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ARTICLE

# Immobilization of Cell Wall Invertase Modified with Glutaraldehyde for Continuous Production of Invert Sugar

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Yeast cell wall invertase (CWI) was modified with dimethyl suberimidate, glutaraldehyde, formaldehyde, and sodium periodate. Retained activity after modification was 45% for CWI modified with formaldehyde, 77% for CWI modified with sodium periodate, 80% for CWI modified with glutaraldehyde, and 115% for CWI modified with dimethyl suberimidate. Chemically modified and native CWIs showed significantly broad pH stability (pH 3−11), whereas after incubations at 50, 60, and 70 °C, CWI modified with glutaraldehyde showed the highest thermostability. Optimum pH for CWI modified with glutaraldehyde was between 4 and 5, whereas optimum temperature was at 60 °C. Comparison to CWI modified with glutaraldehyde after immobilization within alginate beads showed broader pH optimum (4.0−5.5) as well as broader temperature optimum (55−70 °C). Column bed reactor packed with the immobilized CWI modified with glutaraldehyde was successfully used for the 95% inversion of 60% (w/w) sucrose at the flow rate of 3 bed volumes per hour, pH 4.9, and 45 °C. A 1 month productivity of 3844 kg of inverted sugar/kg of the immobilisate was obtained.

**KEYWORDS: Cell wall invertase; immobilization; alginate; chemical modification; glutaraldehyde; invert sugar**

## INTRODUCTION

Chemical modifications are a powerful tool for altering biotechnologically important enzymes, either by enhancing their stability, improving their selectivity, or changing their surfaces to improve their reversible immobilization (*1*). Chemical modifications are often shortcuts for obtaining more efficient enzymes and, combined with the mutations, can provide solutions for creating an "ideal" enzyme (*2*).

Invertase is one of the most studied enzymes so far. Yeast invertase is a dimeric glycoprotein and can be found in internal (nonglycosylated) or external (glycosylated) form. The average mass of internal invertase is 59 kDa, whereas the mass range of external invertase (74−121 kDa) depends on the observed glycan masses (the shortest chains are 16 kDa; the longest chains are 62 kDa) (*3*). Invertase is cheap and can be easily obtained in the soluble form (*4*, *5*) or cell wall form (*6*). Soluble invertase preparations have at least four isoforms that have been recently characterized in detail (*7*). Because of enzyme availability, there is no practical interest in its improvement by mutations. However, further improvement of enzyme stability due to its wide industrial applications such as production of invert sugar (*8*), production of fructose (*9*), or alcohol fermentation (*10*) justifies the necessity of employing chemical modifications (*11*).

Either side-chain amino groups or glycosyl residues in invertase molecules can be chemically modified. However, the result of amino group modifications was rarely an enzyme with enhanced stability. Site-directed mutagenesis studies demonstrated that the catalytic residues of invertase are Asp23 and Cys205, functioning as proton acceptor and donor, respectively (*12*, *13*). Because no amino group with a catalytic function was reported, dimethyl suberimidate (DMS), a homobifunctional bisimidoester, was selected as the cross-linker (*14*). Hence, all other chemical modifiers that would not react with Asp and Cys can be used. Because invertase is a highly glycosylated protein, introduction of additional sugars into the enzyme seems to be less justified. Very few previous studies have shown that partial (*15*) or complete removal (*16*) of carbohydrates had no effect on invertase stability. On the contrary, the majority of previous studies have confirmed that deglycosylation enhanced enzyme instability toward denaturants such as chaotropic agents (*17*) or temperature (*18*). The importance of polysaccharides for invertase stability was confirmed when neoglycoproteins were developed by the modificaton of invertase with pectin (*19*), chitosan (*20*, *21*), or carboxymethylcellulose (CMC) (*22*). The result of these chemical modifications was enhancement of thermal stability by 6−10 °C compared to native invertase, and in the case of pectin the half-life at 65 °C was prolonged from a couple of minutes to 2 days (*19*). Chemically modified invertase was further stabilized by immobilization (*23*).

To date, there has been no attempt to chemically modify cell wall invertase (CWI) mainly because these forms of invertase were not exploited until recently, when, in our previous work, reproductive CWI preparation was established for the first time (*6*). Before that, the similar CWI preparation was cross-linked with glutaraldehyde in the presence of polyethylenimine

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(PEI) (*24*). However, the obtained cell wall biocatalyst was substantially less stable than the whole yeast cell biocatalyst (*25*).

In our previous work CWI was successfully immobilized within calcium alginate beads. A biocatalyst with a high enzyme load, without enzyme loss in the gel forming solution, without enzyme leakage, and with a strong "depot" effect was obtained (*6*). The aim of this work was to further stabilize the CWI by chemical modification and immobilization. Various properties (pH stability and optima, temperature optima, and thermal stability parameters) of the biocatalyst were examined. The ability of the biocatalyst to be used for continuous production of invert sugar was tested in a packed bed column reactor.

## MATERIALS AND METHODS

**Chemicals.** Sodium alginate, sucrose, glucose, and fructose were purchased from Sigma (St. Louis, MO). Sodium alginate Grindsted Alginate FD 155 was a generous gift from Danisco (Copenhagen, Denmark). Baker's yeast was kindly donated from Altech Fermin (Senta, Serbia). All other chemicals were of analytical grade (or higher) and were purchased from Sigma and Merck.

**Modification of CWI.** CWI was prepared according to the procedure given in our previous work (*6*). Autolysis of baker's yeast was performed at 37 °C using 3% toluene and 1% $Na_2CO_3$ for 4 h with occasional shaking. The obtained slurry was diluted with an equal volume of 1% sodium chloride and mixed for 30 min. The yeast cell wall was centrifugated, and the pellet was resuspended in 5 volumes of distilled water. The procedure was repeated until proteins could no longer be detected in the supernatant. The resulting solid material was defatted and dried using cold acetone. The pellet was left to dry overnight at room temperature. CWI was suspended in appropriate buffer and modified with DMS, glutaraldehyde (GA), formaldehyde (FA), or sodium periodate (SPI). The final concentration of CWI in the reaction mixture was 250 mg/mL, whereas the final concentrations of modifiers were 0.31% GA, 0.50% FA, 0.30% DMS, and 0.50% SPI. Modifications of CWI with DMS, GA, and FA were performed in 50 mM phosphate buffer, pH 7.2, whereas in the case of SPI 50 mM phosphate buffer, pH 7.2, or 50 mM acetate buffer, pH 4.5, were used. Reaction mixtures were incubated at room temperature for 4 h with constant shaking. Thereafter, reactions were stopped by centrifugation of modified CWIs and subsequent washing with 50 mM phosphate buffer, pH 7.2, or 50 mM acetate buffer, pH 4.5, five times. Excess of activated groups in CWIs modified with DMS, GA, and FA was quenched by the addition of 1% ethanolamine solution, pH 7.2, whereas reaction with SPI was quenched by the addition of 1% ethylenediamine solution. Obtained modified CWIs were denoted according to the chemical modifier used: CWI-S (CWI modified with DMS), CWI-G (CWI modified with GA), CWI-F (CWI modified with FA), and CWI-P (CWI modified with SPI).

**Enzyme Activity Assay.** The assay was performed in a batch reactor at 25 °C. Fifty microliters of CWI or modified CWI suspensions (final concentration = 1 mg/mL) was mixed with 450 $\mu$L of sucrose solution (0.3 M in 50 mM acetate buffer, pH 4.5). After 5 min, the reaction was stopped by the addition of DNS reagent (500 $\mu$L). Reaction mixtures were heated in a boiling water bath for 5 min and then immediately cooled to room temperature. After dilution with 4 mL of water, the amount of reducing sugars was determined spectrophotometrically at 540 nm (*26*) using a Philips UV/vis/NIR PU 8630 spectrophotometer (L. G. Philips-Displays, Blackburn, U.K.). One international unit (IU) of enzyme activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 $\mu$mol of sucrose per minute under the assay conditions.

**pH Stability and Thermal Stability of Native and Modified CWIs.** pH stability was studied from pH 3.0 to 12.0 using Britton−Robinson universal buffer. Ten milligrams of biocatalysts was suspended in 1 mL of 0.04 M buffer of different pH values and allowed to incubate for 150 min at 25 °C. Thereafter, 50 $\mu$L of those suspensions was transferred to 450 $\mu$L of 0.05 M acetate buffer, pH 4.5, and the enzyme activity was monitored as described.

The thermal stability was ascertained at 50, 60, and 70 °C. Half a milligram of biocatalyst was suspended in 0.5 mL of 0.05 M acetate buffer, pH 4.5, and allowed to incubate for various time intervals (totally 21 days at 50 °C, 5 h at 60 °C, and 30 min at 70 °C) during which enzyme activity was monitored as described.

**Optimum pH and Optimum Temperature for Native CWI and CWI-G.** The effect of pH on the activity of native and GA modified CWIs was studied at 25 °C by varying the pH of the reaction mixture in the range of 3.0−8.0 using Britton−Robinson universal buffer. Other reaction conditions and enzyme activity monitoring were as described.

The optimum temperature of native and modified biocatalyst was determined by performing the reaction in the temperature range of 30−70 °C (at 10 °C increments). Other reaction conditions and enzyme activity monitoring were as described.

**Immobilization of CWI-G.** Calcium alginate gel beads were prepared by dropping a mixture containing 300 mL of 2% sodium alginate and 300 mL of CWI-G suspension (100 mg/mL) into 4 L of 2% $CaCl_2$ (solution was prepared by using anhydrous $CaCl_2$) with constant stirring. The beads were left to harden for 2 h in the same $CaCl_2$ solution before being washed with 1 L of 2% $CaCl_2$ and stored at 4 °C in 70% (w/v) invert sugar prior to use.

**Packed Bed Reactor.** Immobilized CWI-G in calcium alginate hydrogel was tested in a packed bed reactor. The experiments were carried out in a 460 mL water-jacketed glass column (50 cm long and 4 cm in diameter) at 45 ± 2 °C; 60 and 70% (w/w) sucrose solutions were used as substrates. Besides the pH of sucrose solution in water (7.8), a 60% sucrose solution was additionally buffered with acetate buffer to pH values of 6.8, 6.2, and 4.9. Substrate solutions were brought to 45 °C before entering the column and pumped through the bed by means of a peristaltic pump. After steady state was attained, the ratio of conversion was evaluated at the end of the column by determining reducing sugars, as described. The concentration of the sucrose solution was checked using a refractometer, SinoTech model 2WAJ.

**Statistical Analysis.** Each data point in the figures represents the mean of three independent assays. The data in **Figures 1−5** are presented as the mean ± standard error of the mean (SEM) and as percentages, taking the control value as 100%.

## RESULTS AND DISCUSSION

**Modification of CWI.** In this work four different types of chemical modifications of CWI were performed. Types of chemical modifications used were chosen according to previously published data about the ability of modifiers to improve thermostability or other important operational parameters of biocatalysts as well as to sanitize immobilized enzymes, which is an important criterion for prolonged use of immobilized biocatalysts (*2*, *11*, *14*, *15*). DMS, GA, and FA modified side-chain amino groups in the protein part of the invertase molecule, with cross-linking effect as a result. The effect of creating the protein network by cross-linking was most significant with GA modification of CWI. On the contrary, DMS was responsible for intramolecular amino acid cross-linking with no protein network creation. FA was capable of both inter- and intra-cross-linking, whereas SPI cross-linking was a consequence of reaction between activated sugar moieties and the neighboring side-chain amino groups.

The obtained modified CWIs differed from each other in terms of the activity retained after the modification (**Figure 1**). Retained activity was 45% for CWI-F, 77% for CWI-P, 80% for CWI-G, and 115% for CWI-S. Similar results were obtained for soluble invertase modified with SPI (*15*). However, due to the inhomogeneity of both commercially purchased or in situ purified invertase preparations, there are very few, inconsistent, literature data about the effects of other modifiers on soluble invertase. In a previous attempt to modify CWI, CWI was cross-linked with glutaraldehyde in the presence of PEI (*24*). However, an unstable derivate of CWI was obtained, probably due to the presence of salt bridges established with PEI that changed the conformation of CWI prior to the addition of GA. The presence of salt bridges had a destabilizing effect during the immobilization of soluble invertase on DEAE as well (*27*).

Unlike the reaction conditions for soluble invertase modification, reaction conditions for CWI modification were easily

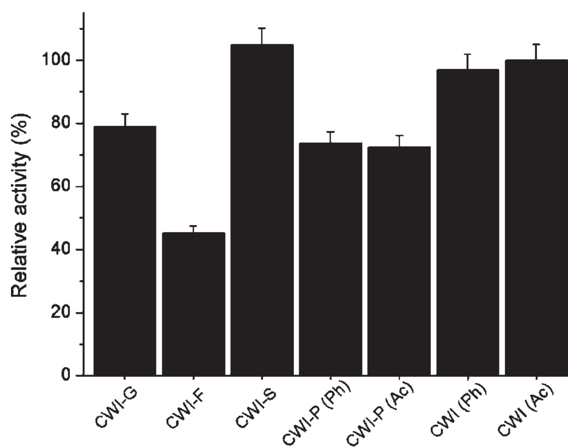


**Figure 1.** Relative activities of native and modified CWIs. CWI, cell wall invertase; CWI-G, glutaraldehyde-modified CWI; CWI-F, formaldehyde-modified CWI; CWI-S, dimethyl suberimidate-modified CWI; CWI-P, sodium periodate-modified CWI; Ph, phosphate buffer, pH 7.2; Ac, acetate buffer, pH 4.5. Each bar represents the mean of three independent assays (standard errors were <5% of the means).

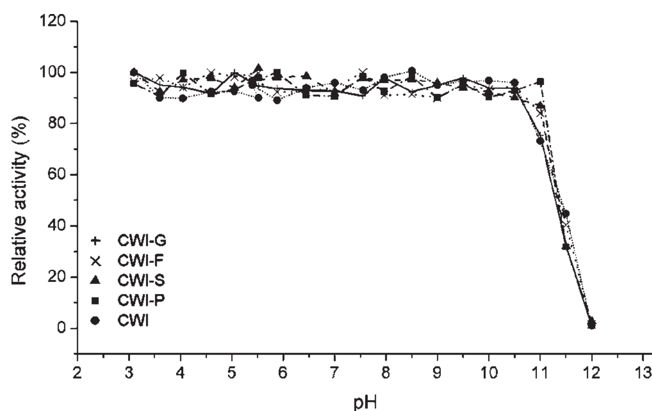


**Figure 2.** pH stability of native and modified CWIs. Each data point represents the mean of three independent assays (standard errors were <5% of the means).

controlled, because reactions were immediately stopped by the removal of CWI from the heterogeneous reactant mixtures by centrifugation and subsequent washing of CWIs. Chemically modified or activated groups were quenched by the addition of ethanolamine in the case of bifunctional protein cross-linkers or by the addition of ethylendiamine in the case of SPI modification according to the previously published procedure (*15*).

**pH Stability and Thermal Stability of Native and Modified CWIs.** pH stability observed in this study was the same for modified and native CWIs (**Figure 2**). However, chemically modified and native CWIs had significantly broader pH stability (pH 3−11) than native and DMS-modified soluble invertase (pH 3−8) (*14*). Chemical cross-linking of enzyme molecules with bifunctional cross-linking agents such as DMS can be used for the reinforcement of the compact tertiary structures, resulting in protein stabilization against pH inactivation (*28*).

Thermostability was higher for modified than for native CWIs (**Figure 3**). Similar to the observation for pH stability, derivates of CWI had significantly higher thermostability than derivates of soluble invertase (*15*). After 120 min at 60 °C, the most stable soluble invertase derivates, invertase modified with periodate followed by ethylenediamine (PEDA-INV), retained 12% of activity, whereas CWI-G and CWI-F retained 45% of activity

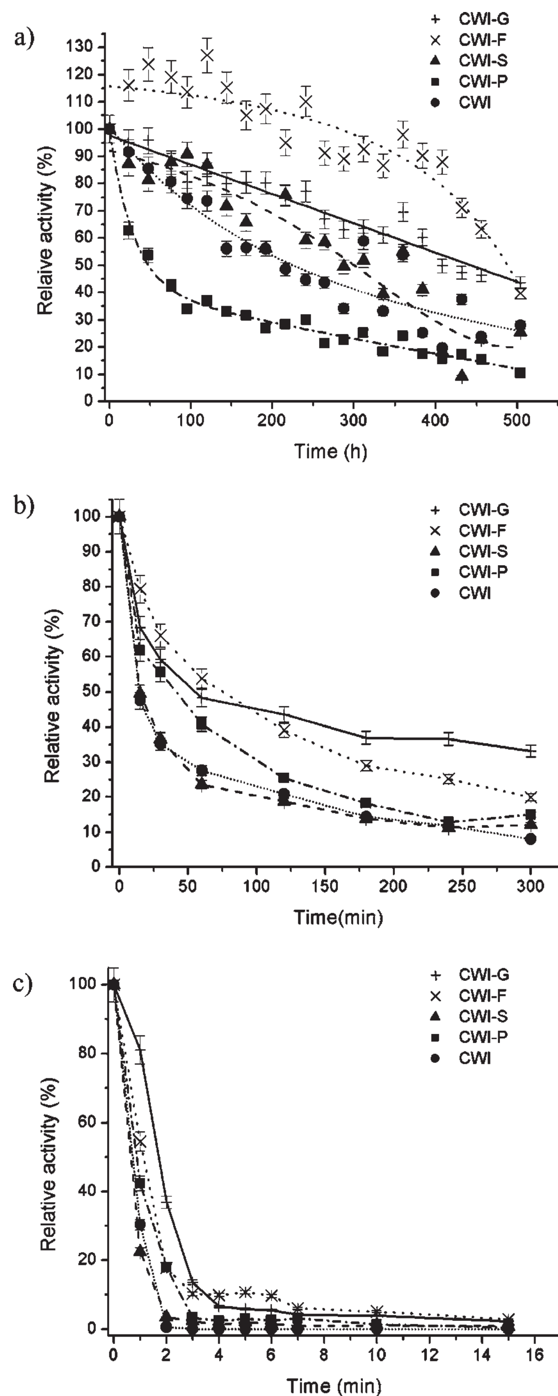


**Figure 3.** Thermal stability of native and modified CWIs: (**a**) 50 °C; (**b**) 60 °C; (**c**) 70 °C. Each data point represents the mean of three independent assays (standard errors were <5% of the means).

at the same reaction conditions (**Figure 3b**). After 300 min, CWI-G still had 40% of activity. There are not sufficient literature data concerning chemically modified soluble invertase. In one study it has been shown that neo-glycoconjugate pectin-PEDA-INV had significantly higher thermostability then PEDA-INV (*19*). Although neo-glycoconjugates as model systems for studying the influence of newly added sugar structure on enzyme functions are interesting, they cannot be applied in real industrial conditions due to the expensive procedure for their preparation. Data about their thermostability after prolonged time are also insufficient. Invertase can suddenly change behavior over time as has been shown for CWI-F (**Figure 3a**). At 50 °C CWI-F lost stability

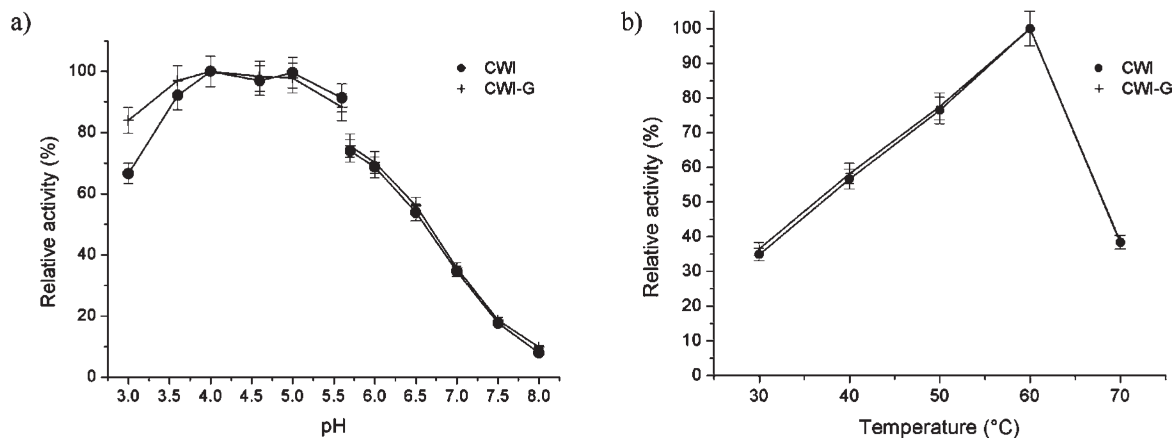


**Figure 4.** Influence of pH (**a**) and temperature (**b**) on the activity of CWI and CWI-G. Each data point represents the mean of three independent assays (standard errors were <5% of the means).

after 300 h, although it was the most stable by that time. After 500 h of incubation at 50 °C as well as after incubations at 60 and 70 °C, CWI-G was the most stable derivate of CWI; hence, it was chosen for immobilization and further studies.

**Optimum pH and Optimum Temperature for Native CWI and CWI-G.** The optimum pH for native CWI and CWI-G was between 4 and 5, whereas no enzymatic activity was detected in buffers above pH 8.0 (**Figure 4a**). Both native CWI and CWI-G retained 55% of the activity at pH 6.5. At the same pH value soluble unmodified invertase retained only 10% of the activity (*29*), whereas chitosan derivate of soluble invertase retained 60% of the activity (*30*).

Temperature-dependent CWI-G and native CWI activity was determined within a range from 30 to 70 °C. CWI-G and native CWI showed almost identical temperature dependence with maximal activity at 60 °C (**Figure 4b**). Although the activity at 60 °C was significantly higher than at 45 °C, due to the increased stability of CWI at 45 °C, this temperature was chosen for investigation of inversion degree of immobilized CWI-G in the column reactor.

**Immobilization of CWI-G.** There is ongoing interest in obtaining stable invertase for use in the sugar industry. Although pure enzymes were immobilized onto different carriers to obtain high specific activity of immobilisate, there is still no definite answer about the best carrier to be used. This is mainly due to the existence of different invertase isoforms that have been immobilized and partly due to the different immobilization techniques and matrices that have been employed. Native CWI is actually a fine powder, having a particle size of 3 $\mu$m, and as such it cannot be separated from the inverts sugar after the completed inversion. This is another reason, apart from biocatalyst stabilization, why immobilization of CWI in alginate is an advantage.

After modification of CWI with GA, the mechanical properties of the obtained CWI-G were slightly better than for native CWI. This was shown by mixing CWI and CWI-G for 15 min at 500 rpm, after which the degree of turbidity as well as the enzyme activity after separation of beads was slightly higher for CWI. Because the particles can be easily suspended in water, the immobilization procedure was the same as in our previous work (*6*). After the immobilization, alginate beads were shrinking in concentrated sucrose solutions, so the radius of beads was about half the value for native alginate beads. This phenomenon was not observed by other authors that have immobilized invertase in alginate (*29*, *31*). This might be a consequence of testing the immobilisate in real industrial conditions (high sucrose concentration, high temperature) or simply because of the nature

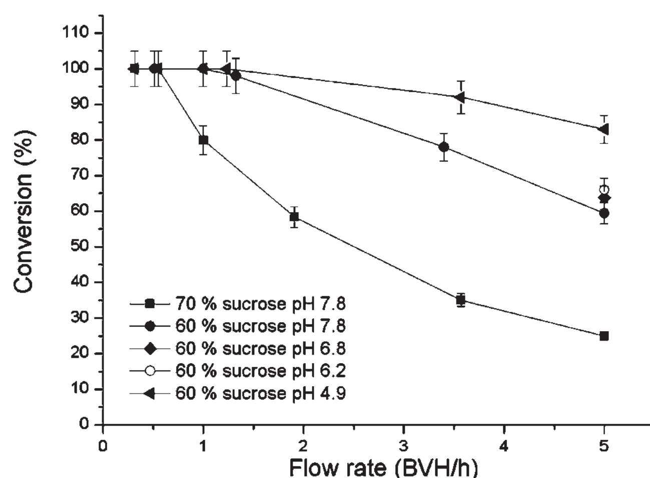


**Figure 5.** Effect of substrate flow rate on conversion in a bed reactor packed with immobilized CWI-G. Each data point represents the mean of three independent assays (standard errors were <5% of the means).

of alginate, used in this work, that might have different properties compared to others. During the osmotic pressure changes that occur when high-concentrated sucrose solutions for testing the immobilisate are used, soluble enzymes can be easily sucked out (*29*), whereas in the case of CWI, this is impossible because the cell wall is a 3 $\mu$m size particle and, unlike the soluble enzyme, alters the mechanical properties of alginate beads, making them harder and more resistant. Immobilized CWI-G when compared with immobilized CWI (*6*) indicates higher industrial potential. The activity of CWI-G was 91 U/g, which is 28% higher than the activity of immobilized native CWI, with pH optimum over a wider range.

**Packed Bed Reactor.** A thermostated bed reactor of 460 mL total volume was packed with 280 mL of CWI-G alginate beads (diameter = 2 mm), previously equilibrated in sucrose; 60 and 70% w/w sucrose solutions were pumped into the column by the means of a peristaltic pump. Inversion of sucrose was monitored at different pH values and flow rates (**Figure 5**). When 70% of nonbuffered sucrose was used, inversion was rapidly decreased with the flow rate. At the flow rate of 0.7 bed volume per hour (BVH), inversion was 100%. At 1 BVH inversion decreased to 80%, whereas at 5 BVH it decreased to 20%. The influence of pH can be more clearly observed with 60% sucrose. At pH 4.9 and flow rate of 5 BVH, inversion of 60% sucrose was 83%. Ninety-five percent of inversion was fulfilled at 3 BVH, pH 4.9, and 45 °C

with 60% of sucrose. Comparison of the idealized experiment setup with 200 mM sucrose, with which no product inhibition occurs, and 30 °C described by others (*21*, *31*) and our real industrial conditions is almost impossible. At pH 4.9 and flow rate of 5 BVH, inversion of 60% sucrose was not changed during the 30 days of testing, having total productivity of 3844 kg of inverted sugar/kg of the immobilisate, that is, beads. This amount was slightly less compared to the productivity of the whole yeast cell immobilized to wool (*32*) but is >2 times higher than the productivity of whole yeast cells immobilized by aggregation and mutual covalent binding (*24*).

Preventing denaturation at higher temperatures in the invert sugar industry is a demand that we have solved by modifying the CWI with reagents that have been approved in the food industry and with subsequent immobilization in alginate. CWI-G modified in this work can be easily immobilized, without enzyme activity loss and with the strong "depot" effect. Immobilized chemically modified CWI has a higher industrial potential then immobilized native CWI, and it is as efficient as highly active, covalently immobilized, soluble invertase (*33*). At 3 BVH and 45 °C, CWI-G had 95% inversion. Finally, the cost of the biocatalyst (U.S. $5.1/kg) is negligibly higher than the cost of the native CWI biocatalyst.

## ABBREVIATIONS USED

BVH, bed volumes per hour; CMC, carboxymethylcellulose; CWI, cell wall invertase; CWI-F, CWI modified with FA; CWI-G, CWI modified with GA; CWI-P, CWI modified with SPI; CWI-S, CWI modified with DMS; DEAE, diethylaminoethyl cellulose; DMS, dimethyl suberimidate; DNS, 3,5-dinitrosalicylic acid; FA, formaldehyde; GA, glutaraldehyde; PEDA-INV, invertase modified with periodate followed by ethylenediamine; PEI, polyethylenimine; SPI, sodium periodate.

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