REPORT

Identification and recombinant expression of a bacterial exolevanase useful for the production of high fructose syrups

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ABSTRACT

The sugarcane endophyte *Gluconacetobacter diazotrophicus* produces levan from sucrose by a secreted levansucrase (LsdA). A levanase-encoding gene (*lsdB*) was identified starting 52 bp downstream of the *lsdA* gene. Recombinant expression of the *lsdB* gene in *Escherichia coli* and *Pichia pastoris* resulted in a functional protein capable of hydrolyzing levan and inulin to free fructose without the formation of oligofructans, indicating exo-type activity. LsdB was efficiently secreted into the *P. pastoris* culture medium driven by the *Saccharomyces cerevisiae* alpha-factor signal peptide using either the methanol-inducible *AOX1* or the constitutive *GAP* promoter. The recombinant protein was not glycosylated at its single potential *N*-glycosylation site. The *GAP* promoter-driven expression of the *lsdB* gene did not cause cell toxicity and provided for a three-fold higher productivity (26.6 U mL^{-1}; 39 h fermentation) than the methanol-inducible system (21.1 U mL^{-1}; 96 h fermentation). We conclude that the *P. pastoris* constitutive system provides a convenient alternative for the large-scale production and secretion of LsdB, an enzyme commercially attractive to convert polyfructans into high fructose syrups.

*I*ntroduction

High fructose syrups (HFS) are widely used as sweeteners in the beverage industry. HFS are currently produced from corn starch in a two step process which requires hydrolysis to glucose and transformation to fructose using the enzyme glucose isomerase. The about 55/45 mixture of glucose and fructose can be enriched by chromatographic separation, but significantly increasing the cost price of HFS [1]. From a technical point of view it is much easier to produce HFS from the natural polyfructans levan and inulin, since this only needs a straightforward hydrolysis of the fructan to fructose. This procedure yields syrups of high purity which can also be used for the production of crystalline fructose with extended food applications.

In nature, several fructan-producing bacteria like *Bacillus subtilis* [2], *Actinomyces viscosus* [3], *Bacteroides fragilis* [4], *Paenibacillus polymyxa* [5] and *Bacillus stearothermophilus* [6] express fructanases under restricted catabolic conditions. Among fructanases, fructose-releasing levanases degrade the β-(2-6)-linked levan and frequently split the β-(2-1) linkages of inulin to successively liberate free fructose from both substrates. Thus, they are potentially useful for the commercial production of fructose syrups of high purity.

Escherichia coli and *Pichia pastoris* have been successfully employed for the large-scale production of a broad range of heterologous proteins used in the industry. Their popularity as recombinant hosts can be attributed to their well-known genetics, fast high-density cultivation and the large number of compatible tools available for biotechnology [7, 8]. Both hosts are particularly well suited to the industrial production of recombinant fructanases due to the lack of endogenous fructan-utilizing enzymes. In addition, *P. pastoris* offers the feasibility of protein secretion driven by native or yeast signal peptides, this aspect and the low levels (about 0.5%) of endogenous proteins in the culture supernatants facilitates the purification of the recombinant protein.

The sugarcane endophyte *Gluconacetobacter diazotrophicus* secretes a constitutively expressed levansucrase, which converts sucrose to β-1,2-oligofructans and levan [9]. The levansucrase structural gene (*lsdA*) was cloned and found to be responsible for sucrose utilization in strains recovered from different host plants [10, 11].

This work describes the isolation and recombinant expression of the *G. diazotrophicus* exolevanase gene (*lsdB*) in the biotechnological hosts *Escherichia coli* and *Pichia pastoris*. The encoded protein successively released the terminal fructose units from the polyfructans levan and inulin, making it attractive for the industrial production of fructose syrups of high purity.

*M*aterials and methods

Microorganisms, media, substrates, and plasmids

Escherichia coli XL-1 Blue (Stratagene, La Jolla, CA, USA) was used as a cloning host and for plasmid propagation. *E. coli* BL21(DE3) and *Pichia pastoris* wild-type strain X-33 (InvitrogenBV, Groningen, The Netherlands) were used as expression hosts. *E. coli* was grown at 37 ºC in LB medium or low salt LB medium prepared as described by Invitrogen [12]. Ampicillin (50 μg mL^{-1}) or zeocin (25 μg mL^{-1}) were added as needed. For fermentation experiments, *P. pastoris* was grown during the batch phase in a minimal medium [2.5% (v/v) glycerol, 0.42% (w/v) KH_2PO_4, 0.37% (w/v) $MgSO_4 \cdot 7H_2O$, 0.023% (w/v) $CaCl_2 \cdot 2H_2O$, and 0.04% (w/v) Na-EDTA] containing vitamins and traces prepared as recommended by Cregg, *et al*. [13]. Solutions of 30% (v/v) ammonium hydroxide and 85% (v/v) *ortho*-phosphoric acid were used to control the culture pH at 5.0 during the batch and the feeding phases.

Levan from *Erwinia herbicola*, inulin from Dahlia tubers, D(+)-melezitose, D(+)-raffinose, and sucrose

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were used as substrates for LsdB activity. All the substrates were from Sigma (St. Louis, MO, USA).

Plasmid pET3d is a ColE1-replicon, ampicillin-resistant vector that contains the T7 RNA polymerase promoter, the gene 10 translation start codon at the *Nco*I site and a T7 transcription terminator [14]. Plasmid pALS3 is a derivative of pUC19 in which the *lsdB* gene was inserted in the *Eco*RI site as a 3.2-kb *Eco*RI fragment from cosmid p21R1, previously recovered from a genomic library of the *G. diazotrophicus* strain SRT4 [10]. Plasmids pGAPZαC and pPICZαC are ColE1-replicon, zeocin-resistant vectors that contain the *GAP* and *AOX1* promoters, respectively (Invitrogen BV, Groningen, The Netherlands).

DNA sequencing and analysis

The 3.2 kb *Eco*RI insert of plasmid pALS3 was digested with *Kpn*I, *Sma*I, or *Sph*I and the resulting fragments were cloned into the corresponding sites in pUC18. Sequencing was performed using pALS3 and the derivatives overlapping clones as the template by the dideoxy chain-termination method [15] with the use of a Sequenase sequencing kit (US Biochemicals) and 7-deazadGTP to clarify regions containing GC compressions. The whole sequence of the levanase gene was determined on each strand using the universal forward and reverse primers and the primer walking procedure with 16-mer oligonucleotides as primers. The nucleotide sequences were compiled by the BioSOS program package [16]. The search for prokaryote promoter sequences was done using the WWW Signal Scan IMD Search Service accessible at http://bimas.dcrt.nih.gov/molbio/matrixs/. The BLAST program [17] and the CLUSTAL W program [18] were used for similarity searches in databases and for multiple sequence alignments, respectively. The cleavage site for signal peptidase was predicted by using the SignalP program (www.cbs.dtu.dk/services/SignalP/).

Construction of vector for levanase expression in *Escherichia coli*

A 1605-bp fragment, consisting of the entire coding region of the *lsdB* gene, was amplified by the polymerase chain reaction (PCR) using cosmid p21R1 as the template and primers containing *Nco*I and *Bam*HI restriction sites flanking the insert. The PCR product was ligated to *Nco*I-*Bam*HI digested pET3d and transferred into *E. coli* XL1-Blue. The resulting plasmid, designated pALS112, was used to transform the expression host *E. coli* BL21(DE3) that contains a chromosomal copy of the T7 RNA polymerase gene under the control of the IPTG-inducible lacUV5 promoter.

Construction of vectors for levanase expression in *Pichia pastoris*

A 1520-bp DNA fragment coding for the predicted mature part of levanase (LsdB) from *G. diazotrophicus* was amplified by PCR using the plasmid pALS5 [10] as template and the primers 5`CTCGGGGC**AT**CG**AT**CGCGGCCGATACG and 5`GGGATTT**TC**TA**A**GCCAGCACCGCCAC with base substitutions (bold letters) to create *Cla*I and *Xba*I restriction sites (underlined). The PCR product (without *lsdB* stop codon) was *Cla*I-*Xba*I digested and inserted in the corresponding sites of the expression vectors pGAPZαC and pPICZαC yielding the plasmids pALS175 and pALS177, respectively. The in-frame fusions of the *lsdB* gene to the alpha factor signal sequence of *S. cerevisiae* at the 5´ end and to the *myc* epitope and the His6 tag at the 3´ end in both constructs were confirmed by DNA sequencing. *P. pastoris* X-33 cells were transformed by electroporation with 5 µg of either *Avr*II-linearized pGAPZαC (control), *Avr*II-linearized pALS175, or *Sac*I-linearized pALS177, following the conditions recommended by Invitrogen [12]. Transformants were selected on YPDS plates supplemented with zeocin (100 µg mL^{-1}) after incubation for 3 days at 30 ºC.

Culture of *E. coli* and preparation of cell-free extracts

E. coli was grown aerobically at 37 °C in LB medium. For levanase expression, BL21(DE3) containing plasmid pALS112 was grown until the culture reached an OD_{600} of 0.6. At this point IPTG was added to a final concentration of 0.4 mM, and shaking was continued for 3 h. Cells from 50 mL cultures were harvested by centrifugation at 5 000 *g* for 10 min at 4 °C, resuspended in 12 mL of distilled water, and disrupted by ultrasonication with a sonifier (Branson). Five sonication rounds were performed for 30 sec on ice, with 1 min intervals on ice. The cell debris was removed by centrifugation at 12 000 *g* for 10 min at 4 °C, and the supernatant fraction, designated soluble extract, was assayed for enzyme activity.

Fed-batch fermentation of *P. pastoris* clones PGAP6 and PAOX12

Fermentations were performed using 7.5-l fermenters (B.E. Marubishi, Tokyo, Japan) interfaced with the computer-based software FERMACS for data acquisition and supervisory control (CIGB, Havana, Cuba). Fermenters containing 3.5 l of the above-mentioned minimal medium were inoculated to an initial OD_{600}~2 with cultures of the clones PGAP6, PAOX12, or the *P. pastoris* transformed with empty vector pGAPZαC. The operation conditions during the batch phase were 30 ºC, pH 5.0, aeration rate constant at a flow of 4l min^{-1}, and agitation 500 rpm. The end of the batch phase upon depletion of glycerol was judged by a sharp increase of pH and pO_2. For constitutive LsdB expression, the feeding medium (50% (v/v) glycerol and trace elements was added with gradually increased flows between 29.5 and 81.2 mL min^{-1} L^{-1} of starting volume. For inducible LsdB expression, the feeding medium [87.5% (v/v) methanol and trace elements] was added gradually at rates between 3.1 and 7.3 mL min^{-1} L^{-1} of initial volume. The operation conditions during the feeding phase were 30 ºC, pH 5.0, aeration rate constant at a flow of 8l min^{-1}, and agitation 700 rpm. The fed-batch phase started at 16 and 18 h in the constitutive and methanol-inducible systems, respectively.

LsdB purification

Purification of LsdB produced constitutively in *P. pastoris* was performed by immobilization-metal affi-

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12. Pichia pastoris expression kit. A manual of methods for expression of recombinant proteins in *Pichia pastoris*, version F. San Diego, USA: Invitrogen Corporation.

nity chromatography (IMAC), using Ni^{2+}-nitrilotriacetic acid agarose beads (Ni-NTA) from QUIAGEN (Hilden, Germany). The culture supernatant (4.8l) of clone PGAP6 was five-fold concentrated in a rotatory evaporator, adjusted to pH 7.0 with sodium phosphate buffer (0.1 M final concentration), and mixed in batch with 25 mL of Ni-NTA resin overnight at 4 ºC to bind the enzyme containing the polyhistidine tag. The agarose beads were recovered by centrifugation at 3 500 rpm for 5 min and packed into a column. Protein elution was carried out with 0.1 M sodium acetate (pH 5.0) containing 0.15 M imidazole. The fractions with levanase activity were pooled and dialyzed against 10 mM sodium acetate (pH 5.0).

Protein quantification and enzyme assays

Proteins were quantified as described by Bradford [20]. LsdB purity in the culture supernatants or the purification fraction was determined by densitometric analysis of 12.5% polyacrylamide gels stained with Coomassie brilliant blue R-250 (Sigma). Levanase activity was measured as the fructose released from levan hydrolysis using the dinitrosalicylic acid (DNSA) test [20]. One unit of enzyme is defined as the amount of enzyme releasing 1 µmol of fructose per min based on initial velocity measurements under the following conditions: 1% (w/v) levan in 0.1M sodium acetate buffer, pH 5.0 at 30 ºC. LsdB reaction products were separated by thin-layer chromatography (TLC) on silica-gel 60 plates (Merck) using acetone water (9:1, v/v) as the solvent. After two runs, the fructose-containing sugars were made visible by spraying the plates with a solution of 3% (w/v) urea, 1 M phosphoric acid in water-saturated butanol, and heating at 80 °C for about 20 min.

Endoglycosidase Hf treatment

Purified LsdB (80 µg) was denatured in 100 µL of 0.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol at 100 ºC for 10 min. After addition of 1:10 (v/v) 1 M sodium citrate buffer, pH 5.5 at 25 ºC, the sample was made to react with endoglycosidase Hf (New England Biolabs, USA) at 0.25 U μg^{-1} of total protein at 37 ºC for 5 h.

Polyacrylamide gel electrophoresis and Western blot

SDS-PAGE in 12.5% gels was preformed according to Laemmli [21]. For Western blots, proteins were electrotransferred onto nitrocellulose membranes (Amersham Pharmacia-Biotech, Uppsala, Sweden) using a Mini Trans- Blot Electrophoresis Transfer (BIORAD, Richmond, CA, USA) at a constant current of 350 mA for 1.30 h. The immunological detection of LsdB was achieved using polyclonal antibodies generated in rabbit against LsdB expressed in E. *coli* and the ProtoBlot AP system (Promega, Madison, USA). The presence of mannose chains in the recombinant LsdB was assayed in Western blot using the Digoxigenin Detection Kit (Roche Diagnostics GmbH, Germany) and *Galanthus nivalis* agglutinin as the digoxigenin-labeled lectin. The B-ribonuclease containing three *N*-glycosylation sites was used as the control glycoprotein.

Nucleotide sequence accession number

The complete sequence of the *lsdA* and *lsdB* genes from *Gluconacetobacter diazotrophicus* SRT4 is available in the European Molecular Biology Laboratory (EMBL) database under accession number L41732.

Results and discussion

Cloning and sequence analysis of *Gluconacetobacter diazotrophicus* levanase gene (*lsdB*)

The nucleotide sequence of the 3.2-kb *Eco*RI fragment generated from pALS3 was determined on both strands. Examination of the sequence revealed the presence of a 1 605-bp ORF, designated *lsdB*, starting 52 bp downstream of the stop codon of the levansucrase structural gene (*lsdA*) [10, 22]. The intergenic region comprises a potential stem-loop structure with a calculated free energy of -24.1 kcal mol^{-1}. No promoter-like sequences preceded the presumptive ATG initiation codon of *lsdB* and no additional ORFs were identified in the region immediately downstream of the *lsdB* stop codon, suggesting that *lsdA* and *lsdB* constitute a two-gene operon.

The *lsdB* gene encodes a polypeptide of 534 residues with a calculated molecular mass of 58.4 kDa and a pI of 6.47. The N-terminal part of the encoded protein has the essential features of a signal peptide for translocation across the cytoplasmic membrane with the peptidase cleavage site predicted between Ala-36 and Ala-37, according to the SignalP program. Removal of the first 36 residues of the presumed precursor LsdB generates a mature protein with a calculated molecular mass of 54.5 kDa and a pI of 5.95.

A BLAST search of the predicted LsdB against available databases showed the highest scores of similarities for microbial β-D-fructofuranosidases of the glycoside hydrolase family 32, most particularly fructanases. Sequence comparisons revealed that LsdB is 49%, 34%, 33%, 32%, and 29% identical to levanases from *Burkhordelia mallei* [23], *Actinomyces naeslundii* [24], *Bacillus subtilis* [25], *Paenibacillus polymyxa* [5] and *Bacteroides fragilis* [4], respectively.

Substrate specificity and action mode of recombinant LsdB produced in *E. coli*

To verify if the *lsdB* gene indeed encodes a β-fructanase, we constructed the plasmid pALS112 containing the *lsdB* coding region under the control of the T7 RNA polymerase promoter. After IPTG induction, *E. coli* BL21(DE3) cells transformed with pALS112 produced a polypeptide with the expected molecular mass of approximately 55 kDa, as deduced by SDS/PAGE (data not shown). Soluble extracts of sonicated cells released free fructose when reacting on sucrose [αglu(1.2)βfru], raffinose [galα(1.6)gluα(1.2)βfru], 1-kestose [αglu(1.2)βfru(1.2)βfru)], inulin [β(2.1)-linked fructan] and levan [β(2.6)-linked polyfructan], but not on melezitose [αglu(1.2)βfru(3.1)αglu] (Figure 1). Lysates of BL21(DE3) cells carrying vector pET3d without insert failed to hydrolyze the above-mentioned substrates.

To investigate the action mode of the recombinant LsdB, samples from the reactions on levan and inulin were collected at 5 min intervals and the products

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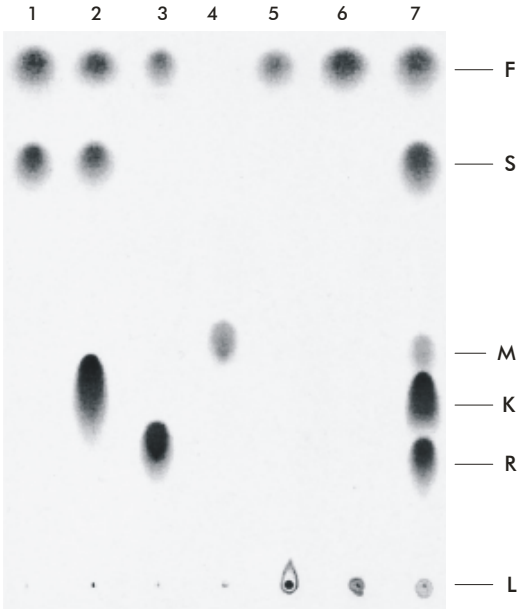

Figure 1. TLC analysis of reaction products from sucrose-containing substrates by LsdB produced in *E. coli*. Soluble extracts from IPTG-induced BL21(DE3) (pALS112) cultures were incubated for 6 h at 30°C with the corresponding substrate in 0.1 M sodium acetate buffer (pH 6.0). Fructose-specific staining of the reaction products was performed as described in Materials and Methods. Lanes 1 to 6 correspond to the LsdB reaction on sucrose, 1-kestose, raffinose, melezitose, inulin and levan, respectively. Lane 7 corresponds to a control mixture of fructose (F), sucrose (S), melezitose (M), 1-kestose (K), raffinose (R), and levan (L).

were characterized by TLC. The hydrolysis of inulin or levan resulted in the successive release of fructose without the formation of intermediate oligofructans, even in the early stages of the reaction (data not shown). The presence of fructose as the sole product of fructan hydrolysis indicates that the action mode of LsdB is exo-type. The rates of sucrose versus levan hydrolysis (S/L ratio) and sucrose versus inulin hydrolysis (S/I ratio) were 0.37 ± 0.21 and 1.40 ± 0.35, respectively. These relatively low values are indicative of a true fructanase with preference for β-(2.6)-linked substrates.

Constitutive and methanol-inducible expression of the *lsdB* gene in *P. pastoris* fermented under fed-batch conditions

The plasmids pALS175 and pALS177 were constructed so as to express the mature LsdB fused at the 5´ end to the alpha factor sequence of *S. cerevisiae* and at the 3´ end to the *myc* epitope and the His_6 tag under the control of the constitutive *GAP* promoter and the methanol-inducible *AOX1* promoter, respectively [26]. The plasmids pALS175 and pALS177 linearized at the promoter region were introduced by electroporation into *P. pastoris* strain X-33.

The zeocin-resistant clones expressing LsdB constitutively or by methanol induction were referred to as PGAP and PAOX clones, respectively. The constitutive clone PGAP6 and the methanol-inducible clone PAOX12 showing the highest LsdB expression levels in shake-flasks experiments were analyzed at a fermenter scale under fed-batch conditions (Figure 2). The clone PGAP6 and the yeast transformed with the empty vector pGAPZαC displayed similar growth curves, indicating that the *GAP* promoter-driven constitutive expression of the *lsdB* gene was not toxic to the host cell. The biomass and the extracellular levanase activity of clone PGAP6 increased proportionally during fermentation reaching the respective maximal values of 59.7 g DW l^{-1} and 26.6 U mL^{-1} at the end of fermentation (39 h) (Figure 2A). The LsdB productivity of clone PGAP6 was 682 U L^{-1} h^{-1}. In Figure 2B it is shown that clone PAOX12 metabolized methanol (Mut^+ phenotype) during the feeding phase and the cell density increased from 28 to 115.2 g DW L^{-1}. After methanol addition the recombinant yeast increasingly secreted LsdB into the culture medium up to a maximal yield of 21.1 U mL^{-1} at the end of fermentation (96 h). The LsdB productivity of clone PAOX12 was 220 U L^{-1} h^{-1}, three fold lower than in clone PGAP6. Following disruption of cells harvested at the end of fermentation it was determined that approximately 95% of the LsdB produced was secreted into the culture media of both the constitutive and the methanol-inducible clones (data not shown).

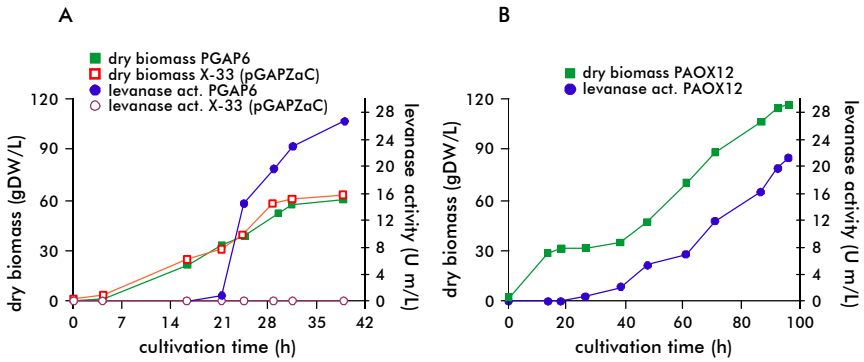

Figure 2. (A) Constitutive and (B) methanol-induced expression of LsdB in *P. pastoris* during 7.5-l fed-batch fermentations. The fed-batch phase started at 16 and 18 h in the constitutive and methanol-inducible systems, respectively. Values represent means ± standard deviations (n = 3).

Purification and characterization of LsdB expressed constitutively in *P. pastoris*

The recombinant LsdB represented 49.5% of the total proteins in the culture supernatant of clone PGAP6. The enzyme containing a C-terminal His6-tag fusion was purified to homogeneity by Ni-affinity chromatography, with a process recovery of 77.3%, and specific activity of 58 U mg^{-1} for the substrate levan at pH 5.0 and 30 ºC (Table 1). The purified protein migrated as a single compact band in SDS-PAGE with an apparent molecular mass of 57 kDa (Figure 3A). This value is consistent with the mass expected for the mature non-glycosylated LsdB fused to the *myc* epitope and the polyhistidine tag. The secreted LsdB did not alter its electrophoretic mobility in SDS-PAGE after being treated with endoglycosidase H (Figure 3A and B) and did not react with the digoxigenin-labeled lectin GNA on a gel blot (Figure 3C), indicating that it is not a glycoprotein. The predicted recombinant LsdB contains a potential *N*-glycosylation site, at position 89, conforming the general rule N-X-T/S, where X is not proline. Our finding confirms the previous as-

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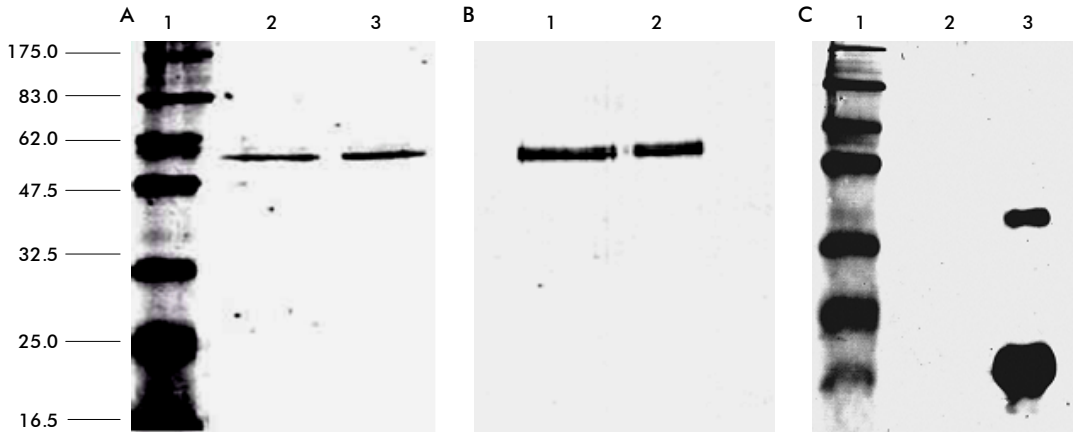

Figure 3. Glycosylation analysis of LsdB secreted by the constitutive *P. pastoris* clone PGAP6. (A) SDS-PAGE analysis of LsdB purified from culture supernatant and treated with endoglycosidase Hf. Proteins were denatured, separated on an SDS-12%-polyacrylamide gel and revealed by Coomassie Blue staining. Lane 1, broad-range protein marker (kDa) (New England Biolabs); lane 2, purified LsdB (1 μg); lane 3, endoH-treated LsdB (1 μg). (B) Western blot for the immunodetection of LsdB. Purified LsdB (1 μg) (lane 1) and endoH-treated LsdB (1 μg) (lane 2) were separated by 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and probed with polyclonal antibodies against LsdB produced in *E. coli*. (C) Western blot for the detection of mannose chains. Proteins were separated by 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and probed with lectin GNA conjugated with digoxigenin. Lane 1, pre-stained broad-range protein marker (kDa) (New England Biolabs); lane 2, purified LsdB (8 μg); lane 3, glycosylated B-ribonuclease (1 μg).

sumption that the presence of this consensus peptide does not always lead to glycosylation [27, 28].

The purified LsdB showed the same product profile as the enzyme produced in *E. coli* when it reacted with sucrose, raffinose, 1-kestose, inulin and levan (see Figure 1). The influence of pH and temperature on the recombinant LsdB activity was examined for the substrates levan and sucrose in the ranges 4.0-8.0 and 20-80 ºC, respectively (Figure 4). The highest levanase activity was achieved at pH 5.0 and 30 ºC, whereas the sucrase activity was maximal at pH 5.0 and 40-50 ºC. For both substrates, the enzyme activity drastically decreased at pH 4.0 and at pH values above 7.0. The recombinant LsdB was irreversibly inactivated at temperatures above 60 ºC. Under optimal conditions the rate of levan versus inulin hydrolysis (L/I ratio) was 3.9 ± 1.1.

The recombinant LsdB produced either in *E. coli* or *P. pastoris* hydrolyzed levan and inulin to free fructose without oligofructans formation. Up to now, there is no commercial system available for the enzymatic production of ultra-high-fructose syrups from polyfructans. LsdB shows an acceptable specific exolevanase activity and appears attractive for this purpose. The *lsdB* gene is not expressed in the natural host *G. diazotrophicus* grown under optimal culture conditions; so recombinant sources are required for the enzyme production at an industrial scale. LsdB was functionally produced in *E. coli*, but the protein resulted in an intracellular enzyme hampering its purification. The expression of the *lsdB* gene in *P. pastoris* under the constitutive *GAP* promoter did not cause cell toxicity and enzyme productivity was three fold higher in comparison with the methanol-inducible *AOX1* promoter system. The high yield and efficient secretion of LsdB fused to a polyhistidine tag enabled the purification of the protein by a simple affinity procedure with a good recovery. The *P. pastoris GAP* promoter system obviates the need to use methanol, which is noxious and flammable, and provides a convenient alternative for the large-scale production of LsdB either in a fed-batch or a continuous fermentation process.

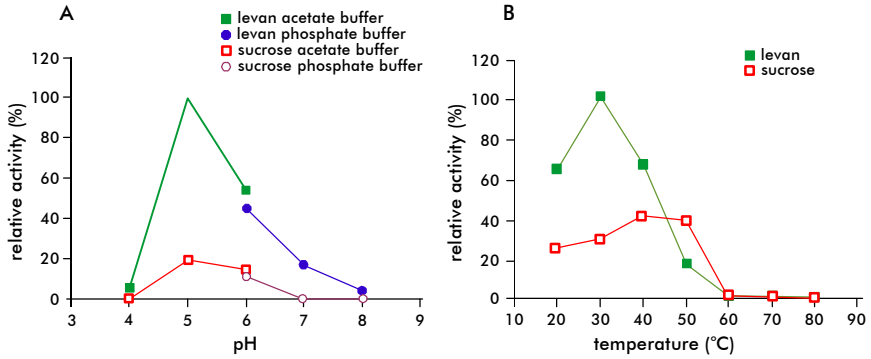

Figure 4. Effect of pH (A) and temperature (B) on the levanase and sucrase activities of LsdB produced in *P. pastoris*. The effect of pH was examined at 30 °C using 0.1 M sodium acetate buffer in the pH range 4.0-6.0 and 0.1 M sodium phosphate buffer in the range of pH 6.0-8.0. The effect of temperature was evaluated within the range 20-80 °C in 0.1 M sodium acetate buffer (pH 5.0). Purified LsdB (5 units) was incubated with 1% (w/v) levan or 0.1 M sucrose for 30 min. Reactions were stopped by heating for 5 min at 100 °C and the enzyme activity was measured as the fructose released from the substrate by using the DNSA method. Values represent means ± standard deviations (n = 3).