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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

DESENVOLVIMENTO DE CERÂMICA A PARTIR DAS CINZAS VOLANTES DE CARVÃO MINERAL PARA IMOBILIZAÇÃO DE ENZIMAS

Aluno: Alessandro Victor Patrício de Albertini
Orientador: Prof. José Luiz de Lima Filho
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Recife, agosto de 2010

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Tese apresentada ao Curso de Doutorado em Ciências Biológicas da Universidade Federal de Pernambuco, para qualificação ao título de Doutor em Ciências Biológicas, Área de Concentração em Biotecnologia.

Orientador: Prof. Dr. José Luiz de Lima Filho

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ATA DA DEFESA DE TESE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS DO CENTRO DE CIÊNCIAS BIOLÓGICAS DA UNIVERSIDADE FEDERAL DE PERNAMBUCO.

Aos vinte e sete dias do mês de agosto de dois mil e dez, às nove horas, no Anfiteatro do LIKA, realizou-se a Defesa de Tese apresentada pelo Doutorando **Alessandro Victor Patrício de Albertini**, intitulada: **"Desenvolvimento de cerâmica a partir das cinzas volantes de carvão mineral para imobilização de enzimas"**. A Banca Examinadora foi homologada em onze de agosto de dois mil e dez, pela PROPESQ, tendo como membros titulares os Professores: **José Luiz de Lima Filho** (Orientador), Doutor em Bioquímica, pela Universidade de Saint Andrews, Escócia, **Danyelly Bruneska Gondim Martins**, Doutora em Ciências Biológicas, pela Universidade Federal de Pernambuco, **Maria do Carmo de Barros Pimentel**, Doutora em Química pela Universidade Estadual de Campinas/SP, **Valdinete Lins da Silva** Doutora em Química, pela Universidade Estadual de Campinas/SP, **Cosme Rafael Martinez Salinas**, Doutor em Ciências Biológicas, pela Universidade Federal de Pernambuco; **Luiz Bezerra de Carvalho Júnior**, Doutor em Bioquímica, pela Universidade de Saint Andrews, Escócia e **Rosa Valéria da Silva Amorim**, Doutora em Ciências Biológicas, pela Universidade Federal de Pernambuco, suplentes. O Prof. José Luiz deu início à Sessão, agradeceu pela presença de todos e passou a palavra ao doutorando para fazer exposição de sua tese, que efetuou em quarenta minutos. Continuando o presidente solicitou à Comissão Examinadora a ocupar seus lugares. A seguir, procedeu-se à argüição na seguinte ordem: Dr. Cosme Rafael Salinas (1º examinador), Dr.ª Valdinete da Silva (2º examinador), Dr.ª Maria do Carmo Pimentel (3º examinador), Dr.ª Danyelly Gondim Martins (4º examinador), Dr. Jose Luiz de Lima (5º examinador). Dando continuidade, o presidente abordou a importância do trabalho desenvolvido pelo doutorando e, em seguida, expressou seus agradecimentos, solicitando aos convidados a gentileza de se retirarem do recinto por alguns minutos, a fim de proceder a avaliação. A Comissão Examinadora atribuiu a **Alessandro Victor Patrício de Albertini** a seguinte menção: **"Aprovado"** por unanimidade. Face ao resultado o mesmo está apto a receber o grau de Doutor em Ciências Biológicas, Área **Biotecnologia**, pela Universidade Federal de Pernambuco. Nada mais havendo a tratar, a sessão foi encerrada e para constar, eu Adenilda Eugênia de Lima, Secretária, lavrei, datei e assinei a presente ata que também assinam os demais presentes. Recife 27 de agosto de 2010.

O Senhor é o meu Pastor, nada me faltará.
Deitar-me faz em verdes pastos, guia-me mansamente às águas tranquilas;
Refrigera a minha alma, guia-me pelas veredas da justiça por amor do seu nome,
Ainda que eu andasse pelo vale da sombra da morte não temeria mal algum, porque tu estás comigo, a tua vara e o teu cajado me consolam;
Preparas uma mesa perante mim na presença dos meus inimigos, unges a minha cabeça com óleo, o meu cálice transborda;
Certamente que a bondade e a misericórdia me seguirão todos os dias de minha vida, e habitarei na casa do Senhor por longos dias.
Amém.

Dedico, a meus pais e irmãos, e em especial a minha avó materna (*in memorian* – Salmo 23, eu coloquei em sua homenagem acima).
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Resumo

Suportes vítreo-cerâmicos foram desenvolvidos a partir da sinterização das cinzas volantes de carvão mineral da Termelétrica Presidente Medici, Candiota-RS/Brasil para imobilização covalente de enzimas. A enzima invertase (β-fructofuranosidase, E.C. 3.2.1.26) foi tomada como exemplo. Invertase foi covalentemente imobilizada em suportes cerâmicos de vidro (GCS), obtida a partir de cinzas volantes de carvão (CFA) com polivinilpirrolidona (PVP) e estearato de magnésio adicionado como aditivo temporário. As amostras GCS contendo Pb (CH_3COO) 2 , (GCS*Pb*) ou $ZnSO_4$ (GCS*Zn*) foram obtidos em várias temperaturas de sinterização (1000-1200 °C) e tempos (1-3 h) . Os valores aparentes de porosidade, absorção de água, densidade e resistência à compressão uniaxial foram estudados por planejamento fatorial e diferenças morfológicas por microscopia eletrônica de varredura. A melhor covalente imobilizado derivado invertase foi em GCS*Zn* (1200 °C / 1 h) com 4398 ± 59,75 U / g GCS. No entanto, 95,83 % de actividade invertase imobilizada foi obtido usando um protocolo económico com a concentração de 0,636 mM de 3-aminopropiltrietoxi -silano (3-APTES) e 0,576 mM de glutaraldeído. O derivado GCS*Zn*-invertase manteve 100% da atividade inicial após nove reutilizações (5.476,19 ± 155,49 U / g GCS*Zn*).

O derivado escolhido contendo a cerâmica com enzima ligada covalentemente (invertase-GCS*Zn*) foi demonstrada por difracção de raios - X (XRD). Não houve qualquer alteração no pH óptimo (4.6) , mas aumentou a temperatura óptima de 55 °C para a invertase livre a 60 °C durante derivado imobilizado . A energia de ativação diminuiu após a imobilização (37,31 ± 3,40 kJ / mol) , apesar de invertase livre (51,34 ± 5,21 kJ / mol). Houve uma melhora na constante de Michaelis - Menten para hidrólise de sacarose após a imobilização ser 15 vezes menor em relação ao de invertase livre (0,30 ± 0,01 mmol). Após dez reutilizações a 25 ± 2 °C , a invertase imobilizada perdeu apenas 9 % da actividade inicial , mas à temperatura óptima (60 °C) , a redução atividade foi cerca de 70 % , o que é economicamente viável sob o ponto de vista energético para aplicação industrial. De acordo com as propriedades físicas obtidas em cerâmica que foi preparada com álcool polivinílico foi também desenvolvida para ser testadas em um inédito sistema de fluxo contínuo. Esse sistema permitiu a desestabilização de caminhos preferenciais ou “zonas mortas” da solução de sacarose entre cerâmicas no bioreator e nas suas regiões interiores; permitindo assim, uma melhor performance do biocatalisador imobilizado e consequentemente a total hidrólise da sacarose em menor tempo de reação.

A cerâmica obtida do resíduo, cinzas volantes de carvão mineral, mostrou ser uma alternativa biotecnológica promissora como suporte de imobilização invertase para a produção de açúcar invertido e devendo ser usada para imobilização outras enzimas de interesse industrial.

Palavras-chave: cinzas volantes de carvão mineral, cerâmica, invertase, imobilização.

Abstract

Glassy-ceramic supports have been developed from the sintering of fly ash from coal Thermoelectric President Medici, Candiota-RS / Brazil for covalent immobilization of enzymes. The enzyme invertase (β-fructofuranosidase, EC 3.2.1.26) has been taken as an example. Invertase was covalently immobilized on glass ceramic substrates (GCS) obtained from coal fly ash (CFA) with polyvinylpyrrolidone (PVP) and magnesium stearate added as a temporary additive. The GCS sample containing Pb $(CH_3COO)2$, (GCS*Pb*) or $ZnSO_4$ (GCSZn) were obtained at various sintering temperatures (1000-1200 °C) and times (1-3h). The apparent porosity values, water absorption, density and resistance to uniaxial compression were studied by factorial design and morphological differences by scanning electron microscopy. The best covalently immobilized invertase was derived in GCS*Zn* (1200 °C/1h) with 4398±59.75 U/g GCS. However, 95.83% of immobilized invertase activity was obtained using a cheap protocol with the concentration of 0.636 mM 3-aminopropyltriethoxy silane (3-APTES) and glutaraldehyde 0.576 mM. The GCS*Zn*-derived invertase retained 100% of the initial activity after nine reuses (5476.19±155.49 U/g GCS*Zn*).

The derivative chosen containing the ceramic enzyme linked covalently (invertase-GCS*Zn*) was demonstrated by diffraction X-ray (XRD). There was no change in the optimum pH (4.6) but increased the temperature optimum of 55 °C for invertase free at 60 °C for immobilized derivative. The activation energy decreases after immobilization (37.31±3.40 kJ / mol), although free invertase (51.34±5.21 kJ / mol). There was an improvement in -Menten Michaelis constant for sucrose hydrolysis after immobilization to be 15 times lower compared to the free invertase (0.30 ± 0.01 mmol). After ten reuses at 25 ± 2 ° C, the immobilized invertase lost only 9% of the initial activity, but the optimum temperature (60 °C) reduction activity was about 70%, which is economically feasible from the point of view industrial.

According to the physical properties obtained ceramic that was prepared with polyvinyl alcohol was also developed to be tested in a unique continuous flow system. This system allowed the destabilization of preferred paths or "dead zones of sucrose solution in the bioreactor between ceramics and its hinterland; thus allowing a better performance of fixed biocatalyst and therefore the hydrolysis of sucrose into shorter reaction. The obtained residue ceramics, coal fly ash, proved to be a promising alternative as biotechnological invertase immobilization support for the producing of invert sugar and should be used to immobilize other enzymes of industrial interest.

Keywords: coal fly ash, ceramics, invertase immobilization.

Lista das principais abreviaturas técnicas

°C	grau Celcius
µg	micrograma
µg	micrograma
µL	microlitro
µL	microlitro
3-APTES	3-aminopropiltrietoxisilano
ABNT	Associação Brasileira de Normas Técnicas
Ad	densidade aparente (apparent density)
Ap	porosidade aparente (apparent porosity)
Awa	absorção aquosa aparente (apparent water absorption)
CFA	cinzas volantes de carvão mineral (coal fly ashes)
Da	Dalton
dry*m*	massa seca (mass dry)
EDS	espectroscópio de energia dispersiva (português)
G	grama
GA	glutaraldeído (glutaraldehyde)
GCS	suporte vítreo-cerâmico (Glass-ceramic support)
GCS*C*	suporte vítreo-cerâmico controle (Glass-ceramic support without addictive (control))
GCS*Pb*	suporte vítreo-cerâmico acetate de chumbo (Glass-ceramic support with addition of lead acetate)
GCS*Zn*	suporte vítreo-cerâmico zinco (Glass-ceramic support sulfato de zinco)
imm*m*	massa imersa (mass immerse)
kV	Kilovolte
LOI	perda ao fogo (lost of ignition)
mA	miliampere
mg	miligrama
mL	mililitro
mM	milimolar
MPa	megapascal
PM	peso molecular

PVA	álcool polivinílico
PVP	polivinil pirrolidona (Polyvinyl pyrrolidone)
sat*m*	massa saturada (mass satured)
SEM	microscópio eletrônico de varredura (scanning electron microscope
U	unidade
Ucs	aumento compressivo uniaxial (uniaxial compressive strength)
XRD	difração de raios-X (X-Ray diffraction)
XRF	X-Ray fluorescence (Espectroscopia de Fluorescência de raios-X)

Outras abreviaturas

PR	Paraná
RS	Rio Grande do Sul
USA	United States of America

Sumário

1. INTRODUÇÃO

Muitos países produzem diariamente grandes quantidades de cinzas volantes de carvão mineral, geradas em termelétricas durante a combustão desse material. O efeito causado é desastroso ao meio ambiente e a saúde das pessoas, por conter metais pesados, com partículas abaixo de 2,5 μm de tamanho (TAKUWA *et al.*, 2006). Com isso, tem-se demandado muitas pesquisas para diminuição do impacto ambiental provocado por esse tipo de cinzas. Geralmente, estes resíduos são depositados em aterros sanitários de forma desordenada e podem trazer inúmeros problemas, como contaminação de solos, lençóis freáticos, rios e lagos, além de causar doenças e influenciar negativamente o ciclo biológico da vegetação.

Segundo a Agência Nacional de Energia Elétrica (ANEEL, 2007), aproximadamente 11% da eletricidade gerada no Brasil é proveniente de sete termelétricas existentes nos estados do Rio Grande do Sul, Santa Catarina e Paraná, as quais produzem cerca de 1500 MW. No processo de produção de energia elétrica dessas termelétricas, sobram aproximadamente três milhões de toneladas de cinzas a cada ano, sendo 65 a 85% de cinzas volantes e 15 a 35 % de cinzas pesadas.

Em outros países, a maior parte das cinzas volantes é utilizada na produção de cimento e pavimentos, mas as cinzas pesadas continuam sendo depositadas no solo sem nenhum controle ou preocupação com a integridade do meio ambiente (JHA *et al.*, 2008).

A partir de janeiro de 2006, a resolução 307 do Conselho Nacional do Meio Ambiente (CONAMA) foi estabelecida a obrigatoriedade de um projeto de gerenciamento de resíduos sólidos para todas as obras submetidas à aprovação dos municípios e o licenciamento delas, parte do construtor, nos órgãos competentes. A medida deveria impedir o despejo destes resíduos em aterros sanitários ou a céu aberto. No entanto, a grande maioria dos municípios brasileiros ainda não está preparada para atender a tal resolução, por não possuir tecnologia adequada para a reutilização dos resíduos e não ter locais apropriados para o depósito deles. O fato, entretanto, não isenta o construtor da responsabilidade de desenvolver seu projeto de gestão dos resíduos. Gesso, resíduos de cerâmica, cinzas volantes de carvão mineral, além dos demais materiais de demolição e construção, precisam passar por procedimentos de seleção, identificação, separação e caracterização, que devem ser feitos de forma racional e eficiente, visando um destino posterior.

Pesquisas apontam uma variedade de materiais que podem ser utilizados como suporte para imobilizar biocatalisadores viáveis ao uso em escala industrial (GIRELLI *et al.*, 2007). Suportes inorgânicos são mais usados que os orgânicos devido à maior resistência mecânica e a ataques microbiológicos. Alguns suportes se tornam inviáveis (como o gel, por exemplo) por serem frágeis em processo e necessitarem de uma pressão maior, utilizada em biorreatores de fluxo contínuo (KOSZELEWSKI *et al.*, 2010). Mas, poucos são os estudos com suportes de origem mineral

(SANJAY e SUGUNAN, 2005; 2006b) ou vítreo-cerâmico (IDA *et al.*, 2000; KOVALENKO *et al.*, 2002; DE LATHOUDER *et al.*, 2006). A preparação de um material vítreo-cerâmico requer o emprego de aditivos fundamentais, como à adição de amido (ZIVCOVÁ, CERNÝ, *et al.*, 2009) ou álcool polivinílico; (KIM e JUNG, 2007) denominados de ligantes temporários. Eles facilitam a prensagem ou extrusão da mistura a ser sintetizada. Elementos fundentes (ERSOY *et al.*, 2008) também são inclusos com a finalidade de proporcionar propriedades físicas e, também, auxiliar na sua composição química final, para ativação do suporte vítreo-cerâmico e, posterior, ligação com proteínas.

Vários processos podem ser empregados para minimizar estes efeitos tóxicos. Um deles pode ser a sinterização destes resíduos, mecanismo largamente utilizado na produção de materiais cerâmicos para a construção civil. Esta pode ser uma solução rápida para captura de metais pesados como aqueles usualmente encontrados nas cinzas volantes de carvão mineral das termelétricas (EROL *et al.*, 2008b) ou nos resíduos provenientes de fábricas de cerâmicas esmaltadas (BERNARDIN *et al.*, 2006).

Alguns trabalhos demonstraram resultados significativos quanto à hidrólise de sacarose, usando invertase imobilizada em suporte oriundo de fonte mineral como montmorillonita (SANJAY e SUGUNAN, 2006a) ou suportes cerâmicos complexos. A enzima é ligada covalentemente a uma camada de polímero previamente adsorvida numa membrana de cerâmica com o glutaraldeído para uma ligação covalente-cruzada (MAGNAN *et al.*, 2004). O uso do suporte vítreo-cerâmico obtido das cinzas volantes de carvão mineral, rico em elementos químicos e com posterior ativação, foi sugerido devido a sua constituição química, carência de estudos voltados à aplicação biotecnológica e contribuição para redução da poluição ambiental.

2. REVISÃO DA LITERATURA

2.1. O carvão mineral

O carvão mineral é uma mistura complexa e variada de componentes orgânicos sólidos, fossilizados ao longo de milhões de anos, em um processo semelhante aos demais combustíveis fósseis. Sua qualidade é determinada pelo conteúdo de carbono, varia de acordo com o tipo e o estágio dos componentes orgânicos.

No Brasil, o carvão é responsável por 11% da eletricidade produzida no país (o que corresponde a 1500MW). A maior parte das cinzas resultantes da combustão desse material é usada na produção de cimento pozolânico e na pavimentação de estradas. O restante, no entanto, não é aproveitado, sendo abandonado em locais desapropriados. O fato acaba transformando a questão em

um problema ambiental, devido à lixiviação das substâncias tóxicas presentes nesse resíduo. Há a necessidade de estabelecer processos apropriados e eficientes ao reuso desses resíduos (OLGUN *et al.*, 2005).

2.1.1. Compostos químicos presentes nas cinzas volantes de carvão mineral.

As concentrações maiores de Al_2O_3-SiO_2 são úteis para uma preliminar junção de outros elementos presentes nas cinzas. A alta viscosidade de compostos fundidos, obtida pela adição de óxidos na sinterização, indica provavelmente uma melhor organização deles para a formação de subprodutos (mulita, quartzo, anortita e entre outros). Às vezes, é necessária a adição de outros elementos para que haja a fundição em menor temperatura e menos tempo de sinterização. Peng *et al.*, (PENG *et al.*, 2005), adicionou as cinzas volantes de carvão mineral CaO, Na_2O e B_2O_3 para este propósito.

A adição de compostos fundentes é determinante para a produção industrial de vítreo-cerâmicos devido à diminuição de custos com aquecimento para a sinterização de materiais. Koo *et al.*, (2008) obteve aumento de porosidade quando adicionou diferentes concentrações de zinco em esferas de cobre e a diminuiu quando reduziu a temperatura de sinterização, por se tratar de um elemento que se vaporiza quando aplicado temperatura alta com rapidez.

2.1.2. Adição de aditivos às cinzas volantes de carvão mineral

Amostras vítreo-cerâmicas são produzidas a partir da adição de compostos que facilitam as suas preparações e influenciam, sistematicamente, a preparação de todos os compostos usados e, depois, a prensagem para obtenção dos corpos verdes para a sinterização. O polímero e o aditivo ligante polirridona polivinil (BAUER *et al.*, 1999; WEI *et al.*, 2008) são adicionados para facilitar a prensagem do principal insumo pela homogeneização de todos os compostos presentes, devido a sua solubilidade. Ele evapora sem deixar resíduo durante tratamento térmico (KIM e JUNG, 2007). O amido (ZIVCOVÁ, GREGOROVÁ, *et al.*, 2009) e o resíduo de óleo (MONTEIRO e VIEIRA, 2005) vêm sendo usados com bastante êxito na preparação de vítreo-cerâmicos. São verificados aumentos significativos nas propriedades mecânicas quando comparados com cerâmicas sem aditivos (KIM e JUNG, 2007; TAN *et al.*, 2008).

Para facilitar a mistura, a granulação ou prensagem dos compostos para obtenção dos corpos verdes, é preciso automatizar, principalmente quando se pensa numa escala industrial. Com isso, na aquisição rápida dos corpos verdes através de prensagem de todos os insumos podem-se empregar técnicas semelhantes à produção de fármacos. O estearato de magnésio assim como o estearato de

cálcio é usado na indústria farmacêutica para lubrificar parte da prensa que tem contato com os insumos a serem usados (FUKUI *et al.*, 2001).

2.1.3. Sinterização de cinzas volantes de carvão mineral como suporte vítreo-cerâmico para imobilização de enzimas

Sinterização pode ser definida como a remoção dos poros entre as partículas iniciais, acompanhada por retração da peça combinada (corpos verdes) com crescimento e formação de ligações fortes entre partículas adjacentes, por exemplo, vítreo-cerâmicos. Estes materiais se apresentam com a forma policristalina, de acordo com o controle de cristalização ou de vidros (amorfo) (Figura 1) (YU *et al.*, 2008). Compreendem-se todos os materiais inorgânicos, não metálicos, obtidos geralmente após tratamento térmico em temperaturas elevadas. O material cristalino caracteriza-se essencialmente por uma periodicidade dos compostos químicos presentes, enquanto que, no material amorfo, não há esta disposição.

A calcinação compreende o processo de aquecimento uma substância a altas temperaturas, sem atingir o seu ponto de fusão, em consequência, consegue-se eliminar elementos voláteis. A adição de compostos a base de CaO é usada para eliminar a toxidez de substâncias poluidoras em resíduos (LEROY *et al.*, 2001).

Kim & Kim (2004) utilizou o termo vitrificação para obtenção de vítreo-cerâmico a partir de cinzas volantes de carvão mineral com adição de CaO e TiO_2 como agente nucleante para reduzir o temperatura de fundição e promover cristalização interna.

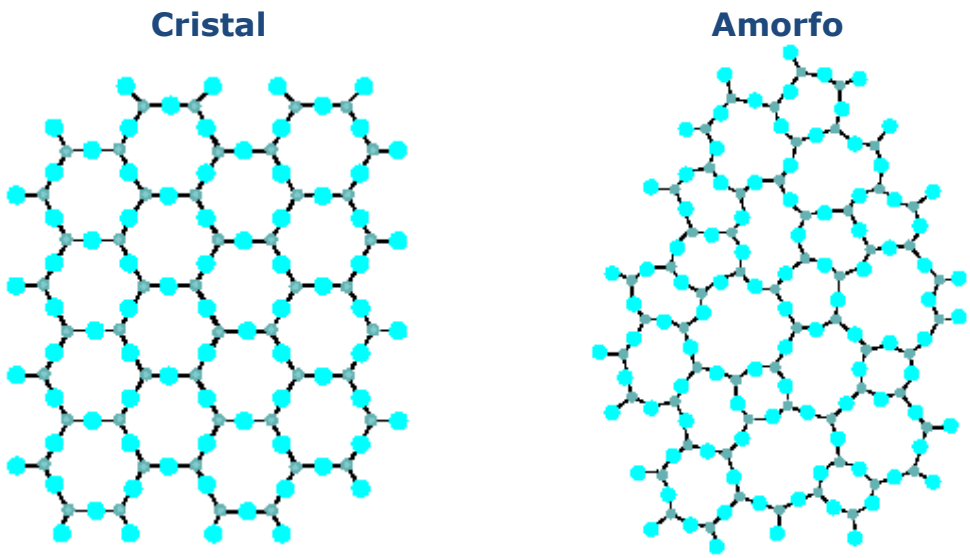

Figura 1. Sílica: cristalina e amorfa (YU *et al.*, 2008).

A produção de vítreo-cerâmico a partir das cinzas carvão mineral tem sido uma alternativa viável para diminuição da poluição ambiental (LEROY *et al.*, 2001). São encontradas nas cinzas volantes de carvão mineral um grande número de elementos conhecidos como CAS (CaO- Al_2O_3-SiO_2), além de possuir, em menor concentração, MgO, Fe_2O_3, presente em forma cristalina (KIM e KIM, 2004); (OLGUN *et al.*, 2005). De acordo com a temperatura e tempo de sinterizações usadas, há uma "organização" dos produtos de maneira amorfa ou cristalina, como: quartzo (SiO_2), mulita (γ-Al_2O_3), coríndon (hexagonal) (Al_2O_3), hematita (Fe_2O_3), anidrita ($CaSO_4$), calcita ($CaCO_3$) e outros em menor proporção. Ou ainda, outros subprodutos unidos com estes como: diopsida ($CaO.MgO.2SiO_2$), wollastonita ($CaO.SiO_2$), albita ($Na_2O.Al_2O_3.6SiO_2$) e outros. Estas constituições, com prévia ativação, são favoráveis à ligação com vítreo-cerâmico→3-APTES↔GA→enzima (Figura 2).

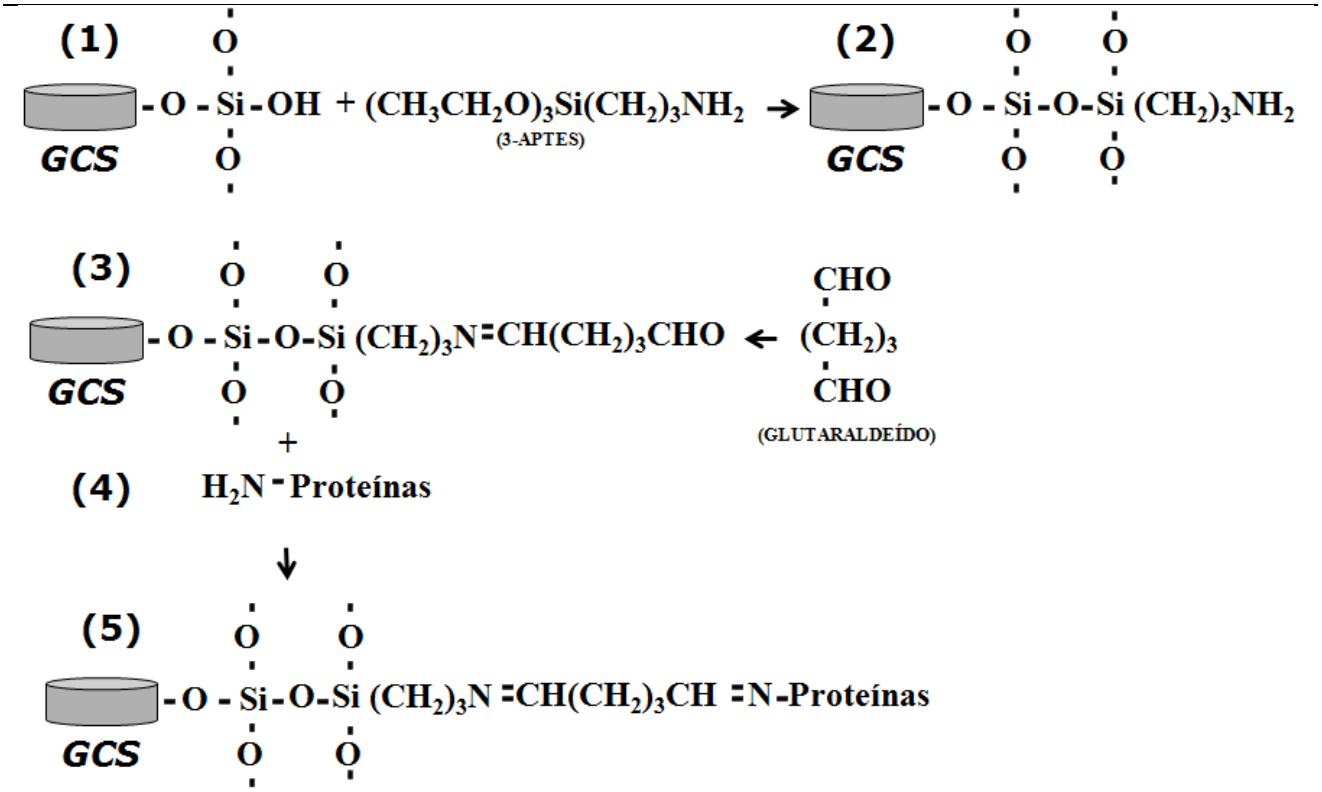

Figura 2. Esquema para ligar proteínas covalentemente. (1) Silanização da superfície do suporte vítreo-cerâmico (GCS); (2,3) ligação grupos de aminas do suporte ativados com grupos carboxílicos do glutaraldeído ($C_5H_8O_2$) para formar uma base de Schiff; (4,5) formação de outra base de Schiff para ligar entre os grupos carbonila do suporte ativado e grupos aminas livres da proteína (LIMBUT *et al.*, 2004).

Um fator importante para a formação cristalina de um material, como citado anteriormente, é verificado pela variação na acomodação estrutural ao comparar amostras de mesma concentração química. Isso acontece porque a energia livre é baixa e, consequentemente, forma uma estável fase cristalina e uma maior densidade vítrea amorfa para a amostra. Então, quando um material vítreo-

cerâmico é considerado não poroso ou com poucos poros, verifica-se um grau de encolhimento dos produtos e aumento da densidade (KARAMANOV e PELINO, 2008).

2.2. Glutaraldeído como agente fixador de enzimas em suportes

O glutaraldeído (1,5 pentanedial – PM 100,12), em altas concentrações (superior a 25%) e sob condição ácida, pode se apresentar em polímeros de múltiplas estruturas cíclicas hidratadas. Após as diluições em soluções aquosas, estes polímeros podem reverte-se à forma monomérica, devido a sua facilidade de hidratação. O glutaraldeído já vem sendo utilizado na imobilização de lactase, o que propicia sua utilização para outras enzimas. Além deste, podem ser usados para produtos alimentares: triacetato de celulose; dietilaminoetil celulose; polietileimina e 1,2 dicloroetano (CIMDINS *et al.*, 2000).

O glutaraldeído é empregado como agente fixador na preservação dos tecidos e das estruturas subcelulares nos estudos de microscopia. Também é empregado como agente esterilizante em material cirúrgico, entre outros. Atualmente, o glutaraldeído vem sendo usado como ativador da superfície de eletrodos para a imobilização de enzimas na produção de biossensores, tanto em ligação covalente (SANJAY e SUGUNAN, 2005) como covalente-cruzada (IKEDIOBI *et al.*, 1998; MAGNAN *et al.*, 2004). Na indústria de processamento de alimentos, o glutaraldeído é recomendado na imobilização de enzimas, em virtude da sua elevada polimerização e baixas concentrações usadas para possibilitar a obtenção de alimentos transformados com baixas concentrações residuais do glutaraldeído (SUZUKI *et al.*, 1991).

Em aplicações industriais, os materiais inorgânicos para suportes enzimáticos são atrativos por serem resistentes à esterilização, com altas temperaturas e pressão e à abrasão mecânica. Outras vantagens para usar o suporte cerâmico são: resistência ao ataque de produtos químicos; resistências à tração; a compressão e dureza (SUZUKI *et al.*, 1991; CIMDINS *et al.*, 2000; IDA *et al.*, 2000).

O desenvolvimento de suportes vítreo-cerâmicos a base de SiO_2 (como cinzas volantes resultantes da queima do carvão mineral ou argilas) para imobilização covalente de enzimas, com ativação dos grupos silanos presentes no mesmo usando o 3-APTES e, posteriormente, o glutaraldeído, é uma tecnologia que possibilita a geração de materiais com maior valor agregado na aplicação industrial para imobilização de enzimas. Por sua vez, pode contribuir com a diminuição dos problemas ambientais associados à deposição de matéria residual de algumas indústrias (termelétricas) brasileiras, e ao mesmo, reduzir os custos com matérias primas necessários ao processo de formulação de suporte convencionais (KNIESS *et al.*, 2007).

2.3. Suportes para imobilização

Foram estudados diversos suportes orgânicos para imobilização de enzimas como: celulose (SANKPAL e KULKARNI, 2002); algodão (GODBOLE *et al.*, 1990); quitosana (GÓMEZ *et al.*, 2006; AZMI *et al.*, 2009); biopolímeros (BAGAL e KARVE, 2006). Apesar de serem muitas as alternativas disponíveis, há um risco eminente de contaminação dos produtos obtidos nos processos de biotransformação pela degradação e/ou erosão mecânica desses suportes (GODBOLE *et al.*, 1990; KOVALENKO *et al.*, 2002). Neste sentido, outras pesquisas estão sendo direcionadas para o emprego de suportes inorgânicos que venham mostrar maiores eficiências nos termos custo/benefício. Alguns exemplos desses suportes para imobilização são: esfera de vidro não-porosa (IKEDIOBI *et al.*, 1998; FARAG e HASSAN, 2004); epóxi (XUE *et al.*, 2008); copolímeros (CHEN *et al.*, 2000; ERGINER *et al.*, 2000; CIRPAN *et al.*, 2003); álcool-polivinílico (AKGÖL *et al.*, 2001); sílica-gel (PARK *et al.*, 2002; LIMBUT *et al.*, 2004); vítreo-cerâmico (IDA *et al.*, 2000; KOVALENKO *et al.*, 2002; MAGNAN *et al.*, 2004); sílica meso-estruturada (SZYMANSKA *et al.*, 2007) resinas de troca-iônica (MARQUEZ *et al.*, 2008); polímeros sintéticos (DIZGE *et al.*, 2008); tereftalato de etileno (AMARAL *et al.*, 2006).

A cerâmica proveniente de materiais denominados de "resíduos", gerados na construção civil e termelétrica (cinzas volantes de carvão mineral), pode ser ativada para imobilização de enzimas devido a sua constituição química. Suportes vítreo-cerâmicos são noticiados para imobilizar acetilcolina esterase (EBRAHIMI *et al.*, 2010), lípase (HUANG e CHENG, 2008), β-galactosidase, devido às suas vantagens, como: reusos do biocatalisador, facilidade de separação dos produtos obtidos.

Em evidência, suporte de origem mineral como a argila montmorillonita (SANJAY e SUGUNAN, 2005) cerâmica a partir de macro estruturas carbonizada (KOVALENKO *et al.*, 2002; KOVALENKO *et al.*, 2009) e material meso-estruturado à base de silício (DAVID *et al.*, 2006; SZYMANSKA *et al.*, 2007) foram obtidos sucessos em seus resultados. Estes suportes ricos em dióxido de silício têm como estratégia no processo de imobilização de enzimas o emprego do composto químico 3-aminopropiltrietoxisilano (3-APTES) com sua ativação em solução de tolueno, acetona ou álcool e, consequentemente, ligação com o agente bifuncional glutaraldeído ($C_5H_8O_2$) (GA).

Esta técnica envolve três etapas: reação de silanização da superfície do vidro com o 3-APTES em sistema não aquoso; ligação do grupo carbonila do GA com o grupo amino da superfície silanizada oriundo do 3-APTES; e finalmente, a ligação covalente das proteínas através dos grupos aminos dos resíduos de aminoácidos e o grupo carbonila disponível do GA (LIMBUT *et al.*, 2004).

2.4. Tipos de ligações para imobilização em suportes insolúveis sólidos

Diferentes métodos são disponibilizados para imobilização de enzimas. Entretanto, alguns deles podem exercer influência nas propriedades do biocatalisador imobilizado. A escolha do tipo de ligação química para esse processo é descrita conforme a propriedade química do suporte (SANJAY e SUGUNAN, 2006b), e para à sua modificação (PARK *et al.*, 2002), além das condições para a reação em biorreatores quanto ao da reação com o substrato (KOVALENKO *et al.*, 2002).

O uso do biocatalisador imobilizado requer ainda fatores que justifiquem o processo, como: extensão da atividade pela proteção do material ativo contra a desnaturação; repetição de sua utilização; redução de custo operacional – principalmente automação; facilidade da separação do biocatalisador como os produtos obtidos da reação. Porém, algumas desvantagens podem ser observadas, tais como: perda ou diminuição da atividade durante o processo de imobilização; resistência difusional ao transporte do substrato ou produto entre o meio reacional e sítios ativos do biocatalisador (BAYRAMOGLU *et al.*, 2003).

Dentre as ligações químicas utilizadas (adsorção, covalente, covalente cruzada e enclausuramento), apenas as duas primeiras são citadas constantemente na literatura para imobilização de enzimas em suportes vítreo-cerâmicos. Há viabilidade apenas nestas devido as suas propriedades físicas que contribuem satisfatoriamente para imobilizar enzimas. Atualmente, suportes orgânicos não têm grande aceitação devido a sua facilidade a ataques microbiológicos e, consequentemente, de decomposição. Invertase foi imobilizada em suporte vítreo-cerâmico ou argila montmorillonita usando a enzima invertase através de ligação adsorção e covalente (IDA *et al.*, 2000; SANJAY e SUGUNAN, 2005; KOVALENKO *et al.*, 2009) (Figura 1).

2.4.1. Ligação adsorção

A ligação de uma enzima por adsorção (Figura 3) em suporte insolúvel é um método simples para imobilização. A técnica tem como base submeter à enzima ao material (suporte), sob condições que favoreçam a ligação da substância com ele. Após um período de incubação, o derivado (suporte/enzima) é filtrado do restante da solução proteica que não se ligou.

Este tipo de ligação favorece o derivado e faz com que ele consiga preservar suas características de origem (nativa) devido às interações físico-químicas adquiridas, as quais não tem efeito sobre a atividade enzimática. Alguns fatores para facilitar a ligação são conferidos, como: o tamanho da proteína, área do suporte; características do suporte insolúvel (porosidade/absorção aquosa, tamanho do poro, distribuição regular dos poros); concentração da enzima e pH do processo de imobilização (VILLENEUVE *et al.*, 2000; TOMOTANI e VITOLO, 2006).

A principal desvantagem desse tipo de ligação é o fato de o biocatalisador não está fortemente ligado ao suporte. Desta forma, mudanças em quaisquer etapas (antes e depois da imobilização), como pH, força iônica (tampão), temperatura, concentração do substrato, podem desligá-la, desnaturalizando a enzima, favorecendo a contaminação dos produtos ou substrato através de lixiviação (SANJAY e SUGUNAN, 2005). Chen *et al.* (2000), imobilizou invertase covalentemente em partículas e filmes do copolímero de polianilina e ácido acrílico. A enzima imobilizada reteve 20 a 40% de atividade comparada à solúvel e melhorou a atividade da submetida a baixas temperaturas.

2.4.2. Ligação covalente

É a ligação fornecida por grupos funcionais presentes na superfície do suporte e pelos pertencentes a aminoácidos na superfície da proteína (Figura 3), sendo um tipo mais forte que a adsorção. As ligações entre as enzimas e suporte são irreversíveis. Aspectos para o sucesso desse tipo de ligação para imobilização de enzimas são importantes: o suporte deve apresentar superfície interna com boa congruência geométrica e formar fibras finas com a superfície da enzima, que, maiores do que as proteínas favorecem uma intensa interação entre esses dois elementos; precisa apresentar superfície densa com grupos químicos reativos, e quando, houver muitos grupos reativos no suporte para ligar a superfície da proteína, uma intensa ligação em muitos pontos do suporte para reagir covalentemente deve ser alcançada; os grupos reativos do suporte devem reagir com os das proteínas frequentemente localizadas na sua superfície; e envolvem no processo de imobilização de maneira que permita uma longa duração de reação do derivado (GÓMEZ *et al.*, 2006).

Amaya-Delgado *et al.*, (2006) imobilizaram invertase covalentemente em nylon-6, obtendo ampla faixa de pH e temperatura em relação a invertase livre.

Suportes sólidos insolúveis, com composição química compatível para reação com elementos, se ligam às proteínas por uma etapa de ativação de grupos silanos contido nos mesmos, com o composto organofuncional (3-APTES). O processo compreende através de uma intercação no SiO_2 contida na superfície e é utilizado como uma preparação para modificação inicial do suporte que deve se ligar com um agente bifuncional (glutaraldeído). Esse, por sua vez, se liga a enzima. A vantagem principal dessa técnica é evitar a ligação por adsorção. Outras são observadas na ligação covalente, em que, em muitos casos, as enzimas se apresentam estáveis e resistentes a condições extremas (pH, temperatura). No entanto, pode-se observar uma diminuição da atividade inicial do biocatalisador, já que estrutura conformacional da enzima é modificada após o processo. Sanjay e Sugunan (2005) imobilizou invertase em montmorillonita, usando os dois tipos de ligação, adsorção e covalente.

2.4.3. Ligação covalente-cruzada

Na imobilização de uma enzima por ligação covalente cruzada, as enzimas são adsorvidas no suporte e em seguida são ligadas pela utilização de agentes bi ou multifuncionais (por exemplo, glutaraldeído). As ligações de enzimas por este método, também, são irreversíveis, apresentando uma elevada resistência a variações de pH e temperatura, propiciando suportes de alta atividade e resistência. Porém, este tipo de ligação tem a desvantagem devido à sensibilidade de algumas já que entram em contato direto com a gente bifuncional por reação de reticulação (GÓMEZ *et al.*, 2006). Suporte como agregados formados de natureza gelatinosa são dispensados seu uso em reatores tudo tipo "*packed bed*". A atividade alcançada é limitada a problemas difusionais, uma vez que os biocatalizadores estão situados dentro do agregado, dificultando o acesso do substrato. O tamanho do suporte e porosidade em relação a limitações difusionais são características muito importantes na reação enzimática em biorreatores (CHEN *et al.*, 2000).

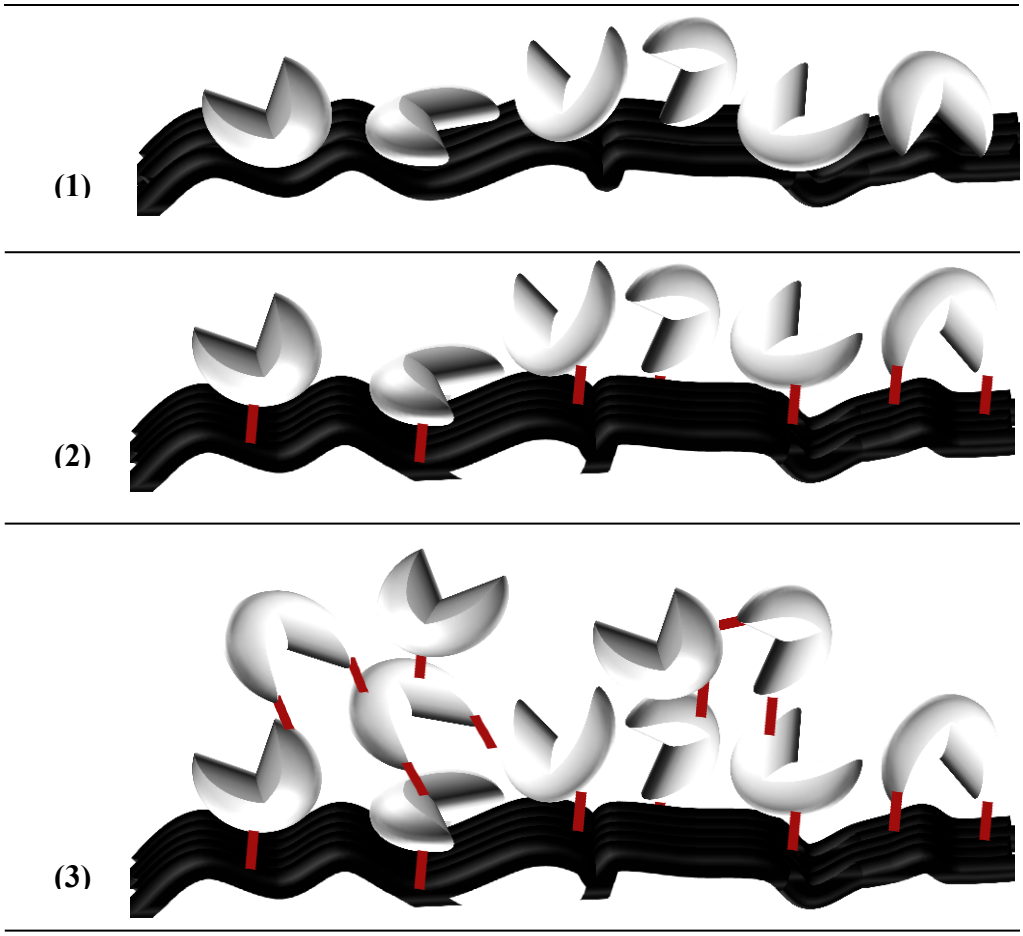

Figura 3. Ilustração dos tipos de ligações químicas usadas para imobilização de enzimas em suportes sólidos inorgânicos porosos ou não-porosos. O esquema da imobilização é demonstrado simbolicamente na figura. A ligação fraca (1) (adsorção) e ligação forte (2 e 3) (covalente e covalente-cruzada) da enzima ao suporte.

2.5. Enzimas de interesse a indústria sucroalcooleira

A indústria sucroalcooleira em países que cultivam a *Saccharum officinarum* (cana-de-açúcar) destina-se a produção de sacarose e álcool etílico, de maneira direta, além de papel e energia elétrica, através de bagaço da planta. A sacarose tem grande relevância socioeconômica e cultural pelo seu alto valor calórico, baixo preço e pela acentuada presença na alimentação em diversos produtos alimentícios e de bebidas (DE MATOS NOGUEIRA *et al.*, 2005). A sacarose O-α- D-glicopiranosil – (1→2) β-D-fructofuranosídeo) é o dissacarídeo mais abundante da natureza e sua hidrólise é catalisada especificamente pela enzima invertase (β-fructofuranosidase, E.C. 3.2.1.26) em α-D-glucose and β-D-frutose, comumente denominado de açúcar invertido (KOVALENKO *et al.*, 2009). O açúcar invertido é bastante apreciado, principalmente pela indústria alimentícia. Seu emprego na preparação de alimentos constitui-se em vantagens como: a preservação da cor dos alimentos em temperaturas altas; a não cristalização quando armazenado em alta concentração; a conservação das propriedades em longo período de tempo em comparação à sacarose (ISIK *et al.*, 2003).

O xarope de açúcar invertido reúne a elevada solubilidade da frutose à difícil cristalização da glicose, aumentando seu poder edulcorante e diminuindo os riscos de cristalização. Essas propriedades contribuem para aumentar o valor desses xaropes para uso em vários produtos alimentícios, sobretudo na indústria de refrigerantes. O produto de maior interesse comercial é aquele com nível de inversão próximo a 55%, pois nessa faixa a solubilidade é máxima possibilitando trabalhar com concentrações em torno de 76,5% de sólidos solúveis, diminuindo assim a susceptibilidade à contaminação microbiana, sem riscos de cristalização (DIZGE *et al.*, 2008). O açúcar invertido possui 20% a mais de poder edulcorante em comparação à sacarose pura (Gratão et al., 2004).

A substituição da hidrólise ácida da sacarose pela enzimática, através do emprego de enzimas ou células livres, é uma alternativa mais aceitável por não gerar resíduos tóxicos ao produto final (DANISMAN *et al.*, 2004). No entanto, resíduos proteicos associados a esta alternativa de hidrólise podem ser restritivos quanto à aplicação desses açúcares obtidos na formulação e na qualidade dos alimentos. O emprego de células pode acarretar ainda, um baixo rendimento de açúcares, pois estes podem ser metabolizados pelas células até a formação de álcool (Voet, 2005). No processo de hidrólise enzimática, há uma perda do material biocatalítico que está dissolvido na solução final de açúcar invertido. Para (ERGINER *et al.*, 2000), a utilização de técnicas que possibilitam a imobilização do biocatalizador em suporte fixo permite o reuso em novos processos e proporcionam a obtenção de soluções de açúcar invertido de melhor qualidade.

Considera-se também como vantagem: a automação do processo para escala industrial e a reação em biorreatores de fluxo contínuo. É sabido, que a atividade catalítica das enzimas livres é superior do que as células livres em contato com a solução de sacarose. Por isso, há anos vêm sendo aprimoradas técnicas para a imobilização da invertase (β-frutofuranosidase frutohidrolase – E.C. 3.2.1.2.6.), que é específica para hidrolisar a sacarose (SANJAY e SUGUNAN, 2005). A invertase é específica à catálise da sacarose convertendo-a em D-glucose e D-frutose. É considerada uma glicoproteína que contém em torno de 50% de carboidratos, possui um peso molecular de 270.000 Da e ponto isoelétrico no pH 4,6 (TANRISEVEN e DOGAN, 2001; TUNCAGIL *et al.*, 2008).

A enzima dextranase (α-1,6-glucanohydrolase, EC 3.2.1.11) catalisa a hidrólise de ligações α-1,6 glicosídicas em polímeros de dextrana em moléculas menores denominadas de isomaltoses. A dextrana é sintetizada pela enzima dextransacarase (α-6-glucosyltransferase, EC 2.4.1.5) presente em diferentes espécies do gênero Leuconostoc, Lactobacillus e Streptococcus que sintetizam em condições apropriadas a esta enzima a produzir cadeias poliméricas de dextrana com alto peso molecular, com o seguinte esquema: SACAROSE n($C_{12}H_{22}O_{10}$) + dextransacarase = DEXTRANA n($C_6H_{12}O_6$) + FRUTOSE n($C_6H_{12}O_6$) (PADMANABHAN e KIM, 2002; KIM *et al.*, 2003).

A presença de dextrana na indústria sucroalcooleira é fator negativo em relação ao aumento da viscosidade no caldo de cana-de-açúcar e, consequentemente, ao baixo rendimento nos subprodutos de interesse (sacarose e álcool etílico). O problema é ocasionado após a colheita da planta. O seu grau de contaminação dependerá do tempo pós-colheita, das condições climáticas para o crescimento dos micro-organismos produtores de dextransacarase ou atraso no processo para obtenção do caldo de cana-de-açúcar (ERHARDT e JÖRDENING, 2007). A presença de dextrana na indústria sucroalcooleira é fator negativo em relação ao aumento da viscosidade no caldo de cana-de-açúcar e, consequentemente, ao baixo rendimento nos subprodutos de interesse (sacarose e álcool etílico). O problema é ocasionado após a colheita da planta. O seu grau de contaminação dependerá do tempo pós-colheita, das condições climáticas para o crescimento dos micro-organismos produtores de dextransacarase ou atraso no processo para obtenção do caldo de cana-de-açúcar (ZAMBRANO *et al.*, 2003) e a micropropagação da planta cana-de-açúcar (ARENCIBIA *et al.*, 2008), a imobilização de invertase já é bastante noticiada na literatura científica, para a qual a produção de açúcar invertido deve ser visto também como alternativa biotecnológica.

2.6. A cinética enzimática

Cinética enzimática é definida como o estudo da velocidade da reação enzimática. Esta é alterada devido a mudanças nas condições experimentais, principalmente em respeito à

concentração da enzima, substrato (e volume), inibidores, ativadores, pH, força iônica (por exemplo:tampão) e temperatura (Voet, 2008).

A concentração e volume da enzima e substrato determinam diretamente a reação. Segundo (COMBES *et al.*, 1981), a atividade catalítica fica comprometida devido às concentrações de sacarose elevadas. O efeito na concentração de sacarose na reação de inversão é limitante à estrutura à associação molecular apresentadas em soluções aquosas em pequenas concentrações. A reação de inversão de sacarose é um consumidor de molécula de H_2O. Por isso, verificar as taxas iniciais de reação para obtenção do produto ou de consumo de substrato determinado período de tempo. Analisar a cinética pode levar a um modelo para a reação enzimática utilizando princípios da cinética enzimática através de equação da velocidade. A relação desses parâmetros possibilita explicar efeitos inibitórios de substrato ou de acesso ao substrato por parte da enzima. O modelo cinético mais usado é o de Michaelis-Mentem ($v=V_{max}.S/K_m+S$), onde o V_{max} indica a velocidade limitante de uma reação catalisada por enzima nas condições ótimas, sendo denominado velocidade máxima de reação, e K_m a constante de Michaelis-Menten, que diz respeito à afinidade da enzima ao substrato. Usualmente, descreve-se que, por exemplo, quando o K_m é maior da enzima imobilizada enzima em relação a livre, que houve limitação difusional ao substrato. Ou seja, uma diminuição na formação o complexo substrato-enzima, e posterior produto (AMAYA-DELGADO *et al.*, 2006).

A equação de Michaelis-Menten é utilizada para modelar diversas reações enzimáticas, mas sabe-se que para se aplicada com sucesso, a concentração da enzima deve ser pequena se comparada em relação ao substrato. Este modelo é impróprio em escala industrial onde as altas concentrações de substrato ou de produtos e em presença de inibidores no meio reacional são constantes.

2.7. Características físicas do suporte insolúvel para processos em biorreatores

Vários suportes, orgânicos ou inorgânicos, têm sido descritos na literatura para imobilização de enzima. Entretanto, sabe-se que não existe um suporte, tampouco um tipo de ligação química, para aplicação imediata. Existem características fundamentais a serem observadas para a escolha de um suporte, que podem afetar em diferentes propriedades e no processo de produção: área de superfície e porosidade – maior difusão dos compostos químicos e biológicos (enzima) e na reação (substrato), grupos funcionais na superfície e estabilidade mecânica e química. Portanto, o bom desempenho da atividade da enzima imobilizada é fundamentado, principalmente, nestes aspectos para uso em biorreator.

A porosidade do suporte é um dos aspectos físicos mais importantes, devido ao aumento na área de superfície do suporte para imobilização de enzimas, e está relacionado à absorção aquosa. A

ativação dessa superfície, do ponto de vista químico, vai proporcionar uma maior eficiência de ligação aos compostos químicos presentes no suporte e posterior ligação com proteínas. Ambas as áreas interna e externa apresentam capacidade de ligação química maximizada, sendo motivo de pesquisa na obtenção de métodos de imobilização mais apropriados. (SUZUKI *et al.*, 1991) apresentou eficiência de atividade catalítica da enzima imobilizada em cerâmica microporosa quando usada em biorreator com sistema de fluxo contínuo. Então, um suporte microporoso com alta retenção (absorção) oferece efeito positivo a difusão do substrato em contato no interior do mesmo para entrar em contato com a enzima imobilizada; determinada pela área de superfície do suporte. O efeito do fluxo ou agitação no sistema batelada em contato com a enzima imobilizada é um ganho significativo à reação no interior do suporte, ou ainda, no tempo de residência usado (KOVALENKO *et al.*, 2002).

Para verificar a porosidade aparente, absorção aquosa aparente e densidade aparente, é usado o princípio de princípio de Archimedes usando relações entre massa seca (Ms), massa úmida (Mú) e massa imersa (Mi). A porosidade aparente (Mú - Ms) / (Mú - Mi) x 100 (%); absorção aquosa aparente (Mú - Ms) / (Ms) x 100 (%); densidade aparente (Ms / (Mú - Mi) (g/cm^{-3}) (PAVESE *et al.*, 2007); (ERSOY *et al.*, 2008; LITTLE *et al.*, 2008). Entretanto, diversos trabalhos utilizam para verificar a porosidade de vítreo-cerâmicos usando porosímetro de mercúrio (EROL *et al.*, 2008a), adsorção de nitrogênio (KORD *et al.*, 2009).

Em complemento, a propriedade física "resistência mecânica" é estudada em ensaios usando diversas pressões diferentes para verificar fraturas nas amostras de vítreo-cerâmicas. Estas pressões podem ser exercidas em diversos pontos simultâneos (FURLANI *et al.*, 2008) ou uniaxialmente (EROL *et al.*, 2008b).

A adsorção de enzimas em suportes insolúveis inorgânicos (cerâmica, bentonita, argilas) permite que materiais baratos, abundantes, resistentes, estáveis quimicamente, sem toxidade, não poluentes sejam facilmente reaproveitados para reuso posterior. A vantagem pode ser atribuída ao seu empacotamento ou em suspensão (biorreator de leito empacotado), causada pela sua densidade adequada, respectivamente em sistema batelada ou em biorreator com leito empacotado, usando fluxos diversos (com alimentação ascendente ou descendente) (TOMOTANI e VITOLO, 2006). O principal fator para diminuição de problemas no rendimento da atividade enzimática nestes sistemas pode ser amenizada usando um suporte poroso (KOVALENKO *et al.*, 2002; KOVALENKO *et al.*, 2009).

Em diversos trabalhos são citados que a constante de Michaelis (K_m) apresentada em maior valor, é, por excelência, fator determinante para explicar a atividade catalítica da enzima imobilizada, porque a técnica de imobilização submetida pode interferir na estrutura

conformacional dela, adquirindo menor afinidade ao substrato quando comprada com a enzima livre (OSMAN *et al.*, 2005; DAVID *et al.*, 2006; EMREGUL *et al.*, 2006; GÓMEZ *et al.*, 2006).

Estudos sobre imobilização de enzimas em suportes inorgânicos que proporcionem aumento na atividade enzimática em relação a suas reutilizações devem ser somados a outros sobre biorreatores com sistema de fluxos (continuo ou descontinuo) e batelada, com o aumento no rendimento final da reação. Com isso, estas variáveis, usualmente, verificadas como fatores principais no melhoramento do sistema, podem ser o diferencial entre os suportes já estudados. Até o momento, não foi noticiado estudos direcionados ao melhoramento da atividade da enzima imobilizada com enriquecimento do suporte por elementos químicos em sua preparação.

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4. OBJETIVOS

Geral

- Desenvolver um suporte cerâmico para imobilização de invertase (E.C. 3.2.1.26.), e dextranase (EC 3.2.1.11) (Novozymes) e suas aplicações em biorreator de sistema batelada.

Específicos

- Sinterizar cinzas volantes de carvão mineral e analisar as propriedades físico-químicas da cerâmica com os aditivos;
- Imobilizar dextranase e invertase (enzima comercial- Novozymes) em suporte vítreo-cerâmico microporoso (CGSM) preparado sem adição de aditivos químicos permanentes;
- Escolher a melhor de atividade de invertase imobilizada (enzima comercial- Novozymes) em suportes vítreo-cerâmicos com formulações diferentes (CGCSMcontrol,CGSMPb e CGSMZn);
-Estudar as melhores condições experimentais para imobilizar invertase (enzima comercial- Novozymes) no suporte vítreo cerâmico microporosos;
- Determinar as propriedades físico-químicas e cinéticas de invertase, livre e imobilizada;
- Verificar a retenção da atividade catalítica e estabilidade operacional da invertase invertase imobilizada.

5. JUSTIFICATIVA

O desenvolvimento de suportes insolúveis de grande resistência física, propriedades químicas e de tecnologia nacional é fundamental quando se pensa na aplicação em sistemas industriais. A Região Nordeste produz atualmente 4,7 milhões de toneladas de sacarose, representando 13,01% da produção nacional (Companhia Nacional de Abastecimento - CONAB, safra/2009). Entretanto, além da obtenção de álcool e açúcar comum, há um mercado em expansão de açúcar invertido. O uso de invertase imobilizada para inversão da sacarose em glucose a frutose, são alternativas para o desenvolvimento do setor açucareiro e, consequentemente, para indústria alimentícia, devido às vantagens operacionais (não cristalização de subprodutos, açúcar claro) e a disponibilização de produto sem resíduos tóxicos, como furfural presente no açúcar proveniente de hidrólise ácida.

No setor de Biotecnologia do Laboratório de Imunopatologia Keizo Asami vem sendo desenvolvido um suporte utilizando cinzas de carvão mineral com aditivos. Esta matriz apresentou

resultados promissores para imobilização de enzimas. Com isso, haverá diminuição dos custos na produção de um suporte para imobilização de enzimas, além da diminuição da poluição ambiental.

A indústria de biotransformação brasileira é muito carente destas tecnologias em grande escala. Apesar de ser o Brasil o principal produtor de sacarose do mundo, o país ainda importa glucose para uso médico devido ao grau de pureza. Desta forma este projeto irá viabilizar duas grandes lacunas na indústria biotecnológica nacional: a primeiramente diz respeito ao uso em grande escala de suporte para imobilização de enzimas; e a segunda, à produção de xarope de frutose para uso médico. Em consequência, pode-se produzir um xarope rico em frutose, que poderá ser utilizado na fabricação de alimentos e bebidas, respectivamente.

6. PRODUÇÃO CIENTÍFICA

6.1. CAPÍTULO I

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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.

Keywords: Coal fly ashes; glass-ceramic supports; invertase; immobilization; performance.

Abbreviations

3-APTES - 3-aminopropyltriethoxysilane

Ad - Apparent density

Ap - Apparent porosity

Awa - Apparent water absorption

CFA - Coal fly ashes

dry*m* - Mass dry

GCS - Glass-ceramic support

GCS*C* - 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

imm*m* - Mass immerse

PVP - Polyvinyl pyrrolidone

sat*m* - mass satured

SEM - Scanning electron microscope

XRD - X-Ray diffraction

XRF - X-Ray fluorescence spectroscopy

Ucs - Uniaxial compressive strength

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].

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 (GCS*C*) 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 ºC / 1 h, 1000 ºC / 3 h, 1100 ºC / 2 h, 1200 ºC / 1 h, 1200 ºC / 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 (Ap), water absorption (Awa) and density (Ad) in GCS*C*, GCS*Pb* and GCS*Zn* were obtained in the five conditions of sinterization. They were determined by measuring the dry mass (dry*m*), immersed mass (imm*m*) and 24 h saturated surface-dry mass (sat*m*). The *Ap* (%) Eq. 1, *Awa* (%) Eq. 2 and *Ad* (g/cm^3) Eq. 3 were obtained respectively by the following relations described by Archimede's principle:

$$Ap = [(satm - drym) / (satm - immm)] \times 100 \quad (1)$$

$$Awa = [(satm - drym) / drym] \times 100 \quad (2)$$

$$Ad = drym / (satm - immm) \quad (3)$$

It was performed studies (in triplicate) about the resistance to uniaxial compressive strength (0.1 to 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 2 θ 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 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 $3x2^2$, 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 Statistic 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 the Figs. 1, 2 and 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 / 1h 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 the Figs. 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 showed 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 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, apparent density. The uniaxial compressive strength was studied to know the mechanical characteristic of immobilized derivatives.

The results were evaluated by factorial design ($3x2^2$) 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 the 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. 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 of 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. The Fig. 4B shows non-linear changes in the uniaxial compressive strength of the GCS*C*, GCS*Pb* and GCSZn sample. The highest mechanical strength values were obtained for: GCS*C* (23.77 MPa sintered at 1200ºC / 1 h), GCS*Pb* (22.33 MPa sintered at 1200ºC / 3 h) and GCS*Zn* (10.67 MPa sintered at 1100ºC / 2 h). The lowest value showed for GCS*Zn* can be explained by preservation of the cenospheres after sinterization process of this sample what it was not observed for the sintered GCS*C* and GCS*Pb* samples (Figs. 1, 2,and 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 $3x2^2$ 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 GCS*C* 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 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 GSC*Zn*.

The 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 GCS*C* (232.02 ± 10.48 U/g GCS*C*) 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 GCS*Zn*).

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

3.4.2. Reusability of immobilized invertase

The average catalytic activity obtained after nine reuses of the immobilized derivative CGS*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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Figure captions

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

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

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

Fig. 4. Effects of additives on the physical proprieties of glass-ceramic supports (GCS) obtained using coal fly ashes. 4A: Apparent porosity labels - GCS*C* (○); GCS*Pb* (●) and GCS*Zn* (▲) and Apparent water absorption columns - GCS*C* (□); GCS*Pb* (■) and GCS*Zn* (■). 4B: Apparent density labels - GCS*C* (○); GCS*Pb* (●) and GCS*Zn* (▲) and uniaxial compressive strength columns - GCS*C* (□); GCS*Pb* (■) and GCS*Zn* (■). 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 - GCS*C* (○); GCS*Pb* (●) and GCS*Zn* (▲) and on protein coupling: columns - GCS*C* (□); GCS*Pb* (■) and GCS*Zn* (■). Experimental conditions described in sections 2.4. and 2.6. Invertase free specific activity used was 2052 U/μg protein.

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

Anexos do Capítulo I

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*	
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].
* Central point.

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					
	GCS*C*	GCS*Pb*	GCS*Zn*	GCS*C*	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**	0.957979
Results of apparent water absorption						
	GCS*C*	GCS*Pb*	GCS*Zn*	GCS*C*	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	0.059616	**0.030599**	0.873689
(B) Results of apparent density						
	GCS*C*	GCS*Pb*	GCS*Zn*	GCS*C*	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						
	GCS*C*	GCS*Pb*	GCS*Zn*	GCS*C*	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	**0.0072228**	**0.017893**	**0.039861**

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

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*
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 ($p < 0.05$).

Figure 1

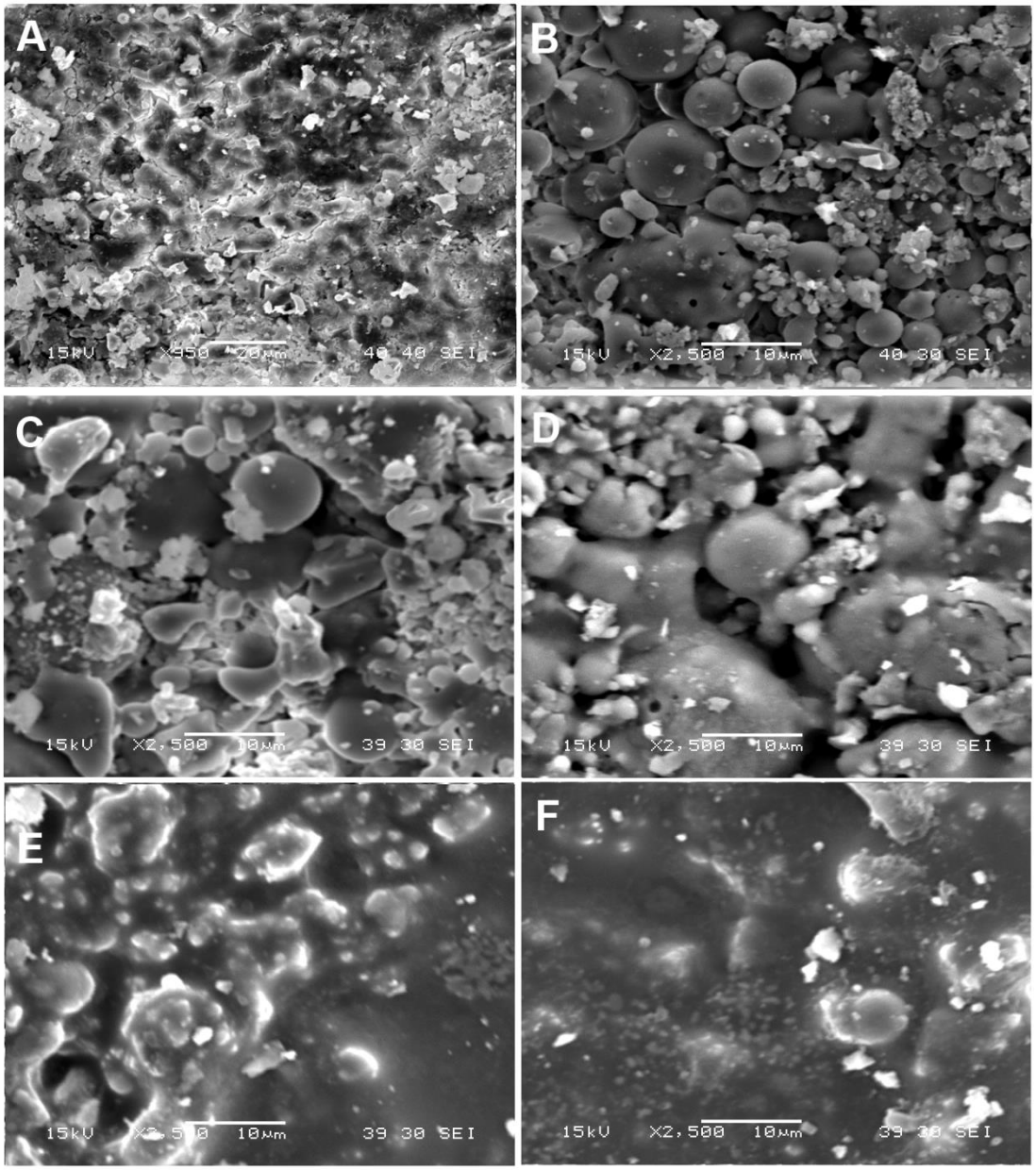

A
15kV X950 20µm 40 40 SEI
B
15kV X2,500 10µm 40 30 SEI
C
15kV X2,500 10µm 39 30 SEI
D
15kV X2,500 10µm 39 30 SEI
E
15kV X2,500 10µm 39 30 SEI
F
15kV X2,500 10µm 39 30 SEI

Figure 2

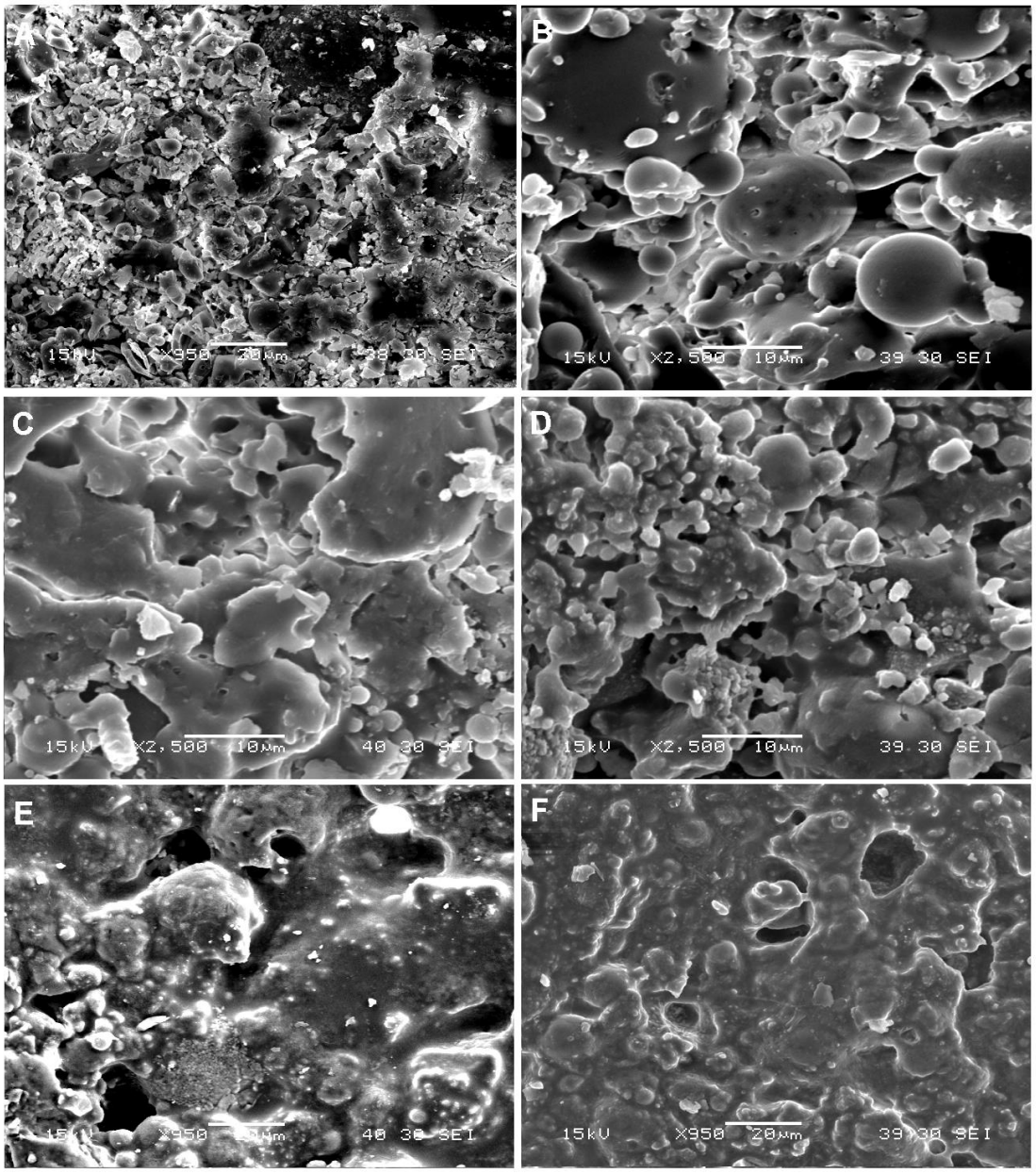

A
15kV X950 20µm 38 30 SEI
B
15kV X2,500 10µm 39 30 SEI
C
15kV X2,500 10µm 40 30 SEI
D
15kV X2,500 10µm 39 30 SEI
E
15kV X950 20µm 40 30 SEI
F
15kV X950 20µm 39 30 SEI

Figure 3

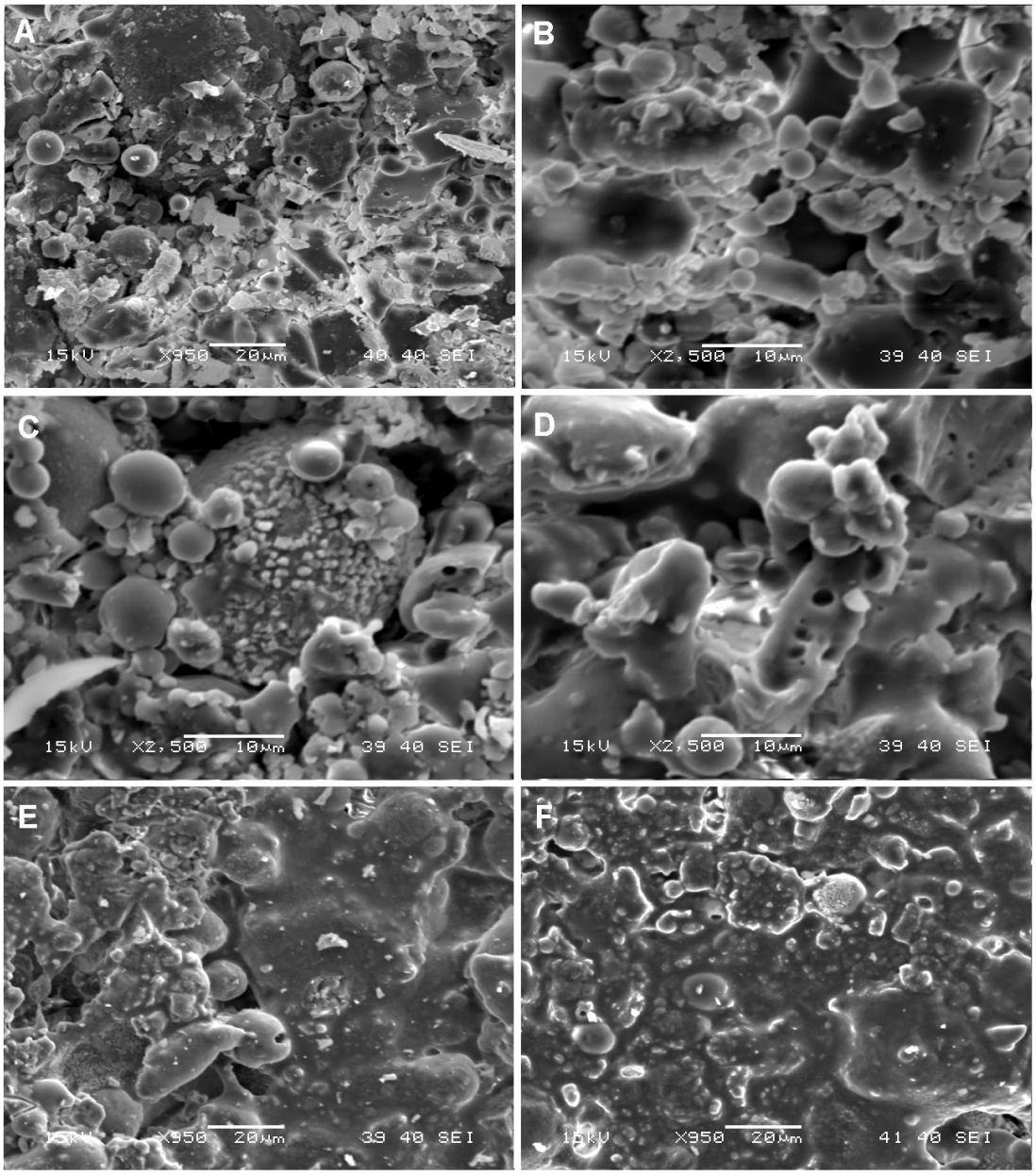

A
15kV X950 20µm 40 40 SEI
B
15kV X2,500 10µm 39 40 SEI
C
15kV X2,500 10µm 39 40 SEI
D
15kV X2,500 10µm 39 40 SEI
E
X950 20µm 39 40 SEI
F
15kV X950 20µm 41 40 SEI

Figure 4

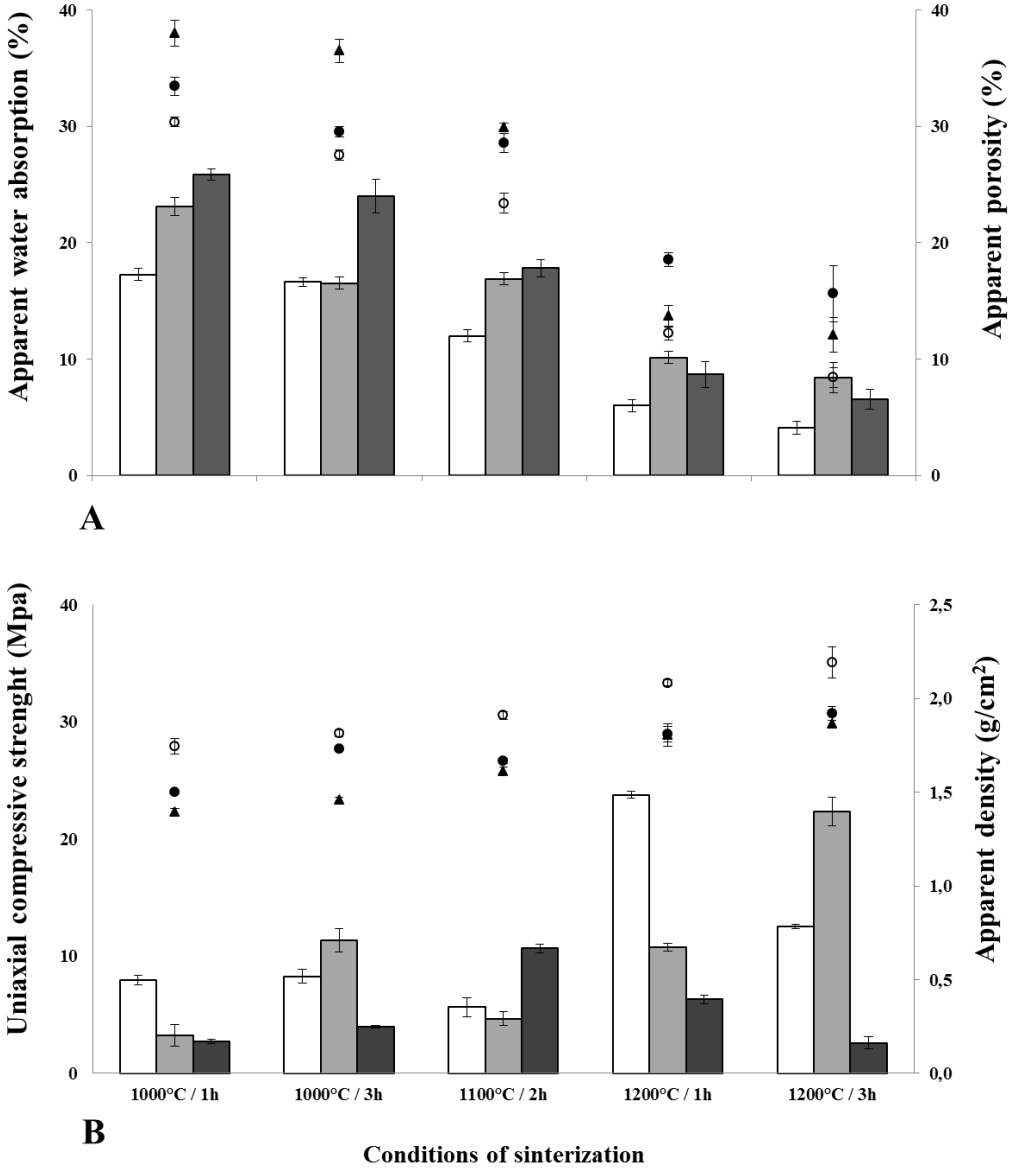

Apparent water absorption (%)
Apparent porosity (%)
A
Uniaxial compressive strenght (Mpa)
Apparent density (g/cm²)
1000°C / 1h
1000°C / 3h
1100°C / 2h
1200°C / 1h
1200°C / 3h
B
Conditions of sinterization

Figure 5

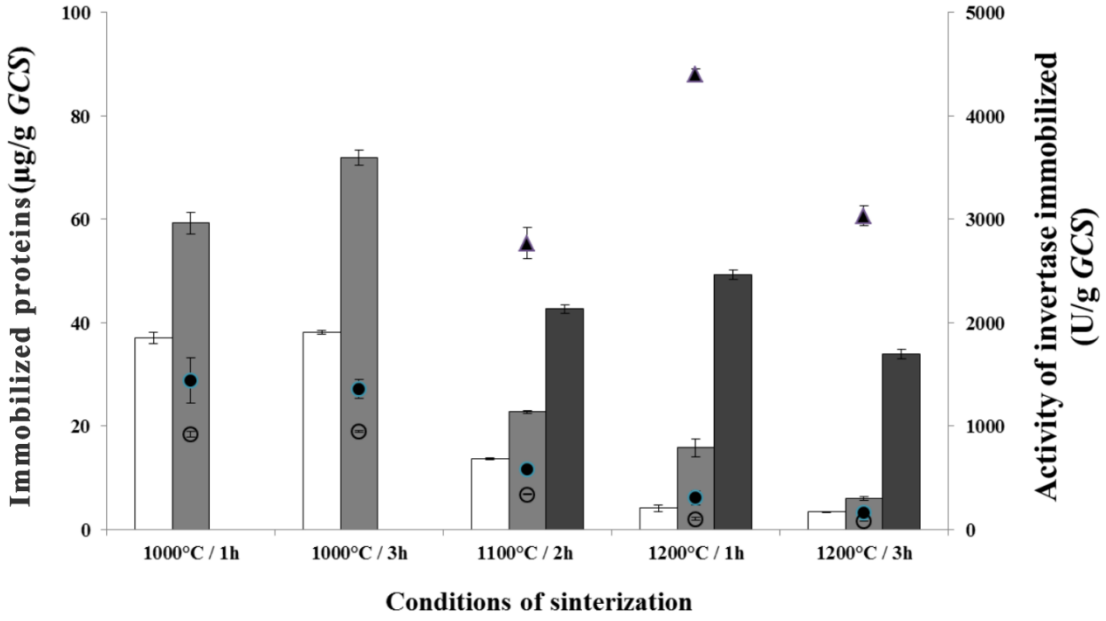
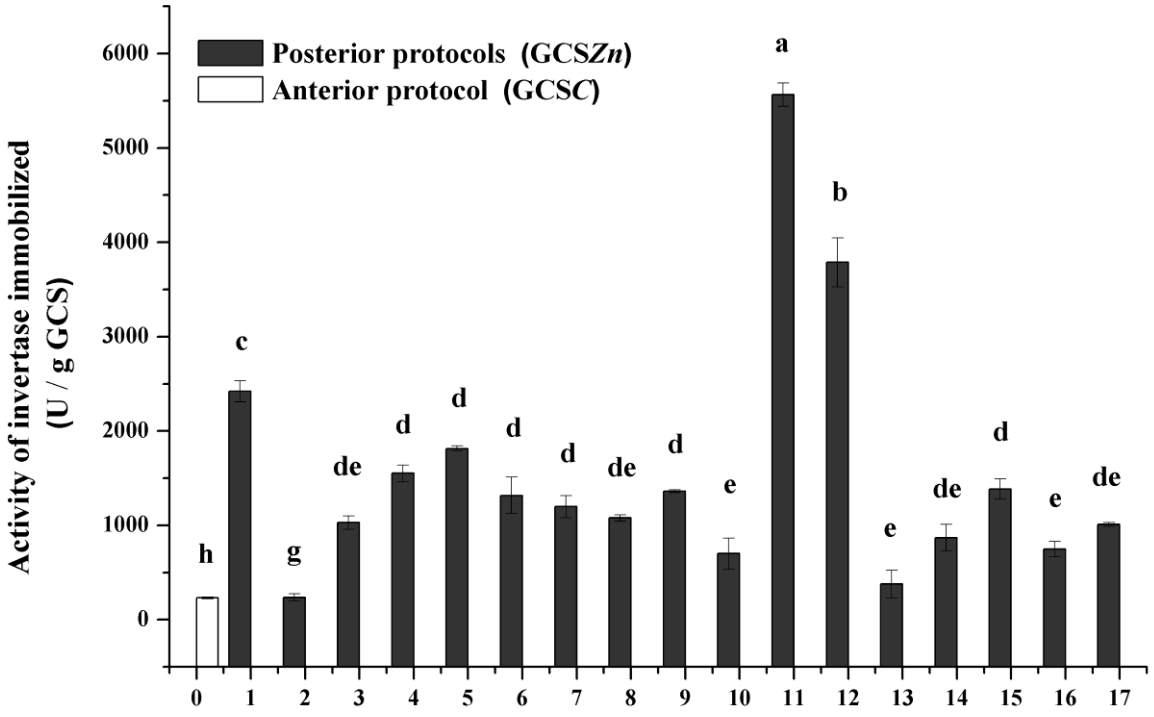

Immobilized proteins(µg/g GCS)
Activity of invertase immobilized (U/g GCS)
100
80
60
40
20
0
5000
4000
3000
2000
1000
0
1000°C / 1h
1000°C / 3h
1100°C / 2h
1200°C / 1h
1200°C / 3h
Conditions of sinterization

Figure 6


Posterior protocols (GCSZn)
Anterior protocol (GCSC)
Activity of invertase immobilized (U / g GCS)
6000
5000
4000
3000
2000
1000
0
h
c
g
de
d
d
d
d
de
d
e
a
b
e
de
d
e
de
0
1
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3
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6.2. CAPÍTULO II

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The new flow system approach in packed bed reactor applicable for immobilized enzyme

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Abstract

Several studies have been directed to development of reactors for chemical-biological reactions have been carried out successfully. "Dead zones" are areas where the substrate has non-contact with catalyst, and this situation in reactors has been seen as inconvenience in relation to productivity. Moreover, a catalyst immobilized in porous support also must be difficult. We have been obtained preliminary results in batch system of invertase immobilized on glass-ceramic support (GCS) (1000 °C / 3 h), were the optimum pH and temperature of free and immobilized enzyme was similar, 5.0 and 45 °C. The Apparent K_m for free invertase was 13.53 times higher than the free invertase. Aiming the work using packed bed reactors to study the activity of invertase in different flow systems, was obtained sigmoid curves using reactor 1 and 2, with the best result 4.75 U / g GCS and 3.09 U / g GCS, respectively. The lab-scale setups from flow system continuous, down-flow, up-flow and alternate-flow were studied. Alternate-flow is the alternation of down-flow and up-flow in previous time mentioned. This innovative flow system has facilitated contact among sucrose solution and immobilized enzyme to obtain the invert sugar production 24 h faster, due elimination of "dead zones" in the reactor in comparison to others flow systems.

Keywords: coal fly ashes, sintering, ceramic, immobilized enzyme, reactor, alternate-flow.

1. Introduction

Several studies have been directed to the development of reactors for catalysis to provide an efficient operation and significant increase of productivity process. These inventions respect to multidisciplinary areas such as biotechnology and hydrodynamic processes. The development of new types of reactors is widely interconnected mass transfer of the substrate to provide heterogeneous catalyst and consequently a decrease in diffusion effect. A significant number of studies designed to eliminate "dead zones" has been performed. In appropriate words, they are the areas where the substrate has not contacted the catalyst. Due to the complicated geometry and high mechanical strength of the ceramic supports, a reactor was proposed. The ceramics were used as a basis for design of a novel type of vortex reactor. It was used to improve substantially the mass transfer of substrate to the immobilized enzyme and eliminate "dead zones" and jet stream in the reactor [1]. Some authors reported problems of flow characterization in a reactor. For example, a high density commercial modules and high area per volume offered by hollow fiber modules is one of their main advantages. The tendency is thereby to minimize the fiber diameter and maximize their number in the bundle. For this reason, the modules offering the highest theoretical area per volume are also the tightest. The consequence is the occurrence of dead zones, back mixing, by passing and channeling, especially on the shell side, causing irregular mass transfer along the module [2]. But, specific experiments to evaluate sludge removal rates from the hollow fiber bundle showed the high capacity of aeration to remove sludge of this device without the occurrence of "dead zones" [3].

Other solution was presented to avoid dead from the bed is supported by a glass-sintered plate placed between the bottom head and the main part of the reactor. The bottom head is packed with glass spheres in order to improve feed distribution and avoid "dead volumes" [4]. Other reactor was investigated palm oil hydrolysis by lipase from *Candida rugosa* immobilized in a biphasic oil / aqueous hollow-fiber membrane, in order to optimize the flow regime in the membrane reactor at different flow rates in lumen and shell zones of the reactor [5].

It was mentioned that the up-flow circulates for sucrose hydrolysis in the reactor using immobilized invertase in compost from chitosan and sodium alginate as the standard for their experiments. Other parameters were studied for using in packed-bed reactors sophisticated with down-flow or up-flow due the differences in hydrodynamic and heat and mass transfer factors [6].

However, packed bed operation has some disadvantages, including "dead zones", channeling, and high pressure drop across the column. Glucoamylase was immobilized on ceramic support from carbonized mesostructured with porosity high to the reaction in packed bed reactor. It was obtained good results due this physical characteristic when the flow velocity was inferior [7].

When the density of the particulate material (e.g., silica aerogels) is less than the density the liquid, inverse fluidization can be applied to disperse the solid particles in liquid [8]. Results were found with these two types of flows in full-bed reactor, due to possible relationship between the chemical composition of support and the activity of the immobilized enzyme due to the formation of "dead zones" [9].

In present literature, there are not studies about the relation between porosity of support and flow system in packed bed reactor as important factor for enzymatic reactions. We have been used a coal fly ashes (CFA) to produce a glass-ceramic support (GCS) at high temperature, it is a nontoxic, compatible with adequate mechanical strength and 3-aminopropyltriethoxysilane-fuctionalization and well-established glutaraldehyde coupling process for most biotechnological applications [10]. And the novelty of our work was using a flow to facilitate of the sucrose solution circulation in contact with the enzyme (β-D-fructofuranidase EC 3.2.1.26) immobilized on the glass ceramic support inside the reactor in order to homogenize sucrose solution. In addition, batch system was carried out further studies on physic-chemical and kinetics for free and immobilized enzyme.

2. Material and methods

2.1. Material

Invertase (EC 3.2.1.26) was donated by Novozymes Latin America Ltda. (Araucaria, PR, Brazil). 3-Aminopropyltriethoxysilane (3-APTES), polyvinyl alcohol (PVA (MM 85,000)) and 3**,**5-dinitrosalicylicacid (DNS) were obtained from Sigma-Aldrich Chemicals Inc. (St. Louis, MO, USA). Glasses bead size 50−100 μm was obtained from Polysciences, Inc., USA. The glutaraldehyde, sucrose, toluene, sodium citrate, citric acid, 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. Chemical characterization CFA and investigation of GCS macro−microstructures

The coal fly ashes (CFA) were analyzed for chemical composition after dried at 110 °C followed by heating at 1000 °C / 2 h, using a Rigaku model RIX 3000, X-ray fluorescence spectroscopy (XRF) unit, according to Maheshwari et al. [11]. The main crystalline phases present in GCS were measured by Miniflex X-Ray Diffractometer (model RIX 3000, Rigaku Americas

Corporation). The detector was scanned over a range of 2θ angles from 19° to 28°, at a step size of 0.02° and a time of 2 s/step.

2.3. Preparation of glass−ceramic support (GCS)

Samples (65 %) 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 ± 2 °C for 12 h under air circulation. These samples were mixed with glass bead (25%) and PVA (0.1 M) using distilled water, homogenized and dry (2 h) at 70 °C for 12 h under air circulation. After cooling, the mixture was used to obtain green bodies in mould, followed the dimensions: 5.5 mm of height and 6 mm of diameter, and dry at 70 ± 2 °C for 12 h under air circulation. Then, the green bodies were submitted to the treatments of sintering: 1100 ºC / 3 h. The furnace was programmed with heating rate of 6 ± 2 °C/min to reach each sintering temperature. After, cooling (25 ± 2 °C) the glass−ceramic supports were stored in airtight containers.

2.4. Physical characterizations of macro−microstructures of GCS

The physical properties were estimated from apparent values of porosity (*Ap*), water absorption (*Awa*) and density (*Ad*) and they were obtained in the five conditions of sintering [12]. They were determined by measuring the dry mass (dry*m*), immersed mass (imm*m*) and 24 h saturated surface-dry mass (sat*m*). The *Ap* (%) Eq. 1, *Awa* (%) Eq. 2 and *Ad* (g/cm^3) Eq. 3 were obtained respectively by the following relations: using Archimedes' principle individual samples were calculated from:

$$Ap = [(satm - drym) / (satm - immm)] \times 100 \quad (1)$$

$$Awa = [(satm - drym) / drym] \times 100 \quad (2)$$

$$Ad = drym / (satm - immm) \quad (3)$$

Studies were done (10 samples) about the resistance to uniaxial compressive strength (2.13−21.28 MPa) of GCS samples in mechanical press (Ronald TOP Ltd. – the USA) with application speed of 0.77 mm/min.

Other studies of microstructures on the surfaces of sintered GCS sample were performed with scanning electron microscope (SEM) (JEOL Model JSM-5600 LV) at setting of 10 and 15 kV.

2.5. Immobilization of invertase on GCS samples

Invertase was immobilized as described by Suzuki et al [13]. The sintered GCS samples at 1100 °C for 3 h (0.20 g, 0.60 cm height, 0.65 cm diameter) was washed with distilled water and sterilized in an autoclave at 121 °C for 20 min to ensure support which is uncontaminated. After, they were submerged in 5 ml of toluene with 3-APTES (0.42 mM) to the period reaction at 85 °C for 6 h under agitation. Posteriorly, the treated samples were washed 3 times distilled water and dried at 100 °C for 1 h. After cooling, the samples were placed in 1 ml sodium phosphate buffer (10 mM, pH 7.5) with glutaraldehyde (0.38 mM) under vertical agitation (3 rpm) at 4 ° for 24 h. Then, the GCS*C* samples activated surface were washed with distilled water and in submerged in 1 ml of phosphate buffer containing invertase (7.17 mg) at 4 °C for 36 h under vertical agitation. After covalent coupling period, the immobilized derivatives (GCS-invertase) were washed 3 times with NaCl (10 mM) and stored in 100 mM sodium citrate buffer pH 4.5 at 4 °C. The amount of immobilized proteins on the surface of activated supports (µg proteins / g GCS) was estimated by measurement of free protein concentration [14] in the supernatant and washing solutions.

2.6. Activity of free and immobilized invertase in batch system

The free enzyme (5 µl containing 36.10 µg of proteins) or immobilized invertase (0.2 g) was incubated in in batch reactor containing 15% (w/v) sucrose solution prepared in 0.1 M sodium citrate buffer pH 5.0 at 25 ± 2 °C and under horizontal agitation (150 rpm). The final volume of reaction enzymatic mixture was 5 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 [15]. A unit of activity (U) was defined as being the amount of enzyme necessary to produce 1 mmol of reducing sugar per min in pH 5.0 at 25 ± 2 °C. The activities were expressed for U/µg proteins and U/µg proteins/g GCS, free and immobilized enzymes, respectively.

2.7. Determination of optimum pH and temperature

The effect of pH was studied at 25 ± 2 °C by varying the pH of the reaction mixture in the range of 3.0−6.0 and 7.0-9.0, 0.1M sodium citrate and sodium phosphate buffer, respectively. The optimum temperature was determined by performing the reaction in the temperature range of 25 ºC−85 ºC. The enzyme activity was represented as a percentage relative to initial activity, called relative activity.

2.8. Kinetic parameters for the invertase enzyme

In order to determine maximum velocity (V_{max}) of reaction and Michaelis–Menten constant (K_m) for free and immobilized invertase system, activity assay was applied for different concentrations of sucrose (29−584 mmol / dm^3). The apparent Michaelis–Menten constant (K_m) was calculated from Lineweaver–Burk plot, according to under condition optimum pH and temperature.

2.8. Packed bed reactor

The packed bed reactors in glass columns have the following dimensions, 63 cm height, 1 cm diameter and 7 cm height, 1 cm diameter, reactors 1 and 2, respectively. The experiments were carried out with the sucrose solution (500 ml, 15% (w / v) in sodium citrate buffer (0.1 M pH 5.0) with immobilized invertase (400 samples = 80 g) at 25 ± 2 °C in each experimental run).

It was carried out two experiments with reactors cited above. The first was studied different flow rates in relation to the enzymatic activity (0.03, 0.06, 0.12, 0.18, 0.24 and 0.30 l / h) using the flow system up-flow. Then the activity of invertase immobilized on GCS seen in better speed and reactor (1 or 2) was chosen to perform the last experiment. It was studied using the types of feeding of reactor the following: down-flow, up-flow and a novel type of feeding called alternate-flow. This type of feeding was used by alternating the two other types of flows in time determined as mentioned below (Fig. 1 and Table 1). The last experiment carried out in reactor observed the best result. The sample for measurement reducing sugars was collected in the container containing the sucrose solution which leaves for reactor (Fig. 1b).

All experiments were performed in triplicate.

3. Results and discussion

3.1. Physical properties of GCS

In previous studies, GCS samples were obtained from CFA and glass beads with different formulations using organic additives (PVA, carboxymethyl-cellulose) uniaxially pressed (255 MPa) or molded. However, the physical and mechanical properties of formulation were chosen with PVA and the green body (molded) obtained a greater preservation of glass beads added and cenospheres, with a regular size and distribution of pores compared to others samples. This initial result in present work was essential to its continuation due these characteristics. Thus the respective summary of physical properties and chemical composition glass−ceramic support is presented in

Table 2 and 3. The parameters such as porosity and water absorption apparent are much more studied when compared with other studies [8, 16].

3.2. SEM and XRD

The microstructural studies of CGS samples were performed using SEM and they are shown in Fig. 2. The samples present qualitatively characteristics that may explain the dense glassy phase formation. There are larger pores and cracks with evident micro and macropores. The dispersion of crystal and amorphous materials, used as addictive (PVA and glass bead), caused some melting regions increasing the nucleation and cenospheres deformation, which influenced in the physical properties. The binders commonly used in spray-drying are water-soluble polymers such as polyvinyl alcohol (PVA), cellulose, polyethylene glycol (PEG), and polyacrylate. Thus the selection criteria for the binder are based on its ability to form granules that readily deform during compaction, to burn-out cleanly before sintering, and to give a high compact density and strength [17]. In addition, small particles of PVA may be joined to other products in CFA and formed small clusters, which in turn is interconnected to other cores to form true internal channels in the ceramic. But, Fig. 2A shows tridimensional structures with characteristics such as cenospheres and glass, probably because of the drying of excipients (additives), cited above, and sintering time/temperature used. And in Fig. 2B cracks are shown in both the glass spheres and cenospheres because the effect provided by the treatment in the green bodies.

Analyses of X-ray diffraction for the samples of GCS to study crystalline phases are shown in Fig. 3. This result concerns to the GCS (black-line). However, the sample glass−ceramics for the peak at (2θ) 20.89°, 26.13° and 26.91° corresponding to the reflection of quartz (SiO_2), 25.02° to

mullite ($Al_6Si_2O_8$) and uncommon result is found to large phase called alunogen ($Al_2(SO_4)_3$. in 21.81° and 24.02°. They were similarly found in the same intensity corresponding stages of the analysis in alunogen found in clay [18] when the acid leaching of malachite in synthetic mixtures of clay and zeolite-rich gangue. According to methodology employed (1100 °C/ 3h) and principal material resource (coal fly ashes) must not a high temperature, for this, they are evidence about crystallization due the alunogen presence [19].

3.3. Immobilization of invertase

The development of methodology for enzyme immobilization is conceived as one of the most important experiments to be performed. Usually, a simple test has been performed to verify

the enzyme coupling — analyses the "wash solution" of derivative. Therefore, analyses of X-ray diffraction for the derivatives still are shown in Fig. 3. There is an interference level very accentuated due to intercalation to the enzyme immobilization into GCS between 19° and 27°. The order to follow is: GCS-functionalized by 3-APTES and glutaraldehyde (light grey−line), GCS-functionalized by 3-APTES and glutaraldehyde with enzyme coupling (grey−line).

The initial experiments were tested with concentrations of glutaraldehyde, 3-APTES with some non-aqueous solvents. Then, it have been tested the concentration of 3-APTES and glutaraldehyde−enzyme to bind to the ceramic (not shown). The efficiency of immobilization of this enzyme was reached under conditions where the salinization reaction was performed in an aqueous solution and cross-linking with the bi-functional agent (glutaraldehyde), used in at 2%. Solution analyses to wash of supports have been showed that the immobilization process was irreversible, when invertase activity was not verified. The amount of immobilized enzyme was 9.76 µg / g GCS. This amount was determined according to the quantification of protein solution in relation to the main protein solution that did not bind to the support and the effluent obtained from washing in the derivative. This small quantity of proteins bond can be partially attributed to the incomplete activation of the amino groups of glutaraldehyde in support, due to diffusion limitations during the activation step [20]. A study of protocols in respect of chemicals (glutaraldehyde and 3-APTES) and biological (proteins) for greater binding of proteins in different sizes of media in the future should be done. Thus, a greater surface area will be implemented in this ceramic. However, after the tenth reuse immobilized invertase activity was around 57%, and its half-life in relation to complete inactivation rate was compromised from one year after the coupling process of the enzyme.

However, some studies have been showed good results as Sanjay and Sugunan [21] used montmorillonite (10 mg proteins / g support), Amaya-Delgado et al. [22] used nylon-6 micro beads (4.95 mg proteins / g support) and Akgöl et al. [23] used magnetic microspheres of PVA (7.18 mg proteins / g support). However, the number of reuses of the immobilized invertase in GCS to hydrolysis of solution sucrose and other studies suggest checking for further understand the relation between amount of protein and support.

3.4. Effect of temperature and pH

Proteins are very unstable when exposed to environmental conditions significantly different from those found in natural conditions. The effect of temperature on the situation of the catalytic activity of free and immobilized invertase was studied in 0.1 M sodium citrate buffer pH 4.5 (25 ± 2 °C) at 25−85 °C (Fig. 4A). The result of the curve shows maximum activity at 45 °C for both free

and immobilized invertase. However, the activity of invertase immobilized showed strong temperature dependence at temperatures below with decreased activity showed critical temperature dependence at temperatures above the optimum temperature. Some authors observed temperature dependence in the activity of invertase immobilized due to varying physical and chemical properties of the enzyme when covalently bound to inorganic supports [23, 24, 25].

Moreover, high temperatures can be an additional cost to achieve industrial-scale or optimum activity may not be the best alternative because this also allows the natural enzyme denaturation**.** Bayramoğlu et al. [26] cited the process of immobilization via amino groups cannot be deteriorative to the structure and conformational flexibility of the enzyme and its own organization to connect to the substrate and impending natural causes due to denaturation temperature employed. Often, enzymes are tested in their optimal pH for appreciable rate of reaction to happen. The pH effect of both free and immobilized invertase was estimated in 3.0−9.0 (Fig. 4B).

The result of the curve also was obtained as the optimum value for free and immobilized invertase at pH 5.0. This result supports the evidence that the system of detention was not as detrimental to the enzyme. The dependence pH activity is to profile the immobilized invertase is expansive to 4.0-6.0 pH. Then, this expansion is possible due to stabilization of invertase molecules as a consequence of multipoint connections on the surface of the ceramic due to the immobilization process [25].

3.5. Kinetic properties

The kinetic parameters of enzymatic reaction can be estimated by direct line of Lineweaver−Burk of the initial rate of hydrolysis of sucrose of the experiment. The result is given by two straight lines as the Michaelis−Menten equation for the reaction. The apparent Michaelis constant (K_m) and V_{max} for free invertase and invertase immobilized invertase were 5.90 ± 0.00 x 10^{-3}mM and 4.11 ± 0.00 x 10^{-3}mmol / min and 7.99 ± 0.01 x 10^{-2}mM and 6.34 ± 0.13 x 10^{-1}mmol / min, respectively. The apparent K_m for free invertase was 13.53 times higher than the free invertase.

However, V_{max} for immobilized invertase is 150 times higher than V_{max} for free invertase. This is an uncommon result, because normally a decreasing on V_{max} for an immobilized enzyme would be expected. According to Tomotani and Vitolo [27], probably, the extra stabilization of supra-molecular aggregates (hexamers and octamers, mainly) acquired through the coupling covalent on GCS, favored the V_{max} . Or greater distribution throughout the ceramic even with the small amount bound proteins. The results obtained can be an indicating to use in packed bed reactor according to circulation of sucrose solution concentration.

For hydrolysis of sucrose with invertase immobilized K_m and V_{max} values were not increased and decreased, respectively. Generally, K_m and V_{max} values of invertase are free are minor and major, respectively. This may be an indication that there was a structural modification of the enzyme after the immobilization process. Amaya-Delgado et al. [22] used nylon-6 microbeads showed an apparent K_m of 1.2 times greater than V_{max} of invertase immobilized similar results were obtained free invertase. The formation of enzyme−substrate complex is more difficult with the invertase immobilized due to porous structure of the support [10,28]. Alternatively, the irregular porosity of ceramics must proportionate an uncommon contact to substrate−enzyme complex. At present, the complex becomes easier to led to the increase in affinity for the substrate and therefore a low K_m value compared with other studies [7,21,23,29].

3.6. Operational stability of invertase immobilized in packed bed reactors, in different flow systems

The proposal to compare the effect of flow rate on invertase activity using the reactors 1 and 2, to verify the best sucrose hydrolysis with the change of flow rate runs with two sizes of reactors. It was obtained sigmoid curves and they are shown in Fig. 5. But, all flow rate, the activities of immobilized invertase were superior using reactor 2.

The physical properties of glass-ceramic support as water absorption and porosity apparent must has influence to obtain this result in the relation to the parameter reactors height / diameter (reactor 1 equivalent to 21:1 and reactor 2 equivalent to 7:1). This may be an indication that gravity must have been an important factor for improved performance in the productivity of invert sugar has been using the reactor 2.

One way the pressure of the flow to enter interior has been facilitated to the sucrose solution to circulate derivative (ceramic / enzyme). The GCS sample obtained a porous structure the enzyme apparently does not suffer influence in the reaction, meaning that the substrate is converted only to a small extent on the support surface. However, the value of the activity was higher in the reactor, probably due to a higher pressure at the entrance of a sucrose solution in a smaller diameter. In addition, the fraction of the sucrose inversion was relatively higher in the long residence time and high effective mass transfer inside the system. This phenomenon is consistent with other results [20]. Other result can be cited in relation this effect when glucoamylase was immobilized in a ceramic support from carbonized mesostructured with porosity high to the reaction in a particular reaction residence time. In other words, the higher velocity flow, the sucrose solution penetrates inside the support, but nonetheless the reaction is reduced [7].

For economic proposition for industrial scale production invert sugar, it is usually preferable to implement the continuous feed flow system. An innovative flow type that we tested type of flow,

which is used both in alternate times. This type of flow is called alternate-flow due the alternation (down-flow and up-flow). Using this flow system may have been a destabilization or elimination of “dead zones”. The results are shown in Fig. 6. Sucrose hydrolysis total (100%) was measured in the alternate-flow 24 h in operational stability, was 16 h later when compared with other type of flow studied (down-flow and up-flow). This can be explained in terms of diffusional resistance and inhibition of by-products or inefficiency in the internal distribution of sucrose solution in reactor using only one type of flow. Then, the innovative flow system facilities the contact among solution sucrose and enzyme immobilized through improved efficiency to obtain inverted sugar for the ‘‘dead zones” elimination in reactor. However, this elimination also can be obtained the “micro-dead zones” elimination in interior of ceramics due their irregular porosity. The alternate-flow allows a substrate circulation through areas which offer greater resistance, and the liquid tends to flow along the wall reactor (lower resistance) and not completely through in packed bed reactor [9]. These observations should also be applied to ceramic due their physical characteristics.

A research carried out involving continuous flow rotary circular or spiral of the solution inside the reactor-vortex reactor. The first named rotor inertial (disposal of the reactor in order horizontal), and the other reactor is immersed (disposal of the reactor in order vertical – relation height / diameter was 10 / 1). Both reactors were respectively 1.2 and 1.5 times better than the reactor of the fixed bed reactor. The use of these reactors were to eliminate "dead zones" and thus obtain better performance, and the relation to the size of molecular weight of the substrate or porosity of the support [1].

4. Conclusion

First, in relation the GCS, obtained using coal fly ashes with PVA, was successfully used as a support for immobilization of invertase by covalent linkage. This can be a viable alternative to reduce environmental pollution by coal fly ashes.

Second, according to the result obtained with the innovative type of flow, the production of invert sugar maybe performed on larger scales in relation to what was used in this study because without the need for greater investments in reactor technology. Another aspect to this technology is to add value to process sugar inverted production from sugar cane juice, and we still can highlight the possibility of using other enzymes or microorganisms of industrial interest.

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Table captions

Figure captions

Fig.1 Scheme of reactor: (a) reactor; (b) container with the solution of sucrose; (c) peristaltic pump, (e) circulation of water for temperature control; (f) GCS-invertase, (h) down-flow; (g) up-flow.

Fig.2 Microphotographs of the ceramics (GCS) from coal fly ash (CFA) sinterization.

Fig.3 X-ray diffraction (XRD) to demonstrate the crystalline phases, glass-ceramic support sample from coal fly ashes (CFA) sintering for the immobilization of invertase process. (black-line) GCS: mullite (Δ), quartz (●), aluminum sulfate (□); (light grey-line) GCS functionalized with 3-APTES and glutaraldehyde; (grey-line) 7.17 mg immobilized protein/g GCS.

Fig.4 Effect of pH and temperature for free enzyme (○) and immobilized (●) in GCS.

Fig.5 Dependence of sucrose hydrolysis rate on the flow different rates for the reactors 1 (○) and 2 (●). Experimental conditions described in Section 2.8.

Fig.6 Operational stability of immobilized invertase in packed bed reactors, in different flows system such as: up-flow (○), down-flow (●), and alternate-flow (▲). Experimental conditions are described in Section in 2.8.

Anexos do Capítulo II

Table 1

Types of flows.

Time (h)	Up-flow	Down-flow	Alternate-flow
1	A	B	A
2	A	B	B
3	A	B	A
4	A	B	B
8	A	B	A
16	A	B	B
24	A	B	A
32	A	B	
40	A	B	
48	A	B	

Table 2
Physical properties of GCS.

Analyses	Treatment 1200 °C/3 h
Mass green body (g)	0.29 ± 0.00
Mass dried (g)	0.20 ± 0.00
Shrinkage (%)	31.03 ± 0.00
Height (cm)	0.65 ± 0.02
Volume (cm^3)	0.19 ± 0.02
Diameter (cm)	0.60 ± 0.00
Apparent porosity (%)	32.15 ± 0.03
Apparent water absorption (%)	23.90 ± 0.01
Apparent density (g/cm^3)	1.47 ± 0.02
Uniaxial compressive strength (MPa)	8.30

Table 3
Chemical composition of glass–ceramic support from CFA by XRF (%).

SiO_2	FeO_3	Al_2O_3	CaO	K_2O	TiO_2	SO_3	P_2O_5	MgO	SrO
58.2	16.2	10.2	7.5	3.2	1.4	0.1	1.1	0.4	0.1

Figure 1

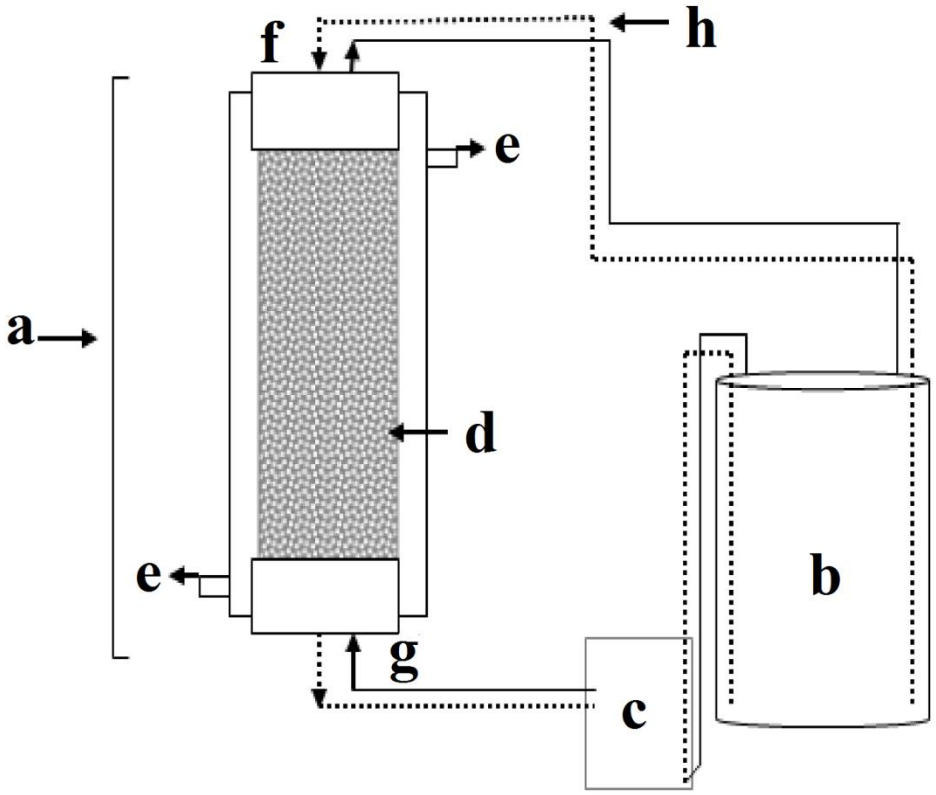


Figure 2

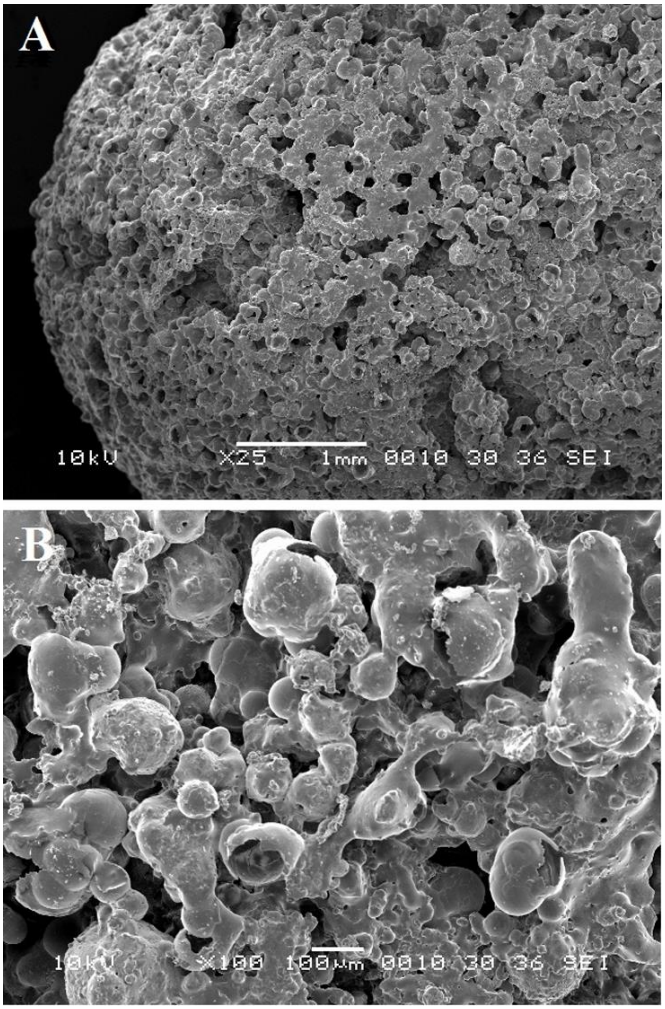


Figure 3

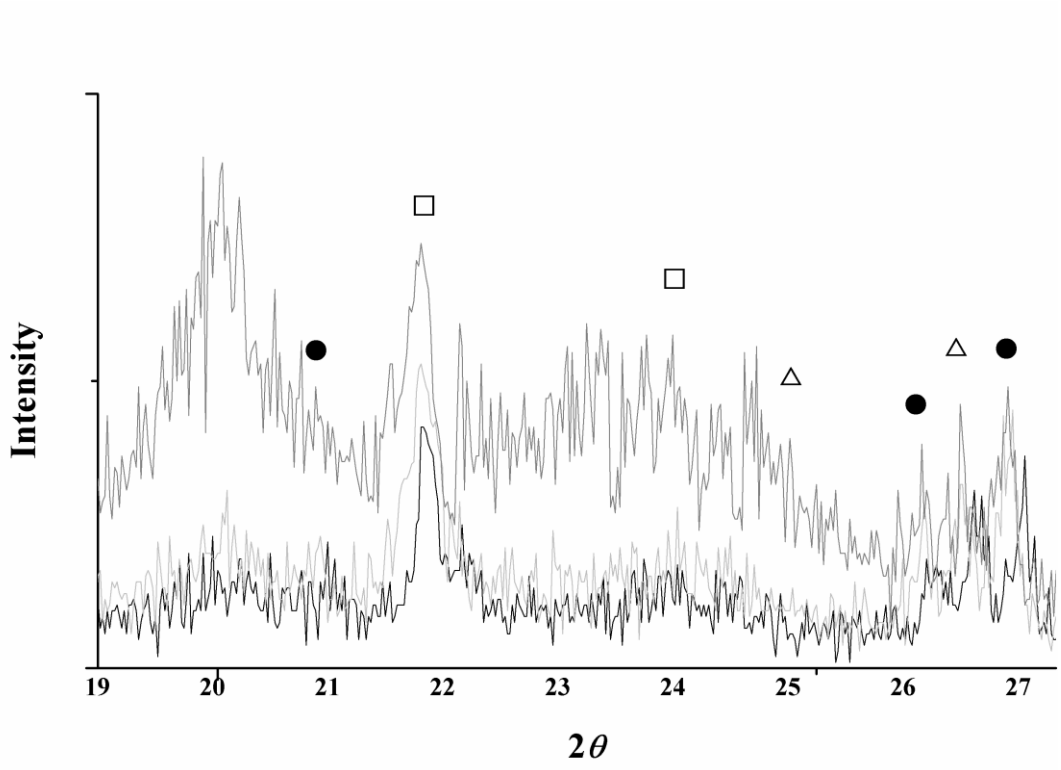


Figure 4

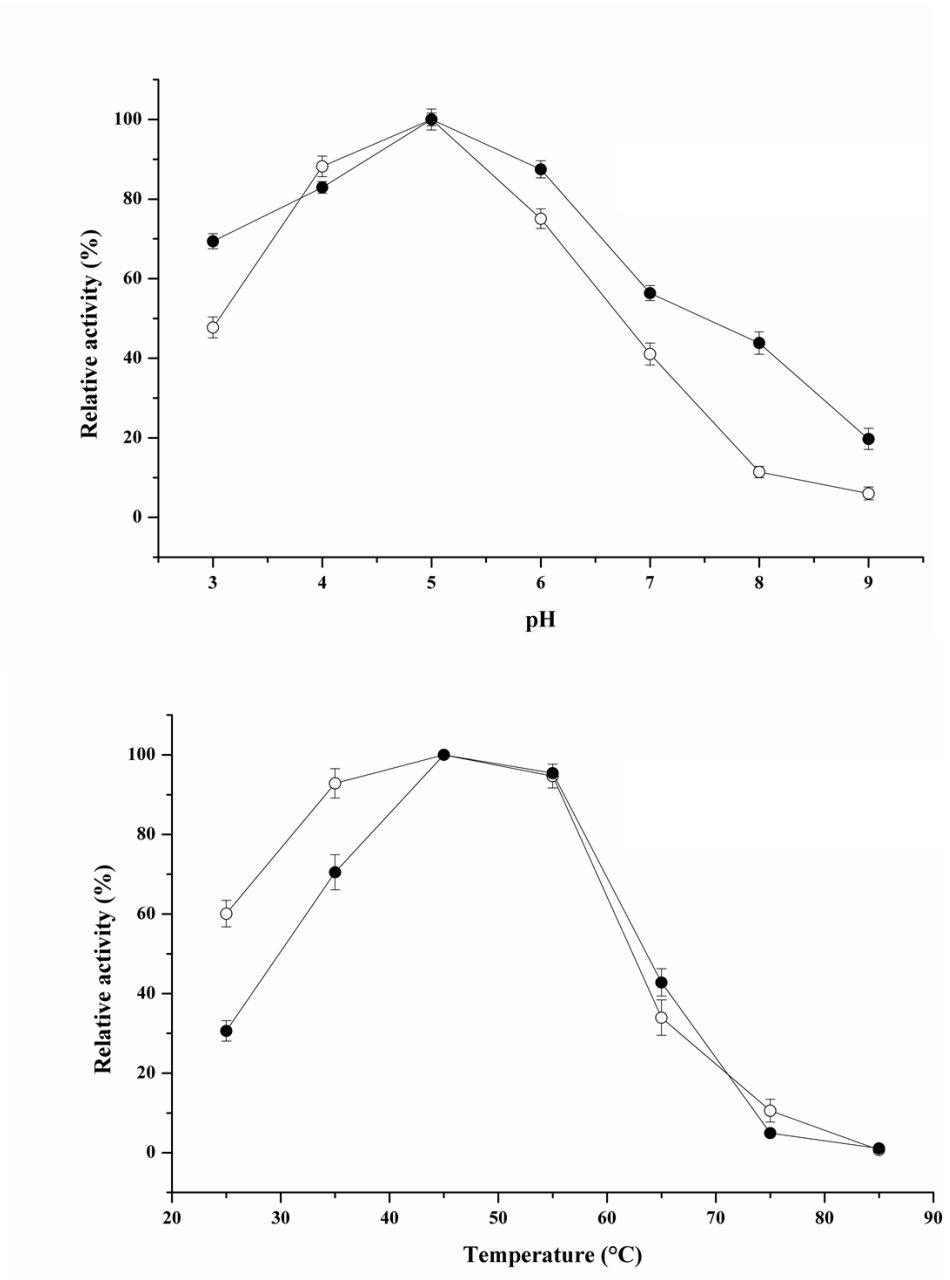

Relative activity (%)
100
80
60
40
20
0
3
4
5
6
7
8
9
pH
Relative activity (%)
100
80
60
40
20
0
20
30
40
50
60
70
80
90
Temperature (°C)

Figure 5

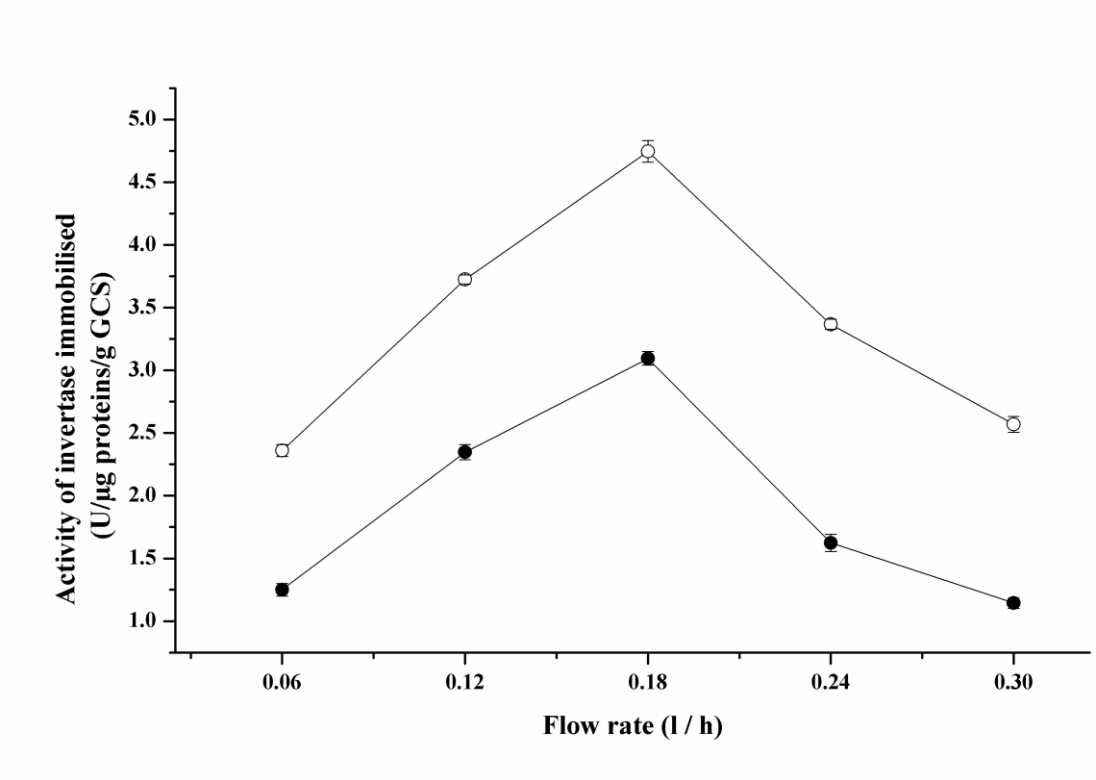
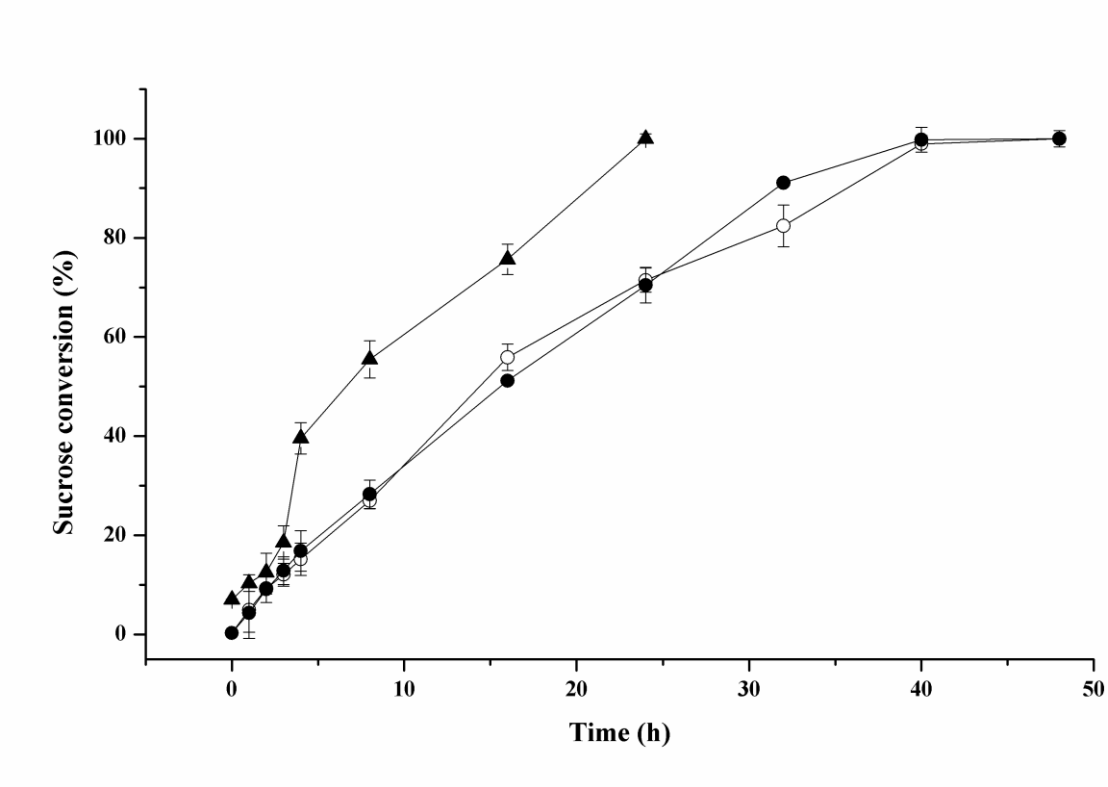

Activity of invertase immobilised
(U/µg proteins/g GCS)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.06
0.12
0.18
0.24
0.30
Flow rate (l / h)

Figure 6



6.3. CAPÍTULO III

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Immobilized invertase studies on glass–ceramic support from coal fly ashes

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Abstract

Invertase was covalently immobilized on new coal fly ashes glass-ceramic support with zinc sulfate (GCSZn). The coupling process of proteins was demonstrated by X-ray diffraction (XRD). There was no change in the optimum pH (4.6) but optimum temperature increased from 55 °C for free invertase to 60 °C for immobilized derivative. The activation energy decreased after immobilization (37.31 ±3.40 kJ/mol) in spite of free invertase (51.34 ± 5.21 kJ/mol). There was an improvement in the Michaelis–Menten constant for sucrose hydrolysis after immobilization being 15 times lower compared to that for free invertase (0.30 ± 0.01 mmol). After ten reuses at 25 ± 2 °C, the immobilized invertase lost only 9% of initial activity, but at the optimum temperature (60 °C), the activity decrease was about 70%, what it is economically feasible under energetic view point for industrial application.

1. Introduction

Invertase, b-fructofuranosidase (EC 3.2.1.26), catalyses sucrose hydrolysis from non-reducing terminal of the b-fructofuranoside residues producing glucose and fructose (invert sugars) [1,2]. The invert sugars syrup is very appreciated by food industry because its sweetness is greater than its own in 20% sucrose, increase of water retention leading to lesser effect of crystallization and viscosity [3]. The common procedure to hydrolyze sucrose is carried out using acids at high temperature that causes dark coloration at the end of process due to by-products denominated of furfurals what can be danger for the health when ingested in the foods and drinks. Immobilized invertase would be a new alternative for production at mild conditions of invert sugars syrup free of furfurals. Several types of supports have been described for immobilization of invertase: montmorillonite by covalent bond [4], resin by adsorption [2], gelatin hydro gel by entrapment [5] and various types of biopolymers, such as corn grifts, gelatin, carbohydrate moieties, porous cellulose beads, and rice husk. Hydrogel polymers have good chemical and mechanical stability, and are not susceptible to microbial attack [6]. Some uncommon supports have been used such as conducting polymer of 1-(4-nitrophenyl)-2,5-di(2- thienyl)-1H-pyrrole [7]. But these supports are sensitive to physic or chemical environment such as organic solvent or phosphate presence, high temperature or pressure.

On the other hand, the use of solid support has been gathering interest recently because of its advantages in mechanical strength and chemical stability. According to de Lathouder et al. [8] enzymatic carriers selection must meet certain criteria: the support must have a high adsorption capacity and hold the enzyme firmly on the surface with high activity and operational stability.

In previous work, Albertini et al. [9] studied the invertase immobilization on different glass–ceramic support to produce the best invertase immobilized derivative.

In this work, the physic-chemical parameters, i.e. optimal temperature and pH, thermal and operational stabilities, Michaelis–Menten kinetics, SEM, EDS and XRD were evaluated.

2. Materials and methods

2.1. Materials

Baker's yeast invertase (EC 3.2.1.26) was donated from Novozymes Latin America Ltda. (Araucaria, PR, Brazil). 3-Aminopropyltriethoxysilane (3-APTS) was obtained from Sigma–Aldrich Chemicals Inc. (St. Louis, MO, USA). All other chemicals were of highest purity

commercially available. For this study, the coal fly ashes glass–ceramic support with zinc sulfate (GCS*Zn*) was prepared and sintered to 1200 °C for 1 h as described by Albertini et al. [9].

2.2. Analysis of CGS*Zn using SEM, EDS and XRD*

Microstructural analysis on the surfaces of sintered GCS*Zn* samples was performed using a scanning electron microscope (SEM) (JEOL Model JSM-5600 LV) at setting of 15 kV. The Archimedes' principle was used to determine the apparent porosity, water absorption and density. The resistance of the GCS*Zn* to uniaxial compressive strength was performed according to Albertini et al. [9]. Thus, the respective summary of physical characterization of the CGS*Zn* is presented in Table 1.The main crystalline phases present in the GCS*Zn* were measured by 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 step size of 0.02° and time intervals of 2 s/step. The phases of the GCS*Zn* samples were analyzed by energy dispersive spectroscopy coupled to a scanning electron microscope (SEM-FEI, Quanta Inspect 200, EDSEDAX PV 9900), which has been used as qualitative way for obtaining the chemical constituents in ceramic samples.

2.3. Immobilization of invertase and enzymatic activity

Invertase was coupled on the GCS*Zn* by covalent bond using the best economical protocol number 11 (0.636 mM 3-APTS; 4 h of silanization silanization; 0.576 mM glutaraldehyde; 4 h activation reaction; 7.73 mg/ml proteins and 18 h covalent coupling – CFIMPS). After covalent coupling period, the immobilized derivatives were washed 3 times with NaCl (10 mM) and stored in water (ultra pure) at 4 °C. Hydrolytic activity of free and immobilized invertase also performed according to Albertini et al. [9]. Sample of the GCS*Zn*-invertase derivative was added to 0.9 ml of sucrose (5%, w/v) prepared in 25 mM sodium acetate buffer (pH 5.0) and 0.1 ml of buffer to keep a final volume of 1.0 ml. This activity measurement was carried out using a conical shape batch bioreactor (25 mm diameter and 50 mm height) under controlled agitation (150 rpm). Whereas, 0.1 ml of free enzyme preparation (26 μg of proteins) was added to 0.9 ml of substrate (final volume = 1.0 ml). The reducing sugars produced were analyzed by DNS method [10]. One unit of activity (U) was defined as being the amount of enzyme necessary to hydrolyze 1 mmol of sucrose in reducing sugars per minute in pH 5.0 at 25 ± 2 °C. The specific activity of free and immobilized enzymes was expressed as U/μg proteins and U/μg proteins/g GCS*Zn*, respectively.

2.4. Determination of optimal pH and temperature

The effect of pH was studied at 25±2 °C varying the pH of the reaction mixture in the range of 3.2–5.4 (25 mM acetate buffer). The optimum temperature was determined in the range of 25–65 °C. The enzymatic reaction conditions were described in Section 2.3. Relative activity was adopted and represented as a percentage in relation to the initial activity.

2.5. Kinetic parameters K_m and V_{max}

In order to determine maximum velocity (V_{max}) of reaction and Michaelis–Menten constant (Km) for free and CGSZn-invertase derivative were used different concentrations of sucrose (13 to 262 mmol/dm^3) at optimal pH and temperature. The apparent Michaelis–Menten constant (K_m) was calculated from Lineweaver–Burk plot.

2.6. Reuses of immobilized invertase at different temperatures

The reuses of immobilized derivative (GCS*Zn*-invertase) were tested at temperature range from 25 °C up to 65 °C. The invertase activity retention was calculated from the initial activity value. The samples were washed three times with ultra pure water under mechanical agitation and drying using sterile absorbent paper.

All experiments were performed in triplicate.

3. Results and discussion

3.1. GCS*Zn analysis using SEM, SEM-EDS and XRD*

In Fig. 1 are showed the SEM micrographs and EDS analysis results of the sample GCS*Zn* for some selected regions, indicated by black circles.

In Fig. 1A and B, it can be noted similar characteristics to the powder particles used as initial raw material before and after the addition of polymer (organic binder) characterizing regions slightly agglomerated. The initial thermal decomposition of the organic binder is mainly due to degradation reactions that occur via inter or intra-chains rearrangements releasing volatile compounds generating microcracks, micropores and crystalline or amorphous phases. However, these phases are not homogeneous because the agglomerations found on the full extent of the GCS*Zn* surface. Maybe this occurs due to the bad distribution of granules formed from the mixture

(CFA, excipients), inefficiency for reaching themelting temperature and subsequent nucleation region of the material. In the images, it was observed the presence of cenospheres structures that are typically present in CFA, its appearance and size (11.5 μm) are still perceptible in accordance with the procedure used for sintering (1200 °C for 1 h) (Fig. 1A). The internal surface of the fractured GCSZn sample was observed in the Fig. 1B, which presents polished irregular internal surfaces and micropores 2.7 times larger than those found in the glass–ceramic support without zinc (90.88 ± 0.54μm^2). The systematic increase GCSZn micropores can be attributed to the large amount of structure irregularities that can lead to possibility of more inter water absorption what will beneficially affect the process of immobilization of the enzyme.

According to Gregerova and Všianský [11] cracks in the ceramic samples may promote appropriating penetration of water and salt solution because occurring a pressure crystallization of salts by filling in the cracks.

The EDS analysis results of GCS*Zn* sample are showed in Fig. 1C and D. Mapping of intact and fractured surface revealed a non-uniform distribution of chemical elements. The black-circles 2 and 3 were from a simultaneous analysis, while the black-circle 1 was from unique analysis. In this order of presentation of these results, distinct rich particles were observed mainly Al, Si and Fe. The amount of zinc was not significant in this analysis to be expressed because its volatilization at the high temperatures, but it can be the differential for this new glass ceramic support. However, there may be a synergistic action to form the irregular porosity on the entire ceramic sample between zinc and organic binder. The absence of mechanical strength of sintered CGS*Zn* sample at 1000 °C 1 h and 3 h, can be attributed to the disappearance of the organic binder and/or volatilization of zinc [9].

The results of the CGS*Zn* analysis by X-ray diffraction are presented in the Fig. 2. The peaks at 2θ = 21.0°, 26.0° and 26.8° correspond to the reflection of quartz (SiO_2). However, the GCS*Zn* presents other crystalline phases such as mullite ($Al_6Si_2O_8$) (26.5°) and large phase of alunogen ($Al_2(SO_4)_3.17(H_2O)$) (22° and 24.3°) (Fig. 2A). This last chemical compound has been cited as product of interaction between inorganic elements and organic sulphur during low-temperature ashing, or it may have been formed in the outcropping coals by processes associated with weathering [12,13]. The mullite and alunogen presences can be justified based on the report about studies of surficial environments, which showed that the salts found on mine wastes, tailings piles, and outcrops, minerals including heavy metals have been detected in solid solutions and some of them are highly soluble. Also, it has been shown that crystalline alunogen presence can be attributed to the weathering of metal-sulfide minerals, typically secondary Al-sulfates are amorphous [14,15].

After GCSZ*n* silanization using 3-APTES, comparing with CGSZ*n* control (Fig. 1A), it can be observed at 2h: more one peak between 16° and 17°; the peaks from 19.5° to 24.5° and 26°–27° partially increased, confirming the efficiency of the APTS treatment (Fig. 2B).

After covalent reaction with the bi-functional agent, glutaraldehyde, there was a small decrease in the peaks in 2θ = 25.5°–27.5° in spite of the Fig. 2B, and an increase of the peaks between 19°and 25° (Fig. 2C).

However, the enzyme coupling using different amount of proteins on GCSZ*n* samples (2.58, 5.15 and 7.73 mg of proteins per g GCSZ*n*) showed profile changes between 15° and 27°, being more affected with the highest protein concentration. These results showed the best amount of protein for immobilization and the protein excess agglomeration on the support surface (Fig. 2D–F). These results were similar to those reported for montmorillonite [4].

3.2. Effects of pH and temperature

The pH effect on both free and immobilized invertase activities was studied from pH 3.8 up to 5.4 as showed in Fig. 3. The optimum pH 4.6 was found for both enzymatic preparations but the plot profiles were different showing that the free enzyme was more stable at the pH range tested while the immobilized derivative showed low activity at the pH lower and higher than optimum value.

This adverse activity of the immobilized enzyme is in agreement with the results of Cadena et al. [16]. When, polyurethane was studied as a support for immobilization of invertase.

The effect of temperature on the activity of free and immobilized invertase is shown in Fig. 4. The reactions were performed in optimum pH (4.6) at temperature range from 25 °C to 65°C. The optimum temperature values for free and immobilized invertase were 55 °C and 60 °C, respectively. The plot profiles show that initially occurred an activity increase until to reach the maximal values and a sharp decrease at higher temperatures, what can be justified due to initially the increase of the kinetic energy of the reaction system and the protein denaturation after the maximal activities. In addition, the results for immobilized CGSZn-invertase derivative suggest a possible epoxydation of the APTS and glutaraldehyde unsaturations at temperature higher than 60 °C by air oxygen due to the mechanic agitation during the assay causing enzyme leaching as reported by Sanjay and Sugunan [2]. These results are similar to those reported by Vujčić et al. [5] and Cadena et al. [16].

The results of the optimal temperature studies allowed calculating the activation energy (EA) of free and immobilized invertase using Arrhenius equation:

$$Lnk = \text{Ln } A - Ea/RT \quad (1)$$

The activation energy of free invertase (51.34 ± 5.21 kJ/mol) was higher than one for immobilized invertase on glass–ceramic CGSZn (37.31 ± 3.40 kJ/mol). These results showed that the immobilization process made the enzyme conformation more favorable for sucrose hydrolysis reaction [16,17]. These results are mean values from three replicates. Average with different letters differs statistically by Tukey test (p = 0.05).

3.3. Kinetic parameters

The K_m and V_{max} were performed under optimum conditions regarding pH and temperature and calculated according to Lineweaver–Burk. K_m values were 0.30 ± 0.01 mM for free invertase and 0.02 ± 0.03 mM for immobilized derivative CGS*Zn*-invertase. The K_m value for immobilized invertase was 15 times lesser than one for free enzyme what can be attributed to easy accessibility of sucrose molecules to the enzyme active center as a result of the spatial distribution of the invertase molecules on the GCS*Zn* surface and conformational changes. The V_{max} value of the free invertase (10.99 ± 0.28 mmol/min) was lower than that one for immobilized invertase (59.98 ± 0.72 mmol/min). These results can be due to the mass transference rate on the support surface which is lesser compared to that into soluble system reactions. These parameters were statically confirmed by Tukey's test ($p < 0.05$). Similar results were reported by David et al. [18] and Cadena et al. [16].

3.4. Reuse stability at different temperatures of immobilized invertase on GCS*Zn*

The reuse stability was studied at different temperature values from 25 °C up to 65 °C (Fig. 5). After ten reuses at the range from 25 °C to 35°C, the invertase activity was lightly affected when compared with the reuses at upper temperatures. These results are very interesting for the industrial application of the immobilized derivative (CGS*Zn*-invertase) for invert sugars syrup production due to reduction in the energy costs. Nevertheless, after ten reuses above 50 °C was observed significant loss of invertase activity mainly at optimum temperature (60 °C) when it was lost 70% of the initial activity.

4. Conclusion

Invertase was covalently immobilized on GCS*Zn* which it was confirmed by SEM, EDS and XRD analysis. The optimal pH and temperature results showed that there was an improvement of the enzyme physic-chemical behavior after immobilization. The immobilized derivative CGSZn-invertase showed higher affinity by the substrate (sucrose) with km value 15 times lesser than that

found for free enzyme. The immobilization process also decreased the activation energy (37.31 ± 3.40 kJ/mol) compared to one for free invertase (51.34 ± 5.21 kJ/mol) make easier the hydrolysis reaction. After ten reuses at 25 ± 2 °C, the immobilized invertase lost only 9% of initial activity, but at the optimum temperature (60 °C), the activity decrease was about 70%. Reactions at low temperatures are biotechnologically interesting making the process in industrial feasible in spite of energy economy leading to reduction operating costs for invert sugars syrup production.

Acknowledgements

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Figure captions

Fig.1 Microphotographs integral and fragmented morphological surfaces (A) and chemical composition (B) from glass–ceramic support from coal fly ashes sinterization by SEM and EDS, respectively. Experimental conditions are described in Section 2.2.

Fig.2 X-ray diffraction (XRD) to demonstrate the crystalline phases, glass–ceramic support (GCS*Zn*) sample from coal fly ashes (CFA) sinterization for the immobilization of invertase process. (A) GCS*Zn*: mullite (Δ), quartz (●), aluminum sulfate (□); GCS*Zn* functionalized with 3-APTES (B); GCS*Zn* functionalized with 3-APTES and glutaraldehyde (C), 2.58, 5.15 and 7.73 mg protein/g GCS*Zn* (D, E and F).

Fig.3 Effect of pH for free enzyme (○) and immobilized (●) in *GCSZn*. Experimental conditions described in Section 2.4.

Fig.4 Effect of temperature free enzyme (○) and immobilized (●) in *GCSZn*. Experimental conditions are described in Section 2.4.

Fig.5 Effect of temperature in reusability for activity of invertase immobilized on *GCSZn*. Experimental conditions are described in Section in 2.6.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2012.10.029.

Anexos do Capítulo III

Table 1

Physical properties of GCSZ*n*.

Analyses	Conditions
	1200 °C/1 h
Green body (g)	0.3
After sinterization	
Weight dried (g)	0.219
Shrinkage (%)	<10.42
Height (cm)	0.286
Diameter (cm)	0.742
Apparent porosity (%)	13.69
Apparent water absorption (%)	8.67
Apparent density (g/cm3)	1.81
Uniaxial compressive strength (MPa)	42.89

Figure 1

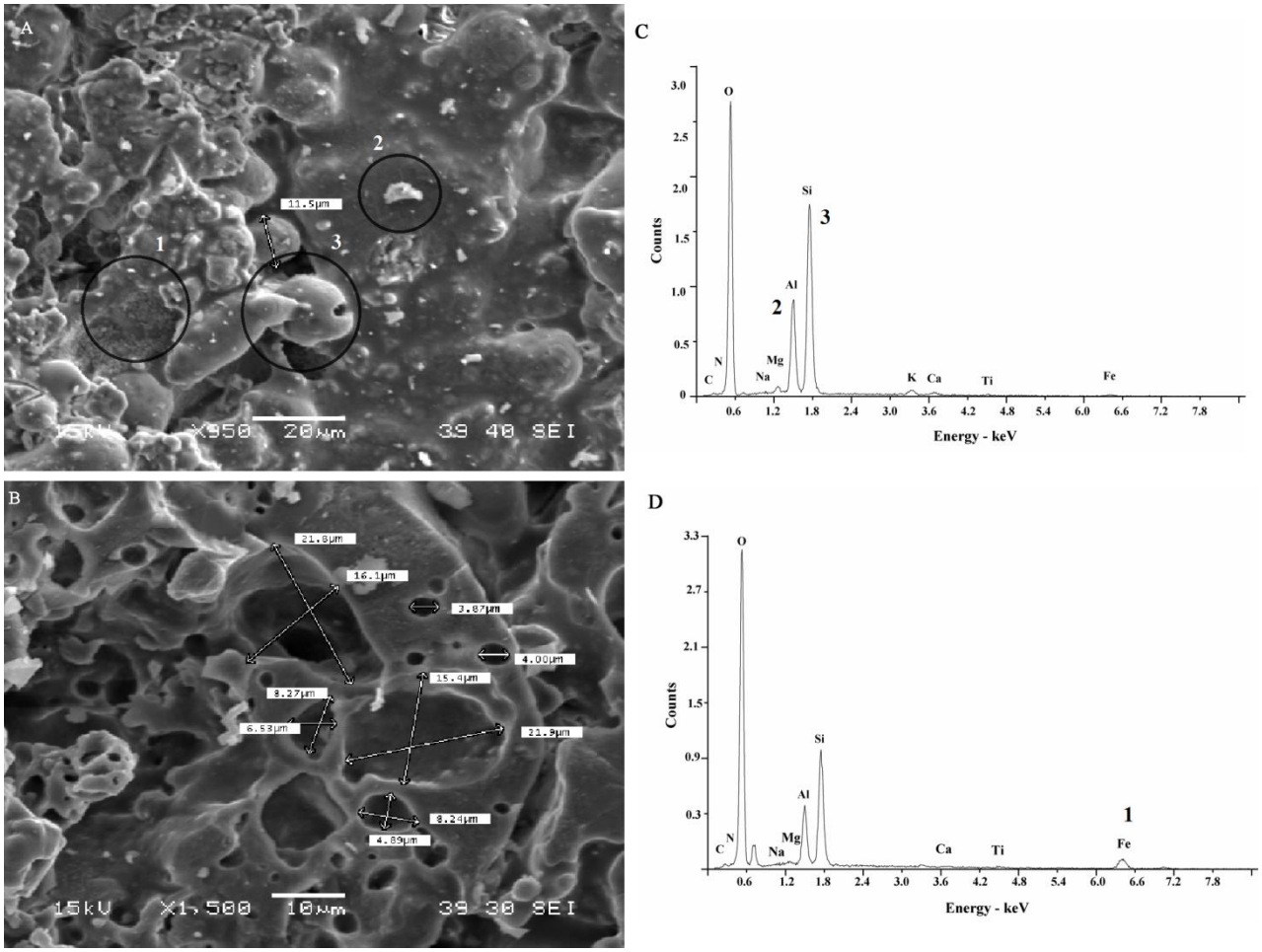

A
1
2
3
11.5µm
15kV X950 20µm 39 40 SEI
B
21.8µm
16.1µm
3.87µm
4.00µm
15.4µm
8.27µm
6.53µm
21.9µm
8.24µm
4.89µm
15kV X1,500 10µm 39 30 SEI
C
Counts
Energy - keV
O
Si
Al
Mg
Na
N
C
K
Ca
Ti
Fe
D
Counts
Energy - keV
O
Si
Al
Mg
Na
N
C
Ca
Ti
Fe

Figure 2

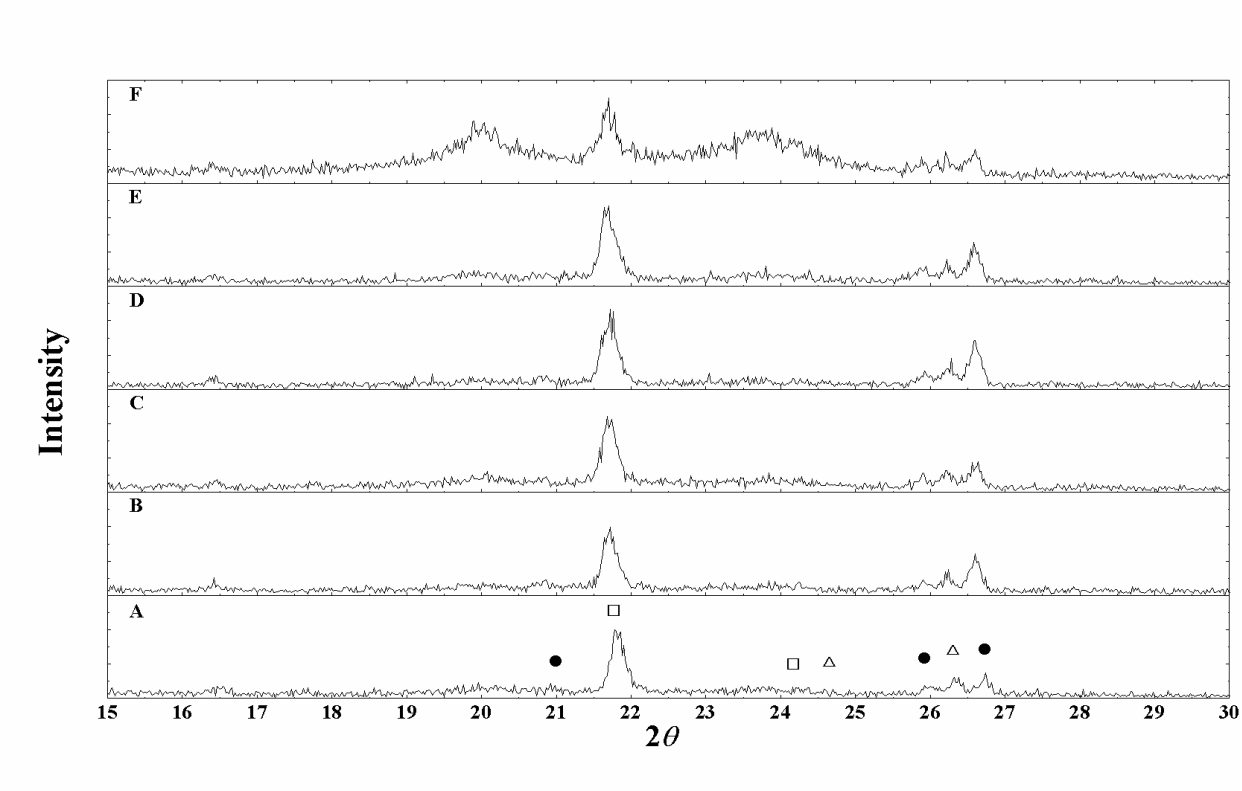


Figure 3

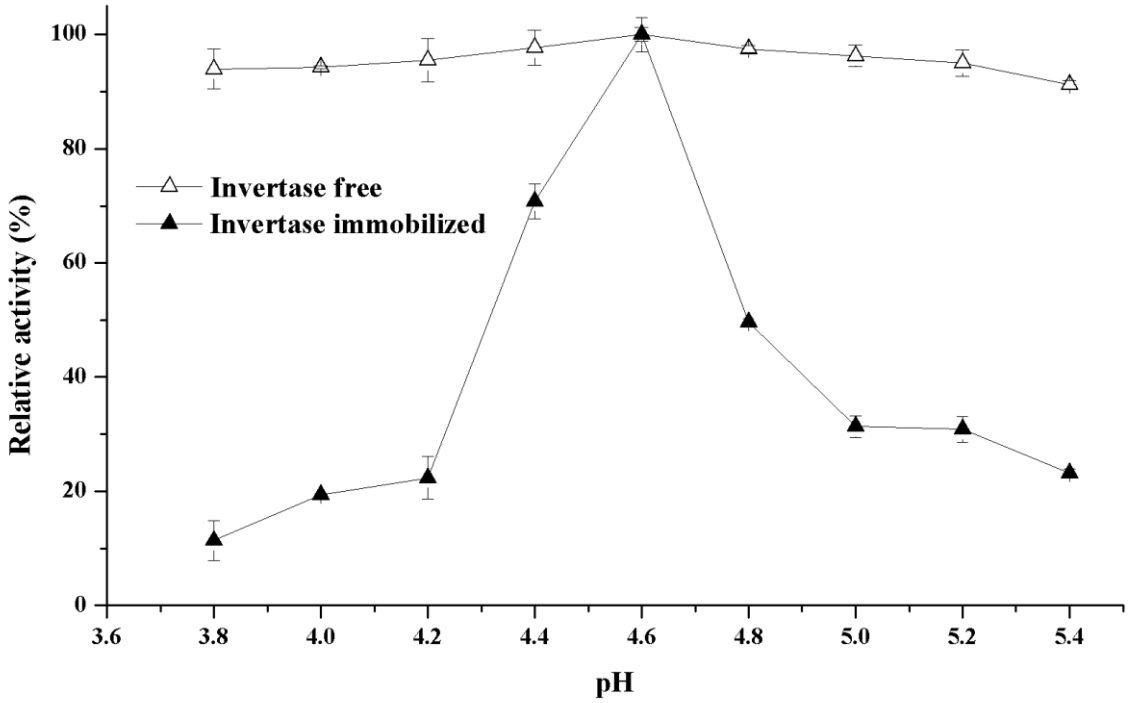

Relative activity (%)
pH
Invertase free
Invertase immobilized
0
20
40
60
80
100
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4

Figure 4

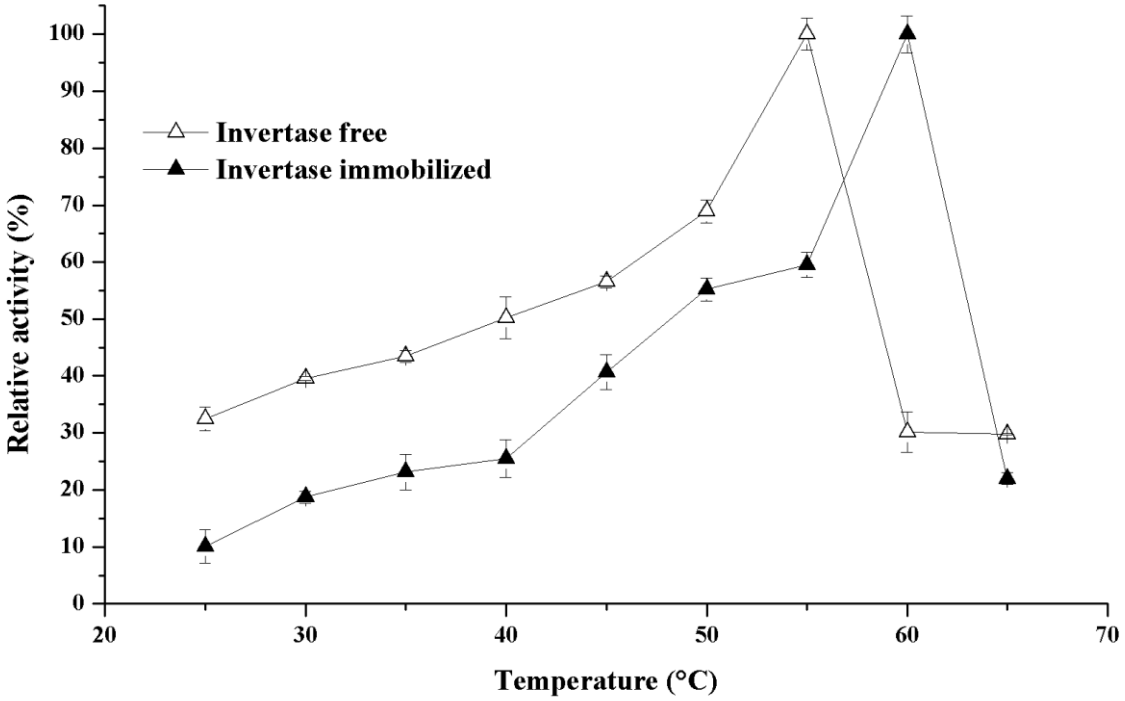
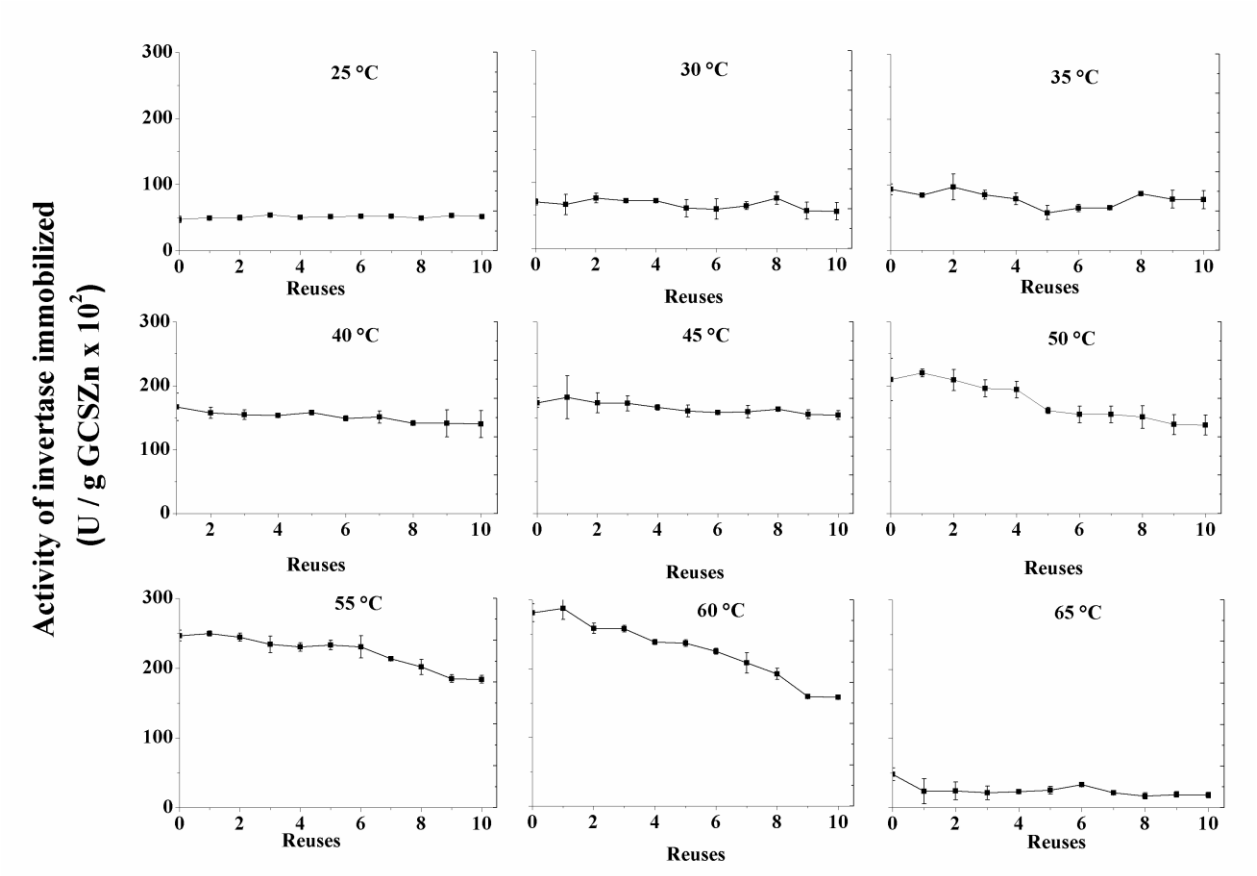

Invertase free
Invertase immobilized
Relative activity (%)
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
20
30
40
50
60
70

Figure 5


Activity of invertase immobilized
(U / g GCSZn x 10²)
25 °C
30 °C
35 °C
40 °C
45 °C
50 °C
55 °C
60 °C
65 °C
Reuses
0
100
200
300
0
2
4
6
8
10

7. ANEXOS

7.1. Trabalho apresentado a SBBq - 2006

Invertase production by entophytic yeasts isolated from root and stem of *Saccharum officinarum*

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Resumo:

The entophytic microorganisms will be the big interest in the enzymes production of biotechnological interest. However, little things in literature about entophytic yeasts invertase producers exist. The aim of this work was isolate endophytic yeasts invertase producers from the intracellular spaces and vacuoles. The yeasts was isolated from the stem (YS) and root (YR) of *Saccharum officinarum* and the culture had been carried out in Erlenmeyers (250 mL) with 10g of sugar cane in 50 mL of distilled water, in orbital shaker (180 rpm) at 30 for 48h. The enzyme was obtained of the supernatant after extraction with a phosphate buffer solution 0,2 M pH 5,0 and centrifuged at 10000 xg for 20 minutes. The analytical determinations had been carried for invertase activity and sucrose consumption. The biomass production was determined through absorbance (600nm). The maximum activities of invertase had been gotten at 36 hours of culture for yeasts, 419 and 379 U/mL for YS and YR, respectively. In the consumption of carbon source, the yeasts had reduced about 30% of the reducing sugar to the end 24 hours. This can be due to affinity to the substrate (high concentration sucrose) of invertase produced by the yeasts. The results demonstrate the potential of these yeasts for invertase production with great affinity for high concentrations of sucrose, what it can make possible process of production of inverted sugar in industrial scale.

7.2. IV Jornada do LIKA 2008

IMMOBILIZATION OF INVERTASE INTO CERAMIC SUPPORT FOR HYDROLYSIS OF SUCROSE

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Resumo:

Ceramic sinterized proceeding from coal fly ashes was used as a support for immobilization of invertase. The attainment of an insoluble support with chemical physical properties to bind invertase into sinterized coal fly ashes is an alternative to decrease environmental pollution with the storage and dissemination through thermal power plants. Of point view, it will be development of sugar market of region due absent invert sugar production from immobilized biocatalyst. The immobilization of enzyme was started with chemical modification of silane groups present with 2% 3-aminopropyltriethoxysilane solution in toluene, through covalent binding immobilization of the alkyl amine to glutaraldehyde and subsequent attachment of the enzyme molecule to glutaraldehyde via Schiff's base linkage. The protein amount and specific activity of the immobilized enzyme on 0.438 M sucrose (pH 5.0 at 45ºC) were 1.37 mg/ 0.25g of particles and 0.67 U/mg protein (19.89 %) of that estimated for the free enzyme, respectively. The apparent *K*m for immobilized invertase was approximately 10-fold lower than free enzyme. Moreover, the results in bioreactor the effect of continuous flow injection (inffluent, effluent and alternated) showed obtained 100% of sucrose inversion at 16h of reaction with alternated flow injection.

7.3. Depósitos de pedidos de patentes

7.3.1. SUPORTE VÍTREO-CERÂMICO A PARTIR DE CINZAS VOLANTES DE CARVÃO MINERAL PARA IMOBILIZAÇÃO DE BIOMOLÉCULAS E/OU MICRO-ORGANISMOS

Esta invenção descreve o inovador processo laboratorial e industrial que envolva o uso de suporte cerâmico a partir da vitrificação das cinzas volantes de carvão mineral para a imobilização ou separação de substâncias biológicas e/ou microorganismos que possam ser utilizadas em fins biotecnológicos de interesse comercial.
Data do depósito: 24/01/2012, protocolo:BR 10 2012 0015773.

7.3.2. SUPORTE VÍTREO-CERÂMICO A PARTIR DE ARGILA PARA IMOBILIZAÇÃO DE BIOMOLÉCULAS E/OU MICRO-ORGANISMOS

Esta invenção descreve o inovador processo laboratorial e industrial que envolva o uso de suporte cerâmico a partir da vitrificação ou sinterização de argila para a imobilização ou separação de substâncias biológicas e/ou microorganismos que possam ser utilizadas em fins biotecnológicos de interesse comercial.
Data do depósito: 26/04/2012, protocolo:BR 10 2012 0097397.

7.3.3. SUPORTE VÍTREO-CERÂMICO A PARTIR DE RESÍDUOS DE CONSTRUÇÃO CIVIL PARA IMOBILIZAÇÃO DE BIOMOLÉCULAS E/OU MICRO-ORGANISMOS

Esta invenção descreve o inovador processo laboratorial e industrial que envolva o uso de suporte cerâmico a partir da vitrificação ou sinterização de resíduos de construção civil para a imobilização ou separação de substâncias biológicas e/ou microorganismos que possam ser utilizadas em fins biotecnológicos de interesse comercial.
Data do depósito: 26/04/2012, protocolo:BR 10 2012 0097419.

8. Conclusão geral

A obtenção da cerâmica vítreo-cerâmica a partir da sinterização das cinzas volantes de carvão mineral com suporte para imobilização de enzima demonstra ser viável economicamente, sobretudo a seu caráter determinante e significativo à redução da poluição ambiental. Em consequência, houve agregação de valor e requinte a técnica biotecnológica com uso deste resíduo tóxico.

Os resultados experimentais permitem concluir que:

1- O conjunto de técnicas usadas para a caracterização física do suporte vítreo-cerâmico (SVC_2) é muito importante para a escolha do suporte vítreo-cerâmico. Porém, ficou demonstrado que a matriz vítrea formada após a temperatura e tempo de sinterização usada foi a que obteve maior resultado significativo para imobilização de enzima em comparação com amostras vítreo-cerâmicas de mesma constituição química e com as amostras obtidas do controle (SVC_0) (sem aditivo permanente) e das amostras com adição de cloreto de chumbo (SVC_1).

2- A análise microestrutural (MEV) evidenciou que o suporte tanto material denso vítreo policristalinos, amorfos e microporos com interligações entre si, propiciando maior área de superfície principalmente para ativação dos grupos silanos para ligar-se a enzima.

3- O efeito maior da atividade enzimática usando o suporte vítreo-cerâmico que teve como corpo verde a adição de sulfato de zinco foi intensificado com o uso de um protocolo com concentrações pequenas de glutaraldeído, 3-aminopropiltrietoxisilano, enzima em relação ao tempo de exposição.

4- A análise das fases cristalinas das amostras vítreo-cerâmicas (XRD) em cada fase do processo de imobilização foi decisiva para demonstrar o sucesso do processo de imobilização da enzima.

5- A caracterização físico-química da enzima imobilizada na amostra vítreo cerâmica foi compatível com trabalhos usando outros tipos de suportes.

6- As propriedades físicas do derivado imobilizado foram significativas quando testado num novo sistema de fluxo contínuo (alternate-flow) em biorreator;

7- O efeito da temperatura em relação aos reusos da enzima imobilizada foi significativo para demonstrar que as condições ótimas nãos são ideias para a produção de açúcar invertido. Atividades enzimáticas da enzima imobilizada em temperaturas entre 25° e 35° são obtidas resultados mais significativos quanto à reutilização do biocatalisador. Os derivados imobilizados submetidos em reações a baixas temperaturas foram biotecnologicamente significantes para tornar o processo viável em escala industrial.

9- As cerâmicas desenvolvidas podem ser uma alternativa viável para reduzir a poluição ambiental por cinzas volantes de carvão.

9. Perspectivas

Atualmente no Brasil há algumas empresas que obtém solução de açúcar invertido com custo baixo a partir de hidrólise ácida. Porém, ainda não há controle neste composto para verificar a existência de resíduos oriundo deste processo. Por se tratar de produto que é integrado a outros, pela indústria de alimentos, bebida e outros, há a necessidade de uma legislação especifica para o caso.

No que se refere à cana-de-açúcar, a retração do preço do petróleo e a crise internacional de créditos que se instalaram em meados do segundo semestre de 2008, fizeram com que o setor sucroalcooleiro revisse seus investimentos, adequando-os a uma nova realidade. Entretanto, essencialmente se destaca a investimentos a manipulações genéticas, para obtenção de espécies mais produtivas e resistentes à praga e clima ou micropropagação em laboratório. O desenvolvimento do setor para desenvolvimento da sacarose para produção de açúcar invertido através da técnica de imobilização da enzima, invertase, é inexistente.

Os resultados apresentados podem servir de base para estudos mais aprofundados à escala maiores à usada.