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ORIGINAL PAPER

Expression, purification and immobilization of the intracellular invertase INVA, from *Zymomonas mobilis* on crystalline cellulose and Nylon-6

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Abstract This paper presents two immobilization methods for the intracellular invertase (INVA), from *Zymomonas mobilis*. In the first method, a chimeric protein containing the invertase INVA, fused through its C-terminus to CBD_{Cex} from *Cellulomonas fimi* was expressed in *Escherichia coli* strain BL21 (*DE3*). INVA was purified and immobilized on crystalline cellulose (Avicel) by means of affinity, in a single step. No changes were detected in optimal pH and temperature when INVA-CBD was immobilized on Avicel, where values of 5.5 and 30 °C, respectively, were registered. The kinetic parameters of the INVA-CBD fusion protein were determined in both its free form and when immobilized on Avicel. K_m and V_{max} were affected with immobilization, since both showed an increase of up to threefold. Additionally, we found that subsequent to immobilization, the INVA-CBD fusion protein was 39% more susceptible to substrate inhibition than INVA-CBD in its free form. The second method of immobilization was achieved by the expression of a 6xHis-tagged invertase purified on Ni-NTA resin, which was then immobilized on Nylon-6 by covalent binding. An optimal pH of 5.5 and a temperature of 30 °C were maintained, subsequent to immobilization on Nylon-6 as well as with immobilization on crystalline cellulose. The kinetic parameters relating to V_{max} increased up to 5.7-fold, following immobilization, whereas K_m increased up to 1.7-fold. The two methods were compared showing that when invertase was immobilized on Nylon-6, its activity was 1.9 times that when immobilized on cellulose for substrate concentrations ranging from 30 to 390 mM of sucrose.

Keywords Enzyme immobilization · Invertase · Nylon-6 · Sucrose · Zymomonas mobilis

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Introduction

Invertase (EC 3.2.1.26, β-D fructofuranosidase) catalyzes the hydrolysis of sucrose in glucose and fructose (inverted sugar syrup). Inverted sugar syrup has been used in food industries as a substitute for sucrose in all industrial processes that use sugar in its liquid phase, for example, in fizzy drinks, juices and liquors. This is mainly due to its physicochemical properties and the high quality of fructosade syrups as sweeteners. Invertase occurs widely in nature and its presence has been reported in bacteria, fungi, higher plants and archeas.

The majority of research concerning invertase has focused on invertase extracted from *Saccharomyces cerevisiae*. This enzyme shows an anticompetitive inhibition through a mechanism involving the reversible binding of substrate on the enzyme molecule at high sucrose concentrations, and experimental evidence of multiple steady states, under defined operation conditions, in a continuous stirred tank reactor for hydrolysis of sucrose by invertase has been reported [29]. The invertase from *S. cerevisiae* is inhibited by low concentration of substrate (5%) and does not represent a thermo stable enzyme. These properties of the invertase are disadvantageous for its employment at an

industrial level. Invertase from *S. cerevisiae* has been immobilized on a number of supports, using physical and chemical methods [1, 3, 9, 18, 21, 25]. Although, this "commercial" enzyme shows a high degree of hydrolytical activity, it contains sludge, besides the fact that its biotechnological application requires an additional stage of immobilization, on an appropriate support. For this reason, it is necessary to produce better biocatalysts with improved characteristics. In this work an intracellular invertase (INVA) [11, 31] was used as a study pattern. INVA was isolated from *Zymomonas mobilis*, a bacteria Gram(−) producer of ethanol from sucrose, glucose or fructose. INVA has been sorted as a Glycoside Hydrolase Family 32 (GH32), (http://afmb.cnrs-mrs.fr/CAZY) [12] and structural information for invertases classified in this family is available [2].

The employment of genetic engineering techniques in order to construct chimeric proteins, containing a catalytic domain fused to a binding domain has permitted the simultaneous purification and immobilization of proteins, by means of affinity chromatography. An example consists in the cbd_{cex} gene from *Cellulomonas fimi*, which is used to design a chimeric gene, fused to any target protein. The recombinant protein is purified and immobilized by affinity chromatography, using cellulose as a support. Cellulose is a chemically inert matrix that is safe to use in food or pharmaceutical applications. Cellulose support is commercially available in different forms; for example, cotton wool, beads, powders, fibers, hydrogels, membranes, and sheets of defined porosity, and it is also relatively cheap [7]. Another type of purification consists in obtaining 6xHis-tagged recombinant proteins for purification on Ni-NTA matrices, so that proteins can be immobilized on several supports. In this case Nylon-6 was used as the support.

We have previously reported the purification and immobilization of *Z. mobilis* INVB-CBD fusion protein to crystalline cellulose (Avicel) [23]. Unfortunately, the Avicel-immobilized INVB-CBD lost about 60% of its catalytic activity compared to the free fusion protein, probably due to unspecific interaction of INVB-CBD with the support, although the immobilized enzyme was more thermo stable than free INVB-CBD and showed an improvement in the affinity for the substrate [23]. Also, we have reported the successful immobilization of the extracellular invertase from *S. cerevisiae* [3] and the extracellular recombinant invertase INVB from *Z. mobilis* on Nylon-6 [27].

In this work, we report the expression of the intracellular invertase INVA from *Z. mobilis* in *Escherichia coli*, and the purification and immobilization of the recombinant enzymes INVA-CBD and INVA on Avicel and Nylon-6, respectively. INVA-CBD and INVA, free and immobilized on both supports were characterized for their enzymatic properties. Data obtained with both immobilization methods were compared.

Materials and methods

Bacterial strains and culture conditions

Z. mobilis CDBB-B603 (Cultivos Microbianos CINVESTAV-IPN, México) was grown in shake flasks at 30 °C for 8 h in static culture in mineral medium base [30] containing (g/L): KH_2PO_4, 2.5; $(NH_4)_2SO_4$, 1.6; $MgSO_4\ 7H_2O$, 1.0; added with yeast extract, 5; sucrose, 100; pH 4.9. *E. coli* DH5α was used for all cloning and storage of plasmids and *E. coli* strain BL21 (*DE3*) was used for expression experiments of the recombinant proteins (Novagen, USA). *E. coli* cells were grown at 37 °C under agitation in LB or 2TY medium [22] supplemented with kanamycin (30 μg/mL) when appropriate.

DNA manipulations

All molecular methods were performed using standard molecular biology techniques [22]. Restriction enzymes were purchased from New England Biolabs (USA). Genomic DNA was isolated from *Z. mobilis* CDBB-B603 by using the DNeasy Kit (Qiagen), following the manufacturer instructions. Expression vectors pET38b(+) and pET30 (Ek/LIC) (Novagen, USA) were used for the expression of the recombinant INVA-CBD and INVA invertases, respectively.

Construction of the plasmid invA-pET30 (Ek/LIC)

The coding region of the *inv*A gene *Z. mobilis* [32] was cloned into the vector pET30 (Ek/LIC) according to the instructions from Novagen (Novagen, USA). First, the open reading frame (ORF) of the *inv*A gene (GenBank accession number NC_006526) was amplified by PCR using *Z. mobilis* genomic DNA as template, the high fidelity Platinum *Pfx* DNA polymerase (Invitrogen) and the primers: sense (5′ GACGACGACAAGATGGAATCCCCCT-CTTAT-3′) and antisense (5′-AGCGAGGAGAAGCCCGGTACA-GGCATCGCTTAAAAA-AATC-3′). The 1.5 kb amplified product was cloned into pET30 (Ek/LIC) vector to obtain the *inv*A-pET30 ((Ek/LIC)) construct, which was then transferred to *E. coli* BL21(*DE3*) strain for protein expression experiments.

Construction of chimerical *invA-cbd* gene

The *inv*A insert was liberated from the *inv*A-pET30 (Ek/LIC) construct and cloned in *Xho* I and *Bgl* II sites from pET38b(+) expression vector. The compatible cohesive ends were ligated using T4 DNA ligase at 16 °C over a 16 h period, in order to produce a chimerical *inv*A-*cbd* gene. pET38b(+) contains a cbd_{cex} tag coding sequence from *C.*

fimi. The construction *inv*A-pET38b(+) was transferred to *E. coli* BL21(*DE3*) strain for protein expression experiments.

Expression of recombinant INVA-CBD and INVA

The proteins were expressed in *E. coli* strain BL21 (*DE3*). *E. coli* strain BL21 (*DE3*) cultures harboring *inv*A-pET30 (Ek/LIC) and *inv*A-pET38b(+) were grown overnight in 2YT medium, containing 30 μg/mL kanamycin, in separate experiments. The overnight culture was diluted 100-fold in 2YT medium with 30 μg/mL kanamycin and grown at 37 °C up to a cell density of $OD_{600\ nm} = 0.6$. Isopropyl-1-thio-β-D-galactoside (IPTG) was added in order to reach a final concentration of 1 mM. Induction was carried out over a 6 h period. Cell culture was harvested by centrifugation (8,000 rpm, 4 °C, 20 min).

The cells were resuspended in lysis buffer (100 mM NaCl; 2 mM EDTA, 50 mM Tris–HCl pH 7.5); with 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and 100 μg/μL lysozyme, at final concentration. The crude extract was incubated at 4 °C for 30 min. The cells were disrupted by passage through a French press Aminco (1,500 lb/in^2) and the cell pellet was removed by centrifugation at 10,000 rpm, at a temperature of 4 °C for 10 min. The supernatant was collected and stored at 4 °C, prior to analysis (soluble fraction). Pellet containing inclusion bodies (insoluble fraction) was directly treated with Laemmli sample buffer [13]. Proteins from both the soluble fraction and the insoluble fraction were analyzed by 10% SDS-PAGE according to the Laemmli method [13].

Purification and immobilization of INVA-CBD on Avicel

All purification and immobilization steps were carried out at 4 °C. INVA-CBD was purified and immobilized on Avicel as described [23]. Immobilization was conducted by gently mixing 5 g of Avicel with 8.5 mL of bacterial lysate [*E. coli* BL21(*DE3*)/*inv*A-pET38b(+) cells] (20 mg of protein/mL) at 4 °C for 18 h on an orbitron Bockel Scientific rotor. Then, the supernatant was recovered by centrifugation (14,000*g*, 4 °C, 1 min) and used to determine residual protein and invertase activity. After washing 5 times with 20 mM Tris–HCl (pH 7.5); 2 M NaCl, and 5 times with 20 mM Tris–HCl (pH 7.5), the immobilized INVA-CBD was stored at 4 °C, prior to being used. Proteins in the Avicel-bound fraction were analyzed by 10% SDS-PAGE and invertase activity.

Purification of INVA

All purification steps were carried out at 4 °C. The recombinant invertase INVA (6xHis-tagged) was purified from the soluble fraction of bacterial lysate [*E. coli* BL21(*DE3*)/*inv*A-pET30 (Ek/LIC)] by nickel affinity chromatography using Ni-NTA resin, according to the instructions from Qiagen. Proteins were eluted from the column with a linear gradient of imidazol (10–250 mM) in 300 mM NaCl, 20 mM sodium phosphate buffer, pH 7.0. Fractions collected were analyzed by 10% SDS-PAGE and invertase activity. Purified INVA was dialyzed against 50 mM Tris–HCl pH 7.0 and then stored at 4 °C for further biochemical characterization in its free form.

Immobilization of INVA on Nylon-6

Purified INVA was immobilized on Nylon-6 microbeads as described [3]. Nylon-6 microbeads were activated with gluteraldehyde, using polyethylenimine (PEI) as a spacer [21]. INVA was covalent immobilized on Nylon-6 microbeads as follows: 1 g of Nylon-6 microbeads were suspended in 5 ml of coupling buffer (0.01 M $MgCl_2$; 0.05 M KCl; 0.05 M sodium acetate pH 5.5; 0.05 M L-cysteine; 5% glycerol) and 20 mg of protein/g of polymeric support were incubated at 4 °C for 3 h. Following this, the microbeads were washed and stored at 4 °C in coupling buffer with 10% of glycerol [3].

Protein assay

Protein concentration was estimated as described by Lowry [15] using bovine serum albumin as a standard. The amount of immobilized protein was determined by measuring the concentration of protein in the coupling buffer, both before and after the immobilization process.

Invertase activity assay

Invertase activity was determined by assessing the amount of reducing sugars released using 3′5-dinitrosalicylic acid (DNS) as described by Miller [17]. The assay was carried out at room temperature, using a 150 mM sucrose solution in 50 mM sodium acetate buffer, pH 5.5 at 30 °C. An equimolar mixture of glucose and fructose was used as standard. One unit of invertase activity was defined as the amount of enzyme required for hydrolysis of 1 μmol of sucrose/min. In many cases, the results were normalized, with 100% enzymatic activity, representing the highest value obtained for each of the series of measurements made.

Effectiveness factor ($F\varepsilon$)

The efficiency of immobilization on both supports was determined by the effectiveness factor ($F\varepsilon$), which make a ratio between the enzymatic activities before and after immobilization. $F\varepsilon$ was calculated as described [1, 3].

Optimal pH and temperature, and kinetic parameters

Optimal pH was determined by incubating the enzyme preparation at 30 °C in 150 mM sucrose solution in 50 mM acetate buffer (pH from 3.5 to 5.5), or in 50 mM sodium citrate phosphate buffer (pH from 6 to 7.5), or in 20 mM Tris–HCl buffer (pH 7.5–8.5). Optimal temperature was determined by incubating the enzyme preparation at different temperatures (17–50 °C) in 150 mM sucrose solution prepared in 50 mM acetate buffer, pH 5.5. Initial reactions rates of sucrose hydrolysis were determined at different substrate concentrations ranging from 30 mM to 2.0 M of sucrose in 50 mM acetate buffer, pH 5.5 at 30 °C. The kinetic constants were determined as described [29] from Lineweaver-Burk [14], Hanes-Wolf and Eadie-Hofstee plots, and then by the numeric method "lineal Newton" using the Excel solver tool.

Thermostability assay

Thermal stability was determined for both free and immobilized INVA-CBD. Enzymes were incubated at different temperatures (25–50 °C) in 50 mM acetate buffer, pH 5.5, without substrate. Residual invertase activity was determined after 10–60 min intervals for up to 7 h.

Results and discussion

Expression of the recombinant INVA-CBD

We have previously reported the expression of the extracellular invertase INVB de *Z. mobilis* in *E. coli* as INVB-CBD fusion protein, and the purification and immobilization of INVB-CBD to Avicel in one single step [23]. Unfortunately, the immobilized INVB-CBD lost about 60% of its catalytic activity compared to the free fusion protein, probably due to unspecific interaction of INVB-CBD with the support. However, since cellulose presents many advantages as support (chemically stable, mechanically resistance, non-toxic, non-pollutant, among others), the catalytic properties of INVA-CBD immobilized on Avicel were investigated in the present work.

A chimerical *invA-cbd* gene was constructed and expressed in *E. coli* strain BL21 (*DE3*) by induction with 1 mM IPTG, in order to produce the INVA-CBD fusion protein. The expression of INVA-CBD was analyzed by SDS-PAGE electrophoresis. One major band of 78 kDa was detected (Fig. 1a). This molecular mass corresponds to the INVA-CBD fusion protein, 57 kDa from INVA and 21 kDa, approximately corresponding to the CDB_{Cex} from *C. fimi*. INVA-CBD was mainly located in inclusion bodies (Fig. 1a) although invertase activity was also found in the soluble fraction of the bacterial lysate.

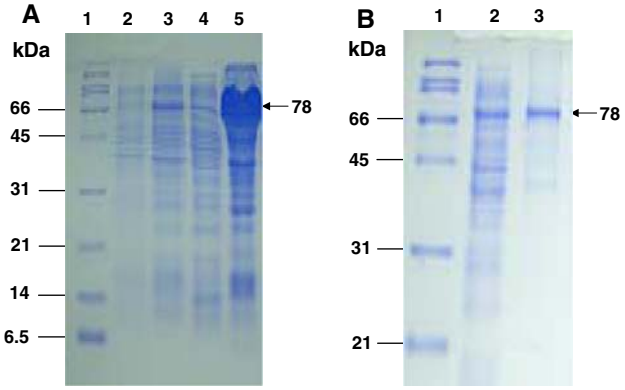

Fig. 1 Electrophoretic analysis of expression, purification and immobilization of INVA-CBD on Avicel by 10% SDS-PAGE. **a** *Lane 1* MW markers, *Lane 2* Intracelular fraction of non-induced cells, *Lane 3* Intracelular fraction of induced cells, *Lane 4* Soluble fraction, *Lane 5* Insoluble fraction (inclusion bodies). **b** *Lane1* MW markers, *Lane 2* Induced cells, *Lane 3* Purified Avicel-immobilized INVA-CBD

Purification and immobilization of INVA-CBD on Avicel

The fusion protein was purified and immobilized on cellulose for enzymatic characterization and the assay was carried out, beginning with the soluble fraction as described above. The CBD_{Cex} from *C. fimi* was used as a cellulose-binding module [6, 10, 19, 20]. Purification and immobilization of INVA-CBD on Avicel was achieved in a single step, as shown by electrophoretic analysis using 10% SDS-PAGE (Fig. 1b). INVA-CBD presented an apparent molecular mass of 78 kDa and this immobilization system was characterized enzymatically, by using the reducing sugar test. The quantity of the enzyme immobilized on cellulose was of 5.3 mg of protein/g of cellulose. Similar Avicel-invertase binding rate has been reported [23]. The enzyme was immobilized with an efficiency factor of 0.99, which means 99% of the immobilized protein retained its enzymatic activity on Avicel.

Enzymatic characterization of free and immobilized INVA-CBD on Avicel

Optimal pH and temperature

INVA-CBD was characterized by measuring the reducing sugars, produced by sucrose hydrolysis. The free invertase was compared with the immobilized invertase. The activity profiles for a number of different pH, ranging from 3.5 to 7.5 were recorded. In both cases, the enzymes were active between pH 5 and 7 and retained more than 60% of their activity in this range of pH. The optimum pH for the immobilized INVA-CBD on cellulose did not present variations when compared to INVA-CBD in its free form, as both enzymes showed optimal activity at pH 5.5 (Fig. 2a). Optimal

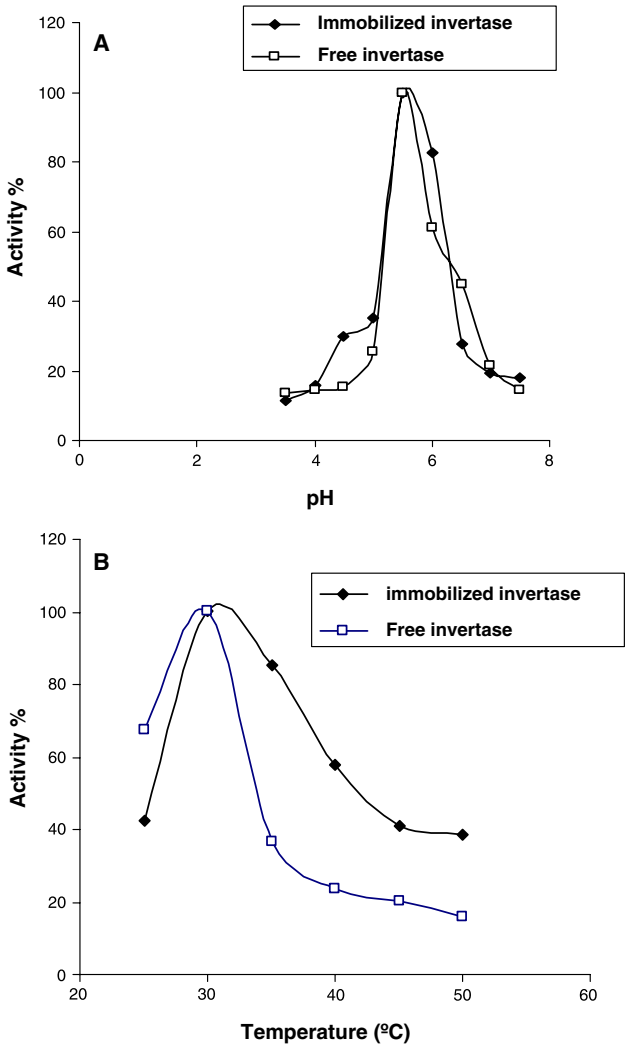

Fig. 2 Effect of pH and temperature on invertase activity of INVA-CBD immobilized on Avicel (*filled dymen*) and free (*open square*). **a** pH. Invertase activity at 30 °C in 0.15 M sucrose solution prepared in 50 mM acetate buffer (pH 3.5–5.5), or 50 mM sodium citrate phosphate buffer (pH 6.0–7.5). **b** Temperature. Invertase activity in 0.15 M sucrose, 50 mM acetate buffer, pH 5.5 at different temperatures (25–50 °C)

pH of both the native INVB of *Z. mobilis* and the recombinant INVB-CBD immobilized on Avicel was also 5.5 [23, 26]. Optimal pH values of 5.0–5.5 have been reported for both free and immobilized invertases from bacteria and yeast [3, 16, 23, 29, 31]. The effect of different temperatures, ranging from 25 to 50 °C was also analyzed. The optimum temperature appeared to be 30 °C for both soluble and immobilized INVA-CBD (Fig. 2 b). Optimal temperature of 45 °C was observed upon immobilization of INVB-CBD on Avicel [23].

Kinetic parameters of free and immobilized INVA-CBD

The effect of substrate concentration on the enzymatic activity of the free and immobilized INVA-CBD on cellulose was analyzed for the same range of sucrose concentrations (30–1,300 mM). Calculated V_{max} was 0.59 µmol/min with a K_m of 51 mM and a K_i of 393 mM. The INVA-CBD immobilized on cellulose showed a calculated V_{max} of 1.75 µmol/min with a K_m of 153 mM and a K_i of 283 mM. Interestingly, the free INVA-CBD was 39% less susceptible to substrate inhibition than when immobilized on Avicel. The kinetic parameter V_{max} showed an increase of threefold upon immobilization of INVA-CBD on Avicel for substrate concentrations ranging from 30 to 390 mM of sucrose. However, immobilization of the enzyme on this support lead to a threefold increase in the K_m value, which indicates that the formation of the enzyme-substrate complex is more difficult for the immobilized enzyme, as it has been reported after immobilization of invertase on other supports [4, 5, 8].

Thermo stability of free and immobilized INVA-CBD

The behavior of INVA-CBD at a number of temperatures is presented in Fig. 3. A total of 90% of the activity was maintained for free INVA-CBD at 25 °C, whereas 45% was maintained for the immobilized enzyme, after 3 h. Free INVA-CBD manifested approximately 60% of enzymatic activity for 3 h at 30 °C and immobilized INVA-CBD maintained just 42% of enzymatic activity for a 3 h period. The percentage of enzymatic activity maintained at 40 °C after 20 min of incubation for the free enzyme, represented 20% of activity and for the immobilized system almost all activity ceased. Thus, the immobilization of INVA-CBD on Avicel had a drastic negative effect in the thermal stability of the enzyme. In contrast, the immobilization of INVB-CBD on Avicel improved the thermo stability of the enzyme compared to that observed for the free enzyme [23]. These results strongly suggest that the interaction of the fusion proteins INVA-CBD and INVB-CBD with the matrix of Avicel is rather different, probably due to the important differences in the amino acid sequence between INVA and INVB from *Z. mobilis*.

Overall analysis of results obtained upon immobilization of INVA-CBD on Avicel showed this method had no effect on optimal pH and temperature; and a threefold increase in the V_{max} value was shown. However, a threefold decrease in the substrate affinity by immobilized INVA-CBD was observed (Table 1) and the thermo stability of the immobilized enzyme was drastically affected at 50 °C (data non shown). In order to find a more suitable method for the immobilization of INVA, the enzyme was expressed in *E. coli*, purified and immobilized on Nylon-6 microbeads, a method that has been successfully used for the immobilization of the extracellular invertase from *S. cerevisiae* [3] and the extracellular invertase INVB from *Z. mobilis* [28].

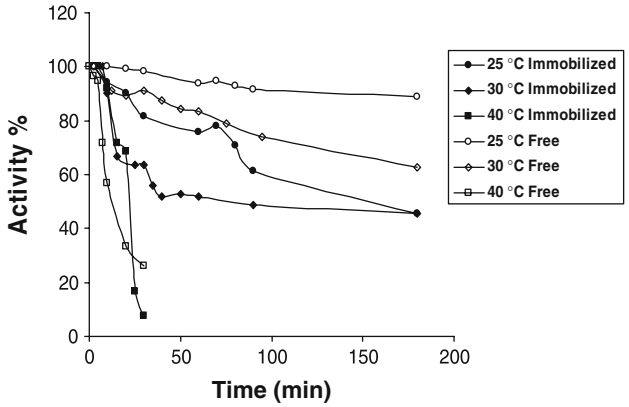

Fig. 3 Thermal stability of INVA-CBD immobilized on Avicel (*filled circle* 25 °C, *filled dymen* 30 °C, filled square 40 °C) and free (*open circle* 25 °C, *open dymen* 30 °C, *open square* 40 °C). Invertase activity in 0.15 M sucrose, 50 mM acetate buffer, pH 5.5

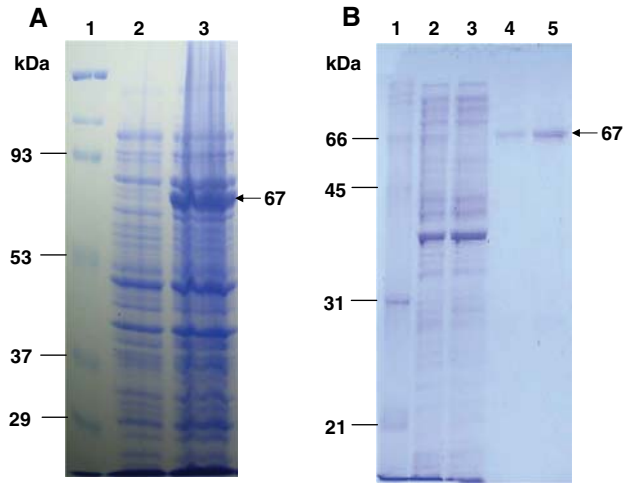

Fig. 4 Electrophoretic analysis of expression and purification of INVA in *E. coli* by 10% SDS-PAGE. **a** *Lane 1* MW markers, *Lane 2* intracellular fraction of non-induced cells, *Lane 3* intracellular fraction of induced cells, *Lane 4* soluble fraction, *Lane 5* insoluble fraction (inclusion bodies). **b** *Lane 1* MW markers, *Lane 2* soluble fraction of bacterial lysate, *Lane 3* wash through fraction, *Lanes 4–5* purified INVA

Expression and purification of INVA

The intracellular invertase INVA from *Z. mobilis* was expressed in *E. coli* BL21(*DE3*)/*inv*A-pET30 (Ek/LIC) cells by induction with 1 mM IPTG. Electrophoretic analysis of total cell lysate before and after IPTG induction showed one major band of approximately 67 kDa; this molecular mass corresponds to the 6xHis-Tagged INVA fusion protein, 57 kDa from INVA and 12 kDa, added from the vector pET30 (Ek/LIC) (Fig. 4a). INVA was purified from the soluble fraction of the bacterial lysate [*E. coli* BL21(*DE3*)/*inv*A-pET30 (Ek/LIC)] by nickel affinity chromatography (Fig. 4b), and the purified recombinant INVA was immobilized on Nylon-6 microbeads, as previously described [3, 28]. This immobilization system was characterized enzymatically by using the reducing sugar test. The quantity of the enzyme immobilized on cellulose was of 1.5 mg of protein/g of Nylon-6. A previous study reported the immobilization of 0.247–0.261 mg of invertase/g of Nylon-6 activated with HCl using glutaraldehyde as cross-linker [24], which corresponds to six times less invertase immobilized per gram of Nylon-6 than that reported in this work. INVA was immobilized on Nylon-6 with an efficiency factor of 0.85, which means 85% of the immobilized protein retained its enzymatic activity on Nylon-6. An efficiency of immobilization for invertase of 93% (Nylon-6) [3], 82% (*Cajanus cajan* lectin) [1] and 57–60% (Nylon-6 activated with HCl using glutaraldehyde as cross-linker) [24] has been reported.

Enzymatic characterization of free and immobilized INVA on Nylon-6

Optimal pH and temperature

The pH activity profile of free and immobilized INVA on Nylon-6 microbeads was studied at pH values from 3.5 to 8.5 (Fig. 5a). A maximum of invertase activity was

Table 1 Enzymatic properties of free and immovilized INVA and INVB from *Z. mobilis* on Avicel and Nylon-6

	Optimal pH	Optimal temperature (°C)	K_m (mM)	V_{max} (U)	Specific activity (U/mg)	K_i (mM)	References
Free INVA-CBD	5.5	30	51	0.59	25.6	393	This work
Immobilized INVA-CBD on Avicel	5.5	30	153	1.75	20.6	283	This work
Free INVA	5.5	30	110	0.36	39	313	This work
Immobilized INVA on Nylon-6	5.5	30	189	2.07	456	259	This work
Free INVB-CBD	5.5	45	193		27.2	ND	[23]
Immobilized INVB-CBD on Avicel	5.5	45	81		10.3	ND	[23]
Free INVB	5.5	45	98		1,981	ND	[28]
Immobilized INVB on Nylon-6	5.5	25	984		317	ND	[28]

ND non determined

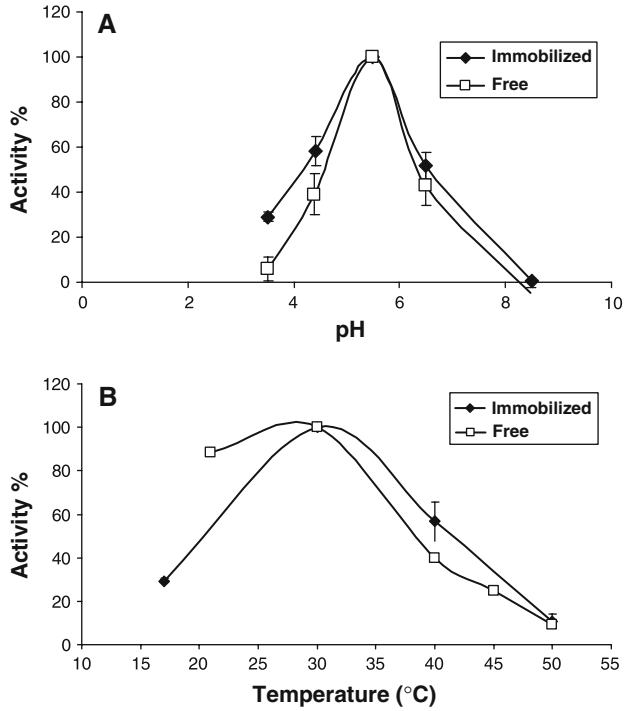

Fig. 5 Effect of pH and temperature on invertase activity of INVA immobilized on Nylon-6 (*filled dymen*) and free (*open square*). **a** pH. Invertase activity at 30 °C in 0.15 M sucrose solution prepared in 50 mM acetate buffer (pH 3.5–5.5), or 50 mM sodium citrate phosphate buffer (pH 6.0–7.0) or 20 mM Tris–HCl buffer (pH 7.5–8.5). **b** Temperature. Invertase activity in 0.15 M sucrose, 50 mM acetate buffer, pH 5.5 at different temperatures (17–50 °C)

observed at pH 5.5 for both INVA immobilized on Nylon-6 and the free form (Fig. 5a). Optimal pH values of 5.0–5.5 have been reported for immobilization of microbial invertases to other supports [3, 9, 16, 23].

The effect of the temperature on the catalytic activity of both free and immobilized INVA on Nylon-6 was analyzed to different temperatures, from 17 to 50 °C (Fig. 5b). A maximum of invertase activity was observed at 30 °C for free and immobilized INVA on Nylon-6. Thus, these results showed neither the immobilization of INVA-CBD on Avicel nor the immobilization of INVA on Nylon-6 modified the optimal pH and temperature of INVA from *Z. mobilis*. Immobilization of INVB on Nylon-6 showed no significant change in the optimal pH, but a difference in the optimal temperature was evident, as that for the immobilized and free enzymes was shown to be 25 and 40 °C, respectively [28].

Kinetic parameters of free and immobilized INVA

Nylon-6 immobilized INVA and the free enzyme were slightly more susceptible to substrate inhibition than Avicel-immobilized INVA and the free form (Table 1). The K_m value of Nylon-6-immobilized INVA and the free form was 189 mM and 110 mM; thus, immobilization of INVA on Nylon-6 lead to a 1.7-fold increase in the K_m value of the enzyme, which indicates that the formation of the enzyme-substrate complex is more difficult upon immobilization of INVA on this support. A decrease in the substrate affinity is generally observed after immobilization of invertase on different supports [4, 5, 28]. The V_{max} value of Nylon-6-immobilized INVA and the free form was 2.07 and 0.36 µmol/min, respectively, thus indicating the catalytic activity of the immobilized enzyme increased 5.7-fold compared to the free form of the enzyme (Table 1) for substrate concentrations ranging from 30 to 310 mM of sucrose. The K_i value of Nylon-6-immobilized INVA and the free form was 259 mM and 313 mM, thus suggesting that free INVA was 20% less susceptible to substrate inhibition than when immobilized on Nylon-6.

Thermal stability of free and immobilized INVA

To evaluate the thermal stability of the INVA immobilized on Nylon-6 and the free form, the behavior of the enzymes was studied at 30, 40 and 50 °C (Fig. 6). Non important differences in the thermal stability of the enzymes were observed at 30 and 40 °C but after 1 h at 50 °C, activity for free INVA ceased, whereas immobilized INVA maintained 70% of its activity for the same time period, at the same temperature (Fig. 6). Thus, immobilization of INVA on Nylon-6 improved the thermal stability of the enzyme. Interestingly, immobilization of INVB on Nylon-6 enhanced the thermo stability of the enzyme by 50% at 30 °C and 70% at 40 °C, when compared to the free enzyme [28].

Overall analysis of results obtained upon immobilization of INVA on Nylon-6 showed that this method had no effect on optimal pH and temperature, but a 1.7-fold decrease in the substrate affinity by immobilized INVA-CBD was

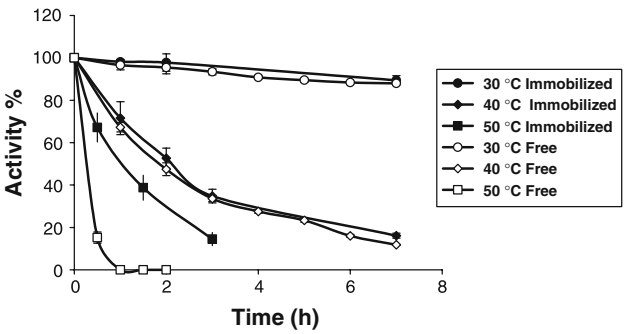

Fig. 6 Thermal stability of INVA immobilized on Nylon-6 (*filled circle* 30 °C, *filled dymen* 40 °C, *filled square* 50 °C) and free (*open circle* 30 °C, *open dymen* 40 °C, *open square* 50 °C). Invertase activity in 0.15 M sucrose, 50 mM acetate buffer, pH 5.5

observed compared to that observed for the free form of the enzyme (Table 1). However, a 1.9-fold increase in the catalytic activity (Table 1) and in the thermo stability of the immobilized enzyme compared to the free form was observed upon immobilization of INVA on Nylon-6. These differences could be due to the treatment of Nylon-6 microbeads with gluteraldehyde and PEI increase the number of reactive centers and the surface area for enzyme immobilization. In contrast, immobilization on Avicel is limited to the interaction between three triptophan amino acid residues from the motif CBD_{Cex} in the INVA-CBD fusion protein.

Conclusions

We report the expression, purification and immobilization of the recombinant intracellular invertase INVA from *Z. mobilis* by two different methods. Immobilized INVA on Nylon-6 showed a 1.9-fold increase in the catalytic activity of the enzyme compared to Avicel-immobilized INVA for substrate concentrations ranging from 30 to 390 mM of sucrose. These data indicated that immobilization of INVA from *Z. mobilis* on Nylon-6 yields a better biocatalyst than immobilization of INVA-CBD on Avicel.

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