Protein Expression and Purification 147 (2018) 29–37

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

High level production of a recombinant acid stable exoinulinase from *Aspergillus kawachii*

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ARTICLE INFO

Keywords:
Exoinulinase
Aspergillus kawachii
Pichia pastoris
Fructosyltransferase

ABSTRACT

Exoinulinases—enzymes extensively studied in recent decades because of their industrial applications—need to be produced in suitable quantities in order to meet production demands. We describe here the production of an acid-stable recombinant inulinase from *Aspergillus kawachii* in the *Pichia pastoris* system and the recombinant enzyme's biochemical characteristics and potential application to industrial processes. After an appropriate cloning strategy, this genetically engineered inulinase was successfully overproduced in fed-batch fermentations, reaching up to 840 U/ml after a 72-h cultivation. The protein, purified to homogeneity by chromatographic techniques, was obtained at a 42% yield. The following biochemical characteristics were determined: the enzyme had an optimal pH of 3, was stable for at least 3 h at 55 °C, and was inhibited in catalytic activity almost completely by Hg^{+2}. The respective K_m and V_{max} for the recombinant inulinase with inulin as substrate were 1.35 mM and 2673 μmol/min/mg. The recombinant enzyme is an exoinulinase but also possesses synthetic activity (*i. e.*, fructosyl transferase). The high level of production of this recombinant plus its relevant biochemical properties would argue that the process presented here is a possible recourse for industrial applications in carbohydrate processing.

1. Introduction

Inulinases—as the name suggests—are enzymes that degrade inulin, a member of the fructans family and a plant energy-storage polymer composed of variable-length linear fructose chains ending in linkage to a β-2-glucose residue [1,2]. According to their mode of catalysis, the inulinases are classified into two types: the endoinulinases that hydrolyze internal 1,2-fructofuranosidic bonds and generate short fructololigosaccharides and the exoinulinases that hydrolyze the polymer from the terminus and release fructose units [3]. Microbial inulinases have many applications in industrial processes such as the production high-fructose syrup via complete inulin hydrolysis, or the synthesis of fructololigosaccharides through the use of highly concentrated solutions of sucrose [4,5]. Fructose is a sweetener of the Food and Drug Administration category Generally Recognized as Safe (GRAS), thus considered as a healthy alternative to sucrose since the monomer possesses a greater sweetening capacity and is furthermore beneficial to diabetic patients [6]. Industrial conditions for the chemical hydrolysis of inulin (*i. e.*, high temperatures, 80–100 °C and acid pHs between 1 and 2), result in product degradation and certain by-products are obtained, such as difructose anhydride, that change the properties of the final product to a dark brown solution with a lower sweetening capability [7]. Nevertheless, fructololigosaccharides have gained attention as functional-food components because of the associated bifidogenic and health-promoting properties [8,9].

Many microorganisms are reported to produce inulinases such as bacteria, yeast, and other fungi. The filamentous fungus *Aspergillus kawachii*, traditionally used in the Japanese alcoholic-beverage industry, produces several enzymes with acid-depolymerase activities such as amylases, xylanases, and proteinases [10–12]. In our laboratory, we found that *A. kawachii* produced, among other enzymes, an exoinulinase with promising properties for a potential use in the food industry [13].

The production of enzymes with potential industrial application through the use of wild microorganisms such as bacteria, yeast, or other fungi is sometimes not feasible because of low productivity levels or the costs associated with a large-scale purification. These considerations indicate the relevance of producing those classes of enzyme in

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https://doi.org/10.1016/j.pep.2018.02.007
Received 12 November 2017; Received in revised form 1 February 2018; Accepted 14 February 2018
Available online 15 February 2018
1046-5928/

heterologous systems, such as *Saccharomyces cerevisiae* and *Pichia pastoris,* in a process having a potential industrial application [14,15].

Pichia pastoris is a eukaryotic microorganism widely used as a heterologous-expression system. In addition to the yeast's productive advantages, such as high culture densities and yields of the recombinant enzyme, an efficient secretion system would point to that microorganism as being an appropriate system to overexpress proteins [16,17].

Although several reports are available about specific aspects of the cloning and overexpression of these kinds of enzymes, we present here the whole procedure—*i. e*, from the initial cloning to the final application of this enzyme to industrial processes—with detailed information about the genetic engineering involved. The specific system under study was the production of a recombinant exoinulinase gene from *A. kawachii* that became overexpressed in *P. pastoris* and possessed a potential for industrial applications. We describe here the successful production, purification, and characterization of the recombinant enzyme and discuss those possible industrial uses.

2. Materials and methods

2.1. Chemicals

Inulin was obtained from Sigma-Aldrich (St Louis, MO, USA); Promega (Madison, WI, USA) provided the enzymes used—namely, the restriction endonucleases (*Xho*I, *Not*I, *Bgl*II, *Avr*II), Taq DNA polymerase, calf intestinal alkaline phosphatase, T4 DNA ligase—along with the cloning and expression systems such as the pGEM™-T Easy Vector, the Wizard SV Gel, and the clean-up kit for the polymerase-chain reaction (PCR). The IllustraTM MicroSpin columns and DNA-purification kit were from GE Healthcare (Fairfield, CT, USA) and the *BsrD*I restriction endonuclease from New England Biolabs (Beverly, MA, USA). Each of these products was used as indicated by the manufacturer. All the other reagents used were of analytical grade.

2.2. Strains, plasmids, and culture conditions

Aspergillus kawachii IFO 4308 strain was kindly provided by the Institute for Fermentation in Osaka, Japan and used as the source of the DNA.

The microbial strains (*Escherichia coli* TOP10F′, *P. pastoris* GS115 and X33) and the plasmids (pPIC9 and pPICZαA) were acquired from Invitrogen Co. Ltd., (Carlsbad, CA, USA).

For the growth of *A. kawachii* cells, the culture medium previously described by Contreras Esquivel and Voget [18], and in the present work containing 10 g/l of inulin as the carbon and energy source, was inoculated with 10^6 spores/ml of the fungus. The cultures were then incubated at 28 °C and 200 rpm for 48 h before harvesting.

Recombinant *E. coli* TOP10F′ cells were grown in Luria-Bertani medium with 0.1 mg/ml of ampicillin or 0.025 mg/ml Zeocin™ when necessary.

Pichia pastoris GS115 and X-33 cells were cultured in medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose (YPD) and supplemented with 0.1 mg/ml of Zeocin™. As suggested by the manufacturer for *P. pastoris* proliferation, the buffered–glycerol-complex medium BMGY was used; whereas, for the induction of the alcohol-oxidase promoter AOX, the buffered–methanol-complex medium BMMY was used. The composition of BMGY was 20 g/l peptone, 13.4 g/l yeast-nitrogen base, 10 g/l glycerol, 10 g/l yeast extract, and 0.4 g/l biotin. The BMMY medium composition was equivalent to that of BMGY but with the glycerol being replaced by 5 g/l methanol. *Pichia pastoris* X-33 transformants were grown on minimal dextrose medium containing 20 g/l agar, 20 g/l dextrose, 13.4 g/l yeast-nitrogen base, and 0.4 mg/l. For cultures performed in the bioreactor (as batch and fed-batch fermentation) basal salt medium BSM (40 g/l glycerol, 6.40 g/l K_2SO_4, 1.80 g/l KOH, 3.40 g/l $MgSO_4.7H_2O$, 0.36 g/l $CaSO_4.2H_2O$, 12 ml/l H_3PO_4) was used supplemented with the *P. pastoris*–growth-promoting salt solution PTM1 (4 ml/l) plus 8 ml/l of a D-biotin solution (0.20 g/l). PTM1 contained 0.5 g/l $CoCl_2.6H_2O$, 2.0 g/l $CuSO_4$, 22.0 g/l $FeSO_4.7H_2O$, 5 ml H_2SO_4, 0.02 g/l H_3BO_3, 0.08 g/l KI, 3.0 g/l $MnSO_4$, 0.2 g/l Na_2MoO_4, 7.0 g/l and $ZnCl_2$.

2.3. Genomic-DNA extraction and the PCR for the amplification of the inulinase gene

The extraction of genomic DNA from *A. kawachii* was performed following a previously described method [19].

The design of proper primers for cloning the *inuAk* gene was based on previous information on the N-terminal-amino-acid sequence of the *Aspergillus awamori* inulinase (fw primer) and on a consensus C-terminal-amino-acid sequence of related enzymes; *i. e.*, the A*spergillus foetidus* fructosyltransferase and the inulinases A and B from *Aspergillus niger* [rv primer] [20].

Genomic DNA was amplified by PCR with the upstream primer P1: 5-TAGTATCT*CTCGAG*AAGAGAGCTCCTCTGTCGAAG-3 (with an added *Xho*I site indicated in italics) and the downstream primer P2: 5-TACT CT*CCTAGG*TCAATTCCACGTCGAAG-3 (with the sequence in italics being the position of an *Avr*II site). The amplification was carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany) with Taq DNA polymerase under the following conditions: 95 °C for 180 s, 30 cycles at 95 °C for 60 s, 56 °C for 30 s, and 72 °C for 60 s, with a consolidation step at 72 °C for 300 s. The PCR product was purified by the DNA-purification kit containing Illustra™ MicroSpin Columns.

Both the amplicon-containing the inulinase gene and the empty pPic9 vector were digested with *Xho*I plus *Avr*II. Calf-intestine alkaline phosphatase was used to dephosphorylate the pPIC9 plasmid so as to prevent self-ligation. The inulinase gene was ligated to pPIC9 with T4 DNA ligase to obtain the recombinant vector pPIC9:*inulinase*. The correct sequence of the open-reading frame (ORF) was confirmed by sequence analysis (Macrogen Inc., Gasan-dong, Seoul, 153-023, Republic of Korea).

2.4. Intron and signal-peptide excision from the inuAk gene

Information in the literature on the inulinase genes was used to design the cloning strategy. We found that the inulinase gene from *A. awamori* was composed of two exons and a 57-bp intron [21]. We then confirmed this information bioinformatically *in silico* and compared the structure with that of the *A. kawachii* genes. From this analysis, we also noticed a possible signal peptide in the sequence. To construct the posttrancriptional region of the *A. kawachii* gene after splicing, both the signal peptide and the intron were excised through the use of PCR and enzymatic restriction.

In order to obtain exon I, a PCR reaction was performed by means of the plasmid vector pPIC9:*inulinase* as a template and P3 and P4 as primers. Primer P3 5′-TGCAGTCG*CTCGAG*AAGAGATTCAACTATGACC AGCCT-3′, with the sequence in italics being a *Xho*I restriction site—was designed to remove the signal peptide. Primer P4 5′-ACGCG*G CAATG*TTTGCGCAACGGGGTAATAGGAAGTATACATGGCG-3′ was designed to span the intron, with the first 19 nucleotides overlapping the 3′end of exon I and the following 16 nucleotides overlapping the 5′ end of exon II, including the recognition site of *BsrD*I (in italics). The reaction was performed with 30 cycles at 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 60 s. The PCR product (383 bp) was gel-purified through the use of a DNA-purification kit Illustra™ MicroSpin Columns, ligated to pGEM™–T Easy Vector and transformed into *E. coli* TOP10F'. The recombinant vectors isolated from the positive transformants were digested with *Xho*I and *BsrD*I.

In order to obtain the second exon, the pPic9:*inulinase* construction was digested with *BsrD*I and *Not*I to release exon II, and that DNA fragment of 1200 bp was then purified by agarose-gel electrophoresis.

Finally, both exons were ligated to pPicZαA (previously digested by

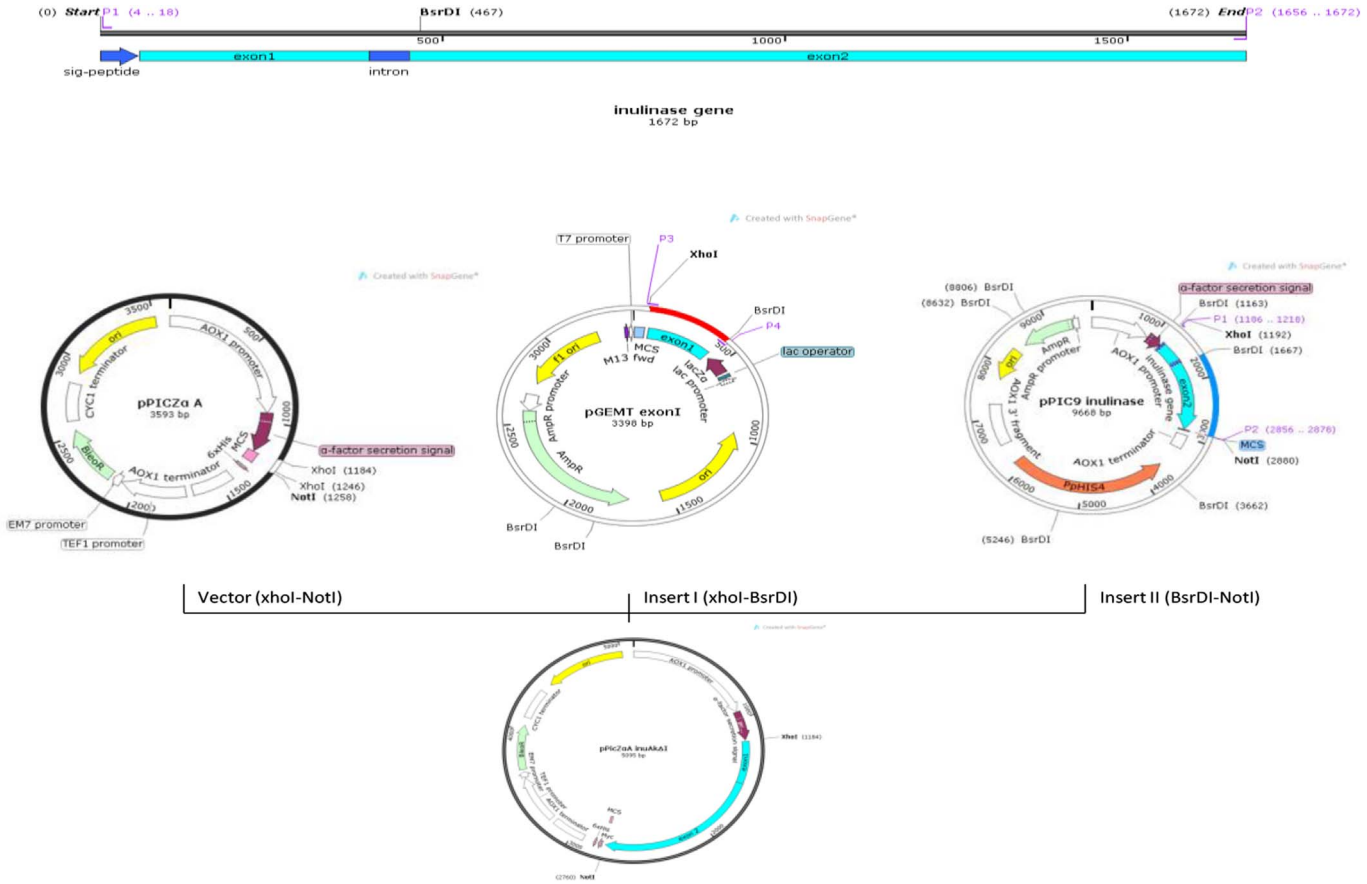

Fig. 1. Cloning strategy of the inulinase gene. Through the use of the construction pPIC9:*inulinase* as a template for PCR with P3 and P4 as the primers, a fragment of 383 bp (exon I) was obtained, purified from the PCR-product mixture, and finally cloned in pGEMT-easy vector. Next, the pPIC9:*inulinase* was digested with *Bsr*DI and *Not*I and a 1200-bp DNA fragment purified on an agarose gel (exon II) after electrophoresis. Both fragments (exons I and II) were ligated to pPICZαA to generate the construction pPICZαa:*inuAkΔI*.

*Xho*I and *Not*I) to generate the pPicZαA:*inuAkΔI* construction. After sequencing a region of the construction to confirm the ORF, this vector was transformed into *E. coli* TOP10F′ for amplification. After a control of the correctness of the sequence, the expression vector was linearized with *Bgl*II. The linear DNA carrying the *inuAkΔI* gene was used to transform *P. pastoris* cells for gene expression. Fig. 1 summarizes this cloning strategy.

2.5. Transformation to P. pastoris GS115 and X33

The recombinant plasmid pPicZαA:*inuAkΔI* was transformed into *P. pastoris* X-33 and GS115 cells by electroporation (MicroPulser BioRad Life Sciences, Richmond, CA). Controls were performed through transformation of *P. pastoris* X-33 and GS115 with the empty vector pPICZαA. After the selection of transformants on YPD plates containing 0.1 mg/ml Zeocin™, the presence of the exoinulinase gene was confirmed by colony PCR with the P1 and P2 primers [22].

2.6. Clone selection and production of the recombinant inulinase in P. pastoris X-33 and GS115

Clones carrying the recombinant plasmid were analyzed for enzyme expression (13 clones of X-33 *P. pastoris* and 13 clones of GS115 *P. pastoris* cells). Positive clones (confirmed by colony PCR) were grown in batch cultures at 28 °C and 200 rpm in 1-L Erlenmeyer flasks containing 100 ml of BMGY medium. Once the biomass reached an OD_{600} = 8–10 (approximately 16–18 h), the cells were pelleted by centrifugation at 3000 × *g* and 4 °C for 5 min and then resuspended in 15 ml of BMMY medium. In order to increase the recombinant-protein production, pure methanol was added to a final concentration of 1% (v/v) during the induction phase. Samples of 1 ml were withdrawn at defined intervals (0, 24, and 48 h), centrifuged at maximum speed for 2 min at 4 °C and the supernatants stored at − 20 °C until use in the analysis of expression levels through measurement of inulinase activity.

2.7. High-cell-density fermentation

Production cultures were performed in a 5-L bioreactor (New Brunswick Scientific, Edison, NJ, USA) with an initial working volume of 3 L of BSM medium supplemented by 4 ml/l of the PTM1 trace-salts solution. The fermentation was carried out at 30 °C and pH 5. The pH was maintained by adding 100% (v/v) ammonium hydroxide (also serving as a nitrogen source for the cultures). The level of dissolved-oxygen tension was maintained at 30% of the oxygen-saturation concentration during the complete process by a cascaded control of the agitation rate. The first step of this procedure was a batch culture with 4% (v/v) glycerol as the carbon and energy source. Upon reaching a total consumption of glycerol, a sudden increase in dissolved-oxygen tension occurred. Next, a 43% (v/v) glycerol solution was added to amplify the cell biomass at a feeding rate of 35 ml/h. At the conclusion of the glycerol feeding, an induction phase was initiated by the addition of methanol at an input rate of 35 ml/h for 26 h.

The outlet-gas composition was measured with a paramagnetic O_2 detector and an infrared CO_2 detector (Servomex 1100, Norwood, MA, USA). The rates of O_2 uptake and CO_2 production were estimated by the method suggested by Cooney [23].

2.8. Enzymatic-activity assays

The inulinase and invertase activities were determined as described

by Gill [24] with inulin or sucrose, respectively, in 50 mM citric acid/25 mM Na_2HPO_4 buffer (CPB), pH 5. The admixture containing 20 µl of an appropriate dilution of enzyme and 180 µl of the respective substrate was incubated at 37 °C for 10 min. The reducing sugars released were determined by the Somogyi-Nelson method with fructose as a standard. One unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugars per minute under the assay conditions [13].

The protein concentration was determined colorimetrically by the method of Bradford [25].

2.9. Purification of the recombinant inulinase

The supernatant of the culture of *P. pastoris* (after centrifugation at 10,000 × g for 30 min at 4 °C) was diluted with five volumes of 20 mM citric-acid buffer pH 3.0 and then loaded onto a SP Sepharose Fast-Flow column (XK 16/20) equilibrated with the same buffer. A linear gradient of NaCl (0–0.5 M, 5 ml/min) was used to elute the proteins, and the inulinase-positive fractions collected were chromatographed on a Sephacryl G25 (XK 16/80) column in 20 mM sodium-acetate buffer pH 5.5. The next purification step involved Q-Sepharose–high-performance (XK 16/20) chromatography; and elution with 20 mM sodium acetate buffer pH 5.5 through the use of a continuous NaCl gradient (0–0.5 M at 2 ml/min). Finally, the fractions containing inulinase activity were pooled and then dialyzed at 4 °C overnight. The purity of the inulinase thus obtained was analyzed by sodium-dodecyl-sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE). The purified enzyme was stored at −20 °C and used for further biochemical-characterization studies. All the purification steps were performed in an Amersham FPLC-U900 system (GE, Fairfield, CT, USA).

2.10. Zymogram

The purified protein was analyzed in a zymogram [26]. The SDS PAGE of the final preparation was carried out under denaturing conditions on a 12% (w/v) polyacrylamide gel. After electrophoresis, the gel was washed twice with a solution of 1% (v/v) Triton X-100 in CPB buffer, pH 5. The washed gel was then incubated for 10 min at 37 °C in a sucrose solution (1% [w/v], CPB buffer pH 5). Finally, the gel was rinsed with distilled water and immersed in a solution of 1% (w/v) 2,3,5,-triphenyltetrazoliumchloride in 0.25 M NaOH. After a 10-min exposure, 5% (v/v) aqueous acetic acid was added to stop the reaction. The sucrolytic activity was visualized as a red band resulting from the reduction of the triphenyltetrazolium chloride.

2.11. Characterization of the purified recombinant enzyme

The stability of inulinase at high temperatures was evaluated by incubating the enzyme in CPB buffer (pH 5.0) at different temperatures (37 °C, 45 °C, 55 °C, and 65 °C) for 180 min. The residual activity was measured after incubation under standard assay conditions. Thermal stability was expressed as the percentage of the original enzyme activity remaining after a given heat treatment.

The stability of the enzyme within a wide range of pHs (2.0–13.0) was further analyzed by measuring the residual activity after incubation at 37 °C for 180 min in a buffer mixture (Tris-HCl, MES, and glycine, at 20 mM each) calibrated at different pH values. The relative inulinase activity was expressed as a percent of the activity of the control at pH 5.0.

The optimal pH of the enzyme was studied as described earlier in the determination of activity by varying the pH of the reaction between 2.0 and 11.0.

The effect of different metal cations (K^+, Ca^{+2}, Hg^{+2}, Mn^{+2}; Zn^{+2}, and Fe^{+3} at 1 mM each) on enzyme stability was studied by incubating the inulinase with each metal for 1 h at room temperature. Enzymatic stability was determined by the activity remaining after the incubation expressed as a percent of the initial value. Similarly, the effect of the cations on inulinase catalytic activity was examined. In this assay, a mixture of 20 µl of suitably diluted enzyme with 180 µl of inulin solution containing a given metal salt (at 1 mM) was incubated under the standard assay conditions and the enzyme activity measured. As with the stability measurements, the effect of a metal ion on inulinase activity was expressed in terms of the residual activity as a percent of the original value.

The kinetic parameters (K_M and V_{max}) were determined from Lineweaver-Burk plots of enzyme activity with inulin as the substrate at different concentrations (0.5–5 mM).

2.12. Determination of mode of action

The *endo* or *exo* mode of action of the recombinant inulinase was analyzed in a 1-h incubation at 37 °C of 200 µL of the enzyme (at 800 U/ml) with 1.8 ml inulin solution (0.5 g/l in CPB, pH: 5) to give a final enzyme concentration of 80 U/ml. Samples were withdrawn every 10 min and the products separated qualitatively by thin-layer chromatography. The mobile phase was composed of a mixture of ethyl acetate/acetic acid/water (3/1/1, v/v/v), and the developed plates were sprayed with 1/1 (v/v) sulfuric acid/methanol reagent and heated for 5 min at 105 °C to visualize the carbohydrates [27]. Inulin, sucrose, and fructose were used as standards.

2.13. Fructosyltransferase activity

Fructosyltransferase activity was measured at pH 5.0 with a sucrose solution of 700 g/l in CPB as the substrate. A mixture of 100 µl of appropriately diluted enzyme (to reach a final ratio of 10 U/g of substrate) plus 900 µl of this solution containing 630 µg of substrate was incubated at 50 °C. Samples were withdrawn at 0, 3, and 24 h and assayed for glucose with a glucose-oxidase kit (Wiener Lab, Argentina). Reducing sugars were determined by the 3,5-dinitrosalicylic-acid method with fructose as a standard [28]. The synthetic activity of the recombinant enzyme was estimated as the difference between the moles of glucose and fructose released, with this difference being equal to the moles of fructose transferred. One unit of fructosyltransferase activity was defined as the amount of enzyme required to transfer 1 µmol of sucrose per min [29]. All the experiments were conducted in duplicate.

3. Results

3.1. PCR amplification of the inulinase gene and sequence analysis

The cloning strategy of the inulinase gene from *A. kawachii* was designed on the basis of| the information obtained from the National Center for Biotechnology Information (based on the BLAST search of the GenBank Database) and comparative bioinformatics analyses. First, the main primers of this strategy were designed and used to amplify the inulinase genewith the genomic DNA of *A. kawachii* as a template, to obtain a product of about 1600 bp. This PCR product was digested and ligated to the *P. pastoris* vector pPIC9. The plasmid thus generated, designated pPIC9:*inulinase*, was used to transform competent *E. coli* TOP10F′ cells. Several positive clones were sequenced and the DNA coding sequence referred to as the *inuAk* gene.

3.2. Bioinformatic analysis of the inuAk gene

Once the *inuAk* gene was obtained by PCR amplification, a bioinformatic analysis was performed. This sequence contained an ORF of 1672 bp, encoding a 537–amino-acid protein. A SignalP examination of this sequence, suggested the presence of a signal sequence that could export the resulting molecule to a secretory pathway (*i. e.*, amino acids 1–19; http://www.cbs.dtu.dk/services/SignalP/). Furthermore, ExPASy analysis resulted in the following theoretical information on

the encoded enzyme: a pI of 5.03 and estimated molecular weight of 59.63 kDa (http://www. ExPASy.org). A potential N-glycosylation-site study (including the signal sequence) resulted in eight potential sites, asparagine residues 49, 67, 111, 112, 254, 300, 398, and 430 (http://www.cbs.dtu.dk/services/NetNGlyc/).

The data on the InuAk sequence obtained was analyzed through the use of the Genbank database. The information from this analysis suggested that InuAk belongs to the glycoside-hydrolase family (GH32). A similarity study revealed that the InuAk amino-acid sequence exhibited a high degree of identity with those of certain *Aspergillus* species: 100% *A. awamori* var 2250 (GenBank accession: CAC44220), 91% *Aspergillus niger* CBS513.88 (GenBank accession: A2R0E0), and 90% *Aspergillus ficuum* JNSP5-06 (GenBank accession: ADM21204.1).

3.3. In-vitro cDNA construction of the inuAk gene

Considering that *P. pastoris* would be incapable of processing the 57 bp intron, we deleted the *inuAk* intron *in vitro* using the following strategy. First, the pPIC9:*inulinase* construction was PCR-amplified as a template through the use of the primers P3 and P4, resulting in a fragment of 383 bp (exon I) Next, the pPIC9:*inulinase* was restricted with *BsrD*I and *Not*I resulting in a DNA fragment of 1200 bp (exon II, Fig. 2). Both fragments (exons I and II) were ligated to the vector pPICZαA and the resulting plasmid pPICZαA:*inuAkΔI* used to transform competent *E. coli* TOP10F′ cells. Colony PCR verified the presence of the correct construction (in 5 of the 5 clones tested). Dideoxy-sequencing of these clones provided further confirmation of the sequence integrity and the absence of any intron. This construction was linearized with *Bgl*II and transferred into *P. pastoris* cells by electroporation. From each strain, 13 clones were chosen for checking the recombination in the yeast genome by colony PCR.

3.4. Production of A. kawachii inulinase in P. pastoris by the recombinant gene

In order to analyze the expression of the exoinulinase, 26 *P. pastoris* clones resistant to 0.1 mg/ml Zeocin™ and transformation-positive by colony PCR were selected. After 48 h of cultivation under inducing conditions, all of these clones manifested inulinase activity, and those that expressed the highest inulinase levels (at *ca*.14.6 U/ml) were selected for further studies (data not shown). When the negative control (*P. pastoris* cells transformed with the empty vector and cultivated under the same conditions) was similarly analyzed, no inulinase activity was detected at any point during the process.

From these experiments, a Pichia pastoris X-33 clone was selected as the producer one and used for further studies.

3.5. Enzyme production and purification

Since one of the principal goals of this work was to design a process for achieving a high level of gene expression, the production of the recombinant inulinase by *P. pastoris* X-33 was further studied in a 5-L bioreactor. At the end of the bioprocess, the final protein concentration detected in the supernatant was 0.7 g/l, corresponding to up to 840 U/ml. This result represented a more than 7000-fold increase compared to the inulinase production by the native microorganism (0.11 U/ml) [13]. The results from an analysis by SDS-PAGE demonstrated that the expression of inulinase increased throughout the induction time to reach that final value of approximately 0.7 g/l of supernatant protein (Fig. 3). Table 1 summarizes the recombinant inulinase purification results. After the chromatographies indicated, the yield achieved was about 42%, while the specific activity became increased by 2.2-fold. These results represent a specific activity of 2,575 U/mg protein. The purity of the final enzyme preparation was confirmed by SDS-PAGE (Fig. 4).

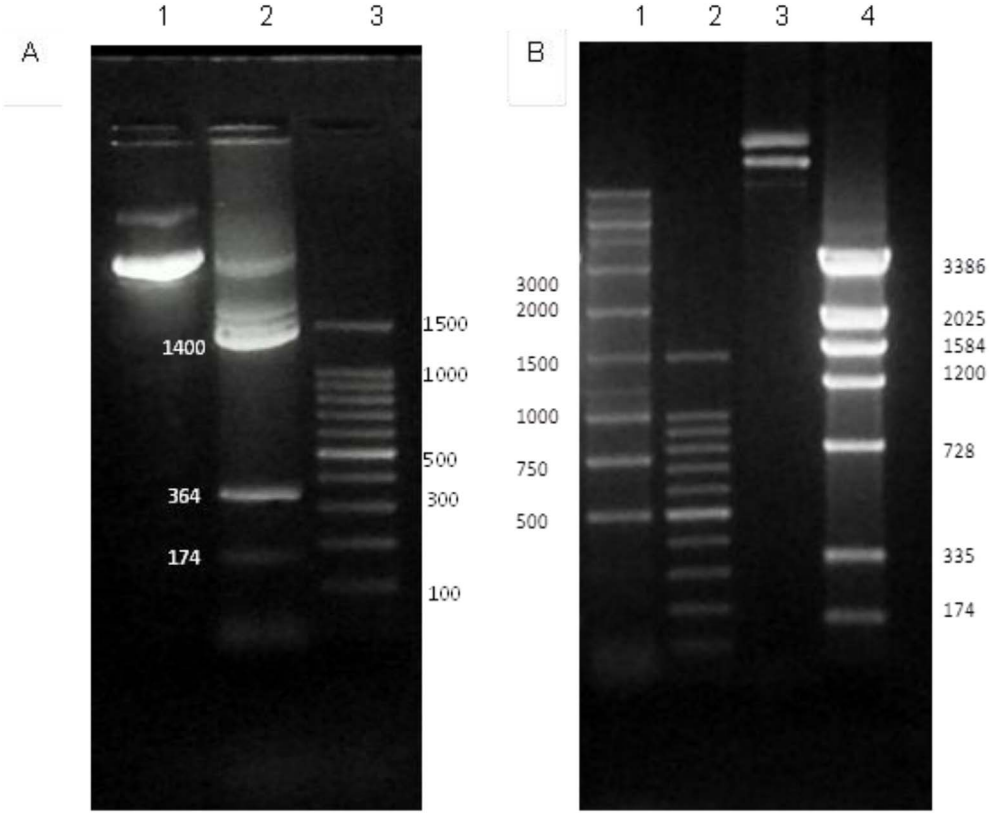

Fig. 2. Agarose-gel electrophoresis of (Panel A) the digestion of the construction pGEMT:exon I to obtain exon I—Lane 1: pGEMT:exon I; Lane 2: pGEMT:exon I digested with *Xho*I and *BsrD*I; lane 3: DNA molecular-weight standard—and of (Panel B) the construction pPIC9:*inulinase* to obtain exon 2 of the *A. kawachii* inulinase gene—lane 1: DNA molecular-weight standard; lane 2: DNA molecular-weight standard; lane 3: pPIC9:*inulinase*; lane 4: pPIC9:*inulinase* digested with *BsrD*I and *Not*I.

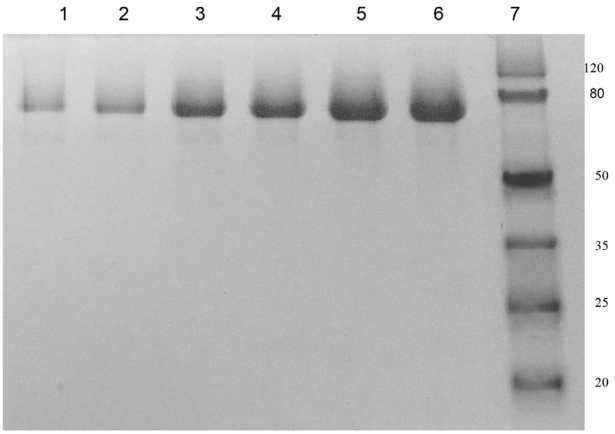

Fig. 3. SDS PAGE of the recombinant inulinase expressed by *P. pastoris* in a fed-batch fermentation process. Lanes 1–6: Samples at times 0, 5, 10, 13, 16, and 26 h after induction with methanol. Lane 7: Protein molecular-weight standard.

Table 1
Analysis of the steps involved in the purification of the recombinant exoinulinase.

Purification steps[a]	Volume	Total protein	Total activity	Specific activity	Fold purification	Yield
Units	ml	mg	U	U/mg	–	%
Crude extracts diluted 1/5	500	72.5	84,000	1158	1.0	100
SP Sepharose FF	190	44.3	68,040	1535	1.3	81
Sephacryl G25	330	33.0	64,680	1960	1.7	77
Q Sepharose HP	60	13.7	35,280	2575	2.2	42

[a] Crude extracts obtained from a bioreactor culture were used to develop the satisfactory chromatographic purification method indicated here.

3.6. Enzyme characterization

According to an analysis by SDS-PAGE, the InuAk gene product has a molecular weight of 97 kDa (Fig. 4), higher than the predicted molecular weight and probably resulting from glycosylation.

The sucrose-invertase/inulinase (S/I) ratio is a useful means of determining if the glycosyl hydrolase under study is an invertase or a true inulinase. If the S/I ratio is higher than 100, the enzyme under study is a *bona-fide* invertase; whereas if ratio is less than 100, the enzyme is a pure inulinase. In the example of InuAk, the S/I ratio was around 2 indicating that the enzyme was a definite inulinase [30].

Investigation of the biochemical characteristics of the recombinant InuAk demonstrated that the enzyme was stable up to pH 9.0, retaining at that high pH more than 80% of the optimum activity recorded at a pH of 5.0 (Fig. 5, Panel A). These results are in agreement with the previously reported pH-*versus*-activity profile for the native enzyme [13]. In addition, catalysis was maintained in brief incubations under the assay conditions over an even wider pH range (*i. e.*, 2.0–11.0), with the optimal pH there being around 3.0 (Fig. 5, Panel B). Finally, the recombinant inulinase activity was highly stable at 55 °C, with 70% of the activity remaining after a 3-h incubation at that temperature (Fig. 6). Moreover, even at 65 °C, the enzyme retained more than 25% of its activity after 15 min.

In view of the possible effect of certain metal ions on the enzyme activity, the positive or negative effect on the recombinant inulinase activity was evaluated. Table 2 shows the relative activities obtained. The presence of Zn^{+2}; Mn^{+2}; and K^{+} increased the enzymic activity only slightly, whereas Hg^{+2} produced a noticeably inactivation.

The recombinant inulinase from *A. kawachii* displayed typical Michaelis-Menten kinetics, at respective K_M and V_{max} values with inulin as the substrate of 1.35 mM and 2673 μmol/min/mg proteins. These values were determined using the Lineweaver-Burk transformation (Fig. 7).

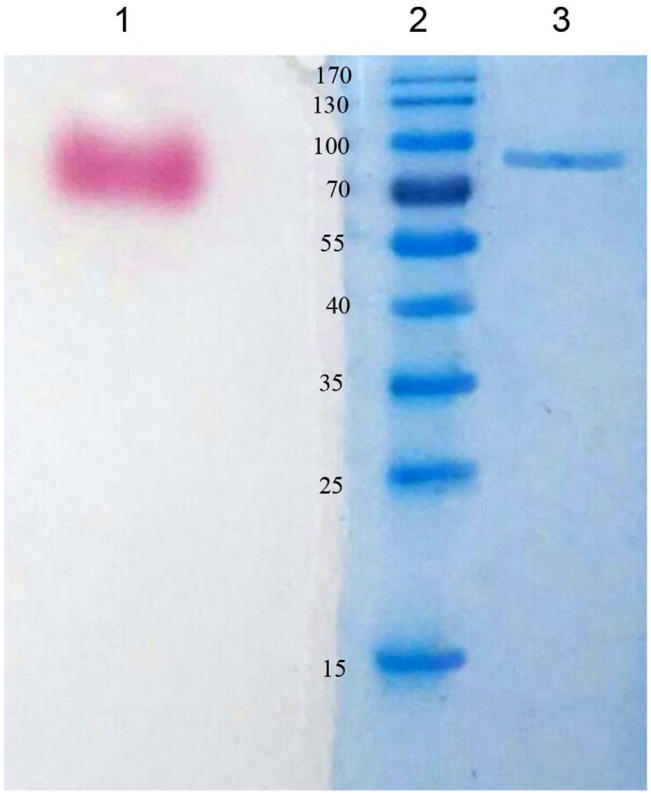

Fig. 4. SDS-PAGE of the purified inulinase. Lane 1: Zymogram showing inulinase activity; Lane 2: Protein molecular-weight standard; Lane 3: The purified enzyme.

3.7. Inulin hydrolysis

Fig. 8 demonstrates that fructose was the sole product obtained after inulin hydrolysis. The addition of other standards such as oligosaccharides was not necessary since no component other than fructose was detected. These results suggest that InuAk has a definite exocleavage mode of action—hydrolyzing inulin from the nonreducing terminus and thus releasing fructose units.

3.8. Fructosyltransferase activity

A concentrated sucrose solution was used for the determination of fructosyltransferase activity. When glucose and total sugars were measured, a difference was observed between the moles of glucose and fructose released, with this difference being equal to the moles of fructose transferred. This quantitative correspondence indicated that the recombinant InuAk possessed fructosyltransferase activity. These results could be relevant to a potential application of the enzyme in the production of prebiotics, which possibility for the food industries and should be explored in detail.

4. Discussion

In the present study, a recombinant construction of the exoinulinase gene from *A. kawachii* IFO 4308, *inuAk*, was successfully overexpressed in *P. pastoris* X-33 in achievement of the original intention to increase enzyme yield along with the continued maintenance of the characteristics of the wild-type protein. Many other successful attempts have been reported on the cloning and expression of inulinase genes from several hosts including bacteria, yeasts, and molds [31]. Along with the example of the yeast inulinases, among other genes that were cloned and

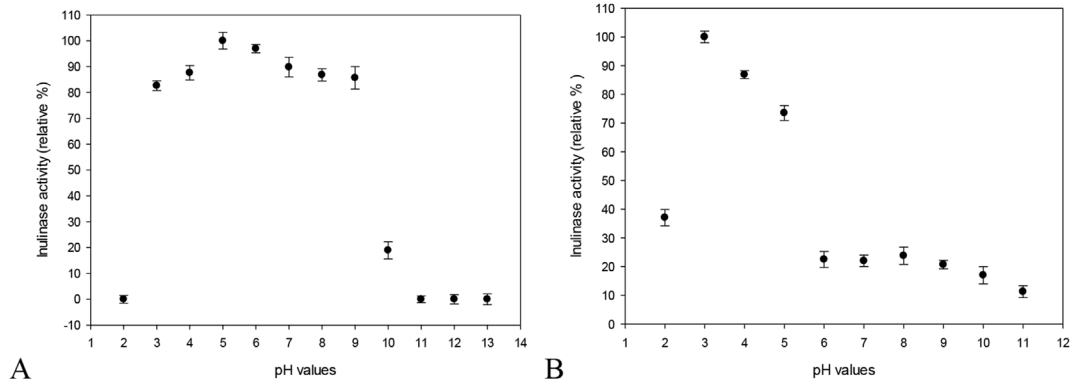

Fig. 5. Effect of pH on the recombinant-inulinase stability and activity. (Panel A) Effect of pH on InuAk stability. In the figure, the inulinase activity, expressed as a percent of the maxiumum value at pH 5, is plotted on the *ordinate* as a function of the pH used during the 180-min incubation on the *abscissa*. (Panel B) Effect of pH on the InuAk catalysis. In the figure, the inulinase activity, expressed as a percent of the maxiumum value at pH 3, is plotted on the *ordinate* as a function of the pH used in the assay. In both panels, the data represent the means of three determinations each ± the SEs.

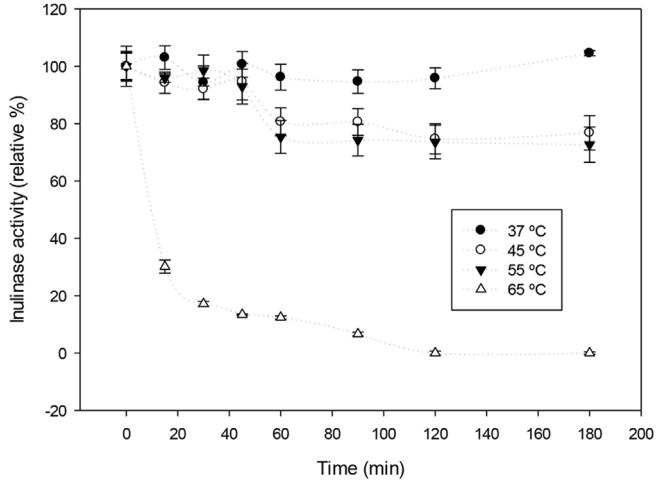

Fig. 6. Effect of temperature on the recombinant-inulinase stability. In the figure, the inulinase activity at the indicated temperatures, expressed as a percent of the value at 37.0 °C, is plotted on the *ordinate* as a function of time in min on the *abscissa*. Key to the temperature symbols: closed circles, 37 °C; open circles, 45 °C; inverted triangles, 55 °C; triangles, 65 °C. The data represent the means of three determinations each ± the SEs.

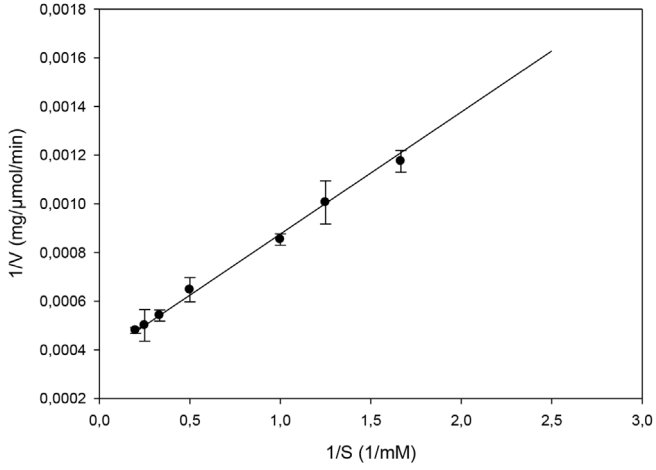

Fig. 7. Lineweaver–Burk plots to estimate kinetic parameters of *A. kawachii* recombinant inulinase, using inulin as substrate. The reactions were developed in 50 mM citric acid/25 mM Na_2HPO_4 buffer, pH 5, and concentrations of inulin ranging from 0.5 to 5 mM. Each point represents the average of three experiments.

Table 2
Effect of metal cations at a concentration 1 mM on recombinant *A. kawachii* inulinase activity (data are shown as residual activity (%) ± SD).

Metal cations	Residual activity (%)
None	100 ± 3.1
Ca^{2+}	99.84 ± 3.8
Fe^{3+}	86.19 ± 1.5
Hg^{2+}	23.35 ± 1.6
K^{+}	103.41 ± 2.2
Mn^{2+}	105.39 ± 1.7
Zn^{2+}	106.06 ± 0.8

overexpressed in the *P. pastoris* system, we can cite the exoinulinase (*kcINU1*) from *Kluyveromyces cicerisporus* CBS4857, the expression of which recombinant resulted in a concentration of 45.2 ± 0.6 U/ml in the culture supernatant [32]. Likewise, when the inulinase gene from *Pichia guilliermondii* was expressed, the maximum productivity of the enzyme under optimal conditions was 286.8 U/ml after 120 h [33]. Moreover, with respect to the fungal-inulinase genes, another striking example is the exoinulinase from *Penicillium janthinellum* B01, which locus was cloned and correctly expressed in *P. pastoris* to produce a level of expression as high as 272.8 U/ml [34]. In the work reported here, the production of the *A. kawachii* exoinulinase, after an engineered recombination and transfection into *P. pastoris*, reached an inulinase activity of 840 U/ml upon high-cell-density fermentation (*i. e.*, a 72-h cultivation): the productivity and yield of this recombinant enzyme were thus considerably higher than those previously reported. To the best of our knowledge, the expression levels in inulinase-activity units attained in this study are the highest thus far reported. These results could be attributed to a synergic combination of the choice of the expression system and the establishment of the production conditions.

The biochemical properties of a recombinant enzyme are one of the main topics to take into account when potential biotechnological applications are being evaluated. A comparison of| the optimal pH of the *A. kawachii* inulinase with other fungal inulinases indicated that, whereas the pH of most fungal inulinases was between 4.5 and 7.0 [35], the optimal pH of the recombinant InuAk was below this range, thus conferring on this enzyme the capability to be used under acidic-pH conditions, where microbial contamination would be prevented. Exoinulinases are commonly applied in the production of high-fructose syrup, in which process a pH of *ca.* 4.0 is desirable because of the high stability of fructose under acidic conditions. Most of the fungal inulinases display a wide range of pH stability (*i. e.*, 3.0–8.0); and as the present inuAk retains more than 80% of the maximal activity at the highest pH within that range after a 3-h incubation, this recombinant would thus constitute a potential candidate for use in that kind of process.

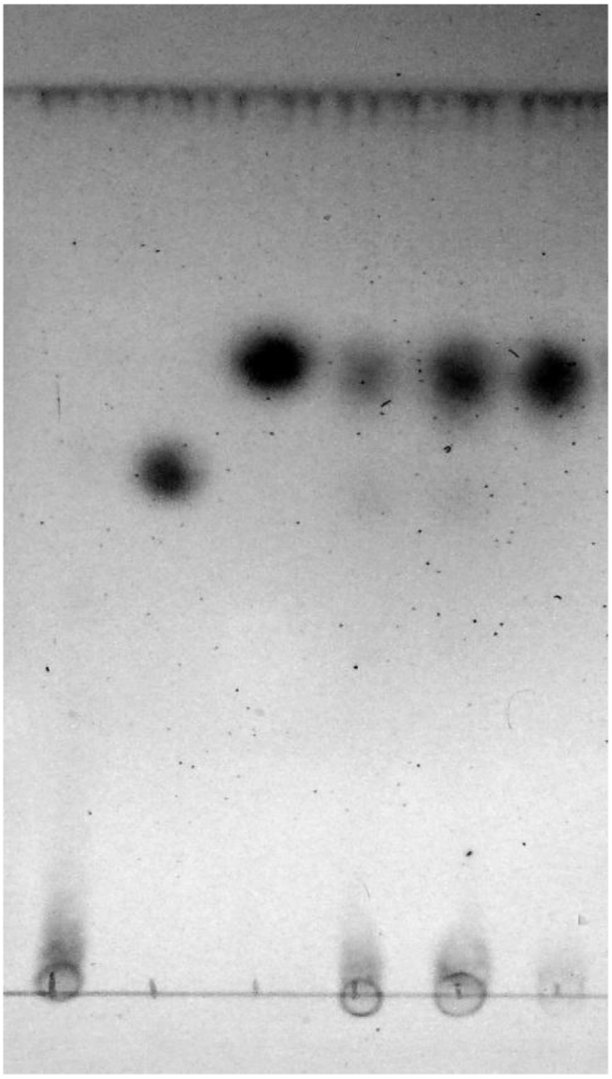

Fig. 8. Thin-layer chromatograph of the hydrolysis products after inulin digestion by the inulinase from *A. kawachii*. Lane 1: inulin; Lane 2: sucrose, Lane 3: fructose; Lanes 4–6 inulinase-hydrolysis products.

Metal ions can either activate or inactivate inulinases: for example Mn^{+2} is usually an activator of those enzymes, whereas Fe^{+3} generally effects a stringent inhibition [36,37]. The activity of the recombinant InuAk was not strongly influenced by the presence of those types of cations, except for Hg^{+2}, where a complete inhibition occurred. That effect by Hg^{+2}, moreover, suggested the possible implication of thiol groups in the catalysis of the enzyme; and, in agreement with that observation, Gao et al. had previously reported a similar effect of Hg^{+2} on the *Paenibacillus polymyxa* ZJ-9 exoinulinase [38].

There are reports about kinetic parameters of recombinant inulinases evaluated using inulin as the substrate, such as the exoinulinase gene from *Kluyveromyces cicerisporus* expressed in *Pichia pastoris* X-33, which K_M and Vmax values for inulin were 0.322 mM and 4317 μmol/min/mg protein, respectively [39]. In the case of exoinulinase gene from *Aspergillus ficuum* expressed in *Escherichia coli*, the K_M and V_{max} values for inulin were 7.1 mM and 1000.0 ± 0.1 μmol/min/mg protein, respectively [36]. Moriyama et al. reported that the inulinase P-I from Penicillium sp. Strain TN-88 had an apparent K_M of 92.6 μM [40].

5. Conclusions

The production of high-fructose syrup, fructooligosaccharides, and bioethanol generation are some of the industrial processes where inulinases can be applied. The recombinant exoinulinase InuAk was successfully overexpressed in *P. pastoris* to give the highest productivity thus far reported. The remarkable characteristics of this recombinant, such as the acidic-to-alkaline–pH stability, would thus point to the suitability of InuAk for those types of industrial applications.

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgements

Funding: This study was funded by CONICET (PIP 01422) and UNQ (National University of Quilmes, PUNQ 1445/15). Dr. Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

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