Glucose and Fructose Production by *Saccharomyces cerevisiae* Invertase Immobilized on MANAE-Agarose Support

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ABSTRACT

Invertase from *Saccharomyces cerevisiae* was immobilized on agarose beads, activated with various groups (glyoxyl, MANAE or glutaraldehyde), and on some commercial epoxy supports (Eupergit and Sepabeads). Very active and stable invertase derivatives were produced by the adsorption of the enzyme on MANAE-agarose, MANAE-agarose treated with glutaraldhyde and glutaraldehyde-agarose supports. At pH 5.0, these derivatives retained full activity after 24h at 40 ºC and 50 ºC. When assayed at 40 °C and 50 °C, with the pH adjusted to 7.0, the invertase-MANAE-agarose derivative treated with glutaraldehyde retained 80% of the initial activity. Recovered activities of the derivatives produced with MANAE, MANAE treated with glutaraldehyde and glutaraldehyde alone were 73.5%, 44.4% and 36.8%, respectively. These three preparations were successfully employed to produce glucose and fructose in 3 cycles of sucrose hydrolysis.
Keywords: Glucose and fructose production. Invertase immobilization. Invertase. *Saccharomyces cerevisiae*.

INTRODUCTION

Invertases (β-D–fructofuranoside fructohydrolase, EC 3.2.1.26) are enzymes especially important in food technology for the production of fructose syrup from sucrose solutions. The use of immobilized invertase for continuous processes is attractive, since the final product is of high purity, typical of enzymatic processes, and there is no problem regarding the pollution of environmental water, which is very common in chemical processes (Husain et al., 1996). Moreover, the use of immobilized invertase in industrial processes could reduce process costs by reducing the quantity of enzyme required, since the immobilized derivative can readily be recovered at the end of a hydrolysis cycle and reused. This advantage depends on the enzyme remaining active for many reaction cycles (Kotwal & Shankar, 2009).

Yeast invertases generally are very stable and active at acidic pH (3.5 to 5.5), and can be used at temperatures between 65 and 70 ºC when the sucrose solution is concentrated. In the immobilized form, this enzyme can be used for sucrose inversion on a large scale (Uhlig, 1990).

Saccharomyces cerevisiae produces intracellular and extracellular invertases. The extracellular invertase is a highly glycosylated oligomer, occurring mainly as the dimer, tetramer and octamer (Burger et al., 1961; Esmon et al., 1987), and each polypeptide chain is about 60,000 Daltons, with oligosaccharide units attached. According to Gascón et al. (1968), the molecular mass of intracellular invertase is usually 130kDa, whereas that of extracellular invertase is 270kDa.

Invertases have been immobilized on several supports by a variety of methods, and various stabilities and recovered activities are reported (D'Souza & Godbole, 2002; Torres et al., 2002; Sanjay & Sugunan, 2006; Goulart et al., 2008). Multipoint covalent attachment requires a support and immobilization conditions that favor the establishment of many enzyme-support linkages (Fernandez-Lafuente, 2009; Mateo et al., 2007b; Pedroche et al., 2007). Supports having large internal surfaces, such as agarose or Sepabeads, and activated with epoxy groups (Mateo et al., 2002; Mateo et al., 2007a; Pedroche et al., 2007), glyoxyl groups (Mateo et al., 2005; Mateo et al., 2006) or glutaraldehyde (Betancor et al., 2006; López-Gallego, 2005), may fulfill these requirements and thus yield an intense multipoint covalent attachment and promote a high level of stabilization of the enzyme by increasing the rigidity of its tertiary structure (Mateo et al., 2007b). In this study, we immobilized an invertase by multipoint or multi-subunit covalent attachment and analyzed the activity and stability of the resulting derivatives.

MATERIAL AND METHODS

Materials

Invertase from *Saccharomyces cerevisiae* was obtained from Sigma Aldrich (St. Louis, Mo). CL6B agarose was from GE Healthcare. Eupergit-C 250L was

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obtained from Rohm Pharma (Darmstadt, Germany). Sepabeads FP-EC3 was a kind gift from Resindion S.r.l. (Mitsubishi Chemical Corporation, Milan, Italy). All other reagents were of analytical grade.

Methods

All experiments were performed at least in triplicate and the results are presented as their mean values. Experimental error never exceeded 5%.

Support activation

The glyoxyl-agarose support was prepared as described by Mateo et al. (2005; 2006). Monoaminoethyl-N-aminoethyl-agarose (MANAE-agarose) was prepared as described elsewhere (Fernandez-Lafuente et al., 1993). The glutaraldehyde-agarose support was prepared as described by Betancor et al. (2006).

Enzyme Assays and protein content

Standard activity was determined by adding 50μL of a 0.5mg/mL invertase solution to 5mL of 1% sucrose in 50mM acetate buffer at pH 5.0 and incubating for 30 min. at 40 ºC. For the invertase derivatives, assays were performed with 100mg of each derivative in 1mL of 1% sucrose under the same conditions as above. After the sucrose hydrolysis, 10μL of the reaction mixture was added to 1mL of Trinder reagent (Glucose Monoliquid, GL-303, Ben S.r.l., Milano, Italy), containing the enzymes glucose oxidase ($\geq$1000 U.mL^{-1}) and peroxidase ($\geq$150 U.mL^{-1}) and a chromogen, and incubated for 10 min. at 37 ºC. The glucose produced by the hydrolysis of sucrose was determined in a colorimeter set at λ 505nm. Protein was determined with the Bradford reagent (Sigma-Aldrich Co., St. Louis, Mo), using bovine serum albumin as a standard (Bradford, 1976).

Effect of temperature and pH on soluble invertase activity and stability

The effect of temperature on the activity of commercial invertase was determined by conducting assays at 5°C intervals from 25 to 80°C, in sucrose solution prepared in 50 mM acetate buffer 50mM (pH 5.0). Thermal stability was investigated by incubating the enzyme without substrate at temperatures from 25 to 80 °C for 1h, before assaying it. The assays were carried out at 40 °C in 50mM sodium acetate (pH 5.0), with 1% sucrose as substrate. The effect of pH on activity was determined in McIlvaine buffer (pH 3.0 to 8.0) at 40 °C. For pH stability determination, the enzyme was incubated for 1h at each pH tested, from 3.0 to 8.0, at 25 °C. These assays were performed at optimum temperature and pH.

Thermal stability of the derivatives

Aliquots of the derivatives were incubated in 50mM sodium acetate buffer at pH 5.0 and 40 ºC or 50 ºC.

Periodically, samples of these suspensions were withdrawn, placed in an ice bath for 30s, and the remaining enzyme activity was assayed as described above. Residual activity was expressed as a percentage of the initial activity at the given incubation time.

Determination of kinetic parameters

Kinetic parameters of soluble commercial and immobilized invertase were determined with sucrose solutions ranging from 5 to 75mM in 50mM McIlvaine buffer at pH 5.0 and 40 °C. Apparent K_m and V_{max} values were estimated from Lineweaver-Burk plots.

Invertase immobilization on glyoxyl, MANAE and glutaraldehyde-agarose supports

Enzyme solutions were prepared by dissolving 0.35mg protein.mL^{-1} in 100mL immobilization buffer (100mM sodium carbonate buffer pH 10.2 for glyoxyl immobilization, 5mM sodium phosphate buffer pH 7.0 for MANAE-agarose immobilization and 200mM sodium phosphate buffer pH 7.0 for glutaraldehyde immobilization), 10g of support was added and the suspension gently stirred until the dissolved activity was constant. The buffers were chosen to prevent prior adsorption of the enzyme on the support and favor covalent bonding with the most reactive amines (Betancor et al., 2006). For supports bearing aldehyde groups, to terminate the enzyme-support reaction, the derivatives were reduced with 1mg.mL^{-1} sodium borohydride, stirred for 40min and then washed with abundant distilled water and pH 5.0 sodium acetate buffer.

Treatment of invertase adsorbed on MANAE with glutaraldehyde

An aliquot of the MANAE-agarose derivative was incubated with 0.5% glutaraldehyde in 5mM sodium phosphate buffer at pH 7.0 and 25 ºC for 2h, to produce the derivative MANAE-agarose-glutaraldehyde (López-Gallego et al., 2005).

Invertase immobilization on Eupergit or Sepabead supports

Ten grams of wet Eupergit-C 250L or Sepabeads FP-EC3 were added to 100mL of enzyme solution (0.2mg protein.mL^{-1}) in 1.0M potassium phosphate buffer at pH 7.0 (Mateo et al., 2007a). The suspensions were gently stirred at 25 ºC for 24h. To increase the number of enzyme-support covalent attachments, the derivatives were then incubated in 100mM sodium bicarbonate buffer at pH 10.0 for 24h. The remaining reactive groups were blocked by incubation in 3M glycine at pH 8.5 (4mL/g of support) and 25 ºC for 24h (Mateo et al., 2002). The derivatives were then washed with distilled water. During these immobilizations, samples of all suspensions and supernatants were periodically withdrawn and enzyme activity was determined.

RESULTS

Characterization of the soluble commercial invertase

Figure 1 shows the temperature profiles of the activity and stability of the soluble enzyme. Maximal activity was observed at 40 °C, and the energy of activation (E_A) for sucrose was 6.65kcal.mol^{-1} (27.80kJ.mol^{-1}). Invertase exhibited stability from 25 ºC to 50 ºC, with full activity after 1h of incubation at all temperatures in this range. The optimal pH for invertase was 5.0, but over 80% of the maximal activity (at pH 5.0) was observed in the range of pH 4.0 to 6.0 (Figure 2). Maximum enzyme stability was also at pH 5.0, but between pH 4.5 and 7.0 the residual activity was over 60%. Given these data, it was possible to choose the most suitable protocol to maintain the invertase active under the conditions necessary for immobilization.

Effects of immobilization on the activity and kinetic parameters of the enzyme

The enzyme was quite robust under a wide range of conditions (Figures 1 and 2), but our objective was to prepare an immobilized biocatalyst and, in the process, try to further improve some of its properties. To this end, invertase from *S. cerevisiae* was immobilized on various supports (Table 1), with acceptable yields (from 60% to 95%) in all cases: the relative activity ranged from 0.5% to 73.5%. The best results in terms of activity were obtained simply by adsorbing the enzyme on MANAE supports by ion exchange (21.6U.mg^{-1}). When this derivative was treated with glutaraldehyde, the activity was 13.1U.mg^{-1}, and a similar activity (10.8U.mg^{-1}) was obtained by immobilizing the enzyme on agarose supports pre-activated with glutaraldehyde. The amount of protein immobilized on commercial epoxy supports (Sepabeads and Eupergit) was higher than on other supports, but the expressed specific activity was under 10% of that of either free or MANAE-agarose immobilized invertase.

Figure 1: Effect of temperature on activity (□) and stability (■) of soluble invertase. The assays were performed in 50mM sodium acetate buffer at pH 5.0, with 1% sucrose as substrate.
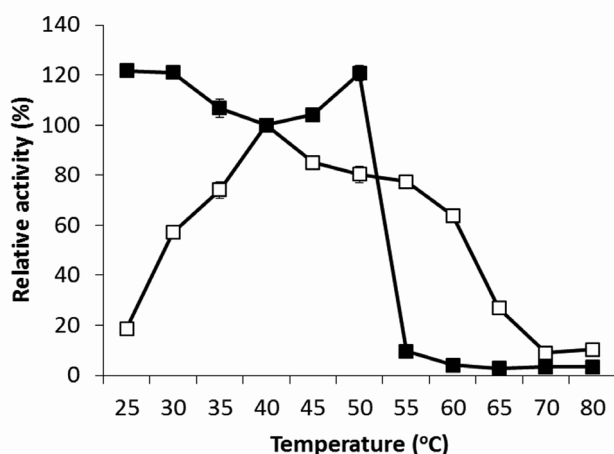

Figure 2: Effect of pH on activity (□) and stability (■) of soluble invertase. The assays were performed at 40 °C, with 1% sucrose as substrate.
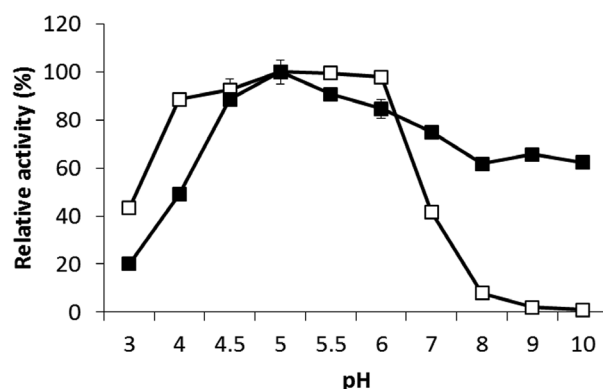

Table 1: Immobilization yields of invertase from *S. cerevisiae* on various supports.

Enzyme preparation	Immobilized Protein		Activity (V0)	
	(mg protein.mL^{-1} derivative)	Immobilization yield (%)	U.mg^{-1}	Recovered activity (%)
Soluble (3.5mg prot.mL^{-1})	-	-	29.5	100.0
Glyoxyl-agarose	2.2	62	1.7	5.8
MANAE-agarose	2.1	60	21.6	73.5
MANAE-agarose + glutaraldehyde	2.1	60	13.1	44.4
Glutaraldehyde-agarose	3.3	92	10.8	36.8
Sepabeads	3.4	94	2.0	6.8
Eupergit	3.4	95	0.2	0.5

Assays performed in 50mM sodium acetate buffer at pH 5.0 and 40 ºC, with 1% sucrose as substrate.

The effect of the temperature on the activity of the derivatives was studied. The optimal temperatures were a clear maximum at 55 ºC for the MANAE derivative, and a plateau from 45 °C to 55 ºC for the glutaraldehyde-treated derivative. Derivatives showed E_A values of 22.11kcal.mol^{-1} (92.49kJ.mol^{-1}) for MANAE, 7.95kcal.mol^{-1} (33.28kJ.mol^{-1}) for glutaraldehyde and 7.22kcal.mol^{-1} (30.22kJ.mol^{-1}) for glyoxyl-bound enzyme.

Table 2 shows a more detailed analysis of the changes in the kinetic properties of the enzyme after immobilization. Immobilization on MANAE-agarose left V_{max} almost unaltered (145U.mg^{-1} versus 154U.mg^{-1} for the soluble enzyme), while the K_m increased only 1.7-fold (perhaps partly due to diffusion limitation). The treatment of the enzyme adsorbed on MANAE-agarose with glutaraldehyde reduced the V_{max} by 40%, while K_m was increased 1.8-fold. A V_{max} over 81% of that of the soluble enzyme was attained with the pre-activated glutaraldehyde-agarose support, although K_m increased 2.9 fold. Again, this increment in the K_m could be partly due to diffusion or steric effects (suggesting a different orientation from the adsorbed derivative).

Table 2: Kinetic parameters found for soluble and variously immobilized invertase from S. cerevisiae for sucrose hydrolysis.

Enzyme preparation	K_m (mmol.L^{-1})	K_m increment factor	V_{max} U.mg^{-1}	V_{max} reduction factor
Soluble	90.9	1.0	153.8	1.0
Glyoxil-agarose	135.0	1.5	4.4	35.0
MANAE-agarose	154.5	1.7	144.9	1.1
MANAE-agarose + glutaraldehyde	161.5	1.8	90.1	1.7
Glutaraldehyde-agarose	263.2	2.9	125.0	1.2
Sepabeads	222.2	2.4	3.0	51.2
Eupergit	76.9	0.8	3.1	49.6

Assays performed in 50mM sodium acetate buffer at pH 5.0 and 40 ºC, with 1% sucrose as substrate.

Effects of immobilization on enzyme stability

Another key criterion in choosing an enzyme immobilization protocol is biocatalytic stability; therefore, this parameter was studied under a wide range of conditions. Figure 3 shows the thermal inactivation of invertase derivatives when incubated at pH 5.0 and 40 °C or 50 °C. In general, the immobilization procedures resulted in a very significant improvement of invertase stability. All invertase derivatives were very stable and retained about 100% of the activity after 24 hours of incubation at 40 °C (Figure 3A) or 50 °C (Figure 3B), while the soluble enzyme exhibited only 75% and 40% of its original activity after similar incubations.

Figure 3: Thermal inactivation of various immobilized invertase preparations. Inactivation was performed at (a) 40 ºC, pH 5.0 and (b) 50 ºC, pH 5.0, as described in Materials and Methods. Symbols: (●) Soluble enzyme; (□) Glyoxil-agarose immobilized enzyme; (△) MANAE–agarose immobilized enzyme; (X) MANAE–agarose-glutaraldehyde immobilized enzyme; (○) Glutaraldeyde-agarose immobilized enzyme.
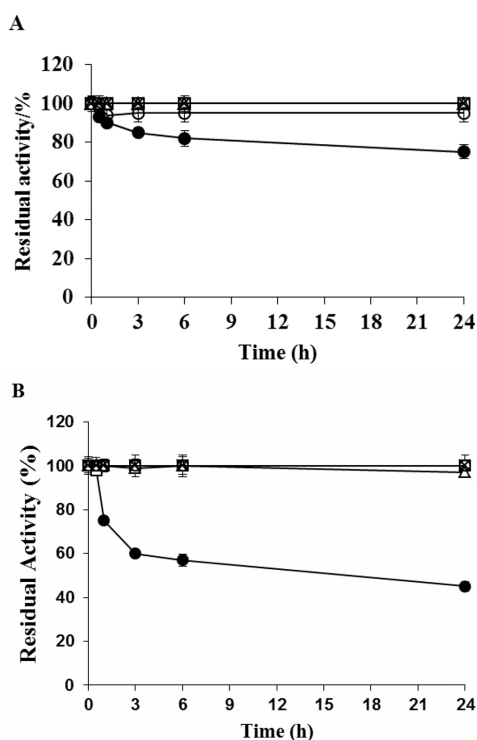

Figure 4: Thermal inactivation of various immobilized invertase preparations. Inactivation was performed at (a) 40 ºC or (b) 50 ºC, pH 7.0, as described in Materials and Methods. Symbols: (●) Soluble enzyme; (□) Glyoxil-agarose immobilized enzyme; (△) MANAE–agarose immobilized enzyme; (X) MANAE–agarose-glutaraldehyde immobilized enzyme; (o) Glutaraldeyde-agarose immobilized enzyme.
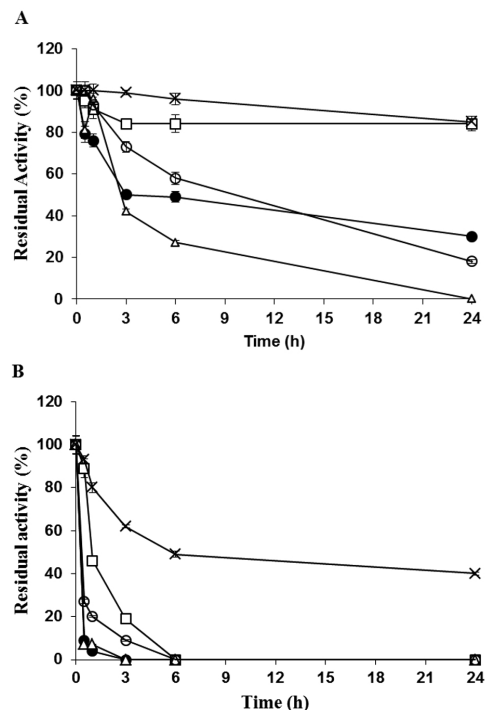

At pH 7.0, at both temperatures, only the adsorbed invertase-MANAE-agarose showed a stability as low as that of the soluble enzyme (Figure 4). This could be due to enzyme desorption from the support at these temperatures, because the pK of the amino group of the support is 6.9 (Fernandez-Lafuente et al., 1993). Bradford (1976) analysis of the supernatant also showed the presence of dissolved enzyme. The treatment of the invertase-MANAE-agarose with glutaraldehyde made the immobilized enzyme the most stable among the biocatalysts under study, at 40 °C and pH 7.0 (84% of activity was observed after 24h). The same preparation incubated at 50 °C and pH 7.0 retained about 50% of its activity after 24 hours, while these conditions fully inactivated the soluble enzyme after this time (Figure 4b) and all the other immobilized preparations after 6h.

Figure 5 shows the possibility of reusing some of the invertase derivatives. Invertase-MANAE, Invertase-MANAE treated with glutaraldehyde and invertase-glutaraldehyde pre-activated derivatives maintained their full activities for a sequence of 3 cycles. Moreover, the three preparations retained full activities after one month of storage at 4 ºC. The glyoxil derivative decreased in activity after the first utilization, retaining about 50% of its original activity after being used three times. Epoxy supports lost all activity after the first cycle.

Invertase immobilization based on MANAE and glutaraldehyde chemistry performed best among all the assayed immobilization methods. While only the enzyme bound by ion-exchange offered very good activity and stability, the stability was further improved by treatment of

the enzyme with glutaraldehyde (giving much better results than immobilization on glutaraldehyde supports). This preparation was very stable at pH 5.0 and 7.0, retaining 100% of the initial activity after 3 cycles of reuse.

Figure 5: Reusability of various *S. cerevisiae* invertase derivatives for three reaction cycles. The assays were performed in 50mM sodium acetate buffer at pH 5.0 and 40 ºC, using 1% sucrose as substrate.
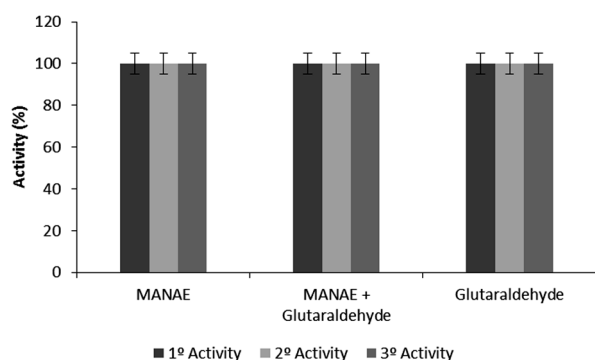
DISCUSSION

Invertase seems to be a hard enzyme to be immobilized. Some techniques that are usually successful in terms of stability and activity recovery, such as glyoxyl or epoxy immobilization (López-Gallego et al., 2005; Mateo et al., 2002; Mateo et al., 2005; Pedroche et al., 2007), led to severe drops in enzyme activity and poor stabilization. This may be due to the multimeric and glycosylated nature of the enzyme. The first implies that changes in the subunit assembly may incur a cost in activity and/or stability of the enzyme; the second hinders the formation of a strong covalent attachment.

Other authors have obtained similar results on invertase immobilization, in which the immobilized enzyme showed higher K_m values and lower V_{max} values than the soluble (Amaya-Delgado et al., 2006; Ettalibi & Baratti, 2001; Sahmetlioglu et al., 2006; Sanjay & Sugunan, 2005; Tümtürk et al., 2000). By comparison, it may be suggested that the enzyme immobilized on the glutaraldehyde-preactivated support at high ionic strength is different from that immobilized by ion exchange. The former method fixes the enzyme to the support by a covalent bond, which at pH 7.0 would involve the most reactive amino groups on the enzyme surface (Mateo et al., 2005), while ion exchange is based on the formation of multiple ionic bridges, involving the area where these are readily formed (very likely immobilizing the enzyme via a face that involves a large number of subunits in the immobilization).

It is remarkable that the invertase adsorbed on MANAE-agarose and later treated with glutaraldehyde was more stable than the enzyme immobilized on glutaraldehyde-activated agarose. Although at first glance the two methods seem similar, because they are both founded on glutaraldehyde chemistry, it is likely that the two preparations have different orientations with respect to the support and different multipoint covalent attachments, which may greatly affect the final properties of the enzyme (Hernandez & Fernandez-Lafuente, 2011). In the pre-activated support, using pH 7.0, the enzyme is immobilized via the terminal amino groups, which will be the most reactive under these conditions (Betancor et al., 2006). After this first immobilization, a direct reaction between amino groups of the external Lys residues (with a pK of 10.7) and the glutaraldehyde groups on the supports is necessary and, under these conditions, the reactivity of the Lys will be very low.

The adsorption of the enzyme on the ion-exchange support is by the area of the protein where a strong multipoint adsorption occurs most readily (where there is a higher density of negatively-charged groups) (Pessela et al., 2004), so it may be oriented on the support differently from the glutaraldehyde-bound enzyme. The treatment of the adsorbed enzyme with glutaraldehyde under the conditions utilized modifies each primary amino group of the enzyme and the support with a glutaraldehyde molecule (López-Gallego et al., 2005). This means that multipoint covalent attachment would require enzyme-amino-glutaraldehyde groups to react with glutaraldehyde-amino groups on the support, and this has been reported to occur easily at neutral pH (Mateo et al., 2006; Fernandez-Lafuente et al., 1995). This would involve a chemical modification of the whole surface of the enzyme, which in this case seems not to be very negative, and even could be partially responsible for the improved stability.

The higher stability of the adsorbed and then crosslinked preparation may be related to the high reactivity of amino-glutaraldehyde with other similar groups (Fernandez-Lafuente et al., 1995), although the different orientation or the chemical modification of the enzyme surface cannot be discarded as potentially responsible for these improved results.

From the above results, we conclude that the invertase-MANAE-agarose derivative showed the highest activity relative to the soluble enzyme (73.5%). This same derivative showed full activity after 24h at pH 5.0, at 40 ºC and 50 ºC. The invertase-MANAE-agarose derivative treated with glutaraldehyde and invertase-glutaraldehyde-agarose derivative activities were similar (44.4 and 36.8% of the soluble enzyme, respectively) and retained full activity after 24h at pH 5.0, at 40 ºC and 50 °C. At the same temperatures and pH 7.0, invertase-MANAE-agarose treated with glutaraldehyde was the most stable, retaining about 80% of the initial activity. The invertase-glyoxyl-agarose derivative and the epoxide derivatives (Sepabeads and Eupergit) showed no significant activity and stability. The derivatives invertase-MANAE-agarose, MANAE-agarose treated with glutaraldehyde and agarose pre-activated with glutaraldehyde were tested in 3 reaction cycles and retained full activity, and will be studied further to improve their hydrolytic efficiency.

ACKNOWLEDGEMENTS

RESUMO

Produção de glicose e frutose por invertase de Saccharomyces cerevisiae imobilizada em suporte MANAE-Agarose

Invertase de *Saccharomyces cerevisiae* foi imobilizada em agarose ativada com diferentes grupos (glioxil, MANAE ou glutaraldeído) e suportes epóxidos comerciais (Eupergit e Sepabeads). Derivados de invertase ativos e estabilizados foram produzidos pela adsorção da enzima em suportes MANAE-agarose, MANAE-agarose tratado com glutaraldeído e glutaraldeído-agarose. Em pH 5,0 estes derivados retiveram total atividade até 24h a 40 ºC e 50 ºC. Quando os ensaios foram a 40 °C e 50 °C com o pH alterado para 7,0, o derivado invertase-MANAE-agarose tratado com glutaraldeído apresentou 80% da atividade inicial. As atividades recuperadas dos derivados foram 73,5%, 44,4% e 36,8%, respectivamente para MANAE, MANAE tratado com glutaraldeído e glutaraldeído. Essas três preparações foram empregadas com sucesso em 3 ciclos de hidrólise da sacarose para produzir glicose e frutose.
Palavras-chave: Produção de glicose e frutose. Imobilização de invertase. Invertase. *Saccharomyces cerevisiae*

REFERENCES

Amaya-Delgado L, Hidalgo-Lara ME, Montes-Horcasitas MC. Hydrolysis of sucrose by invertase immobilized on nylon-6 microbeads. Food Chem. 2006;99(2):299-304. DOI:10.1016/j.foodchem.2005.07.048

Betancor L, López-Gallego F, Hidalgo A, Alonso-Morales N, Mateo G, Fernandez-Lafuente R, et al. Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions. Enzyme Microb Technol. 2006;39(4):877-82. DOI:10.1016/j.enzmictec.2006.01.014

Bradford MM. Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-54.

Burger M, Bacon JSD, Bacon EE. Some observations on form and location of invertase in yeast cell. Biochem J. 1961;78(3):504-11.

D'Souza SF, Godbole SS. Immobilization of invertase on rice husk using polyethylenimine. J Biochem Biophys Methods. 2002;52(1):59-62.

Esmon PC, Esmon BE, Schauer IE, Taylor A, Schekman R. Structure, assembly, and secretion of octameric invertase. J Biol Chem. 1987;262(9):4387-94.

Ettalibi M, Baratti JC. Sucrose hydrolysis by thermostable immobilized inulinases from Aspergillus ficuum. Enzyme Microb Technol. 2001;28(7-8):596-601.

Fernandez-Lafuente R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. Enzyme Microb Technol. 2009;45(6-7):405-18. DOI:10.1016/j.enzmictec.2009.08.009

Fernandez-Lafuente R, Rosell CM, Rodriguez V, Guisan JM. Strategies for enzyme stabilization by intramolecular cross-linking with bifunctional reagents. Enzyme Microb Technol. 1995;17(6):517-23.

Fernandez-Lafuente R, Rosell CM, Rodriguez V, Santana C, Soler G, Bastida A, et al. Preparation of activated supports containing low pK amino-groups – A new tool for protein immobilization via the carboxyl coupling method. Enzyme Microb Technol. 1993;15(7):546-50.

Gascón S, Neumann NP, Lampen JO. Comparative study of properties of purified internal and external invertases from yeast. J Biol Chem. 1968;243(7):1573-77.

Goulart AJ, Benedetti ACEP, Tavano OL, Marques DP, Contiero J, Carmona EC, Monti R. Multipoint immobilization of invertase on agarose: stability and kinetic properties. Curr Trends Biotechnol Pharm. 2008;2:462-70.

Hernandez K, Fernandez-Lafuente R. Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. Enzyme Microb Technol. 2011;48(2):107-22. DOI:10.1016/j.enzmictec.2010.10.003

Husain S, Jafri F, Saleemuddin M. Effects of chemical modification on the stability of invertase before and after immobilization. Enzyme Microb Technol. 1996;18(4):275-80.

Kotwal SM, Shankar V. Immobilized invertase. Biotechnol Adv. 2009;27(4):311-22. DOI:10.1016/j.biotechadv.2009.01.009

López-Gallego F, Betancor L, Mateo C, Hidalgo A, Alonso-Morales N, Dellamora-Ortiz G, et al. Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports. J Biotechnol. 2005;119(1):70-5. DOI:10.1016/j.jbiotec.2005.05.021

Mateo C, Abian O, Ernedo MB, Cuenca E, Fuentes M, Fernandez-Lorente G, et al. Some special features of glyoxyl supports to immobilize proteins. Enzyme Microb Technol. 2005;37(4):456-62. DOI:10.1016/j.enzmictec.2005.03.020

Mateo C, Abian O, Fernandez-Lorente G, Pedroche J, Fernandez-Lafuente R, Guisan JM. Epoxy sepabeads: A novel epoxy support for stabilization of industrial enzymes via very intense multipoint covalent attachment. Biotechnol Prog. 2002;18(3):629-34. DOI: 10.1021/bp010171n

Mateo C, Grazu V, Pessela BCC, Montes T, Palomo JM, Torres R, et al. Advances in the design of new epoxy supports for enzyme immobilization-stabilization. Biochem Soc Trans. 2007a;35(pt 6):1593-601. DOI:10.1042/BST0351593

Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques.

Enzyme Microb Technol. 2007b;40(6):1451-63. DOI: 10.1016/j.enzmictec.2007.01.018

Mateo C, Palomo JM, Fuentes M, Betancor L, Grazu V, López-Gallego F, et al. Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins. Enzyme Microb Technol. 2006;39(2):274-80. DOI:10.1016/j.enzmictec.2005.10.014

Pedroche J, Yust MD, Mateo C, Fernandez-Lafuente R, Giron-Calle J, Alaiz M, et al. Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: Correlation between enzyme-support linkages and thermal stability. Enzyme Microb Technol. 2007;40(5):1160-6. DOI:10.1016/j.enzmictec.2006.08.023

Pessela BCC, Munilla R, Betancor L, Fuentes M, Carrascosa AV, Vian A, et al. Ion exchange using poorly activated supports, an easy way for purification of large proteins. J Chromatogr A. 2004;1034(1-2):155-9. DOI:10.1016/j.chroma.2004.01.061

Sahmetlioglu E, Yuruk H, Toppare L, Cianga I, Yagci Y. Immobilization of invertase and glucose oxidase in conducting copolymers of thiophene functionalized poly(vinyl alcohol) with pyrrole. React Funct Polym. 2006;66(3):365-71. DOI:10.1016/j.reactfunctpolym.2005.08.009

Sanjay G, Sugunan S. Invertase immobilised on montmorillonite: reusability enhancement and reduction in leaching. Catal Commun. 2005;6(1):81-6. DOI:10.1016/j.catcom.2004.11.003

Sanjay G, Sugunan S. Enhanced pH and thermal stabilities of invertase immobilized on montmorillonite K-10. Food Chem. 2006;94(4):573-9. DOI:10.1016/j.foodchem.2004.12.043

Torres R, Mateo C, Fuentes M, Palomo JM, Ortiz C, Fernandez-Lafuente R, et al. Reversible immobilization of invertase on Sepabeads coated with polyethyleneimine: Optimization of the biocatalyst's stability. Biotechnol Prog. 2002;18(6):1221-6. DOI: 10.1021/bp020082q

Tümtürk H, Arslan F, Disli A, Tufan Y. Immobilization of invertase attached to a granular dimer acid-co-alkyl polyamine. Food Chem. 2000;69(1):5-9.

Uhlig H. Hydrolysis of protein, fat, and cellulose, and inversion of sucrose. In: Gerhartz W. Enzymes in industry: production and applications. New York: VHC; 1990. p. 129-30.