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Improvement of catalytical properties of two invertases highly tolerant to sucrose after expression in *Pichia pastoris*. Effect of glycosylation on enzyme properties

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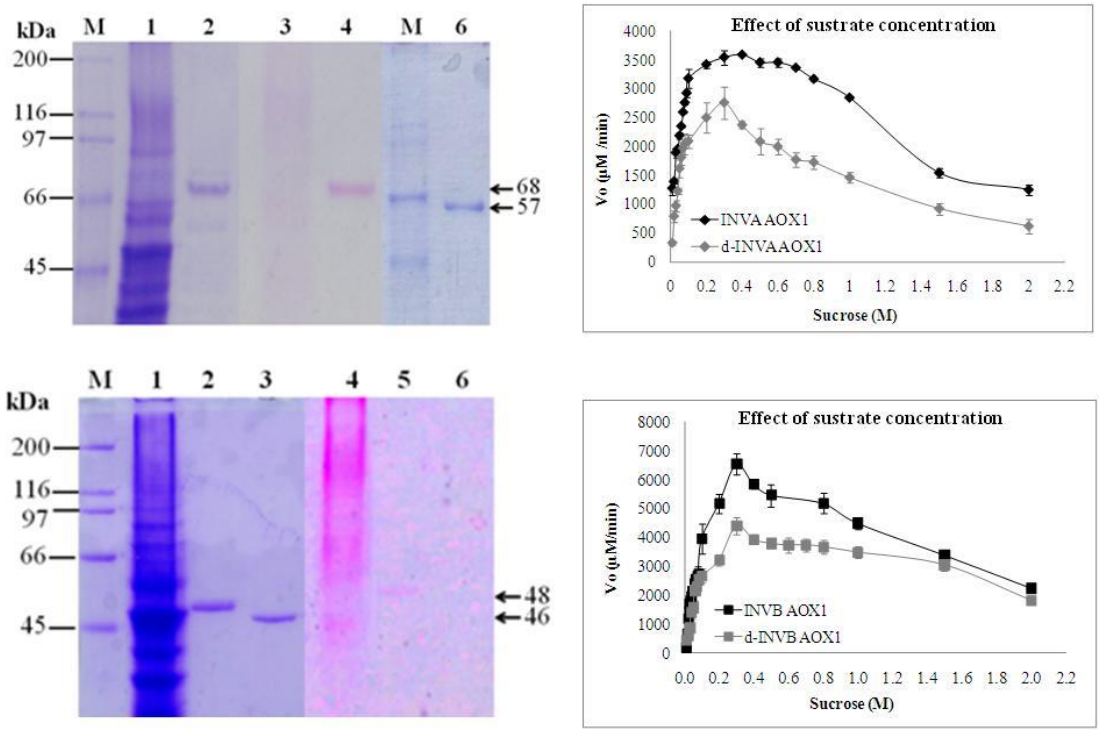
Graphical Abstract



Highlights

- Successful expression of invertases with high sucrose tolerance (up to 300-400 mM)
- Production of recombinant invertases in yeast is higher than that of native enzymes
- Recombinant invertases expressed in P. pastoris show different carbohydrate content
- Glycosylated recombinant invertases display improvement of kinetic properties
- Glycosylation plays a key role in high sucrose tolerance of recombinant INVA.

Abstract

Zymomonas mobilis genes encoding INVA and INVB were expressed in *Pichia pastoris*, under the control of the strong AOX1 promoter, and the recombinant enzymes were named $INVA_{AOX1}$ and $INVB_{AOX1}$. The expression levels of $INVA_{AOX1}$ (1660 U/mg) and $INVB_{AOX1}$ (1993 U/mg) in *P. pastoris* were 9- and 7-fold higher than those observed for the native INVA and INVB proteins in *Z. mobilis*. $INVA_{AOX1}$ and $INVB_{AOX1}$ displayed a 2- to 3-fold higher substrate affinity, and a 2- to 200-fold higher catalytic efficiency (k_{cat}/K_M) than that observed for native INVA and INVB from *Z. mobilis*. Positive Schiff staining of $INVA_{AOX1}$ and $INVB_{AOX1}$ suggested a glycoprotein nature of both invertases. After deglycosylation of these enzymes, denoted d-$INVA_{AOX1}$ and d-$INVB_{AOX1}$, they exhibited a 1.3- and 3-fold lower catalytic efficiency (107 and 164 s^{-1} mM^{-1}, respectively), and a 1.3- to 5-fold lower thermal stability than the glycosylated forms at temperatures of 35 °C to 45 °C. After deglycosylation no effect was observed in optimal pH, being of 5.5 for $INVA_{AOX1}$, $INVB_{AOX1}$, d-$INVA_{AOX1}$ and d-$INVB_{AOX1}$. The invertase activity of both enzymes increased in 80% ($INVA_{AOX1}$) and 20% ($INVB_{AOX1}$) in the presence of Mn^{2+} at 1 mM and 5 mM, respectively. $INVA_{AOX1}$ and $INVB_{AOX1}$ were highly active at sucrose concentrations of up to 400 and 300 mM, respectively; however, the tolerance to sucrose decreased to 300 mM for d-$INVA_{AOX1}$. Our findings suggest that glycosylation of $INVA_{AOX1}$ and $INVB_{AOX1}$ plays an important role in their thermal stability, catalytic efficiency, and tolerance to sucrose. In conclusion, the expression of INVA and INVB from *Z. mobilis* in *P. pastoris* yields new catalysts with improved catalytic properties, making them suitable candidates for a number of industrial applications or for the improvement of ethanol production from cane molasses.

Keywords Intracellular/extracellular invertases, *Zymomonas mobilis*, *Pichia pastoris*, AOX1 promoter, protein expression, effect of glycosylation.

Introduction

For several years, three types of glycosyl hydrolases have been studied in *Zymomonas mobilis* [1]. Two of them belong to the invertase or β-D-fructofuranosidase group (EC.3.2.1.26), which are involved in catalyzing the release of D-glucose and D-fructose in an equimolar mixture from non-reducing ends of sucrose or β-D-fructofuranoside [2,3]. These none-crystal-forming sugars are important in the food industry, primarily because they can be used in confectionery in soft-centered chocolates and as sweetener (fructose) for diabetics [4]. Currently, *Aureobasidium* spp., *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, and *Saccharomyces carlsbergensis* are the main yeast species that produce commercial invertases [4], and so far, production of commercial invertases from bacteria has not been reported.

The invertases from *Z. mobilis* have been intensively studied. Different research groups have cloned the intracellular invertase A (INVA) in *E. coli* [5-7], sequenced [5], purified, characterized [5-7,11], and immobilized on Avicel [11]. The extracellular invertase B (INVB) was first characterized and purified by Preziosi *et al.* [1] and O'Mullan *et al.* [8]. Then, this enzyme was cloned in *E. coli*, characterized and sequenced [9-11], expressed, produced [12-15] and immobilized on Avicel [14] and Nylon-6 [15], using *E. coli* as bacterial expression system. An advantage of these enzymes is their high tolerance to sucrose (300-400 mM) compared to the commercial invertase from *Saccharomyces cerevisiae* (150 mM) [16, 44]. However, the expression levels of both INVA and INVB in *Z. mobilis* are very low [8, 10], limiting their use at the industrial scale.

The *Pichia pastoris* expression system has been widely used as an alternative way to increase the expression levels of target proteins, because it is very efficient at highly expressing heterologous genes when it grows in the presence of methanol as an energy

source [17,19,28]. Additionally, the expression of proteins of interest in *P. pastoris* can be scaled to higher levels than those achieved with *E. coli*, and in many cases, it is also superior to those achieved with *S. cerevisiae*. For this reason, more than 1000 proteins have been expressed in *P. pastoris* over the past three decades [17-19,28].

Currently, there are several studies on invertase expression under the control of the AOX1 promoter in *P. pastoris* (**Table 1**). Invertases from *Bambusa oldhammi* [25], *Ipomoea batatas L.* [22, 24], *Beta vulgaris L.* [23], *Allium cepa* [26] and invertase Suc2 from *S. cerevisiae* [20-21], among others, have been successfully expressed in *P. pastoris* under the control of the AOX1 promoter, and characterized. Most recombinant invertases are glycosylated, because O- and N- linked glycosylation is the most common post-translational modification performed by this host [17,27,28].

The aim of this work was to express and characterize the INVA and INVB invertases from *Z. mobilis* in *P. pastoris*, under the control of the AOX1 promoter, and to evaluate the effect of glycosylation on the resulting recombinant proteins.

2. Materials and methods

2.1 Strains, plasmids, and culture media

A *Zymomonas mobilis* CDBB-B603 strain was obtained from the "Colección de Cultivos Microbianos" (CINVESTAV-IPN, México) and was grown as described by Bekers et al. [29]. *E. coli* DH5α (Novogen, USA) cells were used for vector propagation and construction. *P. pastoris* X-33 and pPICZαB (Invitrogen, Carlsbad, CA, USA) were used as host and vector, respectively, for heterologous expression, according to the manufacturer's instructions. *E. coli* DH5α was grown at 37 °C with shaking at 250 rpm for 16-18 h in LB medium supplemented with 25 μg/mL Zeocin (Invitrogen).

Yeast cultures were grown at 30 °C with agitation at 200 rpm for 3-5 days in YPD or YPDS medium. *P. pastoris* transformants were selected on 2,3,5-triphenyltetrazolium chloride (TTC)-indicator plates according to the method of Bochner and Savageau [32] with slight modifications, MM medium with 1% (v/v) methanol, MD with 1% (w/v) dextrose, BMGY with 1% (v/v) glycerol supplemented with 100 μg/mL Zeocin when necessary, and BMMY with 1% (v/v) methanol to achieve enzyme induction [30].

2.2 Subcloning of the invertase *invA* and *invB* genes in pPICZαB

The YeaStar™ Genomic DNA Kit (Zymo Research, Irvine, CA, USA) was used to isolate *Z. mobilis* DNA and to amplify the ORF of the *invA* and *invB* genes (without initial methionine) by PCR, using *Pfu* DNA Polymerase (Fermentas, Germany) and the primers listed in **Table 2**. INVA-F and INVA-R primers were used for the amplification of the ORF *invA*, while the ORF *invB* was amplified using the primers INVB-F and INVB-R. The forward primers INVA-F and INVB-F included a PstI restriction site (underlined), while the reverse primers INVA-R and INVB-R contained an XbaI restriction site (underlined) and a stop codon (bold), to avoid the translation of the C-terminal peptide, including the c-myc epitope and polyhistidine tag.

The PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, Germany) and cloned into the PstI and XbaI restriction sites of the pPICZαB vector (Invitrogen) to generate the constructs pPICZαB-*invA* and pPICZαB-*invB*, which were used to transform *E. coli* DH5α cells by electroporation. Positive clones were selected by screening for Zeocin resistance (100 μg/mL).

2.3 Invertase and protein assays

Invertase activity was determined by measuring the release of reducing sugars using 3,5-dinitrosalicylic acid (DNS) as described by Miller et al. [31]. The assay mixture contained 0.05 M sucrose in 0.05 M acetate buffer at pH 5.0 (INVA) or 5.5 (INVB), and enzyme preparations were incubated at 35 °C for INVA and 40 °C for INVB. The reducing sugars that formed after incubation were quantified at 540 nm. The enzymatic activity was expressed in U/mL, with one unit equivalent to the hydrolysis of 1 μmol glucose/min, under the assay conditions specified above.

The protein concentration was measured using Bradford reagent (Sigma, St. Louis, MO, USA) with bovine serum albumin (Life Technologies, Grand Island, NY, USA) as the standard.

2.4 Transformation and selection of the *P. pastoris* clones with invertase activity

An amount of 10 μg of the recombinant purified plasmid (pPICZαB-*invA* or pPICZαB-*invB*) was linearized with SacI restriction enzyme and the corresponding linearized plasmid was transferred into cells of the *P. pastoris* X-33 strain by electroporation for 10 ms with a field strength of 1.5 kV, using the Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA, USA). After transformation, the cells were inoculated onto YPDS agar plates with Zeocin (25 μg/mL) and incubated at 30 °C for 2 days. The colonies from YPDS-Zeocin plates were grown on YPD plates containing Zeocin (1000 μg/mL) and stored at 4 °C for further studies.

The primary selection of transformants of each construction was performed in several steps. First, transformants were screened for the methanol utilization plus (Mut^+) phenotype

according to the *Pichia* Expression Kit Manual [30] by using MM/MD agar media. Next, positive (red) colonies were selected via a functional bioassay screening using TTC-indicator plates [5, 32]. Finally, the presence of the ORF of *invA* and *invB* in red positive colonies and Mut^+ colonies was confirmed by PCR using 5′ AOX and 3′ AOX alcohol oxidase primers (**Table 2**).

Secondary selection was made based on small-scale fermentation according to Calik et al. [18] with slight modifications to identify clones with greater extracellular invertase activity.

2.5 Production of native INVA and INVB by *Z. mobilis*

To evaluate the production of native INVA and INVB invertases, *Z. mobilis* was grown as described by Bekers et al. [29], in 1-L flasks containing 100 mL culture medium. Cultures were incubated at 30 °C for 24 h without shaking. During the incubation, samples were taken every 2 h in the first 12 h and then every 6 h until 24 h. Samples were centrifuged (8000 rpm at 4 °C for 5 min), and the culture supernatants were assayed for catalytic activity and protein concentration of INVB. For INVA, we measured protein and invertase activity from the pellet in 50 mM acetate buffer. Biomass was determined by the dry-weight method using the centrifugation pellet.

2.6 Production of $INVA_{AOX1}$ and $INVB_{AOX1}$ in *P. pastoris*

For both enzymes, the production procedures were similar. Cultures were set up in a 1-L baffled flask with 100 mL BMMY medium and 1% (v/v) methanol. Cultures were incubated at 28 °C with shaking at 180 rpm for 5 days. Samples were collected every 12 h and centrifuged (6000 rpm at 4 °C for 5 min), and the culture supernatants were assayed for invertase activity and protein. Biomass was determined by the dry-weight method.

2.7 SDS-polyacrylamide gel electrophoresis

Protein analyses were carried out by 10% SDS-PAGE, according to the method of Laemmli [33]. Proteins in the gel were stained using Coomassie Brilliant Blue R-250. The molecular weight (MW) of the proteins was estimated with reference to a broad range molecular weight protein standard (Bio-Rad). Gels were recorded and analyzed using a gel documentation system (DigiDoc-It Imaging System, UVP). Glycoproteins were detected with acid-Schiff staining following the Segrest and Jackson method [36] using *S. cerevisiae* invertase (Sigma) as positive control.

2.8 Purification of recombinant $INVA_{AOX1}$ and $INVB_{AOX1}$ invertases

2.8.1 $INVA_{AOX1}$ purification

The recombinant invertase was purified from *P. pastoris*/pPICZαB-*invA* cells. Briefly, 300 mL of crude extract was concentrated by ultrafiltration at 4 °C by using a polyethersulfone membrane with a cut-off weight of 30,000 Da (PALL, NY, USA). When a 30-fold concentration was reached, the ultrafiltrate was dialyzed overnight at 4 °C against buffer A (25 mM acetate buffer pH 5.2, 25 mM KCl, 0.1 mM PMSF, and 5 % (v/v) glycerol). Five milliliters of dialysate was loaded onto a UNOsphere^{TM} S (Bio-Rad) column (9.5 × 1.6 cm) with a packaging volume of 17 mL, which had been equilibrated with buffer A. Adsorbed proteins were eluted with a linear gradient of KCl (0.025–1 M) in buffer A at a constant flow rate of 2 mL/min. Fractions of 2.0 mL were collected and those manifesting invertase activity were pooled and analyzed by 10% SDS-PAGE. Fractions of purified $INVA_{AOX1}$ invertase were stored at 4 °C for further study.

2.8.2 $INVB_{AOX1}$ purification

Culture supernatant (300 mL) was concentrated by precipitation in 60% $(NH_4)_2SO_4$ at 4 °C overnight. The protein was recovered by centrifugation (8000 rpm at 4 °C for 1 h), and the pellet was resuspended in 50 mM acetate buffer pH 5.5 and desalted by overnight dialysis at 4 °C against buffer B (25 mM L-histidine buffer pH 5.5, 25 mM KCl, 0.1 mM PMSF, and 5 % (v/v) glycerol). Five milliliters of dialysate was loaded onto a UNOsphere^{TM} Q (Bio-Rad) column (19.5 × 1.6 cm) with a packaging volume of 12.5 mL, which had been equilibrated with buffer B. Adsorbed proteins were eluted with a linear gradient of KCl (0.025–1 M) in buffer B at a constant flow rate of 2 mL/min. Fractions of 2.0 mL were collected and those manifesting invertase activity were pooled and analyzed by 10% SDS-PAGE. Fractions of purified $INVB_{AOX1}$ invertase were stored at 4 °C for further study.

2.9 Characterization and effect of deglycosylation of $INVA_{AOX1}$ and $INVB_{AOX1}$

2.9.1 Optimal pH

The effect of pH on the recombinant enzymes was determined at different pH values, ranging from 4.0-7.5, in 50 mM sucrose solution, prepared in sodium acetate buffer (4.0-5.5), 50 mM citrate-phosphate buffer (6.0-7.0), and 50 mM sodium phosphate buffer (7.5). Reaction mixtures were incubated for 5 min at the optimal temperature for each enzyme.

2.9.2 Optimal temperature

The effect of temperature on the enzymatic activity was estimated by conducting the activity assay at different temperatures ranging from 20 °C to 55 °C, in 50 mM sucrose solution prepared in 50 mM sodium acetate buffer (pH 5.5). Reaction mixtures were incubated for 5 min under the conditions described.

2.9.3 Kinetic parameters

Once the optimal temperature and pH were established for the purified invertases, the rate of hydrolysis was estimated by incubating the enzymes at different sucrose concentrations (0.01-2 M) and evaluating the degree of hydrolysis of the substrate. The K_m and V_{max} values were determined graphically by applying the linear method devised by Hanes-Wolf [34] for $INVA_{AOX1}$ and Lineweaver-Burk [35] for $INVB_{AOX1}$.

2.9.4 The effect of metal ions and EDTA

To evaluate the effect of several metal ions and EDTA on the enzymatic activity of the invertases, samples of the purified enzymes were diluted to an adequate concentration and the invertase activity was determined in 50 mM sucrose solution prepared in 50 mM sodium acetate buffer (pH 5.5), containing $NiCl_2$, $MgCl_2$, $CuSO_4$, $ZnSO_4$, $FeSO_4$, $MnCl_2$, $HgCl_2$, $CaCl_2$, or EDTA at a final concentration of 1, 5, and 10 mM each. Reaction mixtures were incubated at the optimal temperature of each enzyme for 5 min. The reference activity (100 %) was that without metal ions present.

2.9.5 Thermal stability

For thermal stability studies, preparations of purified enzymes were incubated at different temperatures, and samples were taken over a period of time to establish the half-life ($t_{1/2}$) of the enzyme activity (50 % of its initial activity). The remaining activity of the samples was assayed by the DNS method [31].

2.9.6 Deglycosylation of $INVA_{AOX1}$ and $INVB_{AOX1}$

To obtain an active deglycosylated form of the recombinant purified invertases, they were treated under non-denaturing conditions with endoglycosidase H (EndoH), following the instructions of the manufacturer (Roche Diagnostics Co., IN, USA). Briefly, enzymatic deglycosylation was carried out by adding 1 μL of EndoH to purified $INVA_{AOX1}$ (0.77 mg/mL in 50 mM acetate buffer; pH 5.0) and $INVB_{AOX1}$ (0.76 mg/mL in 50 mM L-Histidine buffer; pH 5.5) at 4 °C overnight. Then, aliquot samples of EndoH-treated d-$INVA_{AOX1}$ and d-$INVB_{AOX1}$ (deglycosylated) were analyzed by 10 % SDS-PAGE and stored at 4 °C for further study. Deglycosylated enzymes were characterized on the basis of optimal pH and temperature, kinetic parameters, and thermal stability.

2.9.7 Bioinformatics analysis

The prediction of potential N- and O-linked glycosylation sites in the amino acid sequence of native INVA and INVB from *Z. mobilis* [5,9,10] was performed using the EnsembleGly server http://turing.cs.iastate.edu/EnsembleGly/ [27].

3. Results and discussion

3.1 pPICZαB-*invA* and pPICZαB-*invB* constructs

Twenty clones of *E. coli* DH5α harboring the constructs pPICZαB-*invA* or pPICZαB-*invB* were analyzed by digestion with restriction endonuclease enzymes. Then, two clones, named A31 (pPICZαB-*invA*) and B2 (pPICZαB-*invB*) were selected for further analysis before the transformation of *P. pastoris*, including: digestion with PstI and XbaI restriction endonucleases and PCR using the primers α-Factor and 3' AOX1 (**Table 2**). In all cases, C2 (pPICZαB without insert) clone was employed as negative control (data no show). The

ORF of *invA* (1.5 kb) and *invB* (1.2 kb) genes from *Z. mobilis* were sequenced for confirmation.

Plasmid DNA from clones A1 (pPICZαB-*invA*) and B4 (pPICZαB-*invB*) were linearized with SacI and transferred to *P. pastoris* X-33 cells. Sixty *P. pastoris* X-33 Zeocin-resistant clones (1000 μg/mL) were selected for each construct.

3.2 Selection of *P. pastoris* transformants

It has been reported that *P. pastoris* clones may display Mut^+ and Mut^S phenotypes, and in many cases the Mut^+ phenotype shows greater productivity than the second phenotype [17, 28]. In order to select transformants with the Mut^+ phenotype, approximately 60 *P. pastoris* clones were cultured on MM agar medium and MD agar medium, and 15 clones of each construction were found to display the Mut^+ phenotype, similar to that observed with *P. pastoris* X-33 and *P. pastoris* X-33/pPICZαB that were used as positive controls (data not shown).

The bioassay with TTC-indicator plates has been previously used for the selection of clones with invertase activity [5]. Sixty Zeocin-resistant clones (1000 μg/mL) for each construct were tested using the TTC-indicator plates, depicts clones denoted as AX19, 20, 23, 24, 31, and 56 (pPICZαB-*invA*) and BX3, 5, 12, 26, 29, 32, and 36 (pPICZαB-*invB*), which all have a red color in contrast to the wild type strain cells of *P. pastoris* X-33 and *P. pastoris* X-33/pPICZαB used as negative controls, which appear as pink or white colonies. These clones also showed the Mut^+ phenotype and tested positive for *P. pastoris* genome integration by PCR (data not shown).

To confirm invertase activity in these clones, a small-scale fermentation was performed as previous described [18]. The mutants that showed the greatest activity using this method

were AX24 and BX29 (**Fig. 1**). The wild type strain of *P. pastoris* X-33 (X-33) and *P. pastoris* X-33/pPICZαB (CX11) cells were used as negative controls for all tests because wild type *P. pastoris* has no native invertase activity [20].

3.3 Invertase activity

The specific activity obtained from the native INVA and INVB invertases was 139±8 U/mg (12 h) and 317±21 U/mg (10 h), respectively, with the highest value corresponding to the extracellular invertase activity (**Fig. 2 A**). This behavior is consistent with that reported by Aït-Abdelkader et al. [6] and Yanase et al. [11], confirming that INVB is the enzyme with major saccharolytic activity in *Z. mobilis*.

The AX24 and BX29 clones were selected to evaluate the production of the recombinant $INVA_{AOX1}$ and $INVB_{AOX1}$ enzymes, respectively. These mutants were cultured in a 1-L flask, as described in the materials and methods section, and the obtained data were compared to the production of native INVA and INVB by *Z. mobilis* (**Fig. 2 A**). For the recombinant invertases, the highest production was obtained at 84 h in both cases, with the highest specific activity for $INVB_{AOX1}$ (2278±117 U/mg) compared to $INVA_{AOX1}$ (854±15 U/mg).

Our findings show that the expression of recombinant $INVA_{AOX1}$ and $INVB_{AOX1}$ in *P. pastoris* X-33 cells using 1-L baffled flasks in BMMY medium results in an increase in the specific activity by 6- and 7-fold, respectively (**Fig. 2 B** and **C**), compared to the native *Zymomonas* enzyme production system. The results for $INVA_{AOX1}$ and $INVB_{AOX1}$ in *P. pastoris* are comparable with those of other reports (**Table 1**) presenting similar values of

invertase-specific activity to those of Boβfruct2 (884 U/mg) and Boβfruct3 (1694.5 U/mg) from *Bambusa oldhammi* [25] and higher values than those reported for *I. batatas L.*, *B. vulgaris L.* and *A. cepa* with specific activities ranging from 1.08 to 553 U/mg [22-24, 26]. However, they are about 2-folds lower (3000- 3400 U/mg) compared with those obtained expressing *S. cerevisiae* invertase Suc2 [20-21] using the same promoter.

The conditions under which gene expression was induced may still be optimized, because approximately 50 % of the expressed protein is not secreted (data not shown). Glycosylation may be a factor that causes retention in the periplasm [27]. Some authors, like Amore et al. [43], have mentioned that by decreasing the temperature to 20 °C or less, the expression of heterologous proteins may improve, as cell lysis, protease secretion, and cell death are all decreased.

3.4 Purification of $INVA_{AOX1}$ and $INVB_{AOX1}$

Recombinant enzymes were purified to homogeneity from the culture supernatant of clones AX24 and BX29, after two steps of purification. A summary of the purifications steps is shown in **Table 3**. The specific activity of purified $INVB_{AOX1}$ was 1.2-fold higher than the value found for purified $INVA_{AOX1}$.

Purified invertases were analyzed by 10 % SDS-PAGE. The purified proteins had a MW of 68 and 48 kDa **(Fig. 3 A-B**) and did not migrate at the expected weights of 57 and 46 kDa of native INVA and INVB, respectively. The obtained values are 19 % and 4 % higher than the theoretically expected molecular mass. As these proteins do not have a polyhistidine or c-myc epitope, this weight gain is attributed to potential glycosylation of INVA and INVB, which are both known to undergo post-translational modification [17, 19].

3.5 Deglycosylation analysis of $INVA_{AOX1}$ and $INVB_{AOX1}$

In silico analysis of the potential N-glycosylation sites of INVA and INVB showed the presence of 4 and 2 glycosylation sites, respectively. However, neither exhibited any possible sites for O-glycosylation. To corroborate the prediction of the N-glycosylation sites, Schiff reagent was used to perform an experimental glycosylation assay described by Segrest and Jackson [36]. According to the Schiff assay, $INVA_{AOX1}$ and $INVB_{AOX1}$ (**Fig. 3 A lane 4, B lane 5**) are glycosylated, because a single red band matches the size noted above the purified protein on SDS-PAGE (at 57 and 48 kDa respectively), unlike their native forms produced by *Z. mobilis* [7,14]. The added sugar only represents 19 % and 4 % of the total weight of the respective proteins, which means that a number of GlcNAc monosaccharides were added to both $INVA_{AOX1}$ and $INVB_{AOX1}$.

Once the proteins were treated with EndoH, which cleaves the β(1→4) glycosidic bond between the core GlcNAc monosaccharides in N-linked glycopeptides, complete deglycosylation was achieved in both cases, as the bands of 57 and 46 kDa (**Fig. 3 A lane 6, B lane 3**) correspond to the theoretical MW calculated previously [7, 14].
The biochemical characterization of glycosylated and deglycosylated proteins was performed in order to evaluate the effect of glycosylation.

3.6 Characterization and effect of (de)glycosylation on $INVA_{AOX1}$ and $INVB_{AOX1}$

3.6.1 Optimal pH and temperature

$INVA_{AOX1}$, $INVB_{AOX1}$, d-$INVA_{AOX1}$, and d-$INVA_{AOX1}$ showed optimal activity at a pH of 5.5 (**Table 4**). These data are similar to those previously reported for native INVA (6.0) [7] and the same for native INVB (5.5) [13-15]. On the other hand, $INVA_{AOX1}$, $INVB_{AOX1}$, d-

$INVA_{AOX1}$, and d-$INVB_{AOX1}$ showed optimal activity at temperatures of 35 °C, 40 °C, 30 °C, and 40 °C, respectively (**Table 4**). $INVA_{AOX1}$ presents a 5 °C-increased optimal temperature in comparison to that reported for native INVA (30 °C) [7], whereas d-$INVA_{AOX1}$ shows the same optimal temperature as INVA. $INVB_{AOX1}$ and d-$INVB_{AOX1}$ showed the same optimal temperature as native INVB (40 °C) [14, 15].

These findings indicate that heterologous expression in *Pichia* and enzyme deglycosylation by treatment with EndoH do not substantially affect the optimal pH and temperature for activity of the recombinant enzymes. It has been previously reported that removal of the carbohydrate moiety by treatment with EndoH does not cause an important change in optimal pH and temperature [21,27,37,44], as was indeed observed in this study.

3.6.2 Thermal stability

The thermal stability of $INVA_{AOX1}$ at 35 °C ($t_{1/2}$ of 900 min) and 45 °C ($t_{1/2}$ of 480 min), was 1.8- and 1.6-fold higher than that of d-$INVA_{AOX1}$ at the same respective temperatures (**Fig. 4 A**). In contrast, the thermal stability of $INVB_{AOX1}$ at 35 °C ($t_{1/2}$ of 80 min) and 40 °C ($t_{1/2}$ of 50 min), was respectively 1.3- and 5-fold higher than that of d-$INVB_{AOX1}$ at these temperatures (**Fig. 4 B**). It is generally accepted that the removal of glycosyl groups results in a decreased stability, reflected by a reduction of half-life at the tested temperatures [39], as observed in this study. Several authors have pointed out that carbohydrates confer stability to enzymes by covalent interactions, protecting the enzyme from heat denaturation and premature aggregation of the unfolded enzyme at high temperatures e.g., native invertases [3,44], and recombinant phytase [37] and elastase [38] expressed in *P. pastoris*.

3.6.3 Effect of metal ions

The metal ions Hg^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} showed an inhibitory effect on the activity of both $INVA_{AOX1}$ and $INVB_{AOX1}$ at all tested concentrations (1, 5, and 10 mM). This suggests the presence of thiol groups, which are important for enzyme activity, in the catalytic site of the enzyme [40]. Interactions with –SH or –S on proteins groups may produce conformational changes in the protein structure. Cu^{2+} oxidizes cysteine residues in the protein and causes structural changes and therefore an alteration in the activity [2]. Ca^{2+} (1 mM) increased the activity of $INVA_{AOX1}$ by 20 %, but no positive effect was observed for $INVB_{AOX1}$. Ca^{2+} is an unusual activator of bacterial invertase; however, the ions in solution can interact with charged amino groups in the protein, which may alter the conformational structure of the enzyme, acting directly on the catalytic site and affecting its interaction with the substrate [42]. The presence of metal ion Mn^{2+} increased the enzymatic activity of $INVA_{AOX1}$ (1 mM) and $INVB_{AOX1}$ (5 mM) by 80 % and 20 %, respectively. This activating effect has also been seen for invertases from *Aspergillus ochraceus*, *Aspergillus phoenicis*, and the yeast *Candida guilliermondii* [3,42,44].

3.6.4 Kinetic parameters

The Michaelis constant (K_M), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_M) for $INVA_{AOX1}$, $INVB_{AOX1}$, d-$INVA_{AOX1}$, and d-$INVB_{AOX1}$ were determined (**Table 4**). The affinity of $INVA_{AOX1}$ for the substrate sucrose was 2.6-fold higher than that of INVA from *Z. mobilis* [7], similar to what was previously reported [6]. The affinity of $INVB_{AOX1}$ for sucrose was 1.7 to 2-fold higher than that of INVB from *Z. mobilis* [7,13-15]. The affinity of d-$INVA_{AOX1}$ was 2-fold lower than that observed for $INVA_{AOX1}$, whereas the substrate affinity of $INVB_{AOX1}$ was not affected by deglycosylation.

The k_{cat} of $INVA_{AOX1}$ was 100 to 200-fold higher than that reported for INVA from *Z. mobilis* [6, 7]. The k_{cat} of $INVB_{AOX1}$ was similar than that registered for INVB from *Z. mobilis* [14]. The k_{cat} of d-$INVA_{AOX1}$ and d-$INVA_{AOX1}$ was 1.3-fold lower than that observed for $INVA_{AOX1}$ and $INVB_{AOX1}$.

The catalytic efficiency of $INVA_{AOX1}$ was 100 to 200-fold higher than that reported for INVA from *Z. mobilis* [6, 7], while that of $INVB_{AOX1}$ was similar to the catalytic efficiency registered for INVB from *Z. mobilis* [14, 15]. The catalytic efficiencies of d-$INVA_{AOX1}$ and d-$INVB_{AOX1}$ were 3- and 1.3 fold lower than those observed for $INVA_{AOX1}$ and $INVB_{AOX1}$, respectively. Interestingly, compared to d-$INVA_{AOX1}$, d-$INVB_{AOX1}$ displayed a 1.5-fold higher catalytic efficiency (**Table 4**), an expected finding according to the report of Aït-Abdelkder et al. [6]. However, $INVA_{AOX1}$ exhibited a 1.5-fold higher catalytic efficiency than $INVB_{AOX1}$. These findings may be explained by the fact that INVA (19 %) showed a higher content of carbohydrate than did INVB (4 %), which is in agreement with the higher number of potential N-linked glycosylation sites in the amino acid sequence of INVA.

$INVA_{AOX1}$ and $INVB_{AOX1}$ were highly active at sucrose concentrations up to 400 and 300 mM, respectively, similar to previous reports for native INVA [7] and INVB [14]. Both d-$INVA_{AOX1}$ and d-$INVB_{AOX1}$, however, were highly active at sucrose concentrations of 300 mM (**Fig. 5 A and B**). Interestingly, deglycosylation negatively affected the tolerance to substrate of $INVA_{AOX1}$, again the enzyme with 19 % carbohydrate content. It is worth mentioning that $INVA_{AOX1}$, $INVB_{AOX1}$, d-$INVA_{AOX1}$, and d-$INVB_{AOX1}$, as well as native INVA [7] and INVB [14] exhibit a higher sucrose tolerance than did *S. cerevisiae* invertase, which is inhibited by 150 mM sucrose [16].

In summary, the kinetic properties of $INVA_{AOX1}$ and $INVB_{AOX1}$ were negatively affected by deglycosylation, thus suggesting that glycosylation plays an important role in the

substrate affinity, turnover number, and catalytic efficiency of these enzymes. These finding were particularly evident in the case of $INVA_{AOX1}$, an enzyme that showed a much higher content of carbohydrates (19 %) than $INVB_{AOX1}$ (4 %). The decrease in activity for deglycosylated forms has been previously reported [41, 44]. Schilling et al. [45] expressed the human glutaminyl cyclase in *P. pastoris* and achieved a decrease in K_M and an increase in k_{cat}, compared to that observed when the same enzyme was expressed in *E. coli*; this observation was attributed to a small conformational change that occurred owing to the possible addition of disulfide bonds.

4. Conclusions

The INVA and INVB invertases from *Z. mobilis* were successfully expressed in *P. pastoris* and their production levels in this system, under the control of AOX1 promoter, were 6- and 7-fold greater than those achieved in bacterial expression systems . The recombinant $INVA_{AOX1}$ and $INVB_{AOX1}$ invertases were purified to homogeneity as glycoproteins, with a carbohydrate content of 19 % and 4 %, respectively. Although both $INVA_{AOX1}$ and $INVB_{AOX1}$ recombinant invertases expressed in *P. pastoris* exhibited an improvement in their catalytical properties, $INVA_{AOX1}$ displayed better substrate affinity, turnover number and catalytical efficiency than $INVB_{AOX1}$.The biochemical characterization of $INVA_{AOX1}$ and $INVB_{AOX1}$, and the deglycosylated form of these enzymes shows that glycosylation plays an important role in the thermal stability and kinetic properties of $INVA_{AOX1}$ and $INVB_{AOX1}$ invertases; as well as in the sucrose tolerance of $INVA_{AOX1}$. To our knowledge, this the first study on the effect of glycosylation on the biochemical properties of invertases that are heterologously expressed in *P. pastoris*.

The recombinant invertases obtained in this work exhibit improved kinetic properties compared to those of the native INVA and INVB invertases produced by *Z. mobilis*, as well as a higher tolerance to sucrose compared to commercial invertase from *S. cerevisiae*. Therefore, these enzymes could be valuable in mesophilic processes, such as the fermentation of cane molasses for the production of ethanol. Furthermore, since most industrial processes require enzymes tolerate to temperatures ranging from 55 to 70 °C, the design and selection of thermostable $INVA_{AOX1}$ and $INVB_{AOX1}$ would be an interesting area of future research.

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Legends of Figures

Fig. 1. Invertase activity in culture supernantant from *P. pastoris* cells transformed with construct pPICZαB-*invA* (clones AX19, 23, 24, 31, and 56), and construct (pPICZαB-*invA*) (clones BX3, 5, 12, 26, 29, 32, and 36). *P. pastoris* X-33 wild type (clone X-33), and *P. pastoris* transformed with pPICZαB without insert (clone C11) were used as negative controls.

Fig. 2. Production of invertases. **A**. Natives INVA and INVB invertases from *Z. mobilis*. **B**. AX24 clone expressing $INVA_{AOX1}$. **C**. BX29 clone expressing $INVB_{AOX1}$.

Fig. 3. SDS- PAGE glycosylation analysis of $INVA_{AOX1}$ and $INVB_{AOX1}$. **(A):** Coomasie blue stained gel. **1.** MW marker; **2.** *S. cerevisiae* invertase (positive control); **3.** $INVA_{AOX1}$**. (B):** Deglycosylation of $INVA_{AOX1}$. **1.** MW marker; **2.** $INVA_{AOX1}$ treated with EndoH (d-$INVA_{AOX1}$). **(C):** Schiff stained gel. **1.** *S. cerevisiae* invertase (positive control); **2.** $INVA_{AOX1}$. **(D):** Coomasie blue stained gel. **1.** MW marker; **2.** *S. cerevisiae* invertase (positive control); **3.** $INVB_{AOX1}$; **4.** $INVB_{AOX1}$ treated with EndoH (d-$INVB_{AOX1}$). **(E):** Schiff stained gel. **1.** *S. cerevisiae* invertase (positive control); **2.** $INVB_{AOX1}$; **3.** d-$INVB_{AOX1}$.

Fig. 4. Effect of deglycosylation on thermal stability. **A**. Thermal stability of $INVA_{AOX1}$ (–■–) and d-$INVA_{AOX1}$ (–■–) at 35 °C; and $INVA_{AOX1}$ (–▲–) and d-$INVA_{AOX1}$ (–▲–) at 45 °C. **B**. Thermal stability of $INVB_{AOX1}$ (–■–) and d-$INVB_{AOX1}$ (–■–) at 35 °C; and $INVB_{AOX1}$ (–▲–) and d-$INVB_{AOX1}$ (–▲–) at 40 °C.

Fig. 5. Effect of deglycosylation on reaction rate. **A**. $INVA_{AOX1}$ (–■–) and d-$INVA_{AOX1}$ (–■–). **B**. $INVB_{AOX1}$ (–■–) and d-$INVB_{AOX1}$ (–■–).

Figure 1

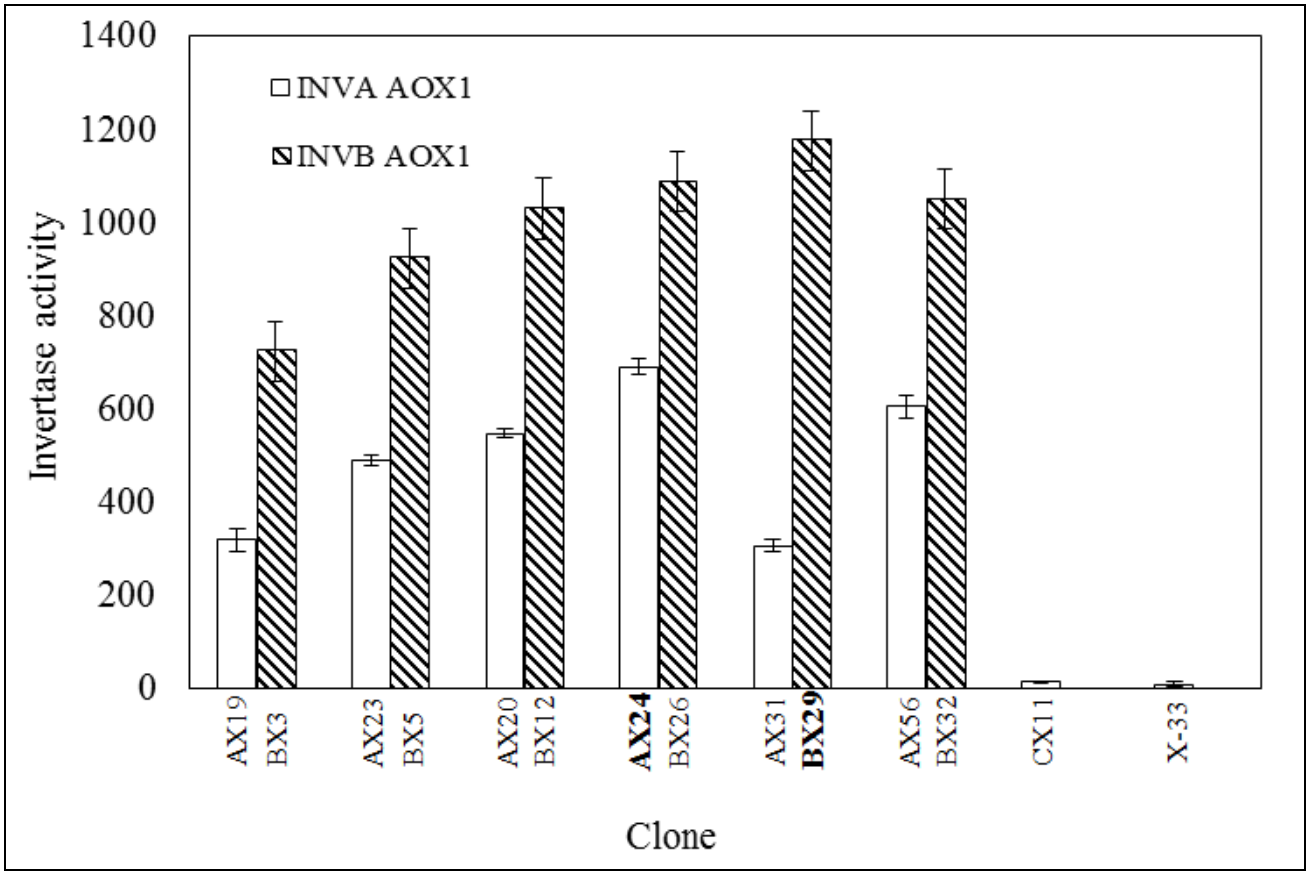
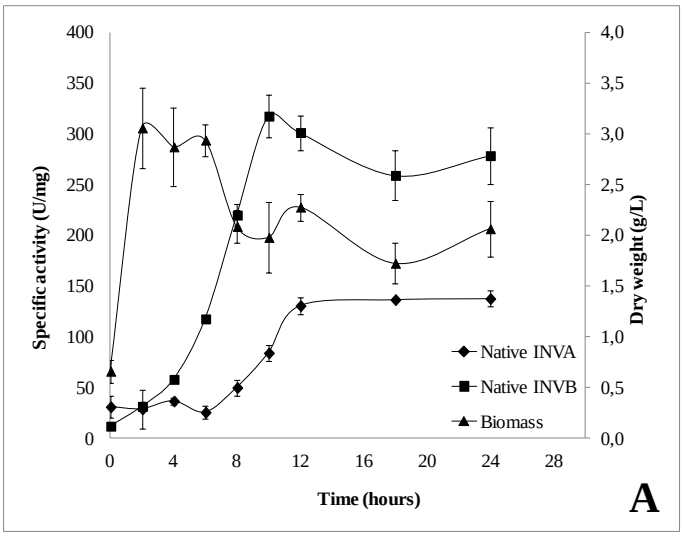
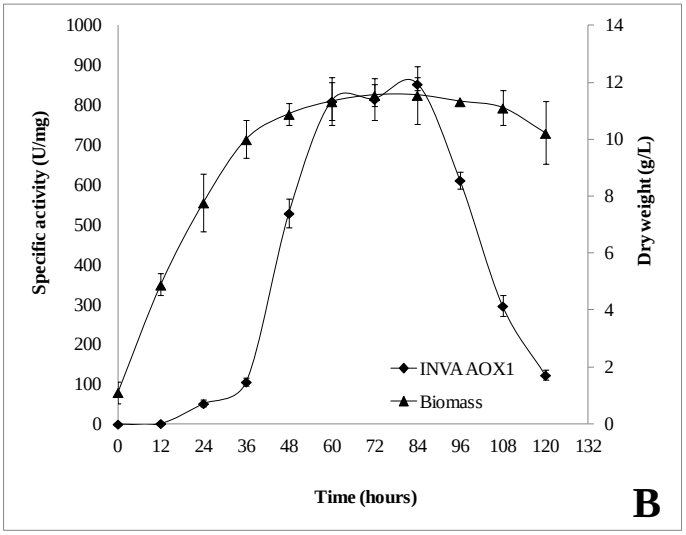


Figure 2





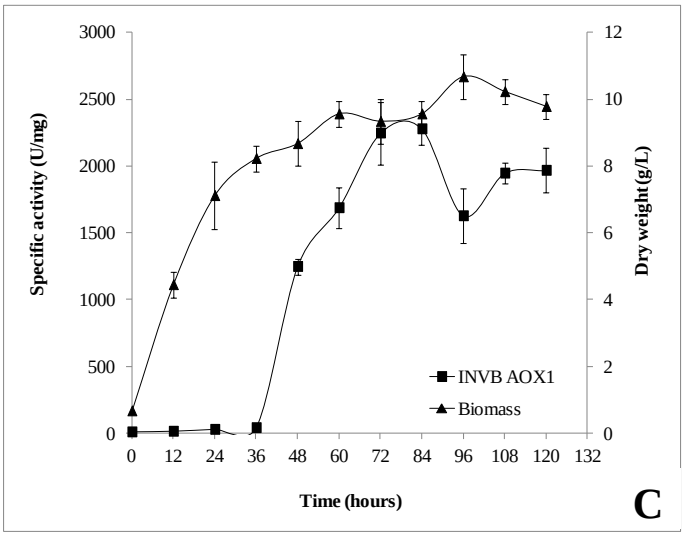


Figure 3

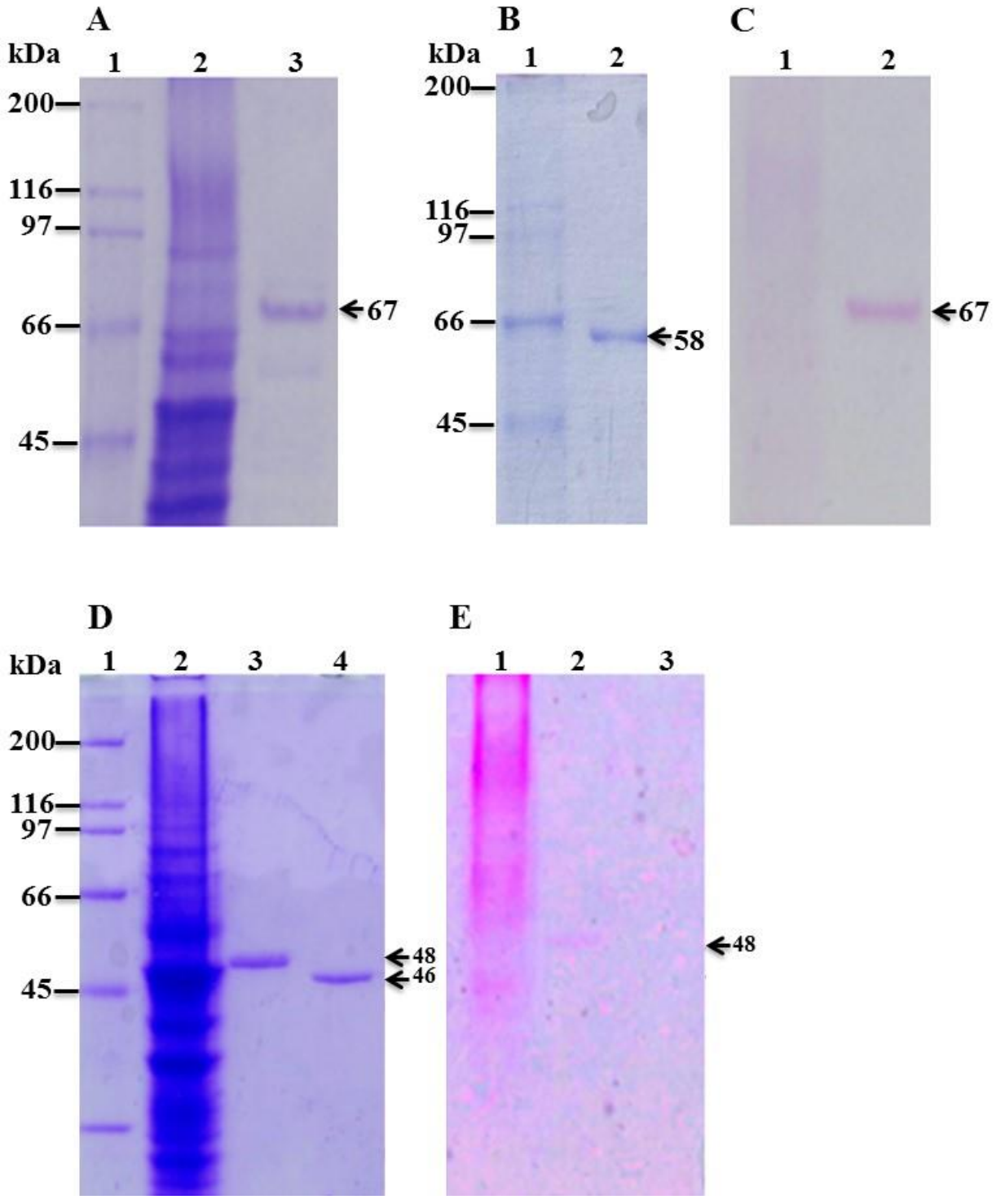
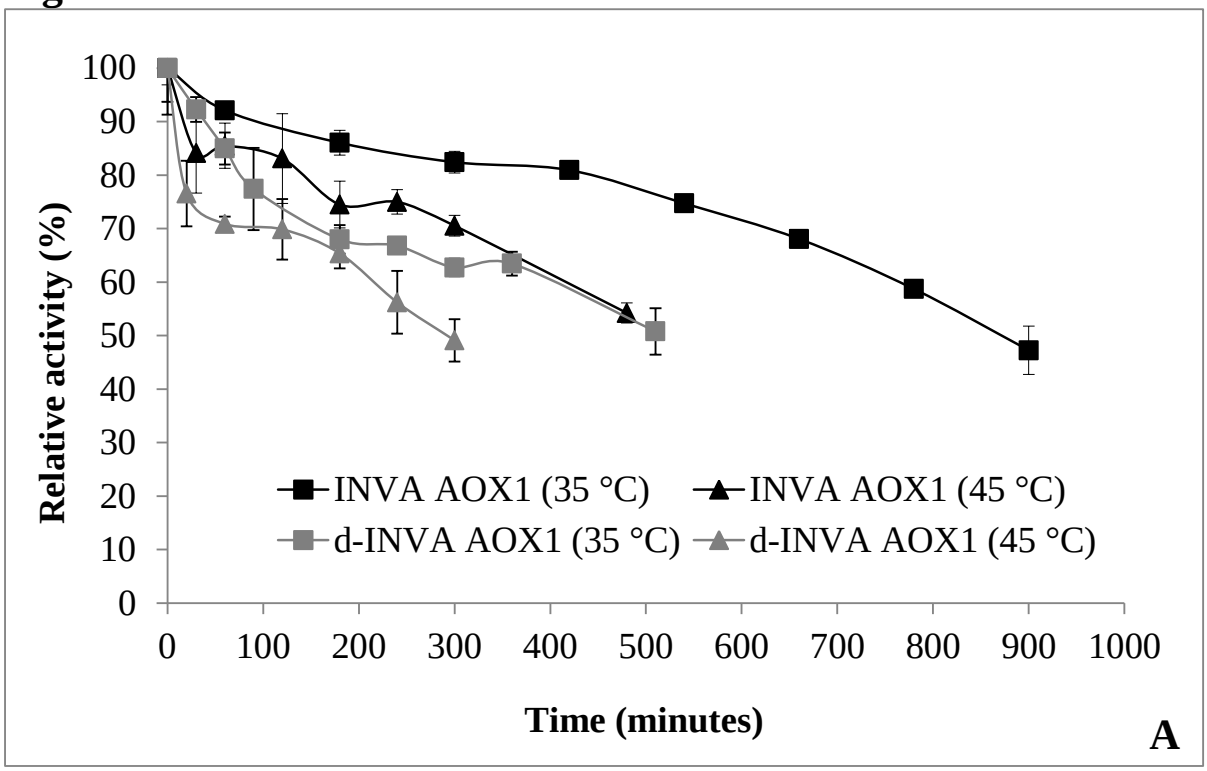


Figure 4



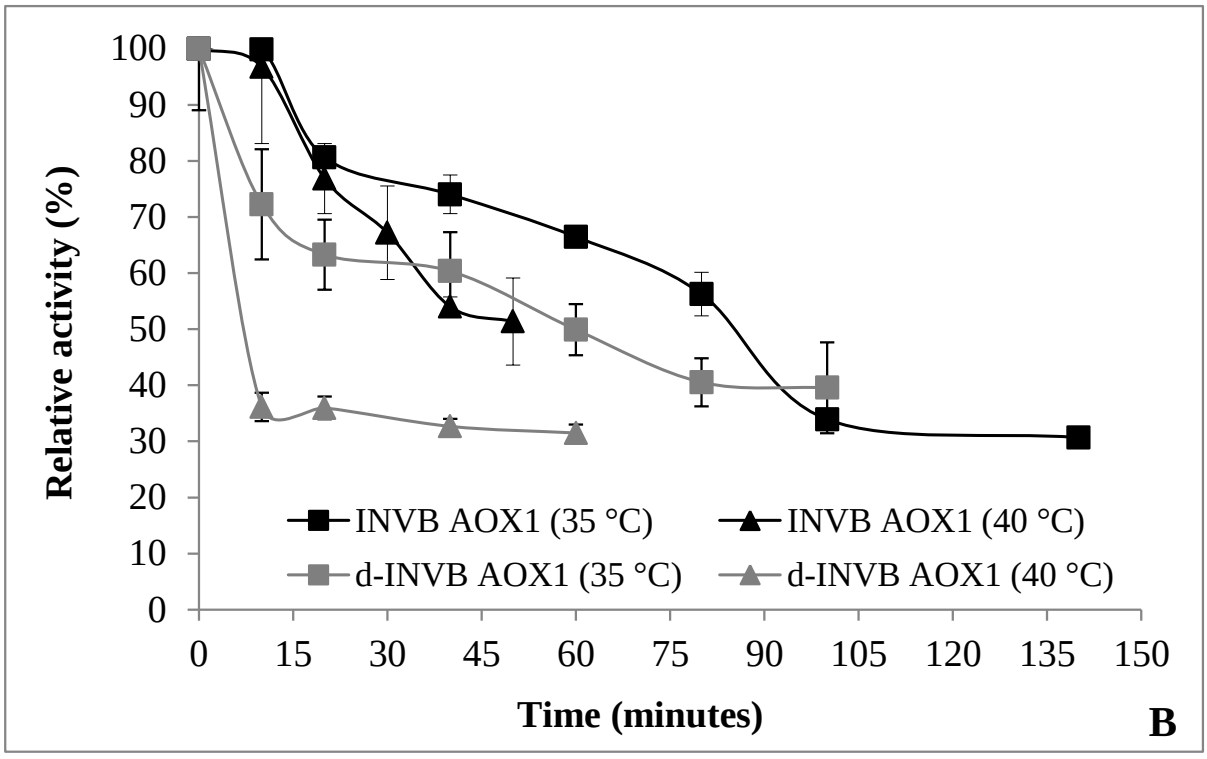


Figure 5

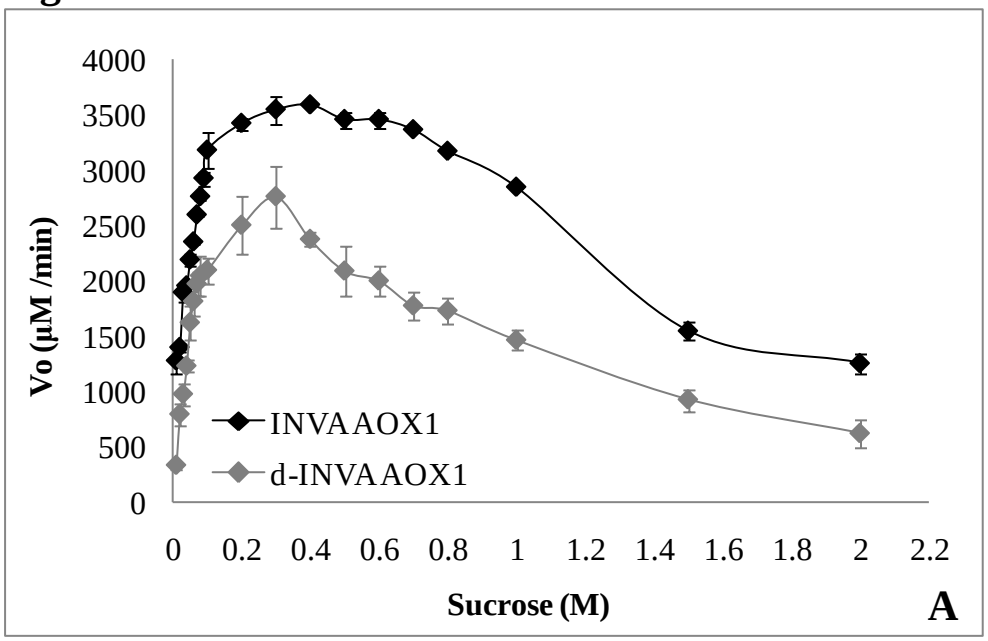


Table 1. Invertases heterologous expressed in *Pichia pastoris* /AOX1 promoter.

Organism Enzime	**Vector**	**MW (kDa)**	**K_M (mM)**	**Optimum pH/temp (°C)**	**Ions Activator/ Inhibitor**	**Specific activity (U/mg)**	**Ref.**
S. cerevisiae SUC2	pGS102 AOX1	85-90 (G)	Nd	Nd	Nd	3000	[20]
S. cerevisiae SUC2	Nd	85 (G)	Nd	6.0, 55	Nd	3400	[21]
I. batatas L Ib*β*fruct1	pPICZαB AOX1	72 (G)	10.69	5.0,40	Nd	1.08	[22]
B. vulgaris L. 6-FEH	pPICZαA AOX1	75	77	5.0, 30	Nd	12.5	[23]
I. batatas L Ib*β*fruct2 Ib*β*fruct3	pPICZαC AOX1	82 (G) 87 (G)	4.97 10.1	5.0 5.0	Nd	553 70	[24]
B. oldhamii Bo*β*fruct2 Bo*β*fruct3	pPICZαB AOX1	77.5 (G) 77.5 (G)	0.42 22.9	3.0, 60 4.0, 50	Ca^{2+},Co^{2+}/ Hg^{2+} None/Hg^{2+}	884.3 1694.5	[25]
Allium cepa GFT-INV INV	pPICZαC AOX1	Nd	1 6	Nd	Nd	45 4.3	[26]

(G): Glycosylated; (Nd): Not determined

Table 2. List of oligonucleotide primers used for PCR studies in this work.

Name	Sequence (5'-3')
INVA-Forward (AF)	GAA CTG CAG GTG AAT CCC CCT CTT ATA AAA ATT TA TC
INVA-Reverse (AR)	TGC TCT AGA **TTA** ACA GGC ATC GCT TGA AAA AGC G
INVB-Forward (BF)	GAA CTG CAG GGT TTA ATT TTA ATG CCA GTC GCT GG
INVB-Reverse (BR)	TGC TCT AGA **TTA** TTT GCG ACG ATC AGG GAA AGG CC
α-Factor Forward	TAC TAT TGC CAG CAT TGC TGC
3' AOX Reverse	GCA AAT GGC ATT CTG ACA TCC

Table 3. Purification of $INVA_{AOX1}$ and $INVB_{AOX1}$ from AX24 and AX29 *P. pastoris* X-33 clones.

Fraction	**Total Activity (10^3 U)**		**Total Protein (mg)**		**Specific Activity (U/mg)**		**Yield %**		**Purification factor %**	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Crude extract	59.8±10	15.81±2	400±2	23.34±0.08	149±10	678±2	100	100	1	1
Protein concentration	8.21±17	11.46±28	8±0.02	7.81±0.07	1007±17	1468±28	13.72	72.48	6.75	2.17
Ion exchange chromatography	4.47±12	7.59±15	3±0.03	3.81±0.01	1660±11	1993±15	7.47	48	11.13	2.94

Notes: **A** is denoted as $INVA_{AOX1}$ and **B** as $INVB_{AOX1}$.

Table 4. Comparison of the biochemical and kinetic properties of native INVA and INVB from *Z. mobilis*, and the recombinant $INVA_{AOX1}$, d-$INVA_{AOX1}$, $INVB_{AOX1}$ and d-$INVB_{AOX1}$ expressed in *P. pastoris*.

Enzyme	MW (kDa)	K_M (mM)	k_{cat} (1/s)	k_{cat}/K_M (1/s mM)	Optimum Temperature (°C), pH	Ions effect (1 mM)		Sucrose inhibition (mM)	Reference
						Activator	Inhibitor		
Native INVA	57	110	56.92	0.517	30, 6.0	ND	Hg^{+2}	400	[7]
$INVA_{AOX1}$	68	41	12149	296	35, 5.5	Mn^{+2}	Cu^{+2} Hg^{+2}	400	**This work**
d-$INVA_{AOX1}$	57	83	8831	107	30, 5.5	ND	ND	300	**This work**
Native INVB	46	98	12000	122	40, 5.5	Mn^{+2} Ca^{+2}	Hg^{+2} Ni^{+2} Cu^{+2} Zn^{+2}	300	[14,15]
$INVB_{AOX1}$	48	50	10396	208	40, 5.5	Mn^{+2}	Hg^{+2} Ni^{+2} Cu^{+2} Zn^{+2}	300	**This work**
d-$INVB_{AOX1}$	46	50	8210	164	40, 5.5	ND	ND	300	**This work**

MW: Molecular weight. The k_{cat} value was calculated assuming protein concentrations of 0.375 mg/ml for $INVA_{AOX1}$, 0.381 mg/ml for $INVB_{AOX1}$, 0.422 mg/ml for d-$INVA_{AOX1}$ and 0.393 mg/ml for d-$INVB_{AOX1}$