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Performance of invertase immobilized on glass–ceramic supports in batch bioreactor

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ABSTRACT

Invertase was covalently immobilized on glass–ceramic support (GCS) obtained from coal fly ashes (CFA) with polyvinyl pyrrolidone (PVP) and magnesium stearate added as temporary additive. The GCS samples containing $Pb(CH_3COO)_2$, (GCS*Pb*) or $ZnSO_4$ (GCS*Zn*) were obtained in several sintering temperatures (1000–1200 °C) and times (1–3 h). The apparent values of porosity, water absorption, density and uniaxial compressive strength were studied by factorial design and morphological differences by scanning electron microscope. The best covalent immobilized invertase derivative was on GCS*Zn* (1200 °C/1 h) with 4398 ± 59.75 U/g GCS. However, 95.83% of immobilized invertase activity was obtained using an economical protocol with concentration of 0.636 mM 3-aminopropyltriethoxy silane (3-APTES) and 0.576 mM glutaraldehyde. The GCS*Zn*-invertase derivative retained 100% of initial activity after nine reuses (5476.19 ± 155.49 U/g GCS*Zn*). The CFA showed to be a promising biotechnological alternative as immobilization support for production of invert sugar and may be for other industrial enzymes.

1. Introduction

Many materials have been studied by different researchers to obtain supports for immobilization of biocatalysts which would be viable for the industrial scale. But, few studies describe the use of mineral sources [1] or waste from biotechnological processes for the development of supports. This may be an alternative to traditional chemical strategies and complicated procedures for immobilization of enzymes. Different supports have been investigated to immobilize enzymes due to the reuse and greater stability of the enzymatic activity. Mineral supports have been described for immobilization of enzymes, such as ceramics monoliths [2], montmorillonite [1], glass porous [3], silica [4], ceramic from carbonized mesostructured [5] and ceramic membrane [6]. These supports were studied due to their chemical characteristics which make easy the process of activation, providing hydroxyl groups to react with 3-aminopropyltriethoxysilane (3-APTES) and followed by cross-linking agent glutaraldehyde. The physical properties of these supports: mechanical strength, resistance to high temperatures, chemicals and microbial contamination, are of great value to industrial application. In addition to these properties, the increase in the surface area due to the porosity of these supports, may allow greater interaction with biomolecules than non-porous supports [2,5,7].

Abbreviations: 3-APTES, 3-aminopropyltriethoxysilane; A_d, apparent density; A_p, apparent porosity; A_{wa}, apparent water absorption; CFA, coal fly ashes; M_{dry}, mass dry; GCS, glass–ceramic support; GCSC, glass–ceramic support without addictive (control); GCS*Pb*, glass–ceramic support with addition of lead acetate; GCS*Zn*, glass–ceramic support with addition of zinc sulfate; M_{imm}, mass immerse; PVP, polyvinyl pyrrolidone; M_{sat}, mass satured; SEM, scanning electron microscope; XRD, X-ray diffraction; XRF, X-ray fluorescence spectroscopy; U_{cs}, uniaxial compressive strength.

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Coal fly ashes (CFA) are mainly sources of SiO_2, Al_2O_3, CaO, Fe_2O_3, and other oxides sources [8]. The sinterization process is well-known in the conventional production ceramic materials for the civil construction industry [9]. The glass–ceramic porous supports from coal fly ashes are obtained at high temperature [10] and have been an alternative to immobilize enzymes due to their chemical composition. Actually, many countries produce considerable CFA in thermal power plants [11]. The coal fly ashes are classified as hazardous pollutants due to its chemical composition and tiny sized, less than 2.5 μm [12]. It has been reported a lot of work about the application of additives (linkers and/or mineral compounds) to improve the pressing of the products using CFA as row material. The pharmaceutical industry applies with efficiency both the polyvinyl pyrrolidone and magnesium stearate to implement the technology pressing drugs [13].

Polyvinyl pyrrolidone has been used as binder to produce deformable granules using dried slurry because it evaporates without leaving residue after sinterization, acting as temporary binder [14]. Leroy et al. [15] used CFA with $CaCO_3$ and Na_2CO_3 as additives to produce glass–ceramic which in some case showed better performances than the conventional ceramic tiles. Others additives as sucrose has been studied to obtain macrostructured carbonized ceramics with carbon layer of different morphology (filamentous) causing higher porosity for immobilization of the glucoamylase inside the support [5].

Invertase (β-D-fructofuranosidase E. C. 3.2.1.26) catalyzes the hydrolysis sucrose in α-D-glucose and β-D-fructose [16]. The immobilization of invertase has been shown a great biotechnological potential because of the relatively inexpensive costs of its substrate, and also its importance in food [17] and drink [18] industry. The fundamental advantages are: the obtainment of white sugar syrup; preventing the crystallization when stored in high concentration; and conservation of the properties of food in long period of time when compared with the acid hydrolysis. The acid hydrolysis causes a product with black color and high concentrations of toxic waste called 5-hidroximethil-2-furfuraldehyde [19–22]. The main proposal of this work was immobilizing invertase on glass–ceramic supports (GCS) obtained from CFA by covalent binding using economical protocols with small concentrations of chemical compounds.

2. Material and methods

2.1. Materials

Invertase (EC 3.2.1.26) was donated by Novozymes Latin America Ltda. (Araucaria, PR, Brazil). 3-Aminopropyltriethoxysilane (3-APTS), polyvinyl pyrrolidone (PVP (MM 50,000)) and magnesium stearate were obtained by Sigma–Aldrich Chemicals Inc. (St. Louis, MO, USA). The glutaraldehyde, sucrose, acetone, ethanol, lead acetate, zinc sulfate, monobasic sodium phosphate, bibasic sodium phosphate were obtained from Vetec fine chemistry Ltda. (Duque de Caxias, RJ, Brazil). Coal fly ashes (CFA) were obtained from the combustion of coal mineral of Thermal Power Plant President Medici (Candiota, RS, Brazil).

2.2. Formulation of glass–ceramic supports

Samples (200 g) of CFA were previously treated using a sieve mesh opening of 3.0 mm and 1.5 mm, followed the standardized samples were dried at 70 °C under air circulation. These samples were mixed with 20 ml of 8 mM PVP solution in ethanol, homogenized and dry (2 h) at 70 °C under air circulation. After cooling, the uniform particles size was obtained using a mechanical granulator (Neuberger, SP, Brazil) – equipped with the sieve (3.0 mm and 1.5 mm of mesh opening) followed by the addition of magnesium stearate (2 g) and homogenized in an enclosed space under axial rotation (100 rpm). The granulated samples were used for obtaining the green bodies control (GCSC) applying uniaxial pressing (764 MPa) with eccentric compressor (Neuberger, SP, Brazil) which camera diameter was 7 mm. Other green bodies were prepared as described above to GCS*C* added of: 7.58 g $Pb(CH_3COO)_2$ (GCS*Pb*) or 0.57 g $ZnSO_4$ (GCS*Zn*). Then, all green bodies were submitted, individually, to following treatments of sinterization: 1000 °C/1 h, 1000 °C/3 h, 1100 °C/2 h, 1200 °C/1 h, 1200 °C/3 h. The furnace was programmed with heating rate of 6 °C/min to reach each sintering temperature. After, cooling (25 ± 2 °C) the glass–ceramic supports (GCS) were stored in airtight containers.

2.3. Chemical and physical characterizations of macro and microstructure of GCS

The physical properties were estimated according to and Ersoy et al. [10] and Little et al. [23]. The apparent values of porosity (A_p), water absorption (A_{wa}) and density (A_d) in GCSC, GCS*Pb* and GCS*Zn* were obtained in the five conditions of sinterization. They were determined by measuring the dry mass (M_{dry}), immersed mass (M_{imm}) and 24 h saturated surface-dry mass (M_{sat}). The A_p (%) in Eq. (1), A_{wa} (%) in Eq. (2) and A_d (g/cm^3) in Eq. (3) were obtained respectively by the following relations described by Archimede's principle:

$$A_p = \left[\frac{(M_{sat} - M_{dry})}{(M_{sat} - M_{imm})}\right] \times 100 \qquad (1)$$

$$A_{wa} = \left[\frac{(M_{sat} - M_{dry})}{M_{dry}}\right] \times 100 \qquad (2)$$

$$A_d = \frac{M_{dry}}{(M_{sat} - M_{imm})} \qquad (3)$$

It was performed studies (in triplicate) about the resistance to uniaxial compressive strength (0.1–30 MPa) of GCS samples in mechanical press (Ronald TOP Ltda – USA) with application speed of 0.77 mm/min.

Studies of microstructures on the surfaces of green bodies and sintered GCS samples for all formulations were performed with scanning electron microscope (SEM) (JEOL Model JSM-5600 LV) at setting of 15 kV.

The coal fly ashes (CFA) were analyzed for chemical composition after dried at 110 °C followed by heating at 1000 °C for 2 h, using a Rigaku model RIX 3000, X-ray fluorescence spectroscopy (XRF) unit, according to Maheshwari et al. [24]. The main crystalline phases present in CFA were obtained in Miniflex X-ray diffractometer (model RIX 3000, Rigaku Americas Corporation). The detector was scanned over a range of 2θ angles from 5° to 80°, at a step size of 0.02° and a dwell time of 2 s per step.

2.4. Immobilization of invertase on samples of GCS

Initially, invertase was immobilized as described by Sanjay and Sugunan [1] called anterior protocol (0). The GCS*C* sintered at 1100 °C for 2 h (0.3 g) was submerged in 2 ml of acetone with APTES (2.12 mM) and at the end of the period reaction, the treated samples were washed 3 times ultra pure water and dried at 80 °C for 1 h. After cooling, the samples were placed in 2 ml sodium phosphate buffer (10 mM, pH 7.5) with glutaraldehyde (1.92 mM), washed 3 times with buffer and dried at 60 °C for 1 h. Then, the GCS*C* samples activated surface were submerged in 2 ml of phosphate buffer containing invertase (2.58 mg/ml) at 4 °C for 1 h. After covalent coupling period, the immobilized derivatives (GCS*C*-invertase) were

Table 1
Protocols for covalent immobilization of invertase on glass–ceramic supports.

Factors			
a	2.120 mM 3-APTES	j	1 h of reaction with glutaraldehyde
A	0.212 mM 3-APTES	J	2 h of reaction with glutaraldehyde
B	0.424 mM 3-APTES	L	3 h of reaction with glutaraldehyde
C	0.636 mM 3-APTES	M	4 h of reaction with glutaraldehyde
d	1 h of silanization with 3-APTES	N	2.58 mg/ml proteins
D	2 h of silanization with 3-APTES	O	5.15 mg/ml proteins
E	3 h of silanization with 3-APTES	P	7.73 mg/ml proteins
F	4 h of silanization with 3-APTES	q	1 h to proteins covalent coupling
g	1.920 mM of glutaraldehyde	Q	6 h to proteins covalent coupling
G	0.192 mM of glutaraldehyde	R	12 h to proteins covalent coupling
H	0.384 mM of glutaraldehyde	S	18 h to proteins covalent coupling
I	0.576 mM of glutaraldehyde		
Organization of protocols			
0	adgjOq	9	BEHLOR[a]
1	ADIMPQ	10	CFGJNS
2	ADGMNS	11	CFIMPS
3	ADGJNQ	12	CDGMPS
4	ADIJPS	13	CDIJNS
5	AFIJNQ	14	CFGMNQ
6	AFGJPS	15	CDGJPQ
7	AFIMNS	16	CDIMNQ
8	AFGMPQ	17	CFIJPQ

The a; d; g; j and q are anterior protocols according to Sanjay and Sugunan [1].
[a] Central point.

washed 3 times with NaCl (10 mM) and stored in ultra pure water at 4 °C.

The processes of covalent immobilization of invertase on GCS*C* and the selected support (GCS*Zn*) were evaluated: 3-APTES concentration (0.212, 0.424, 0.636 mM) and reaction time (2, 3 and 4 h), glutaraldehyde concentration (0.192, 0.384, 0.576 mM) and reaction time (2, 3 and 4 h), invertase concentration (2.58, 5.15 and 7.73 mg/ml) and covalent coupling time (6, 12 and 18 h).

The amount of immobilized proteins on the surface of activated supports (μg proteins/g GCS) was estimated by measurement of free protein concentration [25] in the supernatant and washing solutions. All procedures were performed in triplicate.

2.5. Factorial designs and statistical analysis

A full factorial design 3×2^2, with additional treatment as central point (triplicates), was used to analyze the effects of the mineral additives, sintering temperature and time on the physical parameters of the GCS samples (apparent porosity, apparent water absorption and the uniaxial compressive strength). All immobilized invertase derivatives from factorial design above were selected to study the effects of (1) 3-APTES concentration and (2) time of silanization; (3) glutaraldehyde concentration and (4) reaction time; (5) and enzyme concentration and (6) covalent coupling time. The effects above cited were evaluated based on the amount of protein coupled on GCS (sintered at 1200 °C for 1 h) and the catalytic activity of the immobilized invertase derivative. For this study was used fractional factorial design 2^{6-2} with the central point in quadruplicate. Statistical evaluations were carried out by Statistica software 8.0 (Stat Soft, Inc., 2008, USA). The experimental parameters are shown in Table 1. All data are presented as mean ± standard error of the mean. Comparisons during the study were released by Tukey's test from three replicates. The level of significance was set at $p < 0.05$.

2.6. Activities of free and immobilized invertase

The free enzyme (0.1 ml containing 26 μg of proteins) or immobilized derivative (0.3 g) was incubated in batch bioreactor containing 0.9 ml of 5% (w/v) sucrose solution prepared in 25 mM sodium acetate buffer pH 5.0 at 25 ± 2 °C under controlled agitation (150 rpm). The final volume of reaction enzymatic mixture was 1 ml for both preparations, using polypropylene tubes (25 mm diameter and 50 mm height) with a conical bottom. The reducing sugars produced were analyzed by DNS method [26]. A unit of activity (U) was defined as being the amount of enzyme necessary to produce 1 μmol of reducing sugar per minute in pH 5.0 at 25 ± 2 °C. The activities were expressed for U/μg proteins for free and U/g GCS for immobilized enzymes, respectively.

2.7. Reusability of immobilized invertase

The stability to reuse of the immobilized derivative (GCS-invertase) in batch bioreactor, selected from the statistical analysis of the best protocol, was assayed under the same experimental conditions of invertase activity. After each assay, the biocatalysts were washed three times with ultra pure water with mechanical agitation – vortex, and dry with absorbent paper (sterile). All procedures were performed in triplicate.

3. Results and discussion

3.1. Characterization of the raw material (CFA)

The coal fly ashes (CFA), used to prepare GCS supports, showed the following chemical composition: SiO_2 (55.30%), FeO_3 (18.90%), Al_2O_3 (15.10%), K_2O (4.20%), CaO (2.80%), TiO_2 (1.70%) and smaller quantities of other oxides. These results were similar to those reported by Cheng and Chen [27]; Olgun et al. [8]; Peng et al. [28]; Erol et al. [29]. The yellow to red color is due to the significant presence of Fe_2O_3 (18.90%). According to Vassilev et al. [30], CFA cenospheres showed yellow, red, brown and black colorations due to the presence of Fe and traces of other elements (Co, Cr, Cu, Mn, Ni, Ti, U) within glass.

The main crystalline phases present in CFA by XRD were identified as quartz (SiO_2), mullite ($Al_6Si_2O_{13}$), hematite (Fe_2O_3), anorthite ($CaCO_3$) and amorphous material.

3.2. Microstructural studies of the GCS samples

The microstructural studies of CGS samples were performed using SEM which microphotographs are showed in Figs. 1–3 before and after sinterization (1000 °C/1 h or 3 h, 1100 °C/2 h, 1200 °C/1 h or 3 h) according to factorial design. Figs. 1A (GCS*C*), 2A (GCS*Pb*) and 3A (GCS*Zn*) showed microstructures of green bodies, which had similar characteristics such as cenospheres and cracks, probably because of the drying of excipients. The GCS*C* samples obtained in different temperature and sintering time showed changes on the surface and microstructure compared to green bodies (Fig. 1B–F). The GCS*C* samples obtained at 1000 °C/1 h showed uniform distribution of cenospheres while in higher sintering time there was more deformation and melting causing formation of amorphous microstructures. The images of the microstructures after sinterization at 1000 °C/3 h (Fig. 1C) and 1100 °C/2 h (Fig. 1D) were similar. However, the sinterization at 1200 °C/1 h and 3 h (Fig. 1E–F) showed a dense glass transition phase.

In Fig. 2B–F the morphological differences in samples GCS*Pb* are showed. Qualitatively there are larger pores and cracks with evident microspores in comparison with the control formulation samples (GCS*C*). The dispersion of lead acetate crystals (Fig. 2A), used as additive, caused some lead acetate melting regions increasing the nucleation and cenospheres deformation, which influenced in the physical properties.

The different morphologies in the GCS*Zn* samples are shown below (Fig. 3B–F). There was more preservation of the cenospheres which still can be seen after sinterization at 1200 °C/3 h, in spite of

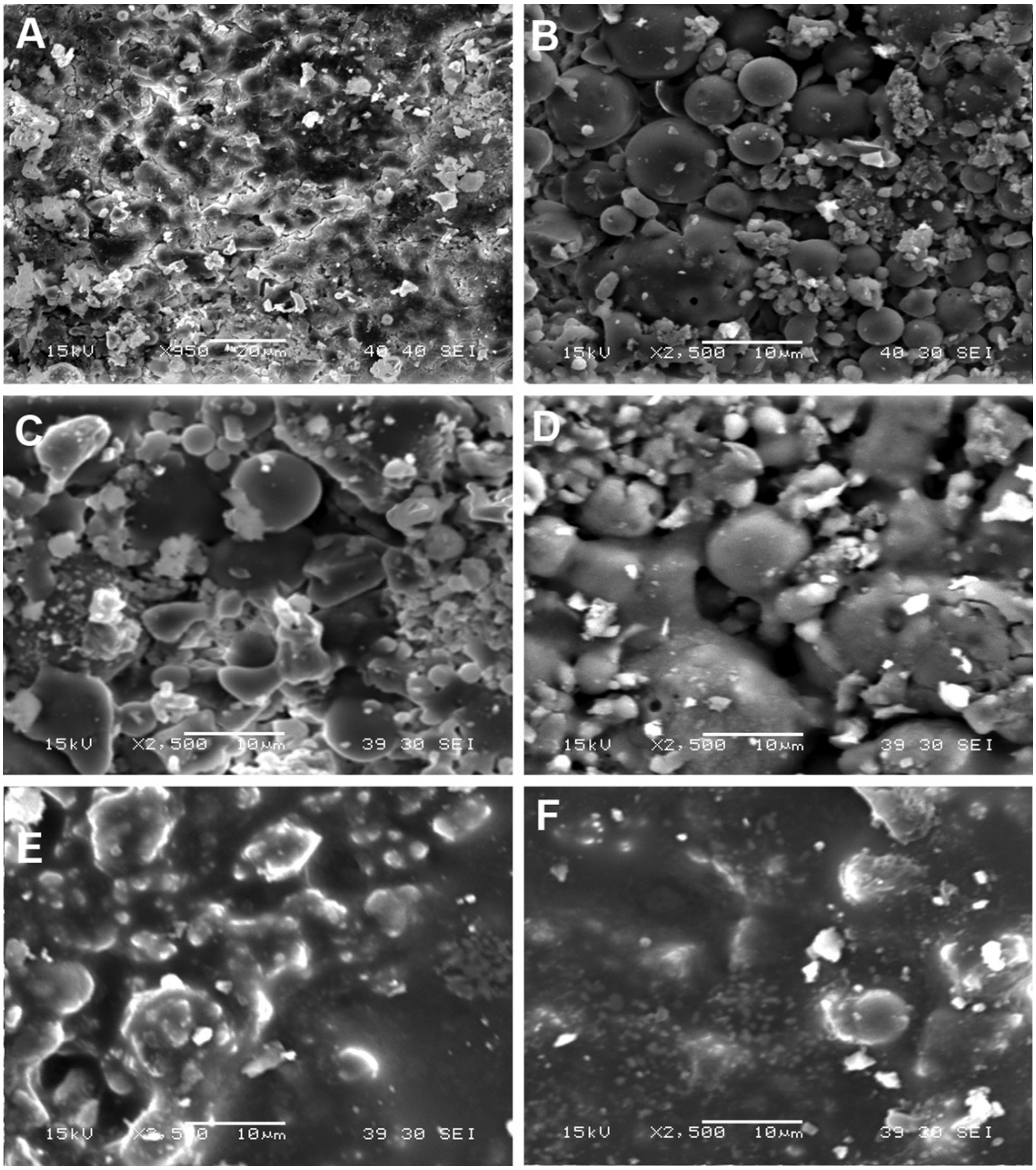

Fig. 1. Microphotographs of the ceramics (GCSC) from coal fly ash (CFA) sinterization. Experimental conditions described in Sections 2.2 and 2.5. Scheme of samples: (A) green body, (B and C) 1000 °C/1 h–1000 °C/3 h, (D) 1100 °C/2 h (central point), (E) 1200 °C/1 h and (F) 1200 °C/3 h.

some nucleation region. This preservation can lead to higher specific superficial area and hydrophilic cenospheres surface exposed to silanization process [31] for covalent immobilization of enzymes.

3.3. Physical and mechanical properties of the GCS samples

The concentration of the additives (lead acetate and zinc sulfate) used in each formulation was previously studied (data not shown) to obtain green bodies without cracks on the surface after uniaxial pressing. The GCS porosity can supply a higher specific superficial area increasing the amount of immobilized enzyme. In this way, the physical and mechanical properties studied were: apparent porosity, apparent water absorption and apparent density. The uniaxial compressive strength was studied to know the mechanical characteristic of immobilized derivatives.

The results were evaluated by factorial design (3×2^2) to select the best GCS formulation (Table 2). In spite of the apparent porosity and water absorption, the temperature was the main factor that affected these physical properties. And the sintering time did not have great influence.

In Table 2B, it can be observed that the temperature factor was the most important and significative positive effect ($p < 0.05$) about apparent density. In addition, the sintering time affected more the sintered GCS*Pb* compared to another sintered samples.

According to Cheng et al. [32], high values of the apparent porosity and apparent water absorption, lead to decrease of the apparent density and mechanical strength. Sintered GCS samples were produced from coal fly ashes, red mud from aluminum production and silica fume. For observing the effect of linkers in the sinterization technique, glass powders were pressed without or with the addition of polyvinyl alcohol (PVA). In spite of physical and mechanical properties (porosity, density and hardness), were produced samples strongly depended on the crystallization degree of the samples [33].

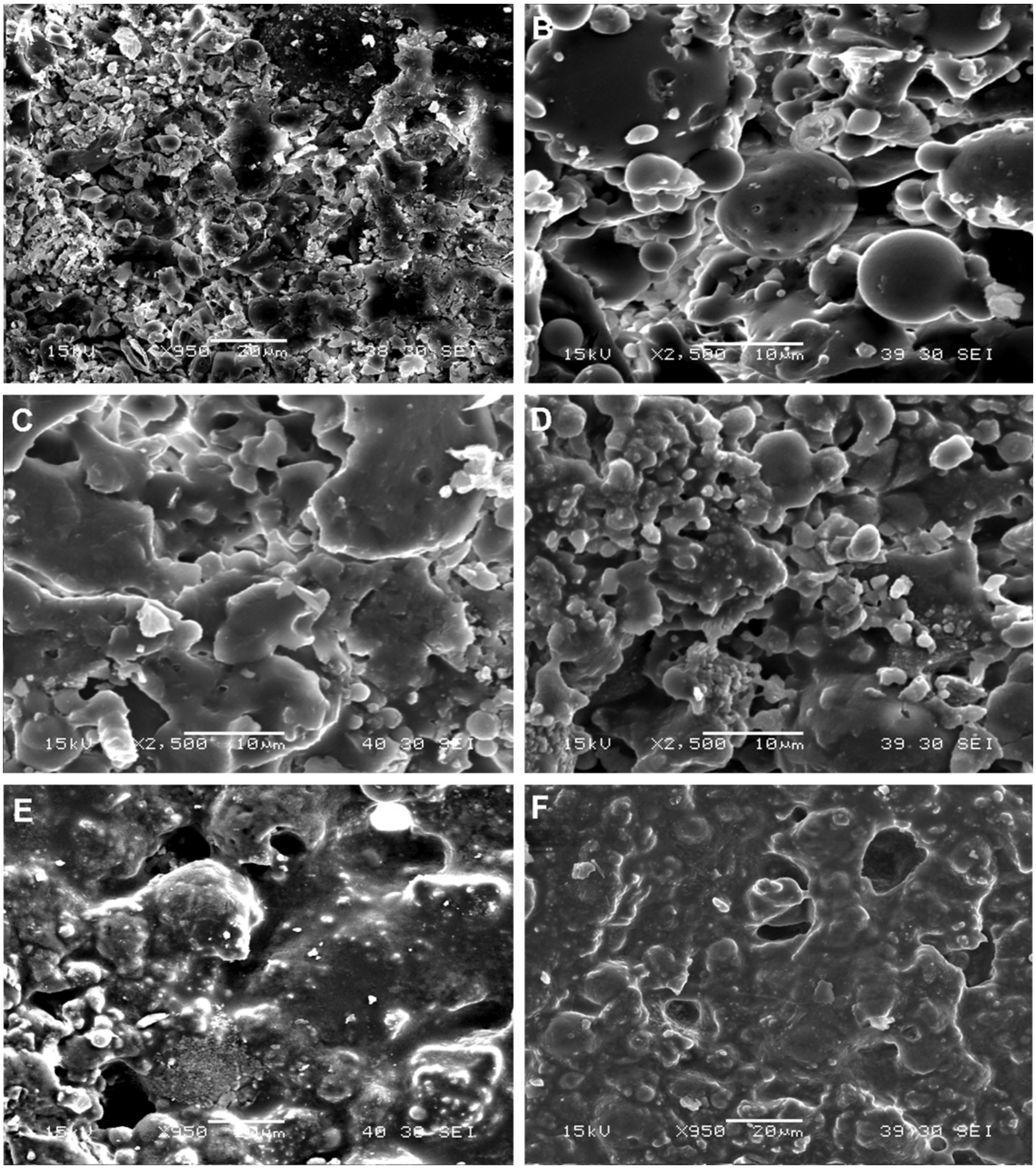

Fig. 2. Microphotographs of the ceramics (GCS*Pb*) from coal fly ash (CFA) sinterization. Experimental conditions described in Sections 2.2 and 2.5. Scheme of samples: (A) green body, (B and C) 1000 °C/1 h–1000 °C/3 h, (D) 1100 °C/2 h (central point), (E) 1200 °C/1 h and (F) 1200 °C/3 h.

Fig. 4A shows that the apparent porosity values of sintered GCS*C* samples were lower than those found to GCS*Pb* and GCS*Zn* sample sintered. It may be due to chemical additives that caused an increase in the apparent porosity. The results showed that the amount of lead added was not enough to cause a decrease in the apparent porosity as reported by Imbrahim et al. [34] that obtained an increase of porosity of a superconducting ceramic formulation.

The GCS*C* samples had apparent porosity of 30.38% and 29.54% (1000 °C/1 h and 1000 °C/3 h) and apparent water absorption of 17.27% and 16.61% (1000 °C/1 h and 1000 °C/3 h). The sintered GCS*Zn* samples had 38.01% and 36.48% (1000 °C/1 h and 1000 °C/3 h) for apparent porosity, and 25.85% and 23.96% (1000 °C/1 h and 1000 °C/3 h) for apparent water absorption. The GCS*C* and GCS*Zn* samples showed a decrease in both physical properties as reported by Koo et al. [35] that studied the effect of zinc to produce high-porous brass hollow spheres sintered from 800 °C to 930 °C. It has been shown that the increasing temperature accelerated the Zn evaporation and sphere densification. The results obtained about apparent porosity and apparent water absorption were in the same magnitude order of those reported by Zimmer and Bergmann [36] and Erol et al. [29].

Regard to mechanical resistance (uniaxial compressive strength), the temperature factor was the significative positive effect for GCS*C* and GCS*Pb*, but was not for the GCS*Zn* samples. The mechanical strength of these materials is influenced by: (1) the density of the glass phase, (2) the density of the polycrystalline phase, (3) the regularity or irregularity of porosity, (4) amount of additives added. Fig. 4B shows non-linear changes in the uniaxial compressive strength of the GCSC, GCS*Pb* and GCSZn sample. The highest mechanical strength values were obtained for: GCS*C* (23.77 MPa sintered at 1200 °C/1 h), GCS*Pb* (22.33 MPa sintered at 1200 °C/3 h) and GCS*Zn* (10.67 MPa sintered at 1100 °C/2 h). The lowest value showed for GCS*Zn* can be explained by preservation of the cenospheres after sinterization process of this sample what

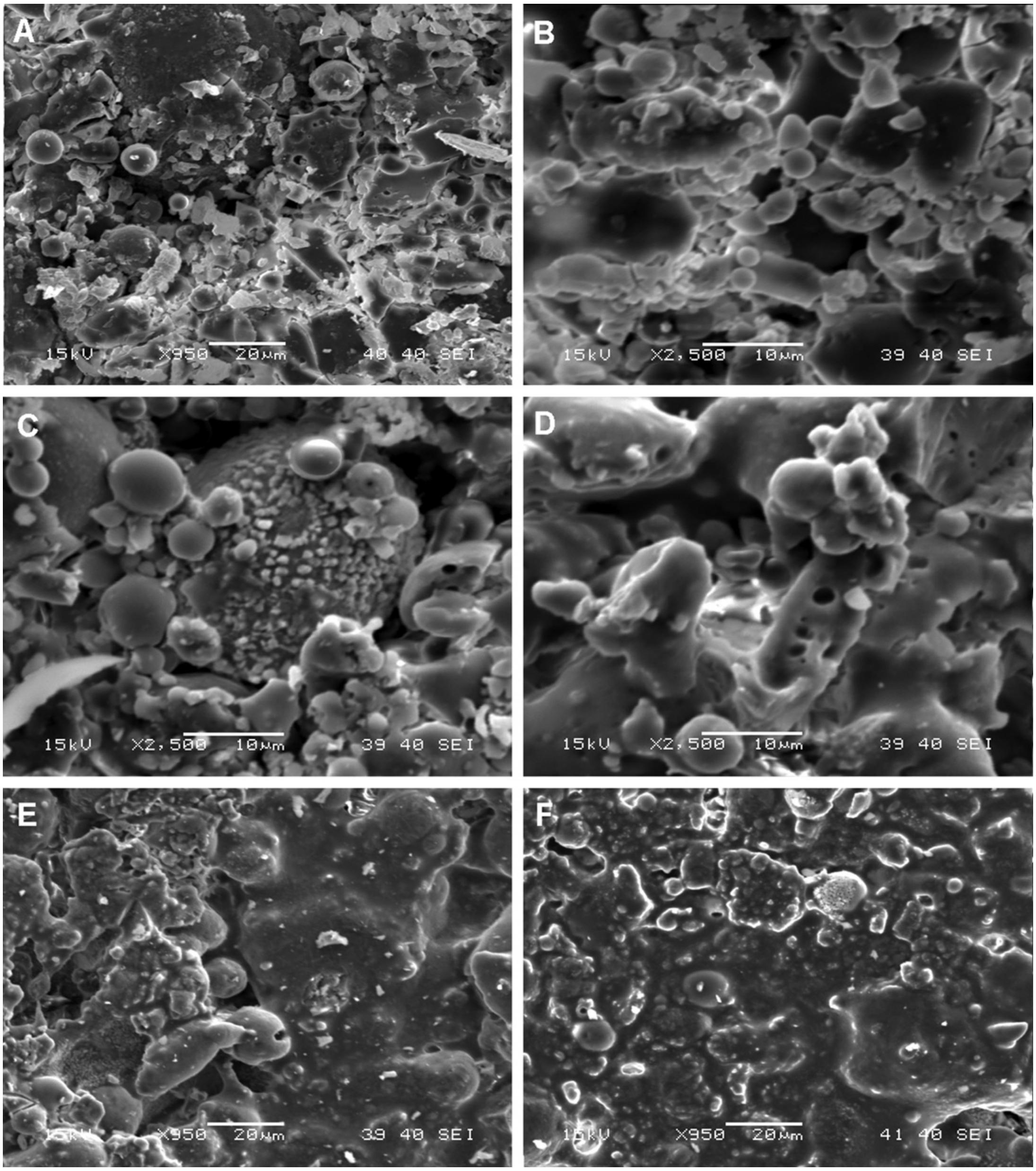

Fig. 3. Microphotographs of the ceramics (GCS*Zn*) from coal fly ash (CFA) sinterization. Experimental conditions described in Sections 2.2 and 2.5. Scheme of samples: (A) green body, (B and C) 1000 °C/1 h–1000 °C/3 h, (D) 1100 °C/2 h (midpoint), (E) 1200 °C/1 h and (F) 1200 °C/3 h.

it was not observed for the sintered GCS*C* and GCS*Pb* samples (Figs. 1–3). These results can be explained by absence of cenospheres in the GCS*Pb* (Fig. 2F) and GCS*C* (Fig. 1E) while in the GCS*Zn* there was preservation of the cenospheres after all the sinterization conditions. Similar results were reported by Huang et al. [37] which showed that the presence of cenospheres cause a decrease of the compressive strength.

3.4. Immobilization of invertase on samples of GCS

All the GCS samples obtained by full factorial design 3×2^2 were used to immobilization of invertase according to Sanjay and Sugunan [1] (anterior protocol (0)). The best results of invertase immobilized by covalent bound obtained were: 951 ± 8.64 U/g GCS*C* (1000 °C/3 h); 1140 ± 211.88 U/g GCS*Pb* (1000 °C/1 h) and 4398 ± 59.75 U/g GCS*Zn* (1200 °C/1 h), which showed that there was no improvement by the lead acetate addition, but in spite of zinc sulfate addition the activity was three times higher, being selected for further experiments (Fig. 5).

Both GCS*C* and GCS*Pb* samples showed similar results about invertase immobilization, reminding that these samples also had similar porosity and water absorption. The study showed that the lead acetate addition did not improve the GCS properties. The best immobilized derivative (GCS*Zn*-invertase) was prepared with the sample that had lower apparent porosity and water absorption. The GCS*Zn* samples obtained at 1000 °C for 1 h or 3 h showed higher apparent porosity, but had no mechanical resistance during the silanization process. The GCSC and GCS*Pb* samples had a decrease in activity retention of invertase with decrease of apparent porosity, being the opposite for GCS*Zn* samples.

According to David et al. [4], invertase activity is strongly inhibited by the presence of iodine, Ag^{2+}, Zn^{2+} and Hg^{2+}. However, this inhibition did not observe for GCS*Zn*-invertase derivative, may be due to the sinterization level which avoids the zinc leaching, added

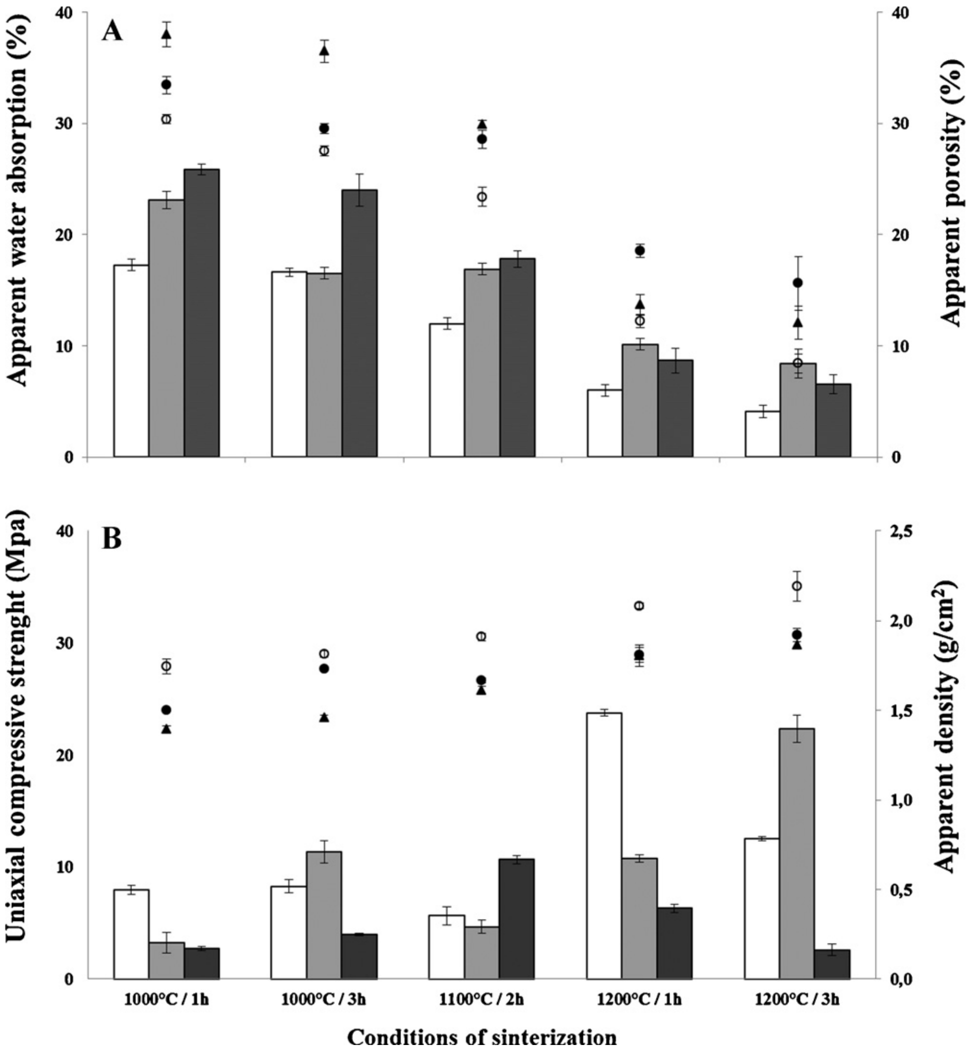

Fig. 4. Effects of additives on the physical proprieties of glass–ceramic supports (GCS) obtained using coal fly ashes. (A) Apparent porosity labels – GCSC (○); GCSPb (●) and GCSZn (▲) and Apparent water absorption columns – GCSC (□); GCSPb (▨) and GCSZn (■). (B) Apparent density labels – GCSC (○); GCSPb (●) and GCSZn (▲) and uniaxial compressive strength columns – GCSC (□); GCSPb (▨) and GCSZn (■). Experimental conditions are described in Sections 2.2 and 2.5.

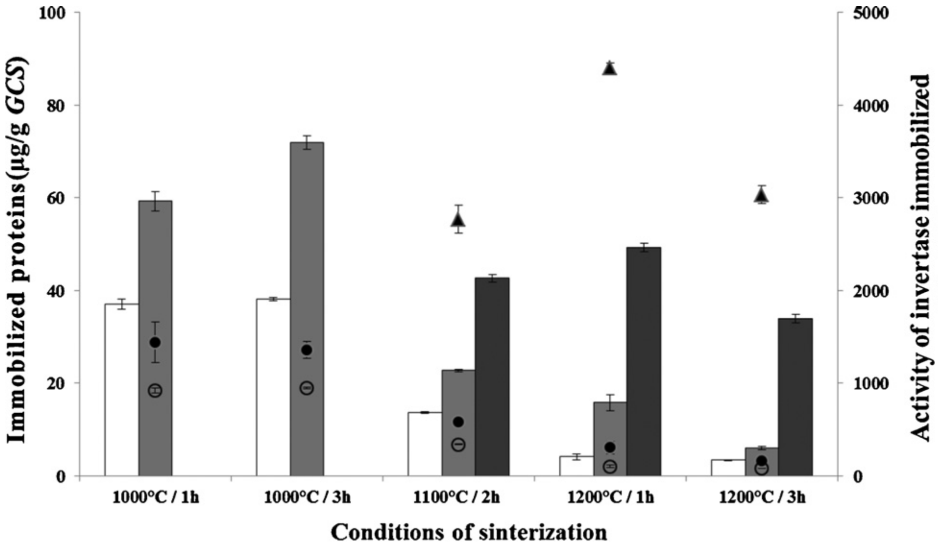

Fig. 5. Effect of sinterization conditions on the relative activity of immobilized invertase: labels – GCSC (○); GCSPb (●) and GCSZn (▲) and on protein coupling: columns – GCSC (□); GCSPb (▨) and GCSZn (■). Experimental conditions described in Sections 2.4 and 2.6. Invertase free specific activity used was 2052 U/µg protein.

Table 2
Experimental parameters using a full factorial design (3×2^2) to study the physical properties of glass–ceramic supports.

Factors	−1	Central point	+1
Temperature (°C)	1000	1100	1200
Time of sinterization (h)	1	2	3

Factors	Effect			*p*		
(A) Results of apparent porosity						
	GCSC	GCS*Pb*	GCS*Zn*	GCSC	GCS*Pb*	GCS*Zn*
Mean	**21.530**	**26.122**	**27.149**	**0.000012**	**0.000002**	**0.000118**
Temperature	**−19.640**	**−14.419**	**−24.365**	**0.000105**	**0.000041**	**0.001022**
Time	**−2.330**	**−3.411**	−1.584	**0.007356**	**0.000729**	0.179208
1 by 2	**−1.490**	**0.485**	−0.046	**0.017696**	**0.034183**	0.957969
Results of apparent water absorption						
	GCSC	GCS*Pb*	GCS*Zn*	GCSC	GCS*Pb*	GCS*Zn*
Mean	**11.414**	**15.538**	**16.914**	**0.000027**	**0.000112**	**0.000227**
Temperature	**−11.902**	**−10.549**	**−17.301**	**0.000173**	**0.001693**	**0.001513**
Time	**−1.278**	**−4.161**	−2.004	**0.014685**	**0.010735**	0.096865
1 by 2	−0.618	**2.427**	−0.121	0.058616	**0.030599**	0.873689
(B) Results of apparent density						
	GCSC	GCS*Pb*	GCS*Zn*	GCSC	GCS*Pb*	GCS*Zn*
Mean	**1.937**	**1.708**	**1.623**	**0.000002**	**0.000060**	**0.000063**
Temperature	**0.401**	**0.250**	**0.411**	**0.000378**	**0.019046**	**0.006867**
Time	**0.046**	**0.171**	0.061	**0.027281**	**0.039602**	0.216417
1 by 2	**0.062**	−0.061	−0.001	**0.015312**	0.230468	0.968659
Results of uniaxial compressive strength						
	GCSC	GCS*Pb*	GCS*Zn*	GCSC	GCS*Pb*	GCS*Zn*
Mean	**9.928**	**8.806**	**6.810**	**0.000353**	**0.000100**	**0.000811**
Temperature	**10.041**	**9.264**	1.110	**0.002407**	**0.000631**	0.163102
Time	**−5.459**	**9.855**	−1.219	**0.008074**	**0.000557**	0.140864
1 by 2	**−5.773**	**1.717**	**−2.493**	**0.007228**	**0.017893**	**0.039861**

Factors in bold were statistically significant ($p < 0.05$).

to coupling process involving silanization and glutaraldehyde as spacers leaving the enzyme distant from support surface.

3.4.1. *Studies of the immobilization processes of invertase*

The statistical analysis of the results from all protocols tested by fractional factorial design 2^{6-2} is shown in Table 3. Based on the results, all factors showed statistically significant positive effect ($p < 0.05$) in the coupling and activity of invertase, the highest levels of the fractional factorial design have to be used to immobilize invertase on GCS*Zn*.

Table 1 shows that the best economical protocol was the number 11 (CFIMPS). The activities of immobilized invertase on GCS*Zn* (1200 °C/1 h) were compared to that obtained using GCSC (232.02 ± 10.48 U/g GCSC) containing immobilized invertase according to Sanjay and Sugunan [1], which after factorial design for optimization of enzyme coupling experimental conditions lead to improvement of catalytic activity of the immobilized derivative (5563.44 ± 123.41 U/g GCSZn).

Other results were statistically significant by Tukey's test ($p < 0.05$), protocols 1 and 12, which showed 43.53% and 68.09% of

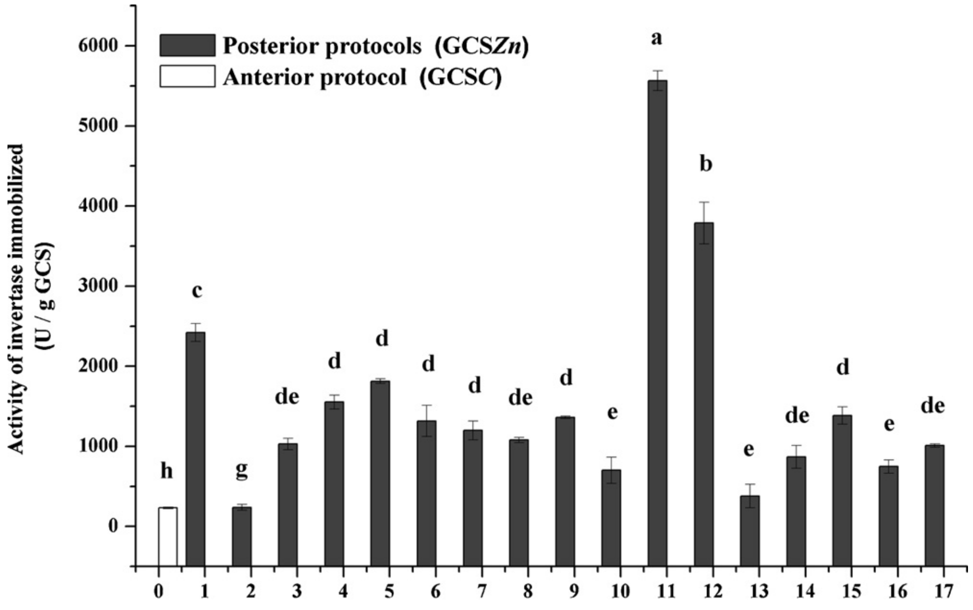

Fig. 6. Effect of enzyme coupling experimental conditions on the relative activity of immobilized invertase on *GCSZn*. Described in Section 2.5.

Table 3
Experimental parameters using a fractional factorial design (2^{6-2}) to study the effect of chemical compounds and protein concentrations and time for immobilization processes on GCS*Zn*.

Factors	−1	Central point	+1
3-APTES (mM)	0.21	0.42	0.64
Silanization time with 3-APTES (h)	2	3	4
Glutaraldehyde (mM)	0.19	0.38	0.58
Glutaraldehyde reaction time (h)	2	3	4
Proteins (mg/ml)	2.58	5.15	7.73
Proteins covalent coupling time (h)	6	12	18

Effects of immobilization processes

	Effect	*p*
	GCS*Zn*	GCS*Zn*
Mean/interaction	**14.14720**	**0.000008**
3-APTES (mM)	**5.42355**	**0.000246**
Silanization time with 3-APTES (h)	**2.71524**	**0.000982**
Glutaraldehyde (mM)	**5.71778**	**0.000222**
Reaction with glutaraldehyde (h)	**8.73768**	**0.000095**
Proteins (mg/ml)	**13.33059**	**0.000041**
Proteins covalent coupling time (h)	**5.50291**	**0.000239**

Results of the factors in bold were statistically significant ($p < 0.05$).

the activity compared to that obtained with protocol 11 (100.0%), respectively. The amount of glutaraldehyde in the protocol 11 was three times higher than that used in protocol 12, showing the needs of this bifunctional agent for the best immobilized invertase activity. Comparing the protocols 1 and 11 can be observed that the amount of APTES, time reaction and coupling time were higher to protocol 11 confirming the effects of these factors in the preparation of immobilized derivative. These results have been in agreement with those reported by Sanjay and Sugunan [1]; Hamerska-Dudra et al. [38]; David et al. [4]; Marquez et al. [20] (Fig. 6).

3.4.2. *Reusability of immobilized invertase*

The average catalytic activity obtained after nine reuses of the immobilized derivative GCS*Zn*-invertase maintained about 100% of the initial activity (5476.19 ± 155 U/g GCS*Zn*). This result was similar to that reported by Sanjay and Sugunan [1] about invertase covalently immobilized on montmorillonite retained 100% of the initial activity after ten reuses.

4. Conclusion

The GCS, obtained using coal fly ashes with PVP and magnesium stareate, was successfully used as a support for immobilization of invertase by covalent linkage. The best support was GCS*Zn* (1200 °C/1 h), which by SEM analysis showed cenospheres preservation important to coupling process. An economical protocol was established to prepare the GCS*Zn*-invertase derivative that can be gradually increased to the industrial scale for invert sugar production. This can be a viable alternative to reduce environmental pollution by coal fly ashes.

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