg total protein (0.4% w/w). The enzyme was purified 107-fold from the crude Pectinex and the activity recovery was about 37%. The N-terminal amino-acid sequence of the purified enzyme was determined to be LDTTAPPXFXLSTLPXXXLF using Edman degradation in a gas phase sequencer. This amino acid sequence showed a significant homology with the N-terminal of β -fructofuranosidase from *A. niger* ATCC 20611 (Yanai et al., 2001) and with an invertase from *A. niger* B60 (Boddy et al., 1993).

Its relative electrophoretic mobility on denaturing and non-denaturing polyacrylamide gels indicates that it has a dimeric structure with an estimated molecular weight of 65 kDa per monomer (Fig. 1). This was confirmed by gel-filtration chromatography on Sephacryl S-100 using standard proteins. Fructosyltransferase purified from Pectinex Ultra SP-L was found to be a glycoprotein. The glycosylation degree of the purified enzyme was deduced from the ratio of the experimentally determined molecular weights of the native protein (assessed by SDS-PAGE: 65 kDa) and the subunit molecular weight of the deglycosylated form obtained after treatment with peptide-N-glycosidase F (PNGase F) (assessed by SDS-PAGE: 52 kDa). This suggests that the enzyme possesses about 20% (w/w) carbohydrate content in the enzyme subunit. The isoelectric point of the enzyme was estimated to be in the range 3.8-4.2 on the basis of relative mobility on a 4% polyacrylamide gel under denaturing conditions.

3.2. General properties of fructosyltransferase from A. aculeatus

3.2.1. Substrate specificity: The substrate specificity of the fructosyltransferase from *A. aculeatus* was estimated using a variety of di-, tri- and tetrasaccharides following the standard assay. The pure enzyme displayed the preference: sucrose > raffinose > 1-kestose > nystose (in a ratio 100:24:10:6).

Other carbohydrates such as turanose, cellobiose, melibiose, leucrose, methyl- α -D-glucopyranoside and stachyose were not substrates of this enzyme. Thus, the enzyme only recognized sugars containing a β -(2-1)-linked fructose in their chemical structure. The trisaccharide raffinose can be considered as a sucrose molecule substituted at the C-6 hydroxyl group, whereas 1-kestose and nystose can be considered as different C-1' hydroxyl-fructosylated sucroses. The specific activity diminished with longer oligosaccharides, being negligible with the sucrose-containing tetrasaccharides stachyose, which suggests limitations for binding of large molecules.

3.2.2. Effect of pH and temperature: The optimum pH of the fructosyltransferase measured with sucrose as substrate ranged from 5.0 to 7.0 (Fig. 2A). No significant activity was found at pH values below 3.5 and above 9.5. The pH stability was assayed in the presence of high concentration of a carbohydrate, to mimic the operational conditions of FOSs synthesis. Thus, we added methyl- α -D-glucopyranoside ($600 \text{ g}\cdot\text{l}^{-1}$), a non-reducing sugar that do not interfere with the standard activity assay and is not a substrate of this enzyme. As shown in Fig. 2B, the enzyme retained >90% of its initial activity after 24 h incubation at neutral and moderately acid pH values (from 4.5 to 7.5). However, it retained only 55% of the initial activity at pH 4.0. In the absence of methyl- α -D-glucopyranoside, the stability was significantly lower, recovering less than 50% of the initial activity in 24 h in the pH range 4.5-7.5. The optimum temperature was in the range 50-70°C (Fig. 3A). The enzyme was significantly stable ($t_{1/2} > 24 \text{ h}$) up to 60°C (Fig. 3B). Temperatures greater than 65°C inactivated the enzyme.

3.2.3. Influence of chemicals: Using sucrose as substrate, purified enzyme displayed activity without addition of any metal ions. However, some differences in susceptibility to mono- and divalent cations were observed. For example, Mn^{2+} , K^{+} and Co^{2+} ions elicited a 1.4-1.9 fold increase in the activity of the enzyme, whereas it showed 36-61% inhibition at low concentrations of Hg^{2+} and Zn^{2+} . The addition of ethylenediaminetetraacetic acid (EDTA) did not result in a decrease in activity, indicating that fructosyltransferase was independent of divalent cations. We further tested the effect of various detergents, i.e. sodium dodecylsulphate (SDS), sodium deoxycholate, Triton X-100 and Tween 80, and reducing agents such as dithiothreitol (DTT) and β -mercaptoethanol. We found that the enzyme was

slightly activated by SDS (1.5-fold at 10 mM), sodium deoxycholate (1.4-fold at 1 mM) and Triton X-100 (1.4-fold at 5% w/v). Moreover, it was resistant to low concentrations (1-10 mM) of DTT and β -mercaptoethanol.

3.3. Kinetic behaviour of fructosyltransferase from *A. aculeatus*

In the presence of sucrose, most of the fructosyltransferases and β -fructofuranosidases (invertases) catalyse both sucrose hydrolysis and FOSs synthesis. The transferase/hydrolase ratio, and therefore the maximum yield of FOSs, depends basically on two parameters: the concentration of sucrose and the intrinsic enzyme properties, i.e. its ability to bind the nucleophile (to which a fructose is transferred) and to exclude H₂O (Ballesteros et al., 2006).

In order to investigate in more detail the behaviour of fructosyltransferase from *A. aculeatus*, we determined the kinetic constants (K_m and k_{cat}) for both hydrolysis and transfer reactions. The reaction rates ($\mu\text{mol mg}^{-1} \text{min}^{-1}$) vs. sucrose concentration up to 1.75 M were plotted (Fig. 4). To discriminate between hydrolytic and transferase activities, reactions were analysed by HPLC. The hydrolytic and transfructosylating initial rates were calculated from the amount of free and transferred fructose, respectively, as described in the experimental section. It is worth noting that most of kinetic studies reported in the literature do not separate both activities. In our work, transferase and hydrolysis profiles did not fit well with Michaelis-Menten kinetics but the adjustment improved substantially when applying the Hill equation. This has been also observed with other fructosyl-transferring enzymes (van Hijum et al., 2004).

The K_m and k_{cat} values for transferase and hydrolytic activities are summarised in Table 2. Regarding the hydrolytic reaction, the K_m value (27 mM) is in the low border of the range reported for β -fructofuranosidases with a low fructosyl-transfer activity [e.g. 26 mM for *S. cerevisiae* invertase (Reddy and Maley, 1996) or 227 mM for *Rhodotorula glutinis* invertase (Rubio et al., 2006)]. However, the k_{cat} for hydrolysis (775 s^{-1}) is lower than those previously described for well-known invertases [e.g. 9430 s^{-1} for *S. cerevisiae* invertase (Reddy and Maley, 1996)]. The K_m for the transfer reaction (535 mM) is higher than those reported for enzymes having fructosyl-transfer activity [e.g.

290 mM for *Aspergillus niger* fructosyltransferase (Hirayama et al., 1989)]. The main feature of fructosyltransferase from *A. aculeatus* is its significantly high k_{cat} value ($1.62 \cdot 10^4 \text{ s}^{-1}$) of transfer reaction. This implies that at high sucrose concentrations (above 1 M, 342 g/l) the rate of transfer is approx. 20-fold that of hydrolysis, which explains the low concentration of free fructose that is observed with this enzyme when assayed under FOSs synthesis conditions.

The pure fructosyltransferase from *A. aculeatus* was assayed for the production of FOSs. A sucrose solution (600 g l^{-1}) containing pure enzyme (5 U ml^{-1}) was incubated at 60°C for 24 h and then analysed by HPLC. The analysis presented a FOSs production of 60.7% (w/w), referred to total carbohydrates in the reaction mixture. In particular, 18.7% (w/w) was 1-kestose, 35.6 % (w/w) nystose and 6.4% (w/w) fructosylnystose. The rest of carbohydrates in the mixture were fructose (minoritary), glucose and remaining sucrose. These values compared well with those reported using the crude Pectinex Ultra SP-L under similar experimental conditions (Ghazi et al., 2005 and 2006). In conclusion, the fructosyltransferase mined from the commercial preparation Pectinex Ultra SP-L has a broad pH, thermal and chemical stability and a higher transfructosylating capacity compared with related enzymes, which are very advantageous for FOSs synthesis. Further studies are required to draw mechanistic conclusions on the nature of kinetics observed with this fructosyltransferase.

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Table 1. Purification of fructosyltransferase present in Pectinex Ultra SP-L.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude Pectinex	168.1	4120	24.5	100	1.0
Dialysis	127.9	3810	29.8	92.5	1.2
DEAE-Sepharose	31.2	3080	98.7	74.8	4.0
Mono-Q HR	2.4	2144	912	52.0	37.2
Sephacryl S-100	0.6	1518	2635	36.8	107

Table 2. Kinetic parameters of fructosyltransferase from *Aspergillus aculeatus* calculated using the Hill transformation of Michaelis-Menten equation.

Reaction	K_m (mM)	k_{cat}^a (s⁻¹)	h	k_{cat}/K_m (mM⁻¹ · s⁻¹)
Hydrolysis	27 ± 3	775 ± 25	2.3 ± 0.8	29 ± 4
Fructosyl-transfer	535 ± 45	1.62 ± 0.09 · 10 ⁴	1.9 ± 0.1	30 ± 4

^a Calculated estimating a molecular mass of 135 kDa for the active enzyme.

Figure legends

Fig. 1. SDS-PAGE for all steps of purification of fructosyltransferase from *A. aculeatus*. Lane 1, crude Pectinex Ultra SP-L; lane 2, dialysed Pectinex; lane 3, DEAE-Sepharose Fast Flow; lane 4, Mono-Q HR; lane 5, Sephacryl S-100; lane 6, molecular weight markers. The gel was silver stained.

Fig. 2. (A) pH-dependency activity curve and (B) pH-stability curve –remaining activity after 24 h incubation- of fructosyltransferase from *A. aculeatus*. Conditions described in experimental section.

Fig. 3. (A) Temperature-dependency activity curve and (B) thermostability curve –remaining activity after 24 h incubation- of fructosyltransferase from *A. aculeatus*. Conditions described in experimental section.

Fig. 4. Kinetic plots (initial rate versus sucrose concentration) of fructosyltransferase from *A. aculeatus*. Hydrolytic activity (○); Transfer activity (●). Reactions were carried out in 0.2 M sodium acetate buffer (pH 5.5) at 60°C.

Fig. 1

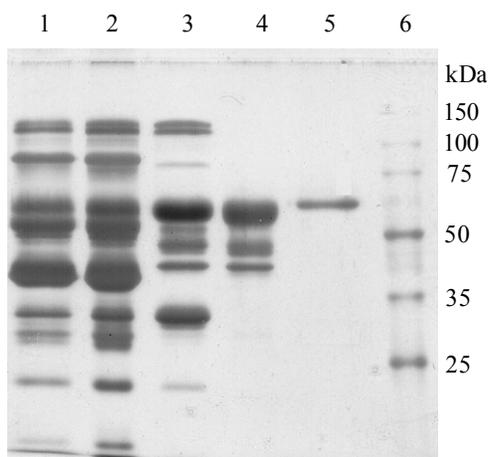


Fig. 2

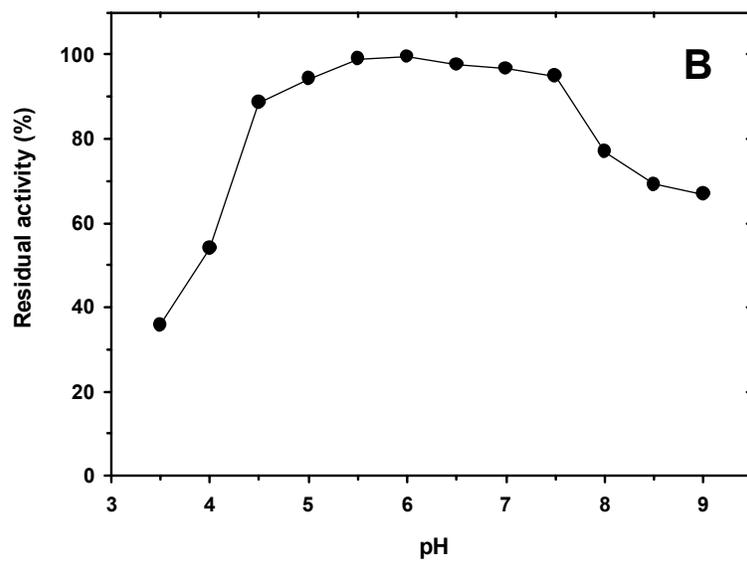
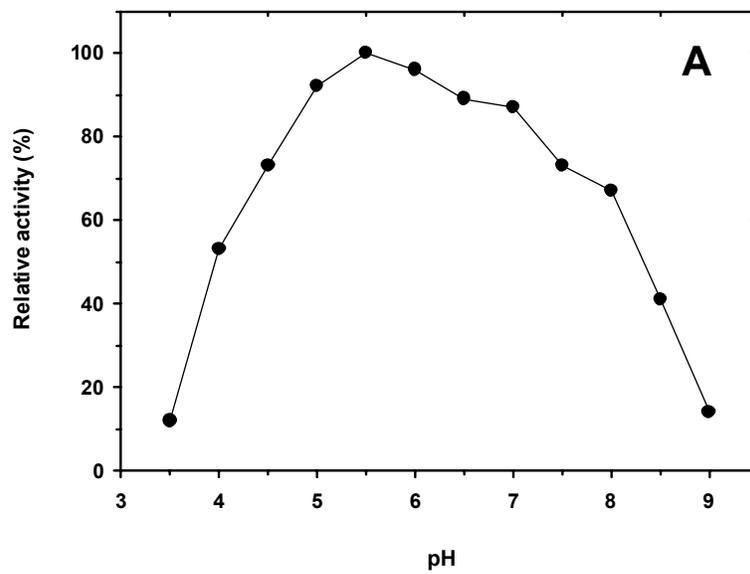


Fig. 3

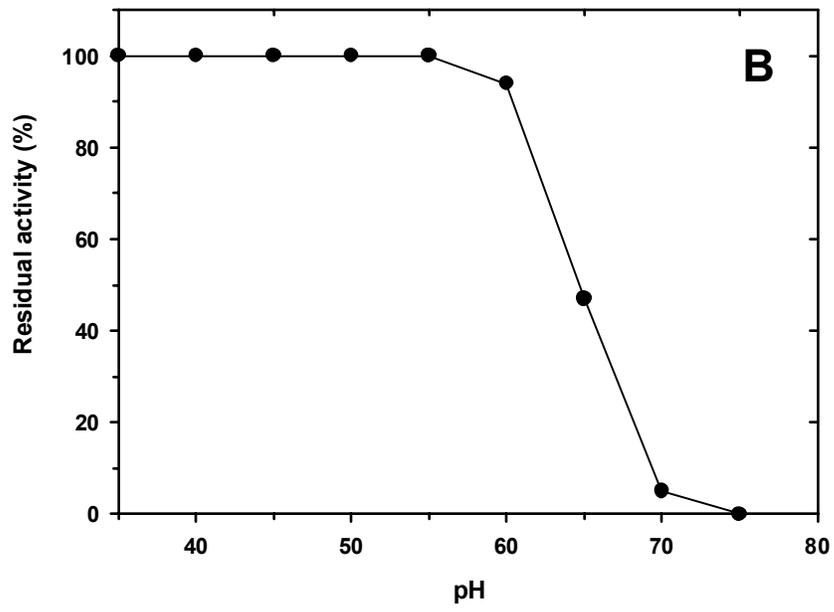
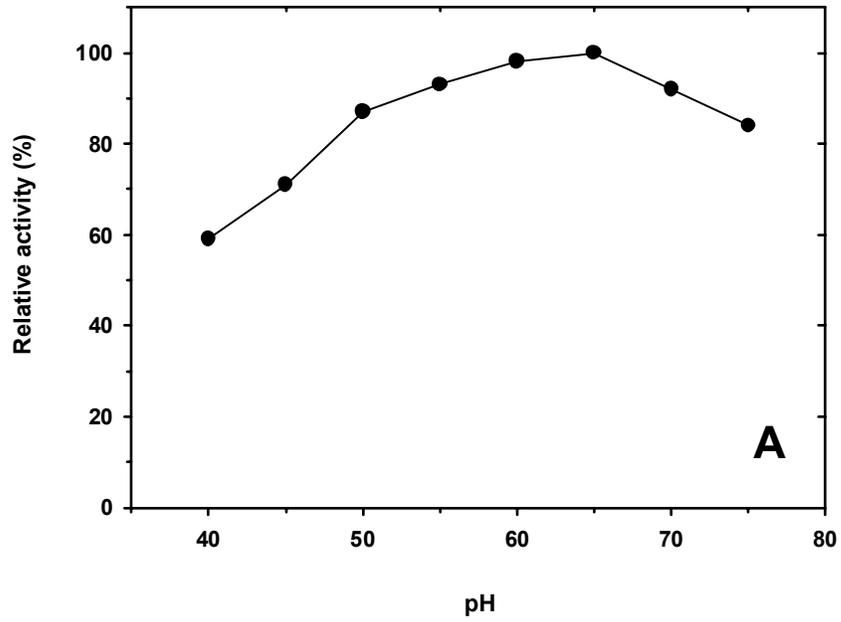


Fig. 4

